BIOTOXICITY POTENTIAL OF ORGANOPHOSPHATE IN COMMON CARP (*CYPRINUS CARPIO)*

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CERTIFICATE

This dissertation submitted by **Ms. Amina Khalid** 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, Pakistan as satisfying the partial requirement for the degree of Master of Environmental Science.

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DEDICATION

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies. Also, this thesis is dedicated to my siblings who have been a great source of motivation and inspiration.

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LIST OF ABBREVIATIONS

μg/ml	Microgram per millilitre
mg/L	Milligram per litre
Vtg	Vitellogenin
HSI	Hepatosomatic index
GSI	Gonadosomatic index
AChE	Acetyl Cholinestrease
OP's	Organophosphates
rpm	Rotation per minute
LD_{50}	Lethal dose 50
GC	Gas chromatography
ECD	Electron capture detector
RBC	Red blood cell
WBC	White blood cell
Hb	Haemoglobin
WHO	World Health Organization
APHA	American Public Health Association
OECD	Organization for Economic Co-operation and Development

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ABSTRACT

Dichlorvos, an organophosphate pesticide, is extensively used for agricultural purposes. Contamination of water by dichlorvos as a consequence of agricultural runoff has been reported in reports. The objectives of the current study were to determine the toxicity and bioaccumulation potential of dichlorvos in Common Carp (*Cyprinus carpio*). The effect was determined on the basis of acute and sub-acute toxicity test results. A semi static toxicity bioassay was performed according to standard method and LD_{50} values determined for 24, 48, 72 and 96 h exposure were recorded to be 34.27, 28.61, 26.12 and 22.51 mg L^{-1}. Sub-acute toxicity test was performed by exposing organism with sub-lethal concentrations (1.3, 1.8 and 2.34 mg L^{-1}) of dichlorvos for 14 days. RBC, WBC, Hb and plasma protein showed significant decrease in levels but plasma glucose increased as the exposure dose was increased. Hepatosomatic index and gonadosomatic index showed decline trend indicating profound effect on liver and gonads of fish. Plasma content of males was analysed to find vitellogenin induction potential of dichlorvos. Male fish were exposed with sub-lethal doses in order to determine vtg concentration. The results showed that dichlorvos has a tendency to feminize the male fish. Bio-accumulation of dichlorvos was observed in muscle tissues through GC-ECD. The results showed 50, 70 and 75 % accumulation of dichlorvos residues when exposed with sub-lethal doses of 1.3, 1.8 and 2.34 mgL^{-1} for a period of 45 days.

Chapter 1

INTRODUCTION

BACKGROUND

Water is an essential element and the basic building block without which survival is impossible. All life forms are dependent on water. Its quality is important for wildlife, human health and environmental stability. As indicated by UNEP (2002), the aggregate sum of water on the Earth is around 1400 million km^3. Around 97.5% of this amount is saltwater and 2.5% is freshwater. The greater portion of freshwater (around 69%) is present in the form of ice and snow cover in the Antarctic, the Arctic and in the sloping districts. Around 30% exists as fresh groundwater and 0.3% of the aggregate sum of fresh water on the earth is in lakes, streams and moderately shallow groundwater basins where it is easily available for commercial needs and for water ecosystems. This small portion is under great stress due to pollution and growing needs. It has now become a global dilemma as all aquatic bodies have been contaminated by pollutants at unprecedented rates (Ghumman, 2011).

Water pollution is defined as deterioration of water quality due to contaminant addition through anthropogenic means up to a level that is not suitable for life. Rivers, oceans, lakes and ground water are affected by water pollution. It is reported as the leading cause of death or disease worldwide. A report on water quality stated that 1.1 billion people lack the access to safe drinking water, 2.5 billion people lack access to safe sanitation and water borne diseases kill more than 5 million people per year that is 10 times the casualties due to war. There is an immense stress on water availability due to rapid growth in population and unsustainable use of water. Industry and agriculture are two main sectors that are over exploiting water resource and deteriorating its quality. This problem is much serious in developing countries than developed. Unavailability of professionals, financial constraints and lack of proper

management are further aggravating the problem. Exponential increase in population puts water availability at stake. Many countries in Africa, South Asia and Middle East will have serious threats of water shortage in the next two decades (Azizullah *et al*., 2011).

The use of pesticide has increased substantially throughout the world during the past four decades. They are chemicals employed to avoid crop damage by insect manifestations in order to improve the yield. Unwanted pests and weeds are killed by these chemicals. It is considered to be the most economical solution. Studies indicate that in year 2000, 5400 million pounds of pesticides were applied worldwide (Zia *et al.*, 2008). Insignificant amount of applied pesticide reaches the targeted pest but a significant amount disperses in the environment resulting in pollution of natural ecosystems that affects the human health and the biota (Pimentel, 1995). Pesticides are also released into environment during manufacturing, handling and transportation. In developing nations like Pakistan this problem is aggravated by using expired pesticides, improper storage and imprudent disposal of pesticide containers. This happens mainly because of lack of awareness and training of farming community as a result of which pesticides enter the ecosystem and contribute to its pollution (Zia *et al*., 2008).

The application methodologies ensure the contact of pesticide with pests avoiding the non-targeted organisms but pests are animal species, sharing many similar characteristics of other animals one of which is sensitivity to toxins. If a chemical is toxic for one, it is toxic for other organisms as well, the only difference is exposure doses (Cocco, 2002). The pesticide applied on pests adversely affect non-targeted organisms by disrupting their functions of hormones and interfering with reproductive system of organism. These also act as xenohormones, mimicking the actions of endogenous hormones or interfering with endocrines processes, hence these have been categorized as endocrine disruptors (Straube *et al*., 2003).

Studies have reported that use of pesticides belonging to organophosphate class has increased tremendously during last decade and entered into the environment. In 1970's and beginning of

1980's, this class completely replaced chlorinated pesticides due to its property of low persistence and less accumulative (Pesando *et al*., 2003).

Dichlorovos (2, 2- dichlorovinyl dimethyl phosphate) was first introduced in 1961. It is extensively used organophosphate in the developing countries. WHO classified dichlorvos as Class IB 'highly hazardous'. It is used to control pests, helminthes and fly larvae (Mennear, 1993). It is released in the environment not only as a parent compound but also in the form of major degradation by-product of other OP insecticides e.g. Trichlorfon, Naled and Metrifinate. Symptoms of intoxication observed are nausea, vomiting, lacrimation, salvation, bradycardia and finally death due to respiratory failure. It is extremely toxic to aquatic organism; it hampers fish health by impairing their metabolic activity which sometimes leads to death (Sovobodova, 1992). Pesticide-induced reproduction failure and dysfunction in Indian fish has been reported in several studies (Singh and Singh, 1982). Acute and chronic toxicity have been observed at only 1 mg L^{-1} concentration of dichlorvos in fish (Roberts and Sheperd, 1986). Dichlorvos also induces behavioral changes in fish. Study reported intense changes in behavior by increasing dichlorvos concentration in guppy (*Poecilia reticulate*) and Common Carp (*Cyprinus carpio*) (Gunde and Yerli, 2012). Similar behavioral changes were reported in Rahu (*Labeo rohita*) (Bhat *et al*., 2012). Histopathological changes are considered to be an important tool in assessing pesticide toxicity, changes have been reported in fish due to dichlorvos exposure (Das and Gupta, 2012). Haematological analysis also showed adverse effects of dichlorvos on fish (Lakshmanan *et al*., 2013).

1.2 OBJECTIVES

Keeping in view the harmful effects of pesticides on fish health, the current study aimed at addressing the eco-toxicological effects of pesticides was conducted for the first time at Institute of Environmental Sciences and Engineering. Dichlorvos, An insecticide, was selected for present research work and its health impacts were observed in Common Carp (*Cyprinus carpio*). Following were the objectives of the research work for the study:

1. Bio-toxicity potential of dichlorvos in Common Carp
    - Acute toxicity
    - Sub-acute toxicity
        - Haematological analysis
        - Gonadsomatic and hepatosomatic index
        - Vitellogenin analysis in males
2. Bio-accumulation potential of dichlorvos in Common Carp
    - Concentration of dichlorvos in muscle tissues using GC-ECD

Chapter 2

LITERATURE REVIEW

Water is an essential component of life. Two third proportion of earth surface is covered by marine water that is unfit for human use. The available form that may be used for drinking purpose is fresh water. It can cater human needs only if it has a high quality as it supports physiological activities of biological cell. Total fresh water present on earth is only 3%, out of which only 0.01% is available for human use (Hinrichsen and Tacio, 2002). This small fraction of fresh water is under great stress due to rapid growth in population, urbanization and unsustainable use in agricultural and industrial sector. In developing countries the condition is aggravated due to improper management, unavailability of professionals, lack of awareness and financial constraints (PCRWR, 2005. Rivers, oceans, lakes and ground water are affected by water pollution. This is not only damaging for an individual specie or population but it also adversely affects biological communities. Adequate treatment of pollutants is necessary in order to remove harmful constituents. Untreated water when discharged into water bodies, causes pollution. It is a matter of concern as it leads to outset of diseases claiming deaths of over 14,000 people every day. In developing countries the condition is worse than developed. Pesticides are one of the reason for water pollution (Agarwal *et al.*, 2010).

2.1. PESTICDE CONTAMINATION OF WATER BODIES

Pesticides are chemicals that are designed to kill pests or insects. The purpose of pesticide application is to achieve better quality by avoiding pest attack (Zia *et al*., 2008). Substantial increase in use of pesticide has been reported in the last four decades. 2.5 million tons is the estimated quantity used in the world annually. These were introduced in 1954 in Pakistan with 254 metric tons of formulations (Tariq *et al*., 2007). 70 thousand tonsof pesticide application is reported with an increasing annual rate of about 6% (WWF, 2007). Increase in population

has changed the dietary patterns. In order to fulfil energy demands more forest covers are converted to agricultural lands. Pesticides are utilized to avoid crop loss due to pests and weeds thus disturbing the natural capacity of environment to act as a buffer. Unplanned urbanization has increased resulting in aggravating the problem of water contamination. The concept of sustainable agriculture is fading away as the population is increasing, the definition of mentioned term is to preserve and maintain the agricultural soil, water bodies and environment ensuring that the future generation can get adequate supply of wholesome and safe food. Pesticides are routine component of agricultural practices, their liberal use at different stages poses a great threat to environmental stability. A little portion of applied pesticide adheres to the crop but unfortunately bulk amount is carried by surface run off from application sites and ends into aquatic ecosystem. A small fraction also gets volatilized, contaminates atmosphere but destined to enter aquatic bodies through precipitation (Ragnarsdottir, 2000). A study stated that only 0.1% of applied pesticide reaches targeted organism and the remaining 99.9% gets dispersed through air, water and soil reaching different ecosystems thus resulting in pollution (Pimentel, 1995). The other factors promoting pesticide contamination of water bodies are improper transportation, handling and careless disposal of pesticide containers resulting in water contamination and effecting biota at different levels.

2.2. SITUATION OF PAKISTAN

Pesticides in Pakistan were first introduced in 1954 with 254 metric tons of formulations (Tariq *et al.,* 2007). Thousand tons of pesticides were imported from Europe and USA in the late 1960's and early 1970's. Seventy thousand pounds of pesticide is applied to cotton crops per year with an increasing rate of about 6% per year. Out of all 70% applied pesticide, 75% is used to protect cotton crops from disease or pest attacks and remaining is used for other crops such as sugarcane, tobacco, maize, paddy rice, fruits and vegetables. Water bodies of Pakistan are contaminated by pesticides as reported by many researchers. The problem has increased

and become more serious as farmers are not properly trained for pesticide handling, there is no regular check and balance in the system. The water bodies of Samundari Faisalabad and Kala Shah Kaku Lahore are contaminated with λ-Cyhalothrin, Endrin and Monocrotophs. Their concentrations range from 0.1 to 60.0 µg L^{-1} (Jabbar *et al*., 1993). The areas of Mardan, chota Lahor, Takhbai and Swabi showed contamination of ground water (at depth of 3.6-5.1 m) with Chlorpyrifos, Dichlorvos, Dimethoate, Endosulfan, Fenitrothion, Lindane, Malathion, Methyl parathion and Phosphamidon. The concentrations ranged from 0.03 to 0.23 µg L^{-1} (Ahad *et al*., 2005). Well water samples collected from Bahawalnagar, Muzafargarh, Dera Ghazi Khan and Rajan Pur districts showed contamination of Bifenthrin, Carbofuran, λ-Cyhalothrin, Endosulfan, Methyl parathion and Monocrotophs. The ranges of pesticide detected in samples were from 2.1 to 7.7 µg L^{-1} (Tariq *et al*., 2004). Samples collected from different areas of Punjab also showed pesticide contamination. The detected pesticide were o,p′-DDD, o,p′-DDE, o,p′-DDT, p,p′-DDD, p,p′-DDE and p,p′-DDT with concentration ranging between 0.181- 1.06 µg L^{-1} (Asi *et al*., 2008). Nowshera showed high concentrations of p-p′ DDT ranging from 70 to 400 µg L^{-1} (Jan *et al*., 2009). These concentrations in water bodies are an evidence of improper agricultural activities in nearby areas which may have detrimental effects in other life forms.

2.3. PESTICIDE CONTAMINATION OF RAWAL LAKE

Rawal Lake Reservoir is situated in the Margalla Hills National Park and is known for its ecological significance. Four major streams and 43 small streams are contributing to its storage and total catchment area of the lake is 268 km^2. Rawalpindi and Islamabad get drinking water from this lake at a rate of about 22 million gallons per day. Proper management of this resource is important if full benefits are to be gained and maintained for future. The lake reservoir is facing deterioration of its water quality. Iram *et al*. (2009) have investigated that there are some agricultural activities in the catchment area of Rawal Lake. The farmers use pesticides and

herbicides in agriculture. The toxic chemicals are washed away by excess irrigation water which enters the Rawal Lake through streams. Ahad *et al*. (2005) have also reported the presence of pesticides residues and pharmaceuticals in Rawal Lake. Possible sources of contamination of reservoir water are human settlements in the catchment area, poultry wastes, recreational activities, agricultural activities, deforestation, pesticides, and erosion in the catchment area (Ghumman, 2011). Iram *et al*. (2009) reported the presence of 21 pesticides in water samples with higher concentrations of 2, 4- DDT, Fenitrothion and Diazinon. Ahad *et al.* (2005) reported presence of 12 pesticides including Dichlorvos, Lindane, Parathion-methyl, Fenitrothion, Malathion, Endosulfan, Azinphos-methyl, Fenpropathrin, Cyhalothrin, α-Cypermethrin, Esfenvalerate and Deltamethrin. Out of them four pesticides were found to be higher in concentrations namely Parathion-methyl, Fenitrothion, Aziophos-methyl and α-Cypermethrin. More concentration of pesticides shows that organisms are at greater risk. Climate change resulting from increased human activities has the potential to harm societies and ecosystems. In particular, agriculture, forestry, water resources, human health, coastal settlements, and natural ecosystems will need to adapt to a changing climate or face diminished functions. So human related activities have strong influences on the quality of freshwater and the ecological integrity of aquatic ecosystems.

