MONITORING OF TOXICOLOGICAL IMPACTS OF LAMBDA CYHALOTHRIN ON FISH USING MULTI-BIOMARKER APPROACH

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In

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Institute of Environmental Sciences and Engineering (IESE)
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CERTIFICATE

This dissertation submitted by **Ms. Tayyaba Bashir** 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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It is certified that the contents and forms of the thesis entitled **"Monitoring of toxicological impacts of Lambda cyhalothrin on fish using multi-biomarker approach"** submitted by Ms. **Tayyaba Bashir**, Registration # 00000206283 has been satisfactory, free of errors and plagiarism for the requirements of the degree of Master of Science in Environmental Science. It is further certified that necessary amendments as pointed out by the GEC members of the scholar have also been incorporated in the said thesis.

Signature: _____

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Dedicated to my parents and beloved siblings for

their love and endless support

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In the name of Allah, the Most Gracious and the Most Merciful, all praises to Him for the strengths and blessing in completing this thesis.

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Tayyaba Bashir

LIST OF ABBREVIATIONS

DMSO	Dimethyl Sulphoxide
ALT	Alanine Aminotransferase
EDTA	Ethylenediaminetetraacetic acid
GC-MS	Gas Chromatography mass spectrometry
LD-50	Lethal Dose 50
MCLs	Maximum Contaminant Levels
µg/L	Microgram per Liter
Ppb	Parts per billion
Ppm	Parts per million
OECD	Organization for Economic Cooperation and Development
APHA	American Public Health Association
ANOVA	Analysis of variance
OMT	Olive tail moment
DNA	Deoxyribonucleic Acid
EtBr	Ethidium Bromide
GCMS	Gas chromatography mass spectrometry

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Agricultural runoff and industrial effluents are directly contributing to the surface water contamination. Pesticides coming from these sources are highly toxic contaminants and their indiscriminate usage is a serious environmental threat especially for aquatic life. These chemical represent a substantial damage to living organisms as damage to reproductive, immune, endocrine and nervous systems. Toxicity of pesticides fluctuates with variation in temperature, as temperature increases it may increase or decrease. The aim of this study was to perform acute and sub-acute toxicity test to determine LD-50 (Lethal dose 50) of Lambda cyhalothrin (a synthetic pyrethroid insecticide) and to evaluate its toxic impacts on grass carp (*Ctenopharyngodon idella*). LD-50 was initially measured for two temperatures because it is regulated by temperature as concentrations of toxic chemicals which are not active at low and moderate temperatures proved fatal with increase in water temperature The results indicated that the LD_{50} at 27 and 13ºC was 1.17 and 1.5 µg/L respectively. Blood glucose and protein levels increased initially with dose and time from 24 to 72 hours and decreased at 96 hours. In the observed values of experimental and control group there was significant difference ($P<0.05$). Triglycerides also have presented some fluctuating trends with increase dose concentration and exposure time while increased ALT level was observed at the end of experiment due to abnormal hepatic functions. Values observed in exposed group were significantly different ($P< 0.05$) from the control group. Oxidative stress due to increased ROS was significantly higher ($P< 0.05$) in brain and gills of experimental group as compared with control group. Respiratory burst activity also shown increasing

trend with increased dose and prolonged exposure in the exposed fish as resulted values were significantly higher ($P < 0.05$) than control. Genotoxicity assessment revealed that there was significant ($P < 0.05$) increase in DNA% damage, tail length and olive tail moment in experimental group as compared to control group. Bioaccumulation was also observed in the muscle tissues after exposure to the Lambda cyhalothrin Morphological and histological alterations observed in fish liver and gills tissues showed different abnormalities in microscopic visualization. Abnormal behavioral patterns including more mucus on gills, surfacing and loss of balance were observed in the exposed fish.

Keywords: *LD-50, Oxidative stress, temperature, comet assay, cytotoxicity, Lambda cyhalothrin, genotoxicity, histopathology.*

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Background

Water is a prerequisite for life on Earth. The aquatic environment plays a vital role in the functioning of ecosystem. All life forms are highly dependent upon water. Both quality and quantity of water is important to ensure proper survival of life. Water has been used as a symbol of life and purity. Therefore, availability of fresh water is essential for the survival of aquatic life and humans as well. Global water distribution contains only 2.5% of fresh water, the remaining 97.5% of water is too salty to use for human consumption. Among fresh water; 69% is present in icecaps and glaciers while underground contain 30% of total. Less than 1% present is present in form of rivers, lakes and swamps which are consumable forms for humans.

Water is a basic unit life and its quality is important to sustain life on the planet. Water quality of reservoirs, natural lakes, and rivers in developing countries is continuously deteriorated because of the contaminated inflows. Waterbodies are a main sink for domestic, industrial and other anthropogenic compound (Somashekar et al., 2015). 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 which are dependent on water for their existence. All over the world, there is an ever-increasing risk of 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 cause environmental pollution especially in aquatic ecosystems as it is increasing day by day due to widespread pesticide usage in agriculture (Wang et al., 2017). Generally, pesticides come into contact with soil and travel to surface water through runoff and ground water through leaching. These pesticides find their way into water reservoirs, river, stream etc. and alter the chemical composition of these water bodies which results in adverse impacts on the fresh water fauna particularly fish.

Fish is an important food item for human diet therefore, it is very important to evaluate the adverse effects of pesticides on fish as it plays a major part in food chain (Xing et 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 deteriorating water quality (Sharbidre et al., 2011). Most of the pesticides are toxic not only to target species because of the structural and physiological similarities between pest and non-pest species. Though, several ecotoxicology studies have been carried out involving fish as model organism, these studies have mostly focused on the impacts of exposure of one pesticide at a time, such as organochlorine, organophosphate, pyrethroid or carbamate insecticides (Clasen et al., 2018).

There is a broad range of environmental contaminants found in the aquatic ecosystems. Among those contaminates, pesticides hardly exist as an individual chemical in the water body. They usually present as mixtures of different substances at relatively

low/mild concentrations. In the current era when there is green revolution, the human population is growing at rapid rate and forests have been consumed and cut down for land clearance for construction, disturbing the environmental equilibrium. We are facing a challenge of evolving and growing problem of contaminants as these contaminants include domestic wastes, semi treated or untreated industrial effluents and different toxic compounds such as pesticides/insecticides which are used in agricultural activities or for safety measures in residential settings. The effects of these contaminants are augmented with the presence of different organic and inorganic compounds, chemicals, pesticides, and heavy metals etc. These pollutants degrade the water quality and this altered water quality badly affects residing aquatic organisms and even leads to death in high concentrations and severe exposures. When these toxic pollutants enter into the water bodies in concentrations higher than the permissible limits this results excessive mortality of aquatic life present in those contaminated aquatic systems. Whereas, in the sub-acute or lower concentrations, these pollutants lead to bio-accumulation in the bodies of aquatic organisms such as fish and become part of food chain (Hassantabar & Babaei, 2013 ; Morel et al., 2014). Use of pesticides is growing progressively as part of novel high yielding crop varieties. Due to immense usage of pesticides these are one of the most detrimental causes of environmental pollution (Uddin et al.,2016). 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).

Various fish species are prone to these lethal pesticides at different concentrations. The changes observed in different body parts of fishes are far different than each other as well as they differ in response to dissimilar insecticides. Pesticide exposures effect all body functions and systems of fish body. This problem should be focused and treated properly in order to guarantee safer fish intake on priority basis (Ullah, 2015). The usage of pesticides in agricultural activities has become a major threat for wild life because of their excessive and indiscriminate usage (Gibbons et al., 2015). Indiscriminate pesticides application leads to deterioration of the water quality of aquatic environment through different ways such as, surface runoff and leaching down in the soil.

When pesticides enter into the water bodies through agricultural run-off generated after rain fall, these deadly chemicals badly effect aquatic systems even used in minor quantities due to their toxicity and persistency (Tennekes, 2011 ; Hladik et al., 2014). The World Health Organization (WHO) indicates that pesticides adversely affect nearly three million people per year; primarily due to organophosphorus insecticides, that ultimately results in 200,000 causalities (Somashekar et al., 2015). Altered fish behavior, changed body's functions, and changes in histology of liver, gills, intestine and kidneys can be produced due to impact of pesticides (Ullah, 2015; Sharmin & Haque, 2015 ; Hossain et al., 2015). Various aquatic organisms such as fish, mollusks, snails and aquatic plants are as bio-indicators in order to evaluate the environmental risks postured to the aquatic ecosystem caused by poisonous contaminants. The impact of pollutants on individuals can be previously indicated via biomarkers before a fatal effect becomes apparent. In addition, these biomarkers are also used to monitor and assess the water

pollution and come up with an eco-toxicological discovery as a warning system. The carp specimens which are subjected to contamination stress have shown significant changes in cellular, molecular, histological and physiological parameters. (Ballesteros et al., 2017).

Fish is directly exposed to contaminants through skin, gills and their diet in the aquatic environment. The residues of pollutants found in fish bodies are considered as a health index for surface water bodies (Beyene et al., 2013). Moreover, this study is relevant due to scarcity of available data about the bioaccumulation of insecticides in edible fish muscles, these insecticides bio-accumulate in bodies of aquatic organisms and bio-magnify along food chains. This progression leads to an increased concentration of insecticides in aquatic organisms and cause a serious health risk to humans as fish is a major cause of human exposure to pesticides which accumulate in the fish bodies when consumed (Mahmood et al., 2014 ; Buah-kwo et al., 2018). Presence of pesticide in the muscles of common carp were reported in a research study where pesticide Lambda cyhalothrin and the fungicide tebuconazole provided the proof of bioaccumulation in muscles of fish (Clasen et al., 2018).

Pesticides are normally categorized into four major classes as Carbamates, Organophosphates and Pyrethroids and Organochlorines. Pyrethroids are widely used to control a broad range of pests in agricultural lands, public health sector, veterinary medicines and residential settings. Recently, the consumption of pyrethroid pesticides has been increasing as restrictions have been placed on numerous organophosphorus

pesticides (Soderlund et al., 2002). The environmental influences of pesticides are determined by toxicity, persistence, biodegradation and ultimately environmental fate.

The occurrence of Geno toxins even in minute quantities effects aquatic as well as non-aquatic organisms by food chain and also through drinking water. Therefore, it is very important to evaluate the cytotoxicity and genotoxicity of compounds at low/mild concentrations. Significant environmental concern have been generated due to the unrestrained agricultural discharge, as these chemicals have potential to spread and wide transference. These persistent pesticides have spread to almost every aquatic environment through contaminated river inputs, surface water runoff and precipitation ending up with accumulation in the marine food chains.

Aquatic organisms such as fish and shellfish are capable of bio accumulating the pesticides in quite higher concentrations than the surrounding exposure water (Muralidharan et al., 2009). Fish food which is polluted with pesticides is a potential source of direct introduction of insecticides in the fish bodies in aquaculture farms (Nardelli et al., 2004). In this vicious cycle, fish becomes a major source of contamination not only for top marine predators but also for human consumers (Wang et al., 2005 ; Campos et al., 2005). For environmental stressors and pollutants fish have been broadly used as bio indicator. In the current study grass carp (*Ctenopharyngodon idella*) was chosen as a model organism to monitor the impacts of a pyrethroid pesticide, Lambda cyhalothrin.

1.2. Study objectives

Keeping in view impacts of pesticides on fish health, this research study was performed at the Institute of Environmental Sciences Engineering (IESE) to monitor the toxicological impacts of Lambda cyhalothrin on fish. Grass carp (*Ctenopharyngodon idella*) was used as model organism for aquatic pollution. Following were the objectives of the study:

1. Investigation of Lethal dose-50 of Lambda cyhalothrin for fish.

2. Validation of biomarkers to assess the toxicity of Lambda cyhalothrin.

3. Evaluation of bioaccumulation potential of Lambda cyhalothrin in fish tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 Present study

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.

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 substances from cultivation 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 & Okocha, 2012). Fish is a valuable bio monitor of water pollution amongst several aquatic organisms. Fish are the top consumers and play a key 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 direct exposure to different pesticides resulting from agricultural lands through runoff (Audu et al., 2015).

Xenobiotic comes into contact with fish, different reactions started 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 bio indicators of aquatic pollution for the quality assessment of the aquatic system. A bottom-dwelling fish, common carp (*Cyprinus carpio*), was selected for research study as it is an important fish for human consumption and it is directly exposed to wide range of environmental pollutants due to its eating habit.

Alterations in the body structure and function of aquatic organism due to sub-lethal exposure of pesticide are more common than mortality. In order to monitor physiological alterations due to different xenobiotic exposure hematology is used as an indicator of fish health (Saravanan et al., 2011). Hematological analysis indicated that Lambda cyhalothrin causes stress and immune-suppression in fish. Lambda cyhalothrin and its metabolites are considered to cause oxidative stress and also bio accumulates in freshwater biota particularly in fish. 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 directly into the environment. The use of insecticides has undoubtedly increased the quantity and quality of agricultural products for the growing world population but with the ever increasing demands of the growing world population upon agriculture, there is a resultant increase in environmental pollution (Lo et al., 2008).

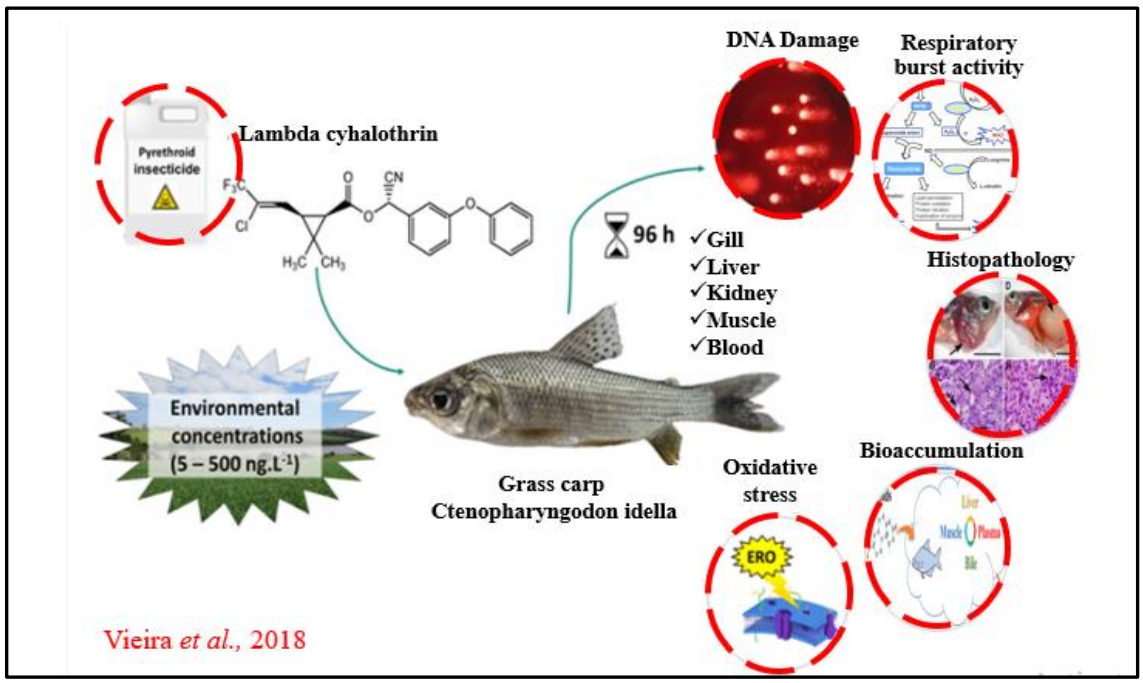


Figure 2.1 Impact of pesticide on fish organs

2.2 Grass Carp *(Ctenopharyngodon idella)*

Grass carp (*Ctenopharyngodon idella*) is one of the largest members of minnow family and closely related to common carp and goldfish, but also has some differences in appearance as well as feeding habits. It is a vegetarian fish native to the Amur River in Asia therefore, also known as the White Amur. It is a native species of large Asian rivers. It is widely found in Pakistan.

Figure 2.2 Grass carp (*Ctenopharyngodon idella*)

2.3 Lambda cyhalothrin

It is an insecticide, registered in 1988 by the U.S. Environmental Protection Agency (EPA). It belongs to pyrethroid class of insecticides. These are artificially prepared substances that are alike to natural pesticide pyrethrins. It is a colourless to beige color solid with a mild odor. Lambda cyhalothrin is a non-volatile compound and has low water solubility. It is used to control a broad range of insects on livestock, crops and in residential settings. There are several other applications of Lambda cyhalothirn as it is used for structural pest management as of termiticide and also in public health in order to control insects such as cockroaches, mosquitoes, flies and ticks which act as disease vectors in household where as in veterinary it is also used as it is applied down the backline of beef cattle for control of horn flies and lice.

