BIOCHEMICAL RESPONSE AND BIOACCUMULATION OF ORGANOPHOSPHATE IN COMMON CARP *(CYPRINUS CARPIO)*

BY

AYESHA MUNIR

NUST201362301MSCEE65213F

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

2015

BIOCHEMICAL RESPONSE AND BIOACCUMULATION OF ORGANOPHOSPHATE IN COMMON CARP *(CYPRINUS CARPIO)*

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

IN

ENVIRONMENTAL SCIENCES

BY

AYESHA MUNIR

NUST201362301MSCEE65213F

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

CERTIFICATE

This dissertation submitted by **Ms. Ayesha Munir** is accepted in its present form, by the Institute of Environmental Sciences and Engineering (IESE), School of Civil and Environmental Engineering (SCEE), National University of Sciences and Technology (NUST), Islamabad as satisfying the requirement for the degree of Masters of Environmental Sciences.

Supervisor: _____

Dr. Imran Hashmi
Associate Dean & Professor
IESE, SCEE, NUST

Member: _____

Dr. Ishtiaq A. Qazi
Professor
IESE, SCEE, NUST

Member: _____

Dr. Muhammad Arshad
Associate Professor
IESE, SCEE, NUST

DEDICATED TO…

MY EVER LOVING HUSBAND

(M. AKMAL JAVED)

&

MY FAMILY

ACKNOWLEDGEMENTS

First and Foremost all praise to ALLAH, the Almighty, for giving me opportunity, determination and strength to do my research.

I would like to thank and express my deep and sincere gratitude to my supervisor Dr. Imran Hashmi, for his continuous support, guidance and encouragement. His positive outlook and confidence in my research inspired me and gave me confidence. His timely and efficient contribution helped me shape this into its final form.

I would like to express my gratitude to my guidance and examination committee for their valuable assistance and suggestions. I would also like to thank Dr. Saud Ahmed Khan who helped me to analyze my data.

I would also like to express my gratitude to NUST R & D for providing additional funds, the pioneers (Ms. Amina Khalid and Ms. Asma Saeed) of my research field, Ms. Saira Khalid for GC training, ASAB for providing disposal facility for dead fish, Dr. Nadeem Akhtar (Optics Laboratories, PAEC) for spectrofluorometric analysis and Ch. Iftikhar Ahmed (Punjab Hatchery, Islamabad) for providing fish to carry out my research.

Finally, I'm forever indebted to my mother and my parents-in-law for their enormous love, support and prayer. As for my husband M. Akmal Javed, I find it difficult to express my appreciation because it is so boundless.

AYESHA MUNIR

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

mg/L	Milligram per liter
μg/L	Microgram per liter
ACT	Acetone
AChE	Acetylcholinesterase
APHA	American Public Health Association
CPF	Chlorpyrifos
DCF	Dichlorofluorescein
DCFH-DA	2,7-dichlorofluorescin diacetate
ECD	Electron capture detector
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
LCL	Lower confidence limit
LD_{50}	Lethal dose 50
MFO	Mixed Function Oxidase System
NBT	Nitrotetrazolium blue chloride
O_2^-	Superoxide radicals
OD	Optical density
OECD	Organization for Economic Co-operation and Development
OPPs	Organophosphorous pesticides
ROS	Reactive oxygen species
UCL	Upper confidence limit
WHO	World health organization

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Chlorpyrifos (CPF) is a member of the group of organophosphate pesticides (OPPs) used extensively for agricultural pest control throughout the world. CPF as a consequence of agricultural runoff enter into water bodies and deteriorate their quality as well as interfere with aquatic ecosystems. Fish have been widely used as biomonitor for environmental pollutants. In current study common carp (*Cyprinus carpio)* was chosen as a model organism to evaluate the effects of chlorpyrifos on immuno-hematology, oxidative stress and to monitor residues levels in muscle tissues. Acute toxicity tests showed 24, 48, 72 and 96h-LD_{50} of CPF for common carp in semi static tanks were 1.53, 1.16, 0.90 and 0.67 mg/L respectively. Sub-acute toxicity test was performed by exposing organism with sublethal concentration (134, 13.4, 1.34 μg/L which corresponds to 1/5, 1/50, 1/500 of the 96h-LD_{50}) of chlorpyrifos for 7 and 14 days. Spectrophotometric nitroblue tetrazolium (NBT) reduction assay showed that respiratory burst activity decreased as a function of CPF dose and exposure time ultimately lead to reduction in immunity. Spectrofluorometric analysis revealed significant augmentation in ROS production in brain and gills of exposed fish by increasing concentration and exposure duration of CPF, which may cause oxidative stress. GC-ECD analysis indicated 60, 68 and 70% accumulated amounts of CPF residues in muscle tissues when exposed with sub-lethal concentrations (134, 13.4, 1.34 μg/L) for 30 days.

Chapter 1

INTRODUCTION

Background

Water, in particular, is at the heart of the resource nexus. About three-quarter of Earth's surface is covered with water and it is the most vital and fundamental component of life on earth. About 97.5% of the earth's total water is salty which is found predominantly in oceans and inland seas and remaining 2.5% accounts for fresh water resources. About 80% of total freshwater is in the form of ice in glaciers and polar ice caps of the world, which leads to the availability of only 0.2% of the fresh water for our use (EPA, 1990).

Water is an essential component for human usage such as drinking, manufacturing, agriculture and sanitation. In developing countries of tropical Asia, rapidly increasing population causes an increase in agricultural production demand (Botte *et al.,* 2012). Development of agriculture is closely linked to the application of pesticides. They are the chemicals that are designed and developed to prevent crop damage by insect-pest manifestation in order to improve crop yield. It is considered to be the most economical and viable solution to kill unwanted pests. Chemical usage has dramatically increased over the last 20 years to control agricultural pest. Studies indicated that in year 2000, about 5400 million pounds of pesticides were applied worldwide (Zia *et al.,* 2008). Pesticides are among the most abundant hazardous pollutants in soil, water, atmosphere and agricultural products. These pollutants have major environmental concerns because of their extensive usage, toxic nature and consequential pollution (Somashekar *et al.,* 2015).

Water is essential to all life so its quality is important. Water quality of reservoirs, natural lakes, and rivers in developing countries is continuously deteriorated because of the contaminated inflows (Ghumman, 2011). Water pollution may be defined as deterioration of water quality that includes alterations in physicochemical properties of water, which cause serious damage to the organisms dependent on water for their existence. Throughout the world, there is an ever-increasing risk about watershed pollution due to the extensive use of pesticides in agricultural areas (Xing *et al.,* 2015). Pesticides play a vital role in increasing the agricultural yield by acting as protective shield for crops against vector-borne diseases and pest attack but at the same time also deteriorating water quality (Sharbidre *et al.,* 2011).

Environmental pollution especially in aquatic ecosystems is increasing day by day due to widespread pesticide usage in agriculture (Wang *et al.,* 2011). Generally, pesticides come into contact with soil and travel to surface water through runoff and ground water through leaching (Somashekar *et al.,* 2015). These pesticides find their way into water reservoirs, river, stream etc. and alter the chemical composition of theses water bodies which results in adverse impacts on the fresh water fauna particularly fish (Wang *et al.,* 2013). Fish is an important food item for human consumption therefore it is significantly important to evaluate adverse effects of pesticides on fish as it plays a major part in food chain (Xing *al.,* 2012). The pesticides impact on water quality and aquatic biota is associated with the different factors, which includes active ingredient, adjutants and contaminants that exist as impurities in the active ingredient.

The World Health Organization (WHO) indicates that pesticides adversely affect nearly three million people per year; mainly due to organophosphorous pesticides, that ultimately results in two hundred thousand causalities (Somashekar *et al.*, 2015). Organophosphorous pesticides (OPPs) harmfulness is a significant public health issue specifically in developing countries. Studies indicated that organophosphorous pesticides are synthetically produced, have been used at a large scale all over the world since the end of the World War II due to their low cost and effectiveness towards pest, weeds and disease control (He *et al.*, 2009). Nearly more than 100 organophosphorous pesticides are in usage that accounts for 38% of the pesticide consumption all over the world. The environmental influences of pesticides are determined by toxicity, persistence, biodegradation and ultimately environmental fate (FAO, 1996).

1.1. Present study

Pollution of surface water channels has been well narrated all over the world and comprises a key issue at indigenous, national, and international levels. Various chemicals from agricultural processes enter into the aquatic environment via atmospheric accumulation, surface run-off, discharge and at the end store in soft-bottom deposits and aquatic entities (Adedeji *et al.*, 2012).

Amongst various aquatic organisms, fish is a valuable biomonitor of water pollution (Xing *et al.*, 2015). Fish are the top consumers and play an important role in aquatic food chain by maintaining a balance in aquatic ecosystem (Li *et al.*, 2013). Fish is an ideal sentinel for monitoring and documenting water pollution, due to their potential to be directly exposed to different pesticides resulting from agricultural fields through runoff (Audu *et al.*, 2015). Xenobiotics come in contact

with fish, different reactions initiated among these chemicals and biological systems that ultimately result into biochemical disturbances (Somashekar *et al.*, 2015). Hence, it is necessary to determine the contaminant action mechanism and potential means to mitigate their impacts. For this reason, fish can be used as bioindicators of aquatic pollution for the quality assessment of the aquatic system. Common carp (*Cyprinus carpio*), a bottom-dwelling fish was selected for the present study because it is one of the most important fish for human consumption and it is directly exposed to wide range of environmental pollutants due to its eating habit.

The pesticide selected for current study is chlorpyrifos, which is a broad-spectrum synthetic organophosphorous insecticide. It was first used after World War II as a nerve gas. In 1969, commercial manufacturing of chlorpyrifos was started. It is mostly used in farming to protect corn, cotton and fruit tress (Halappa and David, 2009). The WHO classified chlorpyrifos as "Moderately hazardous, Class II" pesticide (WHO, 1999). CPF follow a novel mode of action stimulated by contact, ingestion and exploits phosphorylating acetylcholinesterase both in the plasma and at synapse of neurons (Xu *et al.,* 2011). CPF is extremely toxic to aquatic biota; it impedes fish health by impairing their metabolic activity, which occasionally lead to death. Acute toxicity of CPF is found to be 0.6mg/L in fish (Xing *al.*, 2012). Studies reported that sublethal exposure of CPF causes behavioral and biochemical changes in common carp (Banaee *et al.,* 2013). Changes in the structure and function of aquatic organism due to sublethal exposure of pesticide are more common than mortality (Halappa and David, 2009). Hematology is used as an indicator of fish health to monitor physiological alterations due to different

xenobiotics exposure (Saravanan *et al.,* 2011). Hematological analysis indicated that CPF causes stress and immune-suppression in fish (Velisek *et al.,* 2011). CPF and its metabolites have been considered to cause toxic effects related to oxidative stress and also bioaccumulates in freshwater biota particularly in fish (Ural, 2013; Wang *et al.,* 2013).

1.2. Objectives

Keeping in view the harmful effects of pesticides on fish health, the current study was carried out at Institute of Environmental Sciences and Engineering to monitor toxicological effects of chlorpyrifos using Common Carp (*Cyprinus carpio*) as a bioindicator of aquatic pollution. Following were the objectives of present study:

1. Acute toxicity of chlorpyrifos in common carp:
    a. Determination of lethal dose (LD_{50})
2. Sub-acute toxicity of chlorpyrifos in common carp:
    a. Assessment of immuno-hematological parameters
    b. Analysis of oxidative stress
3. Monitoring bioaccumulation of chlorpyrifos in muscle tissues.

Chapter 2

LITERATURE REVIEW

In the last 50 years the use of pesticide has increased dramatically internationally and now more than 1400 different types of pesticides are being used, most commonly in agriculture and many subsequently released into the environment. The use of pesticides has undoubtedly increased the quality and quantity of agricultural products for the growing world population (Manuel *et al.*, 2008) but with the ever increasing demands of the growing world population upon agriculture, there is a resultant increase in environmental pollution.

2.1. Pesticide contamination of water bodies

It is very clear that the main factor contributing to the deterioration of water quality is the liberal application of chemicals in agriculture. In general, only about 0.1% of pesticides that are sprayed reach the target organisms. The remaining 99.9% of pesticides disperse through water, soil and air and this causes the contamination of the natural ecosystem, which ultimately affects human health and other biota (Pimentel, 1995).

Pesticides that have been used in agriculture can enter the environment in multiple ways. Drift, wind erosion and evaporation can transfer these traces of pesticide into the atmosphere and this can ultimately lead to contamination of water bodies via precipitation (Hamers *et al.*, 2001). They can also enter into aquatic system through water runoff following rainfall and other mechanisms such as leaching. With all these ways that pesticides can get into water and cause pollution, studies have

shown that they are now actually widespread in ground water and streams in agricultural and urban areas (Xing *et al.,* 2015).

2.1.1. Pesticide: A threat to aquatic biota

Ecotoxicological research has shown that water pollution caused by pesticide is a threat to aquatic biota. It can cause acute and chronic poisoning of aquatic organism particularly fish (Velmurugan *et al.,* 2007). Pesticides are also found to damage vital organs of fish, their skeletal systems and cause biochemical alterations in the exposed fish (Mishra *et al.,* 2004). Water pollution by pesticides can cause aquatic organisms to die and it has been shown mass mortality of fish and other aquatic biota resulted from pesticide contamination of water bodies (Singh *et al.,* 2009).

2.2. Pesticide prevalence in water bodies of Pakistan

In Pakistan, pesticide market was introduced in 1954 with the import of 254 metric tonnes of formulated product, and since then pesticide consumption has increased at a rate of 25% per annum from 906 metric tonnes (active ingredient) in 1980 to 5519 metric tonnes (active ingredient) in 1992 (Jabbar *et al.,* 1993). It is predicted that at present, a quantity of 70 thousand tonnes of pesticides are sprayed every year in Pakistan that is increasing at an annual rate of about 6% (Azizullah *et al.,* 2011).

Pesticide residues were first detected in cattle drinking water in Karachi where the surface water showed contamination by organachlorine pesticides or their metabolites at concentrations ranged from trace amounts to 16.7 μg/L (Parveen and Masud, 1988). Analysis of shallow groundwater samples from the Summandri area in Faisalabad also revealed the presence of pesticides like cyhalothrin (traces to 0.2

μg/L), monocrotophos (40 to 60 μg/L), and endrine (0. 1 to 0.2 μg/L) (Jabbar *et al.*, 1993). In Multan, an area known for the growing of cotton, it was found that the ground water (at depth of 5.0–18.0 m) was contaminated with carbofuran, cypermethrin, deltamethrin, diazinon, dichlorvos, dimethoate, endosulfan, esfenvalerate, eenitrothion, lindane, malathion, methyl parathion and phosphamidon (Ahad *et al.,* 2001). Furthermore, shallow groundwater samples collected for pesticides residues analysis in four cotton producing districts of the Province Punjab, namely Dera Ghazi Khan, Muzafargarh, Bahawalnagar, and Rajan Pur, detected bifenthrin, carbofuran, λ-cyhalothrin, endosulfan, methyl parathion and monocrotophos in water samples at various concentrations (Tariq *et al.,* 2004). Similarly, water samples collected from different areas of Punjab showed DDT metabolites with concentrations ranging from 0.017–1.06 ng/mL (Asi *et al.,* 2008) and in Rawal Lake, Islamabad, surface water was shown to be contaminated with 4,4-DDD, 4,4-DDE, alpha-HCH, azinphos-methyl, cyfluthrin, deltamethrin, diazinon, endosulfan, esfenvalerate, fenitrothion, heptachlor, lindane, parathion-methyl and α-cypermethrin (Iram *et al.,* 2009).

