STRESS RESPONSES OF STINGING CATFISH *HETEROPNEUSTES FOSSILIS* (Bloch) TO ORGANOPHOSPHORUS INSECTICIDE MONOCROTOPHOS

Thesis submitted to

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY

In

MARINE BIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

By

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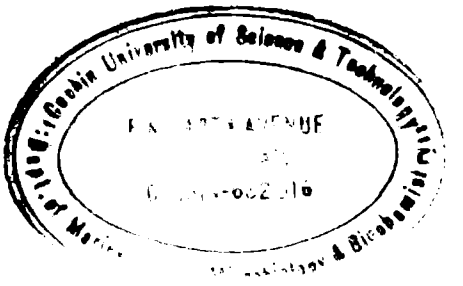
May 2008



Certificate

This is to certify that the thesis entitled "**Stress Responses of Stinging Catfish *Heteropneustes fossilis* (Bloch) to Organophosphorus Insecticide Monocrotophos**" is an authentic record of the research work carried out by Ms. Anupama Nair. P.R under our 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 Marine Biology of Cochin University of Science and Technology and no part of these has been presented for the award of any other degree, diploma or associate ship in any university.

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DECLARATION

I hereby declare that the thesis entitled "**Stress Responses of Stinging Catfish *Heteropneustes fossilis* (Bloch) to Organophosphorus Insecticide Monocrotophos**" is a genuine record of research work done by me under the combined supervision of Prof.(Rtd) Dr. K.Y.Mohammed Salih, Department of Marine Biology, Microbiology and Biochemistry and Dr. Sujatha C.H, Department of Chemical Oceanography, Cochin University of Science and Technology. The work presented in the thesis has not been presented for any other degree or diploma earlier.

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Anupama Nair. .P.R

Acknowledgement

I express my sincere gratitude to my research guide Dr. K.Y.Mohammed Salih, Professor (Rtd), Department of Marine Biology, Microbiology and Biochemistry for his supervision and guidance. I thank him for the kind of freedom I was endowed with, to improvise, plan and implement.

I am forever obliged to Dr. A.V.Saramma, Head, Department of Marine Biology, Microbiology and Biochemistry, for the encouragement and support I received from her throughout the tenure of my doctoral program.

I thank my Co-Guide, Dr. C.H. Sujatha, lecturer, Dept of Chemical Oceanography, for the motivation and guidance I received from her. I take this opportunity to express my thankfulness to her for the way she used to motivate me.

I am thankful to Dr. Mohan Kumar, Dean, faculty of Marine sciences, for his ever inspiring attitude towards constructive research and for the keenness he always showed in improving the quality of research in marine sciences. Sir, I express my boundless appreciation and gratitude for inspiring me to set more goals.

I thank my teacher, Dr. A.A Mohammed Hatha, I could not have imagined having a better educator for my masters and PhD times, and his wisdom, perceptiveness and promptness helped me to become a better scientific person.

I am thankful to my teachers Prof. Dr. R.Damodaran, Prof. Dr. N.R.Menon, Dr.Kuttiyamma, Dr. C.K.Radhakrishnan, Prof. Dr. Babu Philip, Dr. Rosamma Philip, Dr. Bijoy Nandan and Prof. Dr. Anneykutty Joseph for their support and encouragement.

I am grateful to Dr. K.C. George, Principal Scientist, CMFRI, for the information on the histology of fish, and for helping me to interpret the histopathological aspects of my work. I am forever obliged to him for the great lessons on histology he gave despite of his busy schedules.

I express my gratitude to Dr. Nevin Uner, University of Cukurova, Faculty of Science and Letters, Turkey;W Dr. Fousia Begum, Dept. of Zoology, Osmania University, Hyderabad, for their timely help during the preparation of my thesis.

I express my respect and gratitude to Mr. Rajgopal, Director and Dr. Viswanathan Nair, Chief scientist, Sea lab, Seafood Park India Pvt. Ltd. for the kind and friendly attitude which helped me through the Chromatographic aspects of my work.

I am especially grateful to Mr.Stephy Thomas, Dept. of Statistics, CUSAT for his valuable help during data analysis. I am obliged to him for the lessons of statistics.

I am thankful to Mr.Suresh Kumar, Lakeshore hospital for his valuable suggestions during my work.

Dear Rajeev Raghavan, (Conservation Research Group & Department of Aquaculture, St. Albert's College, Kochi), thank you, for all the intellectual support which aided me to come across the most recent information and publications on fish related aspects, which helped me immensely to finish this thesis promptly.

Rekha, wonder how I possibly will find some apt words to thank you!! For making, the past long years of life in CUSAT, happy and peaceful...for the great times of togetherness...for making me feel safe and sound...for pulling me away from hideaways of despair...for always telling me how dazzling the other side of life shall be!

I am short of words when I am to comment on how much I am beholden to my friend Ms. Bindya Bhargavan for her immense help through out my doctoral programme. From basic to advanced, there was not a single thing on toxicology we let not discussed! Thank you my friend, for the kind of support and understanding which always facilitated me to steer across the thick and thin of research days.

Jaleel, I think you would not let me say a word of gratitude, but here I am to say it finally. Your relentless support and assistance spanned from the beginning of Ph.D life, through the acclimation of fishes, to the finishing point of my thesis is forever etched in memory.

Krishna Kumar, how could I thank you for those long time pre treatments and seemed endless periods of acclimation and transportation of my catfishes! I express my appreciation for astonishing me with the valuable information on freshwater fishes of kerala.

Shubhasree, a lot of thanks buddy, for all the well-timed helps, for your positive attitude and for your pleasant company that helped me a great deal to relax and do away with worries. Thanks for being there!

I thank my dear friend Roja Sebastian for her love, care and sweet smile which enabled me to go through the toughest times. I remember the jokes and merry thoughts she shared with me which made me laugh like anything and made my days light and pleasant.

My dear friends Selvan, Anil Kumar, Rejil, Smitha C.K, Sridevi, Manjusha Mohandas, Neil, Nousher Khan, Keshavan Nambudiri, Prabhakaran, Sanal Kumar, Sudha, Beena, Jini Jacob, Subitha, Rejish Kumar, Sudheer, Sreedharan, Remya, Jisha Jose, Aniladevi Kunjamma, Cilla Abraham and Chithra were always there to help me when ever I was in need. I thank them wholeheartedly.

Abhilash, its right time to say a big 'Thank you' for all the help, as you never allowed me to say it earlier.

I specially thank Dr. Soja Louis for her encouragement and valuable comments during the making of thesis.

Smitha V. Bhanu, I am inspired by your openness in knowledge sharing. Keep it up buddy! Thank you so much for all valuable ideas during the making of thesis.

I sincerely thank staff, Department of Marine Biology, Microbiology and Biochemistry for their timely helps.

I thank Mr. Premachandran and Mr. Manuel, librarians, School of Marine Sciences Library for their constant encouragement.

I record my thanks to Mr. Binoop M.R (Indu Photos & Graphics) for his perfect sense of commitment which helped a great deal in the layout and design aspect of my thesis.

I record my gratitude to Mr. Nazar and Mr. Sajeeven (Staff, School of ocean science and technology) for their timely helps which enabled me to carry out work without interruption.

I thank Mr. Stephen, School of Ocean Science and Technology for his well-timed helps during the transportation of my catfishes.

My brothers Bineesh and Anish were always keen about my research and I thank them for their true enthusiasm.

I thank my sister Sharmila, who has always been my best pal, am deeply beholden to her for the long story telling sessions from our childhood, she has always been a powerful source of dynamism to me.

I am deeply beholden to my brother-in-law, Dr. A.P.Mukundanunni, for his enthusiastic attitude which inspired me a lot to pursue my research, his great skill of editing which helped my words with refinement.

Papa and mummy, I thank you for your support, encouragement and understanding which helped me a lot during stiff working hours.

I owe my father and mother, much of what I have become. I thank them for their love, their support, and their confidence throughout the past years. My parents have always put education as a first priority in my life, and raised me to set high goals for myself. They taught me to value honesty, courage, and respect for humanity above all other virtues. I have always needed to work hard to achieve my goals in life and they have always been there for me as an unwavering support. I dedicate this work to them, to honour their love, patience, and support during these years.

Bijeesh, without whose love, understanding and endless impetus, this work would never have been materialized! Especially for all the holidays that he arranged to give me time to write, to think, to improvise and to talk to him about this subject. Had I written this sentence myself, I couldn't have put it any better.

Finally, I have to say 'thank-you' to all my friends and family, at times, it has been good to know they have just been a phone call or an email away.

There are lots of people I would like to thank for a huge variety of reasons...

Peace and harmony,

Anupama.

CONTENTS

GENERAL INTRODUCTION

The field of aquatic toxicology has been defined as the qualitative and quantitative study showing adverse or toxic effects of chemicals on aquatic organisms (Rand and Potrocelli, 1985). The subject of enquiry deals with the study of transport, distribution, transformation, and the ultimate effect caused by the chemicals in the aquatic environment. Today, as a result of the ever booming industrialization, urbanization and the unchecked expansion of modern agricultural practices, excessive load of chemicals, and xenobiotics like pesticides reach the aquatic environment by land runoff and leaching. Insecticides/herbicides are among the most widely used toxic chemicals for various purposes in industry, forestry, horticulture, agriculture and household. Water often serves as a sink for these chemicals after their application in these different fields. These pollutants are often not entirely specific for their target organisms. Non-target species, such as aquatic biota, can be affected because of the universal presence of insecticides in the environment. It is reported that there exists a large amount of circumstantial evidence to indicate a decline in the population level (Houlahan *et al.,* 2000), impairment in growth, and to show an increase in disturbed behaviour (Alvarez and Fuiman, 2005), deformities (Klump *et al.,* 2002) and various diseases in aquatic organisms (Zelikoff *et al.,* 2002). These effects suggest that pesticides at sublethal concentrations are present in the environment (Tanguy *et al.*, 2005; Amando *et al.*, 2006).

Pesticides are the only toxic chemicals deliberately released into the environment in large amounts. Their potential to cause adverse effects to human and wild population has been a subject of intense study and that has led to the development of stringent and encompassing regulations for the risk assessment of novel formulations in order to control the use of compounds currently in use. The organophosphorus insecticides (OPs)

were introduced as replacements for persistent organochlorine insecticides after the tendency of DDT and its metabolites to bioaccumulate in the ecosystem and to cause adverse health effects, particularly in top predators (Woodswell *et al.*, 1967; Peakall *et al.*, 1975; Murphy, 1986). In some countries the latter was legally banned in 1970s and in others only restrictive uses were allowed. The increased use of OPs, originally seen as harmless to the environment due to their rapid breakdown and low persistence, has led to a different range of ecotoxicological problems associated with their high acute toxicity. Evidence gathered from the last twenty years of experiments show that OPs can interfere with immune system and exert immunotoxic effects in laboratory animals through both anticholinergic and non-cholinergic pathways (Wong *et al.*, 1992; Barnett and Rodgers, 1994; Vial *et al.*, 1996).

Way back in 2000, November 23, globally threatened Sarus cranes *Grus antigon* who were resident at Keoladeo National Park World Heritage site and the surrounding area near Bharatpur, Western Rajasthan, India, were joined by wintering common cranes *Grus grus*. Fifteen Sarus cranes and three common cranes were found dead in a field adjacent to the park, where wheat seed had been sown the previous day. Chemical analyses of seed samples from the field and the cranes' alimentary tract contents identified residues of the organophosphate insecticide monocrotophos. Monocrotophos concentrations of 0.8 and 1.8 ppm were found in wheat samples, and 0.2–0.74 ppm in the alimentary tract contents of five of the seven cranes examined. No other organophosphate or organochlorine pesticides were detected. Scientists concluded that the cranes died from Monocrotophos poisoning after eating treated seed.

Monocrotophos (MCP), commonly known as Azodrin, is one of the most extensively used OPs, in agriculture and in animal husbandry. It is a broad spectrum systemic organophosphate insecticide used on a range of crops, primarily cotton, soybeans and rice, and other crops like wheat, potatos, alfalfa maize and sugarcane; also on vegetables (Guth., 1994) and in fruit orchards (Donzel., 1994). Monocrotophos has been used for pest control since the 1960s, and its use has resulted in poisoning of non-target species in large number. It works outs its way systemically. On contact it will be absorbed through skin. It works its way through inhalation and ingestion too. It has been categorized as extremely hazardous (Bhadbhade, 2002). Hydrolysis rates of monocrotophos in soil and aqueous environment are pH dependent and half-lives of monocrotophos in pH 3 and 9 at 25 °C are 13 days and 26 days respectively and it persists in soil in the dark for 30 days at neutral pH (Lee *et al.*, 1990). It is hydrolysed in alkaline conditions. Solubility of monocrotophos in water is 100% (Tomlin., 1995). Monocrotophos sprayed on crops, can remain as soil residue and enter water sources such as rainwater and ground water by leaching through soils.

Monocrotophos was one of the most stable OPs so far studied. In a study on the persistence of pesticides in river water, monocrotophos was found not to degrade (100% recovery as parent) after 8 weeks (Eichelberger and Lichtenberg, 1971). Water used as sample in this study was taken from a small stream into which domestic and industrial waste together with farm runoff had already run in. The sample showed 7.3 as pH value, and it increased to 8.0 after 8 weeks. Dosed fish were kept at laboratory temperature in both natural and artificial (room) light. Analysis was done by proper extraction and Gas Chromatography. However, a study conducted in paddy field showed rapid degradation in the aqueous phase (Osgerby and Page, 1969).

After deliberately killing of 62 000 birds with monocrotophos on a farm in Argentina in 1997, and poisoning thousands of Swainson's hawks with secondary infliction (Goldstein *et al.*, 1999), Novartis, one of the manufacturers of that time, announced they were phasing out all the manufacture and sales of monocrotophos globally (Winegrad, 1998). A number of studies were conducted on the toxicity of monocrotophos on different aquatic organisms, and finally it was adjudged to be a potent neurotoxicant (Qadri *et al.*, 1994; Rao *et al.*, 1991; Rao *et al.*, 1992a; Venkateswara Rao *et al.*, 2001). Several studies confirmed the occurrence of monocrotophos in aquatic systems in concentrations that can be deleterious to various life forms.

Reviewing Overseas Regulatory Actions, it is of importance to observe that, monocrotophos has been voluntarily withdrawn from sale in the US in 1989 following concern on its toxicity to non-target species. It is also banned in Indonesia, Sri Lanka and Philippines; severely restricted in Kuwait (for use on plants in flowering stage only); Malaysia (for use on coconut tree and on oil palm by trunk injection) and Germany (not to be handled by adolescents, pregnant and nursing women). monocrotophos is not used in the United Kingdom. In 1996, the companies selling monocrotophos in Argentina have voluntarily agreed to withdraw the product from the market, and bought back all existing supplies following concerns over bird deaths from its use in grasshopper control (Pesticide Action Network North America Updates Services, 4 November 1996).

As chemical analyses alone may not suffice to explain the adverse effects of the complex mixtures of chemicals present at contaminated sites, determining the extent and severity of water contamination by pollutants is often difficult. In the case of aquatic environment, because of its

peculiarity, biomonitoring by means of biomarker parameters assessed in different native species is a useful tool. It has the advantage of providing a quantitative response as well as valuable information of ecological relevance on the chronic adverse effects caused by water pollution (van der Oost *et al.*, 2003).

Indiscriminate use of pesticides in agricultural operation adversely affects the aquatic environment to a very great extent potentially affecting fish. The responses of fish to such environmental challenges are initially reversible, but prolonged exposure to environmental pollutants brings about permanent (pathological) changes in fish physiology. Sometimes, death of the aquatic living resources such as fish may not occur but they suffer physiological perturbations like altered enzyme activity mainly due to high load of these environmental anthropogenic agents (Racicoot *et al.* 1975). With the increasing awareness of the impact of these pollutants, especially in developing economies, it is essential that sensitive biochemical and/or physiological parameters which could act as early warning signals of pollution at sublethal levels be studied. Hence, changes in the activity of enzymes or other biomarkers (Moore and Simpson, 1992) have been used as possible tools for aquatic toxicological research.

Many circumstances, biotic and abiotic, promote the antioxidant defense response in fish. Factors inherent to the fish itself, such as age, phylogenetic position and feeding behavior, and environmental factors such as types of diet available, temperature, dissolved oxygen, toxins present in the water, pathogens, and parasites can either enhance or decrease the antioxidant defenses. Research in fish has demonstrated that mammalian and piscine systems exhibit similar toxicological and adaptive response to oxidative stress. This suggests that piscine models in addition to traditional

mammalian models may be useful for further understanding the mechanisms underlying oxidative stress response (Kelly *et al.*, 1998). Studies on the oxidative stress in fish open a number of research lines aimed at providing greater knowledge of fish physiology and toxicology. Over and above, such studies would give more accurate information concerning the response of antioxidant defense in different species under different circumstances as well as the regulatory mechanism of the responses. Such future studies will, of course, do good to aspects allied to fish farming and aquaculture production.

Poisoning by pesticides from agricultural fields is a serious water pollution problem and its environmental long-term effect may result in the incidence of poisoning of fish and other aquatic life forms (Jyothi and Narayan, 1999). Fishes like *Heteropneustes fossilis* and *Clarius batrachus* are especially prone to serious pesticide pollution as their habitat is mostly the agriculture area. Though only few studies are conducted in this area, it can be assessed from the local information that, population of such fish is on the verge of vulnerability due to extensive use of pesticides. The knowledge of sublethal effects of xenobiotic compounds on hematological parameters, enzyme activities and metabolite concentrations is very important to delineate the fish health status and provide a future understanding of ecological impacts. These pesticides act by causing inhibition of cholinesterase enzymes (ChE) by formation of enzyme inhibitor complex (O'Brien, 1976) and damaging the nervous system. These effects may result in metabolic disorders. Associated to cholinesterase activities, a study of other enzymes such as phosphatases and aminotransferases close to intermediary metabolite determination provides a wider view of metabolism. Interest in toxicological aspects has grown in recent years and research is now increasingly focused on mechanistic aspects of oxidative

damage and cellular responses in biological system. The term 'biomarker' is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). As biomarker stands for immediate responses, they are used as early warning signals of biological effects caused by environmental pollutants.

The present work attempts to assess the toxicity of organophosphorus insecticide monocrotophos on the experimental organism selected for this study namely stinging catfish (*Heteropneustes fossilis*) (Bloch), and to probe into the stress responses of the organism.

REVIEW OF LITERATURE

Monocrotophos (Phosphoric acid dimethyl (1-methyl-3(methylamino)-3-oxon-propenyl) ester)

The Rotterdam convention on 'Prior Informed Consent' (PIC) promotes the safe use of hazardous chemicals, helps importing countries to identify potential hazards and exclude chemicals they cannot manage safely, and ensures that exporters comply with the requirements. The PIC procedure applies to 'banned chemicals' (all uses banned on environmental or human health grounds) and 'severely restricted chemicals' or 'severely hazardous pesticide formulations' of chemicals. Liquid formulations containing over 600 g/ l monocrotophos (MCP) have been included in the PIC list for some time. The active ingredient itself was made subject to the interim PIC procedure at a meeting of the Rotterdam convention in 2002. Pain (2003) reported that, however, MCP remains a frequently used pesticide in many, mostly developing nations. It is manufactured by more than a dozen firms, mainly in Asia, and is actively traded. PIC listing is an important start in the safer use of MCP, but will not stop its production or regulate use. It is essential that as a first step the agrochemical industry provide appropriate and accessible information to farmers and farm workers to help minimise the human health and environmental impacts of MCP use.

Monocrotophos (phosphoric acid dimethyl (1-methyl-3 (methylamino)-3-oxon-propenyl) ester), commonly known as Azodrin, is one of the most extensively used organophosphorus (OP) insecticide for the control of agricultural pests in India (Ray *et al.,* (1985) ; Qadri *et al.,* (1994) ; Santhakumar *et al.,* (1999) ; Rao *et al.,* (2001)). Monocrotophos is a broad spectrum systemic insecticide and acaricide. As per the observations of Huges and Nemcsok (1988) it is a potent neurotoxicant. As per the details furnished by Waite *et al.,* (1992), monocrotophos has been detected in ground, surface and rain water due to its widespread use all over the world.

Nemcsok (1987) observed that monocrotophos can exert harmful effects in the organs of aquatic organisms. Sultatos (1994) reported that exposure of monocrotophos can produce a variety of biochemical changes in mammals and other experimental animals. Metabolic fate of monocrotophos in plants and animals is documented by Bull and Lindquist (1964), Menzer and Cassida (1965), Beynon *et al.*, (1973) and Mucke (1994). Lee *et al.*, (1990) reported that the hydrolysis rates of monocrotophos are pH dependent in soil as well as aqueous environments and it is 13, 25, and 30 days respectively, at 3, 9 and neutral pHs, at 25 °C.

Effects of organophosphorus insecticides on energy and metabolic parameters

As per observations of Laul *et al.* (1974) it has been shown that changes in carbohydrate and nitrogen metabolism in pesticide exposed fish is induced by the stress occurring from pesticide-induced hypoxia. These changes include depletion of proteins, glycogen and pyruvate stores from fish tissues such as liver and muscle. Rao (2006) observed depletion of glycogen may be due to utilization of stored carbohydrates in liver for energy production as a result of monocrotophos -induced hypoxia in *Oreochromis mossambicus*. Rao (2006) reported a time dependent reduction in the amount of glycogen in fish *O. mossambicus* exposed to RPR-II, one of the RPR series of insecticides based on monocrotophos. Gill *et al.*, (1990, 1991) reported a glycogen mobilization, probably to maintain the glucose level and the glycolysis, as usually observed for other fish exposed to a variety of sub-lethal concentrations of organophosphorus compounds . As per Rao and Rao, (1981) ; Heath, (1995) and Bonga, (1997) the reduced locomotion in OP treated fish can be due to a larger energy demand to support the detoxification processes and/or to respond to stress stimuli.

Glycogen depletion in liver and muscle after toxic stress in aquatic animals has been reported by various authors like Shobha *et al.*, (1989); Bhavan *et al.*, (1997) Aguiar *et al.*, (2004); and Hori *et al.*, (2006). Begum and Vijayaraghavan, (1994, 1995, and 1999) observed similar decrease in glycogen content accompanied by an increase in blood glucose levels in *Clarius batrachus* after the fish were exposed to the OP insecticides dimethoate and Rogor. Nakano and Tomlinson, (1967) reported an increased secretion of catecholamines under stress conditions in rainbow trout, enhancing the utilization of glycogen for energy production. This effect on glycogen levels appears to be linked, at least to some extent, to the detoxification mechanisms, indispensable for metabolism or degradation and elimination of the pesticides from the body. Grant and Mehrle (1973) gave a possible explanation of glycogen depletion in endrin exposed *Salmo gairdneri* that, stress-induced secretion of catacholamine and glucocorticoids can lead to increased glycogenolysis.

As reported by Dezwaan and Zandee (1972) and Reddy (1986) the insecticidal stress has been found to lead to a hypoxic/anoxic condition in organisms leading to anaerobic glycolysis and subsequent reduction in oxidative metabolism necessitating the utilization of carbohydrate resources to meet energy demand. Verma *et al.*, (1983) made similar observations on depletion of muscle and liver glycogen in *C. batrachus* exposed to dichlorvos, an OP pesticide. Soengas and Aldegunde (2002) reported that a reduction of brain glycogen of fish exposed to the sub acute doses points to deranged intermediary metabolism primary to ATP production.

Fishes have a very little amount of carbohydrates; the next alternative source of energy is protein to meet the increased energy demand. Umminger (1970) and Narasimhan and Sundararaji (1971) observed lower

total protein content in *Pundulus heteroclitus* and *Notopterus notopterus* respectively, exposed to various kind of stressors. Reddy *et al.*, (1991) and Yasmeen *et al.*, (1991) reported a decrease in concentration of total protein in the tissues of fresh water crab and *Anabas testudineus* respectively, exposed to endosulfan. During stress condition, fish need more energy to detoxify the toxicants and to overcome stress. As reported by Reddy and Bashamohideen (1995) and Singh *et al.* (1996) the depletion of protein fraction in liver, muscle and gonad tissues may have been due to their degradation and possible utilization of degraded products for metabolic purposes.

Gilbert and O'Connor (1970), Chang and O'Connor (1983) reported that Lipids are alternate sources of energy for organisms particularly in stress conditions and accelerated hydrolysis of lipids occur in order to cope with increased energy demand in stressed conditions. Supportive observations have been made in the fish *S. mossambicus* exposed to methyl parathion and in *Barbus chonchonius* exposed to aldicarb by Rao and Rao (1981) and Pant (1987) respectively.

Knox and Greengard, (1965); Watts and Watts, (1974); and Martin *et al.*, (1983) reported that Aspartate aminotransferases (AAT) and Alanine aminotransferases (ALAT) are functioning as link between carbohydrate and protein metabolism catalyzing the interconversion of strategic compounds like aspartate and α-ketoglutaric acid to oxaloacetic acid and glutamic acid and α-ketoglutarate and alanine to glutamic acid and pyruvic acid respectively. Transaminases are intracellular enzymes which exist only in small amounts in serum. As per Philip *et al.*, (1995) elevation in the transaminases indicates the utilization of amino acids for the oxidation or for glucogenesis and is used to determine liver damage. Friedman *et al.*,

(1996) and Henderson *et al.*, (1983) concluded that plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis. LaDue *et al.*, (1954) observed that these enzymes may leak into the plasma following reservoir tissue damage or dysfunction. Hence, the assay has become an indispensable tool in the clinical determination of the pathological conditions of the reservoir tissues and organs.

Earlier, Rao (2006) reported monocrotophos and RPR-V induced alterations in *O. mossambicus*, where fish exhibited elevated AAT and ALAT activities in plasma and kidney, and reduced activities in gill and liver tissues. As reported by Michael *et al.*, (1987), serum transaminase activity was elavated when *Clarius lazera* was exposed to various toxicants. Similarly, methyl parathion (Rao and Rao, 1984) has been reported to cause increase in the serum transaminases activity in fish and mammals. According to Rajyasree and Neeraja, (1989), Oluah (1998, 1999), Balint *et al.*, (1997) and Zikic *et al.*, (2001) the increase in ALAT and AAT indicate the tissue damages in liver, kidney, and gill. Philip and Rajasree (1996) reported that alanine and aspartate transaminases function as biochemical stress biomarkers and their alteration allows identification of damage in different organs such as the liver.

Rao (2006) observed that acid phosphatase (ACP) and alkaline phosphatase (ALP) activities increased in plasma, gill, and kidney of *O. mossambicus* exposed to monocrotophos and RPR series of insecticides, while a significant reduction in both the activities was observed in liver tissue. Agrahari *et al.*, (2007) observed that acid and alkaline phosphatase activity in blood plasma of monocrotophos exposed *Channa punctata* increased by 64-95% and 60-73% respectively. On exposure to stress,

destabilization of liver cell membrane occurs and varieties of enzymes habitually sited in the cytosol are released into the bloodstream and this is a useful quantitative marker for hepatocellular damage. Yang and Chen (2003) pointed out that increase in alkaline phosphatase activity in blood after the exposure of gallium has been implicated due to the direct toxicity of pesticide in fish liver. Sastry and Sharma, (1980) and Das (1998) reported an elevation of brain acid phosphatase activity in stress induced *Channa punctatus* and *Labeo rohita* respectively.

Pelgrom *et al.* (1995) observed that, increase in the activities of functional enzymes in the blood serum and tissues of fish exposed to toxicants could be attributed to cell membranous system damage leading to changes in membrane permeability and intercellular metabolism. Investigations have shown that changes in carbohydrate metabolism in fish induced by the severe stress resemble the changes displayed by higher vertebrates like mammals. As per Everse and Kaplan (1973) Lactate dehydrogenase forms the centre of a carefully balanced equilibrium between catabolism and anabolism of carbohydrates. Ramesh *et al.*, (1993) given that lactate dehydrogenase (LDH) is a marker of tissue damage and its increased level are reported in liver necrosis. Rao (2006) reported rapid decrease of LDH activity in the gill and brain of *Oreochromis mossambicus* intoxicated with monocrotophos, and increased LDH activity in brain and gill, but decreased activity in liver and muscle of *Oreochromis mossambicus* exposed to RPR series of insecticides based on monocrotophos. Ghosh (1987), Begum and Vijayaraghavan (1997) reported a progressive increase in the activity of lactate dehydrogenase as well as a rapid rate of glycolysis in liver and muscle of *C. batrachus* exposed to three OP pesticides.

Das and Mukherjee (2002) gave explanation on marked elevation of LDH activity observed in brain and liver of *Labeo rohita* fingerlings exposed to cypermethrin. Sivakumari *et al.*, (1997) while working on cypermethrin toxicity in *Cyprinus carpio* explained that changes of lactate dehydrogenase activity in pesticide treated fish may be due to severe cellular damage leading to the release of these enzymes and impaired carbohydrate and protein metabolism.

Effects of organophosphorus insecticides on Haematological parameters.

Shanthakumar *et al.*, (1999) reported that *Anabas testudineus* treated with monocrotophos showed reduction in RBC count, haemoglobin concentration and MCHC, while an increase in MCV values were observed. According to Butler *et al.*, (1978), the increase in MCV of fish exposed to monocrotophos was due to beta adrenergic stimulation brought by pesticide-exposed stress conditions and also it could lead to macrocytic anaemia. Siddique *et al.*, (1991) ; Reddy *et al.*, (1996) ; and Dutta *et al.*, *(1992)* reported that the probable mechanisms for developing anemia in *A. testudineus* exposed to monocrotophos could be due to the loss of erythrocytes as compensatory erythropoiesis could not be observed.

As per Shanthakumar *et al.*, (1999), the decrease in RBC concentration in *Anabas testudineus* intoxicated with monocrotophos is due to decreased erythropoietic activity. In most vertebrates, including fishes, erythropoietic activity is regulated by erythropoietin produced in the kidney. Reddy *et al.*, (1992) have given that erythropoietin promotes erythropoiesis by inducing hemopoietic stem cells to differentiate into erythroblasts (which form RBCs). Erythropoietin also activates pyridoxal phosphate in developing RBCs, inducing hemoglobin synthesis. Structural and functional damage to kidney can also contribute to the low erythropoietin

activity. Murphy (1980) has proved that monocrotophos reduces the ventilator movements and decreases the oxygen intake by impairing neuromuscular transmission through AchE inhibition.

As reported by Wedemeyer *et al.*, (1984) ; Das and Mukherjee., (2003) a gradual increase observed in blood glucose level in pesticide exposed *Labeo rohita* revealed that the fish became hyperglycaemic. Also *Labeo rohita* became anaemic which was attributed to haemodilution resulted from impaired osmoregulation across the gill epithelium.

Effect of organophosphorus toxicity on acetyl cholinesterase activity

Rao (2006) studied the effect of sublethal concentrations of monocrotophos on the AChE activity in the brain, gill and muscle of *O. mossambicus* and reported a continuous decrease in the enzyme activity as the exposure progressed. Similarly, AChE inhibition in brain was reported by other researchers when the fish was exposed to OP insecticides like chlorpyrifos and profenofos (Kumar and Chapman, 2001; Rao *et al.*, 2003a; Rao *et al.*, 2003b). Several researchers like Dutta *et al.*, (1992); Richmonds and Dutta, (1992); Dutta *et al.*, (1995); Guozhong and Dutta, (1998); Dutta and Arends, (2003) investigated the effect of OPs malathion and diazinon on brain acetyl cholinesterase activity in bluegill sunfish and the largemouth bass and reported a striking decline in the activity which influenced the optomotor behavior of the fish that could be disadvantageous to their survival in the environment.

Goodman *et al.*, (1979) showed a negative correlation between both egg production and AChE inhibition at similar concentrations in sheephead minnows (*Cyprinodon variegatus*) chronically exposed to organophosphorus insecticide diazinon.

According to a vast array of literature obtained from past, innumerable studies on the effect of organophosphorus compounds, it has become evident that primary effect of OPs on vertebrate and invertebrate organisms is the inhibition of the enzyme acetyl cholinesterase (AChE), which is responsible for terminating the transmission of the nerve impulse. OPs block the hydrolysis of the neurotransmitter acetylcholine (ACh) at the central and peripheral neuronal synapses, leading to excessive accumulation of ACh and activation of ACh receptors and therefore, accumulation of ACh at synaptic junctions is regarded as a marker for assessing the OP pesticides.

The over stimulation of cholinergic neurons initiates a process of hyper excitation and convulsive activity that progresses rapidly to *status epilecticus*, leading to profound structural brain damage, respiratory distress, coma, and ultimately the death of the organism if the ACh receptor antagonist atropine is not rapidly administered (Shih and McDonough,1997).

Inhibition of acetyl cholinesterase is a precise and sensitive method for assessing effects of OP on non-target organisms and it is extensively employed as a biomarker of exposure to OP insecticides (Fulton and Key, 2001). Dembele *et al.,* (2000) reported that chronic exposure to chlorfenvinphos; chlorpyrifos, diazinon and carbofuran significantly depressed the AChE level in brain of *C. carpio.*

Brzezinski and Ludwicki (1973) proposed that inhibition of AChE is accompanied by an increase in acetylcholine levels. This condition can lead to increase of catecholamines, which can affect the activity of enzymes involved in glycogenolysis and glycogen synthesis. Thus, increase in the levels of catecholamine may produce hyperglycemic condition which can be well reflected by increase in blood glucose level in the exposed animals. Studies

conducted by Van der Wel and Welling, (1989); and Ansari and Kumar, (1984) have shown that high levels of AChE inhibition are needed to cause significant mortality in aquatic species, both after acute as well as after chronic exposure to Ops. It is obvious from the studies of Fulton and Key, (2001) that the fish can endure even after 70% inhibition of AChE in brain when exposed to OPs. According to Mayer and Ellersieck (1986) it is well accepted that a 20% or greater inhibition of ChE in birds, fish, and invertebrates indicates exposure to OP insecticides. Ludke *et al* (1975) reported that some animals are able to survive with more than 50% of ChE inhibition but this is an indication of a life-threatening situation. As per Zinkl *et al.,* (1991) in fish, inhibition of brain AChE between 60–70% can result in death.

Uner *et al* (2006) studied on the significant inhibitory effect of chronic exposures to sublethal concentrations of OP insecticide diazinon on AChE activity in the brain of *O. niloticus* and fish exposed to diazinon were able to survive at the high percentages (up to 93%) of AChE inhibition observed at sub lethal concentrations over 30 days exposure period. Pan and Dutta, (1998) observed that although 91.45% inhibition occurred in the enzyme activity after diazinon exposure, all the exposed fish (*Micropterus salmoides*) survived. Morgan *et al.,* (1980) observed that the inhibition of the fish brain AChE may persist for a long time even after the transference of the animals to clean, unpolluted media. Thus, decreased enzyme activity found in animals captured from water containing no detectable amounts of organophosphorus insecticides can be a reliable marker of a recent exposure to anticholinesterase xenobiotics.

Vale, (1998) observed that OP compounds generally are lipophilic and therefore cross the blood–brain barrier in most cases. Tomokuni *et al.,* (1985) observed the accumulation of OP compound diazinon in the brain

of rats and mice after single intraperitoneal injection. They also found that brain AChE activity was markedly inhibited after injection. Hai *et al.*, (1997); Sancho *et al.*, (2000) observed that *O. niloticus* after exposure to diazinon, exhibited erratic swimming and convulsions. Similar behavior has been exhibited by many other fish species exposed to different OP insecticides. Aguiar *et al.*, (2004) reported that reduction of swimming performance observed in the fish exposed to OP compound methyl parathion could be attributed to the inhibition of AChE. As reported by Balint *et al.*, (1995); Pan and Dutta (1998) the inhibition of AChE activity in fish can be dangerous since it will affect feeding capability, swimming activity, identification, avoidance of predators and spatial orientation of the species.

Effects of organophosphorus insecticides on Branchial ATPases

Adenosine triphosphatases (ATPases) especially sodium, potassium-activated ATPase (Na^+, K^+ - ATPase) is an important component of active transport in teleost gills playing a central role in whole-body osmoregulation (Towle, 1981). Na^+, K^+ - ATPase activity is associated with the active transport system, which is responsible for the reciprocal transfer of Na^+ and K^+ across the plasma membrane (Skou, 1975, Flik *et al.*, 1986; Jagoe *et al.*,1996; Das and Mukherjee, 2000)

Gills are the main osmoregulatory surface organ in aquatic animals and are the primary site of uptake of waterborne pollutants. Therefore, gills may be the first site where the sub lethal effects of chemicals on Na^+, K^+ - ATPase activity would be observed. Haya *et al.*, (1983) stated that environmental organic pollutants usually affect the Na^+, K^+ - ATPase by decreasing its activity. Reddy *et al.*, (1992) stated that aquatic pollutants exert a biological effect on gill ATPase system by partitioning in the

enzyme complex which may cause an allosteric change that result in decreased ATPase activity.

Reddy and Philip, (1994) reported inhibitory effects of the Na^+, K^+ ATPase in carp during acute exposures to pesticides in laboratory studies. De La Torre *et al.,* (1999) observed the same effect in juveniles of *Cyprinus carpio* exposed to polluted water from the Reconquista River. Dalela *et al.,* (1978) reported that the action of toxicants on Na^+, K^+ - ATPase activity in tissues may disturb the osmoregulatory capacity of fish. Na^+, K^+ - ATPase plays a crucial role in branchial epithelial ion transport. As per Rahman *et al.,* (2000) ATPases are enzymes concerned with immediate release of energy and are responsible for a large part of basic metabolic and physiological activities and ATPase activity can be taken as significant indicator of cellular activity and forms a practical toxicological tool. Yang *et al.,* (2002) reported that inhibition of Na^+, K^+ - ATPase activity may produce unfavorable effects in the organism, which may lead to energy-related perturbations and finally affect basic metabolic and physiological activities.

Karnaky *et al.,* (1986), Perry,(1997); Bonga,(1997) reported that the actual cell involved in these transport steps is unknown, but is generally assumed to be the chloride cell, despite its usually reduced population in the freshwater species. Moreover the energizing step for these antiports is also presently unknown, but the chloride cells does contain substantial concentrations of Na^+, K^+ activated ATPase even in fresh water species.

Verma *et al.,* (1983) studied effects of sub lethal concentrations of mercuric chloride on Na^+, K^+ activated ATPase activity in various tissues including gill of *Notopterus notopterus* and found that Na^+, K^+ activated

ATPase activity was inhibited in most tissues of the fish and the enzyme activity did not return to the normal level except at the lower concentration.

Aaltonen *et al.,* (2000) reported that the activities of gill ATPases have been shown to be inhibited in fish species exposed to mill effluents. Desaiah and Koch (1975) also reported that toxaphene inhibited ATPase activity in tissues of catfish, *Ictalurus punctata* with an identical pattern of inhibition in brain and gill. Hazarika and Sarkar (2001) reported that although the main mode of action of organophosphorus pesticides is AChE inhibition, changes in ATPase activity also mediate the toxicity of organophosphates.

The ATPases are considered to be involved in the maintenance of ionic balance in the gills. The alteration in the activities of these enzymes by paper mill effluent as reported by Ahmad *et al.,* (2000) could cause an ionic imbalance and affect other vital processes associated with gills. The effect of xenobiotics on gill ATPase activity has been variable. For example, Leadem *et al.,* (1974) observed that DDT decreased the activity of Na^+, K^+- and residual ATPase in gills of *Salmo gairdneri* and Dalela *et al.,* (1978) reported that exposure to endosulfan for 30 days decreased ATPase activity of *Channa gachua* gills, whereas Verma *et al.,* (1988) reported on stimulated activities of Na^+, K^+ -ATPase in gill homogenates of *Labeo rohita* exposed to low concentrations of the pesticides aldrin and dieldrin. Haya *et al.,* (1985) reported reduced activity of Na^+, K^+ - ATPase in gills of *Salmo salar* exposed to methylphenol for 96 h.

Effects of organophosphorus insecticides on lysosomal membrane stability

Adverse lysosomal reactions appear to provide useful biomarkers that are diagnostic for cell injury and putative indicators for further pathology (Moore, 1990). Several researchers like Depledge *et al.,* (1993); Depledge (1994,1999); Moore *et al.,* (2004) explained that biomarkers include a

variety of measures of specific molecular, cellular and physiological responses of key species to contaminant exposure.

Exposure to different kinds of contaminants, both metals and organic xenobiotics like insecticides, can result in increased radical generation and the intra lysosomal environment is already a site of oxyradical production. The resulting oxidative damage to membranes, proteins and DNA will undoubtedly contribute to decreased protein synthesis, cell injury and patho-physiological dysfunction (Winston *et al.*, 1991; Moore *et al.*, 1992; Krishnakumar *et al.*, 1994; Winston *et al.*, 1996; Livingstone., 2001)

Lysosomal reactions are involved in normal physiological responses as well as many cell injury and disease processes; these include augmented sequestration and autophagy of organelles and proteins and such reactions have been widely documented for many adaptive and developmental physiological and disease processes; and lysosomal responses have been shown to be involved in generalized reactions to environmental stress (Moore, 1985, 1990, 2002; Cajaraville *et al.*, 1995; Klionsky and Emr., 2000; Kohler *et al.*, 2002)

Moore.,(2002) and Moore *et al.*, (2004) reported that lysosomal perturbations have been broadly used as early indicators of adverse effect to various factors, including pollutant exposure. The functional stability of the lysosomal membrane is a good indicator of lysosomal integrity and has been used extensively to evaluate responses to environmental perturbation in fish and mollusks (Lowe *et al.*, 1995; Hwang *et al.*, 2002; Kohler *et al.*, 2002; Moore, 2002; Allen and Moore, 2004; Moore *et al.*, 2004).

Effects of organophosphorus insecticides on enzymatic and non-enzymatic antioxidants of fish.

The oxidative stress resulting from the production of reactive oxygen species (ROS) has gained considerable interest in the field of ecotoxicology (Kappus, 1987; Lemaire *et al.*, 1996). McCarthy and Shugart (1990) recommended that oxidative stress biomarkers could be employed in environmental monitoring programs. Estimation of lipid peroxidation (LPO) in particular has been found to have high predictive importance as revealed from a credible number of research papers describing its use as a biomarker of effect (Lackner, 1998).

In addition to LPO, other parameters of both antioxidant enzymes and non-enzymatic antioxidants have also been successfully employed in aquatic biomonitoring studies as reported by Hasspieler *et al.*, (1994). As per Pandey *et al.*, (2001), since the typical reaction during oxidative stress is peroxidative damage to unsaturated fatty acids, the oxidative stress response could conveniently be used as biomarkers of effect of oxidative stress induced by chemical pollutants. Many classes of environmental pollutants are known to enhance the intracellular formation of reactive oxygen species. (Winston and DiGiulio, 1991). The role of ROS in alterations of physiology, growth and survival of aquatic organisms has been reported by several authors like Schlenk and Rice., (1998); Baker *et al.*, (1997, 1998).

Khrer, (1993) suggested LPO as one of the molecular mechanisms involved in pesticide induced toxicity. Pesticides may cause oxidative stress leading to the generation of free radicals and alterations in antioxidants or free oxygen radical scavenging enzyme systems (Almeida *et al.*, 1997). The antioxidant enzyme activities, the glutathione redox status, the level of lipid peroxidation product and the specific induction of glutathione S transferase

are most frequently used as biomarkers in toxicological evaluations (Doyotte *et al.*, 1997; Oruc *et al.*, 2004).

Alves *et al.*, (2002) stated that the exposure to pesticides can bring out pro-oxidant conditions that set off adaptive responses such as increases in the activity of the antioxidant enzymes. The superoxide dismutase-Catalase (SOD-CAT) system provides the first defense against oxygen toxicity. Usually a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants (Dimitrova *et al.*, 1994). Apart from three major antioxidant enzymes, SOD, CAT and Glutathione peroxidase (GPx) there are certain other antioxidant enzymes which may be useful biomarkers, like detoxification enzymes especially Glutathione-S-transferase (GST) helps in eliminating reactive compounds by forming their conjugates with glutathione and subsequently eliminating them as mercapturic acid, thereby protecting cells against ROS induced damage (Lackner, 1998; Ariza *et al.*, 1991).

Rao (2006) observed that Total reduced glutathione (GSH) content was depleted in liver, brain and gill tissues of *Oreochromis mossambicus*, following 30 days exposure to monocrotophos, compared to the controls while the GST levels in liver increased significantly during the entire period of experiment. As per the observations of Hasspieler *et al.*, (1994) ; Sies., (1999) and Reed and Beatty., (1980), GSH plays an important role in the detoxification and excretion of electrophiles and xenobiotics, and prevention of cellular oxidative stress.

Vaglio and Landriscina, (1999); Pena-Llopis *et al.*, (2002); Ariza *et al.*, (1993); and Hasspieler *et al.*, (1994) reported that glutathione depletion is a good biomarker of environmental stress and the glutathione dependent enzymes are good markers of oxidative stress in fish. GST-mediated

conjugation may be an important mechanism for detoxifying peroxidised lipid breakdown products, which have a number of adverse biological effects when present in high amounts. Julka *et al.*, (1992) observed that organophosphorus insecticide dichlorvos decreased glutathione levels and inhibited AChE and GPx activities in several tissues of rats. In human poisoning cases, organophosphate insecticide malathion decreased the blood GSH content (Banerjee *et al.*, 1999). Though in moderate oxidative stress conditions, GSH levels can be increased as an adaptive mechanism by means of increased synthesis, Zhang *et al.*, (2004) concluded that a severe oxidative stress can reduce GSH content. Hai *et al.*, (1997) found decreased levels of GSH in carp liver and muscle after 24 h exposure to dichlorvos, an organophosphorus insecticide known to induce oxidative damages. Induction in GST activity on exposure to organophosphorus diazinon is reported by Uner *et al.*, (2007). Elia *et al.*, (2002) reported an elevation in GST specific activity in atrazine treated *L. macrochirus*. Pena-Llopis *et al.*, (2003) monitored similar trends in dichlorvos treated *Anguilla anguilla*. According to Leaver and George, (1998) the increase in GST activity is indicative of the role of this enzyme in protection against the toxicity of xenobiotic-induced lipid peroxidation.