2.4. ORGANOPHOSPHATES

Organophosphorus or organophosphates (OPs) constitute a large group of chemicals used over the past 60 years to protect crops, human health, livestock and as warfare agent. Structural modifications divide the class into 13 types including phosphonates, phosphates, phosphinates, phosphonothioates (S=), phosphorothioates (S=), phosphorothioates (S substituted), phosphorodithioates, phosphonothioates (S substituted), phosphorotrithioates, phosphoramidothioates (Gupta, 2006). OPs and their metabolites are most widely used

pesticides worldwide because of their characteristic properties of rapid degradation (relative to organochlorines), low bioaccumulation capability and less persistent (Barr, 2004).

2.4.1. Entry routes of organophosphates

The people dealing with pesticides are vulnerable to get exposed with them. Exposure routes of organophosphates vary depending on the type of work they are involved. In workplaces like industries or manufacturing units the most important route of entry is skin, absorption through skin and thus entering the blood stream. Many OP pesticides get oxidized to a more active compound so impose great threat to health of workers. In case of pesticide application in fields, oral ingestion is observed. The fumes in air are inhaled and enter blood stream via respiratory track. Accidental ingestion may also occur as a result of poor practices and lack of personal hygiene. Absorption through mucous layer and eyes can also occur. The absorption of comparatively toxic OP is very rapid (Dementi, 1994).

2.4.2. Mode of action

Organophosphates act by inhibiting Acetylcholinesterase enzyme in the nervous system. The location and type of cholinesterase vary in the body, but the principle enzymes are acetylcholinesterase (AChE). These are not only present in nervous tissue but also in red blood cells, plasma, liver and serum. AChE under normal condition helps in breakdown of acetylcholine. Acetylcholine is a chemical mediator which acts as a neurotransmitter for transmission of nerve impulse at different sites. In presence of OPs, acetylcholinesterase is phosphorylated and becomes unavailable for breaking of acetylcholine into choline and acetic acid. This causes accumulation of acetylcholine in parasympathetic nerve synapses, the motor end plate and in central nervous system causing toxicity in the target organism after acute poisoning with Ops (Aldridge *et al*., 1952).

2.5. DICHLORVOS

It is an insecticide that was first introduced in 1961. It is manufactured chemically by a reaction between chloral and trimethyl phosphite. It is commonly sold under brand names of Vapona, Nuvan, Atgard, Decovas and Task. DDVP is also used which is an abbreviation of its full chemical name 2,2-dichlorovinyl dimethyl phosphate.

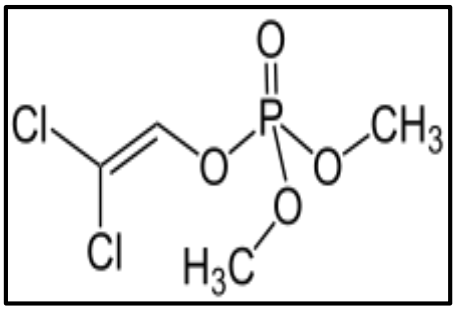

Figure 2.1. Structural formula of dichlorvos

In pure form it is dense liquid that evaporates easily in air, it also readily reacts with water. Dichlorvos is mainly used to control pests in food storage areas, barns, greenhouses and to control parasites in livestock. It is also used by veterinarians to control pest diseases in pets and by salmon farming units to avoid diseases in salmon and to kill salmon lice. It is one of the most commonly used organophosphate in developing countries.

Dichlorvos persists for a longer time on hard dry surfaces such as wood but it breaks rapidly in humid air, soil and water, by both abiotic and biotic processes. The degradation products it converts into are dichloroethanol, dichloroacetaldehyde, dichloroacetic acid, dimethylphosphate, dimethylphosphoric acid and some other compounds. All are water-soluble and eventually get mineralized (USEPA, 1994). The physcio-chemical properties are enlisted in Table 2.1 (Verschueren, 2001).

Characteristics	Information
Color	White to amber
State	Liquid
Odor	Mild aromatic, chemical
Solubility in water at 22 ˚C	10 mg L^{-1}
Molecular Formula	$C_4H_7Cl_2O_4P$
Boiling Point	140ºC/20 mmHg
Molecular Weight	220.97 g/mol
$logK_{ow}$	1.43

Table 2.1: Physico-chemical properties of selected pesticide, dichlorvos

2.5.1. Potential for human exposure

World health organization (WHO, 1992) classified dichlorvos as Class IB, 'highly hazardous chemical'. Self-poisoning of dichlorvos kills approximately 200,000 people per year. Mild irritation to skin, localized swelling, burning sensations or actual burning and muscle contractions are some symptoms of skin exposure to dichlorvos. Respiratory track is effected if inhaled. The symptoms include bloody or runny nose, chest congestion, coughing, short breath or difficulty in breathing along with excess fluid production in bronchial tubes. If dichlorvos comes in contact with eye it causes bleeding, pain, tears, constriction of pupil and blurred vision. After exposure other effects that may begin within few minutes or delayed up to 12 hours include pallor, vomiting, nausea, abdominal cramps, dizziness, headache, pain in eye, salvation, confusion and sweating. On the other hand. Severe poisoning effects central nervous system. It produces incoordination, difficulty in speech, loss of reflexes fatigue, weakness, involuntary muscle contractions, twitching, tremors of eyelids or tongue eventually leads to paralysis of body (Michael *et al*., 2008).

2.6. TOXICITY OF DICHLORVOS IN AQUATIC ORGANISMS

Principal entry routes of dichlorvos include industrial effluents and accidental discharge in water bodies. In addition to that it is also used in Salmon farming as delousing agent thereby effecting non-targeted organisms. It may also enter the aquatic environment through indirect means. Spray drift during its application in agricultural fields and land runoff may cause contamination of nearby water bodies. It is reported to be an extremely toxic pesticide to aquatic organisms as it causes impairment of metabolism and hampers health leading to death of organism.

2.6.1 Acute toxicity of dichlorvos

Acute toxicity is defined as the adverse effect occurring within few hours of exposure (1, 12, 24, 36, 48, 72, 96 hours) following oral or dermal administration of single or multiple doses. It is generally determined as Lethal Dose 50 (LD_{50}) which is the concentration at which half of the population of model organism die.

Acute toxicity of dichlorvos has been determined by many researchers. Its toxicity varies from moderate to high in freshwater and estuarine fish species. The 96h – LD_{50} values ranged from 0.2 to 12 mg L^{-1} for freshwater and estuarine fish (Gupta *et al*., 2008). Studies indicated that fingerlings of European sea bass are resistant to dichlorvos than other species of estuarine and freshwater. However comparison with fathead minnow (*Pimephales promelas*) or mosquito fish (*Gambusia affinis)* indicated that sea bass fingerlings are more sensitive to dichlorvos (WHO, 1989). In another study it was found that 100% of salmon *(Salmon salar)* weighing 100g, survived after 24 hour exposure to 1, 3 and 5 mg L^{-1} of dichlorvos *(*Sievers, 1995*).* Rohu (*Labeo rohita*) was exposed and LD_{50} value was determined to be 16.71 mg L^{-1} *(*Bhat *et al*., 2012*).* Post fertilization LD_{50} values in Common Carp (*Cyprinus carpio*) in semi-static test were 39.75 mg L^{-1} and 0.5- 10 mg L^{-1} after 24 and 48 hours of exposure. The abnormality in behavior is also stated by many researchers. Some of the commonly observed behavioral

impairments after acute exposure are erratic swimming, loss of equilibrium, copious mucus secretion and hitting the walls of the water tank prior to mortality (Bhat *et al*., 2012).

2.6.2. Chronic toxicity of dichlorvos

Chronic toxicity refers to long term exposure with low doses of the chemical. Dichlorvos is reported to be a neurotoxic compound due to its inhibitory effects on acetylcholinesterase. Accumulation of acetylcholine in synapses disrupts the nerve functions and causes death of the organism (Wang *et al*., 2004). Many studies reported inhibition of AChE as a result of sub-lethal dichlorvos exposure. Tissues of European sea bass were examined, significant inhibition of ChE was observed in vitro and in vivo conditions (Galgani and Bocquene, 1990). ChE sensitivity differs with age of fish and the tissue analyzed. Studies reported that European sea bass fingerlings are resistant and can tolerate high levels of muscle and head ChE inhibition before death (Varo *et al*., 2003). Similar results were obtained for pinfish (*Lagodon rhomboides*) and European eel (*Anguilla anguilla*) (Coppage and Mathews, 1975; Sancho *et al*., 1997). Researchers stated that chronic exposure of dichlorvos results in impairment of mitochondrial energy metabolism and neuronal apoptotic cell death in brain (Kaur *et al*., 2007). Pesticide toxicity can be assessed by examining the impacts on histopathology. Researchers have observed effects of dichlorvos on liver health of fish. Hepatic lesions were reported characterized by cloudy swelling of liver cells (hepatocytes), vacuolar degeneration, karyolysis, nuclear hypertrophy and dilation of sinusoids (Velmurugan *et al*., 2009).

Studies were conducted to observe the effects of dichlorvos on reproductive system of fish. Air breathing catfish (*Clarias batrachus*) was when exposed with lethal and sub lethal doses modification in oocyte structure in form of pronounced vacuolation, degeneration and deformation were observed (Benarji and Rajendranath, 1992). Changes in haematological parameters were also observed. Red blood cell, white blood cell and haemoglobin count decreased in Peters fish (*Orechromis mossambicus*) as a result of increase in exposure

concentration (Lakshman *et al*., 2013). Studies also the validated that dichlorvos has potential to induce alterations in immune responses of Common Carp (*Cyprinus carpio*) (Dunier *et al*., 1991).

Studies reported changes in structure and function of fish gonads, although there is not enough data available to validate this. Mir and his colleagues (2012) exposed Common Carp (*Cyprinus carpio*) with sub-lethal doses of dichlorvos. Gonadosomatic index showed decreased in both genders by increasing concentration but increased when exposure time was increased.

Vitellogenin is a predictive biomarker to evaluate reproductive disruption in males. It is a precursor protein that is produced in female fish or egg yolk, it is either absent in males or concentration is insignificant. Environmental pollutants or endocrine disruptors induce vitellogenin production of males which causes organizational and activational effects in fish. Activational effects are usually transient changes in the function, morphology and behavior. These vanishes when the external stimulus is removed. On the other hand organizational effects are permanent changes in morphology and that persist even after external stimulus is removed, affecting subsequent functioning and behavior of all organs (Cheek *et al*., 2001). There is no data available on endocrine disruption of vitellogenin in Common Carp (*Cyprinus carpio*) by dichlorvos so in the current study vtg induction in blood plasma of males was observed as a result of sub-lethal dose exposure.

2.6.3. Bioaccumulation of dichlorvos in fish

Bioaccumulation is biological sequestration of pollutant in an organism. Moderate to high toxicity of dichlorvos has been reported by many researchers but it does not significantly bio accumulate in the body unlike many other organophosphates. Horsberg and Hoy (1990) observed bioaccumulation in salmon (*Salmo salar*) muscle tissues and liver at 4 °C and 12 °C when exposed with 2 mg L^{-1} for several days. It was detected in both samples, muscles and

tissues. The concentration kept decreasing with increasing days of treatment unlike other organophosphates.

Protection of aquatic ecosystem against adverse effects of contaminants as a consequence of anthropogenic activity has been a growing concerns in recent decades. Pesticides are easily washed into water bodies which may affect wide range of non-targeted organisms. Studies have reported massive fish killing due to pesticide poisoning (Oropesa *et al*., 2009). Toxicity potential of dichlorvos cannot be ignored. Recent study was conducted keeping in view the health impacts of dichlorvos on Common Carp (*Cyprinus carpio*).

Chapter 3

MATERIALS AND METHODS

Toxicological study was carried out in Environmental Toxicology Laboratory. Common Carp (*Cyprinus carpio*) was selected as bio-indicator for current work because it has an ability to bear stress and extreme conditions. Fish were exposed to different concentrations of dichlorvos at variable durations to evaluate the health status.

Semi static tanks were used and OECD guidelines 203 and 204 were followed for acute and sub-acute toxicity tests. Experiments were carried out in batches depending on the type of test conducted (acute or sub-acute) and the duration of exposure per test.

3.1. PESTICIDE AND CHEMICALS

Commercial grade dichlorvos (Decovas 50 EC STEDEC) was purchased from local market in order to represent the actual situation. Gas chromatographic analysis was done by using analytical grade standard FLUKA dichlorvos PESTANAL® (45441). The standard was stored at -20 °C. GC grade Acetone, *n*-hexane, diethyl ether and acetic acid were used for extraction of dichlorvos from muscle tissues of fish and for preparation of stock and working solution. Analytical grade anhydrous sodium sulphate was used as a drying agent in muscle tissue extracts. Purification of GC extracts was done by using high purity grade silica gel (60-100 mesh) Davisil®. Analytical grade chloroform was used for tissue extract as well as anesthetic agent to induce unconsciousness in fish.

3.2. PURCHASE AND MAINTENANCE OF EXPERIMENTAL FISH

Healthy specimen of Common Carp (*Cyprinus carpio*) were purchased from Punjab Hatchery Rawal Town, Aquaculture and Fisheries program - National Agriculture Research Centre, Rawal Lake and Peshawar hatchery. Number of batches were dependent on the nature of test

to be conducted. They were brought to laboratory avoiding mechanical injuries and kept in glass aquarium (dimension 3 X 1.5 X 1.5 ft).