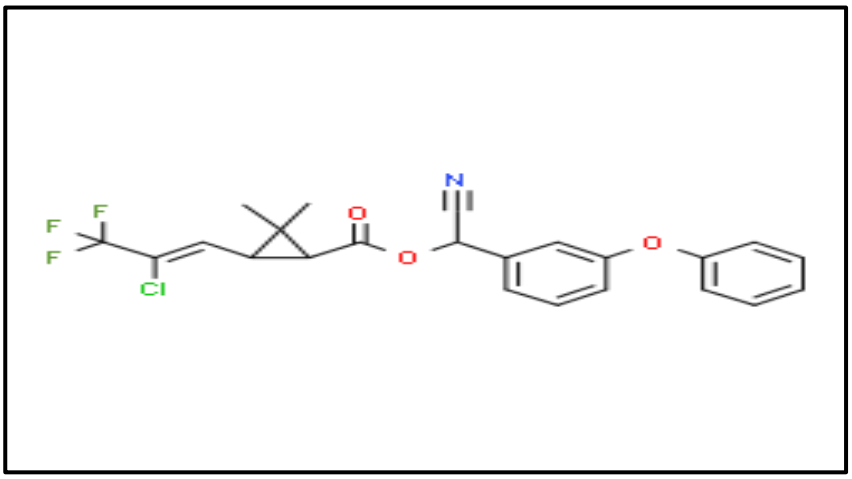


Figure 2.3 Chemical structure of Lambda cyhalothrin

2.4 Characteristics of Lambda cyhalothrin

Physical and chemical characteristics of Lambda cyhalothrin are described in table 2.1.

Table 2.1 Chemical and physical properties of Lambda cyhalothrin

Characteristics	Information
Molecular formula	$C_{23}H_{19}CLF_3NO_3$
Molecular weight (g/mol)	449.8
Physical description	Colourless-to-beige solid
Melting point (°C)	49.2
Solubility in water (mg/L)	5×10^{-3}
Odor	Mild
Octanol/water Partition Coefficient (Log[KOW])	7.00
Vapor Pressure (mm Hg at 20°C)	1.5×10^{-9}
pH	NA 2
Density (g/cm^3 at 25°C)	1.33

2.5 Working of Lambda cyhalothrin

It disturbs the normal body functions and nervous system of organisms, by dis-functioning the nervous system in the insects Lambda cyhalothrin may cause paralysis or death. Its toxicity is influenced by temperature and different outdoor and indoor insects are affected when they come into contact with it and also possess the characteristics of insect repellents.

2.6 Acute toxicity of Lambda cyhalothrin

A study has evaluated the acute effects of four concentrations of the pesticide which were 5, 50, 250 and 500 ng/ for period of 96hour and significant changes were observed in the enzymatic profiles of *P.lineatus* (Vieira & Martinez, 2018). Another study investigated the impact of individual and joint pesticides on zebrafish and it reported that Lambda cyhalothrin had the highest toxicity to the three mentioned life stages as embryonic, larval and juvenile stage among these selected pesticides (Wang et al., 2017).

2.7 Lethal dose-50 (LD-50)

The term LD-50 is defined as the concentration or amount of a substance (usually per body weight) which required to kill 50% of the test population.

2.8 Sub-Acute toxicity of Lambda cyhalothrin

Assessment of toxic effects 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. Increase in the liver-somatic index and hematological changes were monitored in the fish exposed to sub lethal concentrations of Endosulfan and Lambda cyhalothrin and combination of both pesticides for period of 96hour (Bacchetta et al., 2014). Oxidative stress was reported when fish was exposed to Lambda cyhalothrin (Piner & Üner, 2012). A set of different biomarkers is generally used to assess the toxic impacts of pollutants, and these biomarkers considered as indicators of a specific harmful biological endpoint. In order to assess the toxicity in ecotoxicology, histopathological studies and oxidative stress are used (Pandey et al., 2003). Histology of fish tissues is an authentic monitoring tool/method to assess the effects of environmental pollutants and it is one of the most reliable indicator of the health impairment caused by the anthropogenic compounds in the aquatic organisms (Fernandes et al., 2008).

2.8.1 Oxidative stress

Chemical pollution caused by pesticide in the environment is of growing concern due to their widespread uses in agriculture. In freshwater biota (fish) toxic impacts related to oxidative stress has been reported because of exposure to these pesticides and their metabolites (Mi et al., 2014). Oxidative stress happens when these contaminants disturb the vibrant equilibrium between oxidant and antioxidant enzymes due to alterations in antioxidants or enhanced per oxidative processes (ROS) or both, causing damage (Mevlüt, 2013). Variety of defense mechanisms have developed in aquatic organisms to overcome potential danger of the free radicals. The initial defense mechanism against oxidative stress is composed of antioxidant enzymes (such as CAT, SOD, 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 (Mi et al., 2014). The decreased activity of these enzymes will change the redox state of the cell. Therefore, the increase in the activity of these enzymes may help to eliminate these oxidative free radicals from cells caused by pesticide exposure.

The important defense mechanisms of organisms include enzymatic antioxidants and non-enzymatic antioxidants, which can resist environmental pro-oxidants by resisting the influence of reactive oxygen species (Tabrez & Ahmad, 2009). Therefore, antioxidant parameters and oxidative stress index are considered as potential biomarkers and are often used as screening tools to assess the impact of environmental stress. Catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione peroxidase (GPx) are important antioxidant enzymes. In addition, glutathione, vitamins and carotene can also help organisms reduce external pollutants and help protect the organism's enzyme system. A study reported oxidative stress in common carp when exposed to atrazine (Xing et al., 2012). Oxidative stress causes damage to DNA, biomolecules and reduction in antioxidant defense (Jin et al., 2011).

2.8.2 Bioaccumulation potential of Lambda cyhalothrin in fish 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.

2.8.3 Respiratory burst activity

Several changes in hematological parameters may be induced in fish exposed to different types of contaminants which are commonly used to assess fish health. Hematology is

the most common tool used to detect the physiological and pathological changes that lead to different stress conditions. Therefore, hematological parameters are the most commonly used method to assess the sub lethal effects of pollutants (Kumar et al., 2011). Several parameters like phagocytic activity, leukocyte (WBC) count, albumin: globulin ratio, and serum cortisol are pointers of improved immune-competence. Phagocytes respiratory burst activity is an indicator of innate immunity (Muthappa et al., 2014). Generally, it is 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 (Minar, 2003). Augmented respiratory burst activity can be linked with elevated phagocytes activity to kill pathogens and hence a better immunity. Studies reported reduction in carp immunological parameters when exposed with sub lethal concentration of phosalone (Kaya et al., 2014). Decrease in phagocytic activity (NBT/respiratory burst) was reported in *Labeo rohita* exposed to Endosulfan (Muthappa et al., 2014). *Cyprinus carpio* exposed to malathion (a pyrethroid pesticide) also showed similar results (Mi et al., 2014).

2.8.4 Genotoxicity in fish blood

Different studies reported several chromosomal abnormalities induced in aquatic organism as a result of pesticides and pollutants in aquatic environment. Single-cell gel electrophoresis or comet assay is a tool for measuring the number of DNA strand breaks. Comet assays have a place in both genetic toxicology and biological monitoring research, and may be used as a useful tool for evaluating various types of DNA damage and repair. This technique is particularly used to quantify DNA damage in mammalian cells, and today it can be applied to various types of organisms and cells, including fish.

2.8.5 Histopathology of fish tissues

Toxic substances in the aquatic system may induce some histopathological changes in different organs of interacting organisms. In the fish tissues exposed to Lambda cyhalothrin changes in biochemical and histological parameters were reported in a research study (Muthukumaravel et al., 2013). Significant changes in the histology and biochemistry of fishes tissues were caused by exposure of pesticides (Velisek et al., 2009).

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. Grass carp was selected as model organism for current study as it has the ability to tolerate adverse environmental conditions and stresses and high sensitivity towards changing environment. Furthermore, it is a cool to temperate water fish being the main constituent of food chain in many areas of world.

The research study was conducted by exposing grass carp (*Ctenopharyngodon idella*) with commercial grade parathyroid pesticide in semi-static tanks following APHA (2017) and OECD Guidelines No. 203, 204 (OECD 1992). Experimental study was divided into control and experimental groups. Each experimental group contains eight fish per batch for Lambda cyhalothrin exposure.

3.1 Chemicals

Commercial grade Lambda cyhalothrin (EC: 2.5) was purchased from local market, Rawalpindi. Nitrotetrazolium blue chloride (715059 Bio world, USA) and N, N-dimethylformamide (15440 Sigma-Aldrich, USA) were purchased and used for NBT reduction assay. Phosphate buffer saline tablets (Oxoid), Sodium chloride, Low melting point agarose, Normal melting point agarose, Ethylene-diamine-tetra-acetic acid, Tris(hydroxymethyl) aminomethane, Triton X-100 (Dae-Jung, Korea), Dimethyl sulfoxide (DMSO), Sodium hydroxide (NaOH), Boric acid (H_3BO_3), Tris-hydrochloride

and Ethidium bromide (Et Br) were purchased and used for gel and solution preparation for comet assay.

3.2 Purchase and Maintenance of Experimental Fish (Grass carp)

Healthy Grass carp were purchased from Punjab Hatchery Rawal Town (Aquaculture and Fisheries Program and Research Centre), Islamabad. The purchased specimens were transferred to Environmental Toxicology Laboratory of National University of Sciences and Technology (NUST) in aerated plastic bags. Special care was taken during transportation of samples and then kept in experimental tanks having dimension of 3 X 1.5 X 1.5 ft. Experimental tanks were filled with tap water supplied in laboratory. Fish were acclimatized to laboratory conditions for two weeks and fed with commercially available food pellets.

3.2.1 Acclimatization of fish

Before the start of experiment fish were acclimatized to laboratory conditions for a period of two weeks under control conditions. They were fed with commercial food pellets containing soybean, rapseed, rice, bran, corn, wheat and other agricultural by-products, on daily basis. Experimental tanks were filled with 50 liters of tap water

from Environmental Toxicology Laboratory and changed on alternate day, to avoid fouling of tanks dead fish were removed immediately.

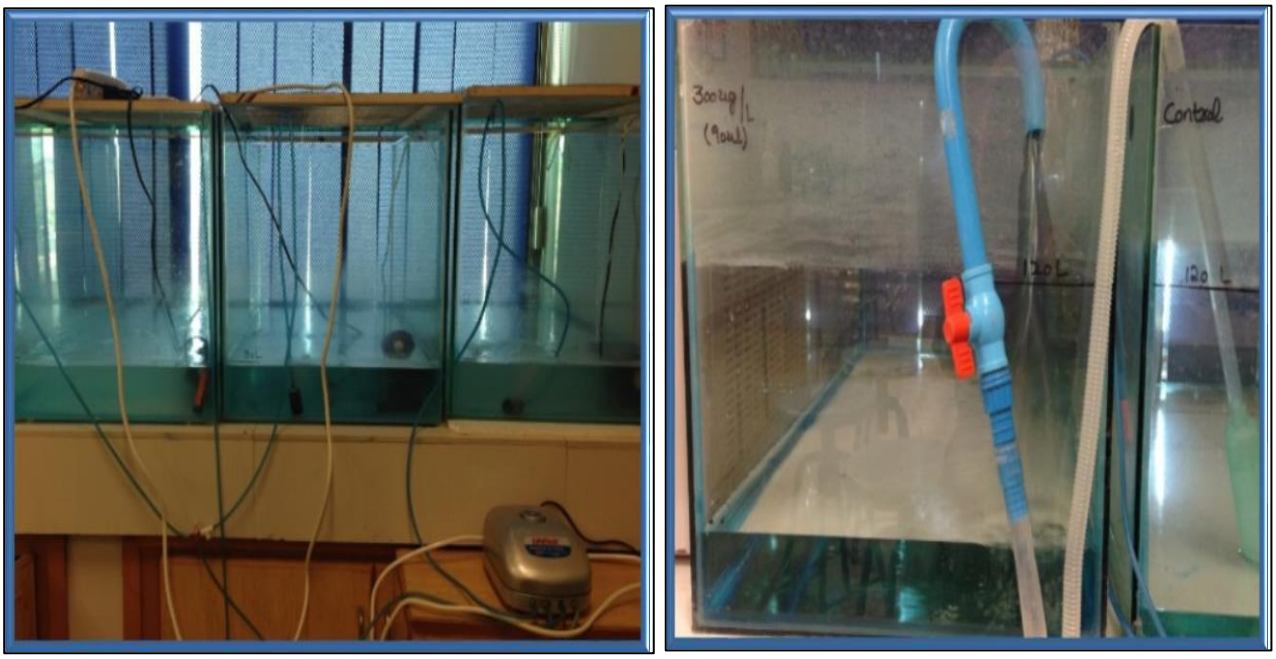


Figure 3.1 Experimental tanks

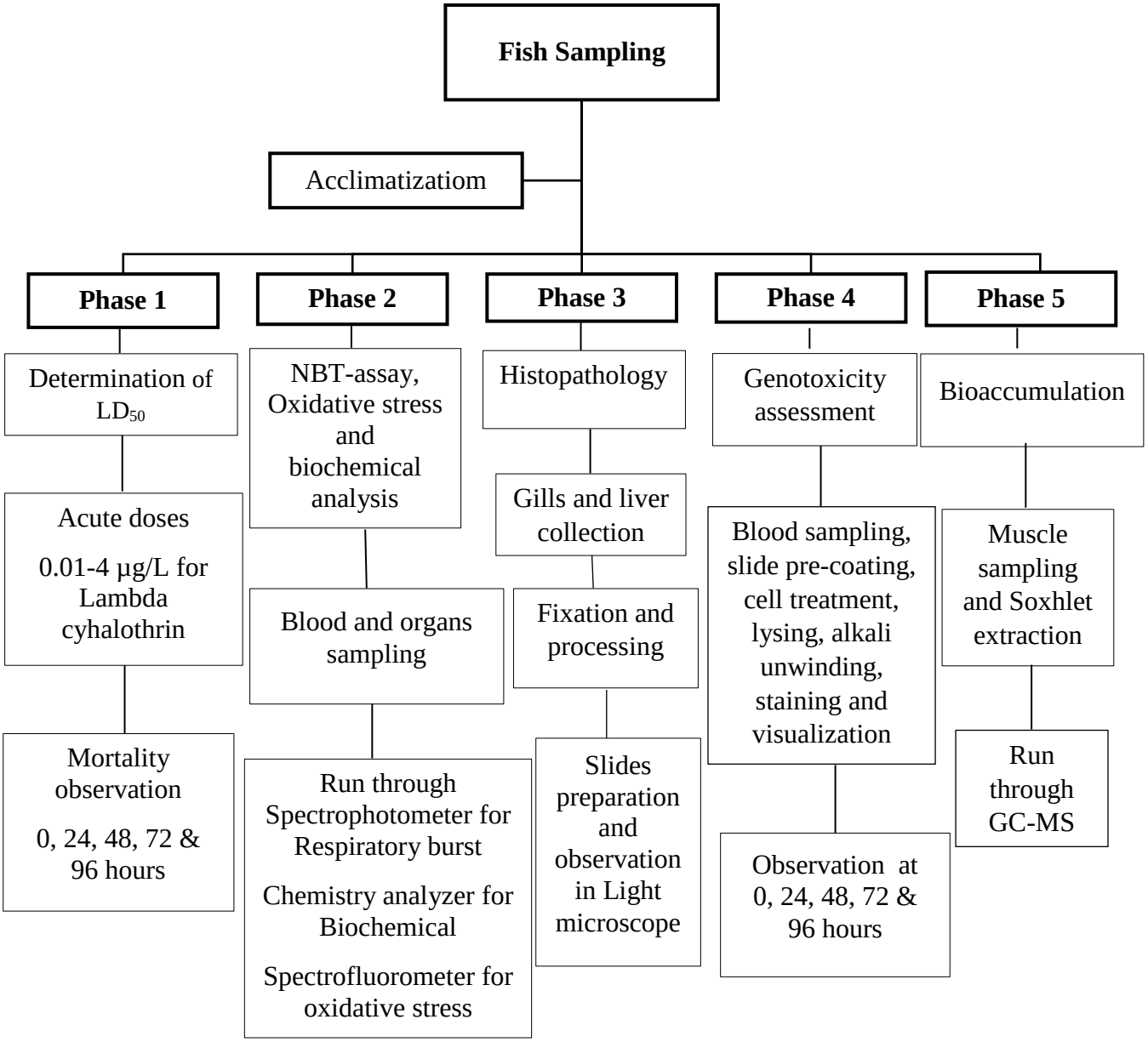


Figure 3.2 Experimental design of research work

3.3 Site description

Rawal Lake is the research area of this study. The lake is located in the east of Islamabad and the northeast of Rawalpindi (33°42′N, 73°07′E). It provides water for two cities and covers an area of 8.8 square kilometers. The storage capacity of the Rawal Dam is 47,500 acre-feet, and the average rainfall can produce 84,000 acre-feet of water. The research site is under tremendous pressure from different human settlements (such as Bhara Kahu, Malpur, Bani Gala and Noorpur Shahan, etc.). Untreated municipal waste (mainly domestic waste and agricultural waste) is dumped directly into the reservoir, which increases the need for disinfection. Therefore, the toxic by-products produced exhibit cellular and genotoxic effects in aquatic organisms. The map below clearly describes the research area.