According to Monteiro *et al.*, (2006) a period of 96 h of exposure to an organophosphate methyl parathion was enough to induce significant alterations in antioxidant enzymes such as SOD, CAT, GST and GPx, as well as the GSH content and LPO levels, resulting in oxidative stress. John *et al.*, (2001) suggested that increases in SOD and CAT activities are probably a response towards increased ROS generation in pesticide toxicity. Dimitrova *et al.*, (1994) described a simultaneous induction response in SOD and CAT activities in carp, *Cyprinus carpio*, exposed to zinc.

However, the activity of these enzymes in fish can also diminish after exposure to xenobiotics, as reported by Livingstone (2001).

GPx plays an important role against the lipid peroxidation, since it is mainly involved in the removal of organic and, in a small extent, hydrogen peroxides. As per Oost *et al.*, (2003), GPx is considered to play an especially important role in protecting membranes from damage due to LPO. While literature observes that environmental pollutants like pesticides could increase the activity of GPx (Almeida *et al.*, 2002; Sayeed *et al.*, 2003; Zhang *et al.*,2004), there are reports showing fall in GPx activity on exposure to toxicants (Fatima *et al.*, 2000; Monteiro *et al.*, 2006). Yarson et al., (1999) observed that organophosphorus insecticide Malathion reduced GPx activity in mice erythrocytes. GPx inhibition was also reported by Oruc *et al.*, (2004) and Oruc and Uner, (2000) in the brain of carp *C. Carpio* and in the liver of *O. niloticus*, after combined treatment with the pesticides 2, 4-D and azinphosmethyl. Tabatabaie and Floyd., (1994) stated that enzyme activity can be decreased by negative feedback from excess of substrate or damage by oxidative modification. According to Remacle *et al.*, (1992), a reduced GPx activity could signify that its antioxidant faculty was outdone by the amount of hydroperoxide products of lipid peroxidation.

Lipid peroxidation (LPO) has been reported as a major contributor to the loss of cell function under oxidative stress conditions (Storey, 1996). LPO is one of the most extensively investigated processes in tissue injury induced by free radicals. Pandey *et al.*, (2003) investigated various biomarkers of oxidative stress, together with lipid peroxidation in gill, kidney and liver tissues of Indian freshwater fish *Wallago attu* and provided interesting results for the rational use of oxidative stress biomarkers in aquatic ecosystems for pollution monitoring. Hazarika *et al.*, (2003)

reported that organophosphates may enhance lipid peroxidation by direct interaction with the cell membrane. Conversely, Yang *et al.* (1996) and Yang and Dettbarn (1996) studied the effects of the diisopropylphosphofluoridate in fish and suggested that cholinergic hyperactivity induced by the inhibition of the AChE initiates the accumulation of ROS, leading to lipid peroxidation, which may cause cell injuries. Ploch *et al.*, (1999); Ahmad *et al.*, (2000); Oakes and Kraak., (2003); Oakes *et al.*, (2004) reported that LPO may be induced by a variety of environmental pollutants. Similar investigations were conducted to measure the adverse oxidative effects of organophosphate pesticides in carp and catfish (Hai *et al.*, 1997). Uner *et al.*, (2007) reported on the alterations in glutathione-related processes and lipid peroxidation in the liver of diazinon exposed *Oreochromis niloticus*.

Histopathological effects of organophosphorus insecticides in fish

The reliability of histological biomarkers as a powerful tool to detect and characterize the biological end points of toxicant and carcinogen exposure were explained by Hinton and Lauren, (1990); Wester and Canton, (1991); Hinton *et al.*, (1992); Moore and Simpson.,(1992). The efficacy of histological lesions as sensitive and consistent indicators of the health of wild fish populations has been demonstrated in several European and North American studies (Kohler, 1991; Kohler *et al.*, 1992; Bucke and Feist, 1993; Vethaak and Wester, 1996; Bogovski *et al.*, 1999).

Several laboratory and filed studies by Varanasi *et al.*, (1987); Stein *et al.*,(1990); Stein *et al.*, (1992); Moore and Myers (1994); Vethaak and Jol (1996) and Vethaak *et al.*,(1996), have also demonstrated contributory links between exposure to xenobiotics and the development of toxicopathic hepatic lesions. Histological changes associated with pesticides in fish have

been studied by Narayan and Singh, (1991). Karan *et al.,* (1998) observed lesions such as epithelial hyperplasia and curling of secondary lamellae on the gills, hepatic swelling, thrombosis at the tips of several secondary lamellae, and club-shaped secondary lamellae.

Vethaak and Rheinallt (1992) have reviewed the epidemiological studies on the occurrence of fish disease in relation to their usefulness in monitoring marine pollution. Heuvel *et al.,* (2000), observed mucous cell proliferation of yellow perch from oil sands reclaimed environments following longer residency periods, suggesting that this type of response may be a long-term adaptation.

Fanta *et al.,* (1995); Mallat, (1985) and Novak, (1992); have reported that the most common effect of any pollutant or water quality change in secondary lamellae is the lifting of it which leads to large increase in the diffusion distance and thereby to less oxygen consumption. Erkmen *et al.,* (2000) reported the lifting of epithelial layer from gill lamellae, necrosis and degeneration of secondary lamellae, shortening of secondary lamellae, and club shaped lamellae in the gills of *Lepistes reticulatus* exposed to cyphenothrin. Epithelial necrosis, fusion of secondary lamellae and lifting of epithelium has also been showed in many fish species (Cengiz and Unlu, 2002, 2003). Ortiz *et al.,* (2003) reported the fusion of secondary lamellae, increased rising of the branchial epithelium and intraepithelial oedema in gills, after an accidental discharge of lindane. Stentiford *et al.,* (2002) reported that the gill epithelium is a major route for the uptake of soluble xenobiotics by fish and he observed the highest prevalence of cellular hypertrophy, hyperplasia and fusion of the secondary lamellae in flounder caught from the polluted estuaries.

Ray and Bhattacharya, (1984); Bhattacharya *et al.*, (1985); and Banerjee and Bhattacharya, (1997) reported on the effect of pesticides in teleost liver. Narayan and Singh (1991) observed extensive degeneration of cytoplasm with pycnosis of nuclei and loss of glycogen in liver tissue of *H. fossils* subjected to acute thiodan toxicity.

Histopathological alterations after the exposure to organophosphorus insecticides in various fish species are described by several authors. Dutta *et al.*, (1993) described the gill alterations such as lifting of the epithelial layer, hyperplasia and necrosis, shortening of the lamellae and frequent epithelial rupture, lamellar fusion and mucous cells hypertrophy in bluegill sunfish, *Lepomis macrochirus.*

The blood–brain barrier (BBB) selectively limits the rates of transfer of soluble substances between blood and brain, isolating the brain from other tissues (Magistretti, 1999). Butt *et al.*, (1990) and Kniesel *et al.*, (1996) reported that brain is very susceptible to environmental stressors including OP pesticides, which being lipophilic may cause neurotoxicity due to their ability to cross the BBB and exert toxic effects on the central nervous system. Although there are differences in fish and mammal BBB (Bernstein and Streicher, 1965), recent study by Miller *et al.*, (2002) showed the existence of P-glycoprotein and Mrp2 transporters in the brain capillary endothelial cells in fish *Fundulus heteroclitus*, which transports the xenobiotics from the CNS to blood as in Mammals.

Bioconcentration of pesticides by fish

In order to elucidate the aquatic behavior of environmental contaminants and to assess exposure of aquatic organisms, fish bioaccumulation markers may be helpful (Oost *et al.*, 2003). The capability of fish to transform pesticides has been reported by Baker *et al.*, (1963);

Creanen *et al.,* (1965); Ludke *et al.,* (1972), who have established the fact that fishes have multi function oxygenase system. Many laboratory studies have been carried out by researchers like Shannon *et al.,* (1972); Muir and Grift, (1981); Schlenk *et al.*,(1992); Barron *et al.*,(1993); Tsuda *et al.*,(1994); and Sancho *et al.*,(1998) on the bioaccumulation of pesticides in fish tissues. Bioconcentration factor gives an idea of the relative uptake of the substance from its medium by the organisms.

Bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystems and when uptake rates are significantly higher than metabolic clearance rates bioaccumulation can still occur even though the substance is readily biodegradable. Pollutant concentrations in tissues and differences in excretion of metabolites can be a function of tissues and conditions controlling the activity of biotransformation enzymes. Bioconcentration of hazardous substances like organophosphorus insecticides causes serious ecological problems when the degree of partitioning of a substance or its transformation products results in translocation to, and storage in critical tissues of organisms (Ramaneswari and Rao, 2000).

Gray and Knowles (1980) reported that organophosphate and carbamate compounds due to their higher water solubility, in general are taken up to a lesser level than organochlorines and are eliminated rapidly. Ramaneswari and Rao (2000) investigated the bioconcentration of monocrotophos in *Labeo rohita* and *Channa Punctata* and observed the bioconcentration of monocrotophos in the form of o-des methyl monocrotophos and hydroxy monocrotophos. However there is a dearth of literature on the bioconcentration studies of monocrotophos in the stinging catfish *H. fossilis.*

Studies conducted by researchers world wide are cited in the review and most of them are concerned about the toxicity of organophosphorus insecticides in aquatic organisms. These studies are pointing to the fact that presence of sub lethal concentrations of monocrotophos and other OPs in the aquatic system, either by land run off, leaching or direct addition of them into hydrosphere can bring about grave deleterious effects in physiological and metabolic functions of the organisms. Such effects challenge their ability to survive in a naturally competitive environment leading to depletion in population as well as reduction in their quality as food for consumption by human. However a dearth of literature on the toxicity of monocrotophos in fish, particularly *H. fossilis* could be observed. *H. fossilis* are of high nutritional and palatability values, fetching fairly high price in market and currently well-known as a promising entrant for aquaculture. It will be of significance to understand the responses of *H. fossilis* to the toxicity of monocrotophos, a widely used organophosphorus insecticide in agriculture especially of paddy, cotton and sugar cane.

Research conducted is crystallized as six chapters with following objectives:

1. To study the effects of monocrotophos (MCP) on some of the important energy and metabolic parameters of *H. fossilis*
2. To evaluate the effects of MCP on hematological parameters of *H. fossilis*
3. To study the responses of membrane bound enzymes to monocrotophos induced stress. This chapter includes three sub chapters as given below.

3A. To investigate the neurotoxic potential of MCP by assessing the Acetylcholine esterase enzyme activity.

3B. To evaluate the responses of Branchial ATPases to MCP induced stress.

3C. To study the effect of MCP on lysosomal membrane enzymes thereby assessing membrane stability.

4. To assess the oxidative stress induced by organophosphorus insecticide Monocrotophos (MCP)

5. To examine the pathological changes in liver, brain and gill tissues of *H. fossilis* in response to MCP.

6. To investigate the bioconcentration of monocrotophos residue in the edible part (muscle) of *Heteropneustes fossilis* (Bloch)

Chapter **1**

EFFECT OF MONOCROTOPHOS ON THE ENERGY AND METABOLIC PARAMETERS

Contents

1.1 Introduction

The responses of fish to environmental challenges are in due course reflected as overall alteration in metabolism. The responses in the beginning are reversible, but long-drawn-out exposure to environmental pollutants brings about enduring pathological changes in the physiology of fish. These alterations are echoed as loss of survival skills, inhibition of growth (George and Nagel, 1990; Alvarez and Fuiman, 2005), reproductive dysfunction (Tyler *et al.*, 1998), and immune suppression (Fatima *et al.*, 2000, 2001). There is widespread scientific consensus that herbicides/insecticides can interfere with the metabolism of fish (Sonnenschein and Soto, 1998). In this chapter an attempt has been made to study the effect of Monocrotophos on some of the enzymatic and non-enzymatic energy and metabolic parameters of stinging catfish *Heteropneustes fossilis.*

Carbohydrates are the primary and immediate sources of energy. When the need arises for energy, glycogen is converted to glucose in the serum. Blood glucose concentration appears to be a sensitive and reliable indicator of stress in fish. Rise in glucose levels in blood and tissues can be used to indicate the toxicity of pollutants in the ambient environment (Elizoic *et al.*, 1987).

Proteins are mainly involved in the architecture of the cell. During chronic period of stress they are also a source of energy (Umminger, 1977). During stress condition, fish needs more energy to detoxify the toxicants and to overcome stress. Since fish have a very little amount of carbohydrates, the next alternative source of energy is protein.

Aspartate aminotransferases (AAT) and Alanine aminotransferases (ALAT) are functioning as link between carbohydrate and protein

metabolism. It has wide distribution in both mammalian and fish tissues (Gaudet *et al.*, 1975, Eze, 1983) existing in two forms viz. M (mitochondrial) and S (cytoplasmic). ALAT catalyzes the conversion of Alanine to pyruvate by the transfer of the amino group to α-ketoglutarate there by converting it to glutamate.

alanine + α-ketoglutarate → pyruvate + glutamate

AAT catalyzes the conversion of aspartate to oxaloacetate and the transfer of amino group to ketoglutarate gives glutamate.

aspartate + α-ketoglutarate → oxaloacetate + glutamate.

Transaminases are intracellular enzymes which exist only in small amounts in serum. When an organ or body tissue is injured, increased levels of AAT and ALAT is released into the blood. The enzyme may leak into the plasma following the damage or dysfunction of reservoir tissue. Hence, the assay of transaminases has become an indispensable tool in the clinical determination of the pathological conditions of the reservoir tissues and organs (La Due *et al.*, 1954). The greater the extent of tissue damage, the greater the quantity of AAT that is released. Therefore alanine and aspartate transaminases activities may be used as sensitive markers in experimental insecticide intoxication in teleost fish (Beyer *et al.*, 1996). Liver is richly endowed with ALAT and AAT and alterations in the plasma level of these enzymes pinpoint liver dysfunction .

Alkaline and acid phosphatases are intrinsic plasma membrane enzymes found in almost all animals, plants and microbes. They catalyses the hydrolysis of various phosphate containing compounds and acts as transphosphorylases at acid and alkaline pHs. Alkaline phosphatase and acid phosphatase have activity optima in alkaline and acidic pHs

respectively. Acid phosphatase is involved in a variety of metabolic processes such as membrane permeability, steroidgenesis, growth and cell differentiation. Alkaline phosphatase is involved in membrane transport and is a good marker of stress, in living systems.

Lactate Dehydrogenase (LDH) is a zinc containing enzyme, which mediates the inter conversion of lactate and pyruvate, depending on the availability of NAD.

(S) – Lactate + NAD^+ ⟷pyruvate + NADH

Lactate dehydrogenase (LDH) forms the center for a precisely balanced symmetry between catabolism and anabolism of carbohydrates (Everse and Kaplan., 1973). The rise in LDH level is the result of cell damage. Enormously high LDH content in the serum can be due to tissue damage, necrosis or trauma (Altman, 1974). A fish under stress preferentially meets its energy requirements through anaerobic condition. LDH can be used as a good indicator in monitoring organophosphorus induced toxicity in fish.

This study was undertaken with a view to using changes in the activity of energy and metabolic parameters in the fish, *H. fossilis,* as a biodiagnostic tool in aquatic ichthyotoxicological work.

1.2 Materials and methods

1.2.1 *Heteropneustes fossilis* (Bloch)

Class – *Teleostomii*

Order – *Cypriniformes*

Family – *Heteropneustidae*

Genus – *Heteropneustes*

Species - *H. fossilis (Bloch)*

Common name - Stinging catfish

Synonyms: *Silurus fossilis* (Bloch 1794), *Saccobranchus singio* (Hamilton) *Saccobranchus fossilis* (Gunther 1864)

Stinging catfish is reported to be highly nutritive, recuperative, and to be possessing medicinal properties. This fish is esteemed for its invigorating qualities (Alikunhi, 1957).Its flesh is rich in protein and iron, and fat content is lower in comparison to that in other fishes.

Heteropneustidae have an elongated, sub cylindrical body; just beyond the ventral fin bases, the body tapers off to the tail. The head is depressed and bony plates cover the top and sides. The snout is depressed, and the nostrils are far apart; the slit-like anterior nostrils are positioned behind the nasal barbules. There are four pairs of rather long barbels, the maxillary pair extending as far as the ventral fins, and the two pairs of mandibular barbels and the nasal barbules reaching the end of the pectoral fins. The mouth is small and terminal. The eyes are relatively small, lateral in position, and with a free orbital rim. The gill openings are wide and the gill membranes are free from the isthmus. There are seven branchiostegal rays. The gas bladder is enclosed in a bony capsule (Burgess, 1989).

The fish is heterosexual. Externally, sexes can be accurately distinguished only during breeding when secondary sexual characteristics become prominent. The best morphological disposition of a good *H. fossilis* brood female is a well-rounded abdomen, the fullness of which widens posteriorly past the pelvic fins. The males, on the other hand, look lean. In a mature female, the genital papilla remains in the form of a raised prominent formation, blunt with a slit-like opening in the middle. Sexual maturity is attained at the end of the first year of life.

During summer when water bodies become dried up, the fish buries itself in the soil to aestivate. A number of fishes live together in an array of pits in large ponds. *H. fossilis* also makes nests in the embankment of ponds and colonizes there. These holes are generally about a foot underneath the water surface in the form of anastomosing tubes having numerous exits. *H. fossilis* is well suited for culture in shallow ponds to 1 m depth. In addition to its aptness for culture in derelict waters, it can also be cultured in conventional ponds for high production. To reach marketable size, a period of six months is usually quite adequate.

H. fossilis is best suited for monoculture but can be cultured in combination with *Clarias batrachus* or *Anabas testudineus*. This fish is popular mainly because it can be cultivated in swampy areas and derelict water bodies without involving expensive reclamation. More fascinatingly, this species is perfect for wastewater aquaculture (Tharakan and Joy, 1996). The maximum recorded size of stinging catfish is 38 cm (Sinha, 1993). Unlike water-breathing fish, air-breathing fish can be easily stored and transported live to consumers. Optimum pH and temperature range for *H. fossilis* are 6.0 to 8.0 and 21 to 25 °C.

1.2.2 Maintenance of Fish

Heteropneustes fossilis (Stinging catfish) of 25.7 ± 3 g were collected from fishermen of Alapuzha district of Kerala and they were acclimatized in the stock tank of 4000 liter capacity containing fresh water for a period of one month. Green algae were allowed to grow in the tank in thick mats to provide natural feeling. Earthen pots were set in the bottom of tank to be used by the fishes as hide outs, as the fish are shade loving ones. They were fed on a commercial fish feed (Higashi, India) two times a day and fresh clam meat thrice a week. The fresh water used had a pH of 7.0 ± 0.45,

temperature of 26 ± 3° C, dissolved oxygen content of 7.8 ppm and a salinity of zero ppt.

1.2.3 Toxicant

Toxicant selected for the study was commercial grade formulation of organophosphorus insecticide Azodrin, manufactured by United phosphorus Ltd, which has 53% W/W monocrotophos technical as active ingredient. Calculated quantity was weighed out to give the desired concentrations in the test medium.

1.2.4 Bioassay method

The bioassay methods adopted in the present study were same as that of Doudoroff *et al.,* (1953). Experiments were carried out in semi static renewal system based on APHA (2005). The lethal toxicity studies provide information about the relative lethality of a toxicant. This test is designed to determine the highest concentration of a pollutant that is sufficient to affect usually 50% of a limited number of organisms. Though the LC50 appears to be a crude method its importance is highlighted by many workers (Duke 1974; Buikema Jr *et al.,* 1982). The acute LC50 value of monocrotophos was determined in the laboratory using the semi-static method (UNEP/FAO/IAEA, 1989) and was found to be 20 ppm, based on the probit analysis (Finney, 1957). For LC50 determination five concentrations of monocrotophos were selected. For each concentration 12 fishes were used. Each experiment was repeated six times at selected concentrations of monocrotophos every time observing the number of fish killed at each concentration up to 96 hours. Experimental tanks were well aerated to prevent hypoxic conditions. The LC50 value was calculated using the software SPSS. Three sublethal concentrations 2 ppm, 4 pm and 6.6 ppm were chosen for further investigations, based on the LC50 value.

For conducting further studies 40 numbers of *H. fossilis* of 25.7 ± 3 g were taken in four fiber reinforced plastic tanks which included the control as well as test (2 ppm, 4 ppm, 6.6 ppm as sublethal concentrations) group of fishes. Duplicates were kept for each experiment. The experimental organisms were exposed to monocrotophos for 21 days. Sampling was done on every 7th and 21st days. Contents of the tanks were replaced daily with same quantity of water and toxicant. During the experimental period of 21 days the animals were fed on the same diet so as to avoid any effect of malnourishment on normal physiological functions and antioxidant status of fish.

1.2.5 Preparation of tissue and serum samples for biochemical studies

Every 7th and 21st day, blood was drawn out by caudal vein puncture in 1 ml plastic syringe and the serum was separated from blood cells by centrifugation at 3000 rpm for 20 minutes and it was used for different biochemical assays. Fish were killed by cervical disjunction and tissues viz. liver, gill and brain were excised, washed with physiological saline and wiped well using blotting paper to remove blood and body fluids. Then they were washed in 0.33 M ice cold sucrose and again blot dried. Desired amount of tissues were weighed and were taken for various assays. Tissues were homogenized using Potter–Elvehjam homogenizer. The homogenates were centrifuged at 5000 g for 10min and the supernatant was further centrifuged at 5000 g for 10 min. The resultant supernatant was used as the enzyme source for the estimation of all the enzyme activities. Protein was estimated by the method of Lowry *et al.*, (1951).

1.2.6 Assays of enzymatic and non-enzymatic energy and metabolic parameters

1.2.6.1 *Estimation of Total carbohydrate* (TCH)

The total carbohydrate was estimated by the method of Carrol *et al.*, (1956)

Reagents

10% trichloro acetic acid (TCA), anthrone reagent.

Procedure

Liver, brain and gill homogenates were prepared in 10% TCA and were centrifuged at 1000 g for 15 minutes. To 0.1 ml of the supernatant, 3 ml of anthrone reagent was added and boiled for 15 minutes. The test tubes were cooled and the absorbance was read spectrophotometrically at 620 nm against a blank that contained 10% TCA and anthrone reagent alone. The values were expressed as mg glucose/g wet wt. of tissue.

1.2.6.2 *Estimation of Glycogen*

Tissue glycogen content was determined as per the method of Carrol *et al.*, (1956)

Reagents

5% trichloro acetic acid (TCA), 95% ethanol, standard glucose, anthrone reagent.

Procedure

0.5% homogenate of liver, brain and gill tissues were prepared in 5% TCA separately and centrifuged at 1000g for 15 minutes. To one volume of the above supernatant, 5 volumes of 95% ethanol was added and allowed to stand overnight in cold. After the precipitation was complete, the tubes were again centrifuged at 1000g for 15 minutes. The supernatants were

decanted and the residual fluid was allowed to drain off by keeping the tubes inverted for about 10 minutes. The residue was dissolved in 1 ml of distilled water. A reagent blank (1 ml of water) and a standard (1ml of glucose solution containing 30 µg of glucose) were also prepared. 5 ml of anthrone reagent was added to each of the above tubes including blank and standard tubes. The tubes were kept for boiling for 15 minutes and then cooled to room temperature. The absorbance was read at 620 nm in a spectrophotometer. The values were expressed as mg/g wet wt. of tissue.

1.2.6.3 *Estimation of Total protein*

Protein content of the tissues was estimated following the method of Lowry *et al.*, (1951)

Reagents

0.1 N NaOH, Alkaline copper reagent, Folin-Ciocalteau reagent, standard protein solution (Bovine Serum Albumin).

Procedure

The homogenates of liver, brain and gill tissues were prepared in 0.33 M ice cold sucrose solution and centrifuged at 1000 g for 15 minutes. To the supernatant obtained, equal volume of 10% TCA was added for precipitating proteins. Contents were set to stand for 30 minutes at room temperature and were centrifuged at 1000 g for another 15 minutes. The sediment was dissolved in 1 ml of 0.1 N NaOH. After appropriate dilution a known volume of the solution was mixed with 4 ml of alkaline copper reagent and was shaken to blend well. The mixture was allowed to stand for 10 minutes. 0.5 ml of folin-ciocalteau reagent was added and the tubes were kept for another 30 minutes. The absorbance was measured at 500 nm in a spectrometer against a reagent blank devoid of sample. Bovine Serum

Albumin (BSA) was used as the standard. Values were expressed as mg/g wet wt. of tissue.

1.2.6.4 *Estimation of Total lipid*

Total lipid content of the liver, brain and gill tissues were determined according to the method of Frings and Dun (1970).

Reagents

Concentrated sulphuric acid, vanillin-0.6%, standard olive oil: 1g % in C_2H_5OH.

Phosphovanillin reagent: 0.6% vanillin dissolved in 100 ml O-phosphoric acid.

Procedure

Accurately weighed samples of liver, brain and gill were homogenized in 10 ml chloroform-methanol mixture (2:1, v/v) and filtered. To the filtrate 2 ml of sodium chloride was added, mixed well and kept it overnight at 4 °C for lipid extraction. Lower phase containing lipid was taken and made up to 10 ml. Definite aliquot of this was dried in vacuum desiccator overnight. To the tubes 0.5 ml concentrated sulphuric acid was added and was kept in boiling water bath for 10 minutes. 0.2 ml of this acid digest was taken and 5 ml of phosphovanillin reagent was added and incubated for 15 minutes. Absorbance was read at 520 nm. The values were expressed as mg/100 gm wet wt. of tissue.

1.2.6.5 *Estimation of tissue and serum alanine aminotransferase (ALAT) activity*

The activity of ALAT was assayed according to the method of Mohun and Cook (1957).

Reagents

Buffered substrate solution: 0.1 M phosphate buffer, pH 7.4; 0.2 M DL-alanine, 0.03g α-ketoglutaric acid.

2, 4-dinitrophenyl hydrazine (DNPH) reagent: 200 mg of DNPH in 85 ml of concentrated hydrochloric acid and made up to 1 L with distilled water.

0.4 N sodium hydroxide

Standard pyruvate: 11.01 mg sodium pyruvate dissolved in 10 ml of distilled water, diluted further to 100 ml with distilled water (Freshly prepared each time).

Procedure

To 1 ml of the buffered substrate, 50µl of the tissue homogenate/serum were added and incubated at 37° C for 30 minutes. The reaction was arrested by adding 1 ml of DNPH and allowed to stand at room temperature for 20 minutes. 10 ml of 0.4 N NaOH was added and the absorbance was read at 540 nm in a spectrophotometer against the reagent blank. The enzyme activity was expressed as µ moles of pyruvate liberated/h/mg protein.

1.2.6.6 *Estimation of tissue and serum aspartate aminotransferase activity (AAT)*

The activity of AAT was assayed according to the method of Mohun and Cook (1957).

Reagents

0.1M phosphate buffer (pH 7.5), 2, 4-dinitrophenyl hydrazine reagent, 0.4 N sodium hydroxide, standard pyruvate solution.

Substrate: Dissolve 300 mg L-aspartic acid and 50mg of α-ketoglutaric acid in 20 ml of the phosphate buffer and add 10% sodium hydroxide to bring the pH to 7.5. Made up to 100 ml with phosphate buffer.

Procedure

To 1 ml of the buffered substrate, 50µl of the tissue homogenate/serum was added and incubated at 37° C for 1 hour. The reaction was arrested by adding 1 ml of DNPH and was allowed to stand at room temperature for 20 minutes. 10 ml of 0.4 N NaOH was added and kept for 10 minutes. The absorbance was read at 540 nm in a spectrophotometer against the reagent blank. The enzyme activity was expressed as µ moles of pyruvate liberated/h/mg protein.

1.2.6.7 *Estimation of tissue and serum acid phosphatase activity (ACP)*

Both serum and tissue phosphatase activity was determined following the method introduced by Anon, 1963.

Reagents

P-nitro phenyl phosphate, 0.1 N NaOH, 0.1 M phosphate buffer.

Procedure

Liver, brain and gill of both control and test fishes were homogenized in isotonic sucrose and were centrifuged at 5000 rpm for 15 minutes. Supernatant obtained was the source of enzymes. 0.5 ml of p-nitrophenyl phosphate was mixed with equal volume of 0.1 M phosphate buffer (pH 4.8). The enzyme was added and incubated for 30 minutes at room temperature. The reaction was arrested by adding 4 ml of 0.1 N NaOH. The absorbance of solution was measured spectrophotometrically at 410 nm. The amount of p-nitro phenol liberated by the acid phosphatase per hour per mg protein gives the specific activity. Protein was determined as per the method of Lowry *et al* (1951).

1.2.6.8 *Tissue and serum alkaline phosphatase (ALP) activity*

Both serum and tissue phosphatase activity was determined following the method introduced by King (1965).

Reagents

0.1 M carbonate-bicarbonate buffer, pH 10.0

Substrate: 0.01 M disodium phenyl phosphate solution in water

Folin's Ciocaltau reagent in distilled water at a ratio 1:10

15% sodium carbonate in water

0.1 M magnesium chloride in water

Standard phenol solution: a solution of distilled crystalline phenol in water, containing 5.0 microgram in 0.1 ml is prepared.

Procedure

1.5 ml of carbonate-bicarbonate buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride and requisite amount of the enzyme source were mixed together. The reaction mixture was incubated at 37° C for 15 minutes. The reaction was terminated by adding 1.0 ml of folin's phenol reagent. Controls were incubated with out adding enzyme source and enzyme source were added after the addition of folin's phenol reagent. 1 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37° C. The blue color developed was read at 640 nm against a blank. Standards also were treated similarly. The enzyme activity was expressed as micromoles of phenol liberated/ mg protein/ hr.

1.2.6.9 *Estimation of tissue lactate dehydrogenase (LDH) activity*

Lactate dehydrogenase activity was measured according to the method of Bergmeyer and Bernt, 1974.

Reagents

50 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, 0.18 mM NADH.

Procedure

Liver, brain and gill tissues were homogenized in phosphate buffer (pH 7.5).The homogenate was centrifuged at 20000 g for 30 minutes in a refrigerated centrifuge at 0°C. The supernatant obtained was used as the source of enzyme. The reaction mixture contained 50mM phosphate buffer, 0.6 mM pyruvate, 0.18 mM NADH and the enzyme source. The reaction was set on by adding enzyme and the assay was carried out at 30 °C. The activity was determined from the rate of oxidation of NADH. The change in absorbance was measured in a spectrophotometer. A standard graph of NADH was prepared and the enzyme activity was expressed as mg of NADH/hour/gm protein. Protein content of the samples was determined as per the method of Lowry *et al.*,(1951).

1.3 Statistical analysis

Statistical analysis was performed using three factor ANOVA, followed by pair wise comparison using LSD analysis using statistical software SPSS-16.

1.4 Result

Most of the parameters pertaining to energy and metabolic status of the fish were at peril for the monocrotophos treated group. Total carbohydrate content of the fish was significantly decreased, both concentration and days wise, with liver as the most affected tissue. There was a significant depletion of glycogen content in all tissues exposed to MCP, with liver recording the maximum decrement. Total protein content of the liver, brain and gill of the monocrotophos treated fish were significantly decreased at both 7^{th} and 21^{st} day intervals.

Activities of enzymes alanine transaminase and aspartate transaminase were significantly altered in different concentrations of monocrotophos treatment. Activities were decreased in liver at all concentrations and time intervals, while brain and gill showed an increasing trend. Serum AAT and ALAT values were significantly increased for both time periods and all the three concentrations of monocrotophos. Acid and alkaline phosphatase activities also were decreased in liver at all concentrations and time intervals, while brain and gill showed an increasing trend. Serum ACP and ALP showed an increasing trend. Lactate dehydrogenase activity was greatly increased in all tissues of monocrotophos treated fish at both 7^{th} and 21^{st} days observation.

Table 1.1 Total carbohydrates in liver, brain and gill tissues of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	**Control**	**7th day**	**21st day**	**7th day**	**21st day**	**7th day**	**21st day**
Liver	49.95 ± 1.59	46.16 ± 1.32	45.25 ± 1.20	41.45 ± 1.42	41.39 ± 0.77	36.97 ± 0.78	34.99 ± 1.63
Brain	24.76 ± 0.47	23.13 ± 0.42	21.67 ± 0.52	18.94 ± 0.64	17.66 ± 0.20	15.41 ± 0.18	14.28 ± 0.31
Gill	9.48 ± 0.24	7.97 ± 0.20	7.73 ± 0.05	6.47 ± 0.19	5.92 ± 0.13	3.96 ± 0.09	2.01 ± 0.04

- Values are expressed as mg/gm wet wt of tissue
- Average of six values in each group

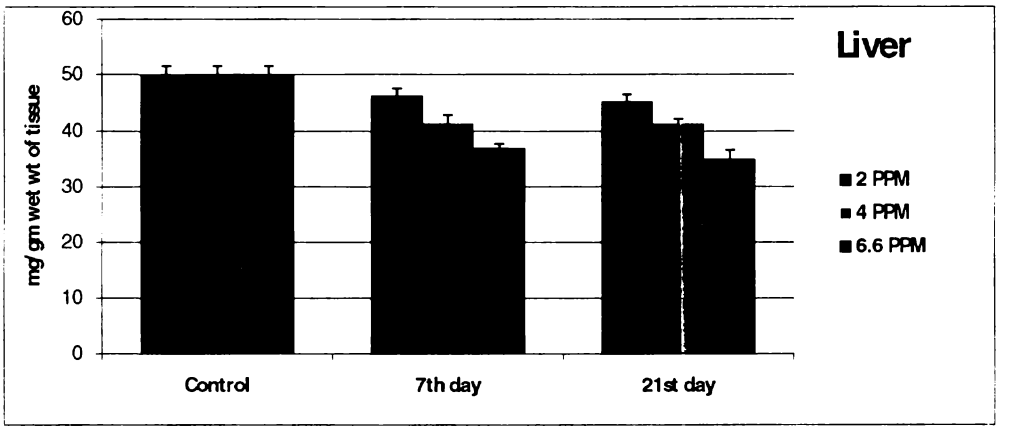


Fig.1.1 Total carbohydrate content in the liver of *H. fossilis* exposed to monocrotophos

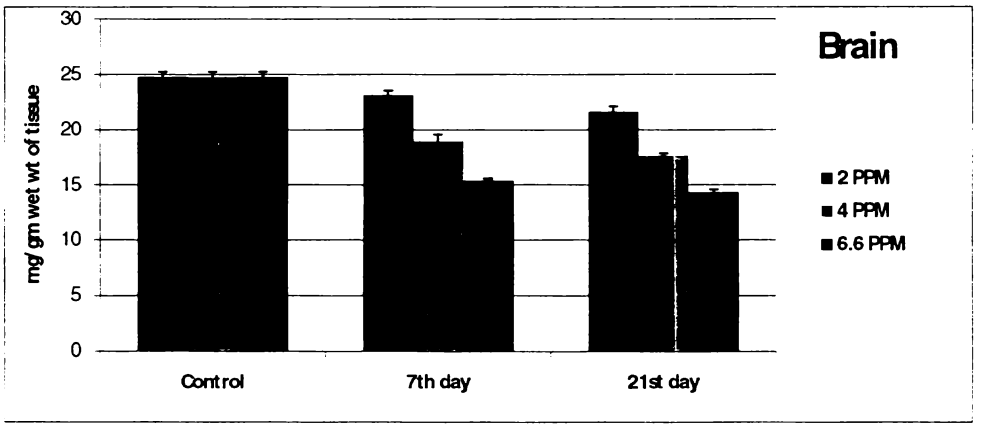


Fig.1.2 Total carbohydrate content in the brain of *H. fossilis* exposed to monocrotophos

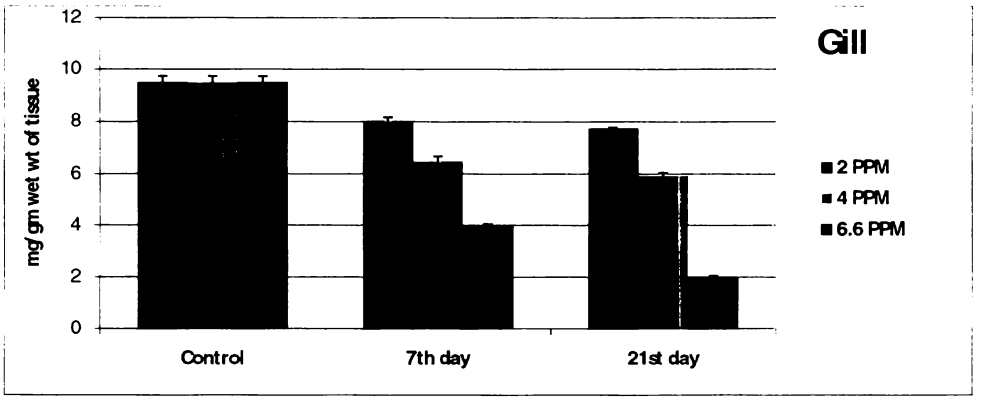


Fig.1.3 Total carbohydrate content in the gill of *H. fossilis* exposed to monocrotophos

Table 1.2 ANOVA table for total carbohydrate content in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	33209.340	2	16604.670	7797.861	0.000
Day	2077.578	3	692.526	325.223	0.000
Concentration	22.108	1	22.108	10.382	0.002
Error	293.855	137	2.129		
Total	35805.604	143			

In the case of total carbohydrate, it can be observed that the p-values are less than 0.05 and hence it can be concluded that the values vary significantly with tissue, concentration and day. Three factor ANOVA revealed an overall significant decrease in total carbohydrate content at different concentration of monocrotophos group compared to control group. 6.6 ppm concentration of the monocrotophos treatment gave significantly higher values when compared to other concentrations. Here liver recorded a high value compared to gill and brain. Brain recorded the least value compared with other under the same conditions. Subsequent comparison by LSD analysis (tables 1.19a, 1.19b) revealed a significant difference ($P < 0.05$) between tissues.

Table 1.3 Glycogen content in liver, brain and gill tissues of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	**Control**	**7th day**	**21st day**	**7th day**	**21st day**	**7th day**	**21st day**
Liver	28.5801 ± 1.003	18.4026 ± 0.704	15.9360 ± 0.632	15.1651 ± 0.397	13.8760 ± 0.156	8.0338 ± 0.1784	6.1062 ± 0.3367
Brain	0.5989 ± 0.0261	0.5037 ± 0.0159	0.3079 ± 0.0097	0.2377 ± 0.0058	0.2013 ± 0.0032	0.1591 ± 0.0034	0.1003 ± 0.0023
Gill	1.5436 ± 0.0637	1.1475 ± 0.0257	0.9030 ± 0.0333	0.8978 ± 0.0276	0.7821 ± 0.0300	0.5623 ± 0.0136	0.2764 ± 0.0105

- Values are expressed as mg/gm wet wt of tissue
- Average of six values in each group

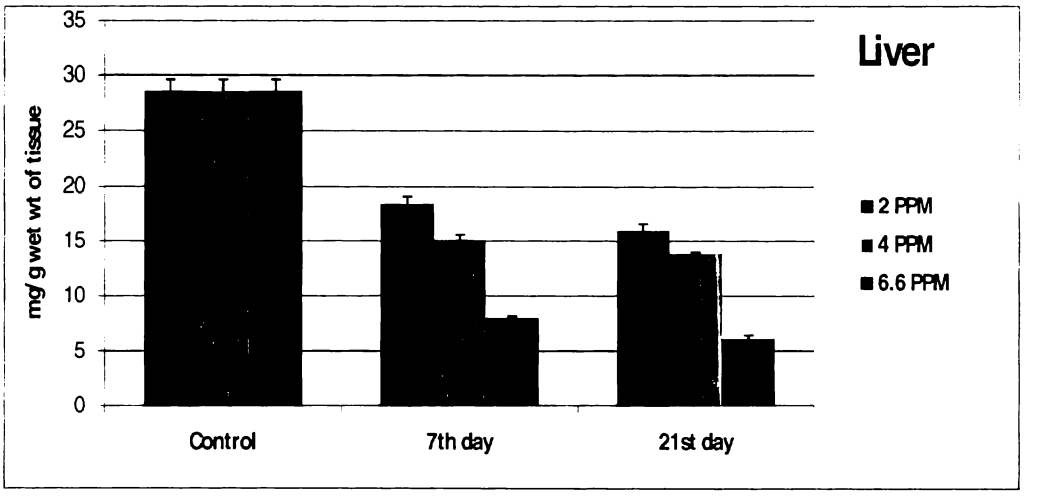


Fig.1.4 Glycogen content in the liver of *H. fossilis* subjected to monocrotophos

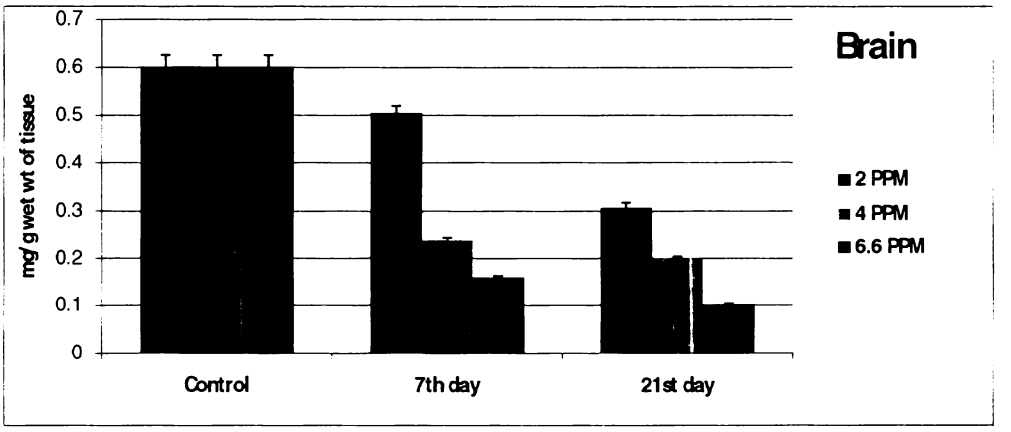


Fig.1.5 Glycogen content in the brain of *H. fossilis* subjected to monocrotophos

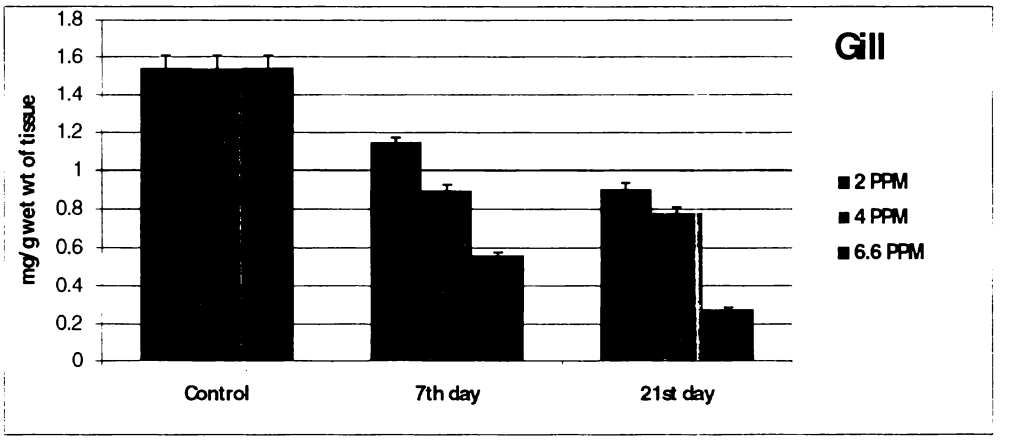


Fig.1.6 Glycogen content in the gill of *H. fossilis* subjected to monocrotophos

Table 1.4 ANOVA Table for glycogen content in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	8394.023	2	4197.012	317.360	0.000
Concentration	1103.108	3	367.703	27.804	0.000
Day	10.958	1	10.958	.829	0. 364
Error	1811.795	137	13.225		
Total	11319.885	143			

The results of the Analysis of Variance procedure comparing the effect of the factors tissue, concentration and day of glycogen data are summarized on table 1.4. Here since the p-value corresponding to the day is greater than 0.05 it can be concluded that there is no significant difference in values between the days. Also it is observed that the values vary significantly with different types of tissues and different levels of concentrations.

All concentration, 2 ppm, 4 ppm and 6.6 ppm give significant difference from the control value as per further comparison by LSD analysis. In the case of pair wise comparison between different concentration groups, there is no significant difference between 2ppm and 4 ppm while all other pairs of concentrations give significant difference. Comparison between brain and gill gives no significant difference in values but all other tissues give significant difference. From the summary table 1.3 it can be seen that liver recorded a higher value and brain recorded the least value when compared with the gill.