2.3. Organophosphates

Organophosphates (OPs) are chemicals that are used extensively for agriculture and public health and which account for 70% of the global pesticide use. This equates to a total consumption of approximately 90 million pounds per year of these substances (Ojha *et al.,* 2011). Chemically, organophosphates are thiol or ester derivatives of phosphoric, phosphonic and phosphoramdidic acid **(**Singh *et al.,* 2006). The universal nature of organophosphates in terms of availability and widespread usage has resulted in ever-increasing concern about their toxicity. The

toxic nature of OPs is a critical community health problem particularly in developing countries, which in turn unexpectedly account for a quarter of their global use (Sharbidre *et al.*, 2011). The general structure of organophosphates is illustrated in Fig. 2.1.

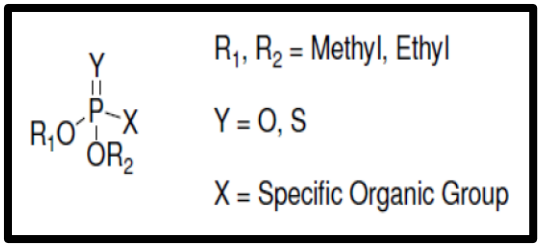


Figure 2.1. General structure of organophosphate

2.3.1. Exposure routes of organophosphate

Living organisms can be exposed to OP insecticides through gastrointestinal, inhalatory and dermal routes (Sharbidre *et al.*, 2011).

2.3.2 Mode of action of organophosphates

The broad mechanism of action of OP insecticides is to affect acetylcholinesterase (AChE) that causes hydrolysis of acetylcholine, which is a key neurotransmitter. Severe intoxication with OP insecticides can result in multiple clinical effects which include irrevocable inhibition of AChE activity in blood and the central nervous system which subsequently resulted in the accumulation of acetylcholine and activation of nicotinic and muscarinic receptors. This can ultimately lead to organism death (Sharbidre *et al.*, 2011).

Acetylcholine is required for the transmission of signals from brain to muscles and other areas of body and the massive accumulation of acetylcholine in synapses initiates the excessive functioning of muscarinic, nicotinic and cholinergic receptors and hyperactivity in the cholinergic pathways (Shenouda *et al.*, 2009).

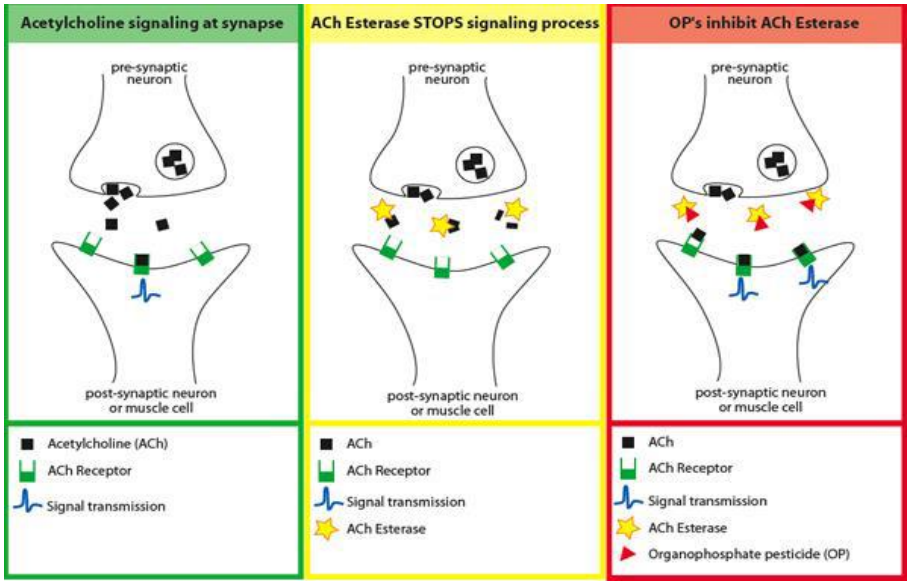


Figure 2.2. Mode of action of organophosphates

There are other effects that result from the inhibition of AChE. Contact with OP pesticides also affects the pancreas, liver, reproductive system and hematological system (Uzun *et al.*, 2009). OP pesticides are considered potentially mutagenic and clastogenic because of their genotoxic, alkylating and clastogenic characteristics (Sarabia *et al.*, 2009). It has been recently hypothesized that OP pesticides have the ability to produce reactive oxygen species in different tissues of fish ultimately leading to oxidative stress (Ojha *et al.*, 2011). Indeed, oxidative stress is fast being recognized as an increasingly important molecular mechanism in OP-induced toxicity (Sharbidre *et al.*, 2011).

2.4. Chlorpyrifos

Chlorpyrifos (IUPAC name: O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) was introduced by Dow Chemical company in 1965. It is a crystalline organophosphate insecticide. It is marketed under various trade names including Lorsban and Dursban (Fig. 2.3).

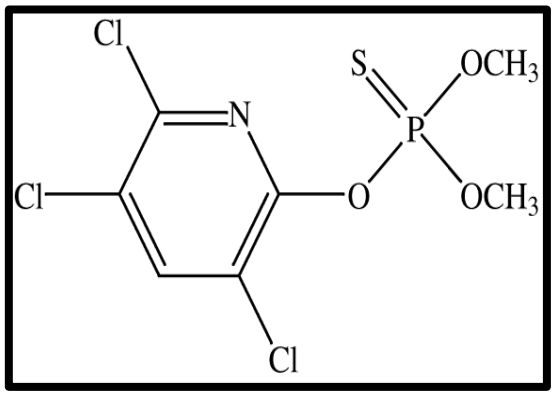


Figure 2.3. Chemical structure of chlorpyrifos

Like other OP pesticides it inhibits AChE activity by acting on central nervous system. Chlorpyrifos (CPF) has been used throughout the world to control pest insects in residential, agricultural, and commercial settings although its use has been restricted in certain residential applications in some countries due to its potential toxicity to people. It should be noted though that CPF is one of the most widely used OP pesticides in agricultural practices throughout the world because it is considered to be relatively safe to humans whilst having a broad spectrum toxicity against insects (Galloway and Handy, 2003).

Chlorpyrifos is one of top ten pesticides used in Pakistan (Khooharo *et al.,* 2008). It is the most common agrochemical of aquatic environment. It is used to control pests associated with vegetables, nuts and fruits (Topala *et al.,* 2014). It has become one of the most prolific environmental contaminants because of its extensive use leading to serious environmental problems (Fu *et al.,* 2013).

Several studies reported that CPF has detrimental effects in different fish species including neurotoxicity via inhibition of AChE (Kokushi *et al.,* 2015), histopathological and biochemical alterations (Xing *et al.,* 2012), genotoxicity (Ali *et al.,* 2009) and oxidative stress (Kavitha and Rao, 2008). In view of the known adverse effects in fish it is clearly necessary to evaluate the effects of this pesticide

on ecological balance that surrounds fish and also on the human health of people that may consume them (Xing *et al.*, 2012).

Characteristics	Information
Chemical formula	$C_9H_{11}Cl_3NO_3PS$
Boiling point	160 °C
Molar mass	350.59 g/mol
State	Liquid
Odor	Mild mercaptan
Color	White to tan
Solubility in water at 25°C	2 mg/L
Log K_{ow}	4.82

Table 2.1. Physiochemical characteristics of chlorpyrifos

2.4.1. Toxicity of chlorpyrifos in aquatic organisms

Runoff from agricultural areas treated with CPF can result in contamination of water bodies by CPF, and there has been shown to be up to 4.3 mg/L in rivers (Nadzialek *et al.*, 2008). When it comes into contact with aquatic ecosystems (as a consequence of spray drift, running off from agriculture, leaching from water and soil,) CPF exerts toxic effects on non-target organisms including fish (Sun and Chen, 2008). For example, neurotoxicity in common carp and Nile tilapia, genotoxicity on Bloch, developmental and reproductive toxicity in zebrafish and

guppy, all of which highlight the necessity to evaluate CPF toxicity on fish and the possible impact on corresponding aquatic ecosystems (Li *et al.*, 2013).

2.5. Acute toxicity of chlorpyrifos

Acute lethal toxicity tests, such as those described by the OECD guidelines 203 (1992) provide a simple index of chemical toxicity for regulatory authorities. One of the properties that describe the acute toxicity to fish in the study of aquatic toxicology is the LD_{50}. LD_{50} values of test chemical for different fish species may vary widely. The LD_{50} is a function of the intrinsic toxicity of the chemical and of its distribution equilibrium between the organism and its environments (Khalil *et al.*, 2013) and they reflect both bio-concentration potential of a chemical and its intrinsic toxicity, i.e. potential of chemical to cause toxicity once inside the organism (Xing *et al.,* 2015). The acute toxicity of chlorpyrifos is due to inhibition of cholinesterase enzymes, including acetylcholinesterase (Gearhart *et al.*, 2007).

Many researchers have determined acute toxicity of chlorpyrifos. Previous result revealed that 96h-LD_{50} of CPF for goldfish was 153 μg/L (Ma *et al.*, 2015), tilapia larvae was 1.57 mg/L (Gul, 2005), zebra fish was 680 μg/L (Ji *et al.*, 2010), Neotropical silverslide was 0.17 μg/L (Oruc, 2010) indicating that CPF is highly toxic to fish.

2.6. Sub-Acute toxicity of chlorpyrifos

Assessment of toxic effect of sub-lethal concentrations as the end point rather than quantitative estimation of mortality can assist the development of bio-sensors to monitor the adverse effects caused by pesticides.

2.6.1. Respiratory burst activity

Fish exposure to different types of contaminants may induce several changes in

hematological parameters, which are commonly used to assess fish health (Modesto *et al.*, 2010). Hematology is most widely used tool for the detection of physiopathological changes resulting different stress conditions. Therefore, hematological parameters are the most frequent method to evaluate the sublethal effects of contaminants (Kumar *et al.*, 2011).

Non-specific mechanisms are important in the defense of all multicellular animals against invading pathogenetic microorganisms. Several parameters like leukocyte (WBC) count, phagocytic activity, albumin:globulin ratio, and serum cortisol are indicators of improved immunocompetence. Phagocytes respiratory burst activity is an indicator of innate immunity (Muthappa *et al.*, 2014). It is generally believed that fish phagocytes have ability to produce free radicals (H_2O_2, O_2^-) during the process of respiratory burst by consuming oxygen intensely (Rehulka and Minarik, 2003). Increased respiratory burst activity can be linked with increased phagocytes activity to kill pathogens and hence a better immunity (Ispir and Doruc, 2005).

Studies reported reduction in carp immunological parameters when exposed with sublethal concentration of phosalone (Kaya *et al.*, 2015). Endosulfan-induced decrease in phagocytic activity (NBT/respiratory burst) was observed in *Labeo rohita* (Muthappa *et al.*, 2014). Similar results were observed when *Cyprinus carpio* was exposed to malathion (Yonar *et al.*, 2014). There is a dearth of data on phagocytic activity caused by chlorpyrifos in common carp so one objective of study was to asses immuno-hematological parameters in carp after CPF exposure.

2.6.2. Oxidative stress

Chemical pollution caused by pesticide in the environment is of growing concern due to their widespread uses in agriculture. Toxic effects related to oxidative stress

has been reported in freshwater biota particularly fish because of exposure to these pesticides and their metabolites (Yonar and Sakin, 2011). Oxidative stress occurs when these contaminants disturb the dynamic equilibrium between oxidant and antioxidant enzymes due to alterations in antioxidants or enhanced peroxidative processes (ROS) or both, leading to damage (Ural, 2013). Aquatic organisms have developed a variety of defense mechanisms to overcome potential danger of the free radicals (Monteiro *et al.,* 2006). The first line of defense against oxidative stress consists of the antioxidant enzymes such as SOD, CAT, GR and GPx, which convert superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and then into water (H_2O) and molecular oxygen (O_2) as well as non-enzymatic antioxidants such as GSH (Yonar *et al.,* 2012). A decrease in the activity of these enzymes changes the redox status of the cells. Thus, it is possible that an increase in the activity of these enzymes contributes to the elimination of theses oxidative radicals from the cell induced by pesticide exposure (Stara *et al.,* 2012).

Xing *et al.,* (2012) observed oxidative stress in common carp when exposed with atrazine. Oxidative stress causes damage to DNA, biomolecules and reduction in antioxidant defense (Jin *et al.,* 2010). Deltamethrin exposure causes reduction in antioxidant activities in liver of common carp (Ensibi *et al.,* 2013). There is no data available on reactive oxygen species (ROS) produced in common carp by chlorpyrifos exposure so in the current study reactive oxygen species production was observed in gills and brain as a result of sub-lethal exposure.

2.7. Bioaccumulation of chlorpyrifos in muscle tissues

Bioaccumulation may refer to gradual increase in quantity of substance in an organism that may be due to an increased rate of uptake than the organism ability

to excrete it. Many pesticides have tendency to accumulate in biological tissues (Caldas *et al.,* 2013). Pesticide residues have been found in aquatic organism is consequence of the pollution of the habitat. Studies have reported that chlorpyrifos has tendency to easily accumulate in aquatic organisms due to its lipophilic nature. Bioconcentration factor of chlorpyrifos ranges between 100-3000 (Varo *et al.,* 2002). Bioaccumulation of chlorpyrifos was observed in kidney and spleen tissues of common carp (Wang *et al.,* 2013). Halim *et al.,* (2006) also observed chlorpyrifos residues (31.6 ppb) in muscle tissues of fish.

Chapter 3

MATERIALS AND METHODS

Toxicological studies were carried out in the Environmental Toxicology Laboratory, IESE, SCEE, National University of Sciences and Technology, Islamabad, Pakistan. The study was conducted by exposing common carp *(Cyprinus carpio)* with commercial grade chlorpyrifos in semi-static tanks following APHA (2012) and OECD Guidelines No. 203, 204.

3.1. Chemicals

Commercial grade CPF (EC: 40) was purchased from local market, Rawalpindi. Analytical grade chloroform was used for fish anesthesia. Nitrotetrazolium blue chloride (715059 Bioworld, USA) and N,N-dimethylformamide (15440 Sigma-Aldrich, USA) were purchased and used for NBT reduction assay. Solution of 2,7-dichlorofluorescin diacetate (D6883 Sigma-Aldrich, USA) was prepared by dissolving in dimethyl sulfoxide (60153 Riedel-de Haen, Germany), 10-μM/L final concentration to quantify ROS. For bioaccumulation studies, GC grade FLUKA chlorpyrifos PESTANAL® (45395) was purchased and standard was prepared. Digestion and extraction of chlorpyrifos from muscle tissues was done by GC grade acetic acid, sodium anhydrous sulphate, acetone, diethyl ether and *n*-hexane. GC grade florisil (60-100 mesh) was used for purification of GC extract.