3.2.1. Acclimatization of fish

Before commencement of experiment fish were acclimatized to laboratory conditions for a period of one week. They were fed commercial dry food pellets, the leftover food in tanks being removed daily when the water in the tank was changed. To avoid fouling of tanks, dead fish was removed immediately. Experimental design of research work is shown in Figure 3.1

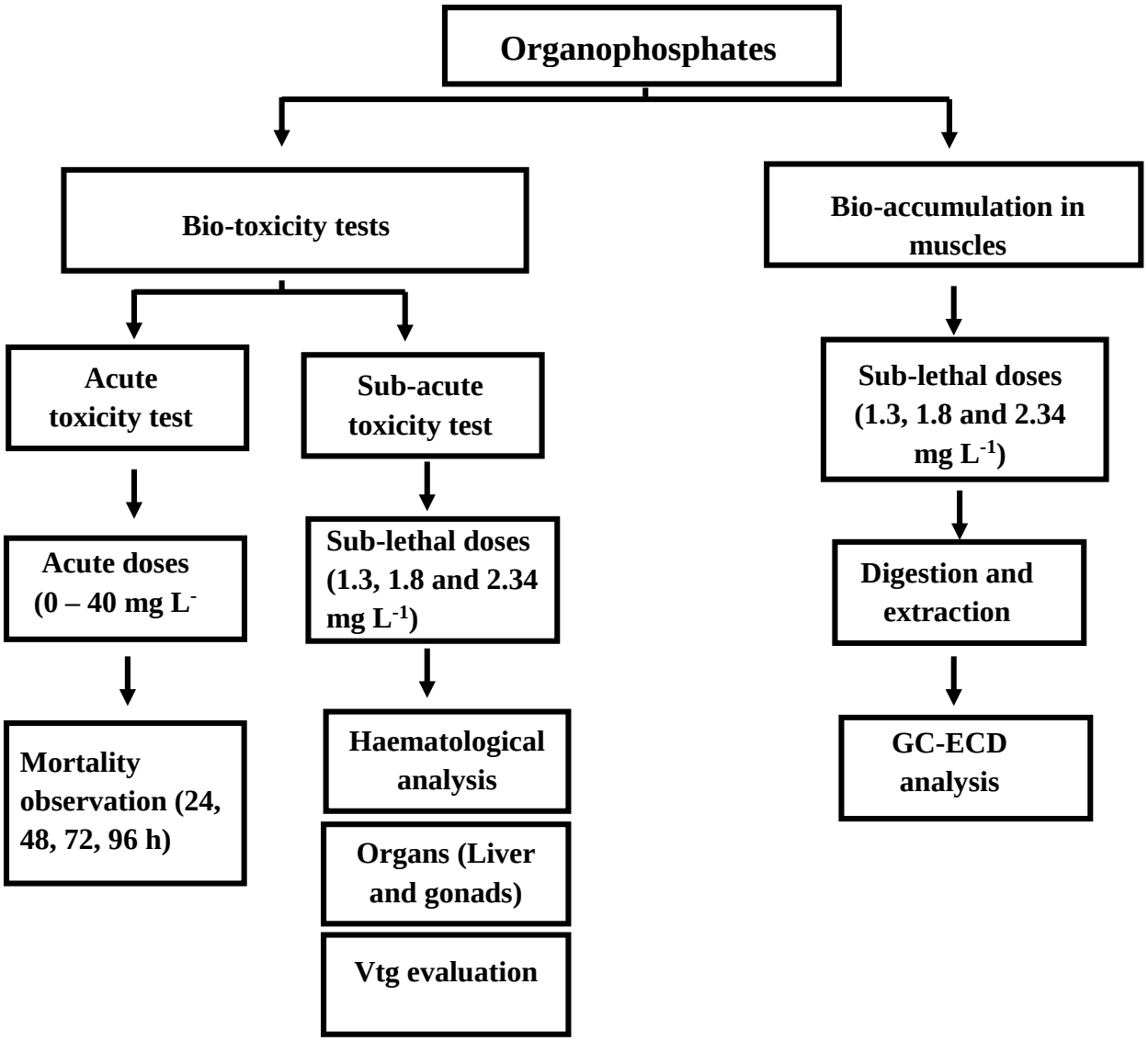

Figure 3.1: Experimental design of research work

3.3. WATER PARAMETERS

Water was changed every alternate day followed by addition of fresh dichlorvos. Temperature, pH, dissolved oxygen, total chlorine and total hardness as $CaCO_3$ were determined according to standard methods regularly (APHA, 2012). Healthy water was provided to fish to avoid any damage to tissues or organs. Table 3.1 shows all values of water parameters were within permissible ranges.

Variables	Mean ± S. D.
pH (units)	7.8 $\pm$ 0.32
Dissolved Oxygen (mg/L)	8 $\pm$ 0.51
Temperature (^oC)	25.15 $\pm$ 2.43
Hardness (mg/L)	139 $\pm$ 6.04
Chlorine (mg/L)	BDL

Table 3.1: Average values of water parameters of dosed tanks

3.3. EVALUATION OF BIO-TOXICITY POTENTIAL OF DICHLORVOS

During experiments, fish were kept in glass aquaria (1.5 X 1.5 X 3 feet) and were divided into experimental and control groups using random selection method. Fish of average weight 20 $\pm$ 2.5 gm and measuring 15 $\pm$ 1.8 cm in length were selected for toxicity tests.

3.3.1. Acute toxicity test

Acute toxicity was determined according to OECD guidelines 203 (1992) for semi-static test. Fish were divided into experimental and control groups using random selection method containing ten fish each. Water was changed every alternate day followed by addition of fresh dichlorvos. Exposure concentrations of dichlorvos introduced initially were 10, 20, 30, 40 and 50 mg L^{-1}. No mortality was observed below 20 mg L^{-1} while not more than 10% fish survived above 40 mg L^{-1}. After finding the range, fish were exposed with final doses of 20, 22.5 25, 27.5, 30, 32.5 and 35 mg L^{-1} respectively for 24, 48, 72 and 96 h. No food was administered 24 h before and during assay. Mortality was recorded in each group shown in Figure 3.2. Dead fish were removed immediately.

Figure 3.2: Mortality of fish after dose exposure

3.3.2. Haematological analysis

Sub-acute toxicity was determined according to OECD guidelines 204 (1984). After finding LD_{50}, the doses causing mortality were found. Low doses were given to find sub-acute/lethal doses causing significant effect on fish health. Three concentrations of 5, 10 and 15 mg L^{-1} were introduced but no survival was observed after 14 days of exposure. The concentrations were reduced to 0.25, 0.5 and 0.75 mg L^{-1} but no significant changes were observed. Literature was consulted and with certain modifications in Mir *et al*. (2012) final doses were selected to be 1.3, 1.8 and 2.34 mg L^{-1}. 14 day of exposure duration was given. Fish were divided into four groups containing 6 fish each. One group served as control. Two replicate tests were conducted containing four fish per tank. At the end of stipulated time, blood samples were taken for haematological analysis by cardiac puncture. It was collected in clean vials and anti-coagulant was added. RBC, WBC and Hb count was measured using Complete Blood Count (Medonic, M-Series Open Vial Analyzer CDS-1400073). Whole blood was centrifuged (Hettich Universal 320) at 12,000 rpm for 2-3 mins in yellow caped vials containing gel and anticoagulant. Protein content was determined using plasma protein analyser (SPAplus® BN II) while glucose was determined using plasma glucose analyser (YSI 2300 STAT Plus).

3.3.3. Gonadosomatic and hepatosomatic index

In order to evaluate the effects of dichlorvos on metabolic and reproductive system, liver and gonads were analysed. Fish were decapitated after collection of blood specimen, dissection was carried out as shown in figure 3.3 followed by collection of required organs (liver and gonad). They were processed for further investigations.

Indices were calculated by using the following formula

$$Gonadosomatic\ Index\ GSI = (weight\ of\ gonads\ /\ weight\ of\ fish) X\ 100$$

$$Hepatosomatic\ index\ HSI = (weight\ of\ liver\ /\ weight\ of\ fish)\ X\ 100$$

Figure 3.3: Dissection of fish for organ collection

3.4. CARP VITELLOGENIN

OECD guidelines 204 (1984) were followed for sub-acute test assay. Three fish per aquarium were distributed by random selection method. A replicate test was also conducted. They were exposed to doses of 1.3, 1.8 and 2.34 mg L^{-1} for a period of 14 days. After stipulated time fish were anaesthetized and blood was withdrawn in yellow capped gel vial for serum separation.

3.4.1. Detection of VTG

CUSABIO Carp vitellogenin ELISA kit was used for vitellogenin detection in blood serum of male fish. It was stored at 2-8 °C. Reagents utilized were provided in the kit. The instruction manual of kit was followed for detection. Fish were anesthetized using chloroform prior to blood withdrawal. Blood was taken in yellow caped gel vials. Plasma was separated immediately to avoid protein denaturation by using centrifuge (Hettich Universal 320) for 3-5 mint at 12,000 rpm and stored at -20 °C to avoid loss of bioactivity and contamination. Reagent preparation and procedure is given in Annexure III.

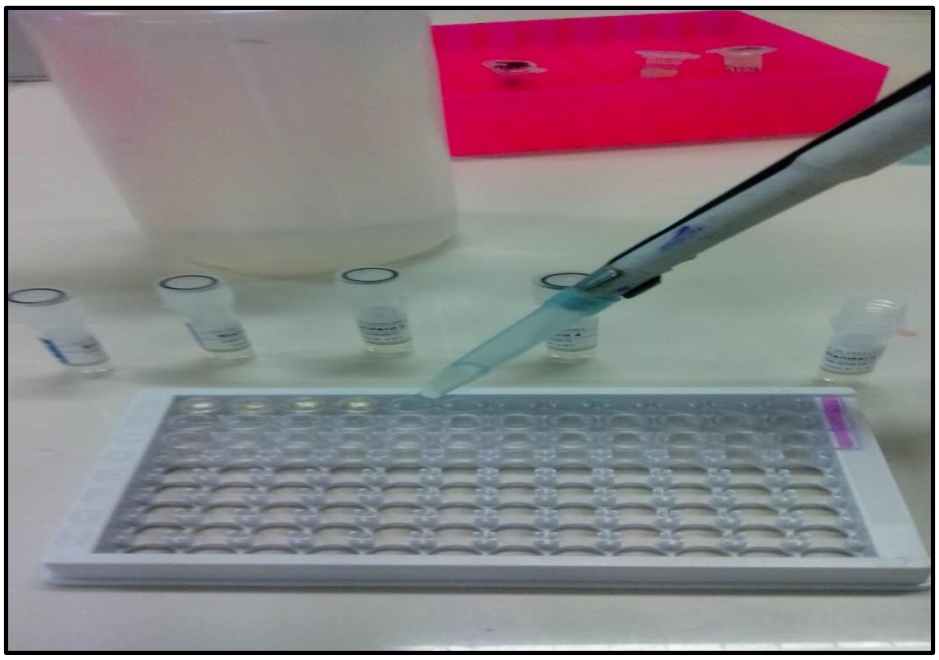

Figure 3.4: Reagents addition for vitellogenin analysis

3.5. BIOACCUMULATION OF DICHLORVOS IN MUSCLE

In order to determine the bioaccumulation potential of dichlorvos, fish of average size 22.146 ± 1.71 cm and average weight of 73.37 ± 4.46 g were taken.

Three fish were taken from Rawal Lake to find dichlorvos residues in their muscle tissues while three fish per aquarium were distributed by random selection method in laboratory and sub-lethal doses of 1.3, 1.8 and 2.34 mg L^{-1} were introduced for a period of 45 days. Replicate test was also done for confirmation. After the completion of exposure duration, fish were decapitated and muscle tissues were extracted.

3.5.1. Digestion and extraction of dichlorvos

A series of steps were followed with some modifications in Abdel-Halim *et al*. (2006). Soft parts were removed and 20 g of tissue samples were taken from dorsal muscle. 10 g of anhydrous sodium sulphate was homogenized in 100 ml of chloroform and acetone (1:1 v/v) using pestle and mortar. The homogenate was acidified by adding 10 drops of acetic acid. It was left for 3 h on shaker (Labcon SPO- MP8) and then filtered. The filtrated was dried using

rotary evaporator at 35 °C for 20-25 mins (Heidolph HB digital, Laborta 4000). The residue obtained after evaporation was re-dissolved in 1 ml of *n*-hexane and acetone (1:1 v/v).

3.5.2. Column chromatography

A column was made for cleaning up of samples it is shown in Figure 3.5. 10g of 1% deactivated silica gel (60-100 mesh) was used as packing material. It was rinsed with 50 ml of n-hexane prior to sample loading. Samples were eluted using 200 ml of 15 % and 50 % of diethyl ether and *n*-hexane. The two fractions obtained after elution were combined, concentrated and completely dried using rotary evaporator. The residues were re dissolved in 0.5 ml of *n*-hexane. (Diaz *et al.*, 1997).

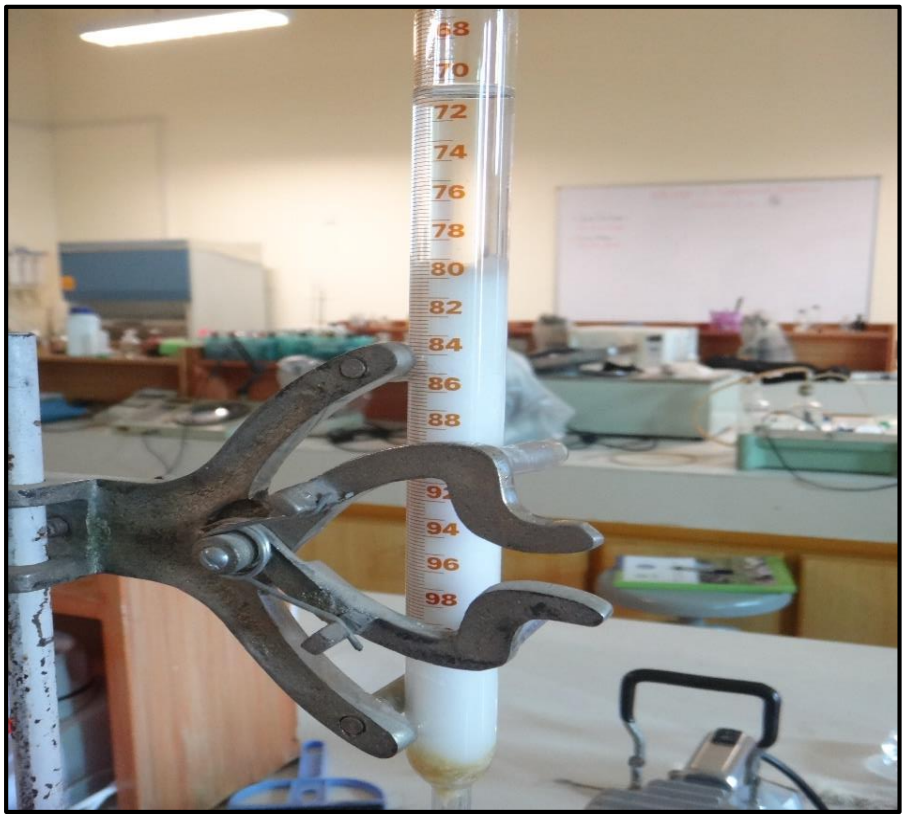

Figure 3.5: Column chromatography for sample cleaning

3.5.3. Standard solutions

500 mg L^{-1} of stock solution was prepared by dissolving standard FLUKA dichlorvos PESTANAL® (45441) in GC grade Acetone. Five dilutions (10, 20, 30, 40 and 50 mg L^{-1}) were prepared to formulate calibration curve and line equation to determine unknown concentration in the sample.

3.5.4. GC-ECD analysis

Shimadzu GC 2010 with Electron Capture Detector (ECD) was used for analysis. 30 m length of TRB-1 column was used as separation column (Figure 3.6). Liquid samples are rapidly vaporized then transported through column by mobile phase.