Table 1.5 Total protein content in liver, brain and gill tissues of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	**Control**	**7th day**	**21st day**	**7th day**	**21st day**	**7th day**	**21st day**
Liver	89.97 ± 1.93	85.50 ± 2.97	78.60 ± 3.50	73.28 ± 1.40	71.92 ± 1.43	66.87 ± 1.08	55.49 ± 1.91
Brain	86.15 ± 2.71	83.65 ± 1.79	73.77 ± 2.07	66.98 ± 1.33	64.71 ± 1.67	56.56 ± 0.53	40.38 ± 0.90
Gill	69.72 ± 1.90	67.63 ± 1.89	62.82 ± 0.59	60.24 ± 0.57	56.91 ± 0.50	52.15 ± 2.60	48.15 ± 1.05

- Values are expressed as mg protein/gm wet wt of tissue
- Average of six values in each group

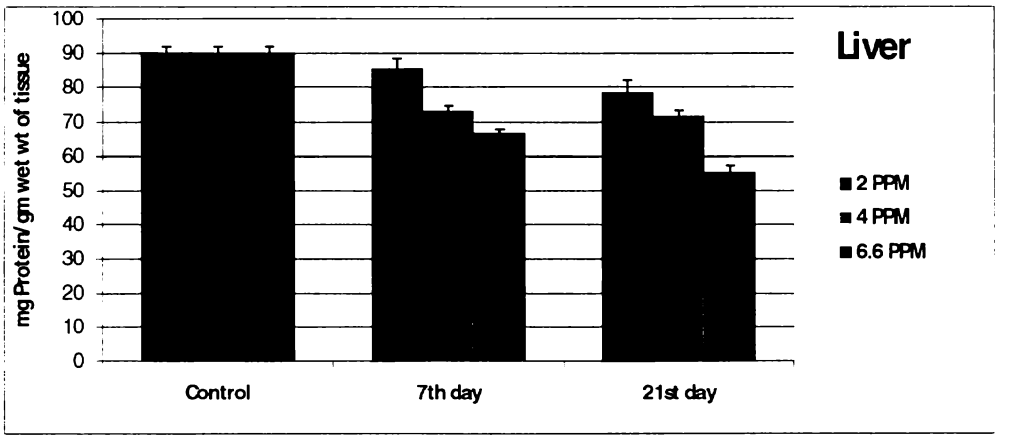


Fig.1.7 Total protein content in the liver of *H. fossilis* subjected to monocrotophos

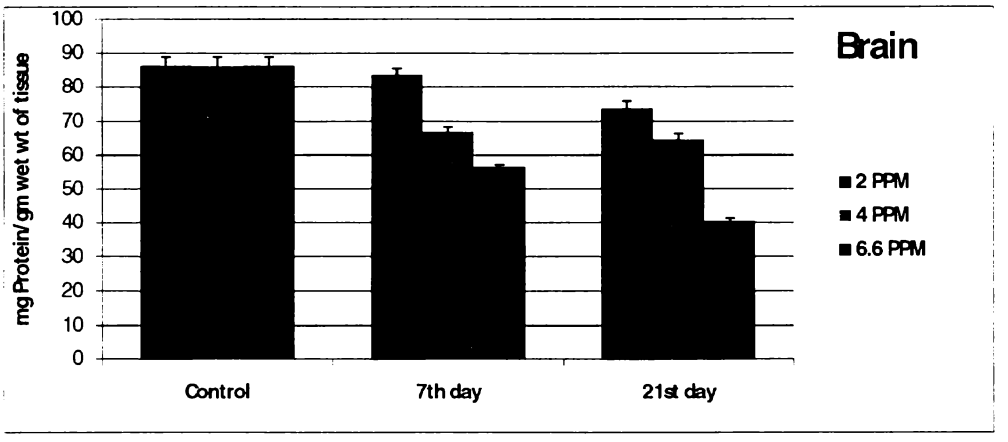


Fig.1.8 Total protein content in the brain of *H. fossilis* exposed to monocrotophos

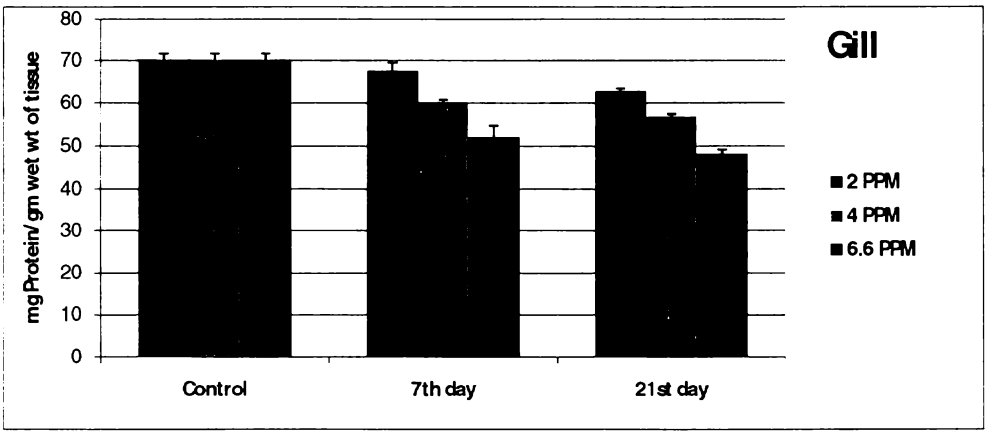


Fig.1.9 Total protein content in the gill of *H. fossilis* subjected to monocrotophos

Table 1.6 ANOVA table for total protein of *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	5829.029	2	2914.514	161.040	0.000
Concentration	16784.233	3	5594.744	309.134	0.000
Day	903.108	1	903.108	49.901	0.000
Error	2479.439	137	18.098		
Total	25995.808	143			

From the ANOVA table 1.6, comparing the effect of tissues, concentration and day on the recorded values, since the p-values are less than 0.05 for all the three factors viz., tissues, concentration and day, it can be concluded that the values vary significantly with these factors. Three factor ANOVA gives a significant decrease on total protein content of the tissues at different concentrations as well as days, compared to control.

Pair wise comparison by LSD analysis gives significant difference between the protein values of different tissues. Comparison between different concentrations also revealed significant difference between 2 ppm and 4 ppm, 2 ppm and 6.6 ppm, 4 ppm and 6.6 ppm. From the summary table 1.5 it can be concluded that the higher values are recorded with liver following brain.

Table 1.7 Total lipid content in liver, brain and gill tissues of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	89.8988 ± 2.353	79.6629 ± 1.947	69.4967 ± 2.902	72.7891 ± 2.234	62.1900 ± 2.078	59.2752 ± 2.402	41.0579 ± 0.939
Brain	82.6477 ± 1.273	74.8246 ± 4.315	64.4631 ± 0.958	68.0185 ± 2.924	59.2625 ± 1.297	55.6502 ± 1.534	45.5035 ± 1.859
Gill	60.4833 ± 0.950	51.5506 ± 1.746	41.4370 ± 1.988	40.7012 ± 3.021	31.6920 ± 0.540	40.4787 ± 0.802	30.6275 ± 0.527

- Values are expressed as mg /gm wet wt of tissue
- Average of six values in each group

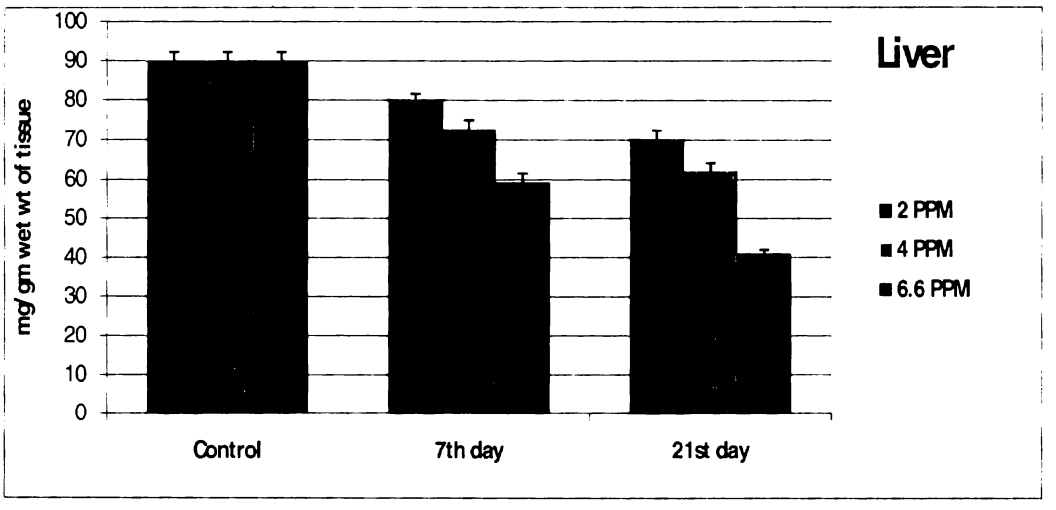


Fig. 1.10 Total lipid content in the liver of *H. fossilis* exposed to monocrotophos

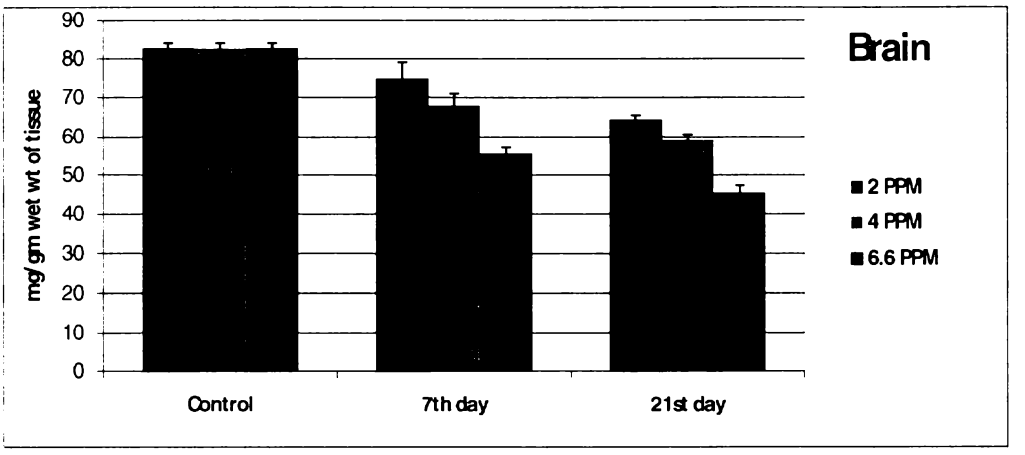


Fig. 1.11 Total lipid content in the brain of *H. fossilis* exposed to monocrotophos

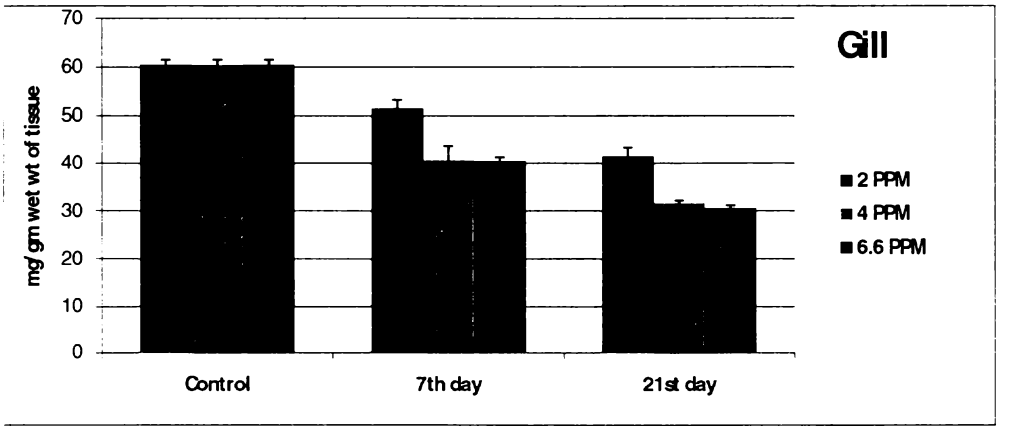


Fig. 1.12 Total lipid content in the gill of *H. fossilis* subjected to monocrotophos

Table 1.8 ANOVA Table for total Lipid of *H. fossilis*

Source of Variation	Type III Sum of Squares	Df	Mean Square	F	Sig.
Tissue	18643.035	2	9321.517	490.246	0.000
Concentration	19936.226	3	6645.409	349.502	0.000
Day	2362.975	1	2362.975	124.276	0.000
Error	2604.911	137	19.014		
Total	43547.148	143			

Since the p-values in the three factor Analysis of Variance are greater than 0.05 for all the factors, tissue, day and concentration, it can be seen that the factors viz, tissue, concentration and days have no impact on the values. It remains the same irrespective of the levels or type of these factors. LSD analysis showed that there was a significant difference between different concentrations and control values, also between various tissues. There was a significant decrease between the total lipid content of different concentration monocrotophos groups and control. The average values in descending order were observed in liver, brain and gill.

Table 1.9 Aspartate aminotransferase (AAT) activity in liver, brain and gill tissues of *H. fossilis* subjected to different concentration of monocrotophos

Tissue& serum	Control	2 ppm 7th day	2 ppm 21st day	4 ppm 7th day	4 ppm 21st day	6.6 ppm 7th day	6.6 ppm 21st day
Liver	0.7107 ± 0.0176	0.6560 ± 0.0055	0.3043 ± 0.0125	0.5120 ± 0.0227	0.1492 ± 0.0031	0.3104 ± 0.0093	0.0210 ± 0.0010
Brain	0.1124 ± 0.0047	0.1407 ± 0.0046	0.1725 ± 0.0071	0.1739 ± 0.0084	0.1918 ± 0.0023	0.2242 ± 0.0034	0.2930 ± 0.0133
Gill	0.4258 ± 0.0183	0.4939 ± 0.0152	0.5229 ± 0.0199	0.5633 ± 0.0134	0.6228 ± 0.0097	0.6648 ± 0.0213	0.7300 ± 0.0479
Serum	1.4376 ± 0.0431	1.7406 ± 0.0534	1.9142 ± 0.0333	1.9719 ± 0.0340	2.0799 ± 0.0448	2.1421 ± 0.1337	3.0805 ± 0.0811

- Values are expressed as units/minutes/mg protein
- Average of six values in each group

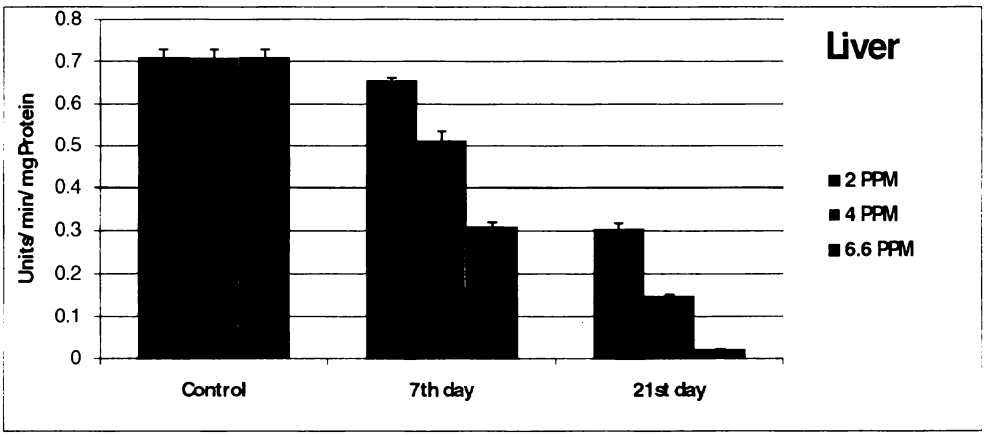


Fig. 1.13 Activity of aspartate aminotransferase (AAT) in the liver of *H. fossilis* subjected to monocrotophos

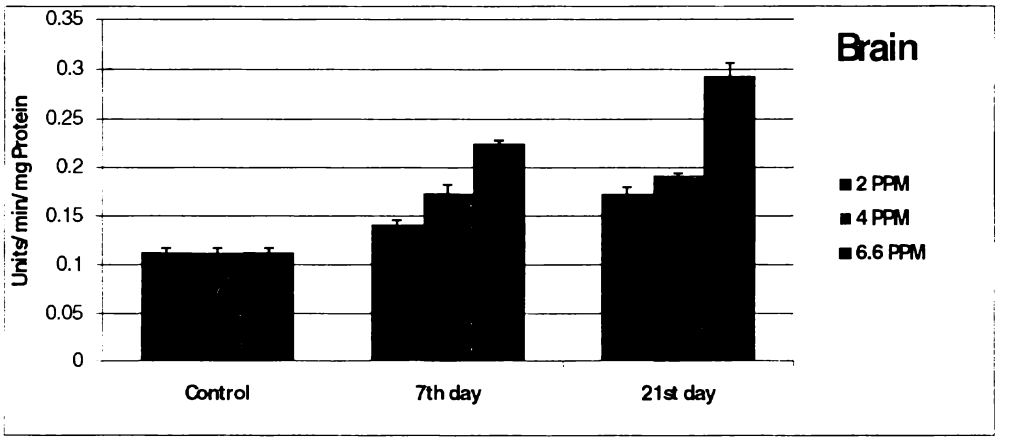


Fig. 1.14 Activity of aspartate aminotransferase (AAT) in the brain of *H. fossilis* subjected to monocrotophos

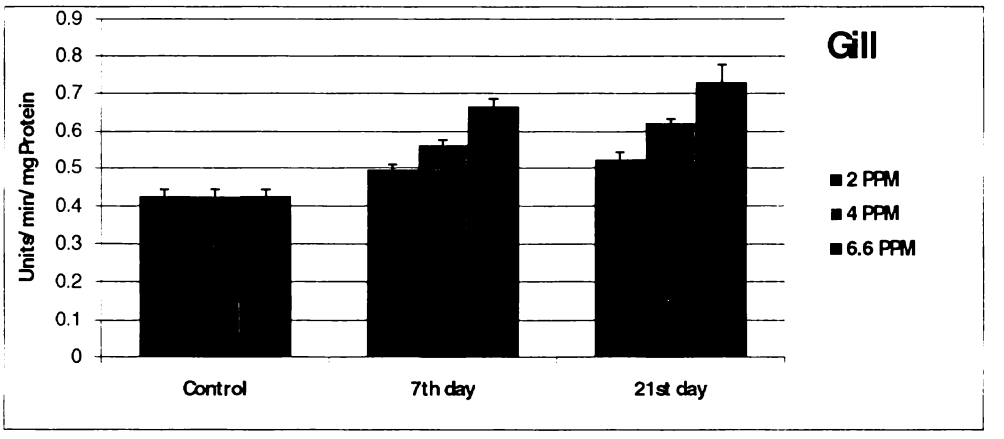


Fig. 1.15 Activity of aspartate aminotransferase (AAT) in the gill of *H. fossilis* subjected to monocrotophos

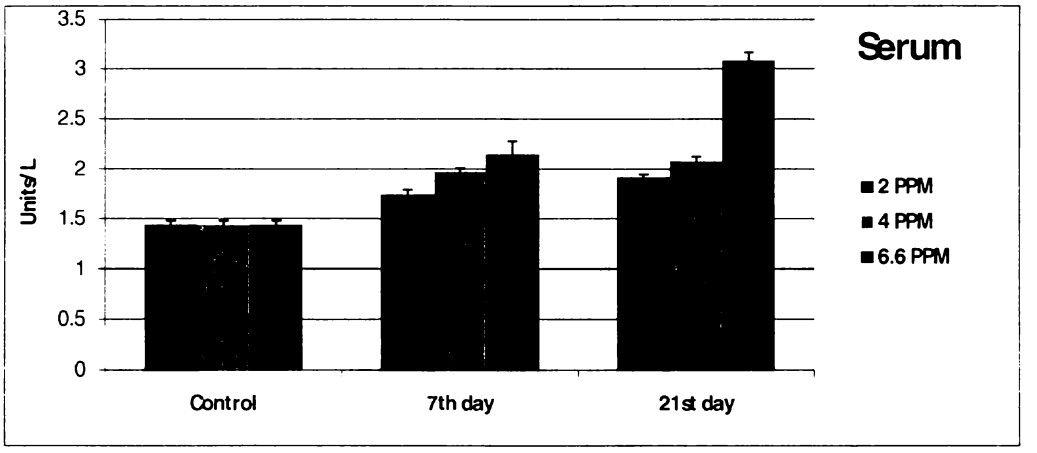


Fig. 1.16 Activity of aspartate aminotransferase (AAT) in the serum of *H. fossilis* subjected to monocrotophos

Table 1.10 ANOVA Table for aspartate aminotransferase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	94.589	3	31.530	432.813	0.000
Concentration	1.755	3	.585	8.031	0.000
Day	.045	1	.045	.614	0.434
Error	13.404	184	.073		
Total	109.793	191			

The results of three factor ANOVA reveals that differences between different days are not significant ($p > 0.05$) in the case of the parameter AAT, while there is a significant difference between the tissue/serum, and between different concentrations. Summary table 1.9 shows a significant reduction in the AAT activity of liver when compared to control, while AAT activity of brain, gill and serum increased significantly with respect to concentration.

Further comparison by LSD analysis shows that there was no significant difference between control and 2ppm, while values vary significantly between control and 4 ppm, as well as control and 6.6 ppm.

Difference between 2 ppm and 4 ppm were not significant, while there was significant variation between 2ppm and 6ppm, as well as 4pm and 6ppm. Under most of the situations serum values recorded a higher value compared to the other three. In almost all the situations brain recorded least values compared to the liver, gill and serum.

Table 1.11 Alanine aminotransferase (ALAT) activity in liver, brain, gill and serum of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue & serum	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	0.3003 ± 0.0214	0.1909 ± 0.0040	0.1703 ± 0.0046	0.1444 ± 0.0030	0.0980 ± 0.0024	0.0747 ± 0.0023	0.0696 ± 0.0012
Brain	0.0357 ± 0.0012	0.0605 ± 0.0019	0.0870 ± 0.0018	0.1192 ± 0.0040	0.1501 ± 0.0019	0.1290 ± 0.0015	0.2237 ± 0.0118
Gill	0.1018 ± 0.0037	0.1344 ± 0.0041	0.2010 ± 0.0031	0.1576 ± 0.0072	0.3071 ± 0.0103	0.1762 ± 0.0100	0.3484 ± 0.0180
Serum	2.5890 ± 0.1187	3.0204 ± 0.1348	3.4367 ± 0.0522	3.6559 ± 0.1560	3.7149 ± 0.0624	3.9091 ± 0.1103	4.2113 ± 0.1394

- Values are expressed as units/minutes/mg protein
- Average of six values in each group

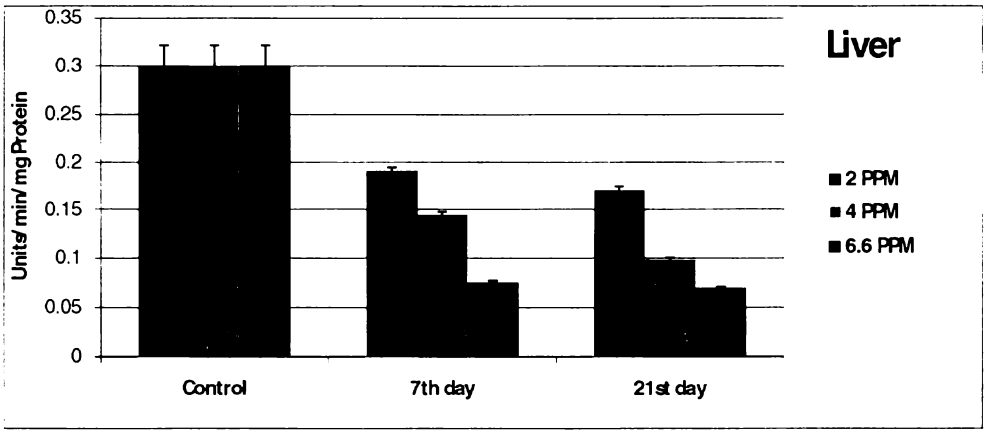


Fig. 1.17 Activity of alanine aminotransferase (ALAT) in the liver of *H. fossilis* exposed to monocrotophos

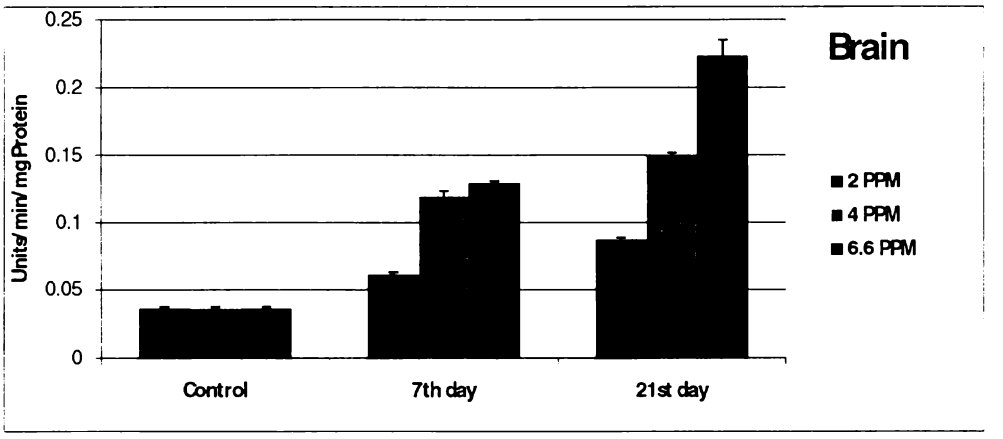


Fig. 1.18 Activity of alanine aminotransferase (ALAT) in the brain of *H. fossilis* exposed to monocrotophos

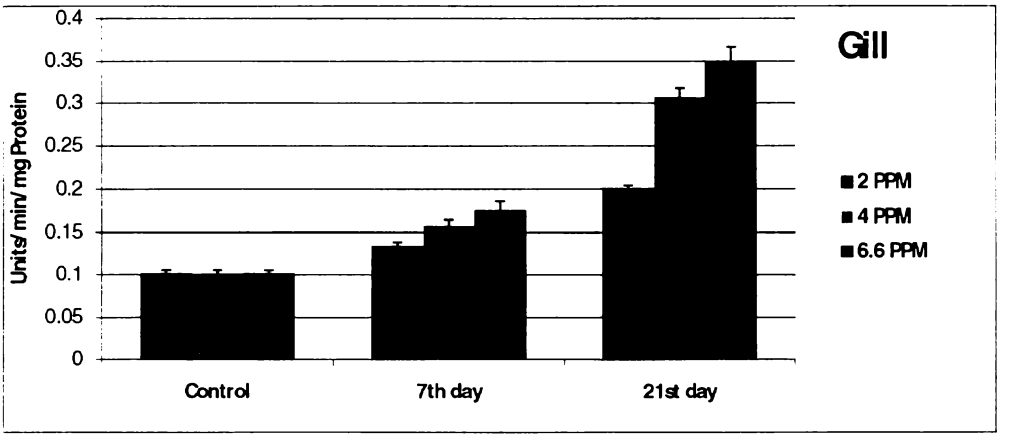


Fig. 1.19 Activity of alanine aminotransferase (ALAT) in the gill of *H. fossilis* exposed to monocrotophos

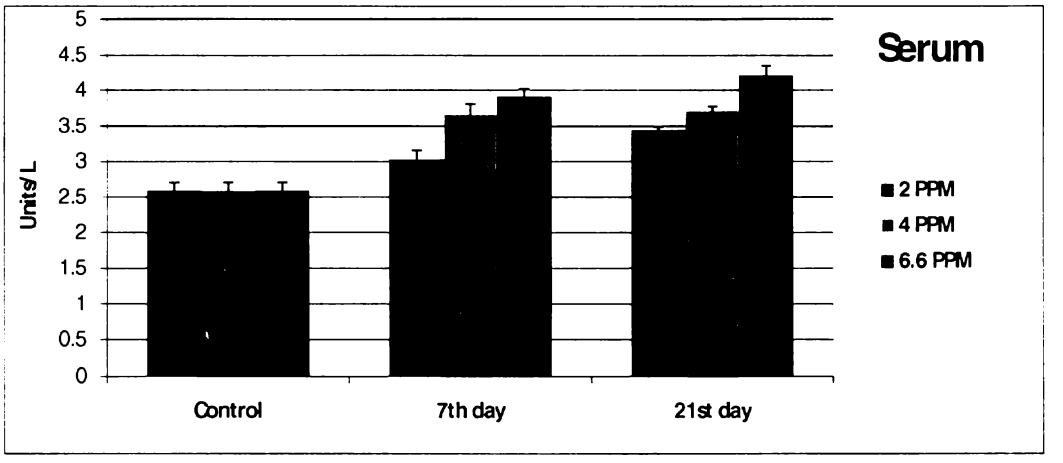


Fig. 1.20 Activity of alanine aminotransferase (ALAT) in the serum of *H. fossilis* exposed to monocrotophos

Table 1.12 ANOVA Table for alanine aminotransferase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	377.147	3	125.716	1869.640	0.000
Concentration	4.026	3	1.342	19.958	0.000
Day	.291	1	.291	4.327	0.039
Error	12.372	184	.067		
Total	393.836	191			

As in the case of AAT, three factor ANOVA shows that the values vary significantly with the factors, tissue and concentration. But here the difference in the values due to day is also significantly different, since the P-value is less than 0.05. Further comparison by LSD analysis shows that there was significant difference between monocrotophos concentration groups and control.

Pair wise comparison between different concentration groups revealed that all groups except 4ppm Vs 6ppm varied significantly. There was no significant difference between liver vs brain, liver vs gill and brain vs gill pairs while values significantly varied between other combinations of tissues. Here brain recorded relatively smallest values compared to other three tissues and serum stands with higher values which can be observed from the summary table 1.11.

Table 1.13 Acid phosphatase activity in liver, brain, gill and serum of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue/serum	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	0.9899 ± 0.0439	0.7080 ± 0.0214	0.4790 ± 0.0185	0.6319 ± 0.0345	0.3717 ± 0.0108	0.4780 ± 0.0079	0.2833 ± 0.0107
Brain	0.5883 ± 0.0192	0.6198 ± 0.0287	0.6878 ± 0.0184	0.8469 ± 0.0141	1.1008 ± 0.0455	1.1231 ± 0.0415	1.1546 ± 0.0251
Gill	0.6307 ± 0.0160	0.7345 ± 0.0221	0.8572 ± 0.0144	0.7118 ± 0.0138	1.1712 ± 0.0301	1.8181 ± 0.0707	2.0494 ± 0.0319
Serum	4.0123 ± 0.0546	4.2030 ± 0.1748	4.9119 ± 0.1275	4.9062 ± 0.1781	5.1279 ± 0.1597	5.1889 ± 0.1681	5.9262 ± 0.3256

- Values are expressed as µmol Pi liberated/mg protein/hr
- Average of six values in each group

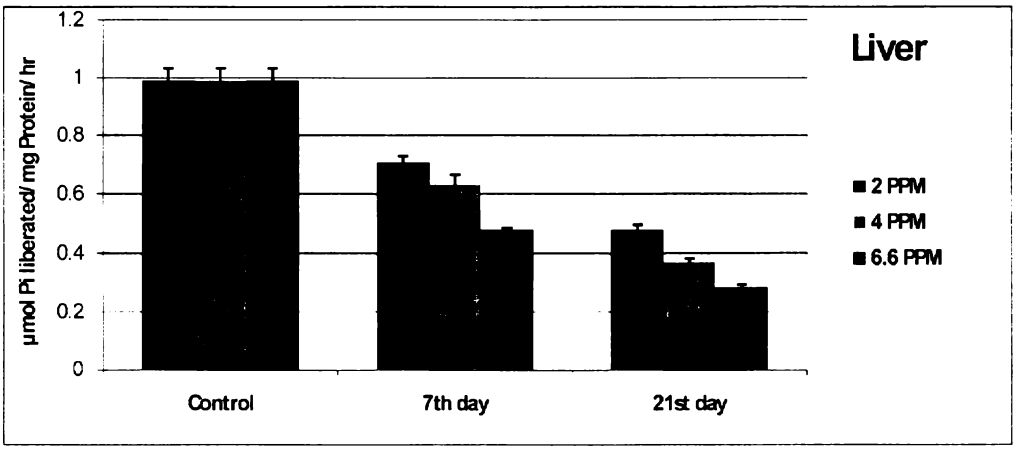


Fig. 1.21 Activity of acid phosphatase (ACP) in the liver of *H. fossilis* subjected to monocrotophos

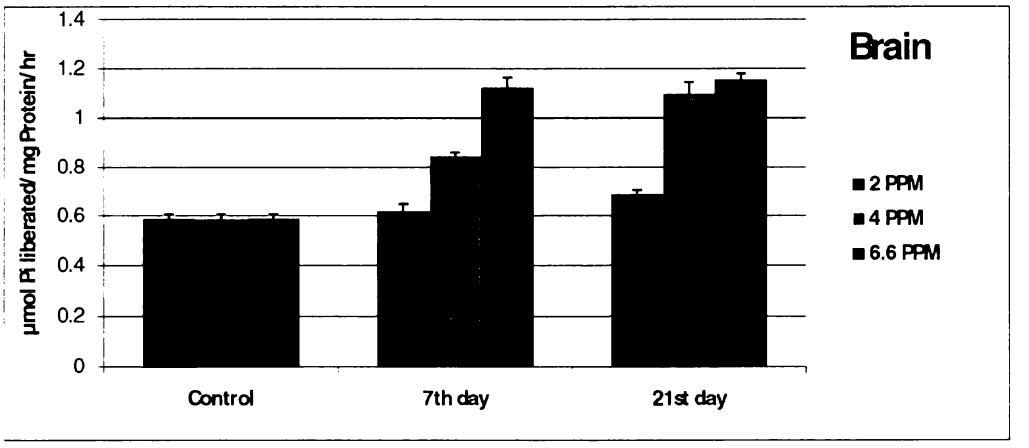


Fig. 1.22 Activity of acid phosphatase (ACP) in the brain of *H. fossilis* subjected to monocrotophos

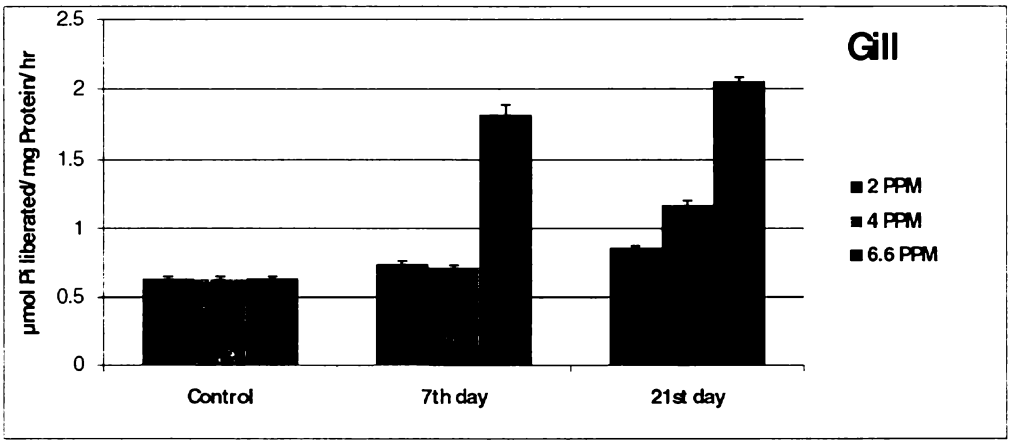


Fig. 1.23 Activity of acid phosphatase (ACP) in the gill of *H. fossilis* subjected to monocrotophos

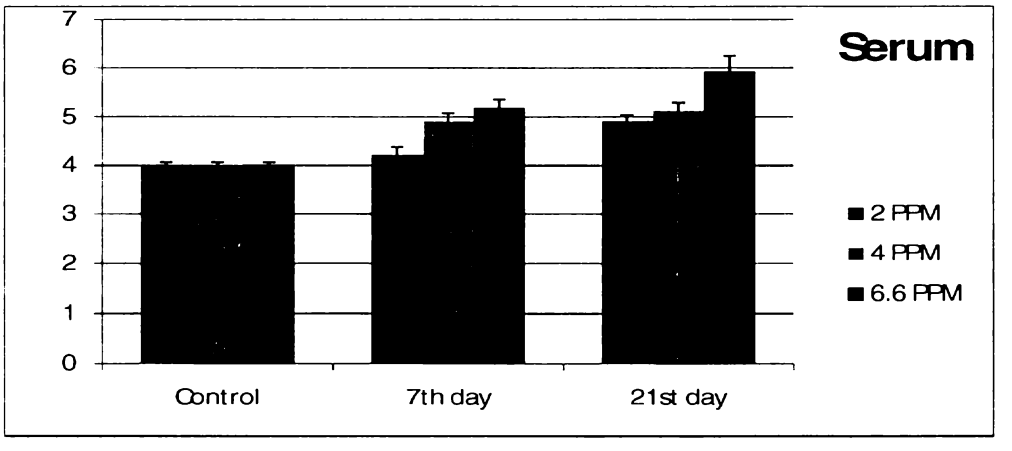


Fig. 1.24 Activity of acid phosphatase (ACP) in the serum of *H. fossilis* subjected to monocrotophos

Table 1.14 ANOVA Table for acid phosphatase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	564.631	3	188.210	1418.925	0.000
Day	13.790	3	4.597	34.655	0.000
Concentration	.867	1	.867	6.539	0.011
Error	24.406	184	.133		
Total	603.695	191			

Since the p-value for all the factors are observed to be less than 0.05 in the ANOVA table 1.14 , it can be observed that the values vary significantly with all the factors i.e., the effect due to concentration, tissue and day. As per LSD analysis, there was no significant difference between control and 2ppm group, while 4 ppm and 6.6 ppm concentration groups varied significantly from the control.

There was significant difference between various tissue/serum groups too. Here too the serum has a highest value and brain has the least value compared to the other tissues. From the summary table (1.13) it can be seen that liver showed a significant decrease in ACP activity among the treated groups, with maximum reduction at 6.6 ppm, where as brain, gill and serum values increased, giving maximum values at 6.6 ppm.

Table 1.15 Alkaline phosphatase (ALP) activity in liver, brain, gill and serum of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue & Serum	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	1.08 ± 0.05	1.02 ± 0.09	0.85 ± 0.10	0.88 ± 0.07	0.89 ± 0.10	0.70 ± 0.03	0.72 ± 0.05
Brain	0.90 ± 0.03	1.03 ± 0.04	1.01 ± 0.10	1.30 ± 0.15	2.00 ± 0.03	2.06 ± 0.08	2.77 ± 0.09
Gill	0.75 ± 0.01	0.85 ± 0.09	0.88 ± 0.06	1.57 ± 0.08	1.93 ± 0.12	2.33 ± 0.12	2.93 ± 0.04
Serum	4.57 ± 0.14	5.09 ± 0.21	5.73 ± 0.11	4.85 ± 0.18	5.94 ± 0.10	6.00 ± 0.02	6.84 ± 0.12

- Values are expressed as μmol phenol formed/mg protein/hr
- Average of six values in each group

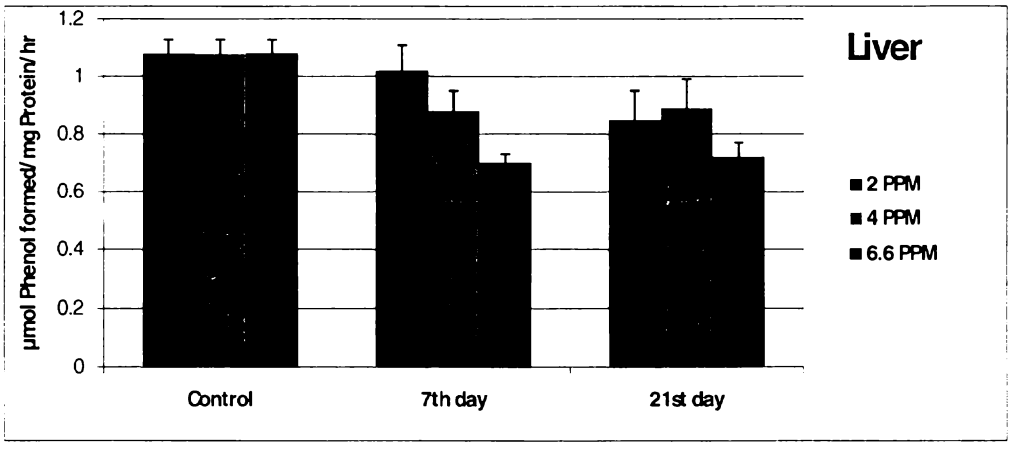


Fig. 1.25 activity of alkaline phosphatase (ALP) in the liver of *H. fossilis* subjected to monocrotophos

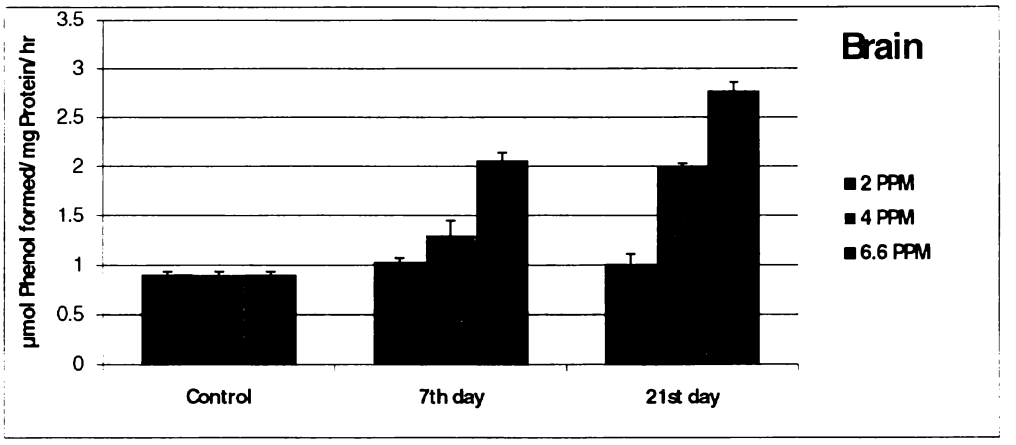


Fig. 1.26 Activity of alkaline phosphatase (ALP) in the brain of *H. fossilis* subjected to Monocrotophos

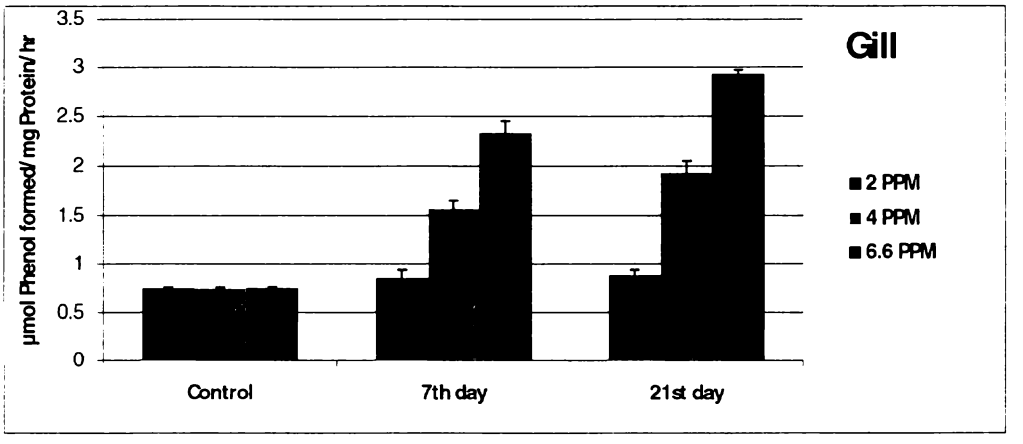


Fig. 1.27 Activity of alkaline phosphatase (ALP) in the gill of *H. fossilis* subjected to Monocrotophos

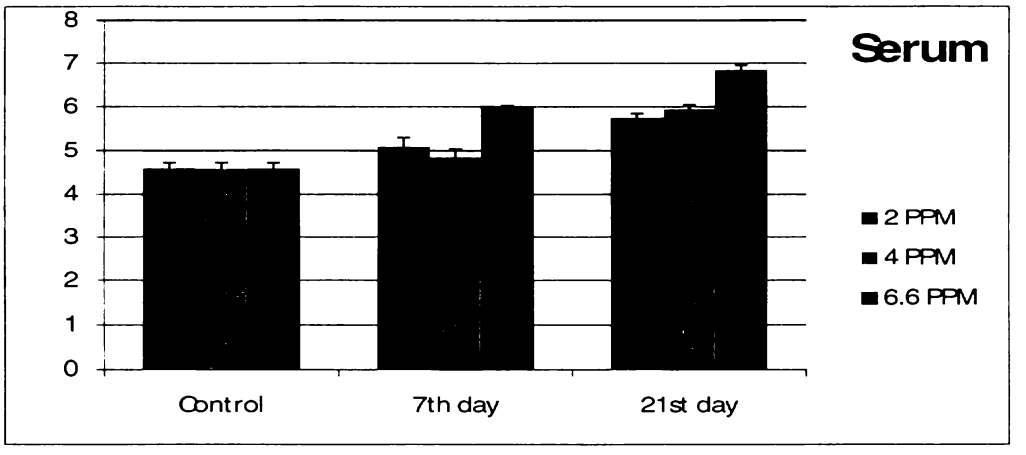


Fig.1.28 Activity of alkaline phosphatase (ALP) in the serum of *H. fossilis* subjected to Monocrotophos

Table 1.16 ANOVA Table for Alkaline phosphatase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	629.900	3	209.967	1222.096	0.000
Day	40.249	3	13.416	78.089	0.000
Concentration	3.899	1	3.899	22.694	0.000
Error	31.613	184	.172		
Total	705.660	191			

Three factor ANOVA reveals that there are significant effects due to the factors tissues, concentration and days on the values since the p-value corresponding to all these factors are less than 0.05 (table 1.16). Further comparison by LSD analysis shows that values were significantly altered between different concentration groups as well as between the control and concentration groups. Difference between liver and gill was not significant, while all other tissue/serum groups give significant difference in values.

From the table 1.15 showing the summary statistics it can be observed that serum and gill respectively recorded the highest and the lowest values respectively among various tissues/serum. Liver ALP activity was significantly decreased compared to control with maximum reduction being observed at 6.6 ppm. Brain, gill and serum ALP values were increased significantly with all of them giving highest value at 6.6 ppm.