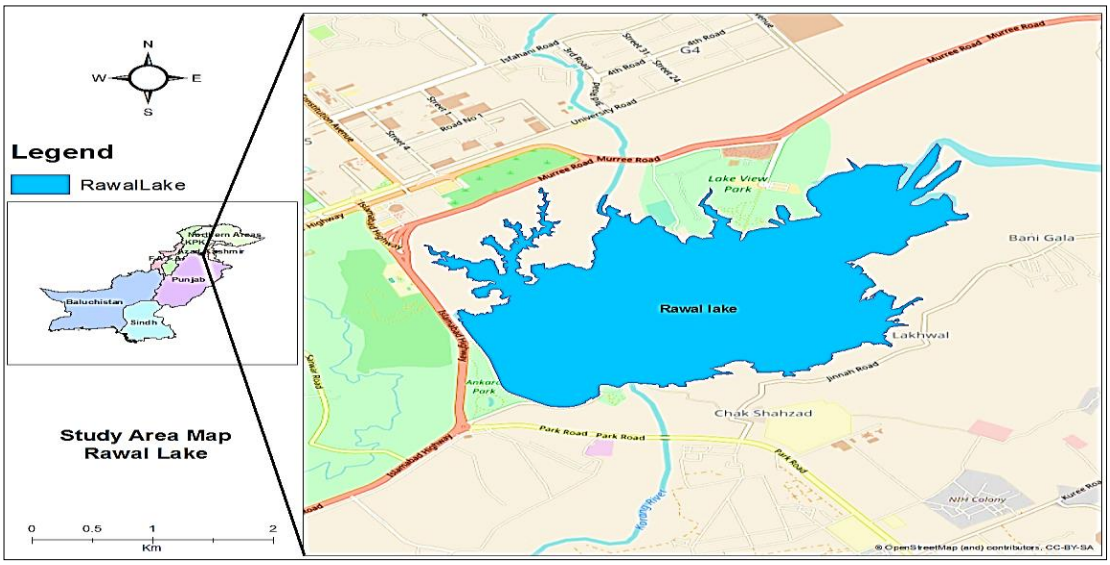


Figure 3.2 Study area for research

3.4 Physicochemical parameters of water

The physicochemical characteristic of experimental tank and lake water was assessed using standard OECD (Organization for Economic Cooperation and Development) guideline method, 203, (1992). Water quality was determined at the start of experiment. The water was renewed at every alternative day using tap water from the Environmental Toxicology Laboratory. pH and temperature were measured using Multi parameter analyzer, Consort- C1020. Dissolved oxygen (DO) was measured using Winkler method, whereas titration method was followed to measure total hardness. Fresh water was provided to fish to avoid any damage to tissues or organs.

Table 3.1 Physicochemical parameters of tank water

Parameters	Values
Temperature (°C)	27.8 ± 0.5
pH	6.94 ± 0.2
DO (mg/L)	5.05 ± 0.1
Hardness (mg $CaCO_3$/L)	380 ± 0.5
Alkalinity(mg/L as $CaCO_3$)	394 ± 0.5

3.5 Acute toxicity test

Acute toxicity test was carried out in semi-static tanks in order to determine lethal dose of Lambda cyhalothrin for grass carp following OECD guidelines 203 (OECD, 1992).

Acclimatized fish were evenly divided into experimental and control groups in 50 L experimental tanks containing 10 fish in each. A range of doses of Lambda cyhalothrin from 0.001µg/L to 4 µg/L were applied according to literature and mortality was noted to determine lethal dose for grass carp. Before two days of the exposure feeding was stopped. Dead fish were removed immediately. The behavior of both control and test fish were keenly observed.

3.6 Sub-acute toxicity test

A set of 10 fish in each tank was exposed to Lambda cyhalothrin for 96hour at following sub lethal concentration: 0.25,0.5,0.75,1,1.25 µg/L which corresponds to 96hour LD-50 of Lambda cyhalothrin for grass carp. Each experimental condition was duplicated along with the control experiment. After every 24 hours, three fish were randomly selected from each tank. Blood samples were collected using heparinized syringes by caudal vein puncture to check respiratory burst activity and biochemical parameters of blood. Fish were decapitated after collection of blood samples. Brain and gill tissues were carefully removed for oxidative stress analysis.

3.7 Comet Assay

Comet assay analysis was performed according to method reported in literature (Singh et al., 1988) with some upgradations (Tice et al., 2000). It can be used to detect single-strand breaks and alkaline unstable damage in the DNA of a single cell. The series of steps followed by the comet assay is as follows.

3.7.1 Preparation of reagents

Agarose preparation

1. Normal melting point agarose (NMPA) was prepared 1 % by mixing powdered agarose (500 mg) with phosphate buffer saline (PBS, 50 ml) in a glass beaker. It was placed in the 90 °C water bath for 10-15 minutes.

2. 0.5 and 1 % low melting point agarose (LMPA) with PBS in the same way as NMA was prepared.

Lysing solution

In order to prepare lysing solution of 500 mL, 2.5 M NaCl ,100 mM EDTA and 10 mM Tris base was used. All these ingredients were added in 350 mL distilled water and mixed properly. 4 g of NaOH was added to the mixture and allowed it to dissolve for 20 minutes. The pH of the solution was adjusted to 10 with concentrated HCl or NaOH. Solution was made 445 ml with distilled water. Finally, at the time of use:

1. 1% Triton X-100 (50 ml)
2. 10% DMSO (5 ml)

were freshly added to the solution to make up 500 ml solution.

Alkaline solution

Stock solutions of NaOH and EDTA were prepared as follows:

1. 10 N NaOH
2. 200 mM EDTA

15 ml of NaOH and 2.5 ml of EDTA were mixed with distilled water to make solution 500 ml. The pH of electrophoresis buffer was also adjusted to >13.

TBE Electrophoresis buffer

For 500 ml alkaline solution following ingredients were added.

1. Tris base (5.4 g)
2. Boric acid (2.7 g)
3. EDTA (0.93 g)

All the given ingredients were added to 500 ml distilled water and mixed well. The pH of alkaline solution was maintained to >13.

Neutralization solution

Neutralization solution was prepared by adding 0.4 M Tris to 400 ml distilled water. The pH was adjusted to 7.5 with concentrated HCl (10 M). More distilled was added to make up 500 ml solution.

3.7.2 Preparation of slides

Slides pre-coating

Microscope glass slides were immersed in 70 % ethanol and burnt over blue flame to remove oil and dust. Slides were then layered with a smooth and very thin layer of 1 % normal melting point agarose (NMPA) and laid in a tray on a flat surface to let drying at room temperature.

Sample pouring

A thin smooth layer of suspension of sample (65µl of 0.5 % low melting point agarose (LMPA) with 20µl of blood) was poured on the previously coated slides and covered with cover slip immediately. Agarose was allowed to cool in refrigerator until it

hardened. Each slide was pre-labeled. Slides were then transferred in a tray and refrigerated for at least 20-30minutes.

Lysing

After solidification of agarose gel, prepared slides were properly submerged in ice cold lysing solution in order to maintain the solidity of agarose gel. Slides were kept in refrigerator for 24hours in a covered slide boxes.

Alkali unwinding

After lysing period, slides were then transferred to highly alkaline buffer (pH>13) for unwinding of damaged DNA fragments for a period of 20-30 minutes.

Electrophoresis

After alkali unwinding, to perform electrophoresis, the prepared slides were placed in Horizontal Gel Electrophoresis tank. The tank was filled with ice cold TBE buffer, making sure that gel on the slides was completely immersed in the solution. The voltage of the chamber was set at 25V and was left to run for 45 minutes.

Neutralization

After electrophoresis, slides were neutralized, using neutralization solution to remove background interferences. Slides were neutralized for 5 minutes and this step was repeated twice or thrice times.

Staining and drying

After neutralization slides were stained with 80µl of 1x ethidium bromide staining solution at room temperature 2-3 times until development of yellowish brown color. After staining slides were rinsed properly with distilled water to remove excess stain. After staining, comet slides were air dried for 30 minutes to remove moisture. Slides were then prepared to be visualized.

3.7.3 Visual Analysis

Slides were visualized to determine DNA damage by observing the stained comets using 100x objective with Trinocular Fluorescent Microscope (Optika- B353FL) equipped with ocular micrometer of 10µm, camera (AIPTEK: AHD-Z600) and white LED/12 V 20 W illuminator. Images were captured and tail length was measured with ocular micrometer.

Image Analysis

The images obtained from each slide were further processed using CASPLAB software. Comets stained with EtBr (red cells on dark background) were analyzed. The image is processed to give values of % DNA damage in numerical form (Konca et al., 2003).

3.8 Oxidative stress

Oxidative stress was determined by methods of (Zhang et al., 2008) and (Driver et al., 2000). Oxidative stress in brain and gill tissues was quantified by measuring reactive oxygen species (ROS) using 2,7-dichlorofluorescin diacetate (DCFH-DA). Brain and gill tissues were extracted carefully and homogenized using homogenizer (Wise TIS 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 5µl of DCFH-DA was added and incubated for 30 min at 37°C. Spectrofluorometer (F4000 Hitachi) was used to monitor the conversion of DCFH to the fluorescent oxidation product

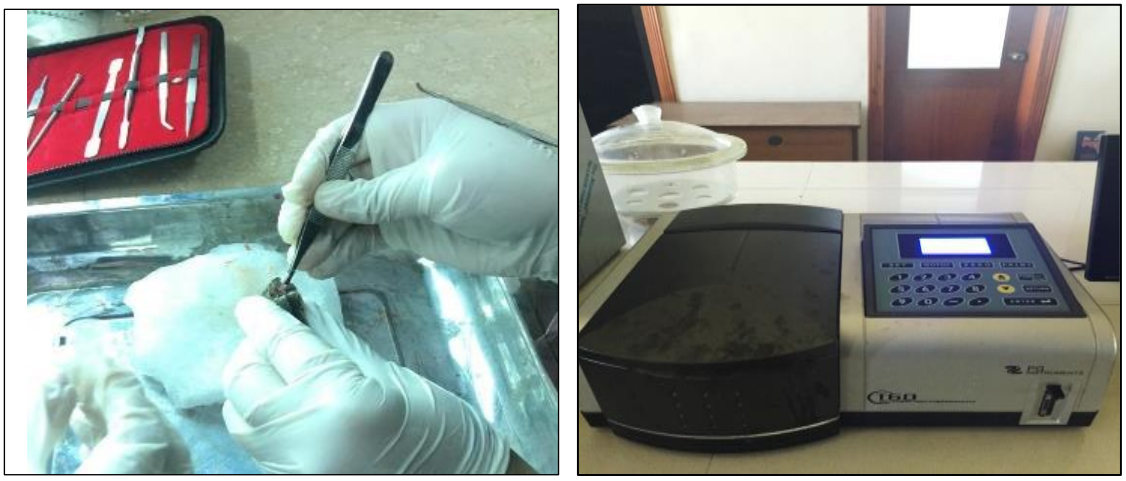


Figure 3.3 Extraction of tissue samples and spectrofluorometer used for ROS measurement

dichlorofluorescein (DCF), with excitation/emission wavelengths of 485/525 nm. By including parallel blanks, background fluorescence is corrected (DCFH is converted to DCF in the absence of homogenate).

3.9 Respiratory burst activity

In order to estimate immuno-hematological parameter, NBT reduction assay was conducted by following the protocol of (Zanuzzo et al., 2014) with some modifications. 0.1 ml of heparinized blood was co-incubated with an equal volume of 0.2% of 21 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.

3.10 Biochemical analysis by chemistry analyzer

Fish blood samples were collected from each tank after 24.48,72 and 96 hours in gel activator tubes. Blood serum was separated by centrifugation of blood samples at 1000rpm for 25 minutes. Blood serum was used further by adding different reagents analyzer using commercially available reagent kits. to check parameters by chemistry

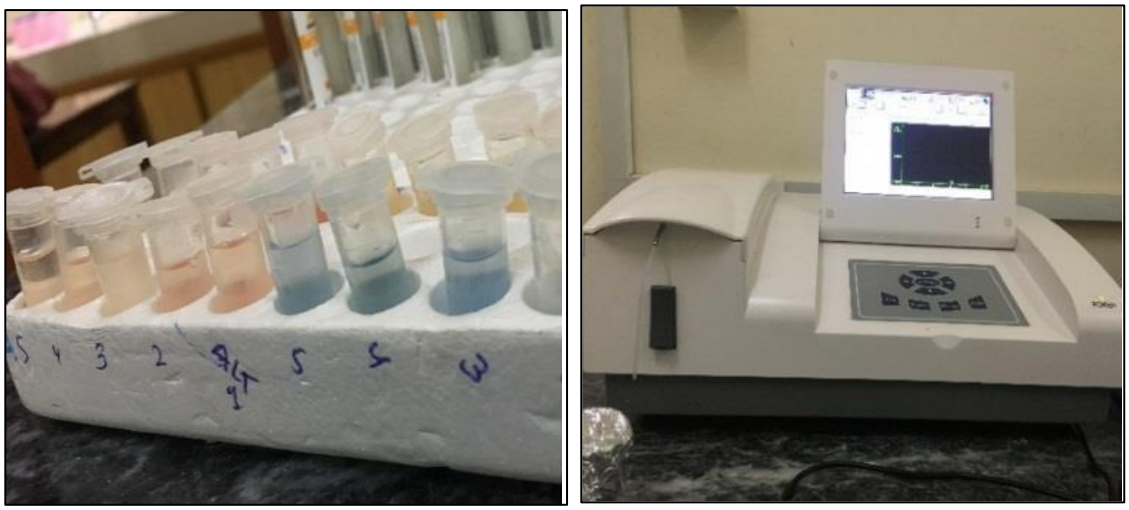


Figure 3.4 Collection of blood, addition of reagents and chemical analyzer

analyzer. Blood glucose, ALT, total protein, triglycerides, calcium and ALH levels were monitored.

3.11 Histopathological studies of gills and liver tissues

Fish from sub-acute toxicity experiment was selected randomly at every 24 of 96 hour exposure. Selected fish was dissected and its gills and liver were removed. Removed tissues were preserved in formalin solution in glass containers for further histopathology analysis.

3.12 Preparation of samples

After exposure of every 24 hours, the fish in the control and experimental groups were dissected. Carefully removed the liver and gills, and immerse them in Bouin's liquid. Once the fish's tissue has been fixed for 24 to 30 hours, the tissue is dehydrated with a series of gradients of ethanol, then removed in xylene and then immersed in paraffin.

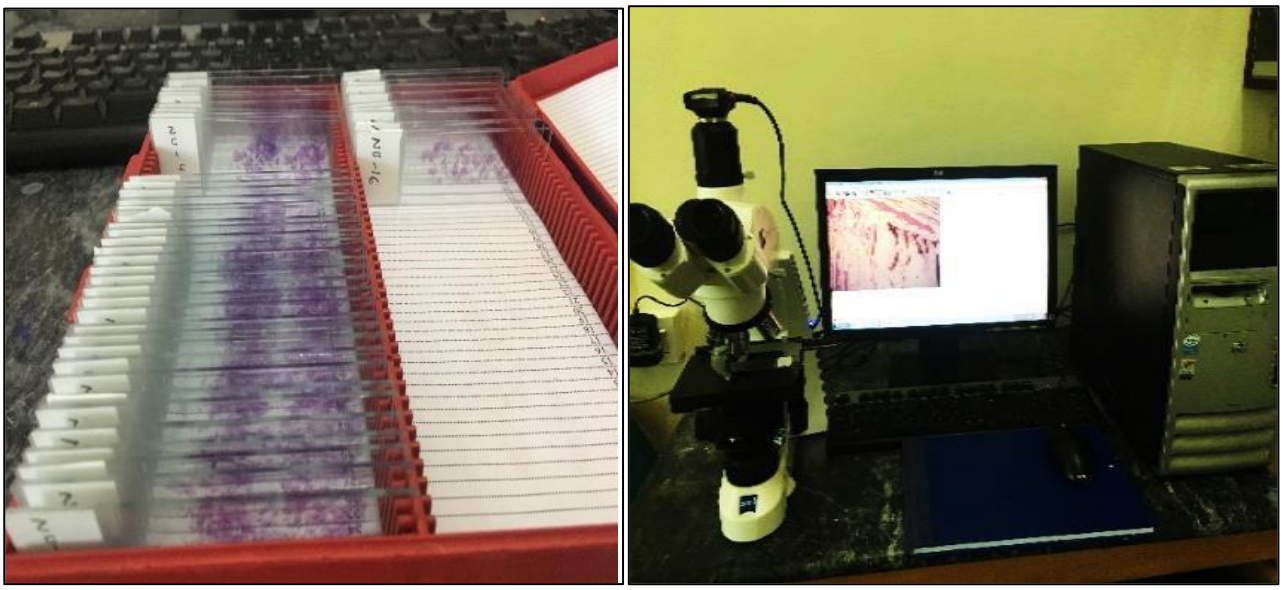


Figure 3.5 Histopathological slides and light microscope for observation of slides

Used a rotary microtome to prepare small sections of 4-6 mm from the paraffin block. Hematoxylin-eosin was used to stain these sections. The histological preparations were randomly inspected 3 times, and the results of each observation were combined into the final result. Examined histopathological lesions and take pictures with an optical microscope.

3.13 Bioaccumulation potential

To assess the bioaccumulation potential of Lambda cyhalothrin in fish tissues, fish was dosed for 96 hours and samples were collected after every 24 hours. For sample collection fish was selected randomly from each of the 5 dosed and one control tank. Fish was sacrificed and its muscle tissue was extracted tissues were preserved in glass containers with formalin to store is for delayed analysis of Gas chromatography mass spectrometry to analyze accumulation of subject pesticide in fish tissues.