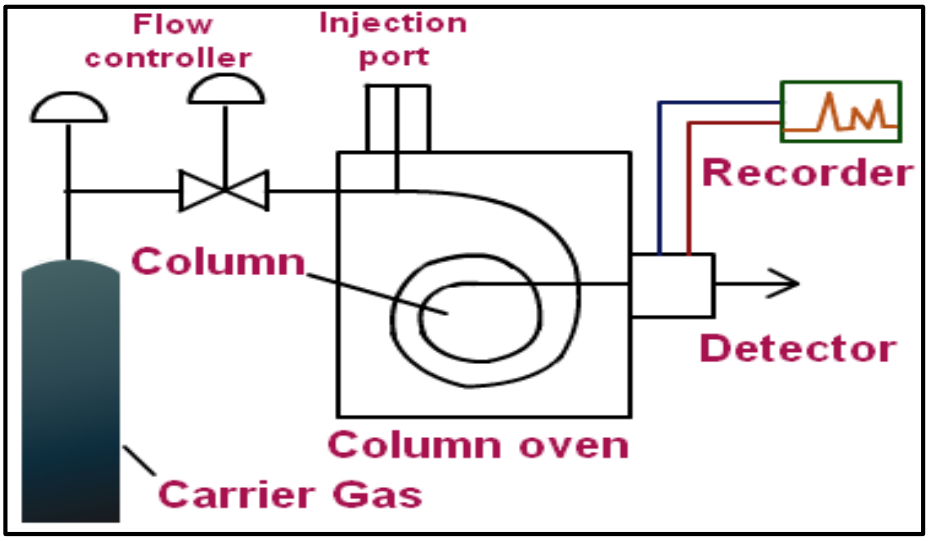

Figure 3.6: Schematic diagram showing Gas Chromatography

The instrument was optimized prior to sample injection. It was done by changing conditions e.g. detector temperature, injection temperature, column oven temperature, split ratio and flow rate. After optimization, retention time of dichlorvos and solvents were identified. 1 µl of aliquot was injected and retention time was analyzed. Operational conditions of GC-ECD are shown in Table 3.2

Conditions	
Initial Temperature	50 °C
Final Temperature	250 °C
Rate	15 °C/min
Run time	15.6 min
Mode	Spit less
Injector Temperature	240 °C
Column Temperature	250 °C
Detector Temperature	300 °C
Gas flow	24.2 cm/S

Table 3.2: Operational conditions of Gas Chromatography

4. STATISTICAL ANALYSIS

The data obtained after tests was subjected to statistical analysis. Cumulative mortality data obtained was further analyzed by using regression equation:

$$LD\ 50\ value\ =\ \alpha\ +\ \beta\ D\ (half\ of\ the\ population)$$

$$\alpha, \beta\ =\ intercept\ coefficients$$

The LD_{50} values were found out by finding regression equation, x^2 denoted the value at which half of population died. Confidence intervals were found statistically.

Significance of data was found out calculated by using Student's T-test. The differences in all experimental and control groups were determined and its significance was observed. A p-value of 0.05 was taken as significant.

Chapter 4

RESULTS AND DISCUSSION

The study aimed to assess toxicity and accumulation potential of organophosphate insecticide, dichlorvos. Acute and sub-acute toxicity was determined by exposing fish with lethal and sub-lethal doses for defined period of time. Sub-acute toxicity was further divided into organ toxicity, hematological analysis and vitellogenin evaluation in males. Bio-accumulation potential varies depending on the properties and nature of pesticides. In the present study an attempt was made to find out the potential of dichlorvos to get absorbed in tissues of Common Carp (*Cyprinus carpio*).

4. 1. ACUTE TOXICITY

4.1.1. Cumulative mortality

It gives the proportion of individuals alive at the start of test and death over the period of test. In the present study cumulative mortality was observed in order to determine LD_{50} of dichlorvos for Common Carp (*Cyprinus carpio*). It is shown in Table 4.1. Dead fish observed after 24, 48, 72 and 96 h of acute exposure depicted that there is a direct relation, as the duration of exposure and doses were increased the die off rate increased.

At 24 h exposure, 40% of population survived at 32.5 mg L^{-1} but at 35 mg L^{-1} death ratio increased and more than half of the population died. In case of 48 and 72 h exposure after 27.5 mg L^{-1} exposure, more than half population did not survive. At 96 h low doses resulted in death of fish as the toxicity increased due to increasing exposure duration. More than half of the population survived at 22.5 mg L^{-1} while but at 35 mg L^{-1} none of them survived.

Organophosphates have a property to inhibit AChE enzymes. Duration and dose of exposure are the factors that significantly affect body of organism. Weiss (1985) observed reduction in

brain AChE activity leading to death of fish. The activity is reported to decline to a level of 30-60% as a result of organophosphate toxicity. Another study conducted by Coppage and Matthews (1974) reported that mortality in Atlantic Croaker (*Micropogonias undulates)* and Pinfish (*Lagodon rhomboids*) occurred when brain AChE levels decreased to 70-90%. Significantly low activity was observed in Tilapia (*Tilapia mossambica*) when exposed with Malathion (Sahib *et al*., 1984). Sanacho *et al*. (1997) reported a sharp decrease in brain activity of European Eel (*Anguilla anguilla*). The dose concentration they applied was 0.04 mg L^{-1} and the decrease they reported was 60% as a result of fenitrothion toxicity.

The cumulative mortality observed in present study suggests seizure of brain activity as the exposure time and dose were increased. High doses of dichlorvos affected the brain resulting in abnormalities and ultimately death of organism. The behavioral changes observed as a result of acute toxicity were; increased operculum movement, swimming on the surface for oxygen, gulping water, hanging statically vertical in water, sudden and erratic movements. These findings were similar to another study which has reported intense behavioral changes by increasing concentration of pesticide in *Cyprinus carpio* and *Peocilia reticulata* including sudden movement in spiral fashion, long periods of motionlessness and rapid gill movement (Gunde and Yerli, 2012).

Exposure doses ($mg\ L^{-1}$)	Exposure duration (Hours)			
	24	**48**	**72**	**96**
0	0	0	0	0
20	0	0	2	3
22.5	0	1	2	4
25	1	2	3	6
27.5	2	3	4	6
30	3	6	6	6
32.5	4	7	8	9
35	6	7	8	10
37.5	6	8	10	10
40	7	10	10	10

Table 4.1: Cumulative mortality of Common Carp (*Cyprinus carpio*)

4.1.2. Lethal dose 50

LD_{50} of dichlorvos in Common Carp (*Cyprinus carpio*) for 24, 48, 72 and 96 h was determined statistically using cumulative mortality. Table 4.2 shows the value of LD_{50} for 24, 48, 72 and 96 h of exposure. The chemical was ensured to be present all the time by changing the water daily followed by addition of pesticide. Toxicity was observed to increase with increasing

dichlorvos concentrations (20, 22.5 25, 27.5, 30, 32.5 and 35 mg L^{-1}) and exposure time (24, 48, 72 and 96 h).

Pesticide	Duration of exposure (Hours)	Lethal dose 50 (LD_{50}) (mg L^{-1})	Confidence limits (mg L^{-1})
Dichlorvos	24	34.278	20.76-47.79
	48	28.61	19.159- 41.06
	72	26.124	16.155-36.095
	96	22.51	15.13-29.88

Table 4.2: LD_{50} of dichlorvos with confidence limits at different time intervals

Mortality of organism depends mainly on sensitivity of organism to the toxicant, concentration and exposure duration. In current study at 96 h, no death was observed in control group but in experimental group the LD_{50} was calculated to be 22.51 mg L^{-1} indicating moderate toxicity of dichlorvos compared to the LD_{50} value of 26.7 mg L^{-1} of diazinon in juveniles of *Cyprinus carpio* (Svobodova *et al*., 1992) and 16.67 mg L^{-1} of dichlorvos in fingerling European catfish (Ural and Koprucu, 2006). 96h LD_{50} of dichlorvos recorded for Atlantic herring (*Clupea harengus*) larvae was 0.12 mg L^{-1} (highly toxic) (McHenery *et al*., 1991). A study reported 24, 48, 72 and 96h LD_{50} of dichlorvos to be 0.73, 0.65, 0.51 and 0.45 mg L^{-1} for fry striped catfish, *Mystus vittatus* (Verma *et al*., 1981).

The toxicity of dichlorvos is reported to be between moderate to high for freshwater and estuarine fish (WHO, 1989). 96 h LD_{50} ranges between 0.2-12 mg L^{-1} for estuarine fish (Demael *et al*., 1990). In a study it was found that 100 % of 100 g Salmon (*Salmon salar*) survived after 24 h when exposed with 1, 3 and 5 mg L^{-1} of dichlorvos (Sievers *et al*., 1995). The LD_{50} values

determined in present study show that dichlorvos is moderately toxic, it is in agreement to the previous study reporting severe damage as a result of acute exposure.

4.2. SUB-ACUTE TOXICITY

4.2.1 Hepatosomatic Index (HSI)

Liver is a vital organ of detoxification that helps in chemical transformations within the cells of an organism. Hepatosomatic index is considered to be a useful biomarker in order to evaluate the toxic effects of environmental stressors on fish health. It also provides an indication on status of energy reserves in an organism. Variation in HSI as a result of pesticide addition is shown in Figure 4.1. The values showed significant differences in both sexes at three concentrations compared to control.

HSI values observed for doses 1.3, 1.8 and 2.34 mg L^{-1} in males were 1.658 ± 0.293, 1.388 ± 0.109 and 0.872 ± 0.188 respectively. All HSI values observed were in decreasing trend as the exposure concentration was increased. The value for controlled group was 2.20 ± 0.214.

In females the values observed for doses 1.3, 1.8 and 2.34 mg L^{-1} were 2.04 ± 1.334, 1.28 ± 0.168 and 0.742 ± 0.196 respectively. HSI value of controlled group was 2.27 ± 0.257. The treated and untreated/controlled group showed significant difference. Moreover P-value observed in all cases was less than 0.05, rejecting the null hypothesis that dichlorvos does not affect fish health.

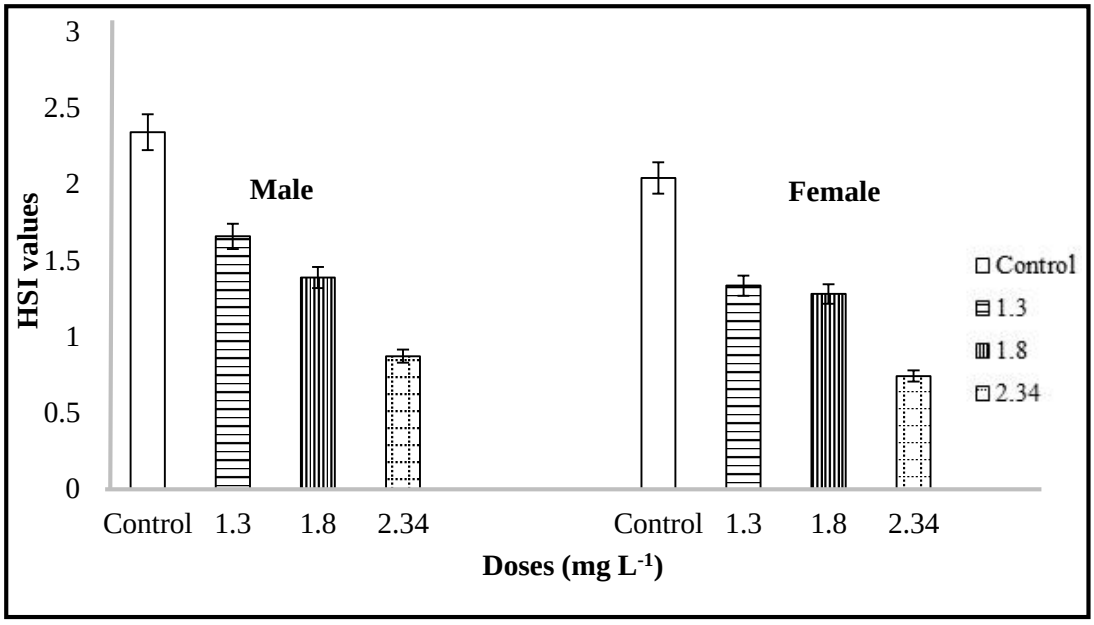

Figure 4.1: Effect of dichlorvos on hepatosomatic index (HSI) on both sexes

Oreochromis mossambicus when exposed to plant nutrient with sub-lethal doses for 45 days, males showed more sensitivity towards chemical than females. Significant decrease was observed in males when exposure time was increased compared to females (Sadekarpawar and Parikh., 2013). HSI values also decreased in rainbow trout when exposed with paraquat after 9 weeks (Akerman *et al*., 2003). Authors reported morphological alterations in liver due to pesticide exposure. Hyperplasia, disintegrated blood vessels, disrupted hepatocytes, hepatic lesions, desquamation, karyolysis and edema of liver tissues were reported in *Labeo rohita* when exposed to Cypermethrin (Sarkar *et al*., 2005). Hyperplasia is a condition in which abnormal growth of cells occur while hepatocytes are liver cells. Desquamation is scraping off or peeling off of layers, karyolysis is a condition in which nucleus of cell gets dissolved. Edema is characterized by swelling of organs which may be due to toxicant response. It occurs when blood vessels become leaky. Cypermethrin resulted in multiple diseases of liver diseases as well as disruption and abnormalities in liver cell. Organophosphates are reported to cause cloudy swelling, bile stagnation, necrosis, atrophy and vacuolization in *Corydoras paleatus* (Fanta *et al*., 2003). Necrosis is dying of body tissues while atrophy is wasting away of organ due to gradual loss of muscle or tissues.

There is a possible chance that in current study dichlorvos resulted in liver infection, as reported by Fanta *et al.* (2003) organophosphate pesticides cause cellular destruction, necrosis and atrophy of liver. Dichlorvos also belongs to organophosphate class, its effect on liver can also be related as HSI values observed in current study decreased at all doses, liver functioning was badly affected as the doses were increased.

4.2.2. Gonadosomatic index (GSI)

Reproductive health of fish is assessed by finding the gonadosomatic index. It gives sexual maturity of organism. In toxicity studies it is used to evaluate effect of toxic chemicals on the mass of gonads.

In present study GSI values decreased when dose was increased. The variation in GSI in both sexes is shown in Figure 4.2.

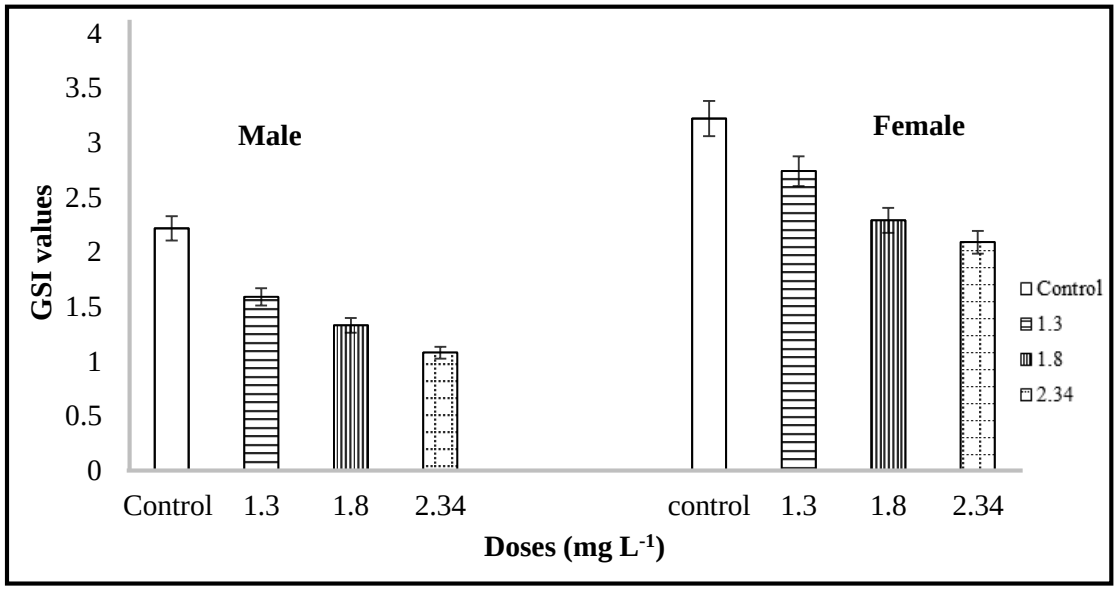

Figure 4.2: Effect of dichlorvos on gonadosomatic index (GSI) on both sexes

Significant change ($p < 0.05$) was observed when dosed group was compared with controlled for all three concentrations (1.3, 1.8 and 2.34 mg L^{-1}) depicting a significant effect of dichlorvos on reproductive system. GSI values observed in males for doses 1.30, 1.80 and 2.34 mg L^{-1} were 1.59 ± 0.4394, 1.33 ± 0.241 and 1.086 ± 0.092 compared to control group 2.216 ± 0.198

whereas in females it was observed to be 2.741 ± 0.172, 2.29 ± 0.209 and 2.09 ± 0.076 compared to control group 3.161 ± 0.221.