Table 1.17 Lactate dehydrogenase (LDH) activity in liver, brain and gill of *H. fossilis* subjected to different concentration of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	1.3886 ± 0.0432	1.8917 ± 0.0636	2.4050 ± 0.0663	3.0866 ± 0.0960	4.2384 ± 0.2710	4.9484 ± 0.2419	7.3930 ± 0.4947
Brain	0.7646 ± 0.0205	3.6724 ± 0.0990	5.2038 ± 0.1511	5.6546 ± 0.1077	7.4268 ± 0.1825	7.4674 ± 0.2367	8.8505 ± 0.2320
Gill	0.8996 ± 0.0145	3.5094 ± 0.0445	4.9530 ± 0.3109	6.5319 ± 0.1437	7.8553 ± 0.1544	7.9885 ± 0.0565	9.2371 ± 0.3489

- Values are expressed as µmol of NADH oxidized /hr/g protein
- Average of six values in each group

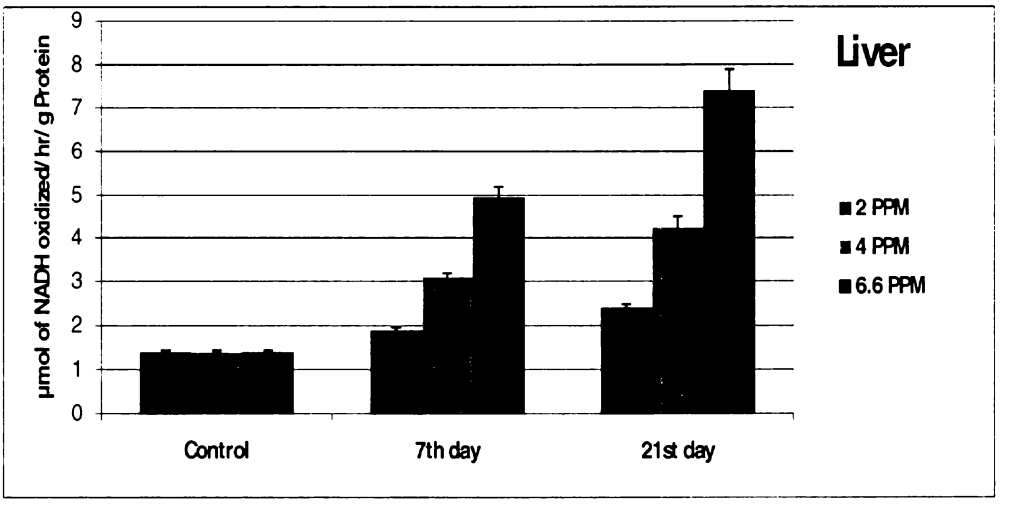


Fig. 1.29 Activity of lactate dehydrogenase in the liver of *H. fossilis* subjected to monocrotophos

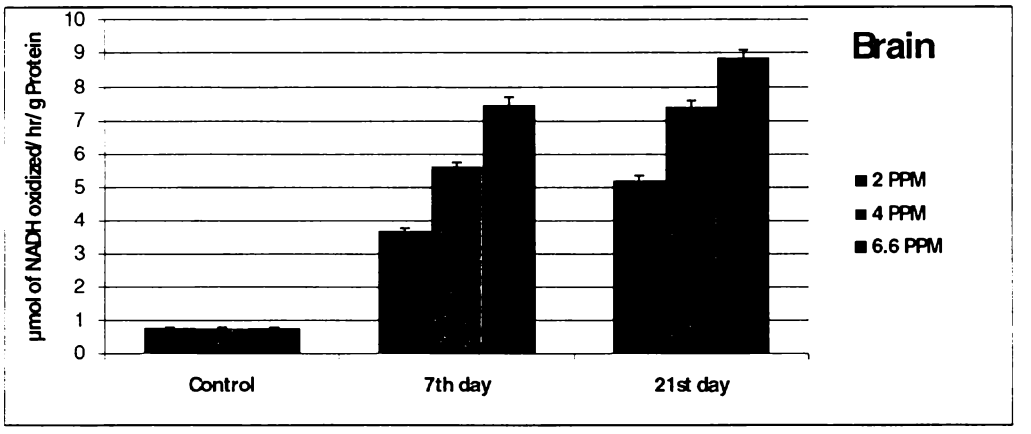


Fig. 1.30 Activity of lactate dehydrogenase in the brain of *H. fossilis* subjected to monocrotophos

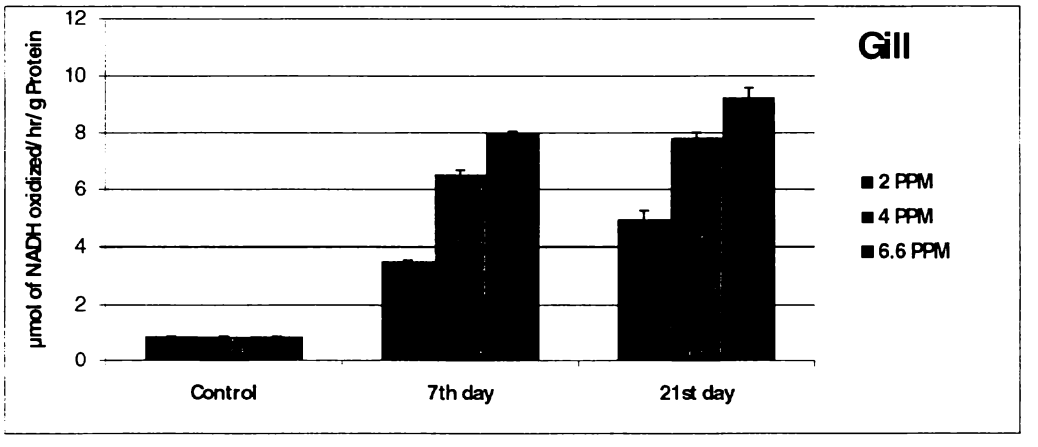


Fig.1.31 Activity of lactate dehydrogenase in the gill of *H. fossilis* subjected to monocrotophos

Table 1.18 ANOVA Table for lactate dehydrogenase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	101.002	2	50.501	77.213	0.000
Day	882.704	3	294.235	449.866	0.000
Concentration	41.037	1	41.037	62.743	0.000
Error	89.605	137	.654		
Total	1114.347	143			

Three factor ANOVA revealed that the values vary significantly with the factors tissue, concentration and day. From the ANOVA table 1.18, it can be seen that the p-values for each of these factors are less than 0.05. Further comparison by LSD analysis shows that values were varied significantly between control and different concentration groups. Values were significantly varied between different tissues, except that of brain vs gill group where the difference was not significant. Here brain recorded relatively high values compared to other two tissues, which can be observed from the summary table 1.17.

In all the above analysis it is observed that the means vary significantly with different types of tissues and different levels of concentrations. Now it is interesting to see whether the value remains the same for any two, in each parameter. Least Significant Difference (LSD) analysis is carried to see which all combinations have significant difference taking two at a time. The results are shown below.

Table 1.19a LSD analysis for liver, gill, brain and serum of *H. fossilis*

	AST	ALT	ACP	ALP
Liver vs Brain	0.000	.232	0.003	0.000
Liver vs Gill	0.017	.671	0.000	0.000
Liver vs Serum	0.000	.000	0.000	0.000
Brain vs Gill	0.000	**0.106**	0.002	**0.918**
Brain vs Serum	0.000	0.000	0.000	0.000
Gill vs Serum	0.000	0.000	0.000	0.000

- Values given in bold face are not significant

Table 1.19b

	Protein	TCH	Glycogen	Lipid	LDH
Liver vs Brain	0.000	0.000	0.000	0.000	0.000
Liver vs Gill	0.000	0.000	0.000	0.000	0.000
Brain vs Gill	0.000	0.000	**0.406**	0.000	**0.185**

- Values given in bold face are not significant

Table 1.20 LSD analysis for different concentration levels of monocrotophos treatments

	AST	ALT	ACP	ALP	Protein	TCH	Glycogen	Lipid	LDH
Control vs 2ppm	**0.196**	0.004	**0.201**	0.004	0.000	0.000	0.000	0.000	0.000
Control vs 4ppm	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Control vs 6.6 ppm	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2 ppm vs. 4 ppm	**0.469**	0.014	0.006	0.000	0.000	0.000	**0.242**	0.000	0.000
2 ppm vs. 6.6 ppm	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
4 ppm vs. 6.6 ppm	0.007	**0.062**	0.000	0.000	0.000	0.000	0.002	0.000	0.000

- Values given in bold face are not significant

Table 1.19a and 1.19b shows the results of the LSD analysis performed for different types of tissues and table 1.20 shows the results of the LSD analysis performed for different levels of concentration. Values in boldface letters show that there is no significant difference between the respective type of tissues and the levels of concentration.

Brain and gill have almost the same values in the case of the parameter ALP (p-value= 0.918) ALT (p-value =0.106), glycogen (p-value= 0.406) and LDH (p-value = 0.185). The difference between the values for control

and 2 ppm group in the cases of the parameter AST and ACP are not significant (p-value 0.196 and 0.201 respectively). The same results hold with other boldface cases too.

1.5 Discussion

In the present study, glycogen content in liver, gill and brain of monocrotophos induced group of fishes were observed to decrease. The glycogen content in the control fish liver, brain, and gill were 28.58, 0.598, and 1.543 mg/gm wet wt tissue, respectively. The glycogen levels of different tissues showed a decreasing trend as exposure progressed. The decrease was greatest in liver as it is the principal organ for glycogen storage. Decline in glycogen content of liver and muscle after toxic stress has been reported in a number of studies with aquatic animals (Shobha *et al.*, 1989; Aguiar *et al.*,2004; Hori *et al.*, 2006). Similar drop off in glycogen content in *Clarias batrachus* was observed after fish were intoxicated with organophosphate pesticide dimethoate and Rogor (Begum and Vijayaraghavan, 1995, 1999). A reduction in brain glycogen of fish exposed to the sublethal concentration, points to deranged intermediary metabolism primary to ATP production. This deprives the nervous system a supply of metabolic fuel resulting in general debility. The depletion of glycogen in the tissues is an indication of typical stress response in fish challenged with pesticides. The observed reduction in glycogen content indicates the utilization of stored glycogen to meet the high energy requirement under the pesticide stress. Significant dose dependent decrease in total carbohydrate and glycogen content of tissues and steady increase of blood glucose levels were observed in all the three sublethal concentrations of monocrotophos.

Carbohydrates represent the foremost and instant energy precursors for organisms exposed to stress. Insecticidal stress has been found to lead to a hypoxic/anoxic condition; this promotes anaerobic glycolysis and a decline in oxidative metabolism necessitating the consumption of carbohydrate to meet energy demand. Arasta *et al.*, (1996) suggested that in stressed fishes, carbohydrates reserve undergoes stark depletion to meet the energy demand. It can be assessed that there is rapid utilization of total carbohydrate and glycogen which constitutes a big share of it, by all the tissues of monocrotophos exposed fish to meet the towering energy demands due to stress.

The decrease in tissue glycogen of the fish exposed to different concentrations of insecticide monocrotophos shows relationship with an increase in blood glucose and maximum increase was observed at the highest dose concentration, indicating an increased break down of glycogen to glucose and its mobilization to other tissues to meet energy crisis (Pickering, 1993). Inhibition of acetyl cholinesterase results in an increase in acetylcholine contents (Singh *et al.*,1996). Increased level of acetylcholine has been shown to enhance the secretion of catecholamine in fish (Nilsson *et al.*, 1976), which may bring about glycogenolysis. Finally glycogenolysis seems to be the result of increased secretion of catecholamine due to stress of insecticide treatment (Singh and Srivastava 1992; Singh and Agarwal 1993). Many studies have suggested that such a change is related to enhanced circulating levels of both catecholamines and glucocorticoids (Nakano and Tomlinson, 1967). Thus, the marked glycogenolysis observed after acute exposure to monocrotophos in the present study was most likely due to stress induced increase in circulating catecholamines and glucocorticoids.

The concentrations of total protein in the liver, gill and brain of *H. fossilis* exposed to monocrotophos were found to be lower than those in controls on all sampling days. Similar findings have been noted in the freshwater prawn *Macrobrachium kistensis* on exposure to naphthalene and pesticides (Jaiswal *et al.*, 1991; Nagabhushanam *et al.*, 1987), in the freshwater field crab *Barytelphusa guerini* and the fish *Anabas scandens* on exposure to insecticides. (Reddy *et al.*, 1991; Yasmeen *et al.*, 1991) and in the fish *Pundulus heteroclitus* and *Notopterus notopterus* exposed to various forms of stress. (Umminger, 1970; Narasimhan and Sundararaji, 1971). Feasible explanation for these observations is that proteolytic activity was induced in these organs due to the stress. Another hypothesis has been advanced to explain the reduced protein level in *B. guerini* exposed to pesticide (Reddy, 1991); physiological compensatory mechanisms are activated to (i) provide intermediates for deriving energy through Kreb's cycle and (ii) compensate for osmoregulatory problems (due to leakage of ions and other vital molecules) by increasing the free amino acid level in blood ; such mechanisms were possibly operative in the test fishes exposed to monocrotophos in the present study.

Lipids are reported to serve as an alternate source of energy in fish, particularly during stress conditions. Thus, in the present study, lower values of the total lipid content in the liver, brain and gill of test fishes in comparison to the quantities of lipid in the same tissues of controls, might reflect an accelerated hydrolysis of lipid in order to cope with the high energy demand occurring due to monocrotophos toxicity. Similar observations have been made in the fish *Oreochromis mosambicus* exposed to methyl parathion (Rao and Rao, 1981) and in *Barbus chonchonius* exposed to aldicarb (Pant *et al.*, 1987).

Activity of both ALAT and AAT increased in plasma, gill and brain of the monocrotophos intoxicated *H. fossilis.* But drop in activities of both enzymes were observed in the liver tissue. Similar observations were made by Rao, (2006) in *O. mossambicus* sub-acutely exposed to monocrotophos. Similar increases in ALAT and AAT activities were reported in liver and muscle tissues of *C. batrachus* after short-term exposure to dimethoate (Begum and Vijayaraghavan, 1995) and in *O. mossambicus* sub-acutely exposed to RPR series of OP insecticides. (Rao, 2006). Decrease in activity of ALAT and AAT in liver can be due to excessive damage to tissue and subsequent release of hepatic ALAT and AAT into serum. Enhanced activity of the transaminases provides the oxaloacetic acid and pyruvate, ketoglutarate and glutaric acid to meet the energy demand due to monocrotophos induced stress. The oxaloacetic acid, pyruvate and ketoglutarate might have been channeled into the citric acid cycle. The increase in ALAT and AAT activities in this study supports earlier findings (Oluah *et al.,* 1998; Zikic *et al.,* 2001) and serves as indicator of tissue damage. When the liver cell membrane is damaged, variety of enzymes normally located on the cytosol is released into the bloodstream. This can be regarded as a useful quantitative marker for hepatocellular damage. Plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis (Friedman *et al.,* 1996; Henderson *et al.,* 1983). The observed increase in the serum aspartate aminotransferase activity in the fish exposed to monocrotophos agrees with earlier reports on the effects of various insecticides, chemical irritants, industrial effluents and heavy metals on serum enzymes of fish (Rao and Rao, 1984; Berstein, 1968; Racicoot *et al.,* 1975; Michael *et al.,* 1987; Hilmy *et al.,* 1981; Oluah and Amalu, 1998).

Lactate dehydrogenase (LDH) is a marker of tissue damage and its increased level is reported in liver necrosis (Ramesh *et al.*, 1993). The higher LDH activity was observed in brain and gill. LDH forms the center for a delicately balanced equilibrium between catabolism and anabolism of carbohydrates (Everse and Kaplan 1973).The elevation in the level of LDH activity in test fish suggests that there was an increased conversion of pyruvate to lactate and a budge in respiratory metabolism from the aerobic to the anaerobic form following exposure to monocrotophos. Stimulation of LDH and the rapid rate of glycolysis observed in the present study indicate that the end product of glycolysis i.e. pyruvate, was not routed through Kreb's cycle but through the lactic acid cycle under hypoxic conditions, which in turn lead to the accumulation of lactic acid.

ACP and ALP activities were increased in both gill and brain of the monocrotophos exposed fish on both 7^{th} and 21^{st} days of observation. Elevation of ACP activity in brain was reported earlier in stress exposed rohu (Das, 1998). Verma *et al.*, (1984) reported an increase in serum ACP in *Mystus vittatus* (Bloch) exposed to different pesticides. Acid phosphatase is a lysosomal enzyme that hydrolyses the phosphorous esters in acidic medium. So it is rational to explain that the enzyme is hydrolytic and is one of the several acid hydrolases involved in the process of autolysis after cell death.

A significant reduction of both ACP and ALP activities was observed in the liver during 7^{th} as well as 21^{st} day observations. This can be possibly due to the direct inhibitory action of monocrotophos on these hepatic enzymes. Such a possibility was suggested in the fish *Oreochromis mossambicus* exposed to monocrotophos (Rao, 2003) and in *Macrobrachium malcomsonii* exposed to endosulfan (Bhavan and Geraldine, 2001). The

decline in phosphatase levels observed in the liver suggests that the rate of transphosphorylation was severely affected, a phenomenon that has been noted in the fish *Notopterus notopterus* exposed to phenol and dinitrophenol (Verma, 1980) and in *Labeo rohita* and *Cirhinus mirigala* exposed to phosphomidon (Medda, 1993)

Increase in alkaline phosphatase suggests an enhancement in the lysosomal mobilization and cell necrosis due to pesticide toxicity. Elevation of ACP activity in brain was reported earlier in stress-exposed *Channa punctatus* (Sastry and Sharma, 1980) and *Labeo rohita* (Das, 1998). Increased activity of acid phosphatase and alkaline phosphatase in blood plasma can be taken as an indicator of hepatic tissue damage and dysfunction in its normal performance which supports earlier findings in many species of fish following the exposure of monocrotophos (Agrahari, 2007) and fungicides (Ram and Sathyanesan, 1987).

1.6 Summary

From the above discussion, it is clear that monocrotophos toxicity seriously impairs various metabolic functions of the fish *Heteropneustes fossilis*, reflected as alterations in various biochemical constituents. AAT, ALAT, ACP and ALP activity in plasma which are conventional indicators of liver injury are observed to be increased in the monocrotophos exposed *H. fossilis.* Plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis. The present biochemical alterations in *H. fossilis*, sublethally intoxicated with monocrotophos, suggest that the treated fish faced a severe metabolic crisis. The increase of biomarker enzymes in plasma might be due to the necrosis of liver. The results revealed that monocrotophos affects the intermediary metabolism of *H. fossilis* and the assayed enzymes can work as good

biomarkers of organophosphorus contamination. The above results clearly indicate that monocrotophos is toxic to fish and a comprehensive appraisal of pesticides involving all the important non-target species representing different ecosystems should be completed before their commercialization. However, it is a matter of concern that in test fishes the concentrations of major biochemical constituents were lower than in controls, since this reflects a loss of nutritive value. In effect, OP toxicity ultimately lowers the nutritive value of the catfish *H. fossilis* and poses a grave threat to its potential use as food. Proper measures should be taken to ensure that OPs including monocrotophos do not pollute fisheries, aquaculture farms, and natural resources.

Chapter 2

EFFECT OF MONOCROTOPHOS ON HAEMATOLOGICAL PARAMETERS

Contents

2.1 Introduction

In order to evaluate the subtle, deleterious effects of sublethal concentrations of monocrotophos (MCP) on the physiology of fish, it is indispensable to monitor certain clinical parameters. The use of haematological methods as indicators of sublethal stress can provide valuable information concerning the physiological reaction of fish in a changing environment. The physical and chemical parameters of fish blood are very sensitive to environmental changes (Huges and Nemcsok., 1988). The blood parameters are excellent indicators of toxic stress (Handy and Depledge., 1999). Its role as a supplier of essential nutrients, ions, gases, and endocrine factors, coupled with its mission as a pool for excretory products of metabolism, means that shift in blood parameters are often reflective of the overall toxic impacts of environmental contaminants. "Since a change or lack of change in blood picture is a fundamental characteristic of practically every physiologic or pathologic state, haematologic findings are among the most valuable and most generally useful of all laboratory diagnostic aids" (Wells, 1956). Blood parameters have been used as biomarkers of exposure to organophosphorus compounds. Hematological changes in fish such as an increase in blood leucocytes, and decreased mean weight of haemoglobin per erythrocyte have been found in response to insecticide contamination of the aquatic environment (Matthiessen, 1981). Simplicity of most blood sampling techniques probably accounts for the wide spread use of haematological studies as a means of assessing the state of health of teleost fish (Pickering, 1986).

Air breathing fishes form an economically important group of food fishes in many parts of South Asia and Africa. They are known for their nutritive, invigorating and therapeutic qualities and are recommended by physicians as diet during convalescence. The present study has been aimed

to assess the effect of different sublethal concentrations of organophosphorus insecticide monocrotophos on hematology of *Heteropneustes fossilis* (Bloch) an important food fish of India and other South East Asian nations. The various parameters studied included Hb concentration, RBC count, WBC count, PCV, RBC constants and blood glucose content.

2.2 Materials and methods

Collection, acclimation and dosing experiments of/on *Heteropneustes fossilis* were the same as that described in chapter 1. Fishes were exposed to three selected sublethal concentrations of monocrotophos for twenty one days and sampling was done on 7th and 21st day intervals to study the effect of monocrotophos on the hematological profile of *H. fossilis*.

2.2.1 Collection of Blood

Fish from each experimental and control group were bled from the caudal vein into sterilized glass vials containing the anticoagulant 1% ethylene diamine tetra acetic acid (EDTA). Hematological parameters were estimated by standard methods; Cyanmethaemoglobin method was used for the estimation of haemoglobin. Packed cell volume (PCV) was determined as per microhematocrit centrifugation method. Red blood corpuscles (RBC) count was done with a Neubauer chamber as described by Sohn and Henry (1969). WBC count was done as per the method by Hunter and Bomford (1963). RBC constants were calculated by means of standard formulae. Blood glucose was estimated as per the method of Mark, (1959).

2.2.2 Estimation Hemoglobin

Blood (0.03 ml) was mixed with 5 ml of Drabkins diluent solution and was allowed to stand for 5 minutes for the formation of cyanmethaemoglobin. Absorbance was measured against a reagent blank

which consisted of 5 ml of diluent solution. Standard calibration curve was composed employing cyanmethaemoglobin standard, from which values of hemoglobin can be read directly as g/dl.

2.2.3 Red blood corpuscle (RBC) count

Red blood corpuscles (RBC) count was done with a Neubauer chamber as described by Sohn and Henry, (1969).

Procedure

The blood was taken in a vial containing 2% ethylene diamine tetra acetic acid (EDTA) as anticoagulant. Blood was drawn up to 0.5 mark in RBC pipette and immediately, the diluting fluid (Hayem's solution-Glaxo) was drawn up to the 101 mark (thus the dilution is 1:200). Pipette was shaken thoroughly and diluted blood was charged into the counting chamber, after discarding two drops. The solution was allowed to settle for few seconds and the number of RBCs was counted in five small squares of the RBC column under high power microscope and the number of RBCs per cubic mm was calculated.

$$\frac{\text{No. of cells} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

2.2.4 White blood corpuscles count

WBCs were counted according to the method described by Donald Hunter and Bomford, (1963).

Procedure

Blood was collected in vials containing 2% EDTA as anticoagulant. The blood was drawn up to 0.5 marks of WBC pipette and immediately diluting fluid was drawn up to 11 mark above the bulb. Solution was mixed thoroughly and was allowed to stand for 2 minutes. Solution was expelled

and a drop of fluid was allowed to flow under the cover slip. It was allowed to stand for 2 minutes and the WBCs were counted in the 4 corner square millimeters. The number of WBCs per cubic millimeter was calculated accordingly.

$$\frac{\text{No. of cells} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

2.2.5 Determination of Packed Cell Volume (PCV)

PCV was determined employing microhematocrit centrifugation method.

Procedure

Blood was collected by caudal vein puncture, with a plastic syringe using EDTA as anticoagulant. It was allowed to run ½ to ¾ lengths of heparinized evenly bored capillary tubes and the tubes were sealed on the opposite end using sealing wax. The tubes were then transferred to a high speed microhematocrit centrifuge and were placed in the grooves of capillary head. Centrifugation was done for 15 minutes at 12000 rotations per minute. PCV was measured directly on a microhematocrit reader associated with the centrifuge as volume present.

2.2.6 Estimation of Blood Glucose

The blood was centrifuged for 15min at 400 rpm. The activity of glucose was determined by the method described by Mark, (1959).

Reagents

O-Toluidine (1%), 0.3 N NaOH, 0.15 M acetate buffer (pH 5.0), 0.02 % peroxidase solution, glucose oxidase, standard glucose, 5% $ZnSO_4.7H_2O$, fermocozyme (a stable liquid preparation of glucose oxidase)

Procedure

To 1.2 ml of 0.9% sodium chloride, 0.4 ml of 5% $ZnSO_4.7H_2O$ solution and 0.4 ml of 0.3 N NaOH was added. To this 0.2 ml of blood was added, mixed well and centrifuged. The supernatant was separated and 1 ml of it was taken in a test tube, and into another two test tubes containing 1 ml of water as blank and 1 ml standard glucose solution. 3.5 ml of glucose oxidase reagent was added to each tube at 30 seconds intervals and mixed gently for next 10 seconds. The absorbance was read at 625 nm in a spectrophotometer.

The values were expressed as mg glucose/dl.

2.2.7 Calculation of RBC constants

RBC constants, which provide quantitative information about Red blood corpuscles, were calculated based on the results of RBC, Hb and PCV measurements.

2.2.7.1 *Mean Corpuscular Volume (MCV)*

MCV is the volume of average cell or the average cell volume of all the RBCs.

$$MCV = \frac{PCV\ \%}{RBC\ in\ million/mm3} \times 10 \text{ expressed in } \mu 3$$

2.2.7. 2 *Mean Corpuscular Hemoglobin (MCH)*

MCH is the amount of Hb in the average RBC or average amount of Hb per cell in all the red cells.

$$MCH = \frac{Hb\ (g/dl)}{RBC\ in\ million/mm3} \times 10 \text{ expressed in pg}$$

2.2.7.3 *Mean corpuscular hemoglobin concentration (MCHC)*

MCHC is the portion of the average RBC containing Hb or the concentration in the average cell.

$$\text{MCH} = \frac{\text{Hb (g/dl)}}{\text{PCV (\%)}} \times 100 \text{ expressed in \%}$$

2.3 Statistical analysis

Data were statistically analyzed by three factor ANOVA followed by LSD analysis, using statistical software SPSS-16.

2.4 Results

The results of the present investigation showed various anomalies in the blood of *H. fossilis,* during prolonged exposure to monocrotophos. No significant changes were observed in the measured variables of fish maintained in uncontaminated water (controls). As a result of progressive exposure to three sublethal concentrations of monocrotophos for 7-21 days, *H. fossilis* showed significant decrease in Hb concentration and RBC count (Fig.2.1 and Fig.2.2). After 7 days, the alteration in the measured blood variables was greater in fish exposed to highest concentration of monocrotophos. Packed cell volume (PCV) was increased considerably in a dose dependent manner (Fig. 2.4). Red blood cell indices such as mean cell volume (MCV) and Mean corpuscular hemoglobin (MCH) exhibited alterations during prolonged exposure to monocrotophos (Fig. 2.5 and Fig.2.6). Mean cell volume increased significantly; however, MCHC was reduced significantly compared to the value of control (Fig.2.7). Blood glucose levels of monocrotophos treated fish showed a sharp increase, in a dose dependent manner (Fig. 2.8)

Table 2.1 Hematological parameters of *H. fossilis* subjected to different concentrations of monocrotophos

Parameters		2 ppm		4 ppm		6.6 ppm	
	Control	7th day	21st day	7th day	21st day	7th day	21st day
Hb(g)	12.2841 ± 0.120	11.6410 ± 0.336	11.0444 ± 0.360	11.1073 ± 0.322	10.1923 ± 0.259	9.8395 ± 0.1919	9.0485 ± 0.2334
RBC(Million)	3.0775 ± 0.0945	2.6788 ± 0.0637	2.5274 ± 0.0426	1.9510 ± 0.0460	1.7762 ± 0.0261	1.6916 ± 0.0214	1.6174 ± 0.0569
WBC(Million)	3.7311 ± 0.1186	3.1586 ± 0.1124	3.0627 ± 0.1173	3.0903 ± 0.0842	3.1030 ± 0.0383	3.0134 ± 0.0680	2.8817 ± 0.0517
PCV (%)	42.7216 ± 0.549	44.1677 ± 1.291	45.4452 ± 0.759	46.6167 ± 0.618	47.1377 ± 1.840	48.9577 ± 1.145	50.4142 ± 0.682
MCV (mu)	140.2808 ± 1.34	164.0780 ± 5.91	170.0034 ± 4.06	228.9372 ± 3.06	245.4660 ± 7.53	278.9261 ± 3.67	299.4943 ± 4.99
MCH(pg)	39.3068 ± 0.679	43.7119 ± 1.521	42.4415 ± 1.273	56.6323 ± 1.554	54.6689 ± 1.484	56.9318 ± 1.255	59.7683 ± 1.194
MCH (%)	28.1123 ± 0.561	26.7244 ± 0.551	23.4752 ± 0.764	23.7149 ± 0.381	20.8150 ± 0.501	19.9195 ± 0.248	17.5506 ± 0.359

- Average of six values in each group

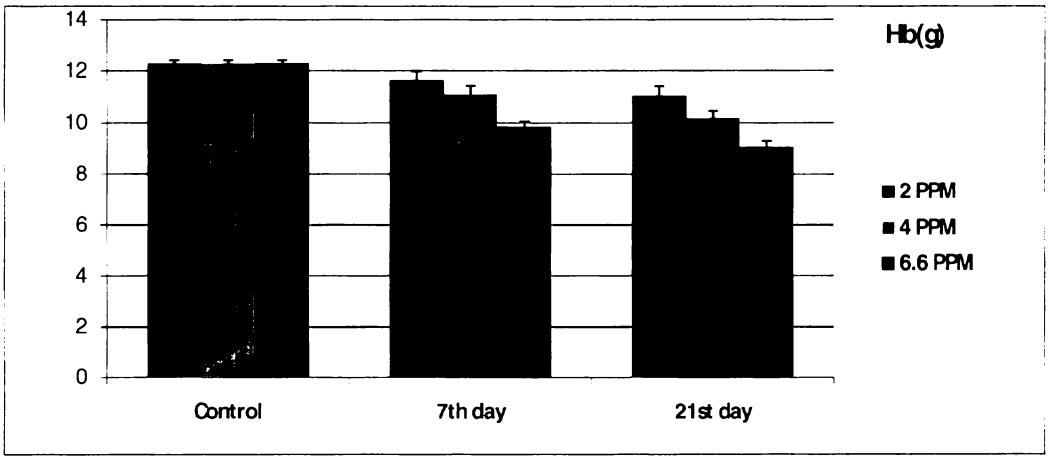


Fig.2.1 Hemoglobin of *H. fossilis* exposed to monocrotophos

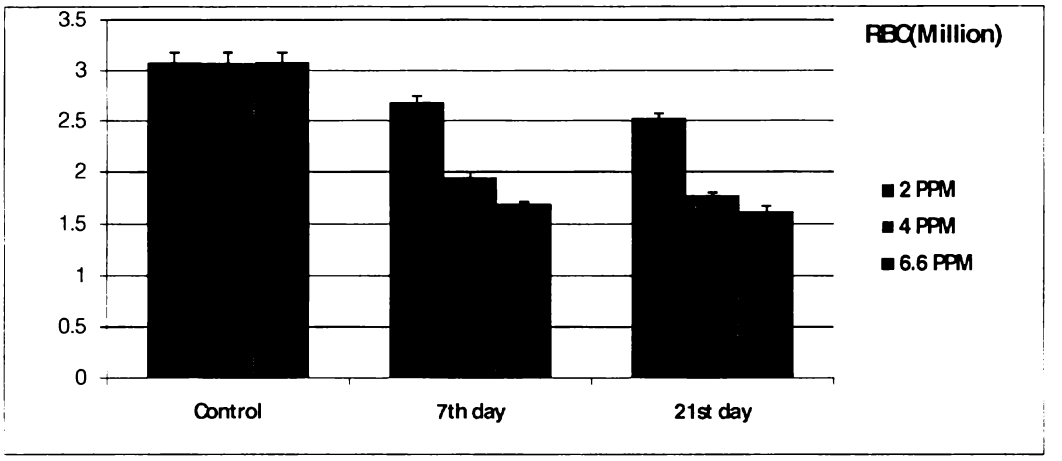


Fig.2.2 RBC of the *H. fossilis* exposed to monocrotophos

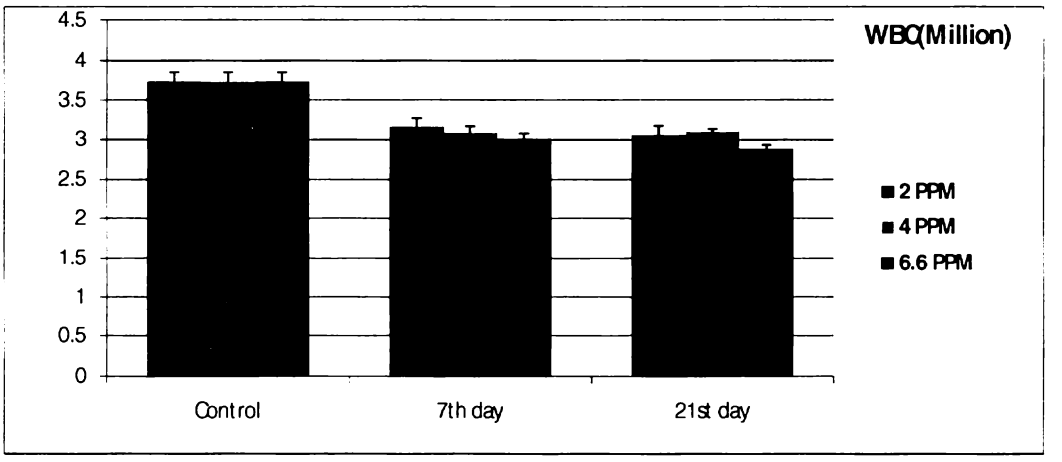


Fig 2.3 WBC of the *H. fossilis* exposed to monocrotophos

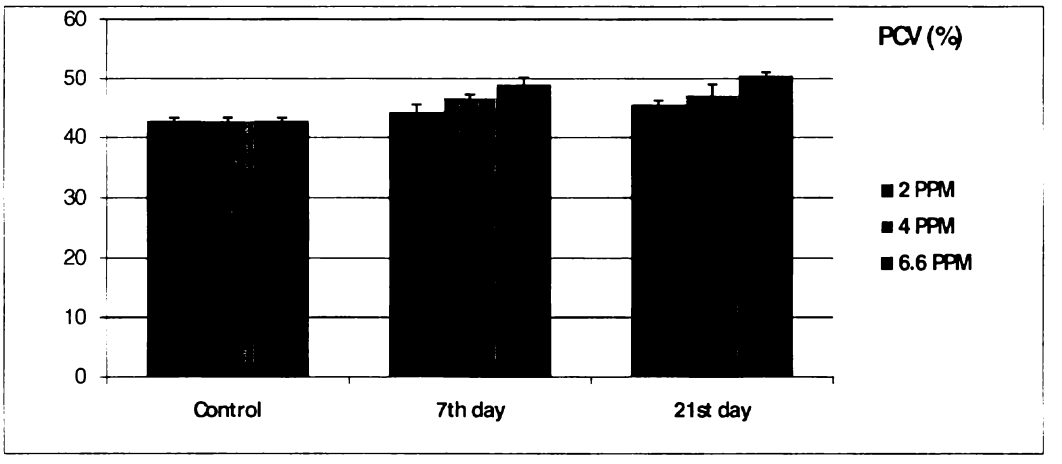


Fig.2.4 Packed Cell Volume of the *H. fossilis* exposed to monocrotophos

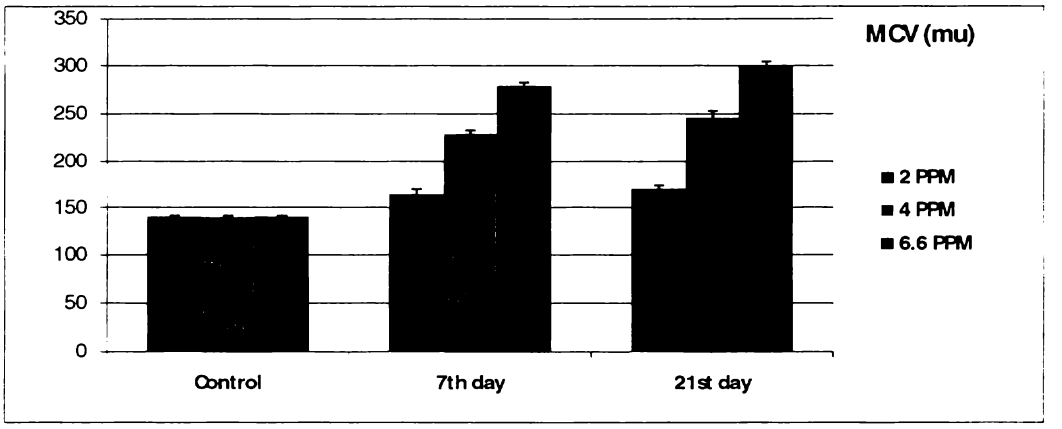


Fig. 2.5 Mean corpuscular volume of the *H. fossilis* exposed to monocrotophos

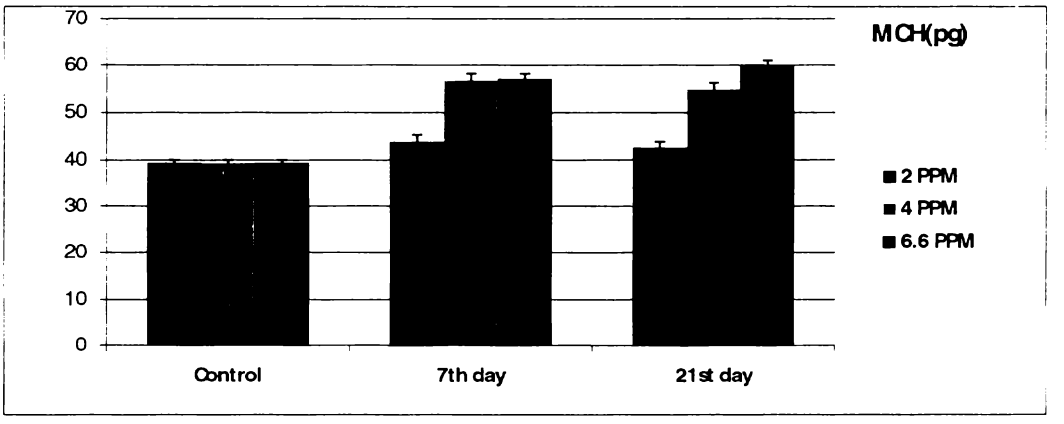


Fig. 2.6 Mean corpuscular haemoglobin of the *H. fossilis* exposed to monocrotophos

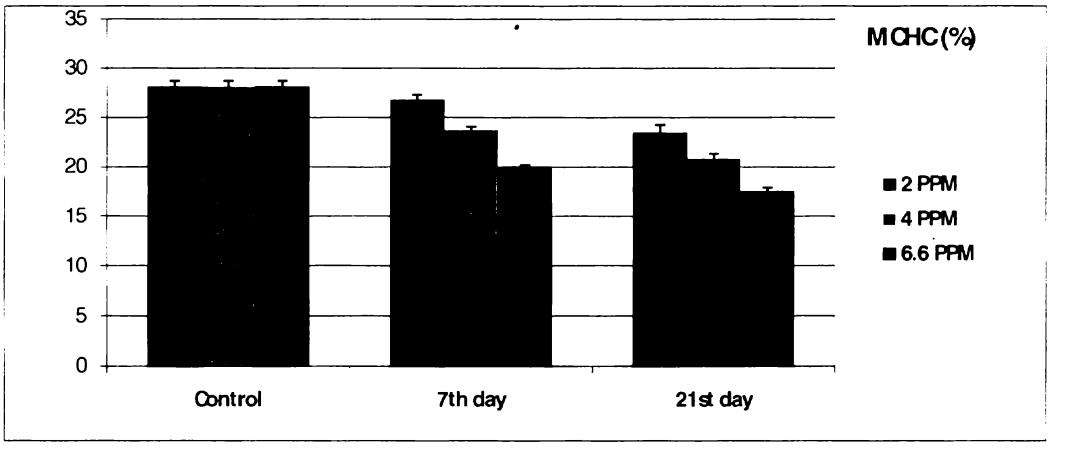


Fig.2.7 Mean corpuscular haemoglobin concentration (%) of the *H. fossilis* exposed to monocrotophos

Table 2.2 ANOVA Table for hematological parameters of *H. fossilis* subjected to monocrotophos

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Parameter	1526460.703	6	254410.117	574.864	.000
Concentration	27906.447	3	9302.149	21.019	.000
Day	127.114	1	127.114	.287	.592
Error	143830.951	325	442.557		
Total	1698325.215	335			

Results Analysis of Variance (ANOVA) procedure for hematological parameters are summarized in table 2.2. Since the p-value corresponding to the factor day is greater than the significance level 0.05, the differences in mean values of 7th and 21st day are not statistically significant. But the difference in mean value for different parameters and concentrations are significant at 0.05 level of significance. Further comparison by LSD analysis shows that difference between the values of different concentration groups and control are statistically significant. That means there is significant difference between 2 ppm and control, 4 ppm and control as well as 6.6 ppm and control. But the difference between each monocrotophos concentration groups, i.e. 2 ppm vs 4 ppm, 2 ppm vs 6.6 ppm and 4 ppm vs 6.6 ppm, is not statistically significant. The results of the LSD analysis for the pair wise comparisons of concentration groups are shown in table 2.6. LSD analysis for the pair wise comparison of different hematological parameters shows that in most cases there is significant difference between different parameter groups. LSD analyses for pair wise comparisons of mean values for different parameters are shown in table 2.5. (The p-values shown in boldface letters correspond to those ones whose mean values are not statistically different)

Table 2.3 Blood glucose content of *H. fossilis* exposed to monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Parameter	Control	7th day	21st day	7th day	21st day	7th day	21st day
Blood	40.5512 ± 0.900	45.3732 ± 1.702	52.9957 ± 1.591	50.7154 ± 0.489	63.5903 ± 1.506	55.8411 ± 1.165	70.2534 ± 2.017

- Values are expressed as mg/100 ml of blood
- Average of six values in each group

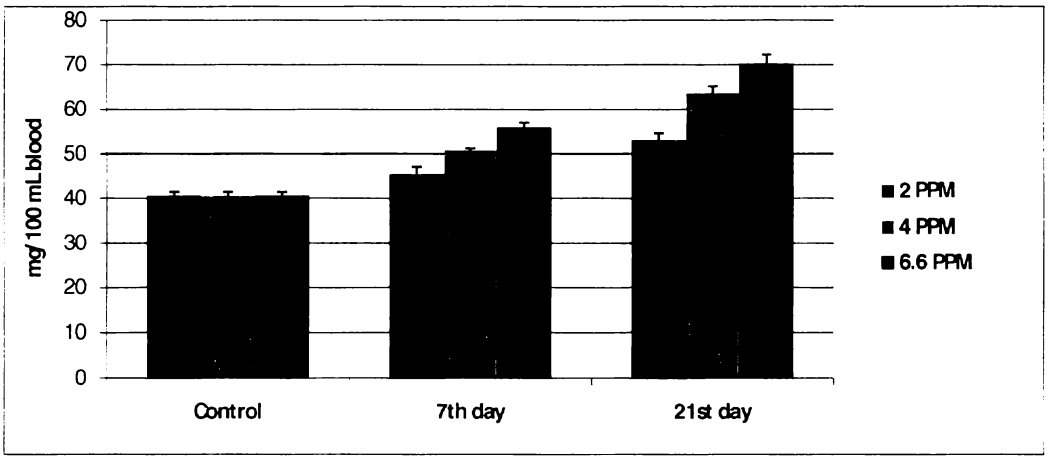


Fig.2.8 Blood glucose levels in *H. fossilis* exposed to monocrotophos

Table.2.4 ANOVA Table for Blood Glucose

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Concentration	3439.924	3	1146.641	108.204	.000
Day	914.017	1	914.017	86.252	.000
Error	455.671	43	10.597		
Total	4809.612	47			

Since the p-value for concentration and the day is less than 0.05, the mean value for each of the concentration and day vary significantly. From summary table 2.3 it can be seen that the recorded values increase with concentration. Since there are only two groups in the case of days there is no need for performing an LSD analysis. The results of the LSD analysis performed for the concentration is summarized in table 2.6. Since the p-values are less than 0.05 for all the pair wise comparisons in the case of blood glucose, it is concluded that values vary significantly from control.

Table 2.5 LSD analysis for pair wise comparison of various parameters

Parameters	p-value
Hb vs RBC	0.045
Hb vs WBC	**0.074**
Hb vs MCV	0.000
Hb vs MCH	0.000
Hb vs MCHC	0.004
RBC vs WBC	**0.830**
RBC vs PCV	0.000
RBC vs MCV	0.000
RBC vs MCH	0.000
RBC vs MCHC	0.000
WBC vs PCV	**0.830**
WBC vs MCV	0.000
WBC vs MCH	0.000
WBC vs MCHC	0.000
PCV vs MCV	**0.830**
PCV vs MCH	0.000
PCV vs MCHC	0.000
MCV vs MCH	0.000
MCV vs MCHC	0.000
MCH vs MCHC	0.000

Table.2.6 LSD analysis for pair wise comparison of various concentrations

	Hematological Parameters	Blood Glucose
Control vs 2 ppm	.000	0.000
Control vs 4 ppm	.001	0.000
Control vs 6.6 ppm	.000	0.000
2 ppm vs 4 ppm	.758	0.000
2 ppm vs 6.6 ppm	.327	0.000
4 ppm vs 6.6 ppm	.198	0.000

2.5 Discussion

The results of the present investigation showed various anomalies in the blood of *H. fossilis*, during prolonged exposure to monocrotophos. The decrease in hemoglobin content observed in this study (Fig.2.1) may be due to the disruptive action of the pesticides on the erythropoietic tissue as a result of which the viability of the cells might be affected. The RBC count showed a decreasing trend (Fig.2.2) with increasing concentrations and exposure time to monocrotophos were attributed to decreased erythropoietic activity. Alterations in the hematological parameters were brought about by OPs as an anemic condition due to decreased synthesis of red blood cells in bone marrow equivalents (Morgan *et al.*, 1980). Svoboda *et al.*, (2001) reported a decrease of non-specific immunity in *Cyprinus carpio* after acute exposure to organophosphorus pesticides.

Decline in Hb concentration as observed in the present investigation can also be due to the decreased rate of production along with increased destruction of RBCs. Decreased erythrocyte count and hemoglobin content in *Cyprinus carpio* after acute exposure to OP insecticide diazinon

were also reported by Svoboda *et al.*, (2001). Changes in erythrocyte profile was induced by acute effect of OP insecticide dichlorvos in *Clarias batrachus* (Benarji *et al.*, 1999), formothion in *Heteropneustes fossilis* (Singh and Srivastava, 1994) and malathion in *Cyprinion watsoni* (Khattak, 1996).

WBCs are inextricably involved in the regulation of immunological functions and a prolonged exposure of *H. fossilis* to monocrotophos may inflict immunological deficiency. Decrease in WBC observed in this study (Fig.2.3) indicates that potential ability of fish to survive any infection or stress is weakened. During stress conditions WBCs undergo autolysis liberating hydrolytic enzymes such as phosphatases, pepsin, trypsin and lipase in the plasma, thereby decreasing the number of WBCs (Wlasow and Dabrowska, 1990).