3.13.1 Sample preparation

Fish dorsal muscle tissue was weighed 5g and mixed with sodium anhydrous sulfate for dehydrating the sample tissue and cleaned properly from salt. After dehydration its dry

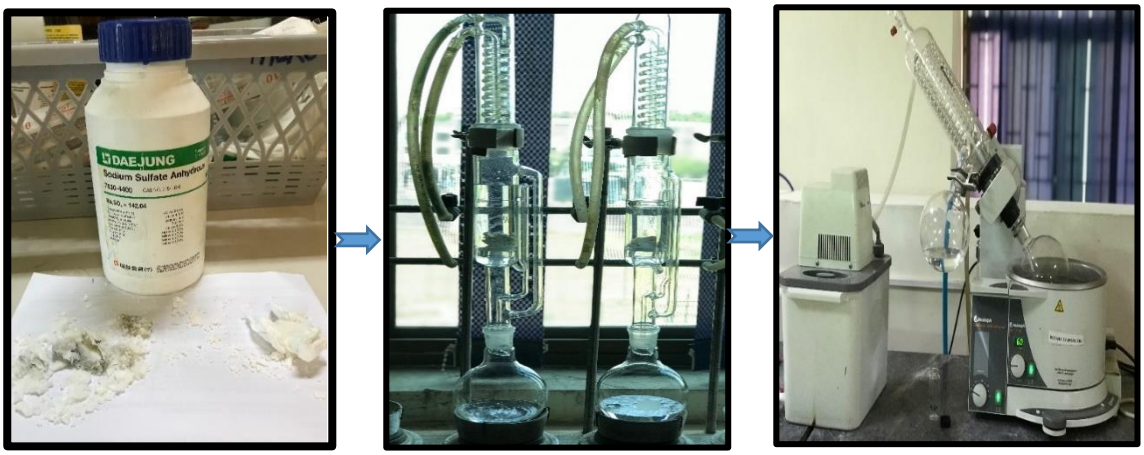


Figure 3.6 Sample extraction for GC-MS analysis by Soxhlet extraction method

weight was measured. Samples were placed in glass thimble. Acetone and chloroform (1:1) were added in Soxhlet flask to carry out sample preparation by Soxhlet extraction method. Samples and flasks were placed in Soxhlet assembly and process carried out for 5 hours (1 cycle/ hour). After Soxhlet extraction solvent was further placed in rotary evaporator for drying.

3.13.2 Chromatographic conditions

Pesticides residues in muscle tissues have been detected by GC/MS system (Shimadzu GC/MS-QP2010 Plus, Japan) equipped with a capillary column DB-5MS (30m length, 0.25 mm thickness, 0.25m diameter). The temperature of injector was 250ºC. The temperature program of oven started with 60ºC. Hold 6 min at 7ºC/min to 200ºC, at 5ºC/min to 280ºC. Hold 20 min; carrier gas, helium; flow rate, 1.2 ml/min; injection port temperature, 250ºC; injection volume, 1ul; injection mode, split less, purge on after 1.5 min; ionization voltage, 70 eV; GC/MS interface temperature, 250ºC.

3.14 Standard solution of Lambda cyhalothrin

250 µg/L of stock solution was prepared by dissolving standard Lambda cyhalothrin PESTANAL^{TM} analytical standard (31058- Sigma Aldrich) (purity ≥ 95%) in GC-MS grade n-hexane. Six dilutions (0.1, 0.25, 0.5, 0.75 and 1.25 µg/L) were prepared to formulate calibration curve and line equation to determine unknown concentration in the sample.

Table 3.2 Chromatography conditions of GC-MS

Column	DB-5MS
Injector temperature	250^{o}C
Carrier gas	Helium
Flow rate	1.2 ml/min
Injection port temperature	250^{o}C
Injection mode	Split less
Ion source temperature	220^{o}C
GC/MS interface temperature	250^{o}C

3.15 Statistical analysis

Statistical analyses were conducted using SPSS software package version 16.0. Data was analyzed using one-way analysis of variance (ANOVA) followed by Lowest significant difference (LSD). All data were expressed as mean ± STD for all experimental and control animals. $P < 0.05$ was considered significant compared to control.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1Determination of LD-50 of Lambda cyhalothrin

To determine acute toxicity, test was performed in semi-static tanks to fix lethal dose of Lambda cyhalothrin for grass carp following OECD guidelines 203,204 (1992). Acclimatized fish were evenly divided into experimental and control groups in 50L experimental tanks containing 10 fish in each. A range of doses of Lambda cyhalothrin from 0.001 to 4 µg/L were applied according to literature and mortality was noted to determine lethal dose for grass carp. Temperature influenced the experiment by showing different mortalities at same dose of pesticide at different temperatures. Due to seasonal variation LD-50 determined at 27 and 13ºC was 1.17 and 1.5µg/L respectively. LD-50 was determined at two different temperatures as toxicity of pesticides vary with the change in temperature of water as reported in the literature.

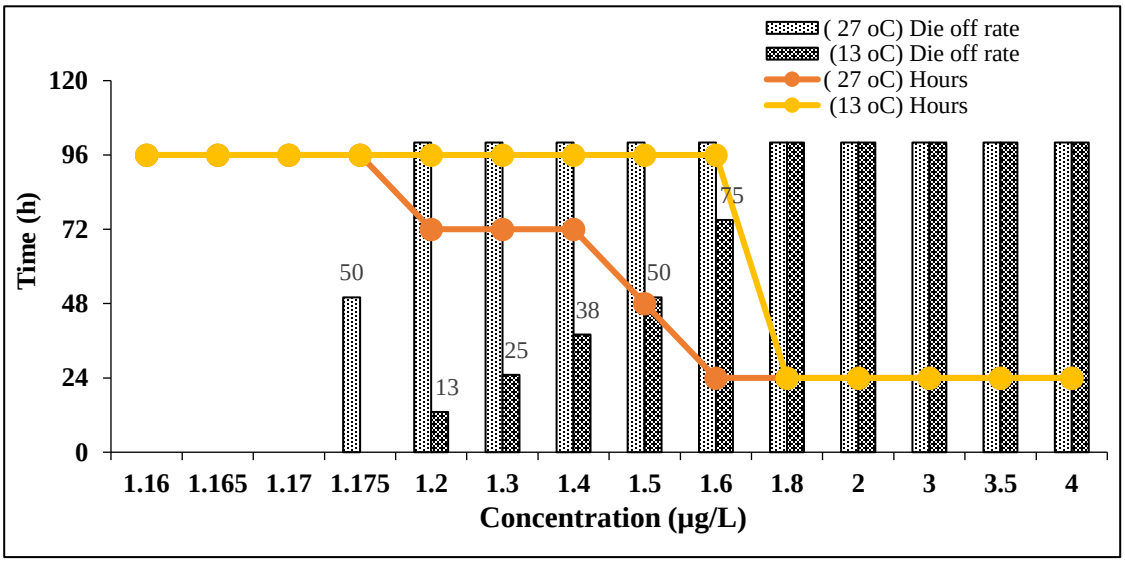


Figure 4.1 LD-50 of Lambda cyhalothrin at two different temperatures

The 96 hours LC-50 values of cypermethrin (a pyrethroid) were found to be 2.1μg/L and 1.4μg/L in T and T2 (30°C), respectively. The 96 hours LC values of cypermethrin at T2 (30°C) was found to be significantly (*P<0.05*) lower compared with calculated LC50 values of cypermethrin at T1 (25°C) transformation of percentage mortality and log concentration of cypermethrin (MH Uddin, et al., 2018). Endosulfan was more toxic to silver perch at 30°C and 35° C than at 15°C, 20°C and 25°C during short exposures of 24 h, but at 96 hours, temperature had no effect on toxicity (Patra et al., 2015). The malathion (a pyrethroid) concentration that resulted in 50% mortality (LC-50; 274.1 μg/L) of the Chinook salmon at 19° C was significantly less than the LC-50 at 11° C (364.2 μg/L) (Dietrich et al., 2014). The concentrations of toxic contaminants which are not active at low and moderate temperature proved lethal with increase in water temperature (Mehta, 2017). Dissolved oxygen decreased and free CO_2 increased significantly (*P<0.05*) with increasing temperature, while the pH of the water was almost unchanged throughout the study period. The present study indicated the impact of increased temperature on pesticide toxicity in the aquatic ecosystem (Uddin et al., 2018).

4.2 Respiratory burst activity in fish blood

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. Results were analyzed statistically by SPSS 16.0. analysis of variance (ANOVA) was applied to check the level of significance among experimental and control groups. There was significant ($P < 0.02$) increase in respiratory burst activity was observed in the exposed group as compared with the control.

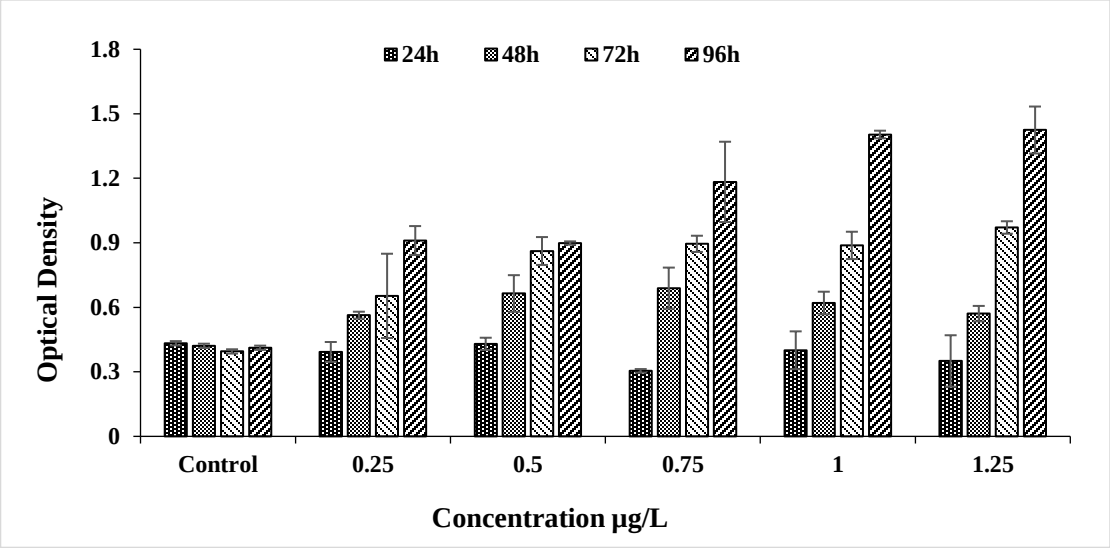


Figure 4.2 Respiratory Burst Activity in fish blood by NBT Assay

4.3 Oxidative stress in brain and gills

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 carboxylation

and DNA damage (Xing et al., 2012). Results of the current study indicated that ROS production augmented as a function of lambda cyhalothrin dose and exposure time both in brain and gills of exposed fish. The values for experimental group were significantly higher *(P < 0.02)* than the control group. Lambda-cyhalothrin caused oxidative stress in fish (Piner & Üner, 2012). 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).

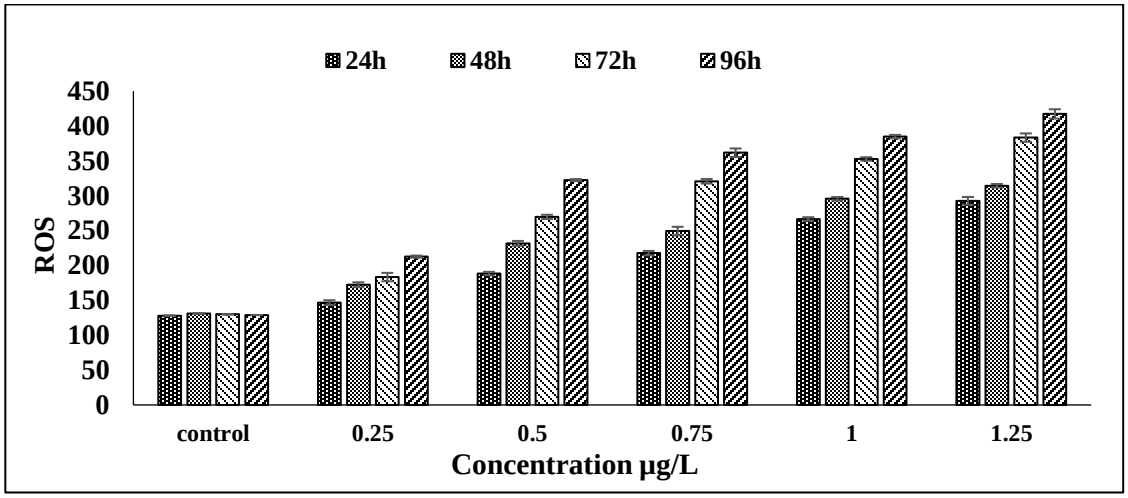


Figure 4.3 Oxidative stress in brain due to Lambda cyhalothrin toxicity

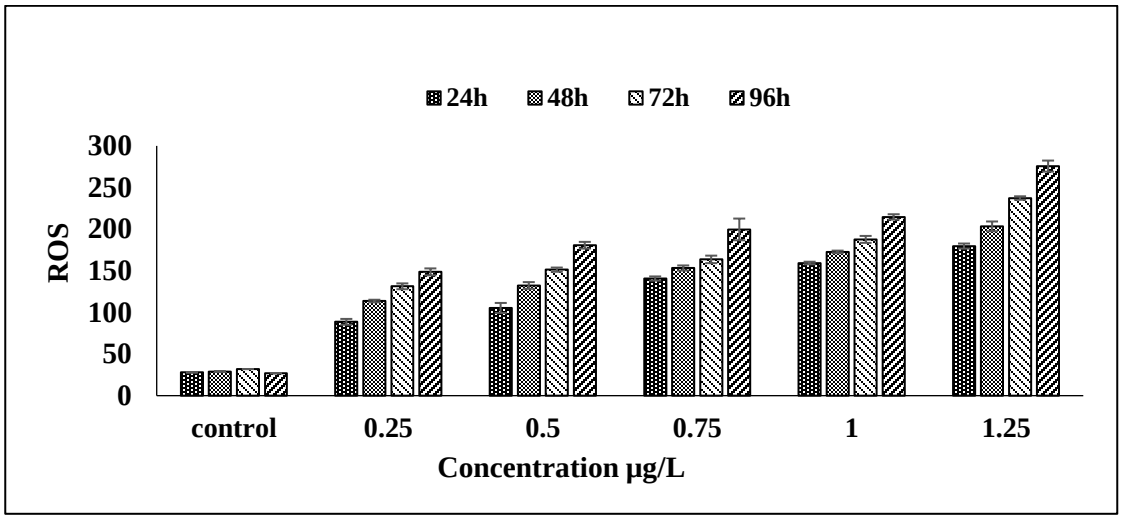


Figure 4.4 Oxidative stress in gills due to Lambda cyhalothrin toxicity

4.4. Biochemical analysis of fish blood

In this study, blood glucose level in exposed fish was increased with the concentration and time period till 72 hours. Then, decline in blood glucose level was observed at 96 hours. Increase in blood glucose level is a general response of fish to acute pollutant effects including organophosphates and pyrethroids (Lusková et al., 2002). There was a significant increase ($P<0.05$) in blood glucose level (mg/dl) in 0.25 µg/L compared to 0 µg/L concentration of cypermethrin at both treatments. The quantity of protein is dependent on the rate of protein synthesis, or on the rate of its degradation. The quantity of protein may also be affected due to impaired incorporation of amino acids in the poly peptide chains (Singh et al., 1996). The results of sub-lethal exposure to Lambda cyhalothrin indicated dose dependent elevations glucose, and triglyceride levels in the serum. The control values of protein and cholesterol were lower than in exposed fish. In this study the blood sugar gradually increasing with increase in exposure period.

There were time dependent elevations in the serum values of glucose, protein and ALP and there was time dependent significant inhibition in cholesterol, triglyceride, GOT and GPT serum enzymes (Auta, 2007).