3.2. Fish

Common carp used in this study were purchased from Punjab Hatchery Rawal Town, Islamabad and transported to the lab in oxygenated polyethylene bags and were held in continuous aerated tanks prior to the experiment.

3.2.1. Acclimatization of fish

The fish were acclimatized for 7 days to the laboratory conditions using dechlorinated tap water whose physicochemical characteristics (pH, dissolved oxygen, temperature, total hardness as $CaCO_3$) were determined according to standard methods mentioned in APHA (2012) regularly. Water was changed on every alternate day. The fish were fed with commercial dry food pellets during acclimatization periods. Dead fish was removed immediately. Table 3.1 shows physiochemical parameters of water.

Parameters	Mean ± S. D	Permissible Limits
pH	7.74 ± 0.41	6 – 9
Dissolved Oxygen (mg/L)	7.35 ±0.56	> 5
Temperature (^oC)	24.37 ± 1.97	18 – 26
Hardness (mg/L)	234 ± 3	< 250
Chlorine (mg/L)	BDL	0

Table 3.1. Mean values of physiochemical parameters of dosed tanks

3.3. Experimental design

Fish of average size 18 ± 2.1 cm and weight 45 ± 2.3 g were kept in semi-static tanks (dimension 3 X 1.5 X 1.5 ft) with continuous aeration. Using random selection method fish were divided into experimental and control groups. Experiments were carried out in batches depending on the test type and exposure duration as shown in Fig. 3.1.

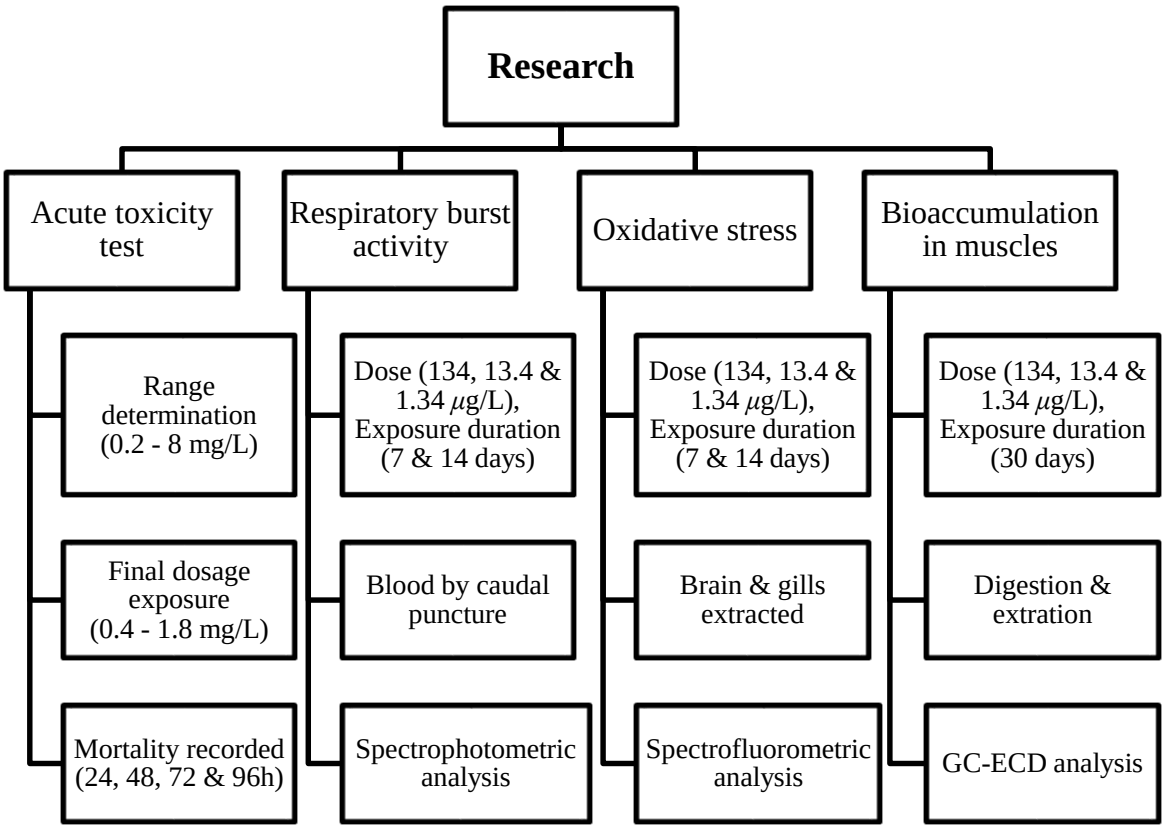


Figure 3.1. Experimental design of research work

3.4. Acute toxicity test

Acute toxicity bioassay was performed in semi-static tanks to determine the 24, 48, 72 and 96 h lethal dose of chlorpyrifos for common carp following OECD guidelines 203 (1992). Healthy and active fish were evenly divided into experimental and control groups in 120 L glass aquaria containing twelve fish in each. Water was changed on every alternate day followed by addition of fresh chlorpyrifos. Primarily, a range finding test with different concentrations (0.2-8 mg/L) was performed to determine the range to be followed in the definitive test. Fish were exposed to final doses of 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6 and 1.8 mg/L. Mortality was recorded in each group at 24, 48, 72 and 96h after the start of the

test. Feeding was stopped two days prior to the exposure. Dead fish were removed immediately. The behavior of both control and test fish were keenly observed.

3.5. Sub-acute toxicity test

A set of 10 fish in each tank was exposed to chlorpyrifos for 7 and 14 days at following sublethal concentration: 134, 13.4, 1.34 μg/L which corresponds to 1/5, 1/50, 1/500 of the 96h-LD_{50} (670 μg/L) of chlorpyrifos for common carp following OECD guidelines 204 (1984). Each experimental condition was triplicated along with the control experiment. After completion of stipulated time, four fish were randomly selected from each tank and anesthetized using chloroform. Blood samples were drawn by caudal vein puncture using heparinized syringes for respiratory burst activity. Fish were decapitated after collection of blood samples. Brain and gill tissues were carefully excised on an ice-cold plate, washed with ice-chilled phosphate buffer saline solution, immediately frozen in liquid nitrogen and stored at -80°C until oxidative stress analysis.

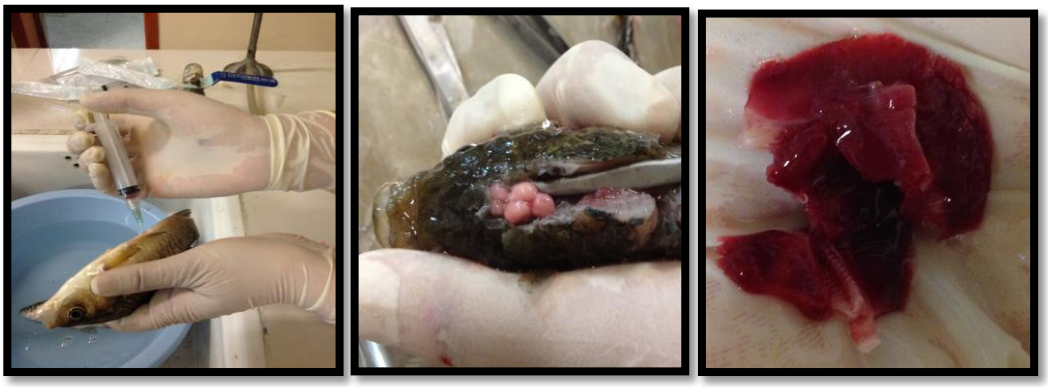


Figure 3.2. Fish dissection for sample collection (Blood, brain and gills)

3.5.1. Respiratory burst activity

In order to evaluate immuno-hematological parameter, NBT reduction assay was performed following the method of Zanuzzo *et al.* (2015) with some modifications. 0.1 ml of heparinized blood was co-incubated with an equal volume of 0.2% of

NBT in phosphate buffered saline solution at room temperature for 45 min. 50 μl from the resultant suspension was added to 1 ml of N,N-dimethylformamide and centrifuged for 10 min at 2000 X g. The optical density (OD) of supernatant was measured on UV-Visible spectrophotometer at 540 nm. The blank consisted similar steps and components excluding blood that was replaced with distilled water.

3.5.2. Oxidative stress

Determination of oxidative stress was based on methods of Zhang *et al.* (2008) and Driver *et al.* (2000). Oxidative stress in brain and gill tissues was quantified using 2,7-dichlorofluorescin diacetate (DCFH-DA) by measuring reactive oxygen species (ROS). Brian and gill tissues were homogenized using homogenizer (WiseTIS HG-15D) in ice-cold Locke's buffer (100 mg tissues/ml buffer). After that, 0.5 ml of homogenate was left for 5 min to warm to room temperature and then 5 μl of DCFH-DA was added and incubated at 37°C for 30 min. The conversion of DCFH to fluorescent oxidized product dichlorofluorescein (DCF) was monitored using spectrofluorometer (F4000 Hitachi) with excitation/emission wavelength of 485/525 nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks.

3.6. Bioaccumulation of chlorpyrifos in muscle tissues

A set of 6 fish in each tank was exposed to chlorpyrifos for 30 days at following sublethal concentration: 134, 13.4, 1.34 μg/L which corresponds to 1/5, 1/50, 1/500 of the 96h LD_{50} (670 µg/L) of CPF for common carp. Each experimental condition was replicated along with the control experiment. After completion of exposure

time, fish were randomly selected from each tank, decapitated and edible muscle tissues were extracted.

3.6.1. Digestion and extraction

Digestion and extraction was done following the method of Halim *et al.* (2006) with a slight modification. 20 g of muscle tissues from dorsal muscle were taken and mixed with 10 g of sodium anhydrous sulphate. 100 ml of acetone and chloroform (1:1 v/v) was added and homogenized using pestle and mortar. Ten drops of acetic acid were added to acidify the homogenate. It was then sonicated (JAC Ultrasonic 1505) for 30 min and left for 3 h on the shaker (Labcon SPO-MP8). The homogenate was filtered and the filtrate was vaporized to dryness using rotary evaporator (Heidolph HB digital) at 35°C for 25-30 min. The residues were re-dissolved in 1 ml of acetone and *n*-hexane (1:1 v/v).

3.6.2. Column chromatography

In order to clean samples, a column was prepared by using 1% deactivated florisil (60-100 mesh) as adsorbent. Before loading the sample, column was rinsed with 50 ml of *n*-hexane. Samples were eluted with 200 ml of 15 % and 50 % of diethyl ether in *n*-hexane, respectively. The two fractions were combined, concentrated and completely vaporized using rotary evaporator. The residues were re-dissolved in 0.5 ml of *n*-hexane (Diaz *et al.,* 1997).

3.6.3. GC-ECD analysis

Shimadzu 2010 series gas chromatograph equipped with Electron Capture Detector (ECD) was used for analytical studies (Fig. 3.3). The separation column used was fused silica capillary column (TRB 1 column) having 0.5 μm film thickness, 0.32 mm diameter and 25 m length. Helium was used as the carrier gas with a constant

column flow of 1.69 ml/min. Linear velocity for the gas flow was 30.2 cm/sec with purge flow of 3 ml/min.

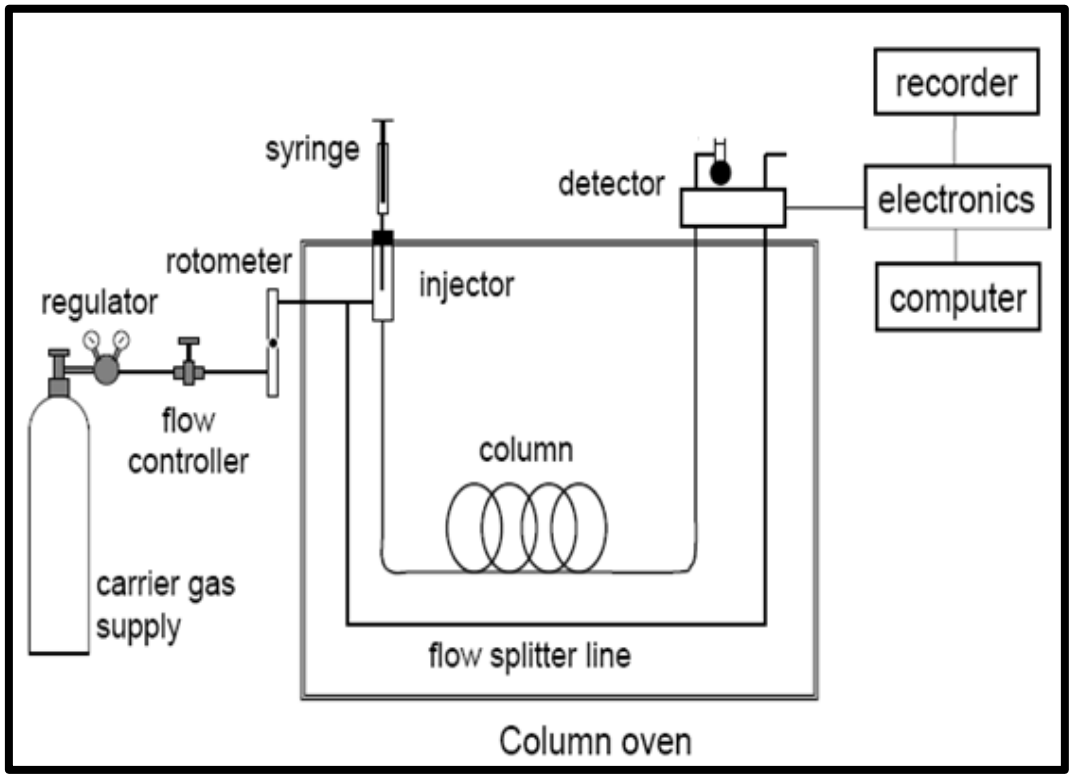


Figure 3.3. Schematic diagram of Gas Chromatography

Before running the real sample, the GC apparatus was optimized by changing various parameters such as injector temperature, detector temperature, column temperature, split ratio and flow rate etc. 1 μl of each standard solution was injected into the GC injection port using glass syringe to analyze retention time of chlorpyrifos and solvent. Table 3.2 shows the optimized parameters of GC-ECD.

3.6.4. Standard solution

Standard FLUKA chlorpyrifos was dissolved in GC grade acetone to prepare stock solution of 300 μg/L. The subsequent dilutions (5, 10, 25, 50, 100 and 150 μg/L) were prepared to formulate calibration curve and line equation. This line equation was used to determine the unknown concentration in the sample.