The increase in MCV of fish exposed to monocrotophos can be due to beta adrenergic stimulation brought by pesticide-exposed stress conditions (Butler *et al.* 1978). Shanthakumar *et al.*, (1999) reported a steady induction in MCV and reduction in MCHC values in *Anabas testudineus* exposed to monocrotophos supports the findings of present investigation. As per available literature, frequently observed haematological responses to the effect of monocrotophos and other organophosphorus compounds were the significant increment of mean corpuscular volume (MCV) associated with increment of PCV and drop of MCHC. Similar trend was observed in the present investigation, with a dose and time dependent increase in PCV (Fig. 2.4) and MCV (Fig.2.5) along with a drop in MCHC (Fig. 2.7).

The increase in PCV observed in this study (Fig.2.4) following exposure to monocrotophos appear to be due to changes in blood cell volume rather than total blood volume, as previously observed by Houston *et al.*, (1971) and Casillas and Smith, (1977). Alterations in PCV values are

directly linked with change in erythrocyte volume. The severe damage to gill observed in monocrotophos treated fishes as explained in the histopathological part of the present study (chapter-5) along with inhibition of branchial ATPase enzyme activity as explained in the chapter-3 might have caused harsh osmoregulatory stress in *H. fossilis*. As demonstrated in previous studies, damage to gill results in hypoxic conditions within the blood of fish (Oris and Giesy., 1985). Gross osmoregulatory stress and hypoxic conditions in the blood of *H. fossilis* must have collectively resulted in the swelling of RBCs which in turn lead to the rise in PCV values and decline in Hb concentrations.

Abrupt drop in blood oxygen content can further influence thyroid function and possibly could have caused considerable increase in noradrenaline production. Increase in noradrenaline can set off the release of more erythrocytes together with immature erythrocytes of much larger size, primarily elevating PCV (Alkindi *et al.*, 1996). As per the observations made by Zbanyszek and Smith (1994); and Davidson *et al.*, (1993) increase in noradrenaline content in the blood can well again explain the increase in PCV.

Stress-induced elevations in blood glucose have been reported in fish earlier by Srivastava and Srivastava (1985). In stressed fish, the release of adrenaline and nor-adrenaline increases to trigger the secretion of catecholamine. Catecholamine increases the conversion of liver glycogen to blood glucose to deliver the greater energy demand by the increased cell metabolism (Srivastava and Srivastava 1985). Somewhat similar may have occurred in *H. fossilis* in the present study, since the blood glucose increased progressively with the increasing concentration of monocrotophos. These changes in the blood glucose level may further be attributed to the changes

in the respiration and activity of the animals, as pointed out by Ghosh (1987).The progressive buildup of the blood glucose suggests a stress-induced hyperglycemic condition in the fish as a result of the incomplete metabolism of blood sugar due to impaired osmoregulation, as suggested by Omoregie *et al.*, (1990).

2.6 Summary

Changes in physical and chemical environmental conditions do affect the physiology of fish and reflect themselves as alterations in peripheral blood profile. Despite the fact that the organophosphorus insecticide pollution of the aquatic environment is a major present day threat, only a little attention have been given to the study of the hematological aspects of their toxicity in fish. Under the light of this study, it can be concluded that exposure to low concentrations of monocrotophos results in significant haematological alterations in stinging catfish, *H. fossilis*. These changes may be potentially disruptive to the survivability of the catfish in their natural environment. This fact should be taken into consideration when monocrotophos is used for pest control in the agricultural fields adjoining their natural freshwater reservoirs.

Chapter 3

EFFECT OF MONOCROTOPHOS ON MEMBRANE BOUND ENZYMES

Contents

Part –A

Effect of monocrotophos on acetyl cholinesterase activity

3. A.1 Introduction

Organophosphate compounds (OPs) continue to occur in the aquatic environment as a consequence of their still widespread usage in agriculture. Concentrations of some of OPs regularly and substantially exceed the maximum permissible concentrations in many countries and are, therefore, indicated as 'priority pollutants'. Due to their ongoing presence in the environment and potential toxicity, OPs comprise a constant hazard for non-target species. Organophosphorus insecticides are known to inhibit acetyl cholinesterase, which plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine to choline and acetate (Eldefrawi, 1985; Soreq and Zakut, 1993). Acetyl cholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are the two enzymes that hydrolyze the neurotransmitter acetylcholine to acetic acid and choline (Chuiko., 2000). The action of OP compounds as insecticides is mainly based on the inhibition of the enzyme acetyl cholinesterase (AChE), which classifies them as compounds with a specific mode of action (Verhaar *et al.*, 1992).

The molecular mechanism of OP toxicity is thought to involve the phosphorylation of the serene hydroxyl group located at the active site of AChE and the formation of an organophosphorus intermediate with AChE (Kwong, 2002) as a consequence of which the inactivated enzyme complex shows little or no activity. The phosphorylated enzyme is much more stable and has a lower rate of hydrolysis and low rate of regeneration of the active enzyme. Due to inhibition of AChE, the neurotransmitter acetylcholine

(ACh) is not hydrolyzed in nerve synapses and in neuromuscular junctions, causing an abnormal amount of ACh at these sites, which leads to over activation of muscular tissue. This over activation subsequently may lead to behavioral effects, such as hyperactivity, asphyxia and finally death. The measurement of AChE inhibition in organisms is widely used as a specific biomarker for exposure to OP insecticides (Walker, 2001).

Behavioral effects of OPs have been determined in quite a lot of studies, even at concentrations that are much lower than those which cause other overt effects. Reduced activity and food intake on exposure to OP insecticide parathion were observed both in fish (Rand, 1977; Banas and Sprague, 1986) and invertebrates (Detra and Collins, 1991) where a positive correlation between AChE inhibition and behavioral responses could be established. Several studies have shown that high levels of AChE inhibition are needed to cause significant mortality in aquatic species, for both acute and chronic exposures (Van der Wel and Welling, 1989; Zinkl *et al.*, 1991; Ansari and Kumar, 1984). Acetyl cholinesterase (AChE, E.C: 3.1.1.7) enzyme is widely used as a biomarker tool to forecast early warning of pesticide toxicity (Dutta and Arends, 2003; Pan and Dutta, 1998).

3.A.2 Materials and methods

Collection, acclimation, dosing experiments, toxicant as well as tissue/serum preparation of/on *Heteropneustes fossilis* was the same as that described in chapter 1. Fishes were exposed to three selected sub lethal concentrations of Monocrotophos for twenty one days and sampling was done on 7^{th} and 21^{st} days intervals to study the effect of monocrotophos on the acetyl cholinesterase activity of *H. fossilis*.

3. A.2.1 Assay of tissue and serum acetylcholinesterase enzyme

Acetylcholinesterase activity in liver, brain, gill and serum was estimated as per the method of Ellman *et al* (1961).

Reagents

Tris-HCl buffer (pH 7.4), 2.6 mM, acetylthiocholine iodide, 0.5 mM DTNB (Ellman's reagent).

Procedure

Brain, liver and gill tissues of both test and control fish were dissected out and were homogenized with 1:5 volume of buffer (tris-HCl 50 mM, KCl 0.15 M, pH 7.4). The homogenate was centrifuged in a refrigerated centrifuge at 10000rpm for 20 minutes at 4°C. AChE activity was determined using the Ellman's reagent DTNB (5, 5"-dithio-bis (2-nitrobenzoic acid); 0.5 mM) and acetylthiocholine iodide as substrate (Ellman *et al.*, 1961). 50 µl of the supernatant/serum was taken for assay, and 2.3 ml of 0.5 mM DTNB was added to it. 100 µl of 2.6 mM ACh was added. The rate of change of absorbance was measured at 412 nm. Blank samples were taken to make sure that there was no non-specific esterase or other background activity. Protein was estimated as described by Lowry *et al.*, (1951) allowing the calculation of AChE as U (mmol/min)/ mg protein.

3.A.3 Statistical analysis

Data were statistically analyzed by three factors ANOVA, followed by LSD analysis using statistical software SPSS-16.

3.A.4 Results

Acetylcholinesterase activities of the monocrotophos treated fish were significantly decreased. The AChE activity in the brain, gill and liver showed a continuous decrease as the exposure progressed. Maximum

decrease was observed at the highest concentration of monocrotophos at 21 days exposure. Decrement of the enzyme activity was more intense as the time of exposure increased.

Table. 3.A.1 Acetylcholinesterase (AChE) activity in liver, brain, gill and serum of *H. fossilis* subjected to monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue & Serum	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	0.1003 ± 0.0027	0.0503 ± 0.0027	0.0396 ± 0.0004	0.0303 ± 0.0006	0.0210 ± 0.0009	0.0184 ± 0.0004	0.0100 ± 0.0003
Brain	0.3536 ± 0.0162	0.1097 ± 0.0029	0.0994 ± 0.0012	0.0909 ± 0.0040	0.0730 ± 0.0028	0.0623 ± 0.0023	0.0446 ± 0.0022
Gill	0.1978 ± 0.0057	0.1008 ± 0.0020	0.0805 ± 0.0032	0.0460 ± 0.0010	0.0297 ± 0.0021	0.0214 ± 0.0007	0.0140 ± 0.0003
Serum	0.2524 ± 0.0106	0.2019 ± 0.0085	0.1296 ± 0.0049	0.1473 ± 0.0046	0.0784 ± 0.0033	0.1084 ± 0.0026	0.0399 ± 0.0015

- Values are expressed as μ mol/mg protein/minute
- Average of six values in each group

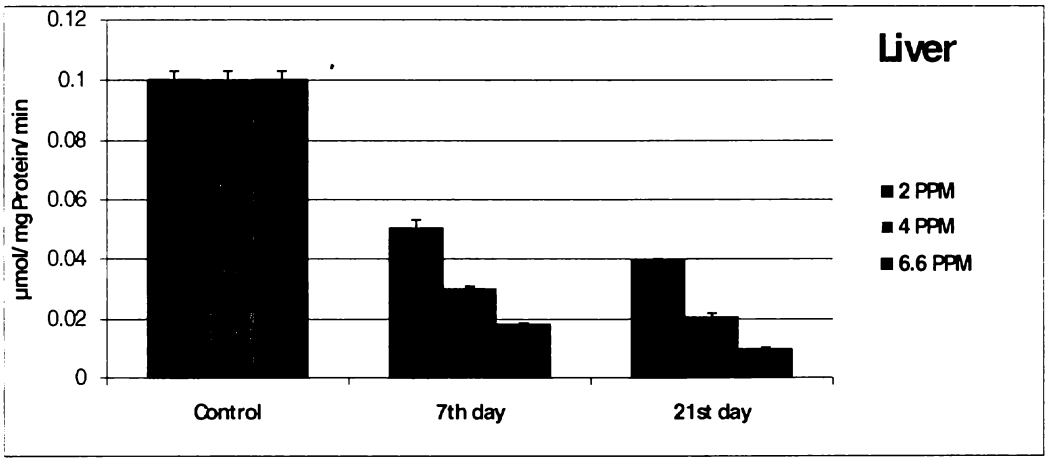


Fig.3.A.1 Activity of acetylcholinesterase (AChE) in the liver of *H. fossilis* subjected to monocrotophos

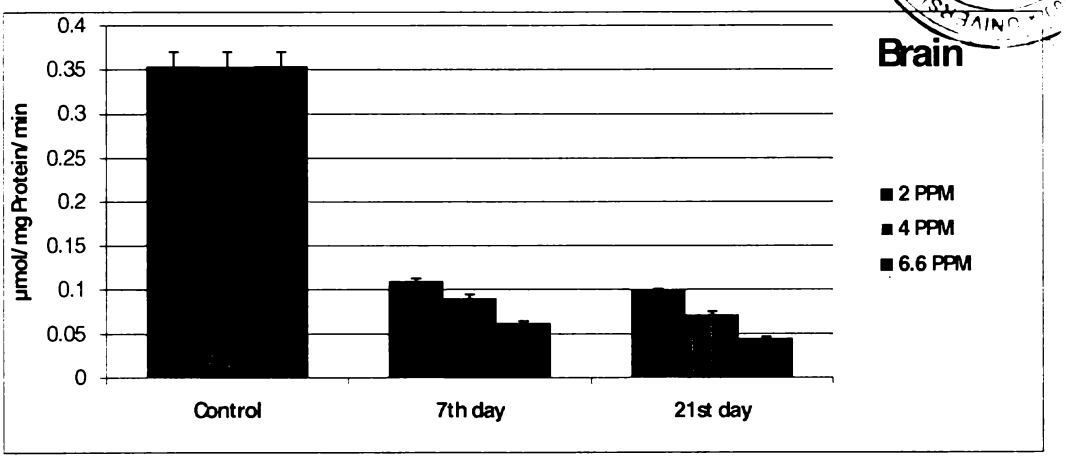


Fig.3.A.2 Activity of acetylcholinesterase (AChE) in the brain of *H. fossilis* subjected to monocrotophos

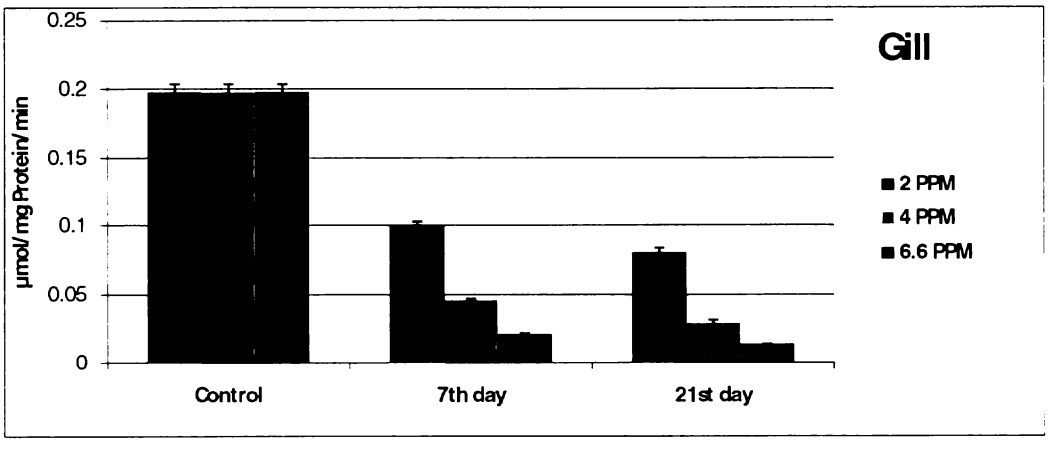


Fig.3.A.3 Activity of acetylcholinesterase (AChE) in the gill of *H. fossilis* subjected to monocrotophos

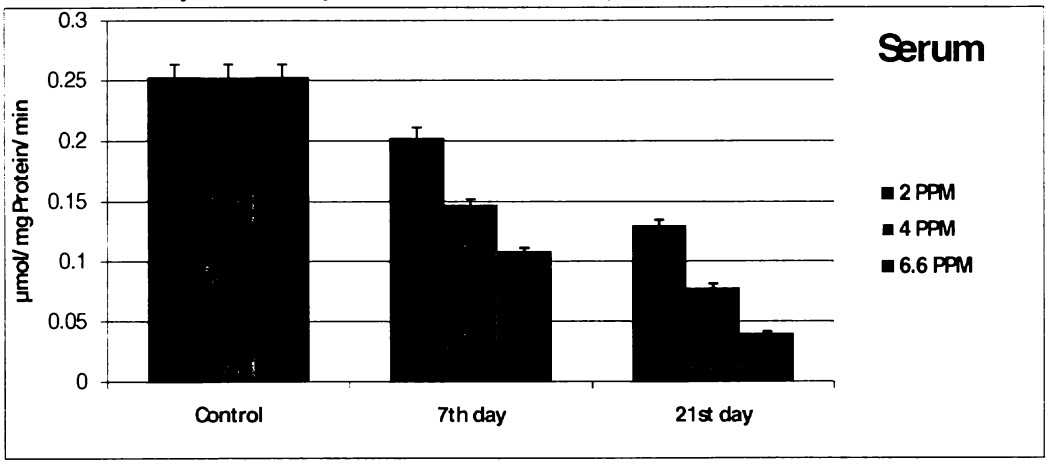


Fig.3.A.4 Activity of acetylcholinesterase (AChE) in the serum of *H. fossilis* subjected to monocrotophos

Table.3.A.2 ANOVA table for acetylcholine esterase (AChE)

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	.374	3	.125	95.401	.000
Day	.984	3	.328	250.678	.000
Concentration	.020	1	.020	15.405	.000
Error	.241	184	.001		
Total	1.619	191			

Since the p-value is less than 0.05, it can be observed that the variables vary significantly with tissue, concentration and day. Further analysis using least significant difference (LSD) procedure is carried out for different tissues and concentration levels. The results are shown in table 3.A.3 and table 3.A.4

Table.3.A.3 LSD analysis for liver, brain, gill and serum

	Liver	Brain	Gill	Serum
Liver		0.000	0.000	**0.694**
Brain	0.000		0.008	0.000
Gill	0.000	0.000		0.000
Serum	**0.694**	0.000	0.000	

Table 3.A.3 gives the p-values of the LSD analysis carried out to check the equality of different types of tissue/serum groups. Here it can be seen that difference between the values of most of the pairs of tissue/serum are statistically significant except for the combination, serum vs liver (p-value=0.694). Further LSD analysis was carried out to check the equality of means for different concentration levels (Table 3.A.4) and it could be seen that all the p-values are less than 0.05 and hence the difference in mean

values between different concentrations, or in other words effect of concentration, are significant even in the case of pair wise comparisons. There is statistically significant difference between the values of different concentration groups.

Table.3.A.4 LSD analysis for different concentrations

	Control	2 ppm	4 ppm	6.6 ppm
Control		0.000	0.000	0.000
2 ppm	0.000		0.000	0.000
4 ppm	0.000	0.000		0.011
6.6 ppm	0.000	0.000	0.011	

3. A. 5 Discussion

This study pointed out significant inhibitory effect of sub lethal exposure to monocrotophos on AChE activity in the brain, liver, gill and serum of *H. fossilis*. A depression was observed in AChE in all exposure groups for the duration of the 21 days of treatment. The maximum reduction in AChE activity of the brain, gill, liver and serum of exposed fish were at the highest concentration of monocrotophos. *H. fossilis* exposed to monocrotophos were able to survive at high percentages of AChE inhibition observed at sub lethal concentrations over 21 days.

Erratic swimming, hyperactivity and convulsions were observed in the test fishes. In general, fish intoxicated with anticholinesterase insecticides show signs of muscle paralysis, especially of the fins and respiratory apparatus, hyperactivity and loss of balance (Sancho *et al.*, 1998). Similar observations are given by Venkateswara Rao, (2006) in *O. mossambicus* exposed to Monocrotophos. Similarly, AChE inhibition in brain, was

observed earlier, when the fish was exposed to other OP insecticides like chlorpyrifos and profenofos (Kumar and Chapman, 2001; Rao *et al.*, 2003a; Rao *et al.*, 2003b). The effect of Malathion, Diazinon and Endosulfan on brain acetyl cholinesterase activity in bluegill sunfish and the largemouth bass was investigated (Dutta *et al.*, 1992; Richmonds and Dutta, 1992; Dutta *et al.*, 1995; Guozhong and Dutta, 1998; Dutta and Arends, 2003) and found a remarkable reduction in the activity which influenced the optomotor behavior of the fish that could be detrimental to their existence in the environment.

Maximum reduction was observed in the brain of the test fish in all time intervals for all dosage levels. Shaoguo ru *et al.*, (2003) reported that monocrotophos inhibited AChE activity in Red Drum *Sciaenops Ocellatus* and the inhibition was progressed in a dose dependent manner. Chronic exposure to an array of OP compounds significantly depressed the AChE levels in the brain of *Cyprinus carpio* (Dembele *et al.*, 2000) supports the present observation. Blood-brain barrier (BBB) selectively limits the rates of transfer of soluble substances between blood and brain, isolating brain from other tissues (Magistretti, 1999). How ever the brain is very susceptible to numerous environmental stressors (Uner *et al.*, 2006). OP Pesticides may cause neurotoxicity due to their ability to cross the BBB and exert toxic effects on the central nervous system. OP compounds are in general lipophilic and therefore cross the BBB in most cases (Brzezinski and Ludwicki, 1973). Tomokuni *et al.*, (1985) observed the accumulation of an OP insecticide diazinon in the brain of rats and mice after single intra peritoneal injection. They found also that brain AChE activity was markedly inhibited after injection.

Inhibition of AChE goes together with an increase in acetylcholine levels (Brzezinski and Ludwicki, 1973). This situation can lead to increase of catecholamines which can affect the activity of enzymes involved in glycogenolysis and glycogen synthesis (Uner *et al.*, 2006). Glycogen depletion observed in this study (chapter-1) can be correlated to the inhibition of AChE activity by monocrotophos. Continuous stress may affect the synthesis site of AChE or reduce the levels of AChE. Mortality of fishes observed in other studies may be due to inhibition of other enzymes, particularly those involved in carbohydrate and protein metabolisms. The inhibitory effects on AChE activity indicates that insecticides might hamper crucial processes like energy metabolism of nerve cells (Ansari and Kumar, 1984). Yang *et al.*, (1996) in their study with an OP compound DVP, pointed out that AChE inhibitor-induced cholinergic hyperactivity initiates the accumulation of free radicals leading to lipid peroxidation, which may be an initiator of AChE inhibitor-induced cell injury. In chapter 2 we have observed a significant elevation of brain malonaldehyde at both 7th and 21st day intervals of exposure. Similarly, Hazarika *et al.*, (2003) observed that LPO induced by OPs malathion and anilophos appears to be mediated through activation of cholinergic receptors.

The inhibition of AChE activity in fish can be hazardous since it will affect feeding capability, swimming activity, identification and avoidance of predators and spatial orientation of the species (Balint *et al.*, 1995; Pan and Dutta, 1998). This also has been observed in many other fishes exposed to OP compounds (Hai *et al.*, 1997; Sancho *et al.*, 2000; Uner *et al.*, 2006) which can be unfavorable to the endurance of fish in an otherwise competitive environment.

3. A.6 Summary

AChE is potentially the most reliable indicator in monitoring monocrotophos pollution of freshwater environments. Monocrotophos is one of OPs containing Oxon structure, which can inhibit the brain AChE more effectively. Biomarkers like AChE activity can be useful as indicators of exposure to chemicals, which have relatively short-half-lives, like organophosphate pesticides. An understanding of how pesticides may influence the enzyme in fishes associated with agriculture areas like paddy fields where pesticides are extensively applied is important to further characterize the role these chemicals may play in the decline of groups such as stinging catfishes.

Part –B

Effect of monocrotophos on Branchial ATPases

3.B.1 Introduction

The gill epithelium is the dominant site of gas exchange, ionic regulation, acid-base balance and nitrogenous waste excretion for fishes, thereby serving a multitude of vital functions for these aquatic animals. Most aquatic animal cells preserve a high intracellular K^+ and low intracellular Na^+ concentration which involves the movement of these ions across the cellular membranes against an electrochemical gradient by active transport process. The uptake by cells, of metabolites such as glucose and amino acids, regeneration of transmembrane potential during nerve excitation, transmembrane movement of Ca^{2+} during muscle stimulation, safeguarding of osmotic equilibrium in cells and control of transcellular ion movement requires the steady maintenance of Na^+ and K^+ gradient (Haya *et al.*, 1985). Gills are the main osmoregulatory surface organ in aquatic animals and are the primary site of uptake of waterborne pollutants. Therefore, gills may be the first site where the sub lethal effects of chemicals would be observed.

Adenosine triphosphatases (ATPases) are a group of enzymes that play an important role in intracellular functions and are considered to be a sensitive indicator of toxicity (Yadwad *et al.*, 1990). They hydrolyze adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate. In this process the energy released becomes available for cation transport (Schuurmans and Bonting., 1981). ATPases are membrane bound enzymes responsible for the transport of ions through biological membranes and thus regulate cellular volume, osmotic pressure, and membrane permeability (Sancho *et al.*, 2003). ATPases are reported to be among the

sensitive bioindicators used for assessing the fragility of branchial membranes. (Stagg *et al.*, 1992). The membrane bound transport enzyme Na^+, K^+-activated ATPase is an integral part of active transport mechanism for cations across the cell membrane (Das and Mukherji., 2000). It is involved in active ion uptake or excretion in both freshwater and seawater adapted fish. Fish are able to uptake and retain different xenobiotics dissolved in water *via* active or passive processes. Freshwater teleost fishes, actively intake ion to maintain homeostasis. In fish, intake of Ca^{2+} , Cl^- and Na^+ ions from the water has greatly been attributed to mitochondria rich chloride cells or ionocytes. (McCormick, 1995; Perry, 1997). A number of studies have demonstrated that the basolateral aspect of the chloride cells contains high concentrations of the transport enzyme, Na^+, K^+ activated ATPase. Freshwater teleosts (and presumably all gilled, fresh water vertebrates) extract needed NaCl from the medium *via* parallel, antiport systems involving exchange of intracellular H^+ or NH_4^+ for external Na^+ and HCO_3 (or possibly OH^-) in exchange for external Cl^-.

Xenobiotics can interact directly with the enzyme or alter Na^+, K^+ - ATPase activity due to disruption of energy producing metabolic pathway (Watson and Beamish, 1980). To preserve ionic homeostasis during exposure to pollutants, fish activates a number of biochemical and physiological processes of detoxification. It has been suggested that assessment of gill ATPases can be used as early warning signals of pollutant induced damage to the osmoregulatory and acid-base regulatory system in gills (Stagg *et al.*, 1992). Environmental organic pollutants usually affect the Na^+, K^+ - ATPase by decreasing its activity (Haya *et al.*, 1985). Interaction of environmental pollutants with ATPases evoked a good deal of interest because the inhibition of this enzyme occurs before gross osmoregulatory dysfunction. This would point to the use of ATPases as a forewarning

indicator of pollutant induced damage to the ionic and osmoregulatory system (Stagg *et al.,* 1992). The ATPase system in fish seems to be a sensitive enzymatic bioindicators for pesticides (Ghosh, 1989; Reddy and Philip, 1994; Sancho *et al.,* 1997).

Although OP insecticides tend to undergo fairly rapid degradation in the environment, with frequent input into the aquatic environment, organisms may be exposed to sub lethal concentrations of the insecticide for an extended period. There has been little study of the effects of monocrotophos on gill ATPase activity of *Heteropneustes fossilis* an important paddy fish of India and South Asia. This study was aimed to explore the effect of different sub lethal concentrations of organophosphorus insecticide monocrotophos on ion transport mechanisms, in particular the ion-dependent Na^+, K^+ - ATPase and Mg^{2+} ATPase of gills of *H. fossilis.* Gills are the main entry points of toxicants, and may be one of the first organs to exhibit symptoms of toxicity. Due to their large surface area and permeability, gills are the primary sites for absorption of xenobiotics including organophosphorus insecticides like monocrotophos.

3.B.2 Materials and Methods

Collection, acclimation and dozing experiments of/on *Heteropneustes fossilis* were the same as that described in chapter 1. Fishes were exposed to three selected sub lethal concentrations of monocrotophos for 21 days and sampling was done every 7^{th} and 21^{st} day intervals to study the effect of toxicant on the branchial ATPase enzymes of *H. fossilis.* The gill tissue was taken from test as well as control organisms, washed in 0.33 M ice cold sucrose and 10% of tissue homogenate was prepared in the ice cold 0.33 M sucrose solution.

Extraction of the enzyme

10% of the gill homogenate was centrifuged at 3000g for 15 minutes and the supernatant obtained was yet again centrifuged in a cold refrigerated centrifuge at 12000 g for 30 minutes. The clear supernatant was over again centrifuged at 35000 g for 30 minutes. The pellet obtained corresponds to the heavy microsomal fraction (Davis, 1970). The pellet was re-suspended in cold 0.33 M sucrose and used as the source of enzyme.

Assay of Na^+, K^+ - ATPase activity (ATP phosphohydrolase)

Reagents

60mM NaCl,

20 mM KCl,

2mM $MgCl_2$,

30 mM Tris-HCl (pH 7.5)

2.5 mM ATP

10% TCA.

Procedure

Na^+, K^+ activated Mg^{2+} dependent ATPase was determined by using the reaction mixture containing 60mM NaCl, 20 mM KCl, 2mM $MgCl_2$, 30 mM tris-HCl (pH 7.5) and 2.5mM ATP. The Mg^{2+} ATPase was measured by substituting sucrose in place of NaCl and KCl. The reaction mixture was incubated at 37°C for 15 minutes. The Na^+, K^+ - ATPase activity was calculated in terms of the difference between the values of total and Mg^{2+} ATPases. After the incubation 2 ml of 10%

trichloro acetic acid was added to the reaction mixture. The mixture was subjected to centrifugation at 1300 g for 10 minutes and the supernatant was separated. The inorganic phosphate liberated from ATP was estimated by the method of Fiske and Subbarow (1925). The specific activity of Na^+, K^+ - ATPase was defined as micromoles of Pi / mg of enzyme protein / hour. Protein was estimated by following the method of Lowry *et al.*, (1951).

3.B.3 Statistical analysis

The obtained data were statistically analyzed by three factor ANOVA followed by LSD analysis using statistical software SPSS-16

3.B.4 Results

The activity profile of Na^+, K^+ - ATPase, Mg^{2+} -ATPase, and total ATPases in the gill of control and test catfishes are listed in Table 3.B.1. Control group of fish did not reveal any significant difference through out the experimental period with respect to gill ATPase activity. Na^+, K^+ ATPase activity was found to be significantly decreased at both 7^{th} and 21^{st} days of exposure (Fig.3.B.1).

Mg^{2+} -ATPase activity also decreased significantly after 7 and 21 days of exposure to monocrotophos (Fig.3.B.2). There was a significant decrease of total ATPase activity in the gill tissue at 7^{th} and 21^{st} days of exposure (Fig. 3.B.3).

Table.3.B.1 Branchial ATPases activity in *H. fossilis* subjected to monocrotophos

	Total ATPase		$Na^+ K^+$ ATPase		Mg^+ ATPase	
Concentration	7th day	21st day	7th day	21st day	7th day	21st day
Control	39.0596 ± 0.445	38.0096 ± 0.764	19.1992 ± 0.422	17.4703 ± 0.268	20.1573 ± 0.599	19.8540 ± 0.527
2 ppm	34.3192 ± 0.568	29.2644 ± 0.479	16.2105 ± 0.343	14.9537 ± 0.446	17.9227 ± 0.763	14.3889 ± 0.531
4 ppm	28.1717 ± 0.510	19.9322 ± 0.297	14.3610 ± 0.398	10.0199 ± 0.328	14.1422 ± 0.221	9.9890 ± 0.2455
6.6 ppm	22.4793 ± 0.583	15.4533 ± 0.244	10.5889 ± 0.222	6.9113 ± 0.2896	12.0170 ± 0.218	8.5315 ± 0.3118

c

- Values are expressed as μmol of Pi liberated/mg protein/hour
- Average of six values in each group

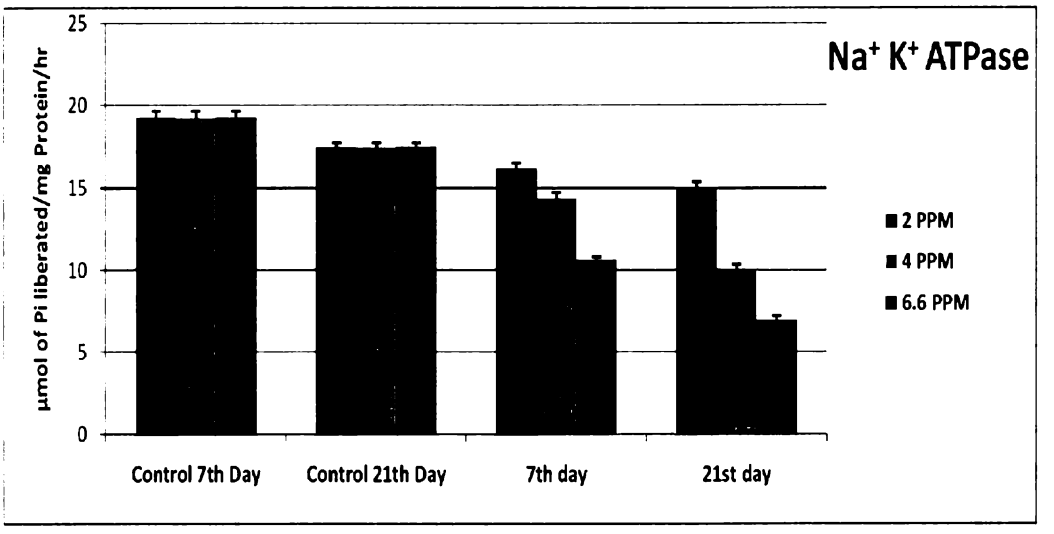


Figure.3.B.1 Activity of Na^+, K^+ - ATPase in *H. fossilis* subjected to monocrotophos

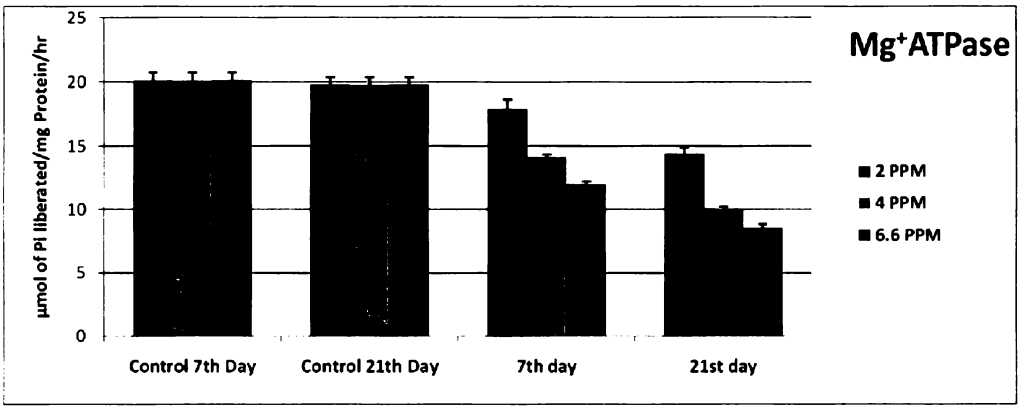


Figure.3.B.2 Activity of Mg^{2+} ATPase in *H. fossilis* subjected to monocrotophos

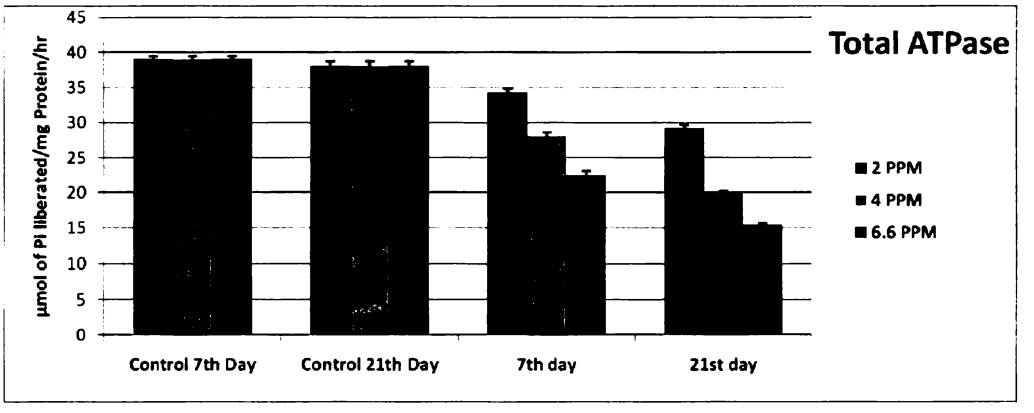


Fig.3.B.3 Activity of Total ATPase in *H. fossilis* subjected to monocrotophos

Table.3.B.2 ANOVA table for ATPases in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Concentration	3496.559	3	1165.520	233.665	.000
ATPase	6441.826	2	3220.913	645.732	.000
Day	480.721	1	480.721	96.376	.000
Error	683.356	137	4.988		
Total	11102.462	143			

A three way Analysis of Variance procedure was carried out to see the effect of concentration, ATPase and day on the recorded value (Table.3.B.2). Since the p-values corresponding to each of these factors are less than 0.05, there is a significant difference in the recorded values for various levels of the factors. The recorded values show a decreasing trend from control to 6.6 ppm. It means that there is significant reduction in values of ATPases as the concentration increases, giving maximum reduction at 6.6 ppm. A Least Significant difference (LSD) procedure to test the significance of difference between each levels of the factors are performed for different ATPases and the concentration and the results are summarized in table 3.B.3 and table 3.B.4 respectively.

Table. 3. B.3 LSD analysis for different ATPases

	p-Value
Total ATPase vs.Na+ K^+ ATPase	0.0000
Total ATPase vs. Mg^+ ATPase	0.0000
Na^+ K^+ ATPase vs Mg^+ATP ase	0.0480

Results of the LSD analysis for different ATPase are shown in figure 3.B.3. Since the p-values are less than 0.05 it is statistically evident that the variation between control and different concentrations are statistically significant. There is significant difference between the values of different ATPases groups also.

Table.3.B.4 LSD analysis for different concentrations

	p-Value
Control vs 2ppm	0.0000
Control vs 4ppm	0.0000
Control vs 6.6 ppm	0.0000
2 ppm vs. 4 ppm	0.0000
2 ppm vs. 6.6 ppm	0.0000
4 ppm vs. 6.6 ppm	0.0000

Since the p-values for each of the pair wise comparisons are less than 0.05 we conclude that all the levels vary from each other.

3.B.5 Discussion

Xenobiotic induced Na^+, K^+ - ATPase inhibition may result in detrimental effects in organism. Two different mechanisms are thought to lead to Na^+, K^+ - ATPase inhibition. These are: 1) direct effect of reactive oxygen species and malonaldehyde; 2) an indirect effect of changes in membrane fluidity. On the other hand, inhibition in ATPase activities initiates free radical production leading to lipid peroxidation (Rauchova *et al.*, 1999). Na^+, K^+ - ATPases are membrane bound enzymes and their activities depend on the phospholipids of the membrane. Therefore, any change in the lipid component of the membrane will directly affect the activities of these enzymes. From chapter- 4, it has become apparent that monocrotophos toxicity resulted in a chain of reactions in *H. fossilis* that lead to failure in antioxidant machinery and subsequently high levels of lipid peroxidation. Although the main mode of action of organophosphorus pesticides including monocrotophos is AChE inhibition, increase in lipid peroxidation and changes in ATPase activity are reported to mediate the toxicity of organophosphates (Hazarika and Sarkar, 2001). It can be understood that decrease in branchial Na^+, K^+ - ATPase in the monocrotophos treated group of fishes can be due to high levels of peroxidation of membrane lipids and the reduction in activity was found to be dose dependent. The effects of lipid-soluble pollutants may often be due to their ability to dissolve in, and alter the integrity of cell membrane, and modification of the permeability of membrane fluid might have influenced the activities of membrane-bound enzymes.

The observed decrease in the activity of Na^+, K^+ -ATPase and Mg^{2+} ATPase (Table.3.B.1) in the gills of pesticide-treated fish in this study

could be the result of direct action of the toxicant on the enzymes. This finding supports the idea that the pesticides can directly interfere with the membrane enzymes. The inhibition of ATPase activity may also be due to primary lethal lesions in gills. Histopathological observations of gills (Chapter-5) carried out as a part of this work proves this to be true, as most of the treated fish gills were severely damaged.

Since Na^+, K^+ - ATPase plays a key role in osmoregulation and maintaining Na^+, K^+ transmembrane gradients, its inhibition may change the ionic concentration of blood and may damage the gill tissue. It is understood that xenobiotics interact with membrane proteins, thereby destabilizing membrane-bound enzymes. Yadwad *et al.,* (1990) suggested that pesticides could interact with membrane-bound enzymes and inhibit ATPase activity.

Mg^{2+} ATPase enzyme is found in association with Na^+, K^+ - ATPase in fish, related to the transport of Mg^{2+} across the gill epithelium, and is also essential for the integrity of the cellular membrane and for the stabilization of branchial permeability (Reddy and Philip, 1994). Mg^{2+} ATPase is responsible for trans-epithelial regulation of Mg^{2+} ions, which are essential to the integrity of the cellular membrane, to the intracellular cements, and to the stabilization of branchial permeability. Inhibition of Mg^{2+} ATPase activity by OP compounds including monocrotophos may reduce ATP production as this enzyme has been reported to be involved in oxidative phosphorylation (Racker *et al.,* 1975).

Inhibition of Mg^{2+} ATPase activity in *Labeo rohita* exposed to cypermethrin (Ghosh, 1989) and the ATPase system of *Cyprinus carpio* under OP stress (Reddy and Philip, 1994) also exemplify the results obtained in the present experiments. The present results suggest that

monocrotophos, even in sub lethal doses, affects the physiological process of osmoregulation in *H. fossilis.* Monocrotophos seems to have altered the ionic profiles, Na^+, K^+ ATPase in particular, thus indicating possible perturbations in the ATPase system along with disruption in the movement of ions across the ionic pumps. Inhibition of ATPase activity could be expected to have metabolic or ionic effects in fish in relation to osmoregulation (Verma *et al.,* 1988).

3.B.6 Summary

Sublethal exposure of *H. fossilis,* to the pesticide monocrotophos produced a significant inhibition of Mg^{2+} and Na^+, K^+ -ATPase activities in gill tissues. In freshwater fishes Mg^{2+} ATPase and Na^+, K^+ - ATPase play a significant role in ionic regulation and aid in salt uptake from the ambient medium (Pfeiler and Kirschner, 1972). Hence, inhibition of these activities in the gill of fish exposed to monocrotophos indicates interruption in its cellular and ionic regulation and salt uptake and could be expected to have metabolic or ionic effects in fish in relation to osmoregulation (Verma *et al.,* 1988).

Part –C

Effect of monocrotophos on lysosomal membrane stability

3.C.1 Introduction

Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms from yeast to humans. Their function in the cellular economy includes the degradation of redundant or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer lived proteins as part of autophagic cellular turnover (Klionsky and Emr., 2000). Lysosomes are also involved in the digestion of materials ingested by endocytosis and phagocytosis (i.e., intracellular digestion). Lysosomal stability is a good indicator of physiological fitness in fish liver (Allen and Moore, 2004). Adverse lysosomal reactions appear to provide practical and helpful biomarkers that are diagnostic for cell injury and alleged indicators for further pathology (Moore, 1990). The functional stability of the lysosomal membrane is a good indicator of cellular integrity and has been used extensively to evaluate responses to environmental perturbation in fish and molluscs (Hwang *et al.*, 2002; Kohler *et al.*, 2002; Lowe *et al.*, 1995; Moore, 2002; Allen and Moore, 2004; Moore *et al.*, 2004). Lysosomes mount up a wide variety of chemical contaminants including pesticides which results in damage of the membrane and leakage of acid hydrolases housed inside, to cytosol and ultimate destruction of cell. Thus the effects of toxicants on lysosome membrane can be assessed by measuring the activity of released enzymes.

Although biomarkers indicative of exposure to pollutants are useful, those indicating deleterious effects on individuals are more ecologically relevant (Depledge, 1994). In this context, lysosomal responses i.e.

lysosomal enlargement and lysosomal membrane destabilization have demonstrated to be one of the most reliable and sensitive biomarkers (Cajaraville., 2000).

The sensitivity of lysosomes to environmental pollutants including organophosphorus insecticides suggests that the lysosomal responses can be considered as early warning systems for detection of the disturbances in the surroundings. The lysosomal stability is measured in terms of lysosomal enzymes assay and the measuring of lysosomal perturbations is a good and reliable tool that can be recommended for biomonitoring (Kohler, 1991). This biomarker can also be used to predict liver damage and tumor progression in fish liver, as well as enhanced protein turnover (i.e. lysosomal autophagy), as a result of radical attack on proteins, and energetic status, as predictive indicator of fitness of individuals within a population.

In this study an attempt has been made to assess the integrity of lysosomal membrane of *Heteropneustes fossilis* exposed to sub lethal concentrations of monocrotophos, by assaying major hydrolases viz. acid phosphatase and ß Glucuronidase, to evaluate its toxic potential, and ascertain the efficacy of lysosomal enzyme assay as a sensitive biomarker for organophosphorus insecticide toxicity.

3.C.2 Materials and methods

Collection, acclimation, maintenance and experimental set up for bioassay is the same as mentioned in chapter-1 (1.2.1, 1.2.2, 1.2.3, 1.2.4)

Preparation of various sub cellular fractions

Liver of both control and test fishes was homogenized in 0.33 M sucrose at 0° C (10%) and the homogenate was centrifuged at 600 g for 10 minutes in high speed refrigerated centrifuge. Sediment of nuclei, unbroken

cells and plasma membranes (nuclear fraction) was separated. Supernatant was again centrifuged at 15000 g for 30 minutes. 15000g sediment (lysosomal fraction) and nuclear fraction were resuspeneded in citrate buffer containing 0.2% Brij-35. Soluble fraction was diluted with equal volume of double strength buffer. Activity of ß-glucuronidase and acid phosphatase was determined in all the three fractions obtained. (Plummer, 1987).

Assay of Acid phosphatase (Anon, 1963)

P-nitrophenyl phosphate (0.5 ml) was mixed with equal volume of 0.1 M phosphate buffer (pH 4.8) in three different tubes, and the three sub cellular fractions added and incubated for 30 minutes at room temperature. The reaction was arrested by adding 0.1 N NaOH. The absorbance of solution was measured spectrophotometrically at 410 nm. The amount of p-nitro phenol liberated by the acid phosphatase per hour per mg protein gives the specific activity. Protein was determined as per the method of Lowry *et al.*, (1951).

Assay of ß glucuronidase (Kawai and Anon, 1971)

P-nitrophenol- ß-D-glucuronide in 0.1 M citrate buffer (pH 4.5) was used as the substrate. Reaction system containing substrate and enzyme source was incubated for 30 minutes at room temperature. The reaction was stopped by adding 0.2 N Na_2CO_3 solution. The absorbance was read spectrophotometrically at 400 nm. The enzyme activity is expressed in terms of µg of P-nitrophenol liberated per hour per gm protein, using P-nitrophenol as standard.

3.C.3 Statistical analysis

Data were statistically analyzed by three factor ANOVA test, followed by pair wise comparison by LSD analysis, using statistical software SPSS-16.