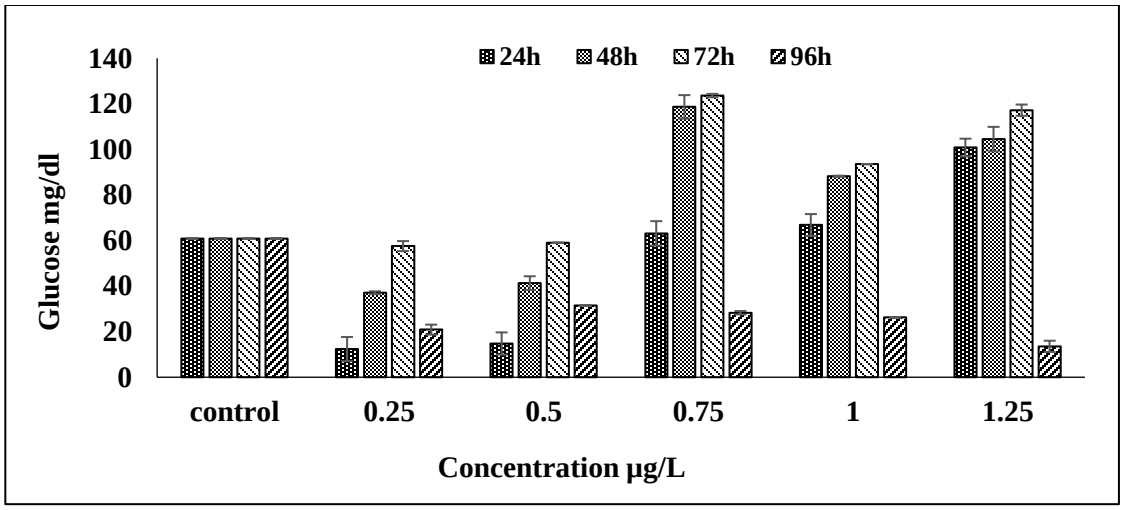


Figure 4.5 Blood glucose level in control and experimental group

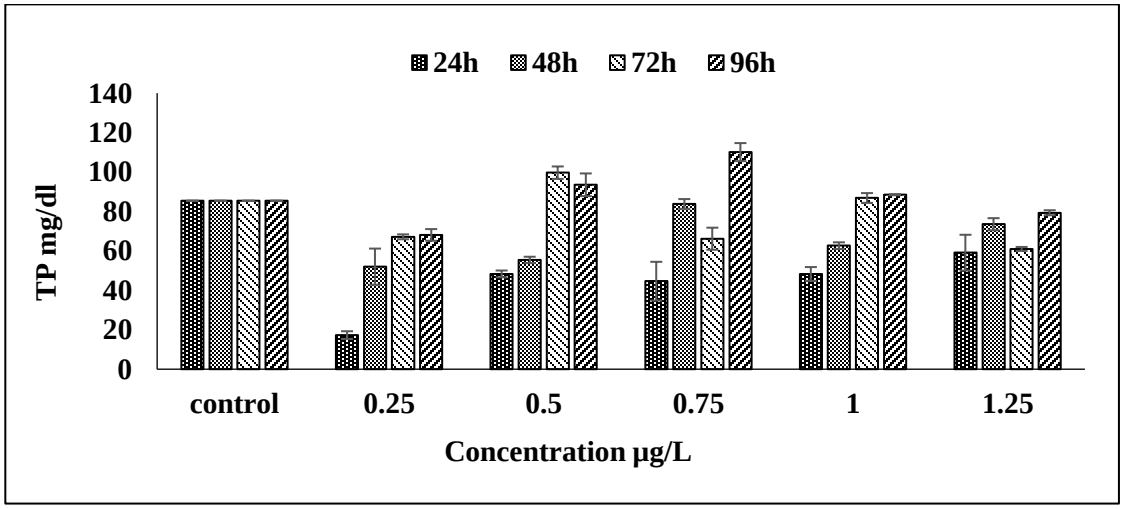


Figure 4.6 Total protein level in control and experimental group

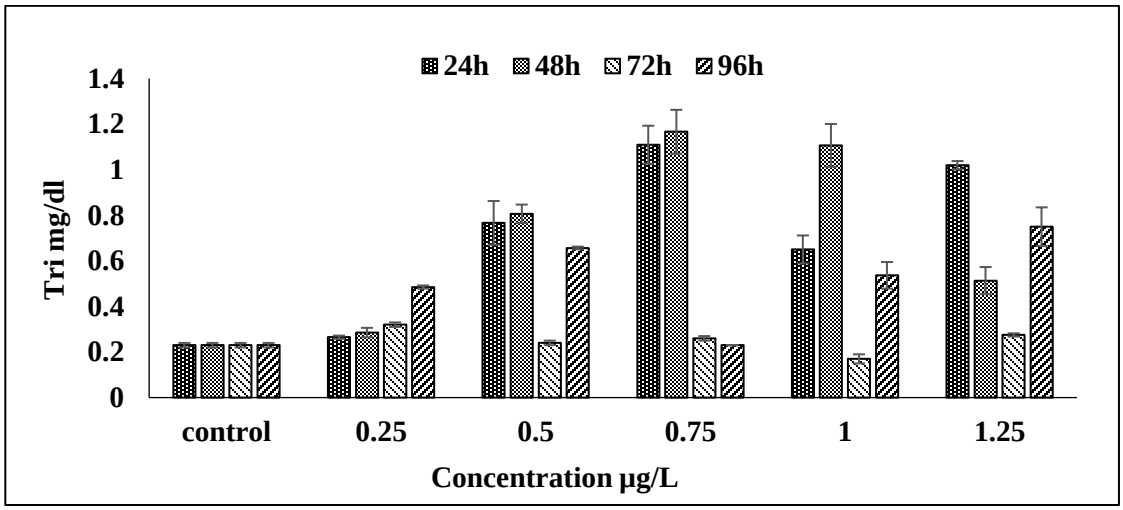


Figure 4.7 Tri glycerides in control and experimental group

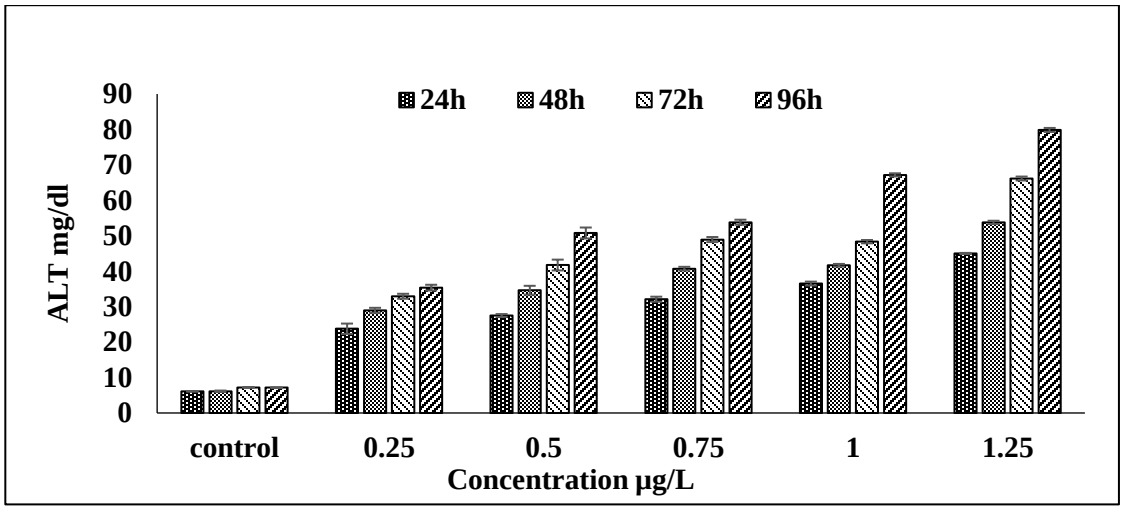


Figure 4.8 ALT level in control and experimental group

4.5. Histopathology of gills and liver

In the present study, the gills and liver of grass carp in control group was shown a normal structure and liver and gills of the Lambda cyhalothrin treated groups showed several pathological changes throughout the experimental period.

4.5.1 Histology of fish liver

In control group of *Ctenopharyngodon idella*, the liver is made up of continuous mass of hepatocytes arranged in irregular cords. The hepatic cells are polygonal in shape with distinct central nuclei. A large number of blood sinusoids were also seen around the hepatocytes.

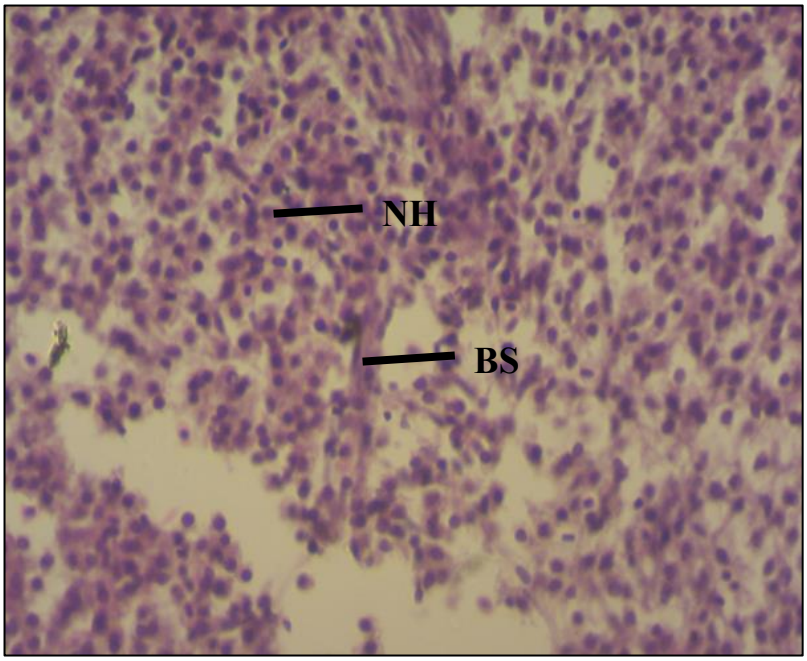


Figure 4.9 Control liver with normal architecture

Histopathology of liver tissue under Lambda cyhalothrin toxicity

1. PN- pycnotic nuclei
2. C- cytolysis
3. V- vacuolization
4. N-necrosis

Pycnotic nuclei were observed in the liver sample of fish exposed to 0.25µg/L of Lambda cyhalothrin after 24 hours. Toxicity directly damages liver tissues as in this study pycnotic nuclei and cytolysis were observed in the liver tissue exposed to 0.5 µg/L

of Lambda cyhalothrin after 24 hours. Vacuolization and cytolysis was observed in 0.75 µg/L dosed fish after 24 hours. Vacuolization, necrosis and Pycnotic nuclei was observed in 1µg/L dosed fish after 24 hours. Cell structure got more ruptured due to pesticide exposure vacuolization and Pycnotic nuclei was observed in 1.25µg/L dosed fish after 24 hours. Cell structure got ruptured due to pesticide exposure and marked toxic effects were observed at the structural and cellular level in the liver.

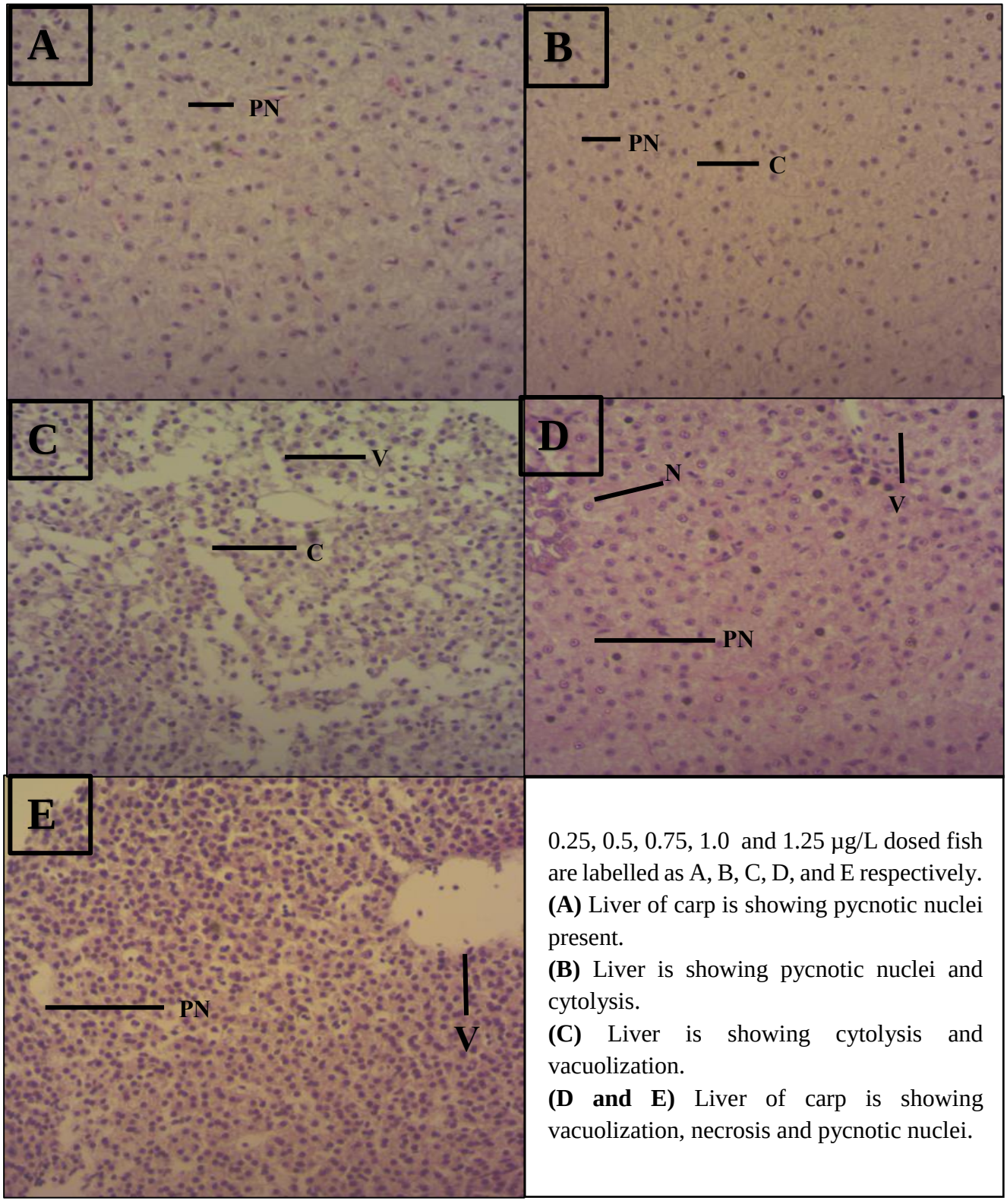
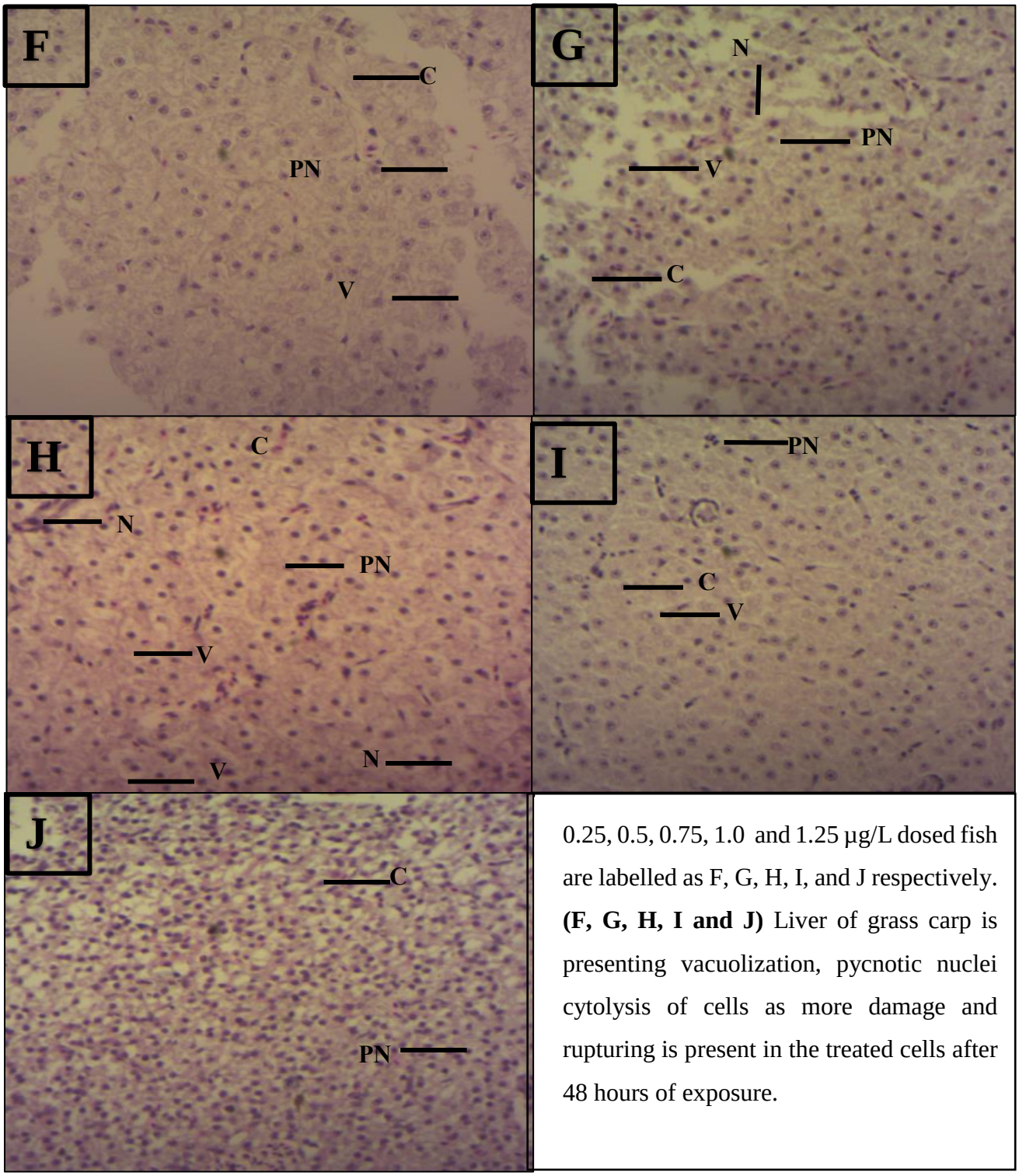