Parameters	Conditions
Initial temperature	50 ^{o}C
Rate	15 ^{o}C/min
Final temperature	300 ^{o}C
Run time	22 min
Mode	Splitless
Detector temperature	330 ^{o}C
Column temperature	150 ^{o}C
Injector temperature	300 ^{o}C
Gas flow	25.3 cm/s

Table 3.2. Optimized parameters of GC-ECD

3.7. Statistical Analysis

Statistical analysis of all data was performed. The data obtained from cumulative mortality was statistically analyzed to determine LD_{50} values by using following regression equation:

$$LD_{50} = \alpha + \beta D$$

where,

α, β = Intercept coefficient

D = Half of the population

One-way analysis of variance with post hoc test was performed to determine significant differences between the means of control and treatment groups. Paired t-test was performed to assess differences between control and treatment groups. Data was expressed as mean value ± standard deviation. Probability level of 5% was considered to be statistically significant.

Chapter 4

RESULTS AND DISCUSSION

Fish is an ideal sentinel organism for toxicological studies because it plays an important role in food web. The fish biomarkers may be used as indicators of pollution because it allows immediate detection of aquatic environmental problems. Therefore current toxicological study was conducted by exposing common carp (*Cyprinus carpio)* as model organism to evaluate the effects of chlorpyrifos on immuno-hematology, oxidative stress and to monitor residue levels in muscle tissue.

4.1. Acute toxicity

4.1.1. Cumulative mortality

In order to determine LD_{50} of chlorpyrifos for common carp, cumulative morality was observed as shown in Fig. 4.1. Generally in toxicological studies, mortality is the decisive criteria because it is easy to evaluate and has obvious ecological and biological significance.

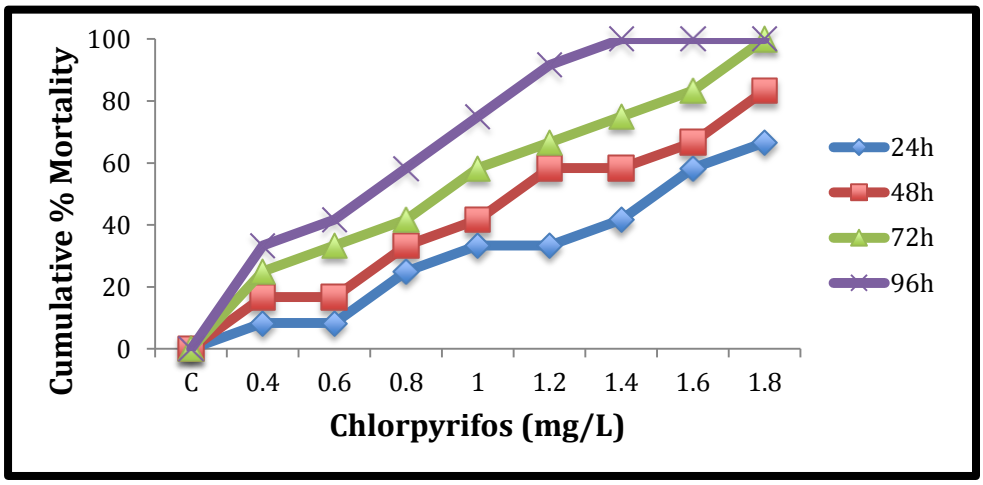


Figure 4.1. Percentage mortality of common carp *(Cyprinus carpio)* exposed to different concentrations of chlorpyrifos for a period of 96h

Mortality of organism mainly depends on sensitivity of organism to the test chemical, its concentration and exposure period. In the present study, 24h of exposure casued 42% population of the test organism to die at 1.4 mg/L but at 1.6 mg/L death ratio increased upto 58%. More than half of the population died at 1.2, 1.0 and 0.8 mg/L after 48, 72 and 96h of exposure respectively. Results of the current study indicate that mortality of test organism is directly dependent on exposure duration and concentration of the test chemical.

4.1.2. Behavioral changes

A promising tool in ecotoxicology is behavior that is considered as an integrated result of endogenous and exogenous processes. The behavior changes observed as a result of acute toxicity in common carp after CPF exposure were rapid gulping of water, swimming on the surface for water, loss of equilibrium and increased operculum movement, sudden and erratic swimming movement, hyperexcitability, mouth swelling and lesion on body (Fig. 4.2). Fish moved to the corners of test aquarium. Stress was observed in fishes before death. They were lethargic, restless and secreted excess mucus all over the body. Similar findings have been reported by Deb and Das, (2013); Halappa and David, (2009) and Sharbidre *et al.* (2011).

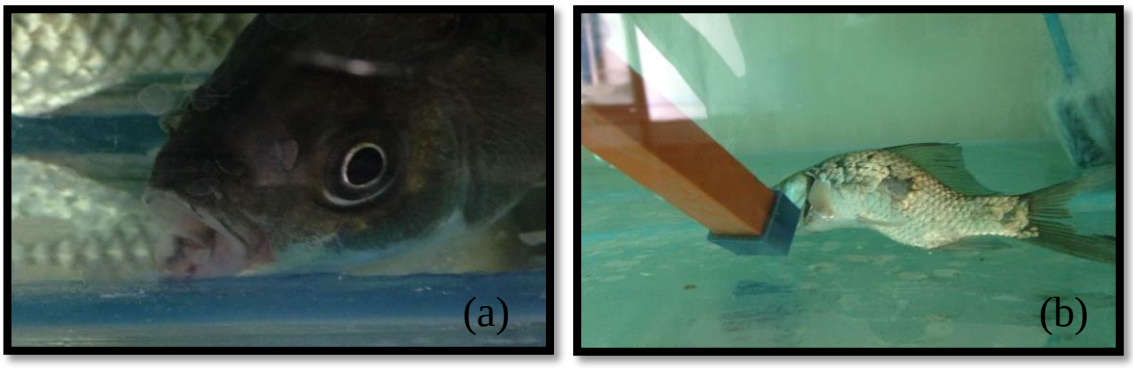


Figure 4.2. Mouth swelling (a) and lesion on body (b) due to chlorpyrifos exposure

4.1.3. Lethal dose 50

For acute toxicity, Lethal dose 50 (LD_{50}) is the most commonly used tool. LD_{50} is concentration of the test chemical at which half of the population died in a particular exposure period of 96h. LD_{50} values of chlorpyrifos corresponding to 24, 48, 72 and 96 h were 1.53, 1.16, 0.90 and 0.67 mg/L determined statistically using cumulative mortality (Table 4.1). Low value of 96h-LD_{50} of 0.67 mg/L suggests that chlorpyrifos is highly toxic to fish.

P	LD_{50} (mg/L)	R^2	LCL	UCL
24	1.53	0.907	0.86	2.20
48	1.16	0.983	0.79	1.54
72	0.90	0.990	0.69	1.11
96	0.67	0.913	0.36	0.94

Table 4.1. LD_{50} of chlorpyrifos with confidence limits at different time intervals for common carp (*Cyprinus carpio)*

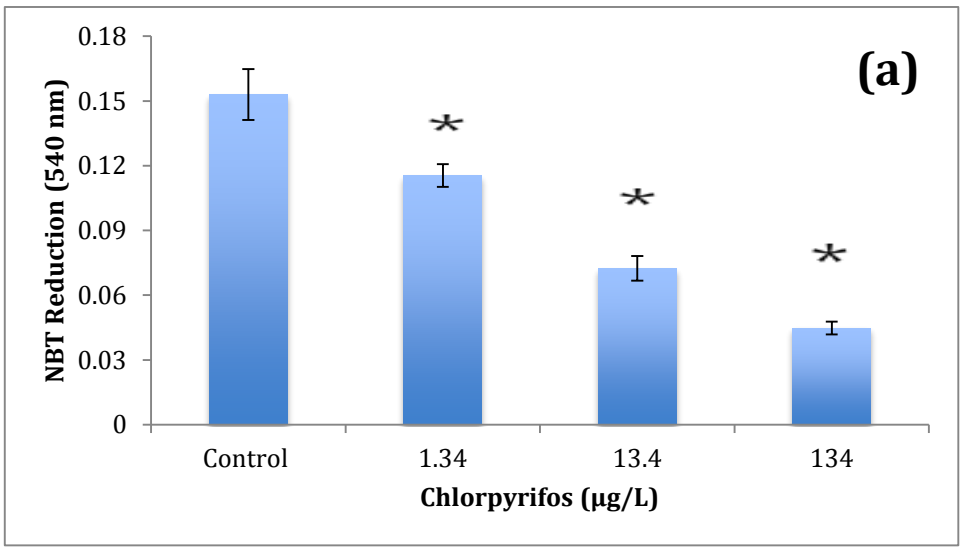
LD_{50} values for 96h of chlorpyrifos to common carp observed in current study was higher from the results reported by Xing *et al.* (2015) and Halappa and David, (2009) that 96h-LD_{50} value of chlorpyrifos for common carp is 0.582 and 0.160 mg/L respectively. 96h chlorpyrifos toxicity reported by Rao *et al.* (2005) to mosquito fish *(Gambusia affinis)* is 0.297 mg/L, Clark *et al.* (1985) to sheepshead minnow (Cyprinodon variegatus) is 0.136 mg/L and Johnson and Finely, (1980) to channel catfish *(Ictalurus punctatus)* is 0.280 mg/L. Chemical toxicity for aquatic organism considered to be affected by size, age, health, and type of species (Farah

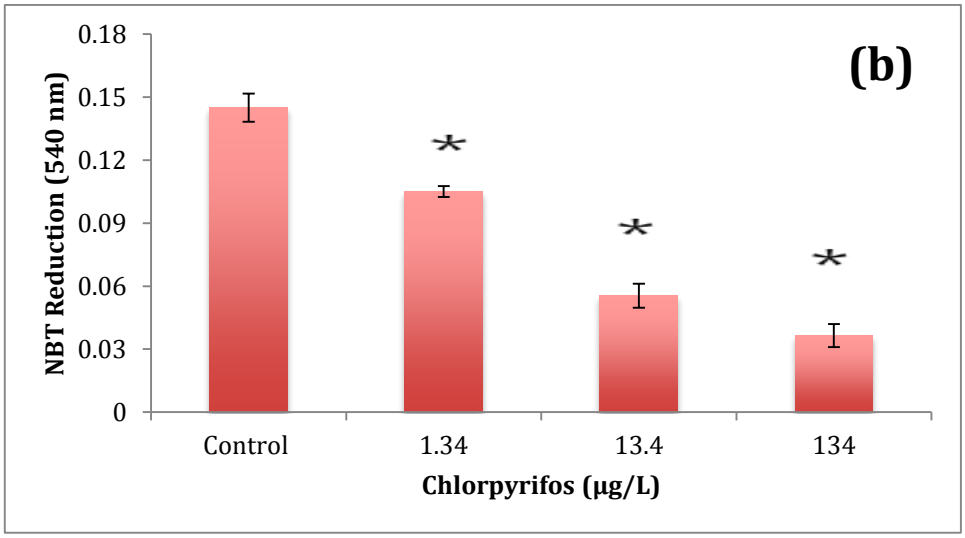
et al., 2004) and physiochemical parameters of water like dissolved oxygen, temperature, pH etc. (Li *et al.*, 2013).

4.2. Immuno-hematological parameters

Fish leukocytes use the respiratory burst as one of their immune mechanism that generates several toxic molecules called oxidative radicals to destroy microorganism (Gomez *et al.*, 2013). An increase in oxidative radicals (H_2O_2, O_2^-) may enhance the immunity of host organism against pathogenic microorganism. Therefore, NBT reduction product obtained after reaction with free radicals (H_2O_2, O_2^-) is a very good biomarker to evaluate the immunity of fish against environmental stressor.

In the present study, immunity of fish decreased significantly ($P < 0.05\%$) in dose and exposure dependent manner when exposed with sublethal concentration (134, 13.4, 1.34 μg/L) of chlorpyrifos for 7 and 14 days. As compared to control, percentage reduction in immunity of common carp exposed to 134, 13.4, 1.34 μg/L was 71, 53, 25% after 7 days and 75, 59, 28% after 14 days respectively as shown in Fig. 4.3.





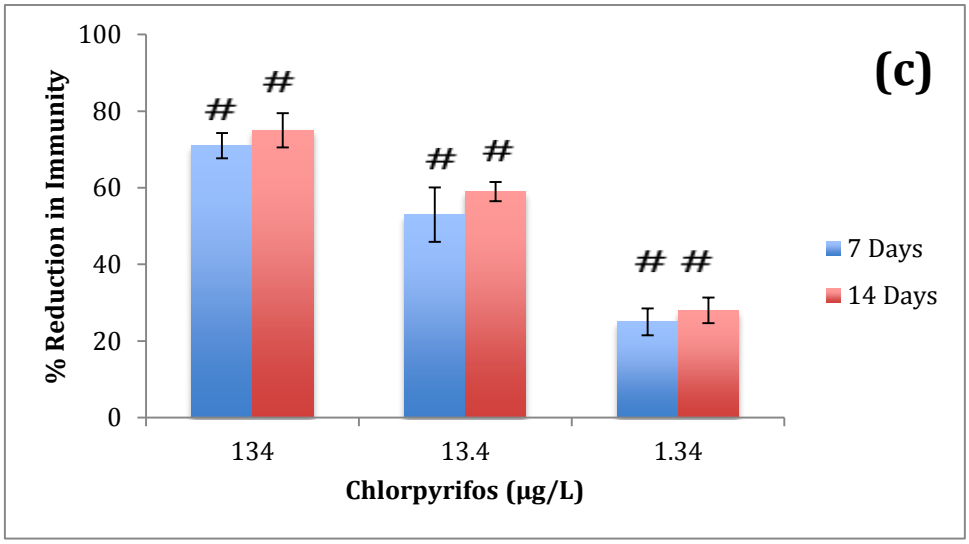


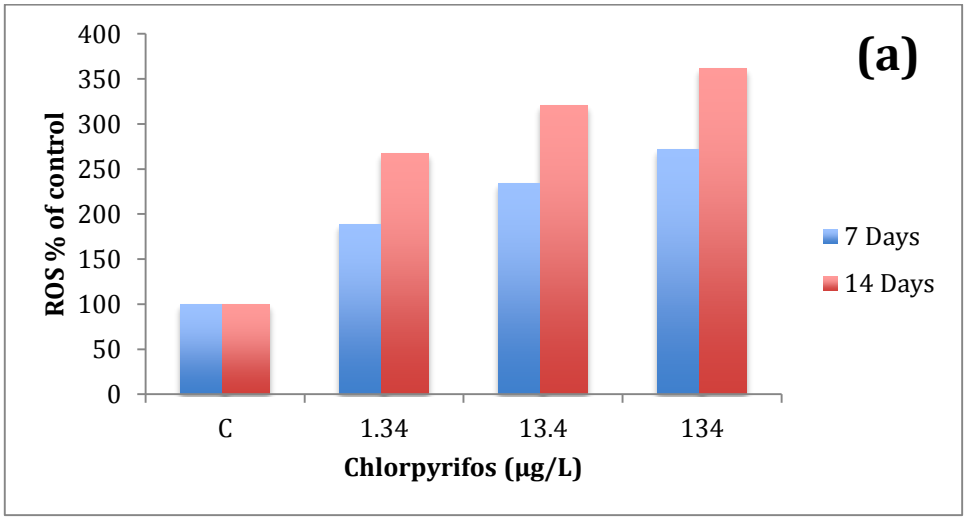
Figure 4.3. NBT Reduction Assay (a) 7 Days (b) 14 day, (c) % Reduction in immunity (Asterisk and hash denotes significant (*P*<0.05) differences from control and within exposure group respectively)

Similar results were observed when common carp *(Cyprinus carpio)* exposed to malathion and Nile tilapia *(Oreochromis niloticus)* exposed to diazinon (Giron *et al.*, 2007). Reduction in immunity is due to inhibition of AChE activity. AChEs are membrane-bounded proteins that assist the immune system to interact with and destroy invading organisms. For example, neutrophils, a type of phagocytic

leukocyte, require esterases to move about by chemotaxis. When organophosphates are present, there may be suppression of these esterases and, therefore, suppression of the chemotactic signals necessary for phagocytic cells (Rabideau, 2001). Tilapia *(Oreochromis mossambicus)* when exposed with sublethal concentration (2.9, 3.3, 3.7, 4.1, 4.5 and 5.0 μg/L) of endosulfan showed a significant reduction (4, 4, 7, 11, 17 and 18%) in immunity. Inhibition in AChE activity in brain is also observed in dose dependent manner (Kumar *et al.*, 2011).