These results were in accordance with previous studies. GSI values in females carp was observed to decrease by increasing exposure time and concentration of dichlorvos (Mir *et al*., 2012). Results of the present study are in agreement with Hanson and his co-fellows (2007), who reported a decrease in GSI values in three species (*Oreochromis niloticus, Clarias gariepinus and Chrysicthys nigrodigitatus*) but more significant change was exhibited by females than males. Same trend was observed in fresh water *Oreochromis mossambicus* when exposed with plant nutrient, Librel^{TM}.

Histological changes have been reported due to chemical exposure in gonads. Studies revealed change in structure and function of gonads due to pesticide exposure. Ovarian wall of *Oreochromis mossambicus* showed progressive thinning and degeneration when exposed to plant nutrient. In addition to that exposure dependent histological changes were also observed in testis morphology. Increase in vacuolization disorganization and distortion of seminiferous tubules increased progressively as a result of exposure to plant nutrient (Sadekarpawar and Parikh, 2013). Cellular proliferation and testes growth was reported to decrease in fish as a result of endocrine disruptors (Hassanin *et al*., 2002).

These findings correlate with the present study. The probable reason for decrease in GSI values may be morphological changes induced by dichlorvos, the changes became more adverse when dose was increased resulting in decreasing the weight of gonads.

4.2.3. Haematological profile

Studies on blood helps in assessing the pathophysiological status of fish and the parameters help in diagnosing the structural and functional changes in fish due to chemical exposure. Figures 4.3, 4.4 and 4.5 show variation in RBC's, WBC's and Hb count after dichlorvos exposure. All doses showed significant change ($p < 0.05$) compared to control.

The levels of all three parameters were decreasing with increasing pesticide concentration. Average red blood cell count observed in doses 1.3, 1.8 and 2.34 mg L^{-1} were 1, 0.44 and 0.053 t L^{-1} as compare to 1.79 t L^{-1} in control group. Normal haemoglobin count of 15.85 g dL^{-1} was observed in group serving as control but it decreased in all three concentrations. 10.5, 8.5 and 2.85 g dL^{-1} were found to be present in fish exposed with doses 1.3, 1.8 and 2.34 mg L^{-1}. Drastic decrease in immunity was also observed in dosed groups. A sharp decrease was observed from a level of 108 to 9 10^3 mm^{-3} of white blood cells (control and 2.34 mg L^{-1}).

Decline in all blood parameters was observed in *Oreochromis mossambicus* when exposed to sub-lethal doses of dichlorvos (0.00375, 0.0075 and 0.015 mg L^{-1} respectively) for a period of 7, 14 and 21 days (Lakshmanan *et al*., 2013). Benargi and Rajendranath (1990) reported decrease in erythrocyte count due to dichlorvos in *Clarias batrachus* as observed in current study. Some other studies also showed same trend when different fish species were exposed with pesticides. Cypermethrin resulted in inducing anaemic conditions in carp fish by decreasing the production of haemoglobin and red blood cells in bone marrow (Saxena and Seth, 2002). Results of the current study are in accordance with the earlier reported study by Svoboda *et al*. (2001) in which decrease in erythrocytes and haemoglobin of Common Carp (*Cyprinus carpio*) was observed when exposed with diazinon. Similar studies by researchers observed the same trend. Decrease in leucocyte level was reported when dose was increases in *Clarias gariepinus* after acute exposure to diazinon (Adedeji *et al*., 2009).

Drastichova *et al*. (2004) reported depressed red blood cells and haemoglobin in fish subjected to stressful conditions. Change in red blood cell profile as a result of pesticide exposure suggests a compensation of oxygen deficiency. The body responds by inhibiting production of red blood cells and increase the rate of destruction of them in hematopoietic organs hence causing decrease in their number. In the present study decrease in red blood cell and haemoglobin count is due to less oxygen content in water which was inturn due to dichlorvos

presence. Further decrease of cell count might have attributed to reduction in erythropoetic activity of kidney or it could also be due to gill damage. Epithelium of gills help in osmoregulation of fluids/water. Damage in gill morphology resulted in impaired osmoregulation. The results of current study are in agreement with Ramesh *et al*. (2009) where atrazine pesticide decreased haemoglobin and red blood cells in Common Carp (*Cyprinus carpio*).

This suggests that dichlorvos decreased the immunity of fish which resulted in inducing anaemic conditions in fish by decreasing red blood cell and haemoglobin production.

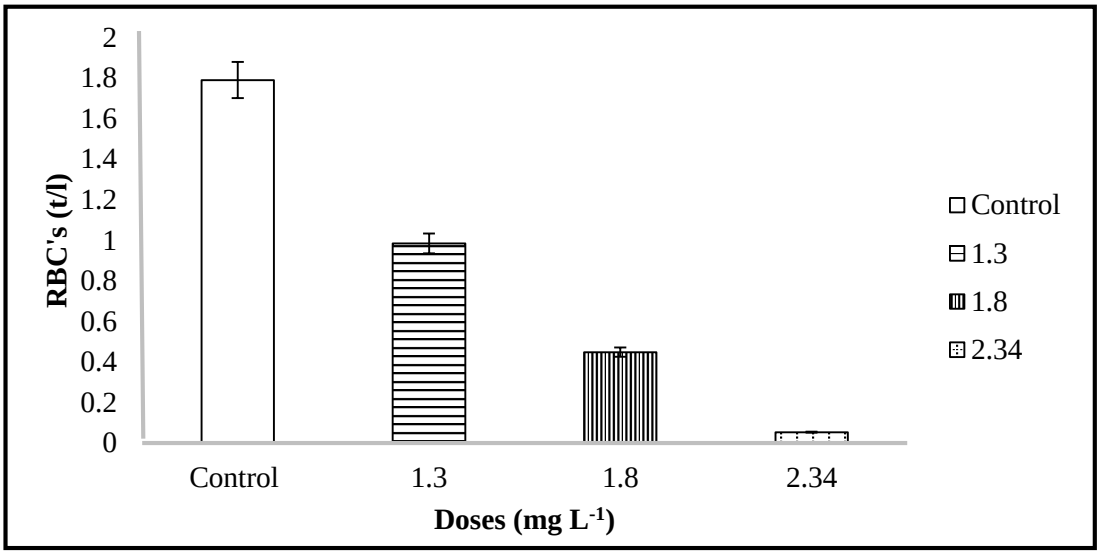

Figure 4.3: Red blood cell count of control and experimental group

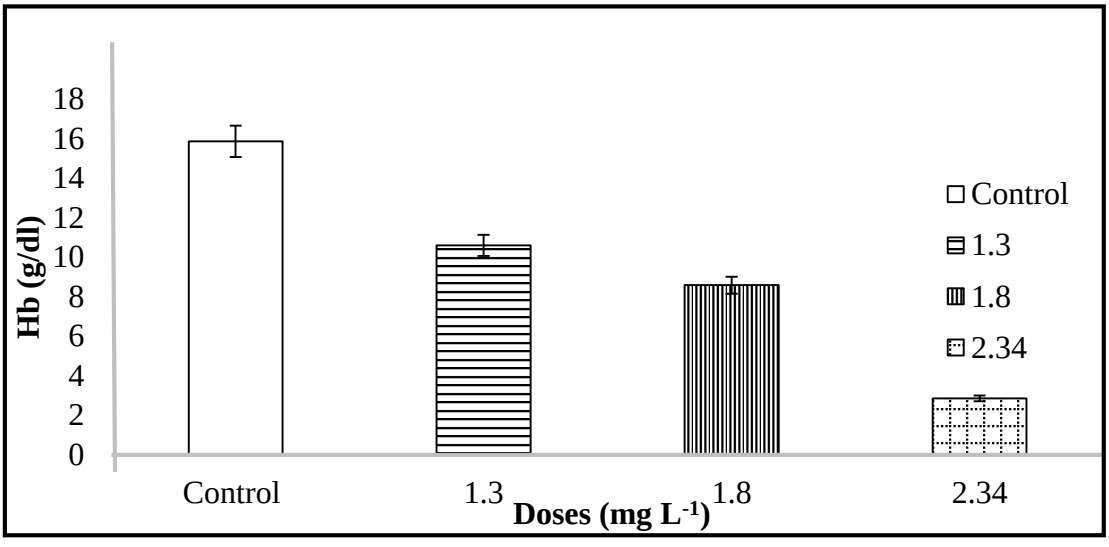

Figure 4.4: Hemoglobin count of control and experimental group

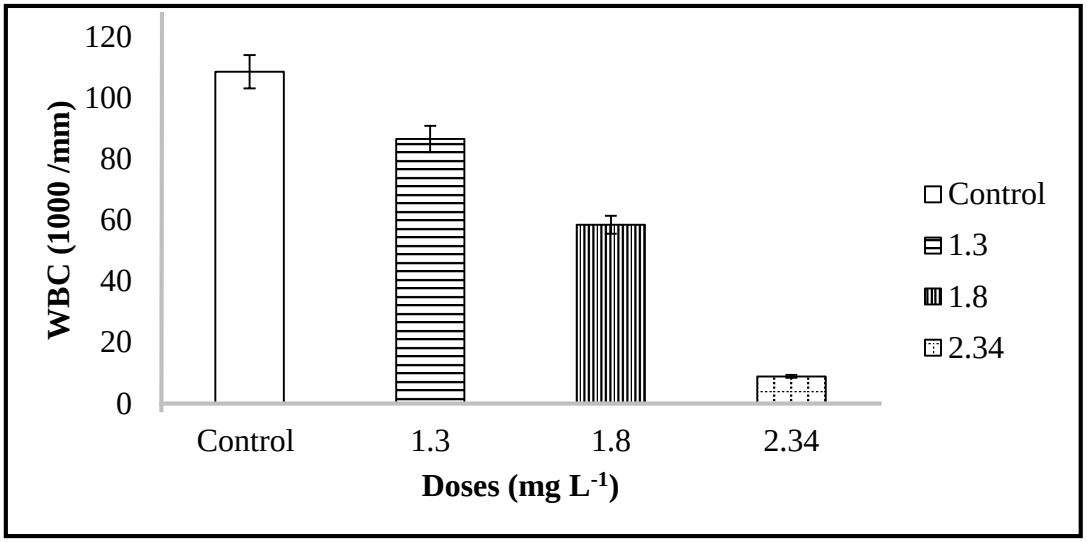

Figure 4.5: White blood cells count of control and experimental group

Results of blood plasma profile are given in Figure 4.6 and 4.7. Relatively higher levels of plasma glucose were observed in fish when dose was increased unlike other parameters. The average values with standard deviation calculated for doses 1.3, 1.8 and 2.34 mg L^{-1} were 70.8 ± 5.58, 71.4 ± 6.94 and 75 ± 6.20 mg dL^{-1} respectively. The average value for control group was 70.6 ± 5.12 mg dl^{-1}.

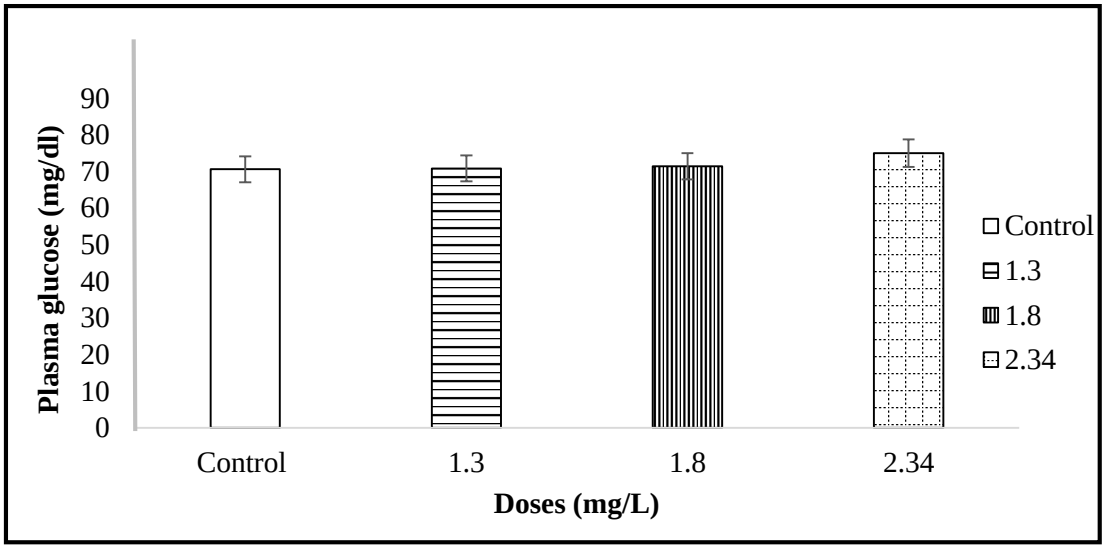

Figure 4.6: Changes in plasma glucose levels

A slight increase in plasma glucose concentration is considered to be a manifestation of stress. Under stressed conditions, the organism needs more energy to cope with the environment. The results are in agreement with Ceron *et al*. (1997) who have reported significant glucose increase in European Eel (*Anguilla anguilla*) when exposed with diazinon. Banae *et al*. (2008) observed increased levels in Common Carp (*Cyprinus carpio*) when exposed with diazinon. A pronounced increased levels were observed by Bhati *et al*. (1972) and Weiss *et al*. (1984).

The observation goes in parallel to AChE inhibition and appearance of cholinergic stimulation due to organophosphate intoxication. Studies reported elevated plasma glucose to be a general response of fish (Svobodova et al., 1992). As exposure damages the internal system, muscular activity needs energy, body starts breaking stored glycogen in liver to fulfil energy requirements of body so elevating glucose levels in blood (Yonar *et al*., 2014)

The concentration of total plasma protein is an indicator of general health status. The effects of dichlorvos on plasma protein observed in current study are shown in Figure 4.7. Exposure resulted in significant decrease. Average values of plasma protein observed in experimental group exposed with 1.3, 1.8 and 2.34 mg L^{-1} were 7.91 ± 0.167, 7.24 ± 0.20 and 6.32 ± 0.50 µg mL^{-1}. Value obtained for controlled group was 8.02 ± 0.35 µg mL^{-1}.