Figure 4.10 Histopathological changes induced in fish liver exposed to Lambda cyhalothirn after 24 hours of exposure

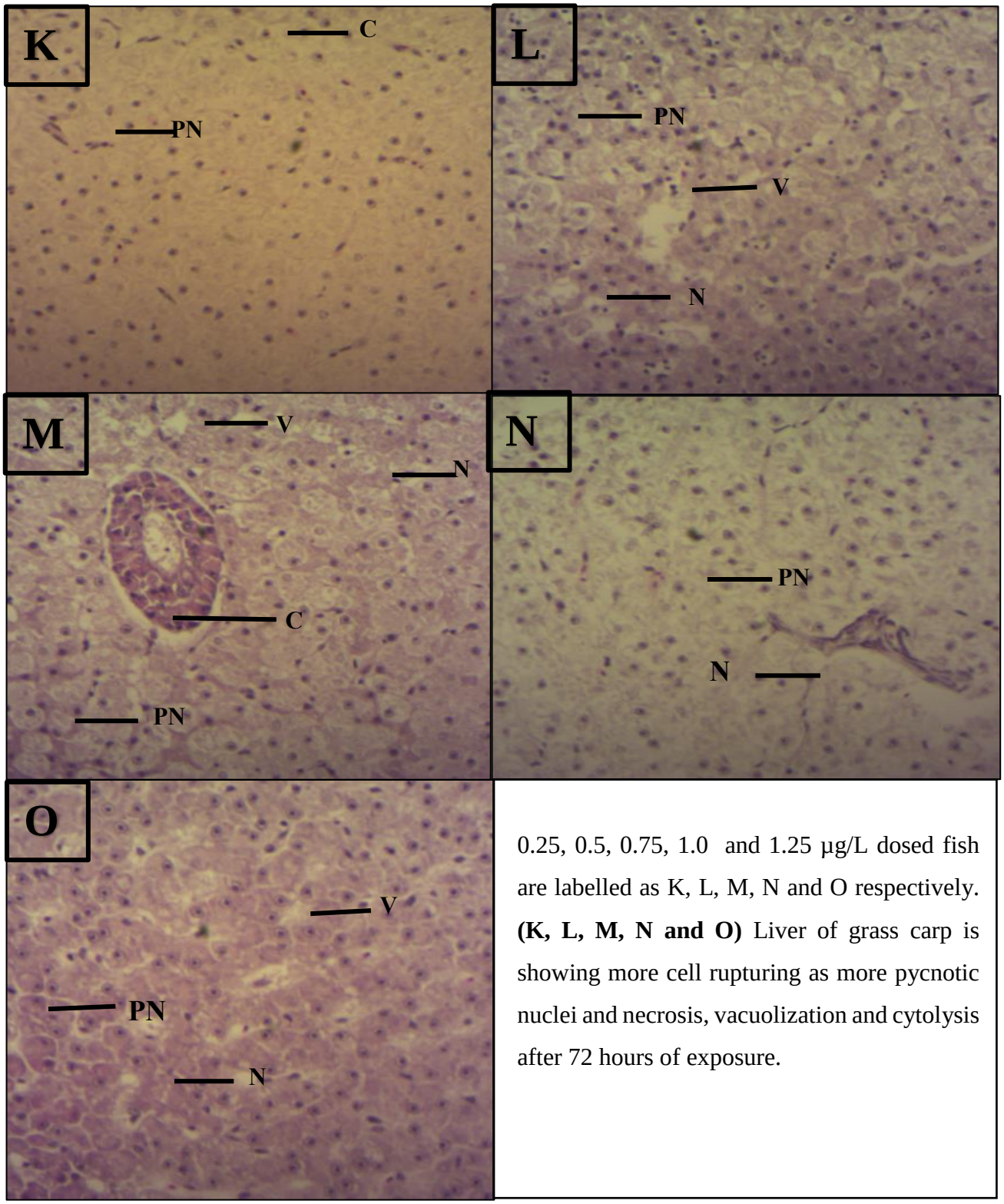


0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as F, G, H, I, and J respectively. **(F, G, H, I and J)** Liver of grass carp is presenting vacuolization, pycnotic nuclei cytolysis of cells as more damage and rupturing is present in the treated cells after 48 hours of exposure.

Figure 4.11 Histopathological changes induced in fish liver exposed to Lambda cyhalothirn after 48 hours of exposure

After 48hours exposure to Lambda cyhalothrin liver tissues were more damaged. There was more vacuolization present along with Pycnotic nuclei and cytolysis of cells. Vacuolization, cytolysis and Pycnotic nuclei was observed in 0.5µg/L dosed fish after 48 hours. Cell structure got ruptured due to pesticide exposure. In this study, it was observed that cell damage in liver tissues increased with increase in concentration and exposure duration. After prolonged exposure, many hepatic cells were completely damaged. Intracellular vacuolation was also apparent. vacuolization, necrosis, cytolysis and pycnotic nuclei was observed in 0.75µg/L dosed fish after 48hours. Vacuolization, necrosis, cytolysis and pycnotic nuclei was observed in 1µg/L dosed fish after 48 hours. More vacuolization, necrosis, cytolysis and pycnotic nuclei was observed in 1.25µg/L dosed fish after 48 hours.

Cytolysis and pycnotic nuclei were observed in 0.25µg/L dosed fish after 72 hours. Cell structure got damaged due to toxicity of Lambda cyhalothrin. Vacuolization, necrosis and pycnotic nuclei was observed in 0.5 µg/L dosed fish after 72 hours. Vacuolization, necrosis, cytolysis and pycnotic nuclei were observed in 0.75µg/L dosed fish after 72 hours. Necrosis and pycnotic nuclei were observed in 1µg/L dosed fish after 72 hours. Cell structure damaged due to toxicity of lambda cyhalothrin. Vacuolization, necrosis and pycnotic nuclei was observed in 1.25µg/L dosed fish after 72 hours.



0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as K, L, M, N and O respectively. **(K, L, M, N and O)** Liver of grass carp is showing more cell rupturing as more pycnotic nuclei and necrosis, vacuolization and cytolysis after 72 hours of exposure.

Figure 4.12 Histopathological changes induced in fish liver exposed to Lambda cyhalothirn after 72 hours of exposure

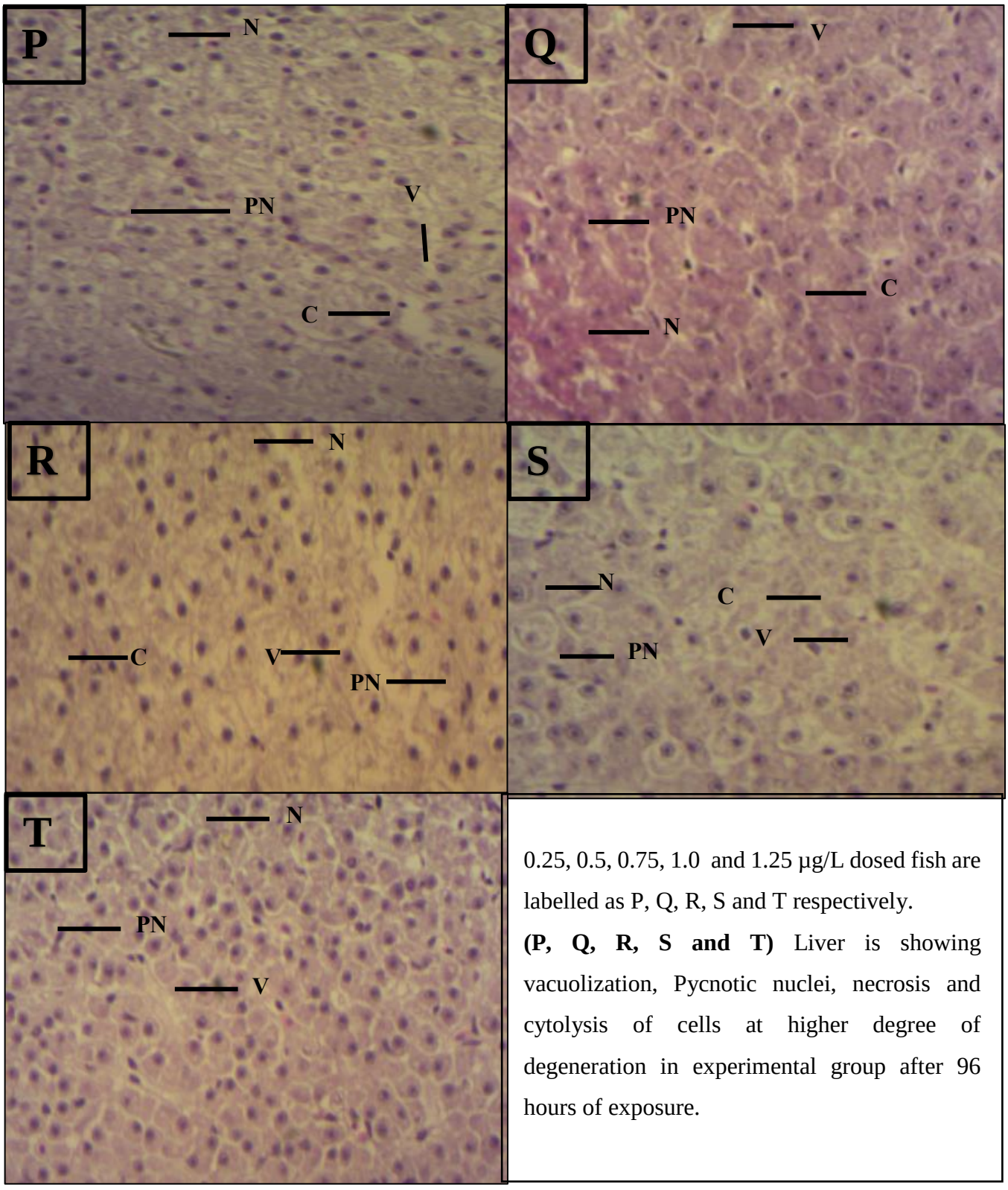


Figure 4.13 Histopathological changes induced in fish liver exposed to Lambda cyhalothirn after 96 hours of exposure

In 0.25µg/L dosed fish Vacuolization, necrosis, cytolysis and pycnotic nuclei was observed after 96hours exposure. In the fish exposed to 0.5µg/L dosed several damages including vacuolization, necrosis, cytolysis and pycnotic nuclei were observed after 96 hours. Structural damages including vacuolization, necrosis and pycnotic nuclei was observed in 0.75µg/L dosed fish after 96 hours. More vacuolization, necrosis, cytolysis and pycnotic nuclei was observed in fish liver exposed 1µg/L concentration after 96 hours. Vacuolization, necrosis, cytolysis and pycnotic nuclei was observed in 1.25µg/L dosed fish after 96 hours. Cell structure got damaged due to toxicity of Lambda cyhalothrin. After 96 hours, the damage to liver cells was obvious. In most liver cells, the integrity of the cell wall is completely lost.

Within 96 hours, the breakdown of cell boundaries and slight dilation of blood sinusoids were observed. Liver cells have many important functions, such as the secretion of bile, detoxification, the synthesis of several components of plasma, the storage of glycogen and the release of glucose in the blood. It was found that the glycogen content in liver tissue was exhausted at all stages after treatment with sublethal doses of Lambda cyhalothrin. The results show that glycogen is a ready-made energy source, and the reduction of glycogen may be due to its faster decomposition (glycogenolysis), which allows glucose to be released into the circulatory system to meet the increased energy demand under stress. Similar observations have been observed in cadmium-treated *Heteropneustes fossils* and *Salmo Gairdneri* (Haux & Larsson, 1984). Morphological and histological changes related to the toxicity of pesticides in fish liver have shown that the substance causes serious damage to liver cells (Ortiz et al., 2003).

The liver is an important organ in the process of detoxification and biotransformation. For these reasons, liver cells have been severely damaged. Several studies have reported degenerative changes in liver tissue caused by various pesticides and insecticide contamination.

Another study reported that degenerative changes in liver of *Channa punctatus* resulted under malathion toxicity.

The literature reported vacuole formation in the liver of *Tilapia mossambica* exposed to fenvalerate, moderate hepatocyte cytoplasmic degeneration, micronuclei and hepatic vascular rupture. The liver of the fish treated with pesticides showed sinusoidal expansion in hemorrhage, vacuolation, cell boundary decomposition and necrosis. The current results are consistent with those observed by many authors who have studied the effects of different pollutants in fish liver (Rios et al., 2003). The liver is an organ that often changes after exposure to sub lethal doses of pesticides. This change may be attributed to the direct toxic effects of pollutants on liver cells, because the liver is the detoxification site for all toxic substances (Gaafar & Soufy, 2007).

4.5.2 Histological alterations in gills

In this research study, the gills of *Ctenopharyngodon idella*, in control group shown a normal structure and gills as proper Primary gill lamellae, Secondary gill lamellae and definite Inter lamellar region was observed while, in the Lambda cyhalothrin treated groups showed several pathological changes throughout the experimental period.