4.3. Oxidative stress

Oxidative stress occurs due to an imbalance between production of reactive oxygen species and the ability of the body to combat and detoxify their harmful effects by antioxidant defense mechanism. ROS are constantly produced under normal conditions as a result of aerobic metabolism (Zapata *et al.*, 2009). Elevated level of pesticides or their metabolites may increase the production of ROS resulting in oxidative stress that impairs the activity of antioxidant enzymes, lipid peroxidation, protein carbonylation and DNA damage (Xing *et al.*, 2012). Results of the current study indicate that ROS production augmented as a function of chlorpyrifos dose and exposure time both in brain and gills of exposed fish (Fig. 4.4).



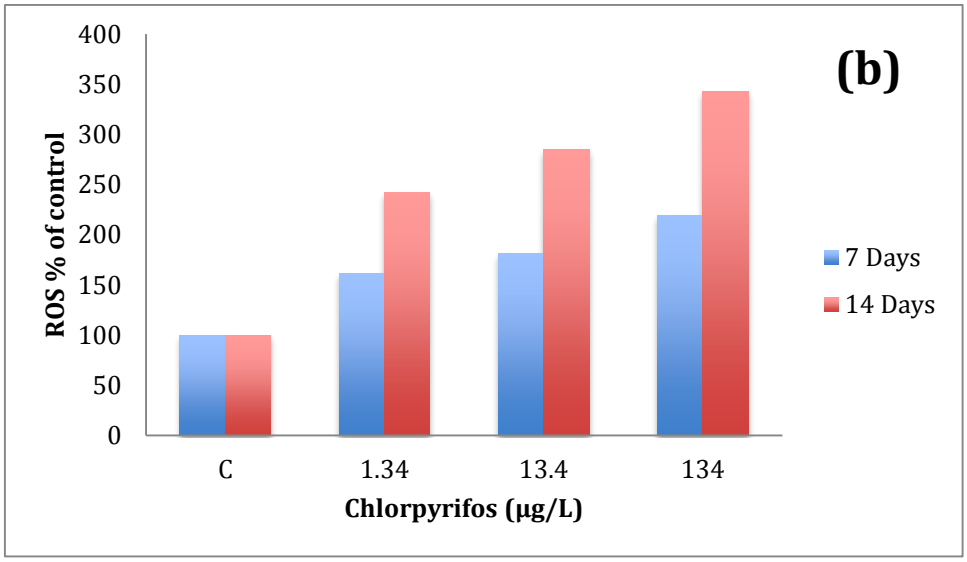


Figure 4.4. Effect of chlorpyrifos on ROS formation (a) Brain (b) Gills

Brain has increased amount of ROS production as compared to gills. Brain has high mitochondrial oxidative metabolism for neural functioning that requires high ATP concentration. Brain may be particularly vulnerable to oxidative stress because it contains huge amount of polyunsaturated lipids that may easily oxidized by ROS leading to lipid peroxidation (Zhang *et al.*, 2008). Common carp when exposed to simazine exhibited similar increasing trend of ROS in brain and gills (Stara *et al.*, 2012). Velisek and his co-workers (2011) also observed oxidative stress in common carp (muscles, gills, brain, liver and intestine) when exposed to terbutryn in the real environment concentration. These findings were also consistent with previous research (Liu *et al.*, 2011; Craig *et al.*, 2007; Parvez and Raisuddin, 2005).

Organophosphate induces Mixed Function Oxidase (MFO) System, which is a chemical metabolizing system that converts OPs into paraxon, which is an AChE inhibitor. MFO uses cytochrome P450 (cyt P450) to interact with toxicant. The general reaction catalyzed by cyt P450 is as follow:

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

RH may be fatty acid, steroid or any toxicant. Through this reaction MFO produces ROH that may be readily excreted. OPs will form enzyme-substrate complex that decreases availability of enzyme thereby overwhelming MPO system resulting into production of free radicals (Rabideau, 2001).

4.4. GC-ECD analysis for bioaccumulation

4.4.1. Standard calibration curve

Calibration solutions (5, 10, 25, 50, 100 and 150 μg/L) were made from 300 μg/L in acetone. 1 μl of all these working solutions were injected in GC injection port using glass syringe to generate signals in form of chromatogram and to detect retention time (Annexure I). Fig. 4.5 shows the calibration curve and line equation.

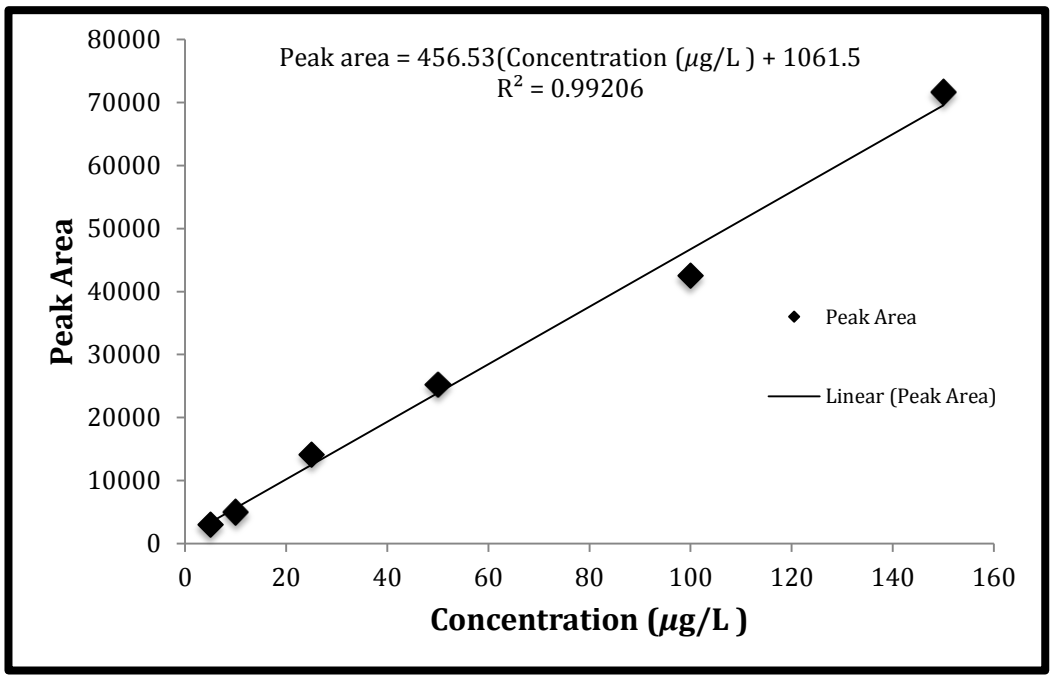


Figure 4.5. Calibration curve and line equation

4.4.2. Retention time of chlorpyrifos

Temperature of injector (300 °C), column (150 °C) and detector (330 °C) were adjusted according to the signals. Calibration solutions were injected at least thrice

times and mean was used to calculate average retention time of acetone and chlorpyrifos (Table 4.2). Peaks of chlorpyrifos and acetone are shown in Fig. 4.6.

Compound	Retention time (min)
Solvent (acetone)	2.61
Chlorpyrifos	18.99

Table 4.2. Retention time of solvent and analyte

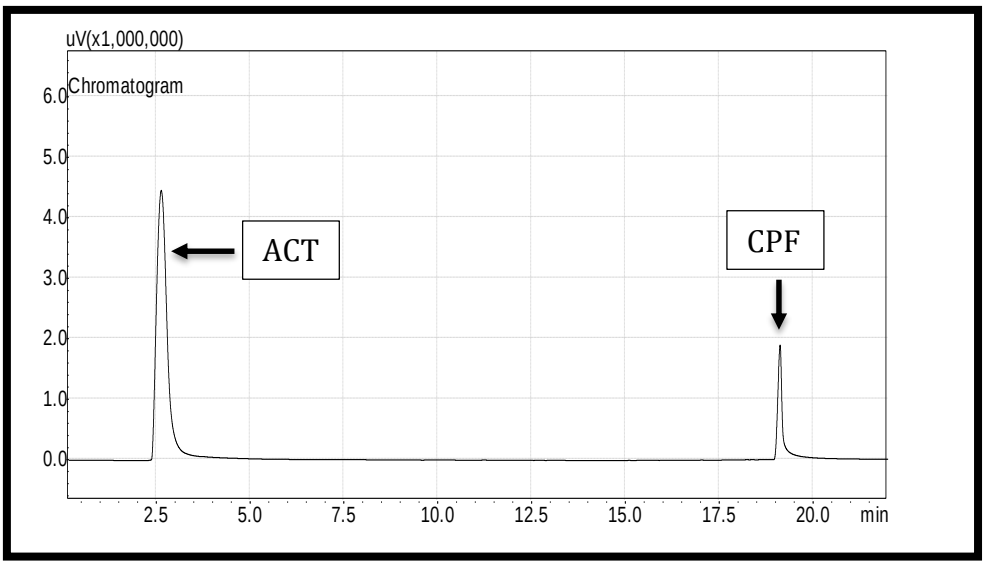


Figure 4.6. Chromatogram showing acetone and chlorpyrifos peak

4.4.3. Bioaccumulation of chlorpyrifos in muscle tissues

The concentrations of chlorpyrifos in muscle tissues of common carp cultivated in pesticide free control water were not detected while treated group showed visible peaks of chlorpyrifos as shown in Annexure II. The percentage accumulation of chlorpyrifos was found to be 60, 68 and 70 % for 1.34, 13.4 and 134 μg/L respectively (Fig. 4.7).

Pesticide accumulation of fish may be biomagnified in every step of food chain upto human. Wang and his co-workers (2013) reported accumulation of chlorpyrifos and its metabolites in head kidneys and spleens of common carp. Chlorpyrifos residues were also detected in Taiwan farmed fish and fishery products (Sun and Chen, 2008).

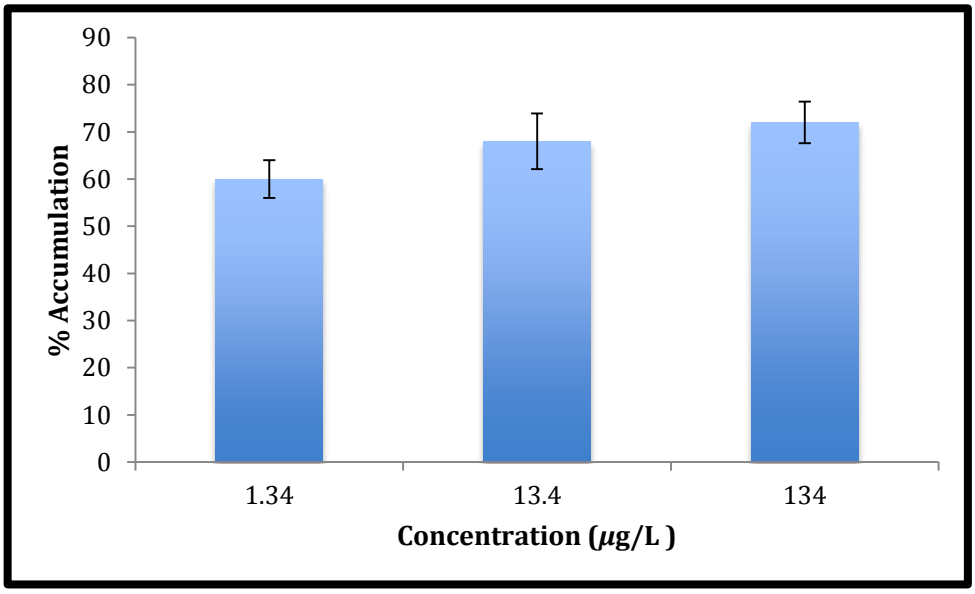


Figure 4.7. Percentage accumulation of chlorpyrifos in muscle tissues

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Pesticides enter into water bodies and deteriorate their quality as well as interfere with aquatic ecosystems. Fish have been widely used as biosensor for environmental pollutants. In the current study common carp (*Cyprinus carpio)* was chosen as a model organism to evaluate the effects of an organophosphate pesticide, chlorpyrifos. Conclusions drawn from the research work are listed below:

1. Cumulative mortality of common carp *(Cyprinus carpio)* depends on exposure duration and concentration of chlorpyrifos.
2. LD_{50} values of chlorpyrifos corresponding to 24, 48, 72 and 96h were 1.53, 1.16, 0.90 and 0.67 mg/L.
3. Respiratory burst activity is an indicator of immunity. A declining trend was observed in respiratory burst activity in dose and time dependent manner. As compared to control, percentage reduction in immunity of common carp exposed to 134, 13.4, 1.34 μg/L was 71, 53, 25% after 7 days and 75, 59, 28% after 14 days respectively.
4. Sub-lethal exposure of chlorpyrifos resulted in significant ROS production augmentation as a function of chlorpyrifos dose and exposure time both in brain and gills of exposed fish.
5. The GC analysis revealed high levels of chlorpyrifos residues in treated fish but no residue was detected in carp cultivated in pesticide-free control

water. The percentage accumulation of chlorpyrifos was found to be 60, 68 and 70 % for 1.34, 13.4 and 134 μg/L respectively after 30 days.

5.2. Recommendations

Following recommendations are important for further study:

1. Antioxidant study to evaluate cellular defense mechanism against oxidative stress.
2. Genotoxicity study to assess DNA damage after chemical exposure.
3. Histopathological study to find morphological alterations due to environmental toxicants.