3.C.4. Results

Both ß-glucuronidase and acid phosphatase activities of lysosomal fractions were lower than that of the control. Nuclear fraction also showed a slight increase. ß-glucuronidase activity and acid phosphåtase activity of the soluble fraction was giving an increasing activity. The reduction as well as induction of enzyme activities was in dose dependent manner. Maximum reduction of lysosomal fraction occurred at 6.6 ppm. At both 2 ppm and 4 ppm concentrations activities of both enzymes of lysosomal fraction showed distinct decrease.

Table.3.C.1 Summary table for ß-glucuronidase Activity

	ß-Glucuronidase Activity in Hepatic Tissue		
Group	**Nuclear Fraction**	**Lysosomal Fraction**	**Soluble Fraction**
Control	5.2028 ± 0.1056	34.1646 ± 0.783	16.0539 ± 0.444
2 ppm	6.9571 ± 0.1483	30.9292 ± 0.752	18.3907 ± 0.470
4 ppm	8.3985 ± 0.2373	25.9905 ± 0.297	20.5651 ± 0.536
6.6 ppm	9.2114 ± 0.2497	18.1060 ± 0.634	27.5291 ± 0.583

- Values are expressed as mg p-nitrophenol liberated/g protein/hr
- Average of six values in each group

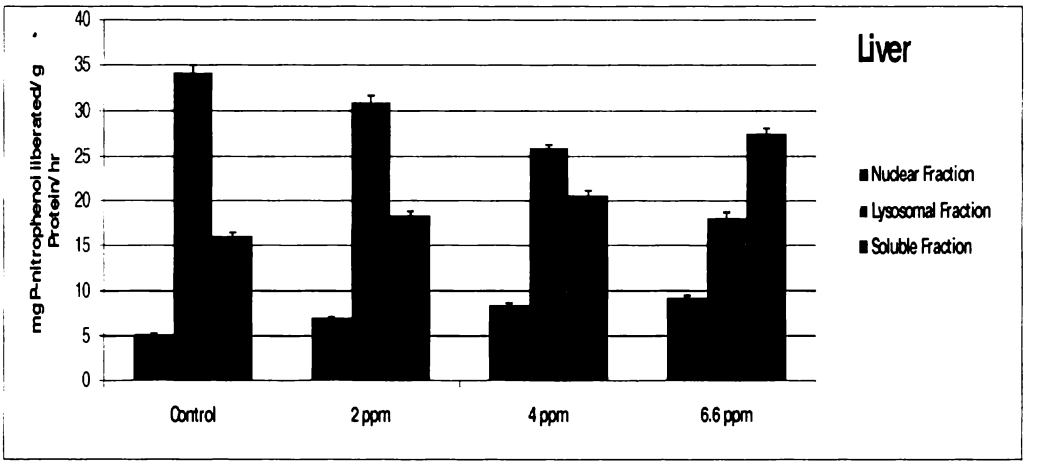


Figure.3.C.1 ß-Glucuronidase activity in the liver of *H. fossilis* exposed to monocrotophos

Table. 3. C. 2 ANOVA table for ß-glucuronidase activity in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Concentration	2.545	3	.848	0.040	0.989
ß-Glucuronidase Activity	4901.240	2	2450.620	116.515	0.000
Error	1388.152	66	21.033		
Total	6291.937	71			

Since the p-value is less than 0.05 for the ß-Glucuronidase activity, it is statistically evident that there is significant difference between various ß-glucuronidase activity groups. From table 3.C.1 it is evident that lysosomal fraction has high recorded value comparing to other counter parts. Since the p-value corresponding to concentration in the ANOVA table 3.C.2 is 0.989, it is statistically proven that there is no significant difference between for different concentration groups.

Table.3.C.3 Acid phosphatase activity in subcellular fractions of hepatic tissue

	ACP Activity in Hepatic Tissue		
Concentration	Nuclear Fraction	Lysosomal Fraction	Soluble Fraction
Control	9.1794 ± 0.3364	17.4543 ± 0.284	9.3320 ± 0.4209
2 ppm	11.6574 ± 0.127	13.2130 ± 0.539	13.5153 ± 0.442
4 ppm	13.0113 ± 0.233	9.2170 ± 0.3354	19.0170 ± 0.574
6.6 ppm	19.1535 ± 0.646	5.5717 ± 0.2744	21.7975 ± 0.528

- Values are expressed as mg p-nitrophenol liberated/g protein/hr
- Average of six values in each group

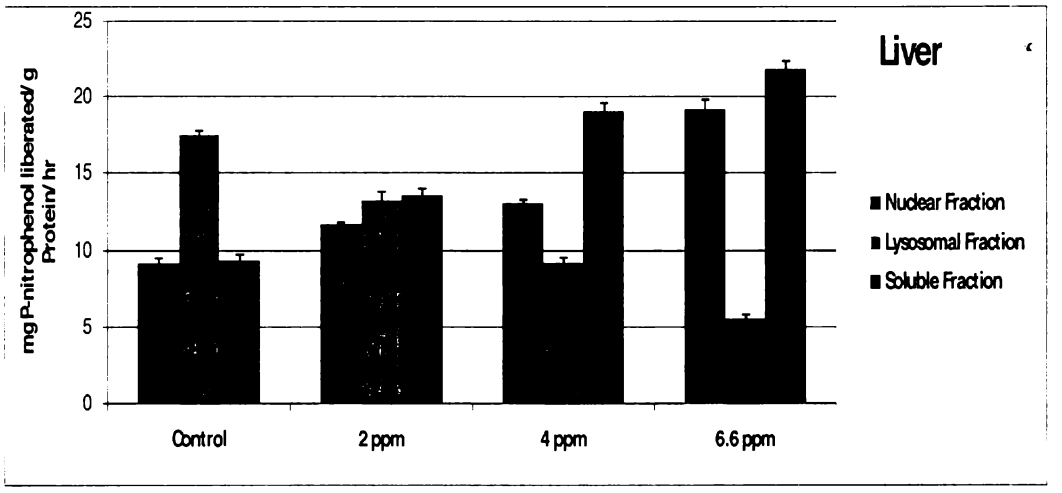


Figure.3.C.2 Acid phosphatase activity in the hepatic tissue of *H. fossilis* subjected to monocrotophos

Table.3.C.4 ANOVA table for Acid phosphatase activity of *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Concentration	123.710	3	41.237	2.189	0.098
ACP Activity	251.016	2	125.508	6.664	0.002
Error	1243.048	66	18.834		
Total	1617.775	71			

Here it is observed that the p-value corresponding to ACP activity is less than 0.05 hence it is statistically evident that there is significant difference in the mean values of the recorded values for different ACP activity group. Since the p-value corresponding to concentration is greater than 0.05 it can be concluded that there is no significant difference between the mean values of the recorded value for different concentration groups. Since, for both the parameters, concentration has the p-value greater than 0.05, it is concluded that the mean value is same for both concentrations

(null hypothesis in the ANOVA is that the mean values are equal). Hence there is no meaning in going for LSD analysis which compares two at a time. LSD for pair wise comparison for different fractions is carried out and the results are summarized in table 3.C.5.

Table.3.C.5 Least significant difference (LSD) analysis for different subcellular fractions

	ß-Glucuronidase activity	ACP Activity
Nuclear vs Lysosomal Fraction	0.000	0.137
Nuclear vs Soluble Fraction	0.000	0.037
Lysosomal vs Soluble Fraction	0.000	0.001

In the case of ß-glucuronidse all the mean values in all solutions vary significantly in case of pair wise comparisons also. The nuclear fraction and lysosomal fraction has statistically same mean values for the ACP activity case. But the mean values vary significantly in the cases of lysosomal fraction and soluble fraction and nuclear fraction and soluble fraction.

3.C.5 Discussion

Lysosomes have an acidic environment maintained by a membrane Mg^{2+} ATPase dependent H^+ ion proton pump (Okhuma *et al.*, 1982). Release of the hydrolytic degradative enzymes from the lysosomal compartment into the cytosol, due to destabilization of the lysosomal membrane may also involve the increased lysosomal fusion with other intercellular vacuoles leading to the formation of pathologically enlarged lysosomes. The consequences of these lysosomal changes would be increased autolytic activity leading to atrophy.

In the present study lysosomal fraction ß-glucuronidase and acid phosphatase activities in monocrotophos intoxicated *H. fossilis* were observed to be lower than that of the control fishes. The reduction in activity was prominent as concentration of the toxicant increased. At the highest concentration of monocrotophos, maximum reduction of enzyme activity was observed in lysosomal fraction. Soluble fraction activities of both enzymes were increased as the dose increased. At highest concentration 6.6 ppm, maximum activities of enzymes were observed in soluble fraction. Nuclear fraction activity of ß-Glucuronidase and acid phosphatase were also increased in a dose dependent manner. The results of lysosomal enzyme assays have shown a considerable escalation in ß-glucuronidase and acid phosphatase in soluble fraction and the alteration in their activities are found to be dose dependent.

This shows that there exists a direct interaction of the toxicant with membrane lipids and proteins. The lysosomal membrane has a lipid content of 25% only, but it is prone to lipid peroxidation. Hazarika *et al.,* (2003) reported that OPs may enhance lipid peroxidation by direct interaction with the cell membrane. As lipid peroxidation continues in lysosomal membrane due to xenobiotic induced reactive oxygen species formation, physical characteristics of the membrane gets modified by the products of peroxidation. When free fatty acids get damaged, membrane conformation is lost and eventually results in gap formation in the membrane. Peroxidation of biological membranes can additionally cause cross links between proteins to form high molecular weight aggregates within the membrane, most probably due to oxyradical processes. This can finally lead to changes in the properties of membrane and loss of its bound enzymes.

Reduced lysosomal stability has previously been reported to contribute to impaired competence of immune system and to loss of body tissues due to autophagy (Farnley *et al.*, 2000). Digestive tissue of mussel *Mytilus edulis*, exposed to toxicants for a period of 30 days showed marked decrease in the stability of lysosomal membrane (Krishna Kumar *et al.*, 1997).

Variation in the lysosomal stability indicates severe dysfunction of lysosomal system. This interferes with the intracellular digestion of food, normal turn over of proteins and regulation of fusion processes associated with the lysosomal vacuolar system (Livingstone *et al.*, 2000). Damage resulting in destabilization of the lysosomal lamina bears a quantitative relationship to the magnitude of toxicant stress response (Bayne *et al.*, 1982). Derangement of cell function brought about by the leakage of lysosomal enzymes into cytoplasm and nucleoplasm hamper the firmness of lysosomal membrane which can be applied as a sensitive measure of the functional state of cell providing an ideal tool for probing into the cellular deterioration in general (Pickwell and Steinert., 1984).

Considerable necrosis and loss of architecture of liver tissue observed in the histopathological examination of the monocrotophos treated *H. fossilis* (Chapter-5) can be due to labilization of lysosomal membrane and thereby the spill of lysosomal enzymes acid phosphatase and ß-glucuronidase to soluble fraction, leading to cell damage and cell death. From the present investigation, it can be assessed that monocrotophos damages the lysosomal system involving loss of integrity which results in escape of enzymes to cytosol. The overall outcome includes loss of membrane fluidity which can result in the leakiness of membrane to substances which in normal conditions are not permitted to cross the membrane.

3.C.6 Summary

In conclusion, *H. fossilis* exposed to different sub lethal concentrations of organophosphorus insecticide monocrotophos exhibited decreased stability of lysosomal and cell membranes in hepatocytes. Reduced lysosomal stability can add greatly to impaired competence of immune system and to loss of body tissues due to autophagy. Lysosomal stability index can be employed as a low cost and effective tool for evaluating the toxicity of different chemical contaminants in the environment.

Chapter 4

EFFECT OF MONOCROTOPHOS ON ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS

Contents

4.1 Introduction

Aquatic environment receives substantial amounts of environmental pollutants which have immense potential to cause oxidative stress in aquatic organisms. The uptake of these pollutants by aquatic organisms can occur from sediments, suspended particulate matter with toxic properties and food sources. Exposure to these contaminants will depend on the particular dietary and ecological life styles of the organisms. Current knowledge and recent advances of oxidative toxicity by xenobiotics in aquatic organisms provide a fertile field for aquatic toxicology studies (Livingstone, 1998). Aquatic organisms were chosen as test species because of their ease of caging and maintenance, and sensitivity to oxidative damage from concerning chronic exposure or sub-lethal concentrations. Aquatic organisms especially fishes can provide model systems for investigation of how reactive oxygen species (ROS) damage cellular components, how cells respond, how repair mechanisms ameliorate this damage, and how oxidative stress can lead to disease (Livingstone *et al.*, 1994; Di Giulio *et al.*, 1989). Aquatic organisms are more sensitive to exposure and toxicity when compared to terrestrial organisms including mammals and in this respect they may provide experimental data for evaluation of subtle effects of oxidative stress, mutagenicity and other adverse effects of pollutants (Lackner,1998). Interest in toxicological aspects of oxidative stress has grown in recent years and research is now increasingly focused on mechanistic aspects of oxidative damage and cellular responses in biological system. Recent researches in fish have focused on the antioxidant status as biomarkers of stress or pollution. Owing to their short time of response, they are used as early warning signals of biological effects caused by environmental pollutants.

Reactive oxygen species play an important role in toxicity of pesticides and environmental chemicals. Endogenous and exogenous oxidative challenges have endowed living cells of aerobic organisms with sophisticated antioxidant systems to regulate oxidative stress. Assaying antioxidant enzymes can offer indications of the antioxidant status of the organisms and can serve as biomarkers of oxidative stress. The non-enzymatic antioxidant systems are mainly substances of low molecular weight, such as vitamin C, vitamin E, urate, retinyl esters, ß-carotene, glutathione (GSH) etc.

Increase in intracellular levels of ROS to such a level that cellular antioxidant defenses are insufficient to maintain these harmful molecules below a toxic threshold level is generally referred to as oxidative stress. Oxidative stress is the disturbance in the pro-oxidant- anti-oxidant balance in favor of the former, which leads to potential cellular damage. Oxidative stress occurs when the ROS generation rate exceeds that of their removal (Sies, 1986). Cellular antioxidant defense systems in biological systems, when exposed to environmental pollutants, are depleted, but levels of these antioxidants may rise also to redress the imbalance caused by oxidative damage (Winston and Di Giulio, 1991). Antioxidant defenses, which are normally ubiquitous in animal species and different tissue types, are found far and wide in aquatic organisms.

Oxyradicals

Oxygen in its molecular state 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). This gives rise to the so called 'aerobic life paradox' or the 'oxygen paradox', where aerobic organisms which has no existence with out oxygen, are nevertheless inherently at risk due to oxidative stress

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. The valence layer of atomic oxygen contains an unpaired electron. When two oxygen atoms coalesce to form molecular oxygen, its electrons remain as two unpaired electrons. A radical is defined as any atom or molecule with one or more unpaired electrons.

Radicals are formed by the loss or gain of an electron from a non-radical. The bi-radical nature of the oxygen molecule allows oxidation/reduction reactions. The tetravalent reduction of oxygen, catalyzed by cytochrome oxidase at the end of the mitochondrial electron transport chain, generates water. On the other hand, monovalent reduction produces several reactive intermediates (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-).

The hydroxyl radical is one of the most potent oxidants known, capable of reacting at random with nearly all organic chemicals including cellular macromolecules leading to protein degradation and enzyme inactivation, lipid peroxidation, DNA damage and finally cell death. (Viarengo *et al.*, 1991).

Lipid peroxidation

Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. Membrane phospholipids of aerobic organisms are continually subjected to oxidant challenges from endogenous and exogenous sources, while peroxidized membranes and lipid peroxidation products represent constant threats to aerobic cells. Lipid peroxidation is considered as the most frequent feature of cellular injury arising from the direct interaction of oxyradicals with membrane lipids. Peroxidised membranes become rigid and eventually loose their permeability and integrity. Lipid

peroxidation progresses by three steps: initiation, propagation and termination. The initiation phase of peroxidation usually proceeds with the formation of conjugated diene bonds generated by abstraction of hydrogen atoms from the methylene group of poly unsaturated lipids. Propagation of lipid peroxidation relies on the interaction of molecular oxygen with carbon centered free radicals to form lipid hydroperoxides. These lipid peroxides can form free radicals to propagate the process of lipid peroxidation. Lipid peroxidation is a major source of cytotoxic products such as malonaldehyde (MDA) produced from the decomposition of lipid hydroperoxides. MDA reacts with nitrogenous bases of DNA to form DNA adducts.

Lipid peroxidation is probably the most extensively investigated process in tissue injury caused by oxyradicals but, because the direct analysis of endogenous lipid peroxidation products is complicated, most methods measure the levels of secondary oxidation products (aldehydes and ketones). The most widely used assays for lipid peroxidation is malonaldehyde (MDA) formation as a secondary lipid peroxidation product and by determining the diene conjugation from the polyunsaturated fatty acids. Estimation of LPO in particular has been found to have high predictive importance as revealed from a credible number of research papers describing its use as a biomarker of exposure (Lackner, 1998). MDA is the main oxidation product of peroxidized polyunsaturated fatty acids and increased MDA level is an important indicator of lipid peroxidation (Elia *et al.*, 2002).

Antioxidant defense system

The damage from ROS includes lipid peroxidation, cross-linking and inactivation of proteins, DNA and RNA breaks, and cell death (Halliwell and Gutteridge, 1984). To prevent damage from oxidative stress, cells maintain these ROS at a steady-state level by a variety of enzymatic and

non-enzymatic antioxidant system which includes specially adapted enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione dependent enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR), glutathione-S-transferase (GST) and non-enzymatic antioxidants such as ascorbic acid and total reduced glutathione(GSH) constitute the antioxidant defense system.

Superoxide dismutase

SOD catalyses the dismutation of superoxide anion free radicals to hydrogen peroxide and oxygen (Babior, 1997).

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD is a metalloprotein found in both prokaryotic and eukaryotic cells. Two forms of superoxide dismutase are present in eukaryotic cells: a form that contains Cu^{2+} and Zn^{2+}, the former serving as the redox center and the latter as a structural element, and a form that contains only one metal, namely Mn^{2+}, which functions as the redox center. The Cu^{2+}/Zn^{2+} form, is found in the cytosol, while the Mn^{2+} form, is located in mitochondria. The Mn^{2}+ form is also found in bacteria, as is a third form of superoxide dismutase containing Fe^{2+} as its redox element. The concentrations of the Cu^{2+}/Zn^{2+} and Fe^{+} forms of superoxide dismutase are unaffected by oxidative stresses, but the Mn^{2+} form is inducible in both bacteria and eukaryotic cells, its activity increasing with oxidative stress (Babior, 1997).

Catalase

The H_2O_2 produced by the dismutation of O_2 or generated by H_2O_2 generating oxidases (e.g., D-amino acid oxidase) is handled by two systems: catalase and a glutathione-dependent antioxidant system that reduces H_2O_2 to water at the expense of NADPH.

$$2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$$

The enzyme primarily occurs in peroxisomes and it is an enzyme of high molecular weight containing a porphyrin nucleus.

The glutathione-dependent antioxidant system

The glutathione-dependent antioxidant system consists of glutathione plus two enzymes: glutathione peroxidase and glutathione reductase. As this system operates, glutathione cycles between its oxidized and reduced forms. During this process 60% of hydrogen peroxide formed due to ROS is scavenged.

Glutathione peroxidase (GPx) is the most important peroxidase enzyme which is involved in the detoxification of hydroperoxides. GPx are localized in the peroxisomes of fish liver cells. The reaction catalyzed by GPx:

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \text{ (catalyzed by GSH peroxidase)}$$

Total reduced Glutathione

GSH is a tripeptide, a low molecular weight sulphur containing compound which is the most abundant thiol in most tissues. It has two fold functions: it is an antioxidant which scavenges ROS and a cofactor for enzymatic reactions like the conjugation of xenobiotics by glutathione-S-transferase (Gallagher *et al.*, 1992). Glutathione is necessary for Glutathione peroxidase which detoxifies organic and inorganic peroxides.

When glutathione scavenges a radical, a thiol radical is generated, which gets dimerised to form oxidized glutathione disulfide or mixed sulfides with proteins. Both the disulfides and mixed sulfides with protein are indicators of increased reactive oxygen species formation and both get

reduced by glutathione reductase enzyme to restore the original glutathione concentrations at the expense of cellular NADPH.

Glutathione-S-transferase

The GSTs are a multigene family of ubiquitous enzymes which catalyze both conjugation of GSH with lipophilic electrophiles and reduction of oxidants by GSH. Besides certain roles in endogenous metabolism, these enzymes are associated with the detoxification of xenobiotics such as drugs, carcinogens and environmental pollutants such as pesticides. On the basis of sequence identity, GSTs are classified into five separate families: one is a trimeric membrane bound form termed microsomal GST; the rest are soluble dimeric proteins called CX, II, 7 and 8.With the exception of 8 class isoenzymes, all other GSTs conjugate the substrate CDNB to GSH. (Ketterer *et al.*, 1989; George, 1994; Timbrell *et al.*, 1994).

Conjugated Dienes and Malonaldehyde

Many of the products initially produced by oxyradical attack on electron rich substrates such as polyunsaturated fatty acids are having short half lives. So the detection of oxidative stress has largely relied on the quantification of conjugated dienes, malonaldehyde and hydroperoxides which are the products of degradation of initial products of free radical attack.

The main rationale of this investigation aims at studying the peroxidative changes in the *H. fossilis* exposed to 6.6 ppm, 4 ppm and 2 ppm concentrations of commercial grade monocrotophos.

4.2 Materials and methods

Collection, transportation, acclimation, dozing experiments, application of toxicant, tissue/serum preparation of/on *Heteropneustes fossilis* were the same as that described in chapter 1 (1.2.1,1.2.3,1.2.4,1.2.5). Fishes were exposed to three selected sublethal concentrations of monocrotophos and samplings were done on 7th and 21st day intervals to study the effect of toxicant on the antioxidant status of *H. fossilis*. Protein was estimated by the method of Lowry *et al.*,(1951).

4.2.1 Estimation of super oxide dismutase

Super oxide dismutase in different tissues was determined by using the method of Kakkar *et al.*, (1984)

Reagents

0.33 M sucrose, n-butanol, 0.052 M sodium pyrophosphate buffer (pH 8.3), 0.0025 M tris HCl buffer (pH 7.4)186 µM phenazine methosulphate (PMS), 300 µM nitro blue tetrazolium (NBT), 780 µM NADH and glacial acetic acid.

Procedure

Tissue samples were washed well and homogenized in 0.33 M sucrose and subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity an initial purification was done by precipitating the protein from supernatant with 90% ammonium sulphate and this fraction was dialysed against 0.0025 M tris HCl buffer (pH 7.4). The supernatant was used as the source of enzyme. Assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate, 0.3 ml of nitroblue tetrazolium, 1.3 ml of distilled water and 0.1 ml of enzyme source. The tubes were kept at 30°C for one minute

and then 0.2 ml of NADH were added and incubated at 30°C for 90 seconds and the reaction was stopped by the addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the chromogen in butanol was measured at 560 nm against n-butanol blank. A system devoid of enzyme was served 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.

4.2.2 Estimation of Catalase

Catalase activity in different tissues was determined following the method of Maehly and Chance (1955).

Reagents

0.01M phosphate buffer (pH 7.0), 30 μ M H_2O_2

Procedure

Catalase activity was estimated spectrophotometrically following a decrease in absorbance at 230 nm. The reaction mixture contained 0.01M phosphate buffer, 30 μM H_2O_2 and the enzyme extract prepared by homogenizing the tissues in phosphate buffer and centrifuging at 5000 rpm. Specific activity was expressed as international units/ mg protein. 1 IU = change in absorbance/ min/ extinction coefficient (0.021). Protein was estimated by the method of Lowry *et al.*, (1951).

4.2.3 Estimation of Glutathione peroxidase

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

Reagents

Tris buffer 0.4 M, (pH 7.0), 10mM sodium azide solution, 10% trichloro acetic acid (TCA), 0.4 mM ethylene diamine tetra acetic acid (EDTA), 0.2 mM hydrogen peroxide, 2mM glutathione solution.

Procedure

Different tissue samples were homogenized in a known volume of tris buffer. To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture, 0.2 ml of GSH followed by 0.1 ml of H_2O_2 solution added. The contents were mixed and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes, the reaction was arrested by the addition of 1.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH. Values were expressed as µg of GSH/min/mg protein. Protein was estimated by the method of Lowry *et al.*, (1951).

4.2.4 Estimation of Glutathione-S-transferase

Glutathione-S-transferase in different tissues was estimated using the method of Habig *et al,* (1974).

Reagents

Phosphate buffer 0.5 M (pH 6.5), 25mM 1-chloro-2, 4-dinitrobenzene (CDNB) in 95% ethanol, 20mM glutathione (GSH).

Procedure

Different tissues were homogenized in 0.5 M phosphate buffer. The reaction mixture containing 200µl phosphate buffer, 20µl CDNB, and 730µl distilled water were taken in the control tube and 200µl phosphate

buffer, 20 µl CDNB and 680 µl distilled water were taken in the test sample tubes. Tubes were incubated at 37°C for 10 minutes.

After the incubation, added 50 µl of GSH in both sets of tubes. After thorough mixing, added 50 µl of tissue extract in the test sample tubes. Increase in absorbance was noted at 340nm for 5 minutes using UV-Visible spectrophotometer. Values are expressed in n moles of CDNB conjugated/min/ mg protein. Extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6 mM^{-1} cm^{-1}. Protein was estimated by the method of Lowry *et al.*, (1951).

4.2.5 Estimation of Total reduced glutathione

Reduced glutathione in different tissues was estimated following the method of Patterson and Lazarov (1955).

Reagents

Alloxan 0.1 M, 0.5 M phosphate buffer (pH 7.5), 0.5 N NaOH and 1 N NaOH

Procedure

Tissue samples were weighed and homogenized in 0.5 M phosphate buffer. The incubation mixture contained tissues extract, 50 µl alloxan, 50 µl phosphate buffer and 50 µl 0.5 N NaOH. The mixture was incubated for 5 minutes at 25 °C. The reaction was stopped by adding 50µl of 1 N NaOH. The absorbance was observed at 305 nm using a UV-Visible spectrophotometer. A control tube was maintained with phosphate buffer instead of extract. The values are expressed in mg/ 100g tissue. Protein was estimated by the method of Lowry *et al.*, (1951).

4.2 6 Estimation of Conjugated Dienes

The concentration of conjugated dienes was estimated as per the method of Retnagal and Ghoshal., (1966).

Procedure

The membrane lipids were extracted and evaporated to dryness. The lipid residue was dissolved in 5 ml of cyclohexane and the absorbance was read spectrophotometrically at 233 nm, using cyclohexane blank. Molar extinction coefficient of conjugated dienes is 2.52×10^4 M^{-1} cm^{-1}. Protein was estimated by the method of Lowry *et al.*, (1951).

4.2.7 Estimation of malonaldehyde

Malonaldehyde was estimated following the method of Niehaus and Samuelson, (1968).

Reagents

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

Procedure

The homogenates of liver, brain and gill were prepared in tris-HCl buffer and was combined with thiobarbituric acid, mixed well and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600 g. The absorbance was read at 535 nm, spectrophotometrically against a reagent blank that contained no tissue extract. The extinction coefficient of malonaldehyde is 1.56×10^{-5}/ M^{-1}/cm^{-1}.

Protein was estimated by the method of Lowry *et al.*, (1951).

4. 3 Statistical analysis

Statistical analysis of the data was performed employing three factor ANOVA test followed by LSD analysis using statistical software SPSS - 16.

4.4 Results

The fish exposed to monocrotophos (MCP group) exhibited lethargic movements with a partial lack of reflexes. Macroscopic findings included discoloration of skin and loss of firmness. The exposure to monocrotophos caused significant alterations in super oxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities in all tissues when compared to the control. Total reduced glutathione (GSH), malonaldehyde (MDA) and conjugated dienes (CD) values were significantly altered in monocrotophos treated group of fishes. SOD activity was increased at both 7^{th} and 21^{st} day observations, for the lowest (2 ppm) and the medium (4 ppm) concentrations while it was decreased sharply at the highest (6.6 ppm) concentration for both time periods. CAT activity was increased at all the three concentrations of monocrotophos for both 7^{th} and 21^{st} days of observations. GST activity was increased at both time intervals for the lowest (2 ppm) and the medium (4 ppm) concentrations while it was decreased sharply at the highest (6.6 ppm) concentration for both time periods. GPx activity was showing a decreasing trend through out the experiment at all the three concentration groups.

Exposure to monocrotophos induced severe depletion of total reduced glutathione content of the tissues when compared to the control group. There was an initial increase in GSH content at 2 ppm and 4ppm for the seventh day observation, while values were decreased at 21st day observation. In the case of highest concentration 6.6 ppm, GSH showed a

sharp decrement at both intervals. MDA and CD in all tissues of MCP induced fishes were increased and the changes were dose dependent. Brain recorded the highest value for both MDA and CD.

Table 4.1 Superoxide dismutase activity in liver, brain and gill tissues of *H. fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	**Control**	**7th day**	**21st day**	**7th day**	**21st day**	**7th day**	**21st day**
Liver	1297.02 ± 7.53	1590.03 ± 14.19	1605.32 ± 7.66	3500.99 ± 22.86	4429.25 ± 40.52	1399.94 ± 10.77	1348.06 ± 8.65
Brain	899.87 ± 5.58	1243.34 ± 8.58	1355.64 ± 7.25	1417.48 ± 19.27	1589.99 ± 11.13	1003.73 ± 11.09	932.12 ± 9.79
Gill	1695.02 ± 16.73	2029.66 ± 11.36	2147.08 ± 27.72	2789.17 ± 32.28	3465.45 ± 20.99	1831.65 ± 20.95	1748.68 ± 14.73

- Values are expressed as units/mg protein
- Average of six values in each group

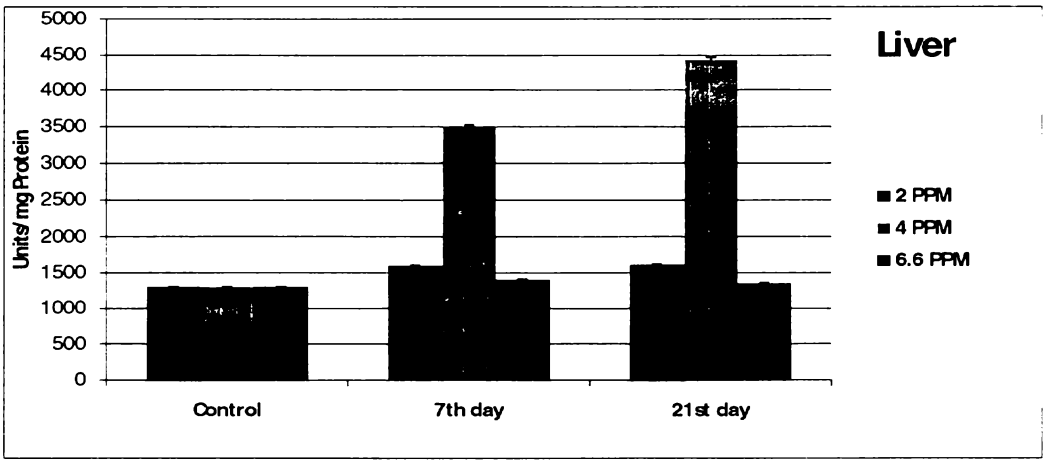


Fig.4.1 Activity of superoxide dismutase in liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

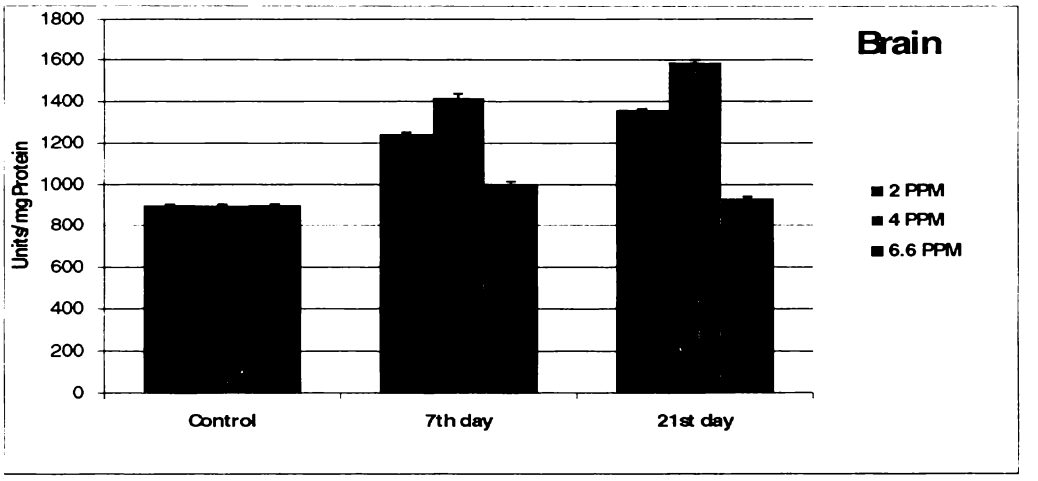


Fig. 4.2 Activity of superoxide dismutase in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

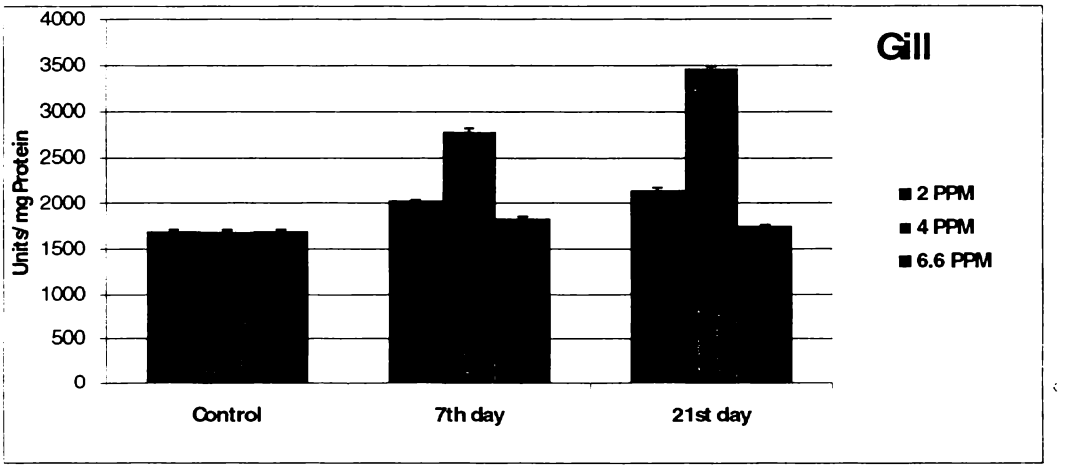


Fig.4.3 Activity of superoxide dismutase in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.2 ANOVA Table for superoxide dismutase activity in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	29151295.198	2	14575647.599	85.064	0.000
Concentration	57076755.922	3	19025585.307	111.034	0.000
Day	824097.920	1	824097.920	4.809	0.030
Error	23474852.545	137	171349.289		
Total	110527001.585	143			

Since the p-value is less than 0.05, it is arrived at the conclusion that the variables vary significantly with tissue, concentration and day. Further comparison by LSD analysis shows that there is significant variation between various concentration group as well as tissues. It can be noted that the difference is not statistically significant between liver and gill tissues while it is significant for all other tissue groups.

Values significantly differed between concentration groups (2 ppm, 4 ppm) and control except for 6.6 ppm. From the summary table (4.1) it is evident that there is sharp increase in the SOD activity in both 2 ppm and 4 ppm concentration groups at both time intervals, while at the highest concentration 6.6 ppm, values were decreasing. It can be seen from table (4.1) that the gill recorded a significantly higher value compared with other tissues. Liver has a slight upper edge compared to that of brain.

Table 4.3 Catalase activity in liver, brain and gill tissues of *H. fossilis* subjected to different sublethal concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	**Control**	**7th day**	**21st day**	**7th day**	**21st day**	**7th day**	**21st day**
Liver	758.93 ± 24.36	956.52 ± 14.15	1029.01 ± 4.84	1135.51 ± 8.89	1212.32 ± 47.80	1326.75 ± 38.66	1407.23 ± 22.54
Brain	522.67 ± 17.98	696.02 ± 15.97	782.22 ± 28.93	792.72 ± 23.16	906.87 ± 4.62	911.34 ± 23.58	997.27 ± 11.22
Gill	579.45 ± 27.14	652.99 ± 4.28	700.91 ± 4.57	695.58 ± 33.91	798.16 ± 26.54	852.43 ± 7.02	979.15 ± 15.00

- Values are expressed as IU/gm tissue
- Average of six values in each group

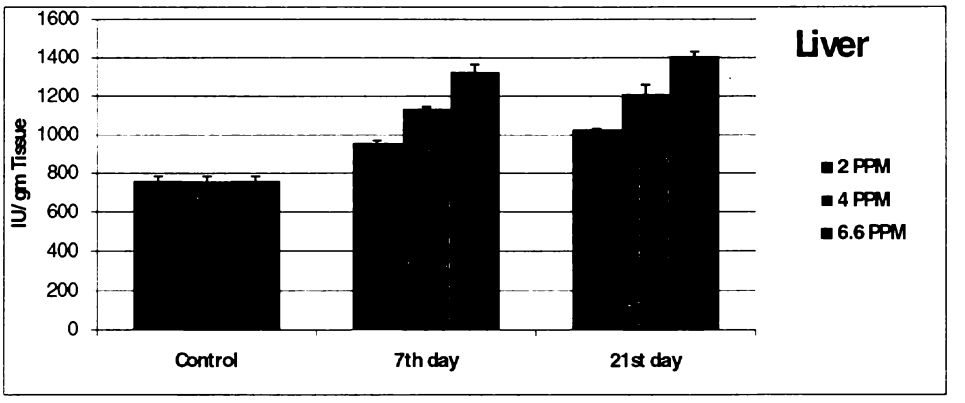


Fig.4.4 Activity of catalase in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

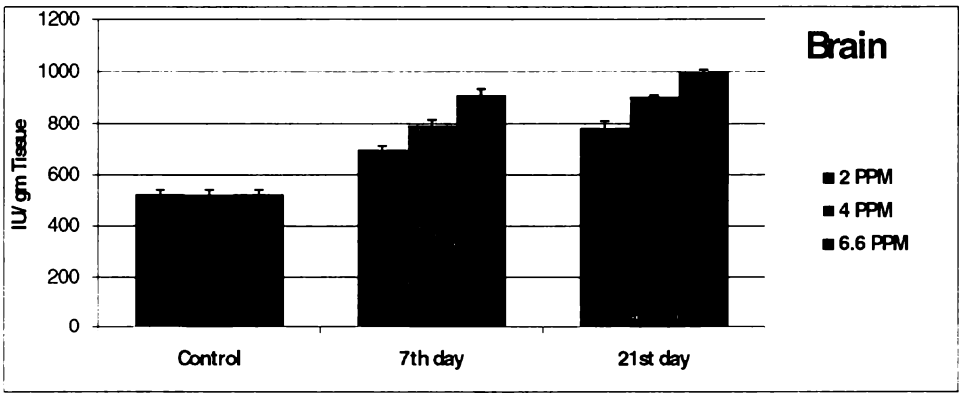


Fig.4.5 Activity of catalase in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

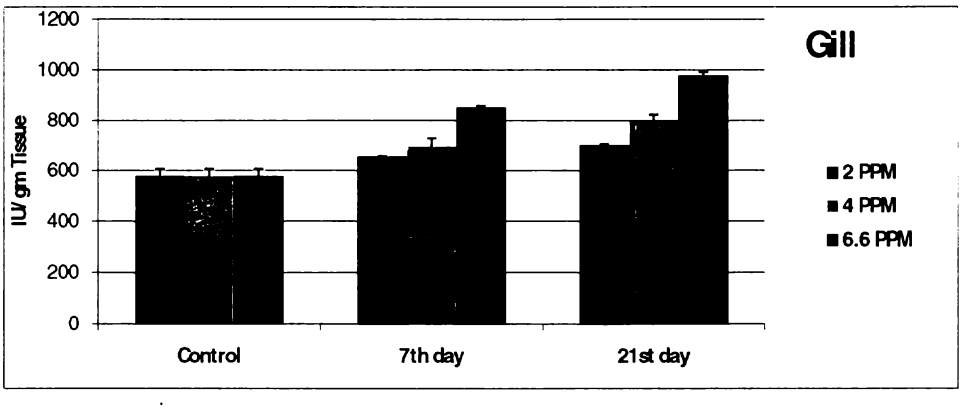


Fig.4.6 Activity of catalase in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.4 ANOVA Table for catalase activity in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	3445928.619	2	1722964.309	537.472	0.000
Concentration	4143919.521	3	1381306.507	430.893	0.000
Day	158583.545	1	158583.545	49.469	0.000
Error	445589.987	139	3205.683		
Total	8100808.646	145			

In the case of catalase, also, it can be observed that the p-values are less than 0.05 for all the factors and hence it is concluded that the values vary significantly with tissue, concentration and day. Further comparison by LSD analysis shows that the difference between the values of control and different concentrations are statistically significant. The difference between tissue groups also is statistically significant. Here liver recorded a high value compared to brain and gill. Brain recorded the least value compared with others under the same conditions.

Table 4.5 Glutathione-S-transferase activity in liver, brain and gill tissues of *H. fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	2.55 ± 0.24	9.24 ± 0.54	6.92 ± 0.60	9.05 ± 0.23	11.40 ± 0.76	3.92 ± 0.27	3.91 ± 0.54
Brain	1.10 ± 0.14	3.55 ± 0.33	5.31 ± 0.28	5.83 ± 0.21	7.68 ± 0.58	4.96 ± 0.44	3.04 ± 0.25
Gill	1.08 ± 0.03	2.92 ± 0.29	4.57 ± 0.39	5.13 ± 0.31	6.35 ± 0.56	4.29 ± 0.18	2.81 ± 0.28

- Values are expressed as nmol/mg protein
- Average of six values in each group

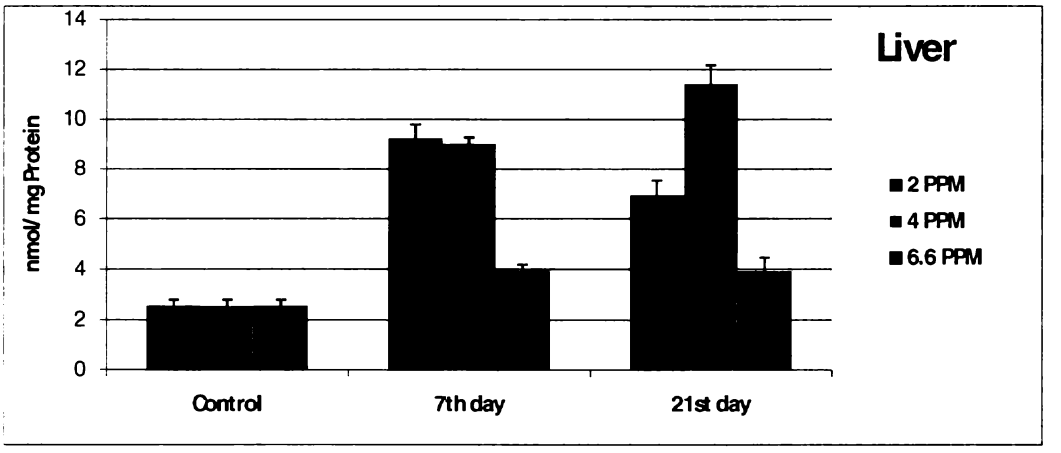


Fig.4.7 Activity of glutathione-S-transferase in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

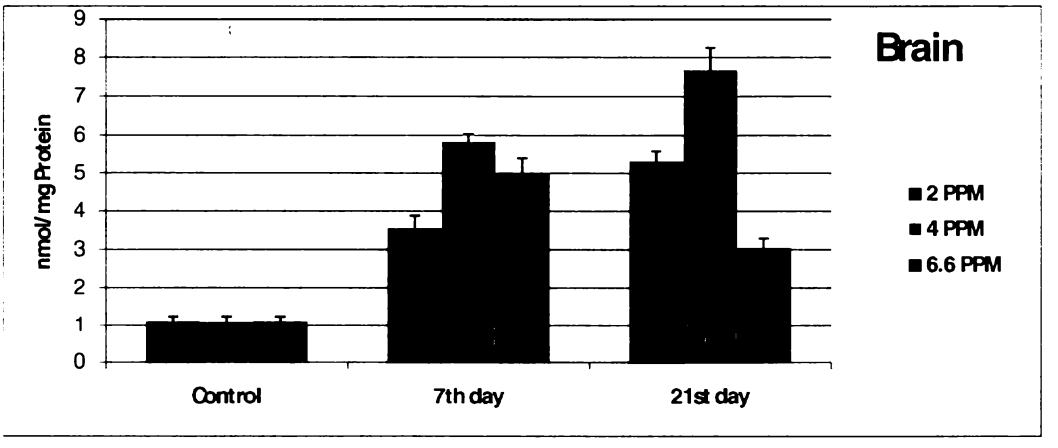


Fig.4.8 Activity of glutathione-S-transferase in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

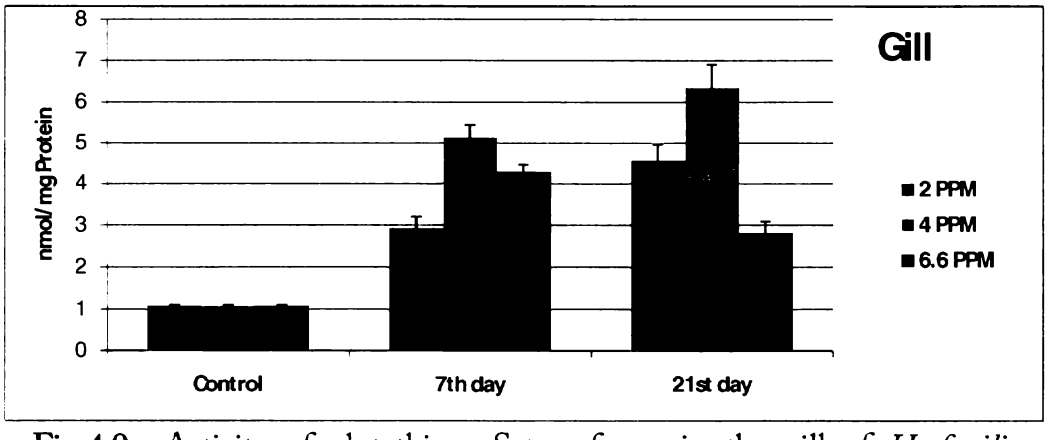


Fig.4.9 Activity of glutathione-S-transferase in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.6 ANOVA Table for glutathione-S-transferase activity in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	194.855	2	97.428	72.909	0.000
Concentration	696.322	3	232.107	173.695	0.000
Day	2.407	1	2.407	1.801	0.182
Error	188.417	141	1.336		
Total	1087.290	147			

There is a significant difference between the tissues and between concentrations and the difference between the days are not significant in the case of the parameter GST. Here, further LSD analysis proved that there exists significant difference between the values of different concentrations and control (P-value less than 0.05). Difference between various tissue groups was also found to be statistically significant (P-value less than 0.05). Under most of the situations liver recorded higher values compared to the other two.