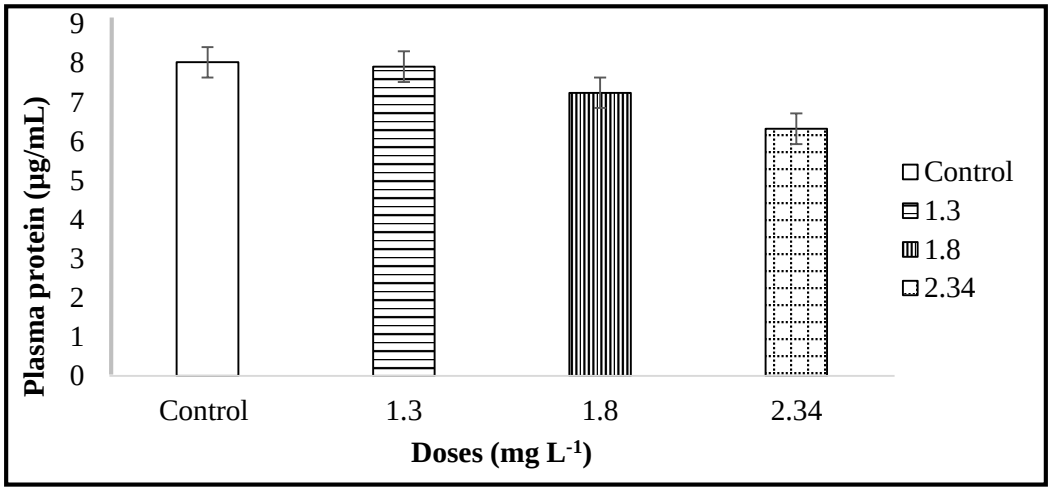

Figure 4.7: Changes in plasma protein levels

Yonar *et al*. (2014) reported decrease in total plasma protein as a result of malathion exposure in Common Carp (*Cyprinus carpio*). Endosulfan also resulted in lowering plasma protein in *Oreochromis mossambicus* (Kumar *et al*., 2011). The findings of current study are in concurrence to Banae *et al*. (2008) who have reported that diazinon decreased plasma protein in Common Carp (*Cyprinus carpio*). 18.73 % decrease in total plasma protein in *Cyprinus carpio* was also reported by Ramesh *et al.* (2009) as a result of Atrazine toxicity.

Starvation and malabsorption/malnutrition are two main factors for lowering total plasma protein. Methylation and phosphorylation of cellular proteins are properties of organophosphates. Intoxication of pesticide causes protein deficiency in body, tissue protein is broken down as a defense mechanism to maintain the protein levels in plasma (Wild, 1975). Das and Gupta (2012) reported that catabolism of protein might have been triggered by high energy demand. Protein catabolism is a process in which both blood and structural protein are broken down to yield energy thereby decreasing serum/plasma protein. They further elaborated that dilution of plasma due to haemolysis and red blood cell are also responsible for reducing protein concentration in plasma. Haemopoietic failure is a characteristic indication of kidney failure. They also reported that pesticides result in kidney failure due to which excess of protein is excreted out of the body so depletion of concentration in plasma occurs.

The reduction in blood profiles after sub-acute exposure in current study indicates toxic effects of dichlorvos on spleen, kidney and liver.

4.2.4. Vitellogenin evaluation in blood plasma of male fish

4.2.4.1. Standard calibration curve

Five standards (125, 250, 500, 1000 and 2000 ng mL^{-1}) were provided in the kit. They were used for calibration curve. Figure 4.8 shows calibration curve and line equation.

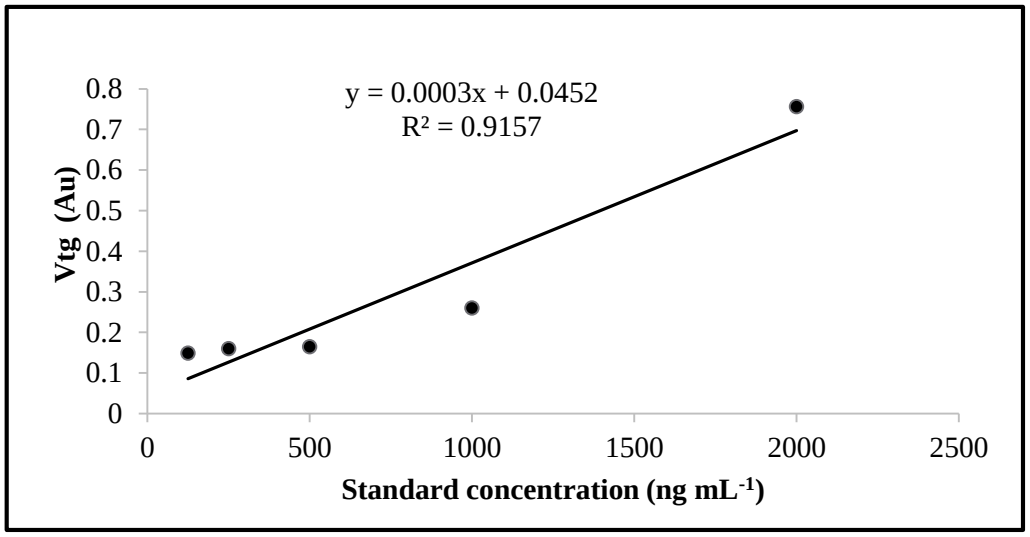

Figure 4.8: Calibration curve and line equation

4.2.4.2. Vitellogenin concentration

The average absorbance for vtg observed when exposed with 1.3, 1.8 and 2.34 mg L^{-1} were 0.038, 0.043 and 1.47 respectively as shown in Figure 4.9. Absorbance increased as doses were increased showing a direct relation.

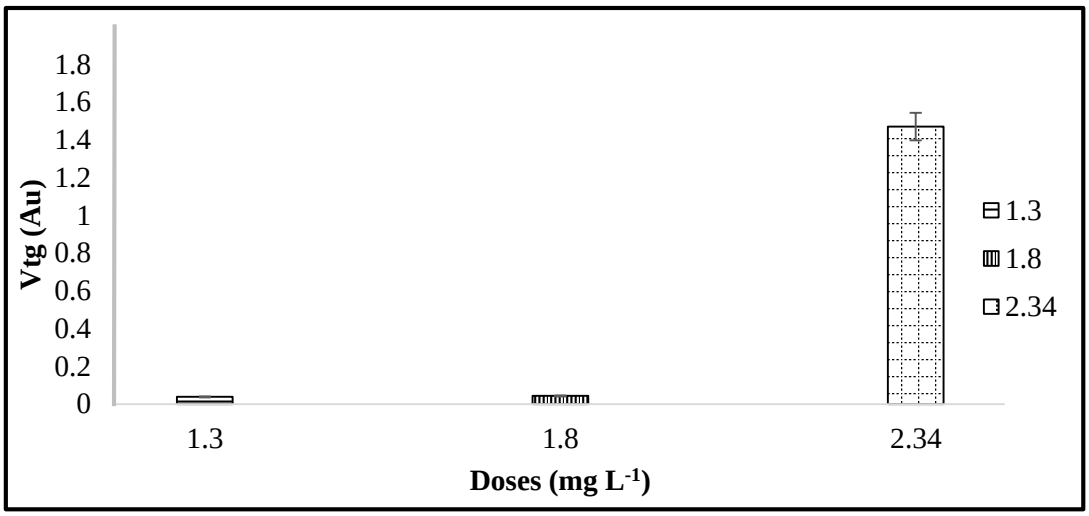

Figure 4.9: Vtg concentration in males after dichlorvos exposure

When male fish are exposed with estrogenic chemicals or pesticides, vtg production is induced. The sensitivity of vtg response is specie and compound specific. Studies have shown that DDT

has pronounced effect on sex differentiation of males. It alters gonads by inducing ovotestes in them due to which vtg concentration increases in male. (Cheek *et al*., 2001).

Wester and Canton (1986) reported effect of lindane on vtg induction in males. An isomer of lindane showed less potency to induce feminization effect, requiring a dose of > 0.18 mg L^{-1} for more than three months of exposure to significantly increase vtg levels. Although it has been stated by them that feminization induction would increase if dose of toxicant and exposure duration would be increased.

In the present study the exposure duration was 14 days. The results showed drastic increase of vtg at sub-lethal doses. A pronounced effect was observed when doses were increased. This helps to conclude that the dose of dichlorvos is directly related to vtg production. A drastic change was observed at higher doses. GSI of males showed decrease in gonadal weight suggesting that the chemical has a tendency to alter functioning of reproductive organs as well as induction of female egg protein in males.

4.3. GC-ECD ANALYSIS FOR BIOACCUMULATION STUDIES

4.3.1. Standard calibration curve

Working solutions were made from 500 mg L^{-1} stock solution in ethanol (10, 20, 30, 40 and 50 mg L^{-1}). They were injected in GC injection port and signal was generated in form of chromatogram. Figure 4.10 shows calibration curve and line equation.

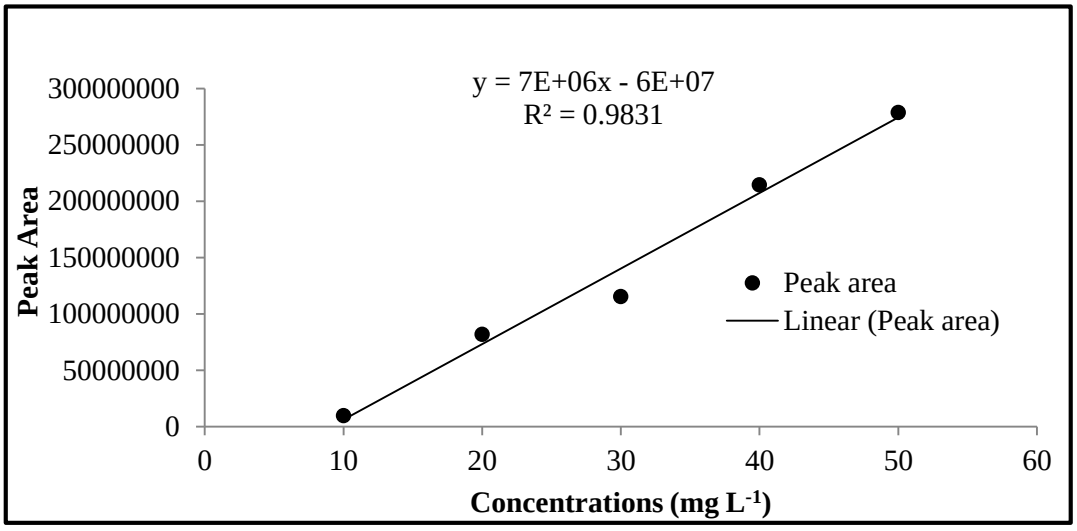

Figure 4.10: Calibration curve and line equation

4.3.2. Retention time of dichlorvos

The instrument was optimized after trial and error. Standard solutions were injected. The mean retention time was calculated after injecting dichlorvos three times. Figure 4.11 shows peak of dichlorvos and acetone. The retention time calculated for dichlorvos and acetone was 4.1 and 2.65 min respectively.

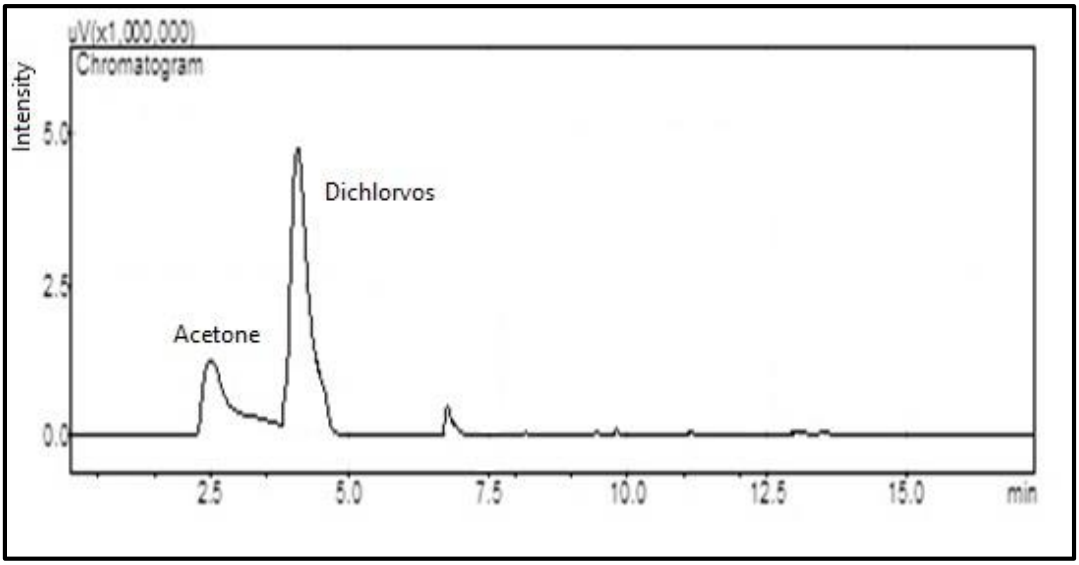

Figure 4.11: Chromatogram showing dichlorvos and acetone peak

4.3.3. Bioaccumulation of dichlorvos in tissues

The fish taken from Rawal Lake showed no accumulation in their body as no visible peak was observed but treated group showed a visible peak of dichlorvos (Annexure I). The percentage accumulation of dichlorvos (shown in Figure 4.12) was found to be 50, 70 and 75 % for 1.3, 1.8 and 2.34 mg L^{-1}respectively.