Chapter 6

REFERENCES

Adedeji, O.B. & Okocha R.O. (2012). Overview of pesticide toxicity in fish. *Advances in Environmental Biology, 6(8),* 2344-2351

Ahad, K., Hayat, Y., Ahmad, I. and Soomro, M.H. (2001). Capillary chromatographic determination of pesticides residues in ground water of Multan division. *Nucleous, 38,* 145-149

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B. and Lakra, W.S. (2009). Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food and Chemical Toxicology, 47,* 650-656

Amin, K.A. and Hashem K.S. (2012). Delta-methrin induced oxidative stress and biochemical changes in tissues and blood of catfish (*Clarias gariepinus)*: antioxidant defense and role of alpha tocopherol. *Veternary Research, 8,* 45-52

APHA (2012), Standard methods for examination of water and wastewater. 22nd addition (Washigton: American Public Health Association)

Asi, M.R., Hussain, A. and Muhmood, S.T. (2008). Solid phase extraction of pesticide residues in water samples: DDT and its metabolites. *International Journal of Environmental Research, 2,* 43-48

Audu, B.S., Ajima, M.N. and Ofojekwu, P.C. (2015). Enzymatic and biochemical changes in common carp *(Cyprinus carpio)* fingerlings exposed to crude leaf extract of *Cannabis sativa* (L.). *Asian Pacific Journal of Tropical Disease, 5(2),* 107-115

Azizullah, A., Khattak, M.A., Richter, P. and Hader, D.P. (2011). Water pollution in Pakistan and its impact on public health — A review. *Environment International, 37,* 479-497

Banaee, M., Haghi, B.M. and Ibrahim, A. (2013). Sub-lethal toxicity of chlorpyrifos on common carp, *Cyprinus carpio* (Linnaeus, 1758):

Biochemical response. *International Journal of Aquatic Biology, 1(6),* 281-288

Botte, E,S., Jerry, D,R., King, S,C., Keune, S,C. and Negri, A,P. (2012). Effects of chlorpyrifos on cholinesterase activity and stress markers in the tropical reef fish *Acanthochromis polyacanthus. Marine Pollution Bulletin, 65,* 384-393

Caldas, S., Bolzan, C.M., Menezes, J., Escarrone, A.L., Martins, C.M., Bianchini, A. and Primel, E.G. (2013). A vortex-assisted MSPD method for the extraction of pesticide residues from fish liver and crab hepatopancreas with determination by GC–MS. *Talanta, 112,* 63–68

Clark, J.R., Patrick, J.M., Middaugh, D.P. and Moore, J.C. (1985). Relative sensitivity of six estuarine fishes to carbophenothion, chlorpyrifos and fenvalerate. *Ecotoxicology and Environmental Safety, 10,* 382-390

Craig, P.M., Wood, C.M. and McClelland, G.B. (2007). Oxidative stress response and gene expression with acute copper exposure in zebrafish *(Danio rerio). Regulatory, Integrative and Comparative Physiology, 293,* 1882–1892

Deb, N. and Das, S. (2013). Chlorpyrifos Toxicity in Fish: A Review. *Current World Environment, 8(1),* 77-84

Diaz, A.N., Parwja, A.G. and Sanchez, F.G. (1997). Liquid and gas chromatographic multi-residue pesticide determination in animal tissues. *Pesticide Science, 49 (1),* 56-64

Driver, A.S., Kodavanti, P.R. and Mundy, W.R. (2000). Age-related changes in reactive oxygen species production in rat homogenates. *Neurotoxicology and Teratology, 22,* 175-18

Ensibi, C. Lopez, P.M., Rodriguez, S.F., Yahya, M.N and Moreno, D.H. (2013). Effects of deltamethrin on biometric parameters and liver biomarkers in common carp *(Cyprinus carpio L.) Environmental Toxicology and Pharmacology, 3(6),* 384–391

FAO, Rome (Italy), The state of food and agriculture 1996. ISBN 92-5-103858-9

Farah, M.A., Ateeq, B., Ali, M.N. and Ahmad, W. (2004). Studies on lethal concentrations and toxicity stress of some xenobiotics on aquatic organisms. *Chemosphere, 55,* 257-265

Fu, Y., Li, M., Liu, C., Qu, J.P., Zhu, W.J., Xing, H.J., Xu, S.W. and Li, S. (2013). Effect of atrazine and chlorpyrifos exposure on cytochrome P450 contents and enzyme activities in common carp gills. *Ecotoxicology and Environmental Safety, 94,* 28-36

Galloway, T. and Handy, R. (2003). Immunotoxicity of organophosphorous pesticides. *Ecotoxicology, 12,* 345-363

Gearhart, D.A., Sickles, D., Buccafusco, J., Prendergast, M. and Terry, A.V. (2007). Chlorpyrifos, chlorpyrifos-oxon, and diisopropylfluorophosphate inhibit kinesin-dependent microtubule motility. *Toxicology and Applied Pharmacology*, *218*, 20-29

Ghumman, A, R. (2011). Assessment of water quality of Rawal Lake by long-time monitoring. *Environmental Monitoring and Assessment, 180*, 115-126

Giron, M.I., Santerre, A., Gonzalez-Jaime, F., Casas, J., Hernandez, M., Peregrina, J., Takemura, A. and Zaitseva, G. (2007). Immunotoxicty and hepatic function evaluation in Nile tilapia *(Oreochromis niloticus)* exposed to diazinon. *Fish Shellfish Immunology, 23,* 760–769

Gomez, L., Cortes, R., Valenzuela, A. and Smith, C. (2013). Kinetics of reactive oxygen species produced by rainbow trout *(Oncorhynchus mykiss)* leukocytes and the effect of the antioxidant astaxanthin. *Acta Veterinaria Brno*, *82(4),* 435–439

Gul, A. (2005). Investigation of acute toxicity of chlorpyrifosmethyl on Nile tilapia *(Oreochromis niloticus L.)* larvae. *Chemosphere, 59,* 163-166

Halappa, R. and David, M. (2009). Behavioural responses of the freshwater fish, *Cyprinus carpio* (Linnaeus) following sublethal exposure to chlorpyrifos. *Turkish Journal of Fisheries and Aquatic Sciences, 9, 233-238*

Halim A.K., Salama, A.K., El-khateeb, E.N. and Bakry, N.M. (2006). Organophosphorus pollutants (OPP) in aquatic environment at Damietta Governorate, Egypt: Implications for monitoring and biomarker responses. *Chemosphere*, *63,* 1491-1498

Hamers, T., Smit, D. and Murk, J. (2001). Biological and chemical analysis of the toxic potency of pesticides in rainwater. *Chemosphere, 46,* 609-624

He, L., Luo, X., Xie, H., Wang, C., Jiang, X. and Lu, K. (2009). Ionic liquid-based dispersive liquid–liquid microextraction followed high-performance liquid chromatography for the determination of organophosphorus pesticides in water sample. *Analytica Chimica Acta 655 (1),* 52–59

Iram, S., Ahmad, I., Ahad, K., Muhammad, A. and Anjum, S. (2009). Analysis of pesticides residues of Rawal and Simly lakes. *Pakistan Journal of Botany, 41*, 1981-1987

Ispir, U. and Doruc, M. (2005). A study on the effects of levamisole of immune system Rainbow trout *(Oncorhynchus mykiss, Walbaum). Turkish Journal of Veterinary & Animal, 29,* 1169-1176

Jabbar, A. and Mallick, S. (1994). Pesticides and environment situation in Pakistan. *SDPI Working Paper Series (22),* 37, Sustainable Development Policy Institute, Islamabad, Pakistan

Jabbar, A., Masud, S.Z., Parveen, Z. and Ali, M. (1993). Pesticide residues in cropland soils and shallow groundwater in Punjab Pakistan. *Bulletin of Environmental Contamination and Toxicology, 51,* 268-73

Ji, J., Xiao, B., Li, Y., Yu, W.L. and Liu F. (2010). Toxicity assessment of two different formulations of chlorpyrifos to four environmental organisms. *Journal of Agriculture and Environmental Sciences, 29,* 1681–1686

Jin, Y.X., Zhang, X.X., Shu, L.J., Chen, L.F., Sun, L.W., Qian, H.F., Liu, W.P. and Fu, Z.W. (2010). Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish *(Danio rerio). Chemosphere, 78,* 846–852

Johnson, W.W. and Finley, M.T. (1980). Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates. U.S. Fish and Wildlife Service Resource Publications, 137, *98*

Kavitha, P. and Rao, J.V. (2008). Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, Gambusia affinis. *Environmental Toxicology and Pharmacology, 26,* 192-198

Kaya, H., Celik, E.S., Yılmaz, S., Tulgar, A., Akbulut, M., and Demir, N. (2015). Hematological, serum biochemical, and immunological responses in

common carp *(Cyprinus carpio)* exposed to phosalone. *Comparative Clinical Pathology, 24(3),* 497-507

Khalil, F., Kang, I.J., Undap, S., Tasmin, R., Qiu, X. and Y. Shimasaki, Y. (2013). Alterations in social behavior of Japanese medaka *(Oryzias latipes)* in response to sublethal chlorpyrifos exposure. *Chemosphere, 92,* 125-130

Khooharo, A., Memon, R. and Mallah, M. (2008). An empirical analysis of pesticide marketing in Pakistan. *Pakistan Economic and Social Review, 46(1),* 57-74

Kokushi, E., Uno, S., Pal, S. and Koyama, J. (2015). Effects of chlorpyrifos on the metabolome of the freshwater carp, *Cyprinus carpio*. *Environmental Toxicology, 30(3),* 253-260

Kumar, N., Prabhu, P.A., Pal, A.K., Remya, S., Aklakur, M., Rana, R.S., Gupta, S., Raman, R.P. and Jadhao, S.B. (2011). Anti-oxidative and immuno-hematological status of Tilapia *(Oreochromis mossambicus)* during acute toxicity test of endosulfan. *Pesticide Biochemistry and Physiology, 99,* 45-52

Li, X., Liu, L., Zhang, Y., Fang, Q., Li, Y. and Li, Y. (2013). Toxic effects of chlorpyrifos on lysozyme activities, the contents of complement C3 and IgM, and IgM and complement C3 expressions in common carp (*Cyprinus carpio* L.). *Chemosphere, 93,* 428-433

Liu, X.X., Cao, H. and Jia, X.Y. (2011). Oxidative damage of cadmium to protein in the viscera tissues of *Cyprinus carpio var color*. *Asian Journal of Ecotoxicology, 6,* 595–59

Ma, J., Liu, Y., Niu, D. and Li, X. (2015). Effects of Chlorpyrifos on the transcription of CYP3A cDNA, activity of acetylcholinesterase, and oxidative stress response of Goldfish *(Carassius auratus). Environmental Toxicology, 30(4),* 422-429

Manuel, A.E., Eugenio, L.P. and Elena, M. (2008). The mobility and degradation of pesticides in soils and the pollution of groundwater resources. *Agriculture, Ecosystems and Environment, 123,* 247-260

Mishra, D., Srivastav, S. and Srivastav, A.K. (2004). Plasma calcium and inorganic phosphate levels of a teleost Heteropneustes fossilis exposed to metacid-50. *Malaysian Applied Biology, 33(2),* 19-25

Modesto, K.A. and Martinez, C.B.R. (2010). Effects of roundup transorb on fish: hematology, antioxidant defenses and acetylcholinesterase activity. *Chemosphere, 81,* 781-787

Monteiro, D.A., Alves J., T. Rantin. and Kalinin, L. (2006). Oxidative stress biomarkers in the freshwater characid fish, Brycon cephalus, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). *Comparative Biochemistry and Physiology, 143,* 141-149

Muthappa, N., Gupta, S., Yengkokpam, S., Debnath, D., Kumar, N., Pal, A.K. and Jadhao, S. (2014). Lipotropes promote immunobiochemical plasticity and protect fish against low-dose pesticide-inducedoxidative stress. *Cell Stress and Chaperones, 19,* 61-81

Nadzialek, S., Spano, L., Mandiki, M. and Kestemont, P. (2008). High doses of atrazine do not disrupt activity and expression of aromatase in female gonads of juvenile goldfish *(Carassius auratus L.). Ecotoxicology, 17,* 464-470

Ojha, A., Yaduvanshi, S. and Srivastava, N. (2011). Effect of combined exposure of commonly used organophosphate pesticides on lipid peroxidation and antioxidant enzymes in rat tissues. *Pesticide Biochemistry and Physiology, 99,* 148-156

Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Effects on Biotic systems. Method 203, Fish, acute toxicity test, Adopted: 17 July 1992

Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Effects on Biotic systems. Method 204, Fish, Prolonged Toxicity Test: 14-day Study, Adopted: 4 April 1984

Oruc, E.O. (2010). Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in *Oreochromis niloticus* exposed to chlorpyrifos. *Pesticide Biochemistry and Physiology, 96,* 160-166

Parveen, Z. and Masud, S.Z. (1988). Organochlorine pesticide residue in cattle drinking water. *Pakistan Journal of Scientific and Industrial Research, 31,* 53-56

Parvez, S. and Raisuddin, S. (2005). Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). *Environmental Toxicology and Pharmacology, 20,* 112–117

Pimentel, D. (1995). Amounts of pesticides reaching target pests: environmental impacts and ethics. *Journal of Agricultural and Environmental Ethics*, *8,* 7-29

Rabideau, C.L. (2001). Pesticide Mixtures Induce Immunotoxicity: Potentiation of Apoptosis and Oxidative Stress. *Verginia Tech, 25-29*

Rao, J.V., Ghousia, B., Pallela, R., Usman, P.K. and Nageswara R.R. (2005). Changes in behavior and brain acetylcholinesterase activity in mosquito fish, *Gambusia affinis* in response to the sub-lethal exposure to chlorpyrifos. *International Journal of Environmental Research and Public Health, 2(3),* 478-483

Rehulka, J. and Minarik, B. (2003). Effect of lecithin on the hematological and condition indices of the rainbow trout *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research, 34,* 617-627

Sarabia, L., Maurer, I. and Bustos, E. (2009). Melatonin prevents damage elicited by the organophosphorous pesticide diazinon on mouse DNA. *Ecotoxicology and Environmental Safety, 71,* 663–668

Saravanan M., Prabhu K.K. and Ramesh M. (2011). Hematological and biochemical responses of freshwater teleost fish *Cyprinus carpio* (Actinopterygii: Cypriniformes) during acute and chronic sublethal exposure to lindane. *Pesticide Biochemistry and Physiology, 100(3),* 206-211

Sharbidre, A, A., Metkari, V. and Patode, P. (2011). Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, Poecilia reticulate. *Pesticide Biochemistry and Physiology, 101,* 132-141

Shenouda, J., Green, P. and Sultatos, L. (2009). An evaluation of the inhibition of human butyrylcholinesterase and acetylcholinesterase by the organophosphate chlorpyrifos oxon. Toxicology and Applied Pharmacology, *241,* 135-142Sarabia, L., Maurer, I. and Bustos, E. (2009).