Table 4.7 Total reduced glutathione (GSH) concentration in liver, brain and gill tissues of *H. fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	1920.99 ± 10.21	1994.31 ± 8.04	1695.00 ± 57.23	2020.69 ± 10.19	1600.56 ± 7.99	1202.02 ± 41.35	1122.30 ± 21.86
Brain	979.47 ± 11.03	1104.12 ± 19.59	913.71 ± 2.70	1037.60 ± 14.35	643.18 ± 24.66	496.42 ± 5.82	313.02 ± 5.93
Gill	1708.17 ± 5.32	1814.58 ± 45.26	1614.50 ± 24.33	1906.27 ± 50.21	1438.02 ± 33.80	998.04 ± 38.31	775.76 ± 22.41

- Values are expressed as nmol GSH /mg tissue
- Average of six values in each group

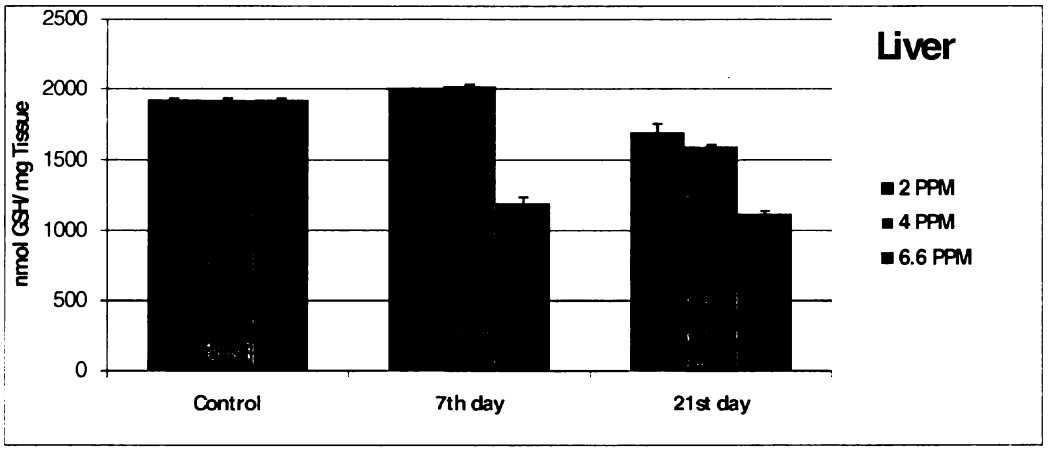


Fig.4.10 Total reduced glutathione in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

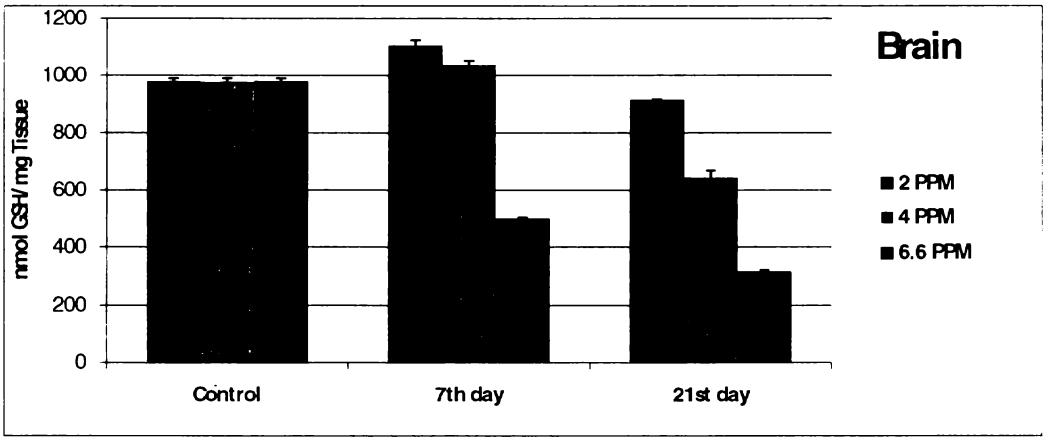


Fig. 4.11 Total reduced glutathione in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

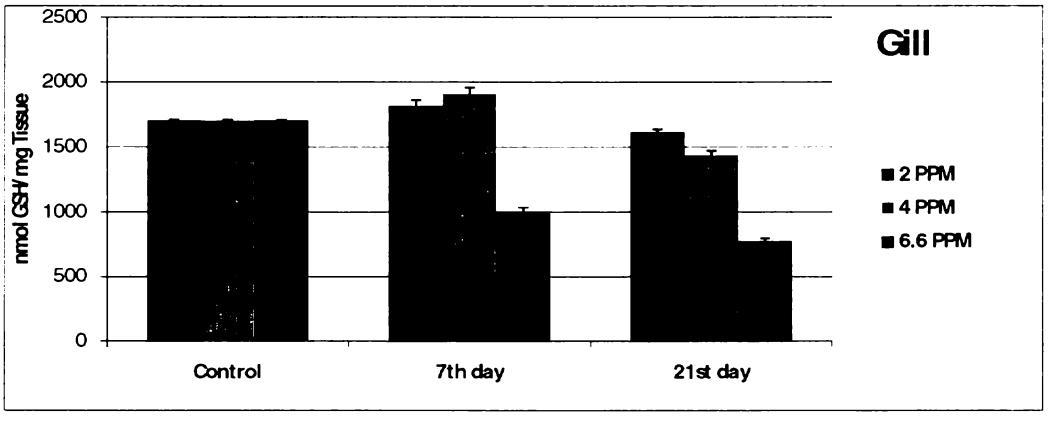


Fig.4.12 Total reduced glutathione in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.8 ANOVA Table for total reduced glutathione concentration in *H. fossilis*

Source of Variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	20385426.399	2	10192713.199	984.992	0.000
Concentration	14085622.603	3	4695207.534	453.730	0.000
Day	1621544.014	1	1621544.014	156.701	0.000
Error	1459070.807	141	10348.020		
Total	37286847.882	147			

Results of three factor ANOVA (table 4.8) for GSH suggest that the values vary significantly with tissue, concentration and day. Further comparison using LSD analysis shows that there is no significant variation between control and 2 ppm, while values significantly differed between control and 4 ppm as well as control and 6.6 ppm. Also there is significant difference between the values of different tissue groups. From the summary tables shown as table (4.7) implies that the liver occupies higher values compared to brain and gill. Gill occupies the next position.

Table 4.9 Glutathione peroxidase (GPX) activity in liver, brain and gill tissues of *H. fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	30.57 ± 0.35	28.52 ± 0.38	26.11 ± 0.25	28.01 ± 0.28	25.17 ± 0.31	23.42 ± 0.09	21.56 ± 0.22
Brain	25.35 ± 0.44	23.94 ± 0.30	23.67 ± 0.31	20.27 ± 0.21	19.48 ± 0.24	17.36 ± 0.10	16.16 ± 0.22
Gill	27.76 ± 0.25	25.39 ± 0.24	23.06 ± 0.17	22.00 ± 0.18	20.13 ± 0.18	18.10 ± 0.23	17.84 ± 0.24

- Values are expressed as unit/mg protein
- Average of six values in each group.

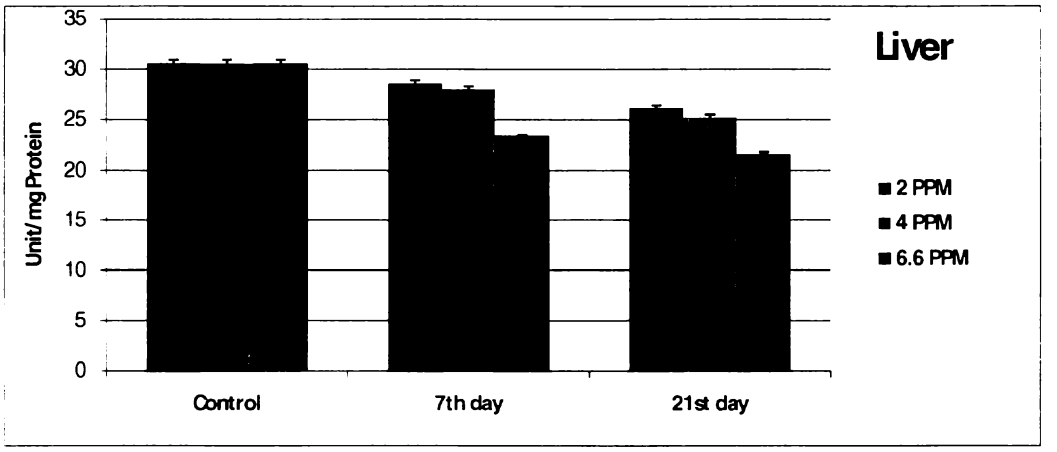


Fig.4.13 Activity of glutathione peroxidase in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

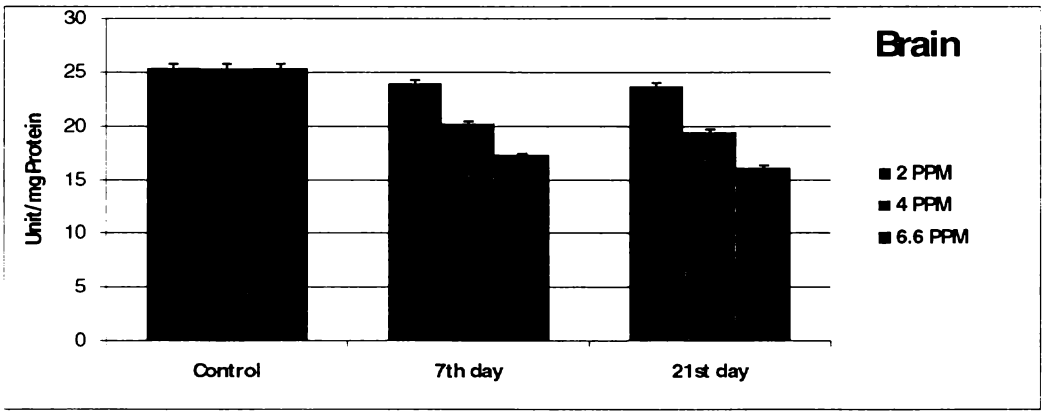


Fig.4.14 Activity of glutathione peroxidase in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

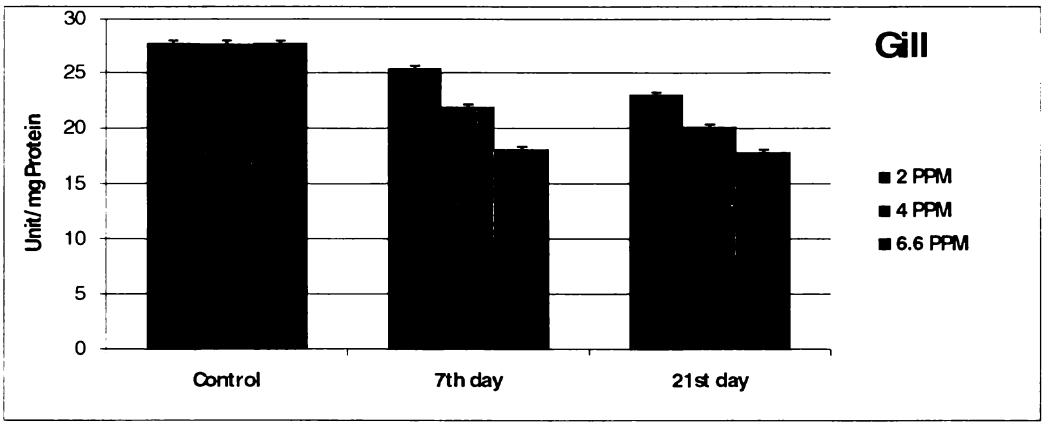


Fig.4.15 Activity of glutathione peroxidase in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.10 ANOVA Table for glutathione peroxidase activity in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	729.753	2	364.876	522.806	0.000
Concentration	1525.211	3	508.404	728.456	0.000
Day	47.715	1	47.715	68.368	0.000
Error	95.615	137	.698		
Total	2398.294	143			

From the table (4.10) comparing the effect of tissues, concentration and day on the recorded values using the concept of ANOVA , since the p-values are less than 0.05 for all the three factors viz. tissues, concentration and day, it can be concluded that the values vary significantly with these factors. Difference between the values of tissue groups also varied significantly. From the summary table (4.9) it can be noted that the higher values are recorded with liver then with gill. Maximum reduction of GPx activity was obtained at 6.6 ppm, for all tissues.

Table 4.11 Malonaldehyde (MDA) concentration in liver, brain and gill tissues of H. *fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	1.08 ± 0.02	1.90 ± 0.04	2.06 ± 0.02	1.96 ± 0.02	2.13 ± 0.02	2.00 ± 0.02	2.63 ± 0.03
Brain	1.10 ± 0.01	2.49 ± 0.03	2.94 ± 0.03	2.98 ± 0.09	3.66 ± 0.08	4.57 ± 0.07	6.47 ± 0.14
Gill	0.90 ± 0.01	1.96 ± 0.04	2.02 ± 0.01	3.18 ± 0.04	3.77 ± 0.07	4.79 ± 0.12	6.89 ± 0.10

- Values are expressed as nmol/mg protein
- Average of six values in each group

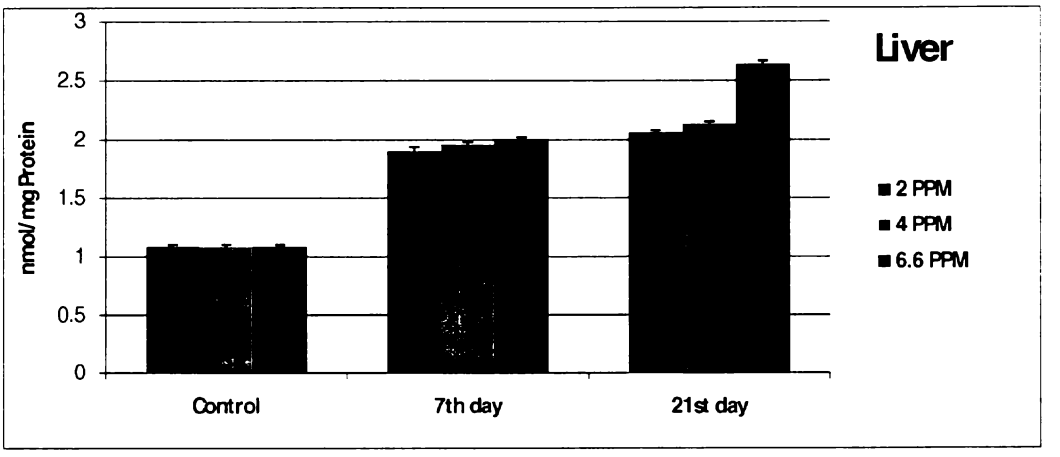


Fig.4.16 Malonaldehyde (MDA) in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

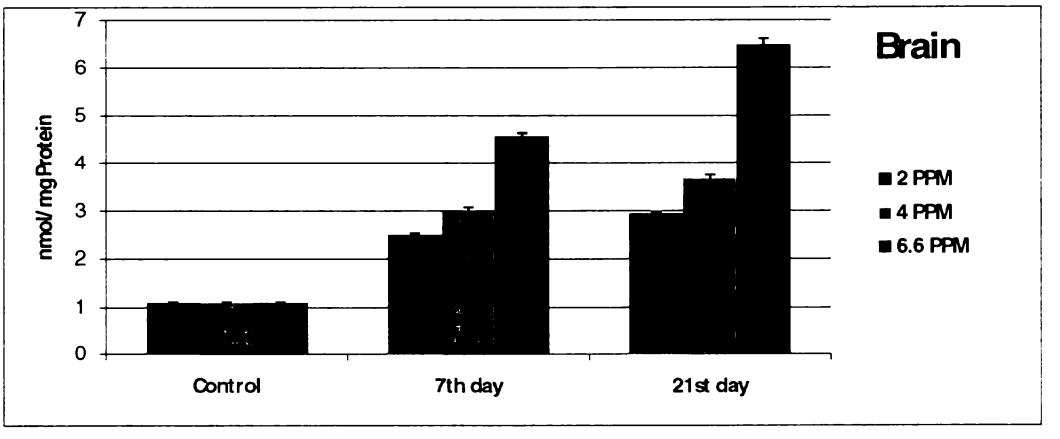


Fig.4.17 Malonaldehyde (MDA) in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

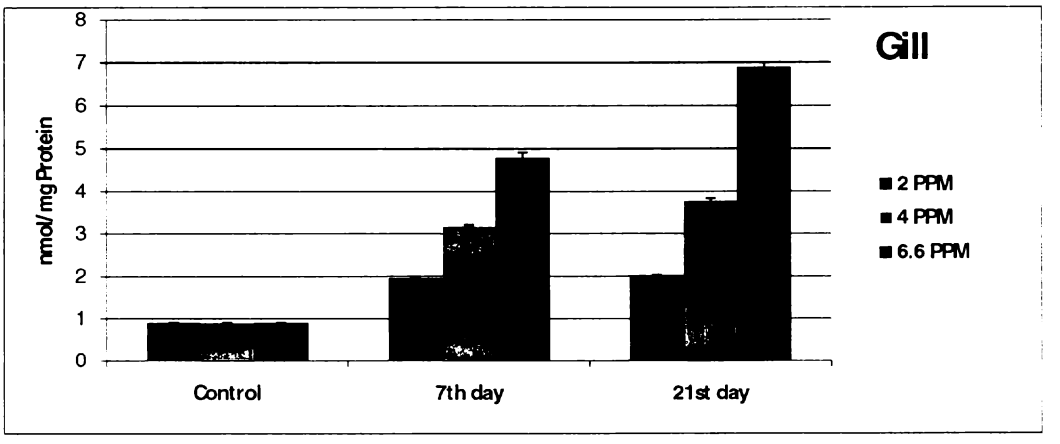


Fig.4.18 Malonaldehyde (MDA) in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.12 ANOVA table for malonaldehyde concentration in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	52.029	2	26.014	44.734	0.000
Concentration	245.263	3	81.754	140.584	0.000
Day	12.319	1	12.319	21.184	0.000
Error	80.252	138	.582		
Total	392.722	144			

Results of ANOVA reveal that the values vary significantly with the factors tissue, concentration and day. From the three factor analysis of variance (table 4.12) it can be seen that the p-values for each of these factors are less than 0.05. Here brain recorded relatively high values compared to other two tissues, which can be observed from the summary table (4.11). Further comparison by LSD analysis shows that values vary significantly between different concentrations. Difference between values of tissue group is also significant except for the comparison between brain and gill, where the difference in values is not statistically significant.

Table 4.13 Conjugated dienes (CD) concentration in liver, brain and gill tissues of *H. fossilis* subjected to different concentrations of monocrotophos

		2 ppm		4 ppm		6.6 ppm	
Tissue	Control	7th day	21st day	7th day	21st day	7th day	21st day
Liver	9.17 ± 0.05	9.39 ± 0.02	9.60 ± 0.02	9.63 ± 0.06	9.78 ± 0.03	9.95 ± 0.07	10.52 ± 0.07
Brain	8.50 ± 0.02	10.01 ± 0.10	12.89 ± 0.06	14.60 ± 0.06	17.87 ± 0.07	27.66 ± 0.13	29.86 ± 0.12
Gill	7.59 ± 0.02	9.65 ± 0.04	9.96 ± 0.05	18.60 ± 0.08	19.17 ± 0.08	19.88 ± 0.05	20.58 ± 0.03

- Values are expressed as nmol/mg protein
- Average of six values in each group

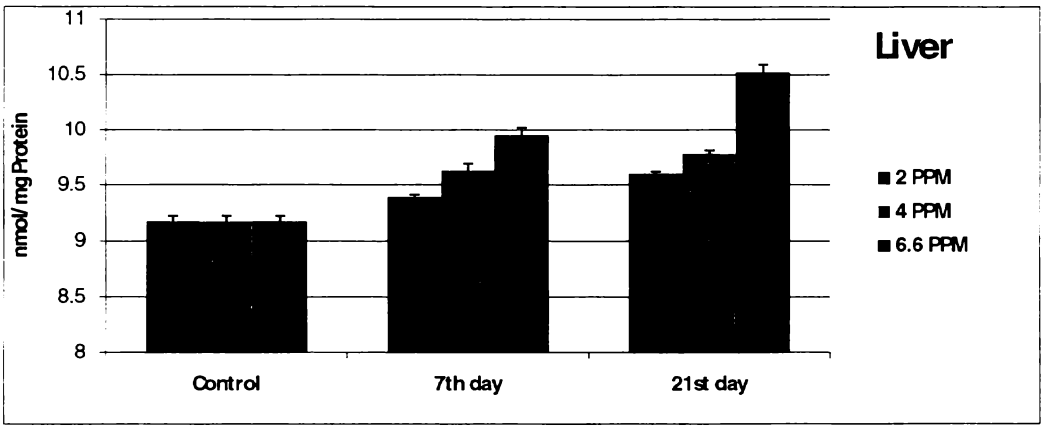


Fig.4.19 Conjugated dienes (CD) in the liver of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

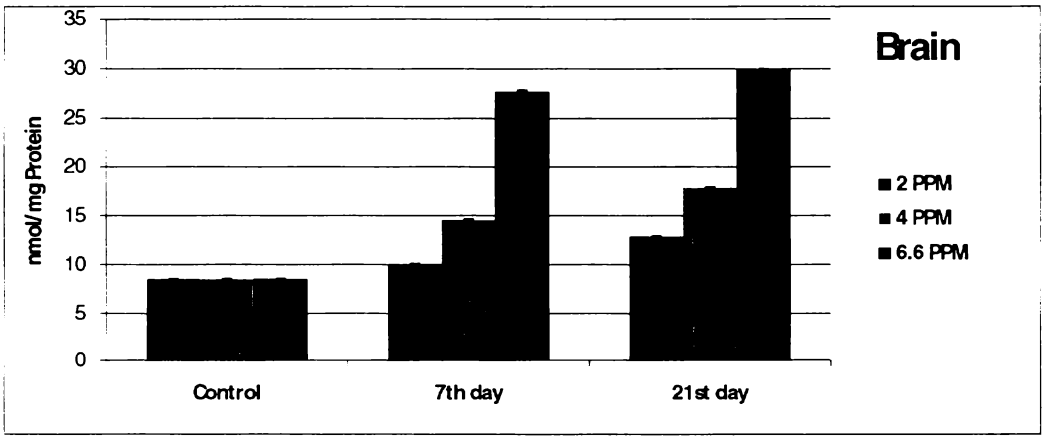


Fig.4.20 Conjugated dienes (CD) in the brain of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

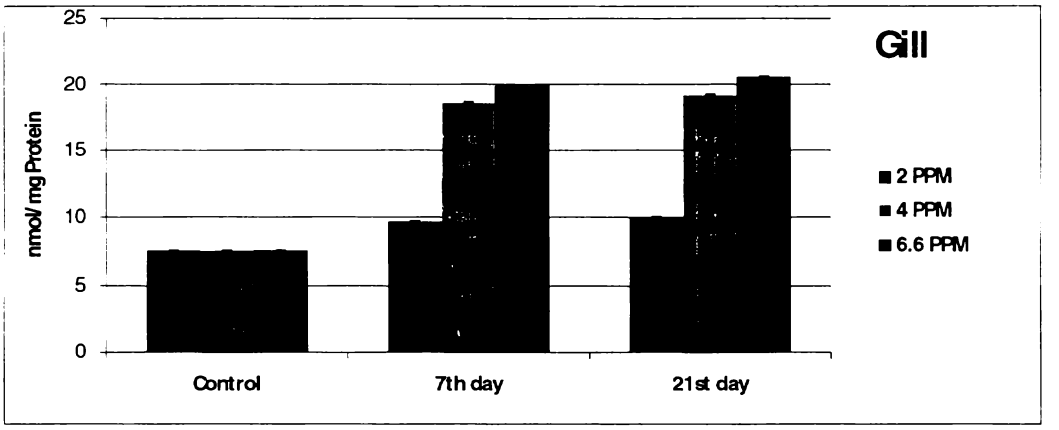


Fig.4.21 Conjugated dienes (CD) in the gill of *H. fossilis* exposed to different sublethal concentrations of monocrotophos

Table 4.14 ANOVA Table for conjugated dienes concentration in *H. fossilis*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Tissue	1085.033	2	542.516	47.073	0.000
Concentration	2849.980	3	949.993	82.429	0.000
Day	29.280	1	29.280	2.541	0.113
Error	1601.970	139	11.525		
Total	5601.831	145			

Since the p-value for all the factors are observed to be less than 0.05, except for the day, in the ANOVA table (4.14), it can be concluded that the values vary significantly with the factors tissue and concentration. Here LSD analysis shows that there is significant difference between different concentration groups and control group. Also the values of different tissue group vary significantly. Here the brain gives higher value compared to the liver and gill. Highest value of conjugated dienes is observed at 6.6 ppm in all tissues.

From each of the conclusions it can be finally wrapped up that the values vary significantly with tissues and concentration. To see is there cases in which two tissues or concentrations are same, LSD analysis were carried out. In LSD analysis the effect of two tissues or concentrations at a time can be compared and see whether they are equal or not. There is no meaning in going for LSD as there is no significant difference between two levels of the any factor. Table 4.15 shows the results of the Least Significant Difference (LSD) for the tissue and table 4.16 illustrates the one for various concentration levels.

Table 4.15 LSD analysis for liver, brain and gill tissues of *H. fossilis*

	SOD	CAT	GST	GSH	GPX	MDA	CD
Liver vs Brain	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Liver vs Gill	**0.169**	0.000	0.000	0.000	0.000	0.000	0.000
Brain vs Gill	0.000	0.021	0.011	0.000	0.000	**0.831**	0.008

From table 4.15, representing the p-values of pair wise comparisons tissues, it can be seen that almost all the p-values are less than 0.05 it can be observed that wherever the p-values are less than 0.05, the difference of mean of the recorded values vary significantly. But in the cases shown with boldface letters i.e., brain and gill in the case of MDA (p-value=0.831) and liver and gill in the case of SOD (p-value=0.169) do not vary significantly in their mean values.

Table 4.16 LSD analysis for various concentrations of MCP treatments

	SOD	CAT	GST	GSH	GPX	MDA	CD
Control vs 2 ppm	0.000	0.000	0.000	**0.594**	0.000	0.000	0.023
Control vs 4 ppm	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Control vs 6.6 ppm	**0.413**	0.000	0.000	0.000	0.000	0.000	0.000
2 ppm vs 4 ppm	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2 ppm vs 6.6 ppm	0.004	0.000	0.000	0.000	0.000	0.000	0.000
4 ppm vs 6.6 ppm	0.000	0.000	0.000	0.000	0.000	0.000	0.000

- Values given in bold face are not significant

For all the pair wise comparison of concentrations for which the p-values are less than 0.05, two concentrations do not come closer in mean of the recorded values. But control and 2 ppm in the case of GSH, control and 6.6 ppm in the case of SOD have almost the same mean values for the recorded values.

4.5 Discussion

Many environmental pollutants have oxidative stress inducing effect in fish. The oxidative stress resulting from the production of ROS has gained considerable interest in the field of ecotoxicology. The present study demonstrated that monocrotophos has a high oxidative stress inducing potential in *H. fossilis*. Exposure to monocrotophos induced significant variation in the levels of SOD, CAT, GST, and GPx as well as the Ascorbic acid and GSH contents and LPO levels resulting in oxidative stress. The drop in swimming performance observed in the fish exposed to monocrotophos could be due to the inhibition of acetylcholine esterase enzyme or to a larger energy requirement to carry out the detoxification processes and/or to respond to stress stimuli.

In this study SOD and CAT activities of monocrotophos exposed group showed significant increases when compared to the control group. The SOD-CAT system provides the first defense against oxidative stress. The increases in the activities of these enzymes were probably a response towards increased ROS generation in pesticide toxicity (Johns *et al.*, 2001). Usually a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants (Dimitrova *et al.*, 1994). The increased SOD and CAT levels induced by monocrotophos in *H. fossilis* indicate an elevated antioxidant status attempting to neutralize the impact of the ROS. These trends substantiate the statement of Alves *et al.*, (2002) that the exposure to pesticides can elicit pro-oxidant conditions that trigger adaptive responses such as increases in the activity of antioxidant enzymes.

Initially there was a slight increase in the SOD activity in lowest concentration, but at the medium concentration of monocrotophos the SOD activity was rapidly increased, and at highest concentration, the

activity was low. The trend was similar for both time intervals. The lowest SOD activity at the highest concentration can be probably due to the rigorous oxidative stress condition which eventually led to the tissue damage thereby directly interfering with enzyme production.

Increased GST activity is involved in xenobiotic detoxification and excretion of xenobiotics and their metabolites including monocrotophos. GST plays an important role in protecting tissues from oxidative damage. In this study, the increased activity of GST in all tissues of monocrotophos exposed group of fish reflects the possibility of a more efficient protection against pesticide toxicity. GST has been reported as a biomarker for assessing the environmental impact of organic xenobiotics generating oxidative stress (Livingstone, 1998; Ariza *et al.*, 1991). In the present study the GST was more sensitive in hepatic tissue than gill and brain, indicating the efficient role of liver in xenobiotic detoxification.

Reduced activity of GPx in all tissues of monocrotophos group in all intervals could be attributed to O^{-}_{2} production or to the direct action of pesticide on the enzyme synthesis in the concerned tissues. Although xenobiotics can increase the GPx activity (Almeida *et al.*, 2002; Sayeed *et al.*, 2003; Zhang *et al.*, 2004), Fatima *et al.*,(2000) reported a low activity of GPx in different fish tissues exposed to paper mill effluent indicating the inefficiency of these organs to neutralize the peroxide impacts. Similarly an organophosphate insecticide malathion reduced GPx activity in mice erythrocytes (Yarson *et al.*, 1999). GPx reduction was also reported after combined treatment with pesticides 2, 4-D and azinphosmethyl in the brain of carp, *Cyprinus carpio* (Oruc *et al.*, 2004) and in the liver of Nile tilapia (Oruc and Uner, 2000). Enzyme activity can be decreased by negative feedback from excess of substrate or damage by oxidative

modification. A reduced GPx activity could indicate that its antioxidant capacity was surpassed by the amount of hydroperoxide products of lipid peroxidation (Remacle *et al.*, 1992).

The relationship between the changes in the GST activity and the GSH levels could imply, to some extent, that there is a limitation of GST activity by the on hand levels of GSH. Though, it is not clear to which extent the decrease in the GSH levels is accountable for the raise in the GST activity. Moreover, the drop in GSH levels was accompanied by drop in GPx activity. GPx catalyzes the reduction of H_2O_2 and organic peroxides to water and their corresponding stable alcohols employing GSH as a reducing agent. The GPx activity depends on the presence of GSH, which is oxidized in this process. Thus, GPx activity is likely to be influenced by GSH levels. The decreased GPx activity may also be related to the decreased availability of the GSH needed to reduce the ROS impact (Cheung *et al.*, 2004).

Lipid peroxidation has been reported as a major contributor to the loss of cell function under oxidative stress condition (Storey, 1996). Present study shows that monocrotophos exposure for a period of 21 days led to severe oxidative stress with significant increase of LPO in gill and brain of *H. fossilis* while compared to the liver. Gills due to their large surface area and permeability are the primary sites for absorption of xenobiotics and may be the first site for the effect of monocrotophos. Therefore it seems obvious that there is high level of LPO in the gill of monocrotophos exposed *H. fossilis*. Since OP compounds are lipophilic substances they penetrate blood-brain barrier and may enhance LPO by direct interaction with the cellular plasma membranes of brain (Hazarika *et al.*, 2003). The brain of fish contains low levels of antioxidants and higher levels of oxidizable

catecholamines and peroxidizable unsaturated lipids (Lasner *et al.*, 1995). It may be therefore more vulnerable to oxidative damage compared with other tissues. Bracardo *et al.*, (2005) demonstrated that an increase in MDA levels in the cerebral cortex and hippocampus of OP insecticide malathion treated rats, in addition to AChE inhibition, indicating that oxidative stress may be a mechanism underlying the central effects of OP exposure. Liver LPO content did not vary significantly at exposure to monocrotophos indicating that this organ resisted the oxidative stress by means of a little more efficient antioxidant system.

The increase in MDA and CD production in the present study implies that ROS induced oxidative stress can be one of the main facets of monocrotophos toxicity. It has been reported that LPO may be induced by a variety of environmental pollutants (Ploch *et al.*, 1999; Ahmed *et al.*, 2000; Oakes *et al.*, 2004). Given that LPO is regarded as a reliable indicator of oxidative damage of cellular components, results of the present investigation suggests that the exposure to monocrotophos enhanced ROS synthesis in the gill, brain and to a lesser extent in the liver of *H. fossilis* and that antioxidant defenses were not absolutely able to effectively scavenge them leading to oxidative stress.

The observation by earlier researchers (Oost *et al.*, 2003; Monteiro *et al.*, 2006), that GPx handle an extremely significant role in protecting membranes from damage due to LPO suggests that the major detoxification function of GPx is the termination of radical chain propagation. In the perspective of present investigation, the significant drop in GPx activity reflects a possible antioxidant failure responsible for the observed increase in LPO levels. In this study results indicate that gills and brain are the most sensitive organs to oxidative stress in comparison to liver.

Reasons for these differences could be the different rates of free radical generation and different antioxidant potentials of the tissues (Winston, 1991; Fatima *et al.*, 2000).

Total GSH is a prospective biological index to indicate contaminant exposure (Stein *et al.*, 1992). In this study, total GSH contents were initially elevated at the 7th day observation for both lowest and medium concentrations and were but depleted towards the 21st day period which indicates an adaptive mechanism for xenobiotic detoxification and later depletion in course of the process. For the highest concentration, it fell sharply at both time intervals indicating their rapid utilization to meet extreme stress level or the pesticide may have directly acted upon its synthesis.

4.6 Summary

Foremost reasons for studying the pro-oxidant and other radical processes in humans are the understanding and treatment of disease, and the understanding of the action of toxins (Halliwell and Gutteridge, 1999). Similar considerations may apply to aquatic organisms, but it is the understanding of action of toxins is of prime relevance, particularly in the perspective of environmental management (Livingstone, 2001). The importance of free radical reactions and reactive oxygen species in the physiological processes of living organisms and in the mechanisms of toxicity by exposure to a variety of environmental pollutants inspired an explosive boost of research and applications into the field of oxidative stress caused by ROS. The resulting oxidative damage to lipids and proteins and the adverse effects on the antioxidant defense mechanisms of aerobic organisms have been used in recent years as biomarkers for monitoring environmental pollution. The current knowledge that such processes of

oxidative damage occur in aquatic organisms conferred the momentum to widen environmental end ecotoxicological studies to aquatic organisms as sentinels of environmental contamination by chemicals.

The findings of the present study provide information on the impact of interaction of monocrotophos with an important non-target organism, fish. Further studies based on the findings of the present investigation would certainly validate the use of enzymatic and non-enzymatic antioxidants as biomarkers of exposure to environmental contaminants including pesticides in aquatic organisms. Also, controlled long-term studies where fish are exposed to contaminants producing low-level oxidative stress are necessary to reasonably mimic exposure to contaminants in the environment. The present work demonstrated that monocrotophos induces oxidative stress in *H. fossilis*. From an ecophysiological viewpoint it is evident that the use of monocrotophos, single largest selling agrochemical in India, in agriculture must be carefully evaluated.

Chapter 5

HISTOPATHOLOGICAL EFFECT OF MONOCROTOPHOS

Contents

5.1 Introduction

The increasing emphasis on the evaluation and monitoring of aquatic ecosystems has highlighted the need to set up appropriate biological indices for these locations. Fish diseases and histopathology, with a wide range of causes, are ever more being used as indicators of environmental stress since they present an explicit biological end-point of past exposure. The aquatic environment is a major sink for potentially hazardous pollutants emitted from agricultural, industrial and domestic sources. Inventory-based chemical monitoring programmes are restricted to identification of a limited range of contaminants and give no information on their biological significance. Recently, greater emphasis has been given to the assessment of the causal relationships between contaminant exposure and observable biological effects in aquatic organisms (De Flora *et al.*, 1991).

In field studies fish can be used as a monitoring tool for the quality of the aquatic environment. Evidently this is done in wild animals, but also the use of semi-field (mesocosm) studies, and more recently, caged sentinels have shown to be successful (Vethaak and Wester, 1996; Harries *et al.*, 1997; Vethaak *et al.*, 2002). The use of fish eco-epidemiology has become predominantly developed in marine and fresh water pollution monitoring programs. Initially, larger animals were screened for visible disease signs/abnormalities on skin and liver in addition to, tissue residue concentrations of toxicants and change in metabolic biomarkers, but as more in depth information was required such as further characterization or screening for early pre-neoplastic changes, histopathology was introduced.

The tool histopathology helps in identifying target organs of toxicity and mechanism of action and this tool was materialised by combining knowledge and experience from fundamental fish biology (anatomy,

physiology, endocrinology), mammalian toxicological pathology and mammalian toxicology. At a quick look this would not seem to be of direct 'ecological relevance'. But other than the specificity of the induced effects histological monitoring has a better sensitivity compared to classical toxicological testing, since effects on the histological level will be visible at lower dosage, compared to toxicological endpoints such as mortality or behavioural changes. Considerable interest has been shown in recent years in histopathological study while conducting sub-lethal tests in fish. Tissue changes in test organisms exposed to a sub-lethal concentration of toxicant are a functional response of organisms which provides information on the nature of the toxicant.

It is generally understood that histopathological biomarkers are important as pointers of the general health of fish and they reflect the effects of exposure to a range of toxicants (Hinton *et al.*, 1992) without any geographic or ecosystem limitations. Acute changes are seen when pollutant levels are amply high, while chronic duration is necessary to determine sub lethal aspects of change (Oost *et al.*, 2003). Many of histological changes persist even after the toxicant exposure has ceased. So that organism's responses to prior toxicity can also be used to determine effects. Responses are relatively easily recognized, provided that proper reference and control data are available.

5.2 Materials and methods

Collection, transportation, acclimation and bioassay of/on *Heteropneustes fossilis* was performed, as described in the chapter I. Sampling was done on 21st day and fish were sacrificed by cervical disjunction. Liver, brain and gill tissues were dissected out and were washed to remove any debris.

5.2.1 Processing of Tissues

The tissue specimens were fixed in 10% neutral buffered formalin for twenty four hours washed thoroughly with water and dehydrated in a graded ethanol series, (20%, 30%, 50%, 70%, 90% and 100%) quickly. Tissues were kept in absolute alcohol for one hour and were cleared using xylene for 3 hours. Infiltered the tissues in 2-3 changes of molten paraffin of melting point 58-62°C. Tissues were embedded in wax (58-60°C) and made into blocks, which were then labelled and stored in polythene covers.

5.2.2 Sectioning and staining techniques

Thin sections were cut from the block samples manually using a rotary microtome. The thickness of the sections was adjusted to 3-4 μm. Mayer's egg albumin glycerol (1.1 v/v) was used as an adhesive for fixing paraffin ribbon on to glass slides. Slides were kept overnight. Slides were cleared off paraffin using xylene and sections were hydrated in alcohol series (20%, 30%, 50%, 70%, 90% and 100%) by giving brief dips. Sections were stained using Ehrlich's haematoxylin and counter stained with 0.5% eosin. Brief dips in 95% and 10% alcohol were given to dehydrate the slides. Finally slides were cleared in xylene. DPX was used as the mounting medium and cover slips were put with out locking any air bubbles. Sections were observed and photographed under light microscope (OLYMPUS BX41TF, Japan).

Composition of fixative and stain used for the study

10% Neutral buffered formalin (pH- 7.0) was used as fixative. It is composed of the following ingredients.

Formalin-10ml

Distilled water- 90ml

Sodium dihydrogen orthophosphate (NaH_2PO_4. H_2O) - 400 mg

Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) – 650mg

Ehrlich's Hematoxylin was used for staining. It is composed of following ingredients

Distilled water- 10 ml

100% alcohol- 100 ml

Glycerin- 100 ml

Glacial acetic acid- 10 ml

Hematoxylin- 2 g

Aluminum ammonium sulphate (or potassium alum) – 20 g

Eosin (0.5%) as counter stain has the following composition

Eosin- 500 mg

70% alcohol- 100 ml

5.3 Results

Liver of the control group of fishes were devoid of any pathological changes and hepatocytes were seen as well arranged structures in histological sections. Liver of the MCP treated animals exhibited various histopathological features such as vacuolated hepatocytes, cell necrosis, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. Small oedema could be found in hepatocytes.

At higher concentration, 6.6 ppm, total loss of architecture of the liver tissue was observed, with more ceroid pigments, more fibrosis and more necrosis. Nuclei were all pycnotic. Other prominent features were swelling of hepatocytes, cytoplasmic degeneration and granulation of cytoplasm. At the medium concentration, 4 ppm, there was an increased occurrence of stromal connective tissue. Vacuolation and some areas of necrosis were also

noted. At the lowest concentration, 2 ppm, observed changes were mainly fibrosis and necrosis.

Brain of the control group of fishes were quite normal without any pathological changes while, brain of the treated animals displayed histopathological features such as gleal cell proliferation, encephalomalacia, total damage to neurons, neuronophagia and loss of neurons. At the highest concentration, 6.6 ppm, gleal cell loss, appearance of cytoplasmic vacuoles, encephalomalacia and neuronophagia were prominent pathological features. At the medium concentration, 4 ppm, encephalomalacia, satellitosis, neuronophagia and disappearance of neurons were observed. At the lowest concentration 2 ppm, focal encephalomalacia and glyosis were the observed features.

Gill of the treated fishes were showing pathological features like hyperplasia, lifting of secondary epithelium, squamous metaplasia, fusion of secondary lamellae, break down of pillar system and hyperaemia of cells. At the highest concentration, 6.6 ppm, areas of hyperplasia, squamous metaplasia, lifting of secondary epithelium and fusion of secondary epithelium was observed as prominent pathological features. At the medium concentration, hyperplasia, fusion of secondary epithelium and break down of pillar system were observed. While for the lowest concentration, 2 ppm, hyperemia, and hyperplasia along with pillar system break down were the main pathological features observed. Slides of histological sections of brain, gill, and liver of *H. fossilis* are given as plates A-Z.

5.4 Discussion

Histopathology is a higher-level response, reflecting prior alteration in physiological and/or biochemical function (Hinton *et al.*, 1992). Studies of responses of stinging catfish gill, brain and liver showed a reduction in cell

membrane integrity, lysosomal function and alterations in the activity of major stress enzymes, at sub lethal levels of monocrotophos exposure, given the liver biochemical activity as reported in previous studies (Rao *et al.*, 2004, 2006) in exposed environments, it was expected that gill and liver histopathology would demonstrate to be sensitive measurement endpoints.

Gills are generally considered good indicators of water quality, being used as models for studies of environment impact, such as of xenobiotics (Rankin *et al.*, 1982; Fanta *et al.*, 2003) being models for environmental impact assessment (Mallat, 1985; Evans,1987; McKim and Erickson, 1991; Laurent and Perry, 1991; Bonga and Lock, 1992). For fish, gills are crucial organs for their respiratory, osmoregulatory and excretory functions. Respiratory distress is one of the early signs of pesticide poisoning (McDonald, 1983). A high rate of absorption of pesticide through gills also makes fish a vulnerable non-target organism of its toxicity (Srivastav *et al.*, 1997).

Tissue damages brought about by waterborne pollutants can be easily observed because the fish gills come into immediate contact with the environment. The gill surface is more than half of the entire body surface area. In fish the internal environment is separated from the external environment by only a few microns of delicate gill epithelium and thus the branchial function is very sensitive to environmental contamination. Hence, fish serve as excellent bioassay animals for toxicological impact studies and have been widely used for this purpose. Water pollution induces pathological changes in fish. As an indicator of exposure to contaminants, histology represents a useful tool to assess the degree of pollution.

The teleost fish gill is covered by a complex epithelium whose function is regulated by perfusion through an intricate vascular system. In

addition to being the site of gas exchange for these aquatic animals, the gill epithelium possesses transport steps which mediate active and passive movements of ions, counteracting dissipative movements down electrochemical gradients between the fish's blood and water. These same transport steps play major roles in acid-base regulation and excretion of unwanted nitrogen in the form of ammonia. A variety of aquatic pollutants produce gross histopathological changes of the gill epithelium, which are often associated with osmoregulatory, acid-base, or hemodynamic malfunction. Since similar pathways and receptors are common to a variety of human tissues, which are affected by environmental pollutants (e.g., kidney, intestine, liver, blood vessel etc.), the fish gill presents an apt model which may be used to examine general epithelial pathologies induced by toxic substances.

In this study, desquamation, necrosis, hyperplasia of epithelial cells, squamous metaplasia and fusion of the secondary lamellae were observed in the gills after exposure to monocrotophos. Karan *et al.,* (1998) observed lesions such as epithelial hyperplasia and lifting of secondary lamellae on the gills, swelling at the tips of several secondary lamellae, and club-shaped secondary lamellae as responses to pesticides. Erkmen *et al.,* (2000) reported the lifting of epithelial layer from gill lamellae, necrosis and degeneration of secondary lamellae, shortening of secondary lamellae, and club shaped lamellae in the gills of *Lepistes reticulatus* exposed to pesticide.