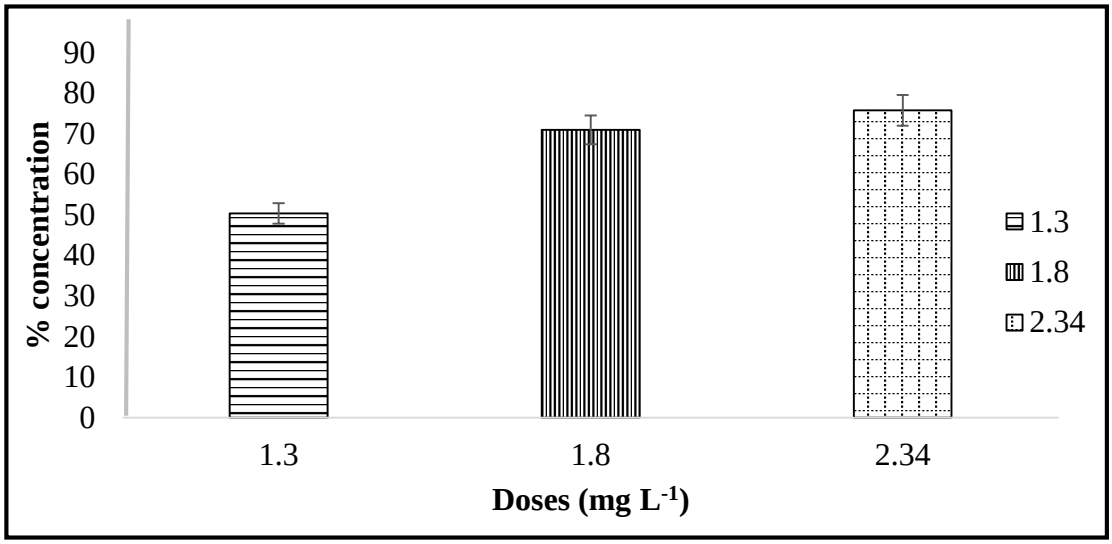

Figure 4.12: Percentage concentration of dichlorvos in muscle tissues

Findings of current study correlates with Horsberg and Hoy (1990). 0.11 µg L^{-1} concentration in muscle tissues was observed in Atlantic salmon immediately after immersion treatment with dichlorvos. At 4°C the residues of dichlorvos in muscle tissues were observed to be 0.13µg L^{-1} immediately after treatment. Low concentration of dichlorvos was detected in muscle tissues longer after treatment. The study reported that liver does not accumulate high concentration of dichlorvos immediately after treatment but presence is detected somewhat longer than in muscle. In current study, tissue samples showed accumulation which increased by increasing concentration. Brandal (1979) found residues of trichlorfon in muscle tissues of Atlantic Salmon up to 12 days after exposing it with 300 mg L^{-1} for 60 min. Organophosphates have tendency to bio-accumulate. Dichlorvos does not bio-accumulate for a longer span, it is excreted out from the body depending on fish species.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Aquatic poisoning by pesticide residues is becoming a serious problem. It results in either death of fish or affecting the health badly. In the current study Common Carp (*Cyprinus carpio*) was selected as a model organism and an insecticide, Dichlorvos, was selected as the toxicant. The conclusions drawn from the research work are as follows:

1. Acute toxicity showed high mortality rate by increasing dose (20, 22.5, 25, 27.5, 30, 32.5, 35, 37.5 and 40 respectively) and exposure duration (24, 48, 72 and 96 h respectively) of dichlorvos thereby exhibiting relatively moderate toxicity of dichlorvos.
2. Haematological analysis depicted that sub-lethal concentrations (1.3, 1.8 and 2.34 mg L^{-1}) have potential effect on organs. Exposure caused severe damage by drastically reducing immunity of fish from 108.6 to 9 10^3 mm^{-3}. Anemic conditions were observed due to low red blood cell and haemoglobin count at 2.34 mg L^{-1} (RBC= 0.053 million mm^{-3} and Hb= 2.85 g dL^{-1}).
3. Sub-acute exposure (1.3, 1.8 and 2.34 mg L^{-1}) also resulted in disruption of reproductive system by decreasing the values of gonadosomatic index and inducing vitellogenin in males. At highest dose the GSI value observed for males was 1.08 (control group = 2.216) while in females it was 2.09 (control group = 3.22). Vtg concentration of 1.02 Au was observed in plasma of male group exposed with highest dose (2.34 mg L^{-1}). It shows that dichlorvos has a potential to induce feminization in male fish by inducing vitellogenin production in them.

4. Decrease in hepatosomatic values depicted liver injection. The male group exposed with 2.34 mg L^{-1} showed 0.87 value of HSI (control group = 2.17) while value calculated in females was 0.742 at dose 2.34 mg L^{-1} (control group = 2). This depicts that dichlorvos causes liver damage at sub-acute concentrations.
5. 50, 70, 75% of dichlorvos residues were found in muscle tissues after 45 days of exposure but samples from Rawal Lake showed no accumulation.

5.2 RECOMMENDATIONS

Following recommendations can be considered while working in aquatic toxicology

1. Histopathological studies for organ damage as a result of dichlorvos exposure.
2. Determination of oxidative stress induced in fish after chemical exposure.
3. Analysis at molecular level to evaluate the extent of genetic modifications due to chemical exposure.

Chapter 6

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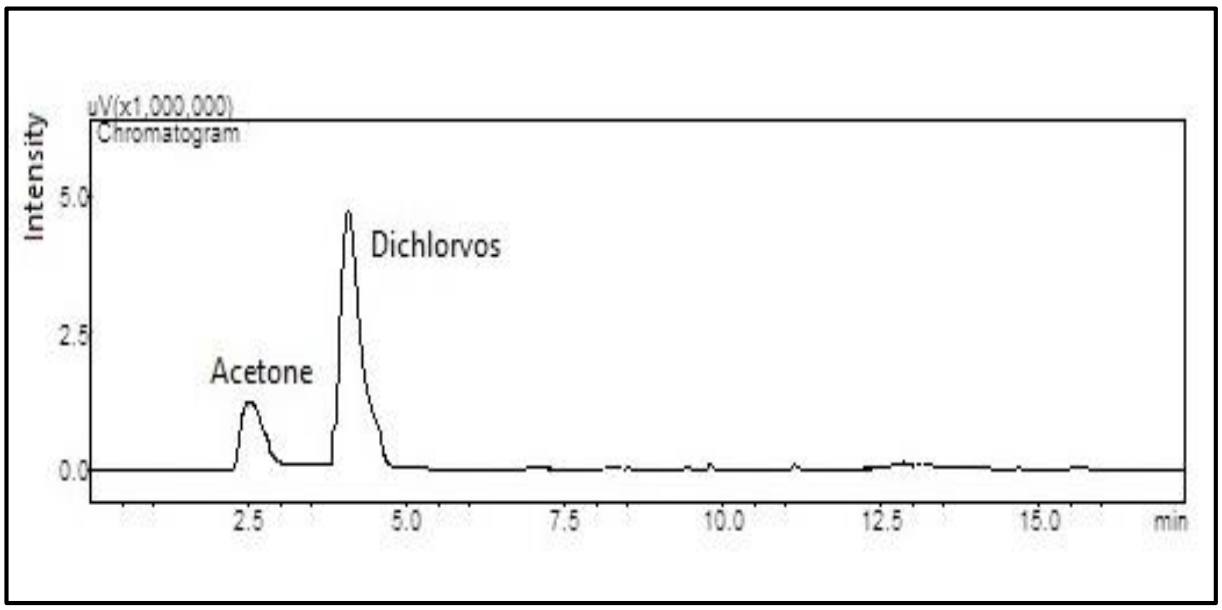
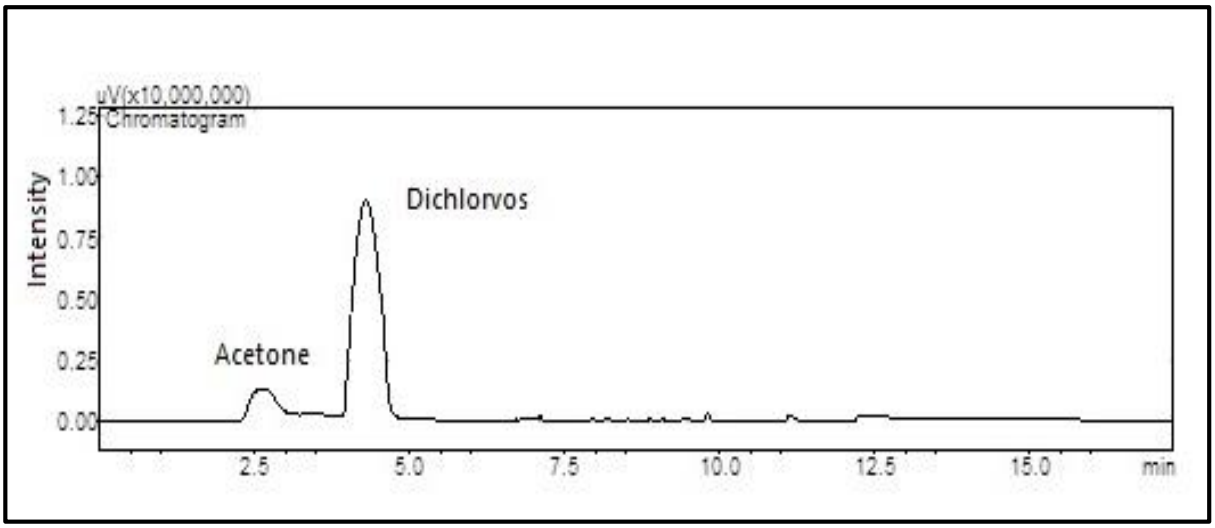
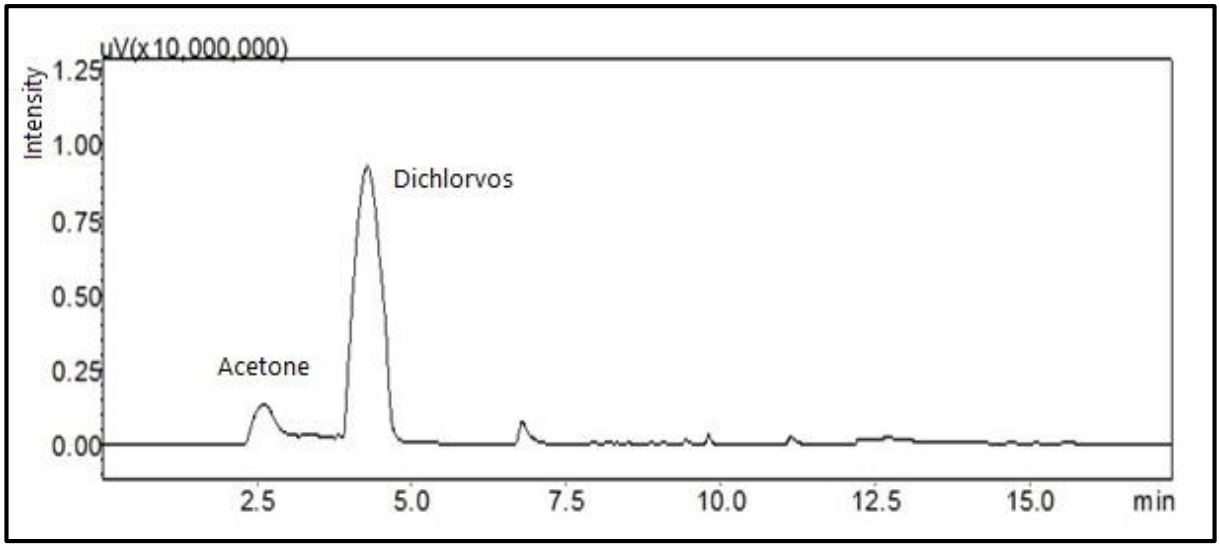
ANNEXURE I



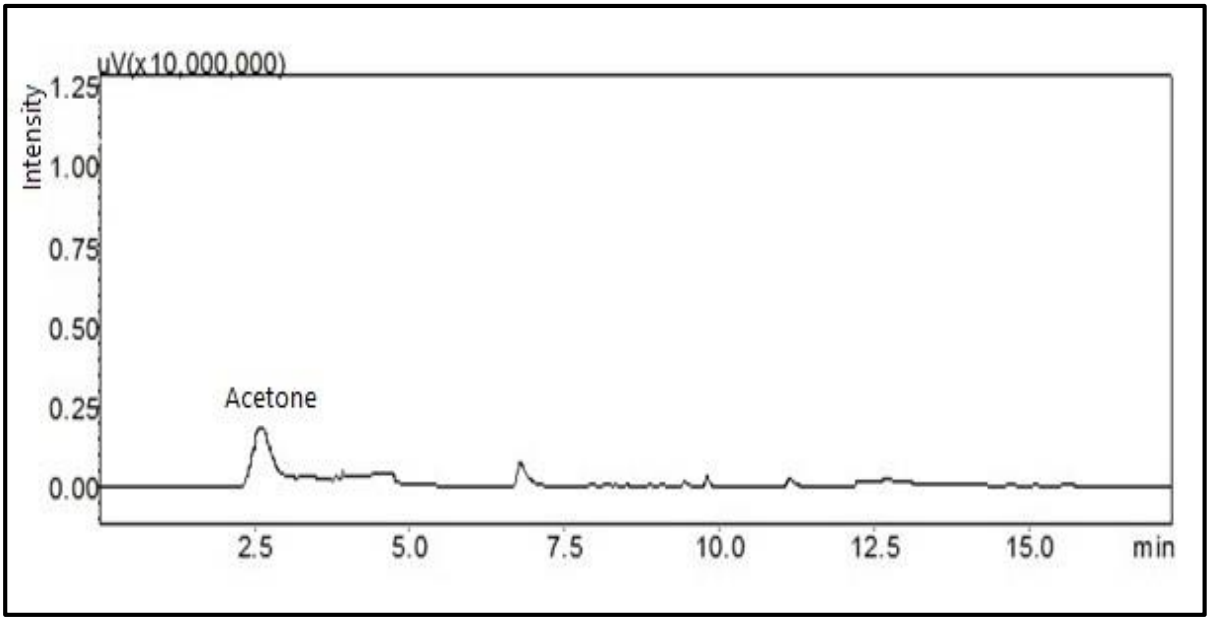
Dichlorvos accumulation in muscle tissues (1.3 mg L^{-1})



Dichlorvos accumulation in muscle tissues (1.8 mg L^{-1})

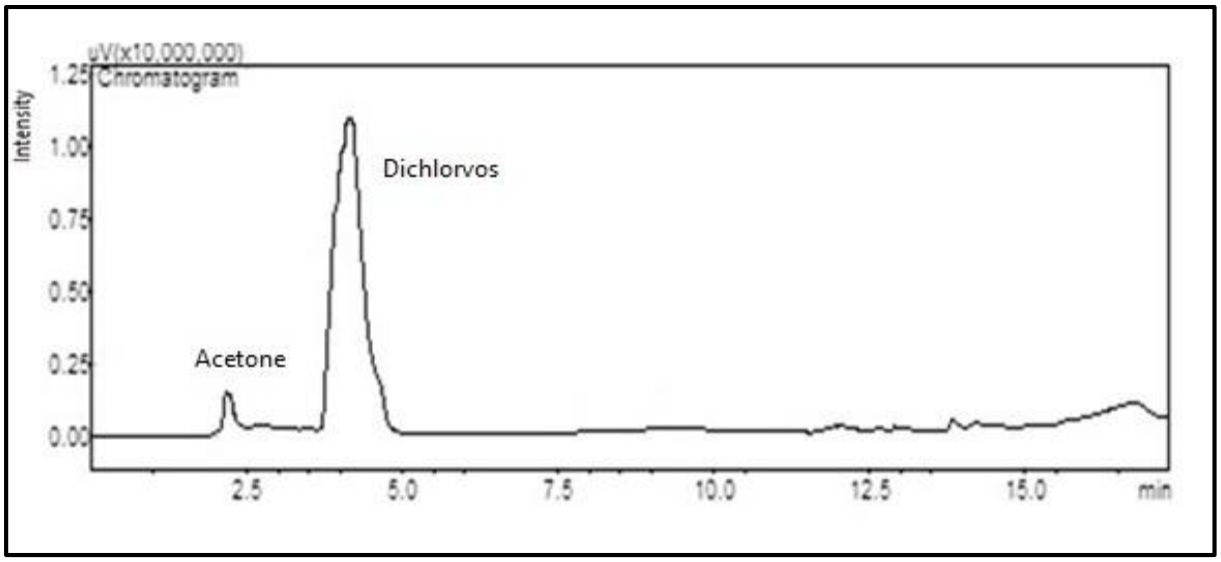


Dichlorvos accumulation in muscle tissues (2.34 mg L^{-1})