Singh, B.K., and Walker, A. (2006). Microbial degradation of organophosphorous compounds. *FEMS Microbiology Review, 30*, 428-471

Singh, R., Pandey, R., Narendra., Singh, N. and Das, V.K. (2009). Acute Toxicity and Behavioral Responses of Common Carp *Cyprinus carpio* (Linn.) to an Organophosphate (Dimethoate). *World Journal of Zoology, 4 (2)*, 70-75

Somashekar, K,M., Mahima, M,R. and Manjunath, K,C. (2015). Contamination of water sources in mysore city by pesticide residues and plasticizer – a cause of health concern. *Aquatic Procedia, 4*, 1181-1188

Stara, A., Machova, J. and Velisek, J. (2012). Effect of chronic exposure to simazine on oxidative stress and antioxidant response in common carp *(Cyprinus carpio L.). Environmental Toxicology and Pharmacology, 33,* 334-343

Sun, F. and Chen, H.S. (2008). Monitoring of pesticide chlorpyrifos residue in farmed fish: investigation of possible sources. *Chemosphere, 71*, 1866-1869

Tariq, M.I., Afzal, S. and Hussain, I. (2004). Pesticides in shallow groundwater of Bahawalnagar, Muzafargarh, D.G. Khan and Rajan Pur districts of Punjab, Pakistan. *Environment International, 30,* 471-479

Topala, A., Atamanalp, M., Oruc, E., Kirici, M. and Kocamanb, E. (2014). Apoptotic effects and glucose-6-phosphate dehydrogenase responses in liver and gill tissues of rainbow trout treated with chlorpyrifos. *Tissue and Cell, 46,* 490–496

United States Environmental Protection Agency. 1990. The quality of our nation's water: A summary of the 1988 national water quality inventory. EPA 440/4-90- 005, 26pp

Ural, M.S. (2013). Chlorpyrifos-induced changes in oxidant/antioxidant status and hematological parameters of *Cyprinus carpio*: Ameliorative effect of lycopene. *Chemosphere, 90*, 2059-2064

Uzun, F.G., Kalender, S., Durak, D., Demir, F. and Kalender, Y. (2009). Malathion induced testicular toxicity in male rats and the protective effect of vitamins C and E. Food and Chemical Toxicology, 47, 1903–1908

Varo, R., Serrano, R., Pitarch, E., Amat, F., Lopez, F.J. and Navarro, J.C. (2002). Bioaccumulation of chlorpyrifos through an experimental food chain: Study of protein HSP70 as biomarker of sublethal stress in fish. *Archives of Environmental Contamination and Toxicology, 42,* 229–235

Velisek, J., Stara, A., Kalarova, J. and Svobodova, Z. (2011). Biochemical, physiological and morphological responses in common carp (*Cyprinus carpio)* after long-term exposure to terbutryn in real environmental concentration. *Pesticide Biochemistry and Physiology, 100,* 305-313

Velmurugan, B., Selvanayagam, M., Cengiz, E. and Unlu, E. (2007). The effects of monocrotophos Cirrhinus mrigala. *Bulletin of Environmental Contamination and Toxicology, 78(6),* 450-454

Wang, A., Xing, H., Jiang, Y., Wu, H., Sun, G., Xu, Q. and Xu, S. (2013). Accumulation, histopathological effects and response of biochemical markers in the spleen and head kidney of common carp to atrazine & chlorpyrifos. *Food and Chemical Toxicology, 62,* 148-158

Wang, X., Xing, H., Li, X., Xu, S. and Wang, X. (2011). Effects of atrazine and chlorpyrifos on the mRNA levels of IL-1 and IFN-γ2b in immune organs of common carp. *Fish Shellfish Immunology, 31,* 126-133

Wang, Y., Chen, C., Zhao, X., Wang, Q. and Qian, Y. (2015). Assessing joint toxicity of four organophosphate and carbamate insecticides in common carp *(Cyprinus carpio)* using acetylcholinesterase activity as an endpoint. *Pesticide Biochemistry and Physiology, 122,* 81-85

World Health Organization (WHO) International Program on Chemical Safety, WHO Recommended Classification of Pesticides by hazard and Guidelines to Classification *1998-1999* (WHO/PCS/98.21/Rev.1)

Xing, H., Li, S., Wang, Z., Gao, X., Xu, S. and Wang, X. (2012). Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. *Chemosphere, 88,* 377–383

Xing, H., Wang, Z., Wu, H., Zhao, X., Liu, T., Li, S. and Xu, S. (2015). Assessment of pesticide residues and gene expression in common carp exposed to atrazine and chlorpyrifos: Health risk assessments. *Ecotoxicology and Environmental Safety, 113,* 491-498

Xu, W., Jilong, L., Houjuan, X. and Shiwen, X. (2011). Review of Toxicology of Atrazine and Chlorpyrifos on Fish. *Journal of Northeast Agricultural University, 4,* 88-92

Yonar, M.E. and Sakin, F., (2011). Ameliorative effect of lycopene on antioxidant status in *Cyprinus carpio* during pyrethroid deltamethrin exposure. Pesticide Biochemistry and Physiology, *99,* 226-231

Yonar, M.E., Yonar, S.M., Ural, M.S., Silici, S. and Dusukcan, M. (2012). Protective role of propolis in chlorpyrifos-induced changes in the hematological parameters and the oxidative/antioxidative status of *Cyprinus carpio carpio. Food and Chemical Toxicology, 50,* 2703-2708

Yonar, S.M., Ural, M.S., Silici,S. and Yonar, M.E. (2014). Malathion-induced changes in the hematological profile, the immune response, and the oxidative/antioxidant status of *Cyprinus carpio carpio:* Protective role of propolis. *Ecotoxicology and Environmental Safety, 102,* 202-209

Zanuzzo, F.S., Urbinati, E.C., Rise, M.L., Hall, J.R., Nash, G.W. and Gamperl, A.K. (2015). Aeromonas salmonicida induced immune gene expression in *Aloe vera* fed steelhead trout, *Oncorhynchus mykiss (*Walbaum). *Aquaculture, 435,* 1-9

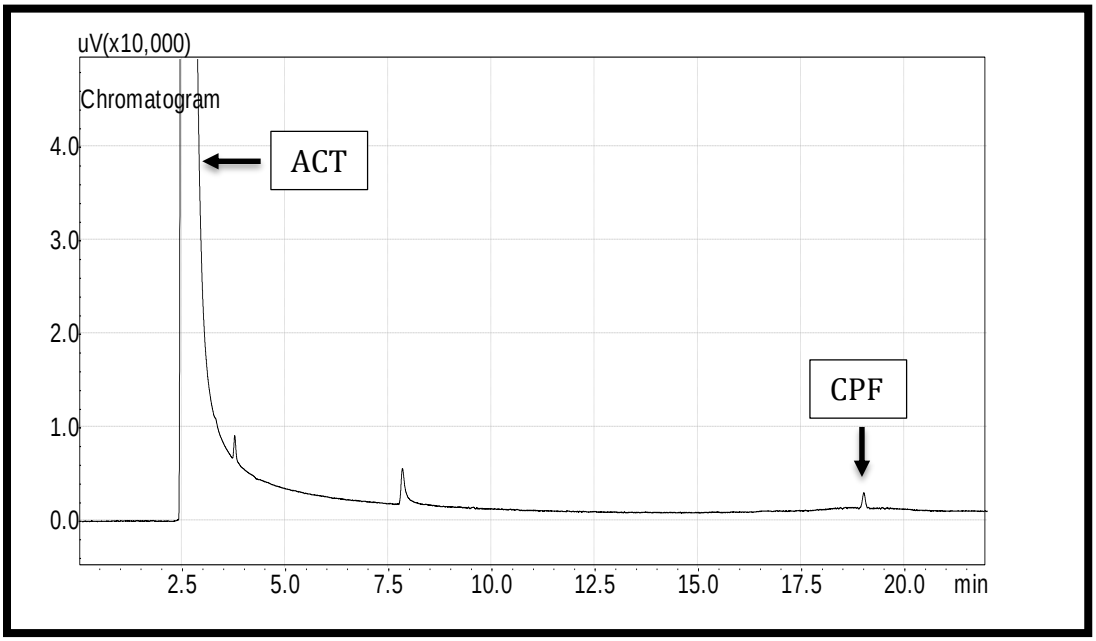
Zapata, M., Tanguy, A., David, E., Moraga, D. and Riquelme, C. (2009). Transcriptomic response of *Argopecten purpuratus* post-larvae to copper exposure under experimental conditions. *Gene, 442,* 37–46

Zhang, X., Yang, F., Zhang, X., Xu, Y., Song, S.L. and Wang, J. (2008). Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (Gobiocypris rarus) exposed to waterborne hexabromocyclododecane (HBCDD). *Aquatic Toxicology, 86,* 4-11

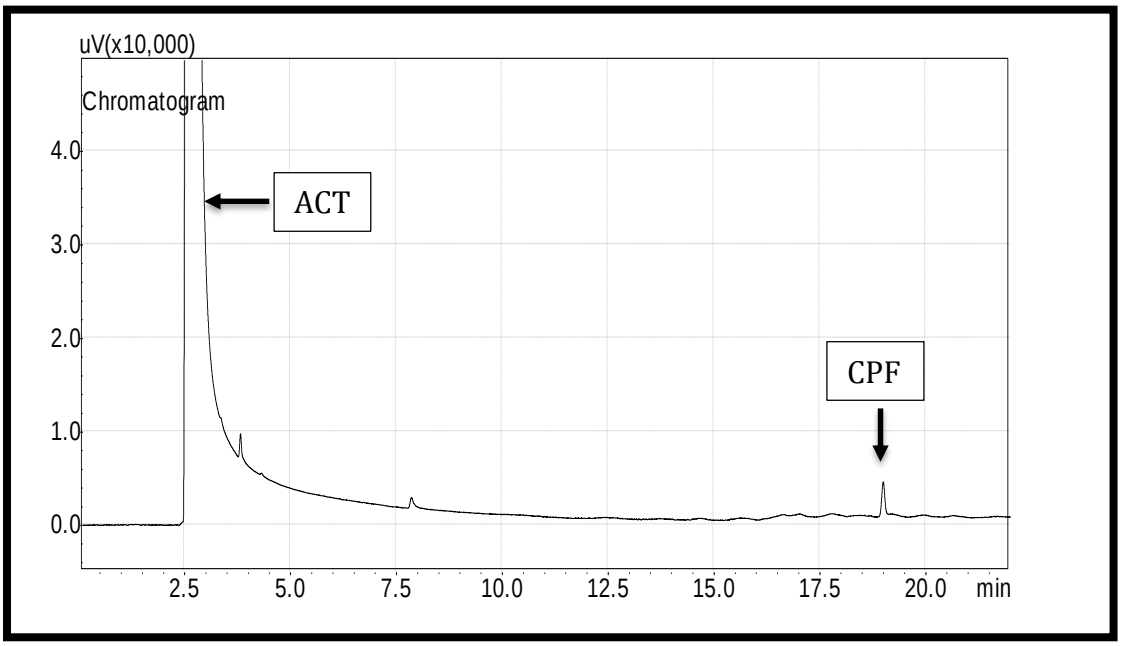
Zia, M, S., Jamil, M., Qasim, M., Rahman, A. and Usman, K. (2008). Natural resources pollution and degradation due to pesticide use in Pakistan. 12th

International conference on integrated diffuse pollution management, Khon Kaen University, Thailand, *226-227*

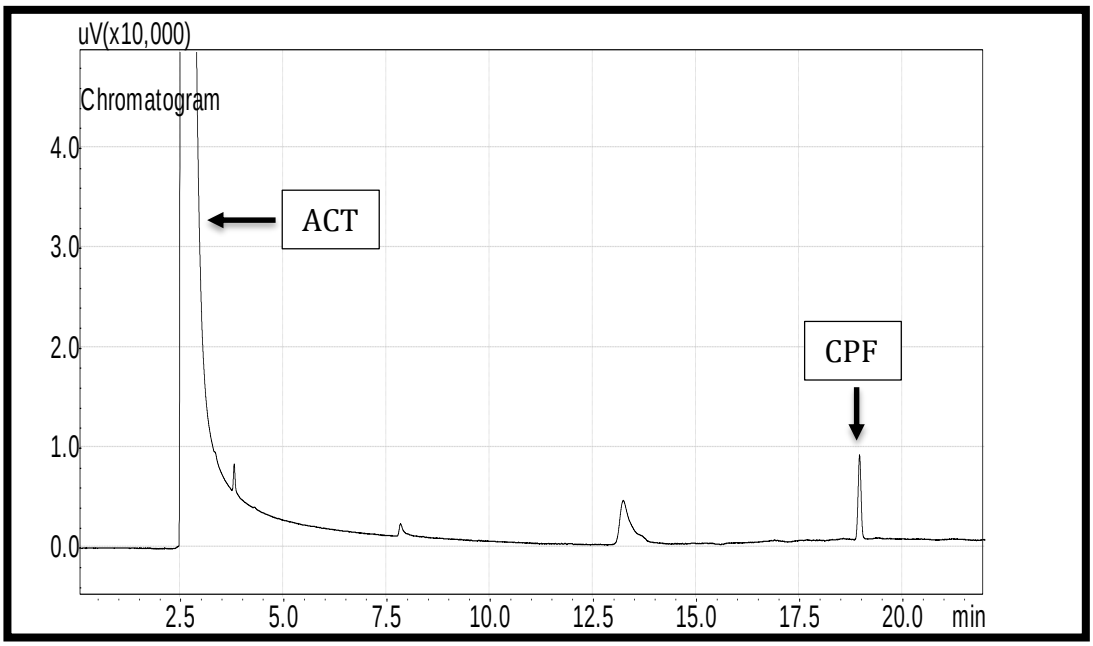
ANNEXURE I



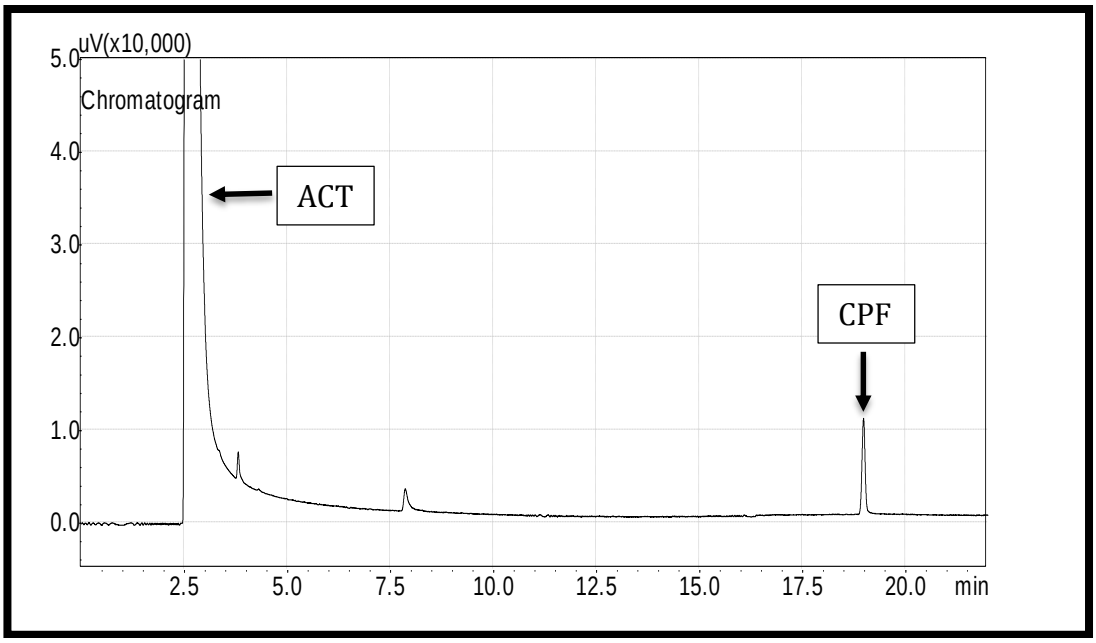
Calibration curve - Chlorpyrifos (5 μg/L)