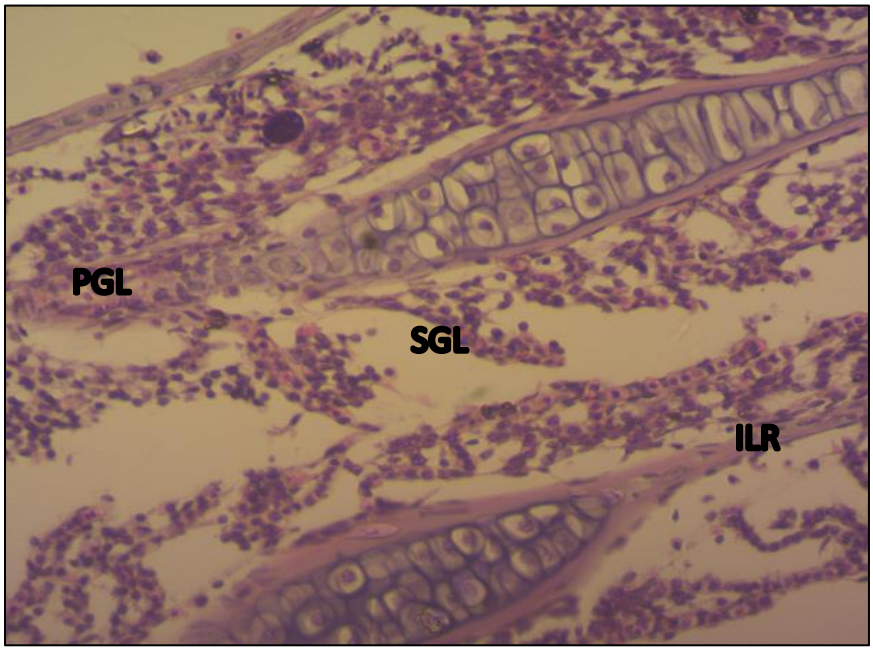
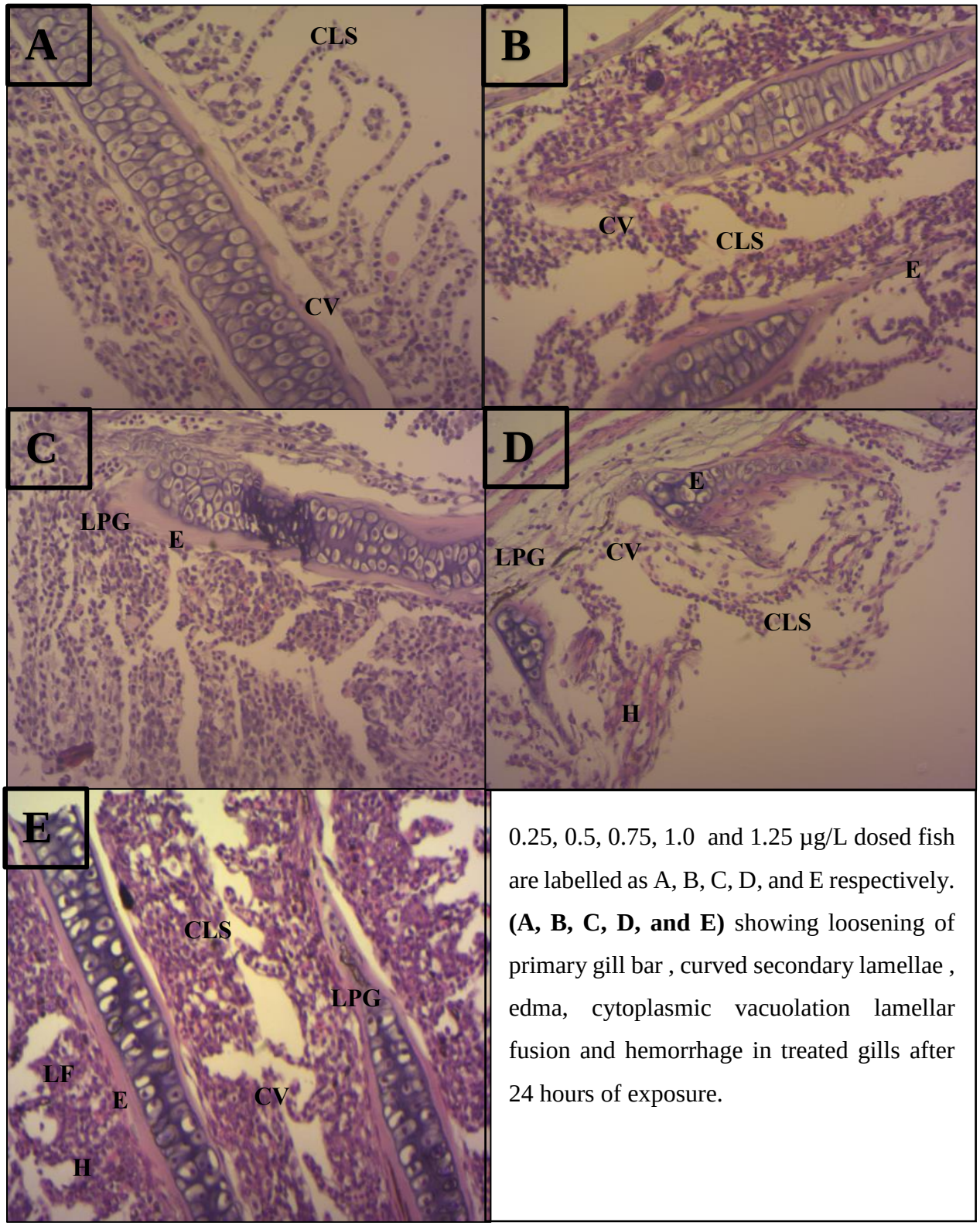
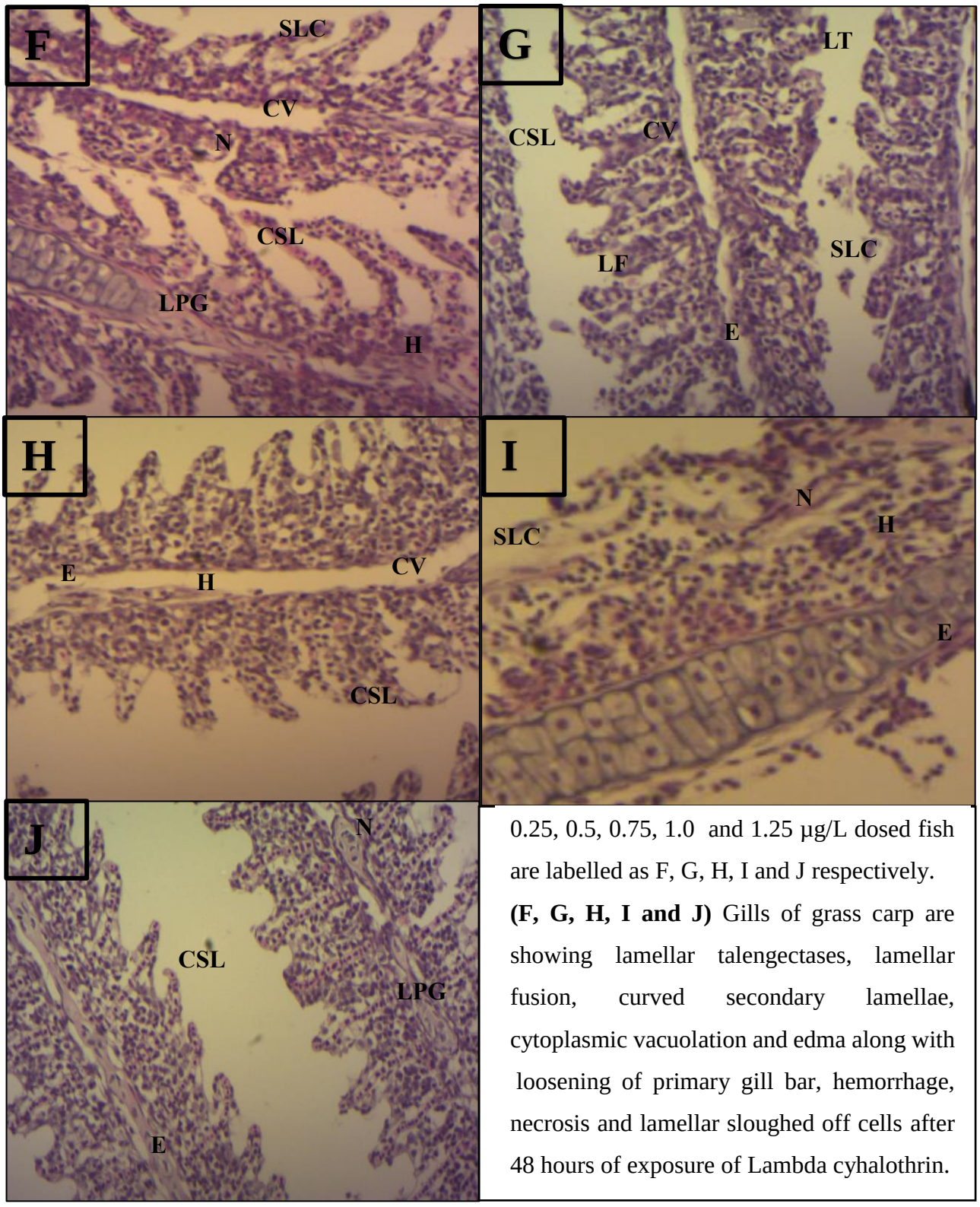


Figure 4.14 Control gills showing proper primary gill lamellae, gill lamellae and Inter lamellar region



0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as A, B, C, D, and E respectively. **(A, B, C, D, and E)** showing loosening of primary gill bar , curved secondary lamellae , edma, cytoplasmic vacuolation lamellar fusion and hemorrhage in treated gills after 24 hours of exposure.

Figure 4.15 Histopathological changes induced in fish gills exposed to Lambda cyhalothirn after 24 hours of exposure



0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as F, G, H, I and J respectively.

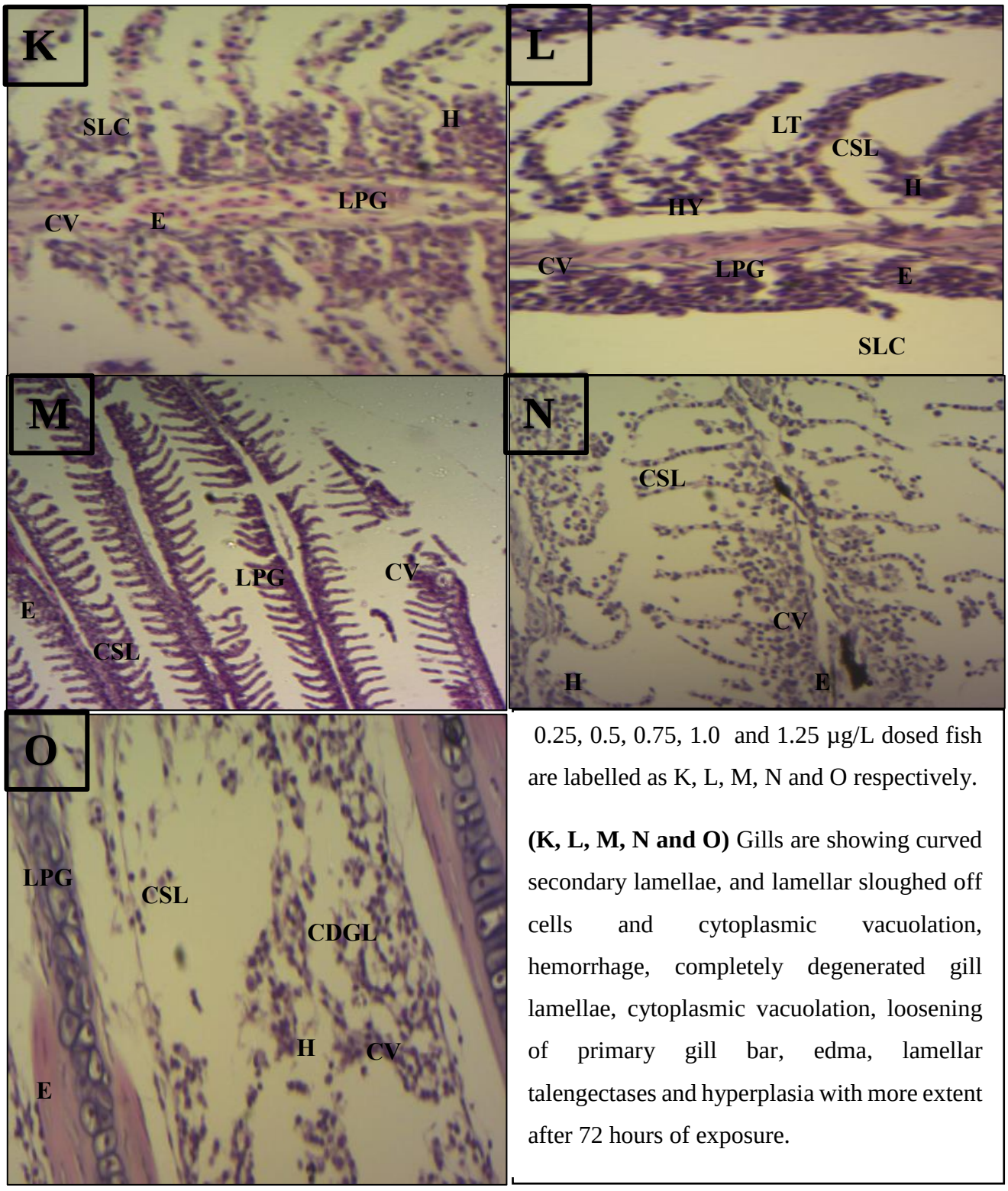
(F, G, H, I and J) Gills of grass carp are showing lamellar talengectases, lamellar fusion, curved secondary lamellae, cytoplasmic vacuolation and edma along with loosening of primary gill bar, hemorrhage, necrosis and lamellar sloughed off cells after 48 hours of exposure of Lambda cyhalothrin.

Figure 4.16 Histopathological changes induced in fish gills exposed to Lambda cyhalothirn after 48 hours of exposure

The fishes exposed to 0.25 µg/L concentration showed edema, hemorrhage of gill lamellae, cytoplasmic vacuolation and curved secondary lamellae 48 hours of experiment. Blood conjugation and increased mucus production were also seen on prolonged exposure to Lambda cyhalothrin. The fishes exposed to 0.5 µg/L lead to edema, hemorrhage of gill lamellae, Necrosis, lamellar sloughed off cells and loosening of primary gill bar after 48 hours of experiment. The fishes exposed to 0.75 µg/L lead to edema, curved secondary lamellae, lamellar fusion and loosening of primary gill bar after 48 hours of experiment. The fishes exposed to 1 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar sloughed off cells, necrosis and loosening of primary gill bar after 48 hours of experiment. The fishes exposed to 12.5 µg/L caused edema, lamellar talengectases, cytoplasmic vacuolation, edma, curved secondary lamellae, lamellar fusion and lamellar sloughed off cells after 48 hours of experiment.

The fishes exposed to 0.25 µg/L caused alterations in structure leading to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells and loosening of primary gill bar after 72 hours of experiment. The fishes exposed to 0.5 µg/L caused edema, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells and loosening of primary gill bar after along with some other alterations after 72 hours of experiment. The fishes exposed to 07.5 µg/L resulted in edema, cytoplasmic vacuolation, curved secondary lamellae, and loosening of primary gill bar after 72 hours. The fishes exposed to 1 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells and

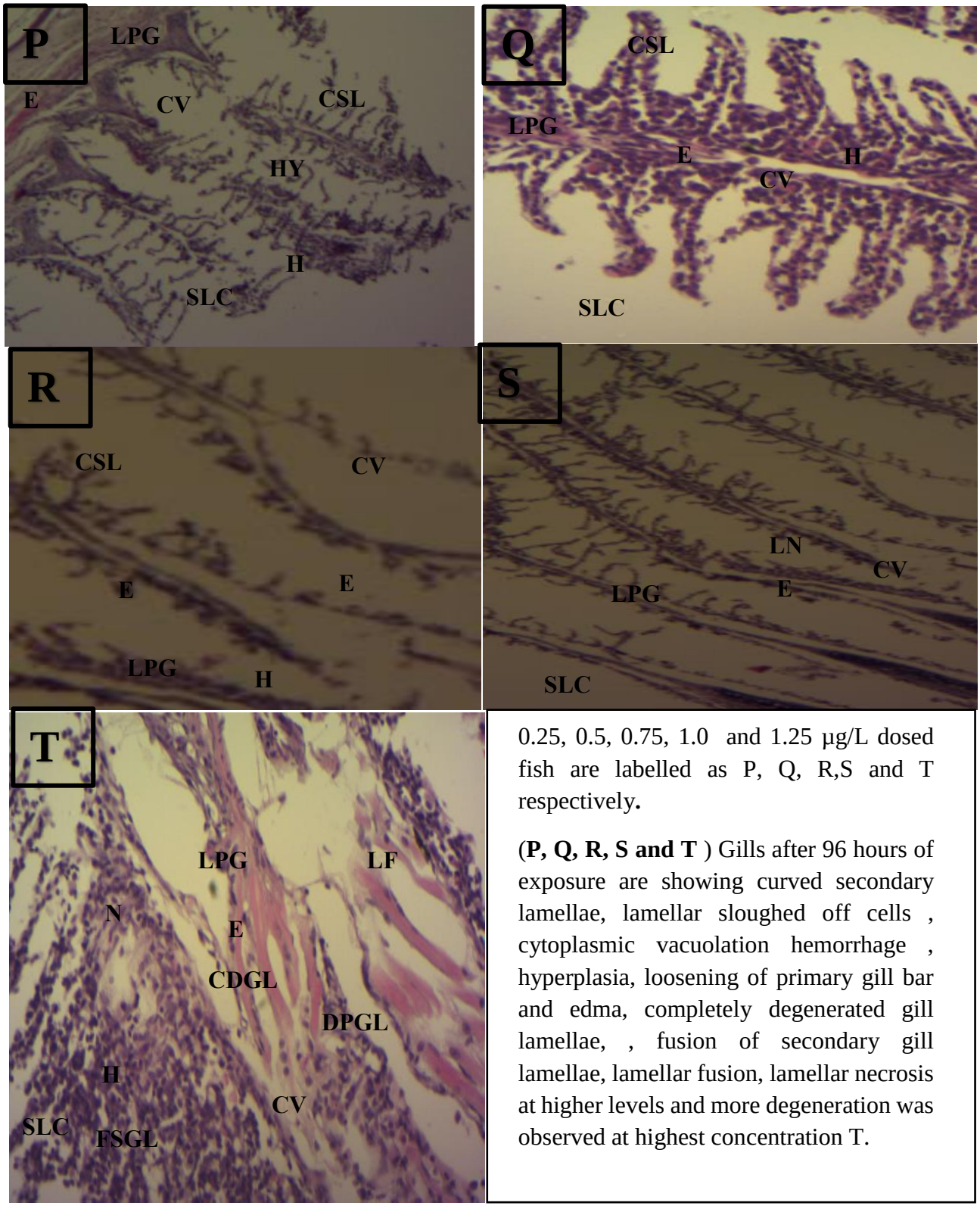
loosening of primary gill bar after 72 hours of experiment. The fishes exposed to 12.5 µg/L resulted in edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, completely degenerated gill lamellae, lamellar sloughed off cells and loosening of primary gill bar after 72hours.



0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as K, L, M, N and O respectively.

(K, L, M, N and O) Gills are showing curved secondary lamellae, and lamellar sloughed off cells and cytoplasmic vacuolation, hemorrhage, completely degenerated gill lamellae, cytoplasmic vacuolation, loosening of primary gill bar, edma, lamellar talengectases and hyperplasia with more extent after 72 hours of exposure.

Figure 4.17 Histopathological changes induced in fish gills exposed to Lambda cyhalothirn after 72 hours of exposure



0.25, 0.5, 0.75, 1.0 and 1.25 µg/L dosed fish are labelled as P, Q, R,S and T respectively**.**

(**P, Q, R, S and T**) Gills after 96 hours of exposure are showing curved secondary lamellae, lamellar sloughed off cells , cytoplasmic vacuolation hemorrhage , hyperplasia, loosening of primary gill bar and edma, completely degenerated gill lamellae, , fusion of secondary gill lamellae, lamellar fusion, lamellar necrosis at higher levels and more degeneration was observed at highest concentration T.

Figure 4.18 Histopathological changes induced in fish gills exposed to Lambda cyhalothirn after 96 hours of exposure

The fishes exposed to 0.25 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells, hyperplasia and loosening of primary gill bar after 96 hours. The fishes exposed to 0.5 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells and loosening of primary gill bar after 96 hours. The fishes exposed to 07.5 µg/L resulted in edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, lamellar sloughed off cells and loosening of primary gill bar after 96 hours. The fishes exposed to 1 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, completely degenerated gill lamellae lamellar sloughed off cells and loosening of primary gill bar after 96 hours. The fishes exposed to 1.25 µg/L lead to edema, hemorrhage of gill lamellae, cytoplasmic vacuolation, curved secondary lamellae, lamellar fusion, completely degenerated gill lamellae, lamellar sloughed off cells and loosening of primary gill bar after 96 hours. After exposure of Lambda cyhalothrin, an excessive amount of mucus was secreted over the gills of *Ctenopharyngodon idella*. It has been indicated that the stress caused by the variations in the environment and pathological agents induced the proliferation of mucus cells and increased secretion (Postal & Federal, 1996). The large quantity of mucus secretion acts as a defense mechanism against several toxic substances.