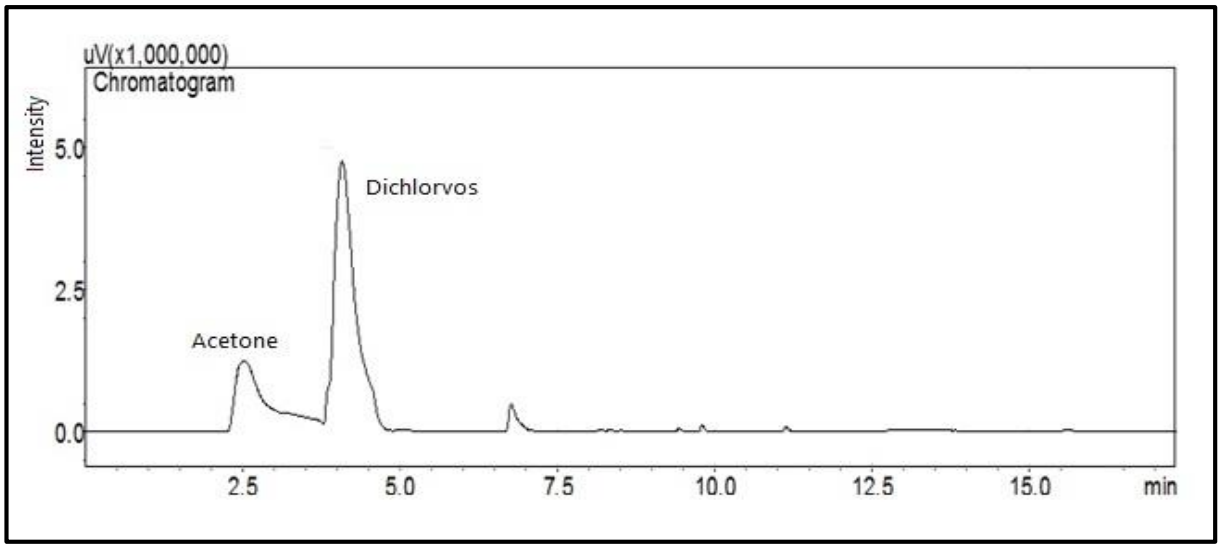


Bioaccumulation of dichlorvos in Rawal Lake sample

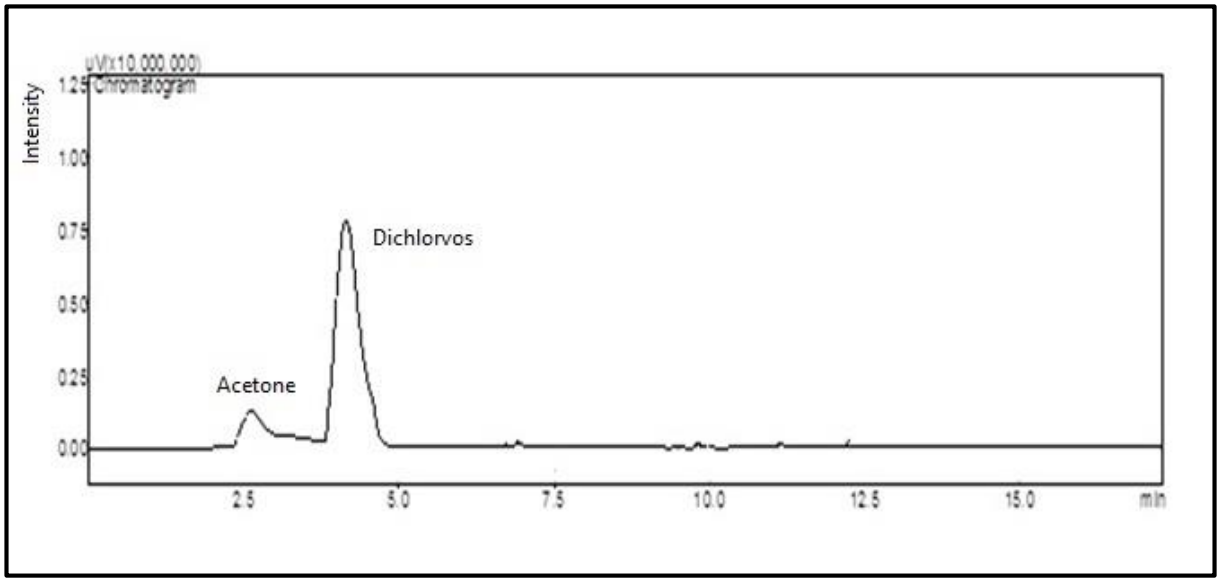
ANNEXURE II



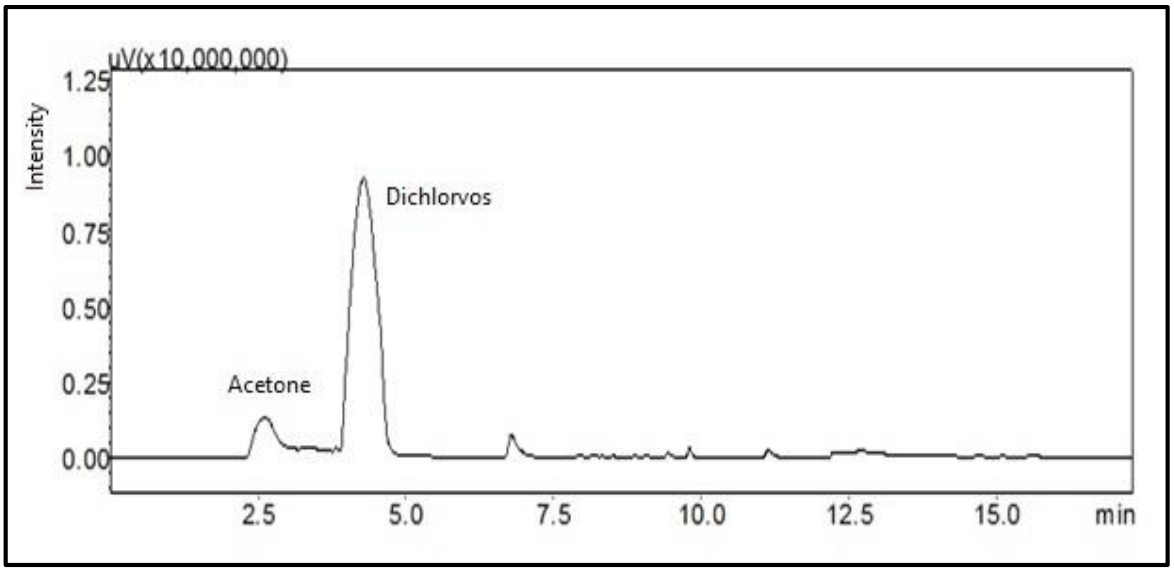
Calibration curve – Dichlorvos (10 mg L^{-1})



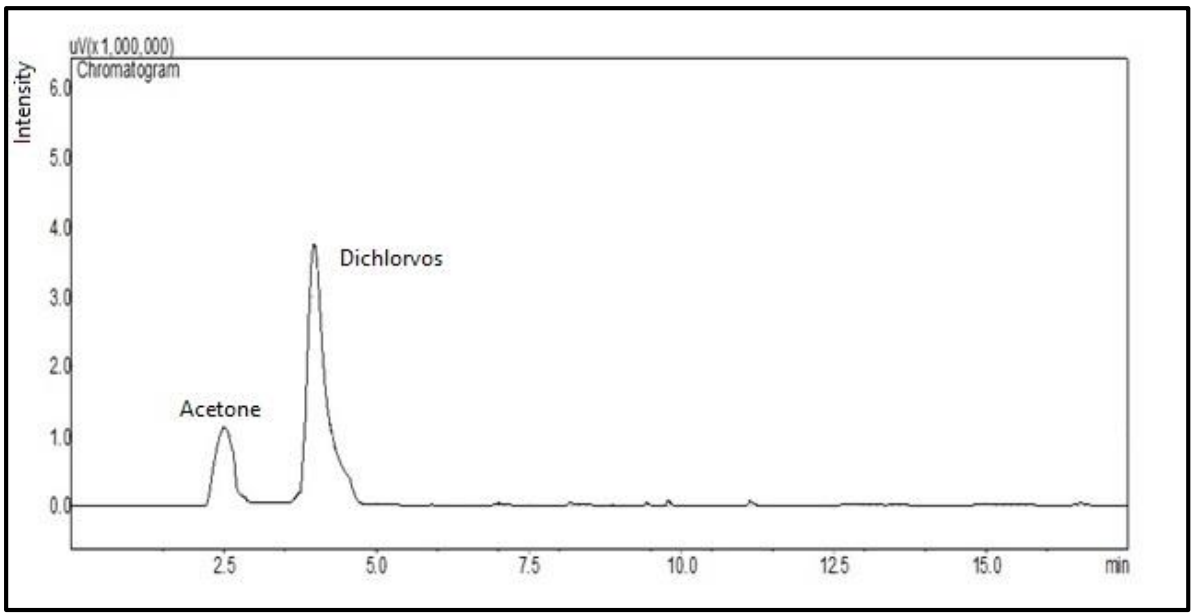
Calibration curve – Dichlorvos (20 mg L^{-1})



Calibration curve – Dichlorvos (30 mg L^{-1})



Calibration curve – Dichlorvos (40 mg L^{-1})



Calibration curve – Dichlorvos (50 mg L^{-1})

ANNEXURE III

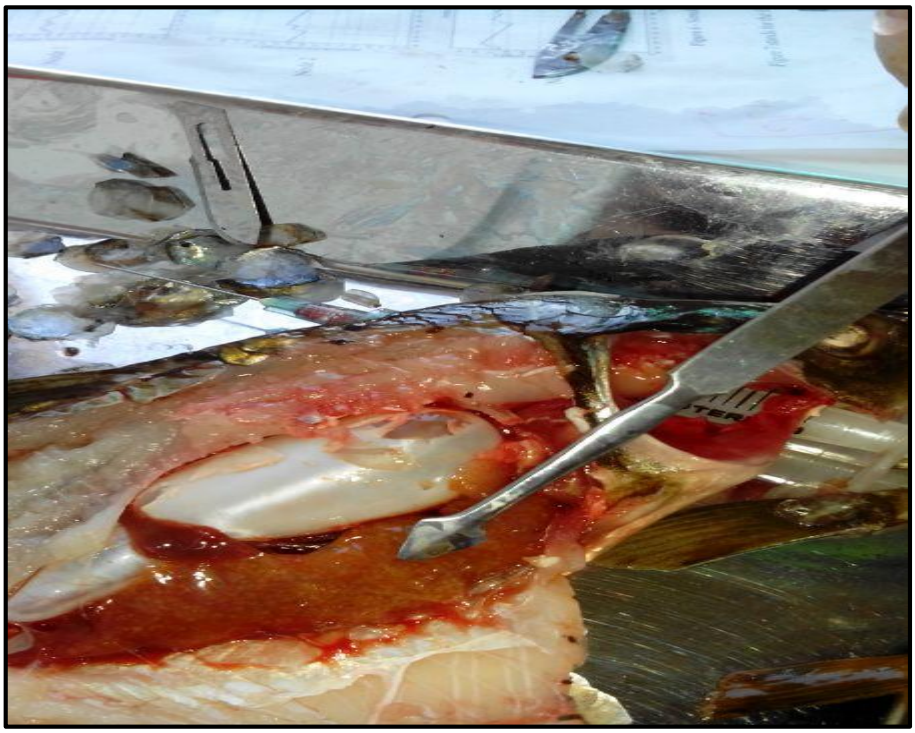
Vitellogenin Analysis:

Reagents were prepared as directed in instruction manual. The reagents provided in the kit are listed below:

- Standards
- Conjugate
- HRP-avidin
- Wash buffer
- Substrate A
- Substrate B
- Stop Solution

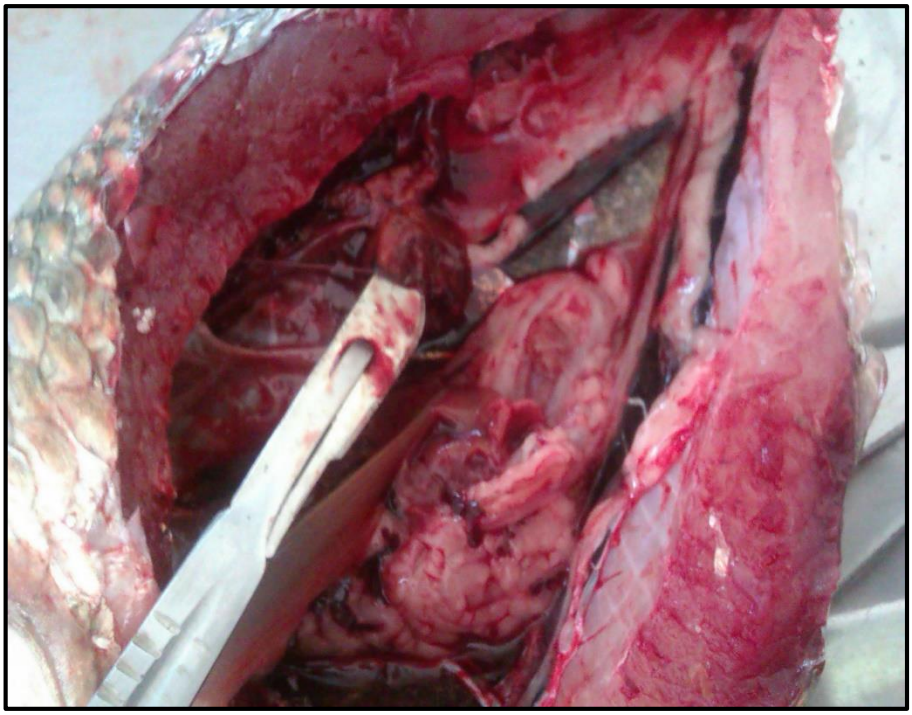
Crystals of wash buffer were formed, it was warmed up to room temperature and mixed gently until the crystals were completely dissolved. 15 ml of wash buffer was diluted up to 300 ml by using distilled water. The samples were thawed and centrifuged again for detection. 50 µl of standard and sample were added per well of assay plate. 50 µl of conjugate was added to each well, mixed and then incubated (Lab Tech LIB-030M) for 60 mints at 37 °C. After incubation each well was aspirated and washed using wash buffer and left the wells dry for 10 secs. 50 µl of HRP avidin was added, mixed and incubated for 30 mints at 37 °C. After incubation the same steps of washing were repeated. 50 µl of both substrate A and substrate B was added, mixed and incubated again for 15 mints at 37 °C in dark. 50 µl of stop solution was added after incubation time. Figure 3.4 shows addition of reagents to samples. Optical density was determined at 450 nm using micro plate reader (BioTek ELx 800).

ANNEXURE IV



Gonads of female fish

Gonads of male fish



Liver of fish