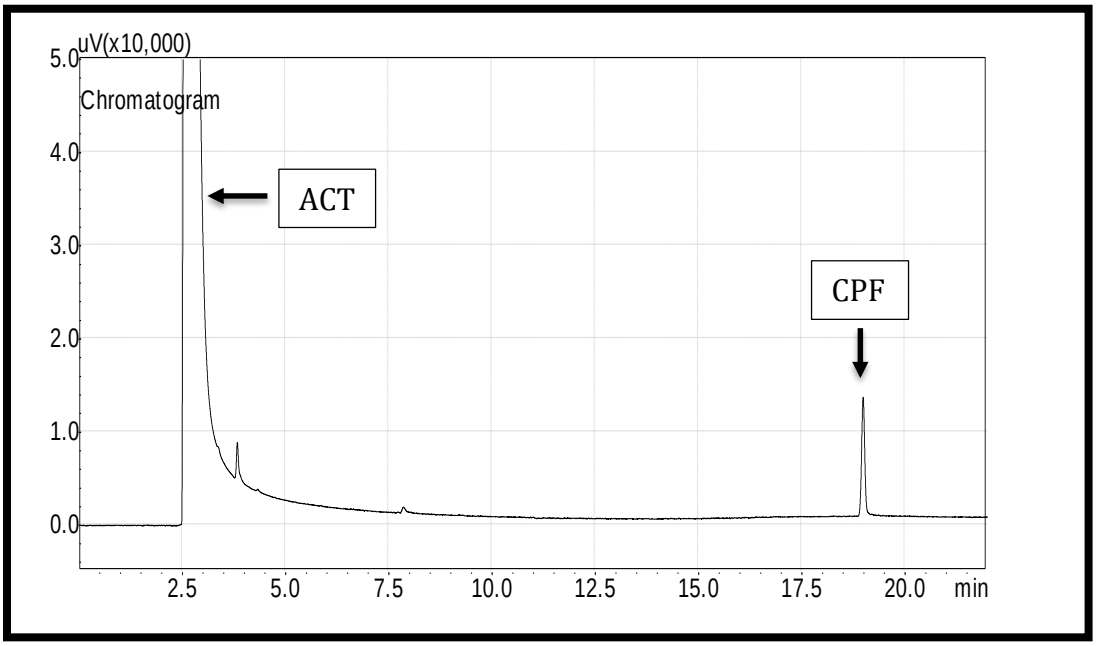
Calibration curve - Chlorpyrifos (10 μg/L)



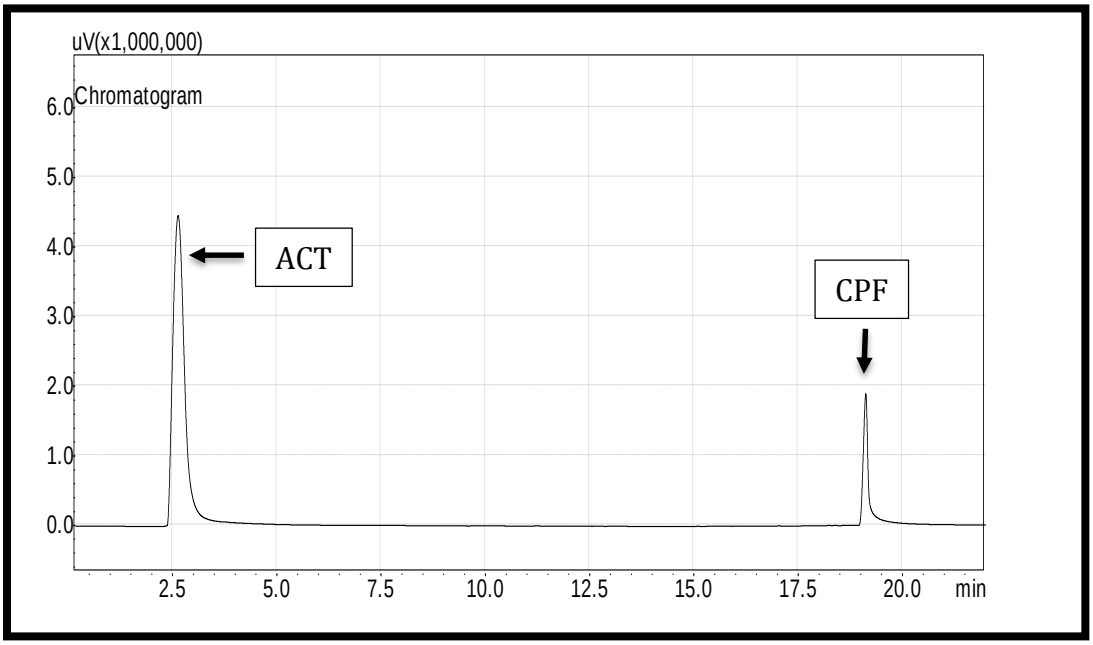
Calibration curve - Chlorpyrifos (25 μg/L)



Calibration curve - Chlorpyrifos (50 μg/L)

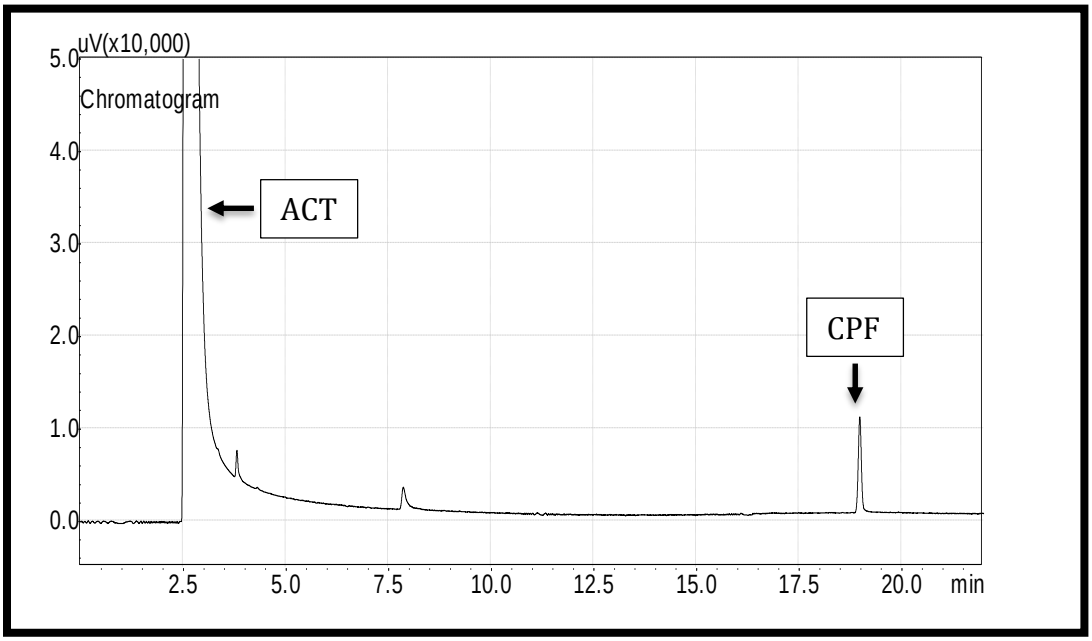


Calibration curve - Chlorpyrifos (100 μg/L)

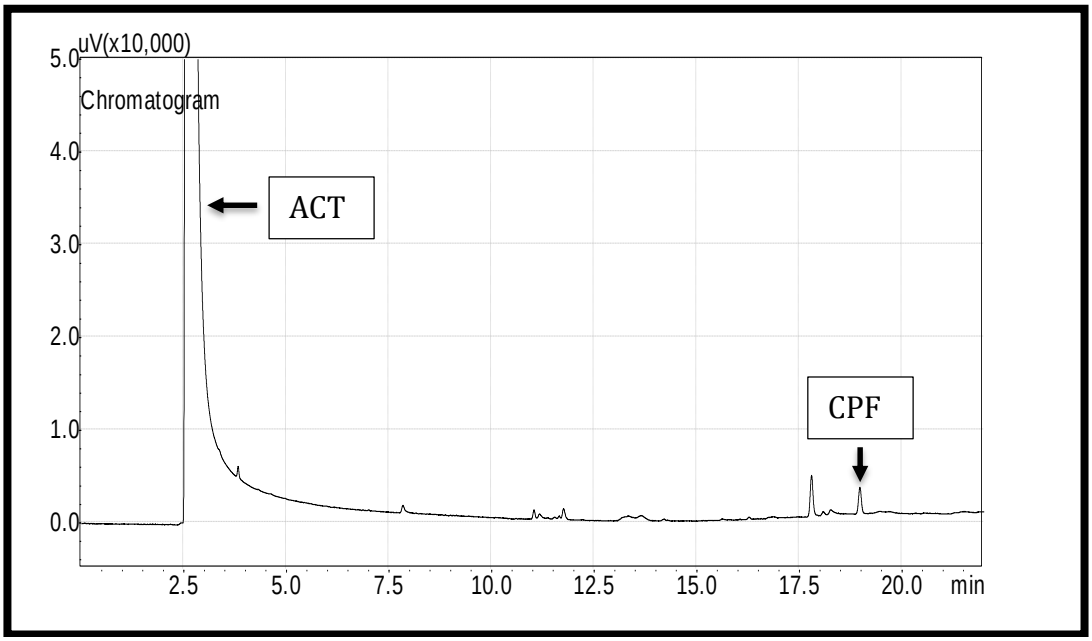


Calibration curve - Chlorpyrifos (150 μg/L)

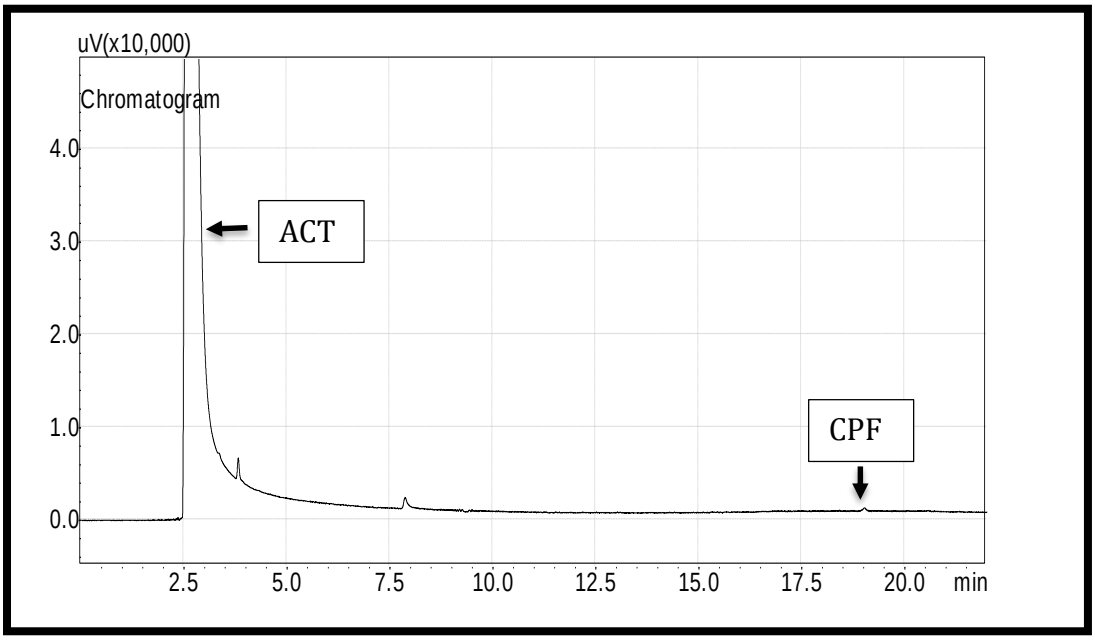
ANNEXURE II



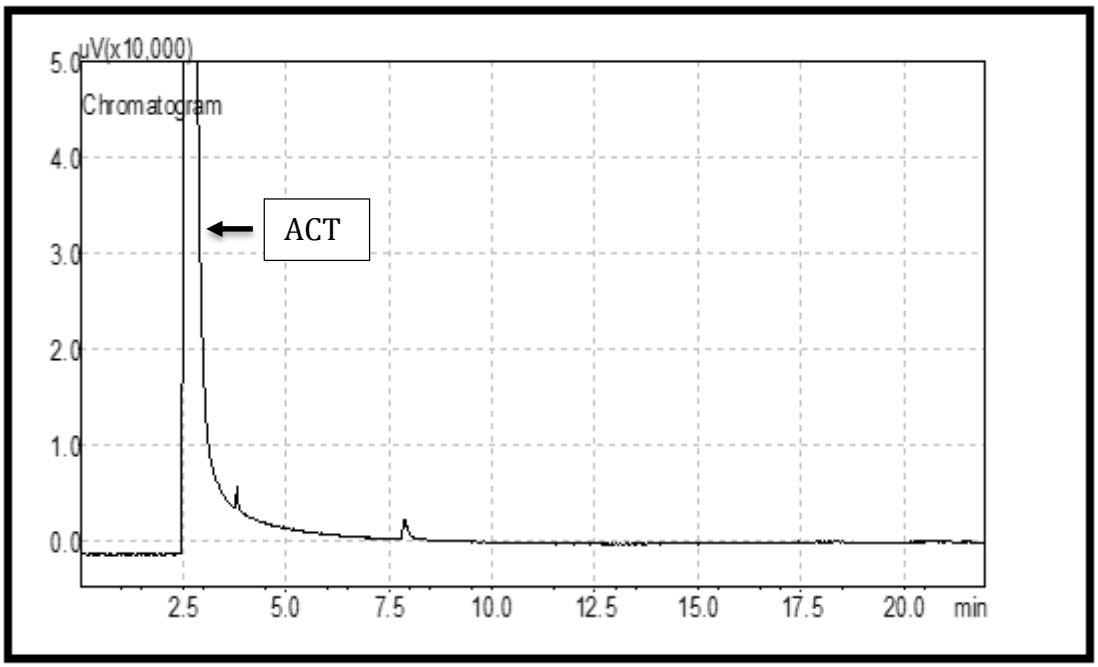
Chlorpyrifos accumulation in muscle tissues (134 μg/L)



Chlorpyrifos accumulation in muscle tissues (13.4 μg/L)



Chlorpyrifos accumulation in muscle tissues (1.34 μg/L)



Chlorpyrifos accumulation in muscle tissues (Control)