Epithelial necrosis, secondary lamellae showing fusion and lifting of epithelium have also been showed in other species (Cengiz and Unlu, 2002, 2003). *Ortiz et al.,* (2003), reported fusion of the secondary lamellae, increased rising of the branchial epithelium and intraepithelial oedema in gills of fish after an accidental discharge of lindane. The observed epithelial

necrosis and desquamation of the gill epithelium are direct responses to the action of pesticide. The defense responses observed are lifting up of the epithelium and hyperplasia. The lifting up of the epithelium increases the distance through which the toxicant has to travel to reach the blood stream (Cengiz, 2006). Collapse of the pillar cell system was observed in this study which lead to the breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward (Alazemi *et al.*, 1996). Such effects on respiration (Schwarzbaum, 1988) and consequent lower levels of oxygen in the tissues impair the health of fish.

Gill hyperplasia might serve as a defensive mechanism leading to a decrease in the respiratory surface and an increase in the toxicant-blood diffusion distance. But the defense responses will take place at the expense of respiratory competence of the gills and finally, the respiratory impairment must outweigh any protective effect against pollution uptake (Cengiz, 2006). Degenerative changes, such as gill epithelial cell necrosis, observed in this study and lesions of this sort are believed to mirror the direct deleterious effects of irritants rather than a compensatory response to pollutants (Mallatt, 1985).

Other predominant pathological responses of *H. fossilis* gills involved basal epithelial cell proliferation and extensive proliferation of mucous cells following exposure to monocrotophos. This is consistent with observations of proliferation of gill mucous cells and basal epithelial cells following exposure to organic contaminants (Spies *et al.*, 1996; Teh *et al.*, 1997). A hyper secretion of mucus is considered a defense response to contaminant exposure rather than a direct effect of toxicants (Mallatt, 1985). Mucous cells contain mucins, polyanions composed of glycoproteins that can be efficient in trapping toxicants and aid in the prevention of toxicant entry

into the gill epithelium (Perry and Laurent, 1993). Although mucous cell proliferation may be helpful in reducing toxicant entry, the end result is an increase in the distance for gas exchange along the secondary lamellae, potentially reducing the efficiency of gas exchange and causing hypoxic conditions (Ultsch and Gros, 1979).

In general, the circulatory system transports required nutrients to different tissues of an animal's body. As the control of rate of blood flow and its allocation to different tissues in a fish is based principally on homeostasis of respiratory system, the cardiovascular responses could be induced as a consequence of the damaged respiratory system, which in turn would badly affect the health of fish and put their life at risk. The morphological findings in this study indicate a string of symptoms in fish gill, which confirms severe damage of fish gills and severely influence normal physiological activities.

The liver holds responsibility for vital functions of basic metabolism and it is also the key organ of accumulation, biotransformation and excretion of contaminants in fish, together with degradation and bioactivation of pesticides (Triebskorn *et al.,* 1994, 1997). The assessment of biochemical and histological changes in fish liver has become an important tool for monitoring environmental exposure of fish to contaminants in experimental studies. As mentioned earlier, majority of the insecticides are biotransformed to metabolites by the liver, through enzymes from the soluble fractions of mitochondria and microsomes, and in some cases the metabolites are more toxic than the original product. Thus, liver is the organ that contains the major concentration of organophosphorus residues (Bender, 1969; Ansari and Kumar, 1987a) and that undergoes different levels of damage as a consequence of this process.

Liver of the monocrotophos exposed group of fishes displayed an array of pathological traits such as vacuolation of hepatocytes, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. There were many regions in the liver where cells were highly vacuolated, leading to a foamy aspect. Shrunk and pycnotic nuclei indicate that the cells became hypo functional, and at the end, necrosis was extensive. Ram and Singh (1988) also showed similar changes in *Channa punctatus* exposed to pesticide carbofuran for six months. The widespread vacuolation might be likely due to accumulation of glycogen in hepatocytes (Wester and Canton., 1986).

At the highest concentration, 6.6 ppm, liver showed total loss of architecture with symptoms including disarray, connective tissue damage, granulation and vacuolation of the cytoplasm, and hypertrophy of the nucleus, necrosis, pycnosis, fatty infiltration, and glycogen depletion. The chain of pathological symptoms portrayed above certainly indicates that the liver is the chief detoxification organ in the organisms. So, the widespread vacuolation of the liver might be a common response in fish hepatocyte to stressor (Chun-Yang Liao, 2006). Leaner and Mason (2004) experimented on toxicant induced stress responses in Sheephead minnow (*Cyprinodon variegates)* and reported that exchange between the blood and the internal organs was relatively slow, with maximum uptake in the liver and gill occurring next to dietary exposure, which demonstrated that fish's liver and gill were common target of aquatic pollutant's action.

Alterations associated with the brain to subtle exposure of monocrotophos are not reported in the accessible literature. The present experiment revealed that monocrotophos can be neurotoxic, as proved by the histopathological changes observed. Brain of the treated fish exhibited

glyosis or glial scarring which is a reactive cellular process that occurs after injury to the central nervous system. As with scarring in other organs and tissues, the glial scar is the body's mechanism to protect and begin the healing process in the nervous system. Although the glial scar does a good job at controlling and suppressing further physical damage, many neuro-developmental inhibitor molecules are secreted by the cells within the scar that prevent complete physical and functional recovery of the central nervous system.

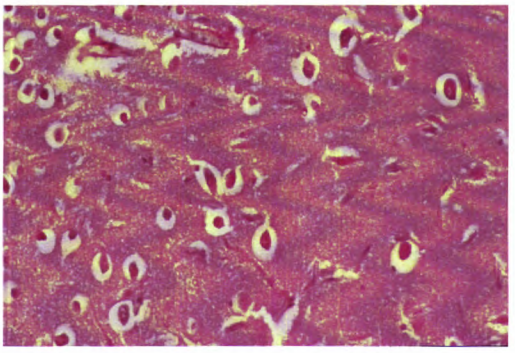
Brain also showed condition of neuronophagia, or phagocytosis of neurons, in which a dying neuron is surrounded by glial cells. Vacuolation may have been due to glycolysis leading to microsomal and mitochondrial dysfunctions. Another pathological feature exhibited by brain was focal encephalomalacia, which in fact is the softening of the brain associated with inadequate cerebral blood flow. Satellitosis, a condition marked by an accumulation of neuroglia cells around the neurons of the central nervous system; often as a prelude to neuronophagia, was also observed in the brain of monocrotophos treated *H. fossilis.*

All the histopathological changes indicate that the exposure to sublethal concentrations of monocrotophos caused destructive effect in the gill, liver and brain tissues of *H. fossilis.* Tissue alterations, such as those observed in this study may result in severe functional problems, eventually leading to the death of fish. The findings of the present histological investigation reveal a direct correlation between pesticide exposure and histopathological disorders observed in tissues. Generally, gill and liver pathological data suggest that degenerative changes were the most prevalent and sensitive changes observed following exposure of *H. fossilis* to monocrotophos. All effects that were observed in the gills, brain and liver

induce behavioural changes as a consequence of the decrease in the general state of health of *H. fossilis*. This will, together with the inhibition of plasma cholinesterase as observed in the present study, negatively influence the prospects of survival of these fish in the natural environment. They will face difficulties in detecting, identifying, and responding in proper way to chemical stimulation in nature.

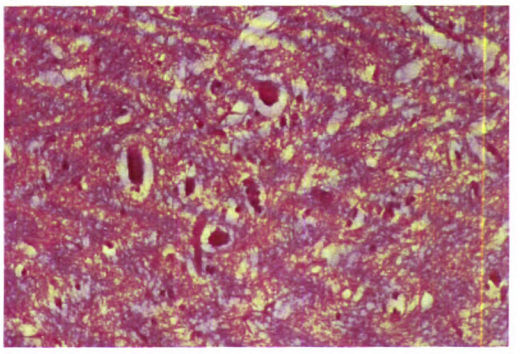
5.5 Summary

In analogy with mammalian toxicology where it is firmly established, fish histopathology ought to have a place in the toxicology toolkit, in particular when the endangered aquatic environment is an issue. Various modern techniques in pathology can equally be applied to fish and these will contribute to its analytical power. As a conclusion, the findings of the present histological investigations demonstrate a direct correlation between monocrotophos exposure and histopathological disorders observed in tissues. Monocrotophos, an extensively used agrochemical in India has caused histopathological changes in *Heteropneustes fossilis* (Bloch) at sub lethal levels of exposure. When these pathological endpoints are measured in combination with other parameters like enzyme responses, haematological parameters, membrane studies and bioconcentration studies, a clearer picture of the complex interactions between anthropogenic and natural environmental modifiers will emerge.



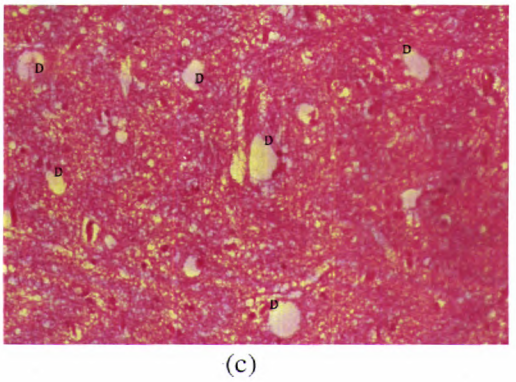
(a)

Brain of *H. fossilis* –control (40x)



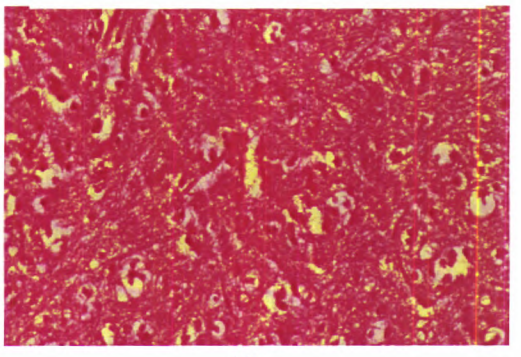
(b)

Brain exposed to 6.6 ppm MCP showing neuronophagia (40x)



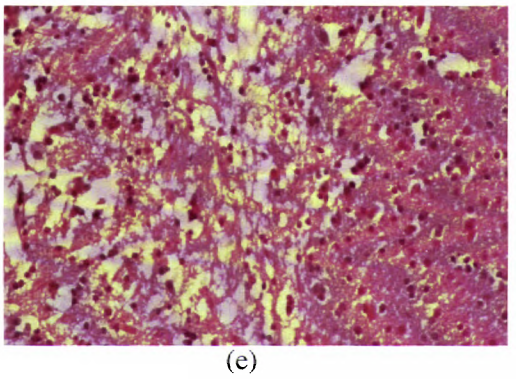
(c)

Brain exposed to 6.6 ppm MCP - neuronophagia and disappearance of neurons (40x)



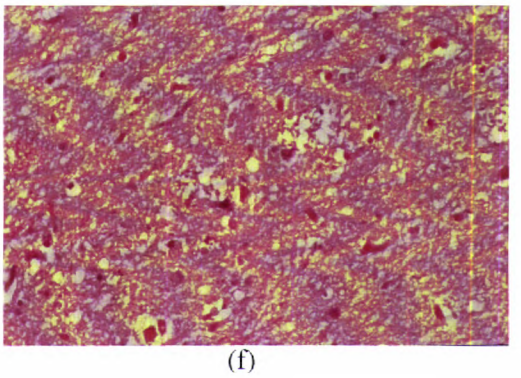
(d)

Brain exposed to 6.6 ppm MCP showing total loss of neurons (40x)



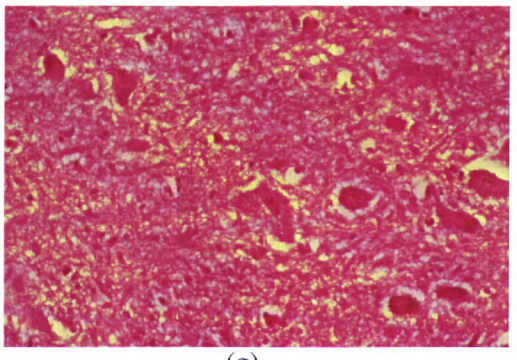
(e)

Brain exposed t o 2 ppm MCP showing necrosis and encephalomalacia (40x)



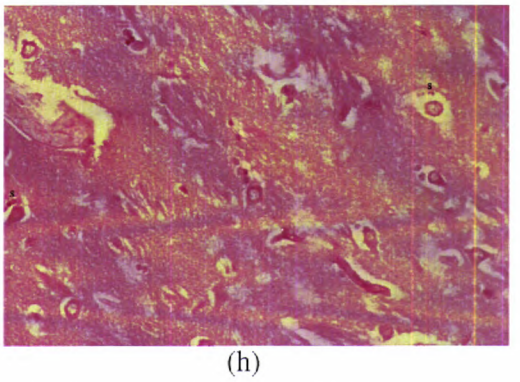
(f)

Brain exposed to 2 ppm MCP showing encephalomalasia (40x)



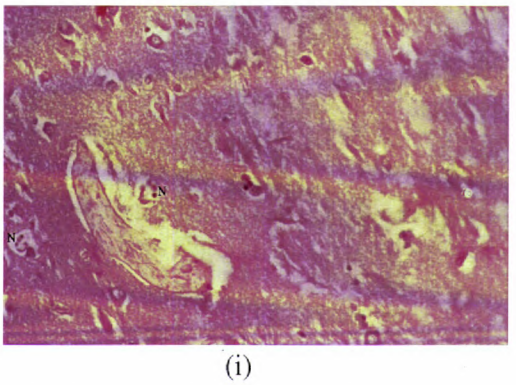
(g)

Brain exposed to 6.6 ppm MCP showing vacuolation (40x)



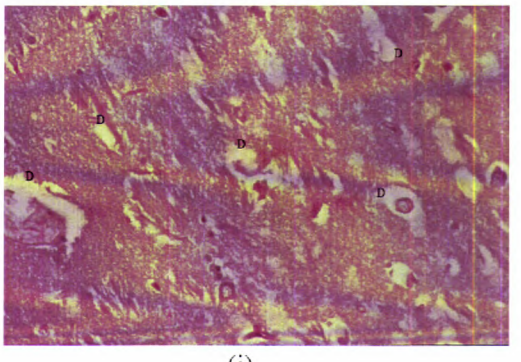
(h)

Brain exposed to 4 ppm MCP showing satellitosis (40x)



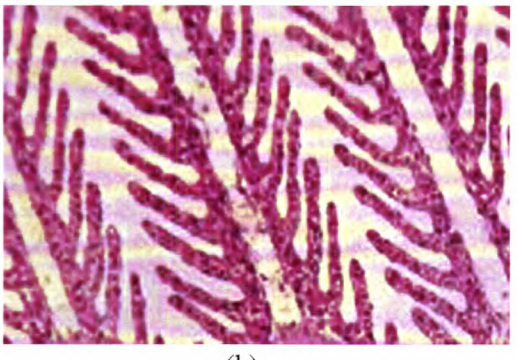
(i)

Brain exposed to 4 ppm MCP showing neuronophagia (40x)



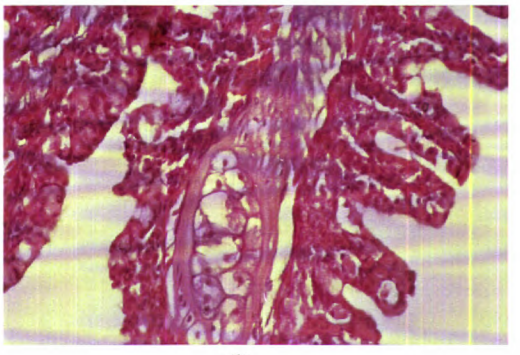
(j)

Brain exposed to 4 ppm MCP showing disappearance of neurons (40x)



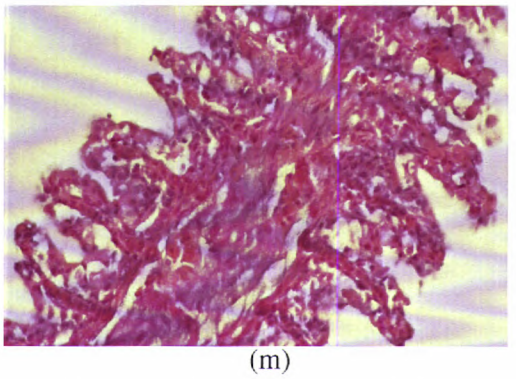
(k)

Gill of *H. fossilis* - control (20x)



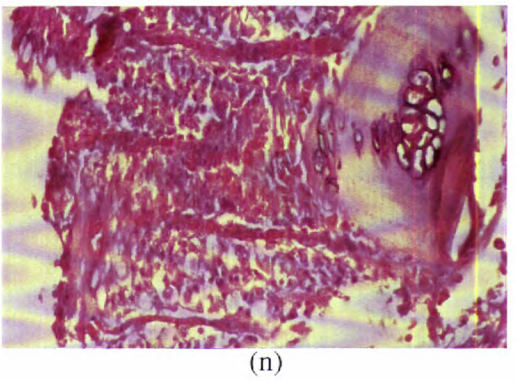
(l)

Gill exposed to 6.6 ppm of MCP showing hyperplasia (40x)



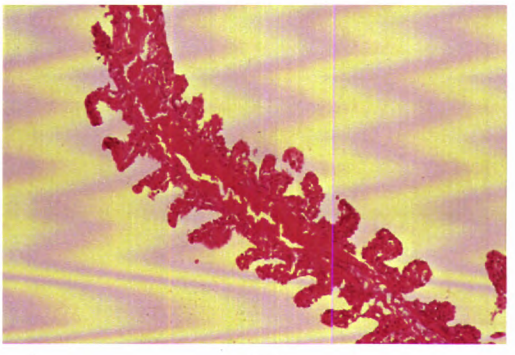
(m)

Gill exposed to 6.6 ppm of MCP showing hyperplasia (40x)



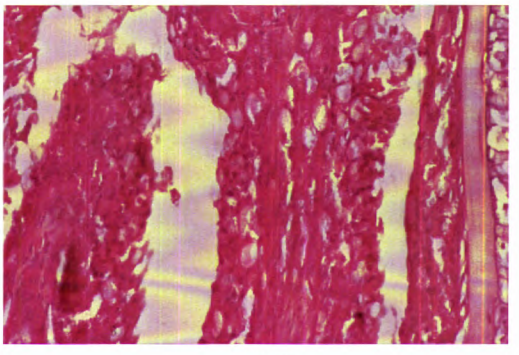
(n)

Gill exposed to 6.6 ppm of MCP showing fusion of epithelium (40x)



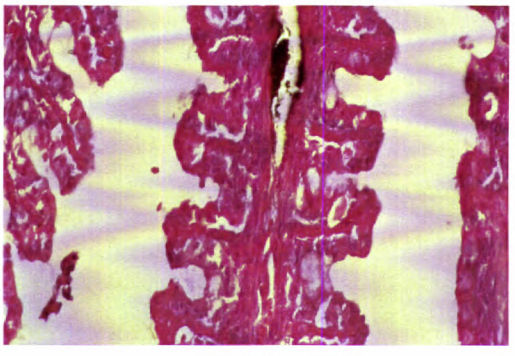
(o)

Gill exposed to 2 ppm of MCP showing hyperaemia (20x)



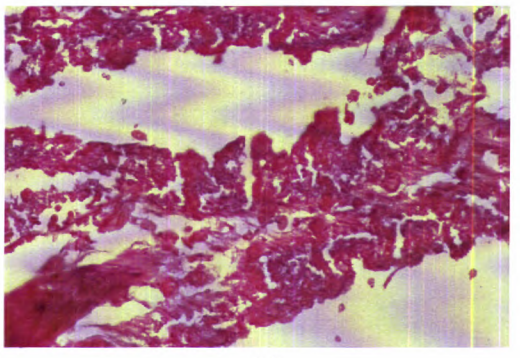
(p)

Gill exposed to 6.6 ppm of MCP showing squamous metaplasia (40x)



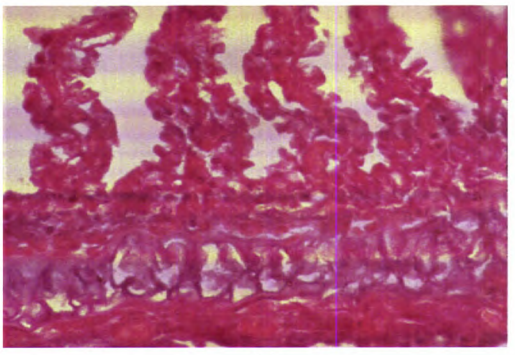
(q)

Gill exposed to 6.6 ppm of MCP showing lifting of secondary epithelium (40x)



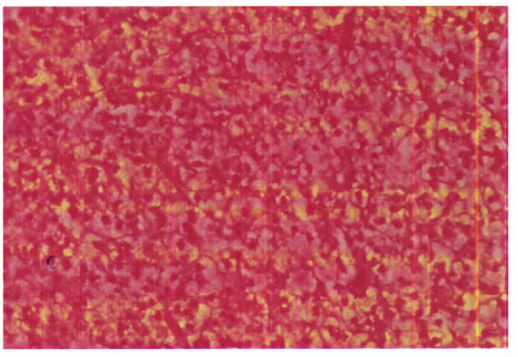
(r)

Gill exposed to 4 ppm of MCP showing fusion of epithelium (40x)



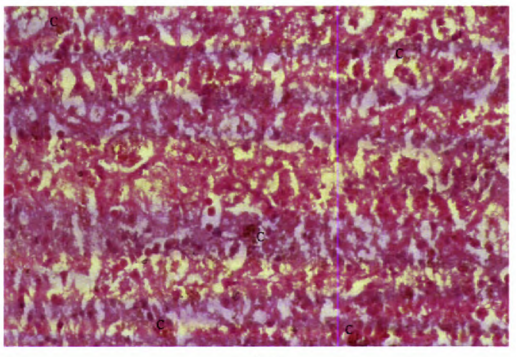
(s)

Gill exposed to 2 ppm of MCP showing hyperplasia (40x)



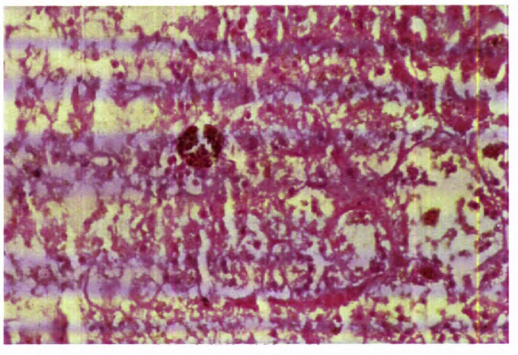
(t)

Liver of *H. fossilis* - control (40x)



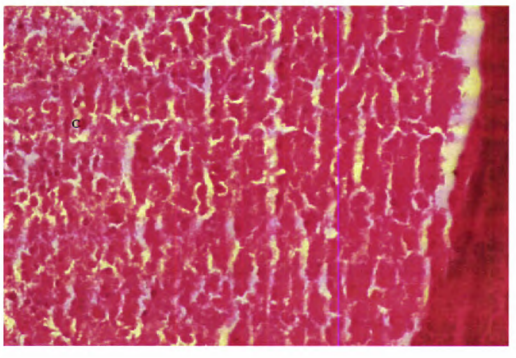
(u)

liver exposed to 6.6 ppm of MCP showing high necrosis, total loss of architecture and increased ceroid pigments. (40x)



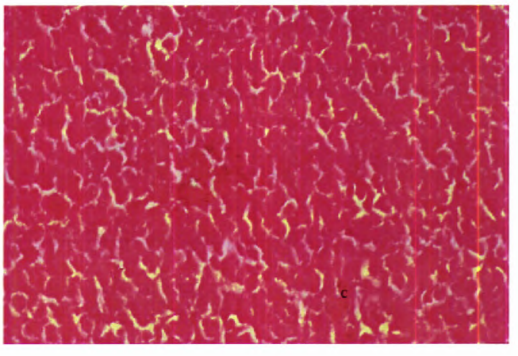
(v)

liver exposed to 6.6 ppm of MCP showing total loss of architecture. (40x)



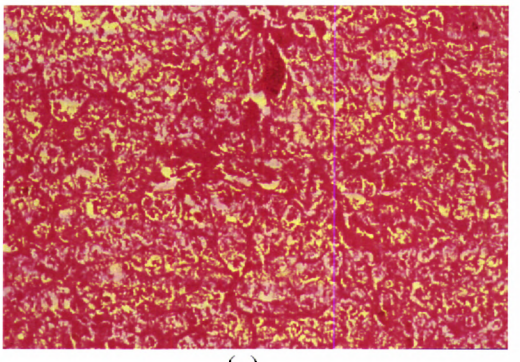
(w)

Liver exposed to 4 ppm of MCP showing necrosis (40x)



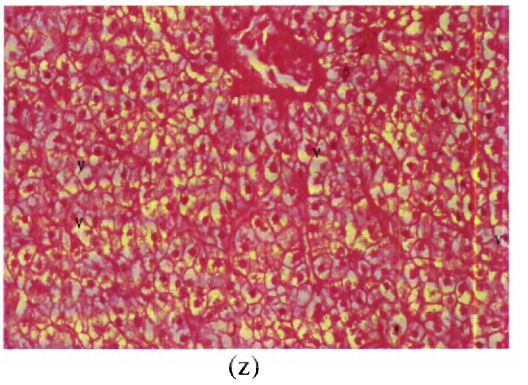
(x)

liver exposed to 4 ppm of MCP showing necrosis (40x)



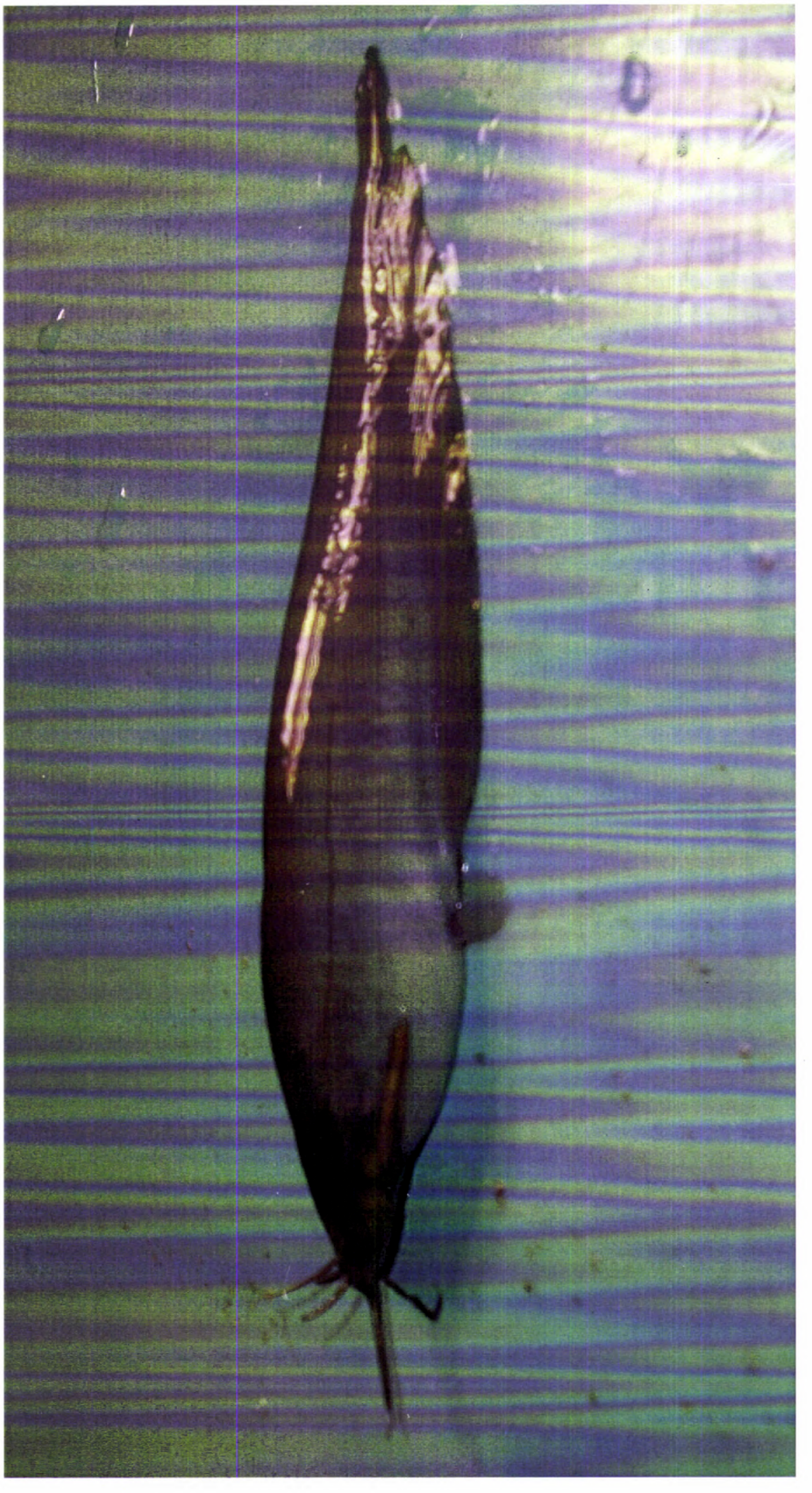
(y)

Liver exposed to 2 ppm of MCP showing mild necrosis (40x)



(z)

Liver exposed to 4 ppm of MCP showing vacuolation (40x)



Stinging catfish, *Heteropneustes fossilis* (Bloch)

Chapter 6

BIOCONCENTRATION OF MONOCROTOPHOS RESIDUE

Contents

6.1 Introduction

The unsystematic use of insecticides can be taken as one of the factors that alter the quality of environment, causing imbalances in the ecosystem, especially to the denizens of the aquatic environment. Organophosphorus insecticides (Ops) cause a non-reversible phosphorylation of esterases in the central nervous system of insects and mammals and act as cholinesterase inhibitors. The level at which a chemical is concentrated in fish depends on the absolute rates of uptake and elimination of the compound (Spacie and Hamelink, 1985). The usage of organophosphorus pesticides is preferred to the usage of other pesticides such as organochlorine compounds, because organophosphorus pesticides degrade much faster in the environment. Hence, there is an increasing demand for developing methods for the determination of such contaminants in food analysis and environmental analysis. It is often assumed that bioconcentration of organophosphate pesticides by aquatic organisms means no risk for the ecosystem and either a low partition coefficient or a high biotransformation rate prevents these chemicals from accumulating through food chains in higher organisms (Bruijn and Hermens, 1991). However the data on their bioconcentration and excretion by fish are useful for the evaluation of their risk to humans and, further, for the assessment of the contamination of fish by pesticides.

6.2 Materials and methods

Collection, transportation, acclimation and bioassay experiments of/on *Heteropneustes fossilis* were carried out as described in the chapter I. *H. fossilis* of 35.5 ± 3 g were subjected to three sub lethal concentrations (6.6 ppm, 4 ppm, and 2 ppm) of monocrotophos. Control group of fish with out toxicant were also maintained. Sampling was done on 21st day to examine the concentration of monocrotophos in the edible part of the fish i.e.

muscle. Whole body with out viscera and internal organs were taken for the analysis.

6.2.1 Extraction

The determination of monocrotophos in the muscle samples was carried out based on the method of Richardson and Seiber (1993), adapted to the current samples as follows: Muscle tissue of the fish was lyophilized and homogenized with 50% HCl (0.01 ml) and 0.5% ethanol-ethyl acetate (20 ml). This homogenate was centrifuged at 1200 rpm (5 min). After centrifugation a drop of 5% decanol in acetone was added to the supernatant. This mixture was dried on a rotary vacuum evaporator. The dried residue was redissolved in 3 ml of a 1: 1 mixture cyclohexane: ethyl acetate and ultrasonicated (1 min). Then, it was filtered through nylon filter by using a Varian Vac-Elut. This volume was redissolved in 3: 1 ethyl acetate: cyclohexane (6 ml) and introduced into a gas permeation chromatograph. The eluate obtained was dried and redissolved in ethyl acetate (1 ml). This final volume was injected into a Perkin Elmer gas chromatograph equipped with an MS detector. Column was Elite-5 ms with a length of 30 m, diameter of 0.25 mm and film thickness 0.25 μm. The detector and injector temperatures were 270 and 250° C. Helium was used as carrier gas with spilt flow at the rate of 0.55 ml/min. All solvents used were pesticide residue analysis grade and were purchased from Sigma-Aldrich Co.

6.3 Results

Constant occurrence of sub lethal concentrations of monocrotophos in the surrounding water for 21 days appears to be physiologically stressful to the stinging catfish. Concentration of monocrotophos in the different concentration groups 2 ppm, 4 ppm and 6.6 ppm were 0.0868 ± 0.0071,

0.1412 ± 0.0057 and 0.1997±0.0033 µg /gm respectively. Chromatograms showing bioconcentration of monocrotophos at 6.6 ppm, 4 ppm and 2 ppm are given as Fig. 6.1, Fig. 6.2 and Fig. 6.3 respectively. Data were statistically analysed and values were expressed as means ± Standard deviation.

Table 6.1 Concentration of MCP in the muscle of *H. fossilis*

Control	2ppm	4 ppm	6.6 ppm
Not Detected	0.0868 ± 0.0071	0.1412 ± 0.0057	0.1997±0.0033

- All values are in µg / gm

6.4 Discussion

Researchers like Shannon, (1977); Muir and Grift, (1981); Barron *et al.*,(1993); Tsuda *et al.*,(1994); and Sancho *et al.*,(1998) reported on the bioaccumulation of pesticides in fish tissues. Bioconcentration of hazardous substances like organophosphorus insecticides causes serious ecological problems when the degree of partitioning of a substance or its transformation products results in translocation to, and storage in critical tissues of organisms. (Ramaneswari and Rao, 2000). It is not simple to model the distribution of a pesticide in fish or measure the time course in the body or a specific organ, but such methods are especially useful for describing residue dynamics over time. Bioaccumulation of chemicals in biota may be a prerequisite for adverse effects on ecosystems and when uptake rates are significantly higher than metabolic clearance rates bioaccumulation can still occur even though the substance is readily biodegradable.

Ramaneswari and Rao (2000) investigated the bioconcentration of monocrotophos in *Labeo rohita* and *Channa Punctata* and observed the bioconcentration of monocrotophos in the form of o-des methyl monocrotophos and hydroxy monocrotophos and the bioconcentration of monocrotophos in *Labeo rohita* and *Channa punctata* were found to be 1.68 and 1.33 µg/g respectively. However there is a dearth of available literature on the bioconcentration studies of monocrotophos in the stinging catfish *H. fossilis*.

In the present investigation, edible parts of the fish were analysed for monocrotophos residue. Bioaccumulation-elimination studies of sub lethal exposure to organophosphate insecticides like monocrotophos in muscle could provide information on the environmental impact of these pesticides. The most obvious signs of monocrotophos intoxication were restlessness, erratic swimming, spasms, and loss of balance. Some of the experimental fish exhibited low motor and sensory activities but all of them endured the set exposure period. These symptoms had been reported in the European eel under sub lethal exposure to other organophosphate insecticides (Da Silva *et al.*, 1993; Sancho *et al.*, 1994a). The results of the present study prove that sub lethal concentrations of monocrotophos bioaccumulates in the muscle of *H. fossilis*. Though the values of concentration were low at all the three sublethal concentration group (0.0868 ± 0.0071, 0.1412 ± 0.0057 and 0.1997±0.0033 µg /gm respectively for 2 ppm, 4 ppm and 6.6 ppm) it was observed to have serious deleterious effects on the metabolic machinery of the fish which could be inferred from studies on various aspects as described in the forgone chapters. Histopathological alterations in the tissues of MCP treated *H. fossilis* (chater-5) supports this observation.

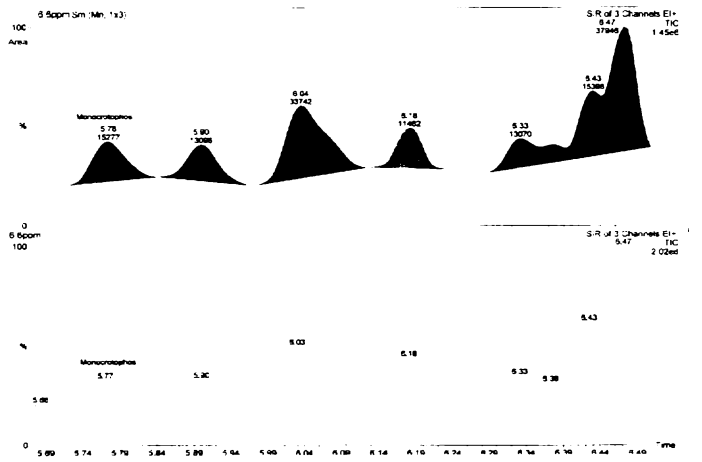


Fig 6.1 Chromatogram- 6.6 ppm

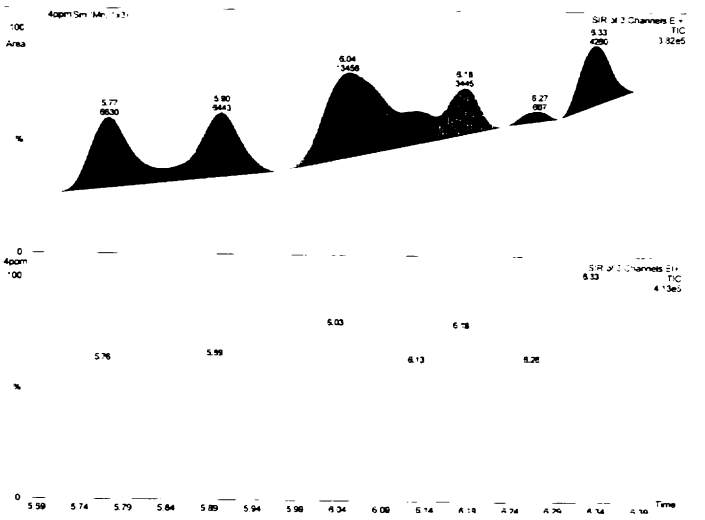


Fig 6.2 Chromatogram- 4 ppm

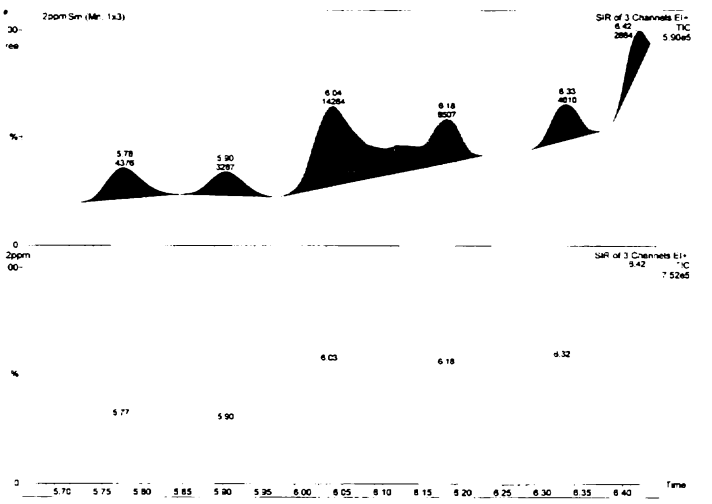


Fig 6.3 Chromatogram- 2 ppm

CONCLUSION

Changes in the chemical composition of natural aquatic ecosystems can distress the non-target organisms, predominantly fish. Fish have been largely used to evaluate the quality of aquatic systems as bioindicators for environmental pollutants. In polluted areas, exposure of fish to xenobiotics leads to interactions between these chemicals and biological systems, which give rise to biochemical disturbances. Despite the fact that tropical ecosystems are threatened by degradation due to ill planned development, little research has been done on the impact of contaminants on tropical ecosystems and tropical fish species. Monocrotophos (MCP) is one of several organophosphorus insecticides (OPs) developed to replace organochlorines. Fish can be exposed to monocrotophos either accidentally or under treatment conditions. Being one of the most extensively used OP insecticide in crop protection programmes, monocrotophos can reach the aquatic systems by land run off or leaching. In fish culture, the treatment with OPs is a common method employed to control larval stages of predator insects that threaten fish larvae. Furthermore, OPs are also used to treat skin and gill infections caused by external parasites.

Observed increase in the frequency of surfacing movements of *H. fossilis* subjected to monocrotophos intoxication, indicates respiratory stress.

Energy and metabolic status of the fish demonstrates that monocrotophos has induced biochemical alterations and caused significant metabolic and the physiological consequences. Reduction in the protein content of fish can be due to the rapid utilization of protein due to pesticide induced stress. The observed decrement in total carbohydrate and glycogen content of the fish and subsequent rise in the blood glucose levels indicates that the carbohydrates were used up to meet energy demand of the body, under stress.

It is an issue of concern that in test fish, the concentrations of major biochemical constituents of organs, particularly the total protein, carbohydrate, lipid and glycogen content, were much lower than in controls, which in turn reflects a loss of nutritive value and poses a grave threat to its prospective use as a foodstuff.

The biochemical changes induced by monocrotophos stress is due to disturbed metabolism manifested as inhibition of enzymes, retardation of growth, damage and dysfunction of the tissues. OPs have their own target sites of action, and most of them are metabolic depressors.

The increase of biomarker enzymes in plasma might be due to the necrosis of liver. The assayed enzymes can work as swift and sensitive biomarkers, for monitoring the impact of organophosphorus pesticides on aquatic biota and eventually entire ecosystem.

The present study reveals that monocrotophos has profound effect on the haematological parameters of stinging catfish *Heteropneustes fossilis.* Exposure to low concentrations of monocrotophos resulted in significant haematological alterations in stinging catfish, *H. fossilis.*

Decline in WBC, RBC and Hb values and increase in PCV of the monocrotophos treated fish raises a serious apprehension a propos the immunity status of the fish. These alterations may be disruptive to the survival capacity of the catfish in their natural environment.

Monocrotophos is a potent acetyl cholinesterase inhibitor that blocks the cholinergic activity of the central ganglion of pest invertebrates. The present study underlines the significance of acetyl cholinesterase as the most reliable marker enzyme in monitoring organophosphorus pollution of freshwater environments. Monocrotophos treated fish exhibited behavioural

signs such as erratic swimming, loss of balance, hyperactivity and convulsions which are common in fish intoxicated with anticholinesterase insecticides.

Exposure to monocrotophos significantly inhibited Mg^{2+} - and Na^{+}, K^{+} - ATPase activities in gill tissues of *H. fossilis*. Inhibition of these activities indicates interruption in its cellular and ionic regulation and salt uptake. The present study further validates that ATPase activity can be taken as a significant index of cellular activity and forms a practical toxicological tool. The ATPase system in the freshwater fish *H. fossilis* (Bloch) seems to be a sensitive enzymatic biomarker of exposure to monocrotophos.

Basically, lysosomal functional integrity is a good diagnostic biomarker of exposure to toxicants. From the present study it can be observed that lysosomal membranes were destabilized and hydrolytic enzymes were released from the cell due to leakage of the membrane. A leaking membrane can perform as open doorway to substances into the cell, which in normal conditions not allowed. Disturbed lysosomal stability, to a great extent add to, the impaired proficiency of immune system and to loss of body tissues due to autophagy.

Antioxidants such as superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, and total reduced glutathione were significantly altered indicating the possibility of direct action of monocrotophos with the enzyme synthesis. Due to the complexity of interactions between pro-oxidant factors and antioxidants, it appears that single responses cannot provide a general marker of oxidative stress. Individual antioxidants can be sensitive and specific but difficult to predict. They are useful as "response biomarkers" indicating a varied pro-oxidant challenge and potentially important early warning signals. Variations of individual

antioxidants are useful for understanding the mode of action of a chemical stressor and the possible molecular interaction with specific responses. However, their value is more limited for understanding the biological effect in terms of health condition of the organisms. However, the adverse effects of oxidative stress on these parameters cannot be overlooked. From an eco-physiological point of view it is apparent that, the use of monocrotophos in agriculture and storage facilities must be carefully evaluated.

Histopathological evaluation points out that exposure to sub lethal concentrations of monocrotophos caused destructive effect in the gill, brain and liver tissues of *H. fossilis*. Liver of the monocrotophos treated animals exhibited various histopathological features such as vacuolated hepatocytes, cell necrosis, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. Brain of the treated animals displayed histopathological features such as gleal cell proliferation, encephalomalacia, total damage to neurons, neuronophagia and loss of neurons. Gill of the treated fishes were showing pathological features like hyperplasia, lifting of secondary epithelium, squamous metaplasia, fusion of secondary lamellae, break down of pillar system and hyperaemia of cells.

Such tissue level alterations can reduce the nutritional value of fish as an important edible commodity which in turn might negatively affect its market demand. It is concluded that the findings of the present histological investigations reveal a direct relationship between pesticide exposure and histopathological disorders observed in several tissues. The current study reinforces the relevance of histopathology as a potent tool for monitoring contamination in aquatic environments.

Studies on bioaccumulation of monocrotophos in muscle, by whole body analysis using gas chromatography proved that monocrotophos

accumulates in the edible part of fish. This can be harmful to consumers in next ecological level, including humans, and can be potent for biomagnification. Levels of bioconcentration were low at all the three sub lethal treatment groups, but it resulted in serious deleterious effects on the metabolic machinery of the fish which could be inferred from studies on various aspects described in this investigation. Possible measures should be taken to make certain that monocrotophos do not contaminate natural water resources, fisheries and aquaculture farms.

Crop protection measures play an important role in agricultural management, particularly in developing countries having high poverty levels. However, there are alternative chemicals other than monocrotophos available. In the light of these findings, it is important that national and international agencies with crop management and environmental conservation expertise should work with pesticide manufacturers, national and local stakeholders in order to find out means of reducing the environmental impacts of such toxic chemicals.

The freshwater fishes constitute one of the major sources of nutritious food for humans. Indiscriminate use of pesticides in agricultural operations can have every possibility to reach aquatic systems and cause deleterious effects to aquatic fauna and flora. Monocrotophos is the most extensively used OPs in India, especially for paddy, cotton and sugar cane. Fresh water fishes are major non-target species for the pesticide action. *H. fossilis*, a fresh water fish, inhabiting in paddy fields and adjacent streams are at risk of population depletion, categorizing them as vulnerable in conservation status (IUCN 1993; CAMP 1998). In this scenario the study conducted on the stress responses of *H. fossilis* to OP insecticide monocrotophos is significant as a source of basic data, for future ecotoxicological studies.

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