4.6. Genotoxicity assessment

Genotoxicity of parathyroid pesticide Lambda cyhalothrin was assessed through comet assay. The results obtained from Comet assay were plotted in a series of bar charts for different comet parameters (tail DNA %, tail length and olive tail moment).

Visual analysis

DNA damage is classified into four different classes in literature; make it sufficient as quantitative utilization for many purposes. DNA damage was classified in 0 to 4 categories as mentioned below in fig 4.51.

This classification was divided as;

- No damage
- Minor damage
- Moderate damage
- Major damage

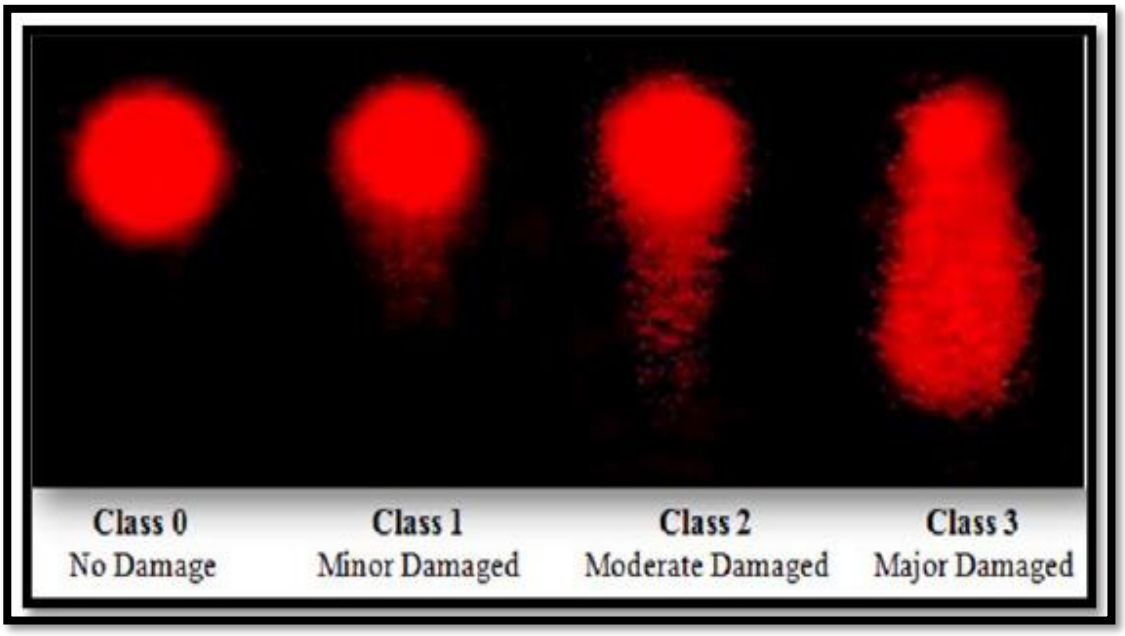


Figure 4.19 Classification of DNA damage

The images of comet parameters for both experimental and control group during the course of current research mostly falls in the classes of 1 to 3, whereas the control groups fall in class 0 i.e. no damage. The images analysis was done using CASP Lab software with focus on three important parameters including tail length, tail DNA % and olive tail moment that are discussed below.

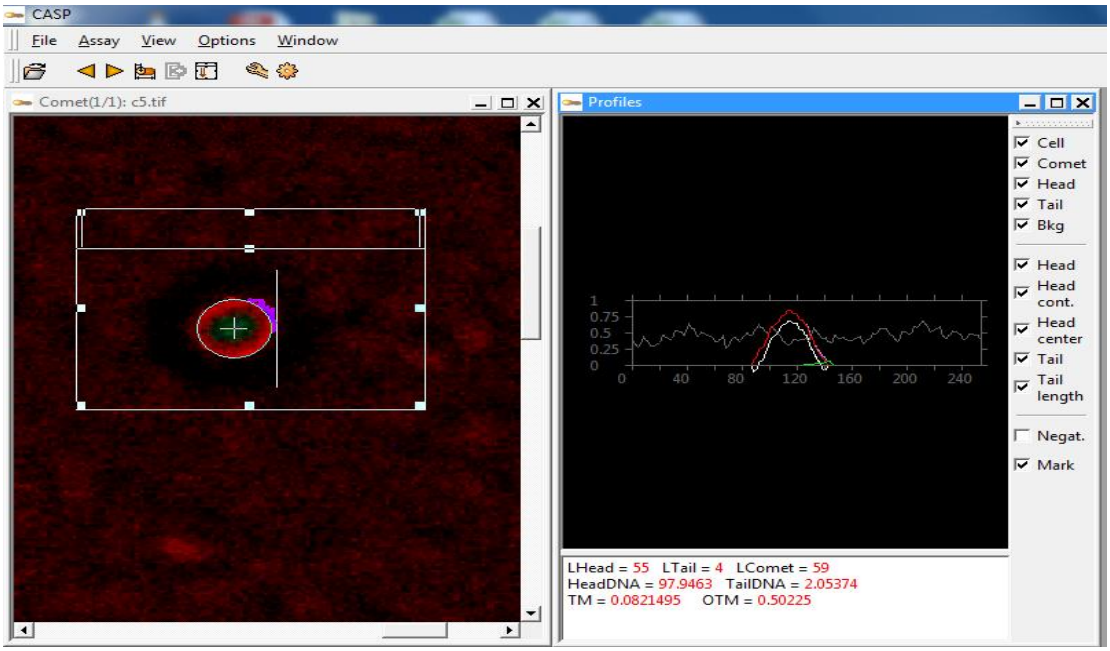


Figure 4.20 CASPLAB for image analysis

Image analysis was done using CASP Lab software focusing on three important comet parameters that are discussed below.

4.6.1 Tail length

The significant increase in the tail length is determined through the migration of DNA towards tail region which is quantified by fluorescence, measured as tail length (µm). The extent of DNA to travel towards anode after applied current during fluorescence depends upon the level of damage occurred in the DNA. This would further determine the level of DNA damage which classified into low, medium, high and very high level. Tail length is considered to be one of the most important parameters to assess the DNA damage (Kumaravel & Jha, 2006).

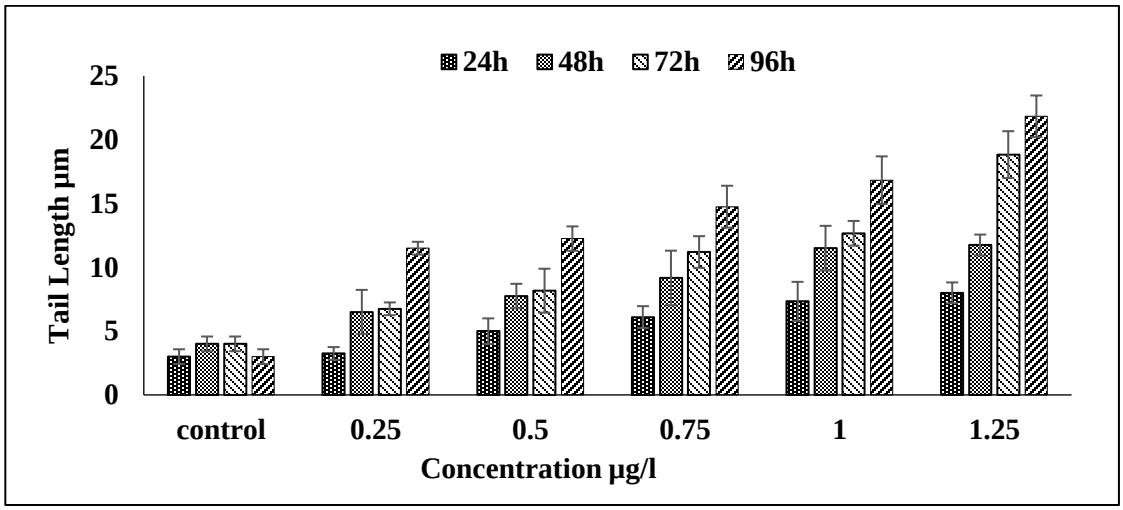


Figure 4.21 Tail length of control and experimental group

The relationship between DNA tail length and varying concentration was observed in Figure 4.20 for Lambda cyhalothrin indicating that the model explains the variability of the response data around its mean. The damage associated with tail length is clearly depicts that a direct relation was showed among dose concentration and tail length damage. It was observed from that there is an increasing trend in dose dependent manner for pesticide Lambda cyhalothrin. The mean tail length values for lambda cyhalothrin was observed at highest dose and longest exposure duration. These values were quite higher as compared to the control groups.

4.6.2 Tail DNA %

Percentage of DNA in tail is also considered as an important index of evaluating DNA damage. The bar charts below presented depicts the relationship between DNA tail length and varying concentrations of Lambda cyhalothrin and exposure time indicating that the model explains well the variability of the response data around its mean.

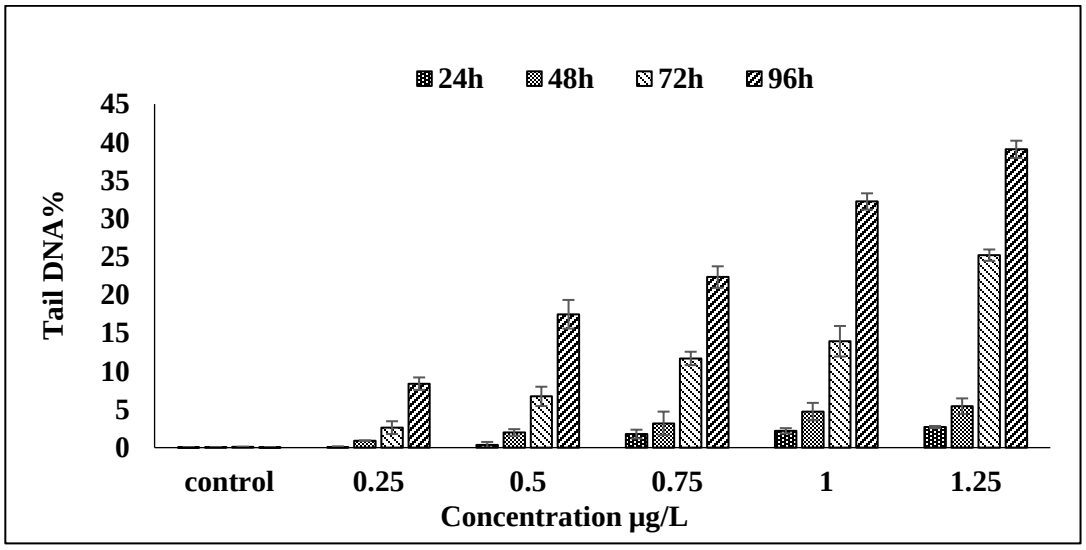


Figure 4.22 Tail DNA% in control and experiment group

Tail DNA % present in the tail region may quantifies the amount of strands breakage, which increases with increase dose concentration. A greater increased tail DNA % and longer DNA tail length reflects the increase level of DNA damage as response towards pesticide. The mean tail DNA % values at the observed doses of experimental group were significantly higher than the control groups (0.03 %) as shown above in the figure.

4.6.3 Olive Tail Moment (OMT)

Another comet parameter is olive tail moment which is commonly used for the genotoxicity evaluation as DNA double strands breakage. The tail moment is defined as the product of the tail length and the total DNA fraction present in the tail region. It combines the minimum detectable size of migratable DNA (reflected in the length of the comet's tail) and the number of loose/broken fragments (represented by the strength of the DNA in the tail).

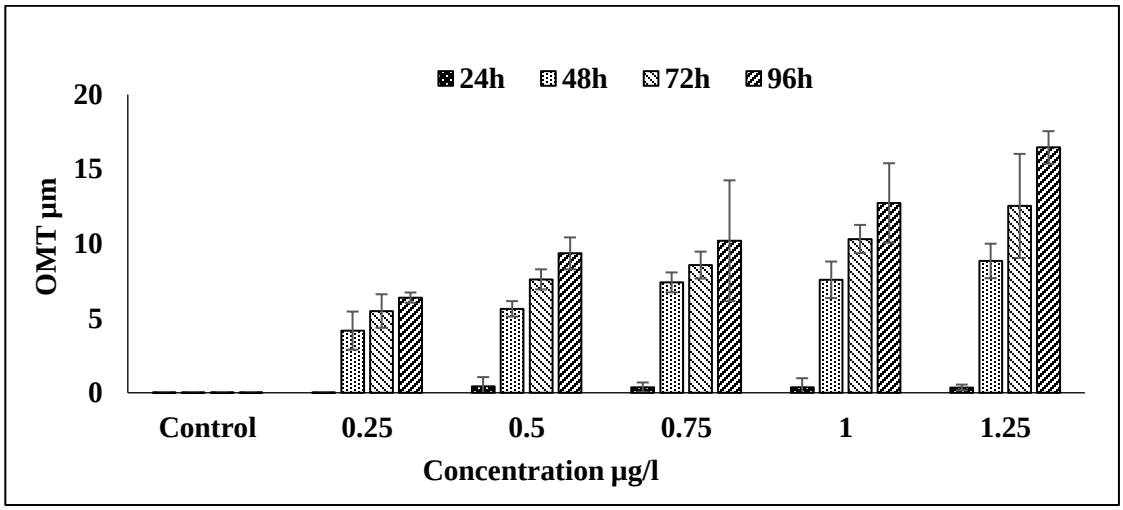


Figure 4.23 Olive tail moment in control and experiment group

It may be clearly seen from the above bar charts that the level of genotoxicity in term of olive tail moment is increasing in a dose-dependent for pesticide Lambda cyhalothrin. The OMT values for all observed doses increased with increase in concentration and exposure time as shown above in fig 4.55. These observed values for this compound was significantly higher in comparison to control group. Thus results obtained from comet parameters (tail length, tail DNA % and olive tail moment) may prove and strengthen the genotoxicity evaluation of pesticide toxicity in fish blood samples using comet assay as promising technique.

The results obtained in the current study shows that the sensitivity of comet assay was relatively higher at higher concentrations for Lambda cyhalothrin, where genotoxic signals increased significantly. Notable damage in comet parameters (tail length, tail intensity and tail moment) was observed in Common carp exposed to pollutants in Lake Mogan (Çok et al., 2011). The damage associated with tail length is clearly depicts that a direct relation was showed among dose concentration and tail length damage. It was

observed that there is an increasing trend in dose dependent manner for Lambda cyhalothrin. These values were quite higher as compared to the control groups. The comparison of data as DNA migration may determines that at the beginning of exposure concentration for Lambda cyhalothrin the damage was statistically significant as compared to control groups. Another study reported extensive DNA damage by increased tail length, % of DNA in tail, tail moment, and olive tail moment in brain and liver at 24 and 48 hours (Yahia, 2018).

4.7 Bioaccumulation of Lambda cyhalothrin

4.7.1 Standard curve of Lambda cyhalothrin

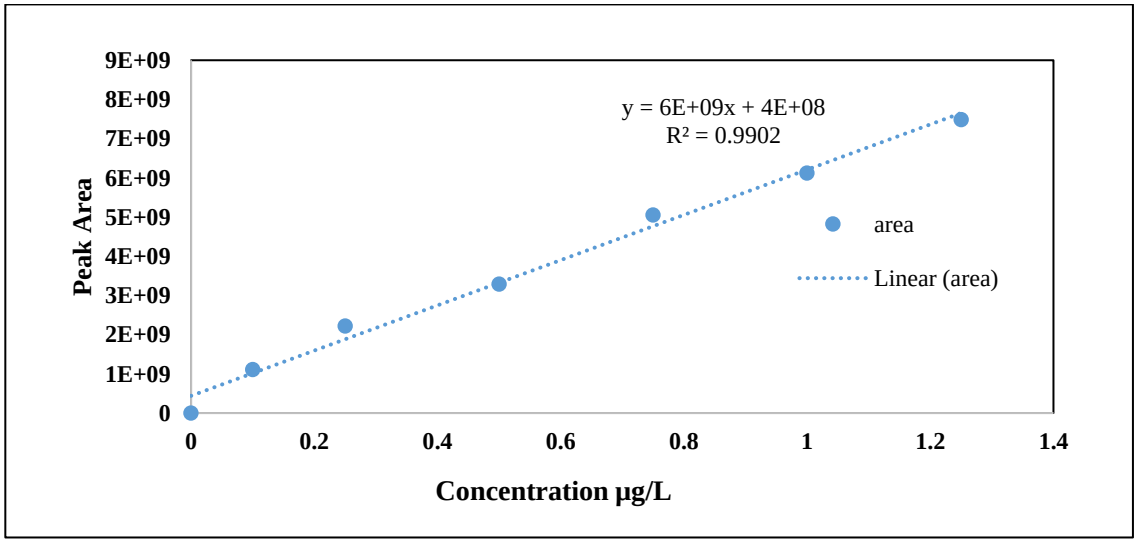


Figure 4.24 Standard curve of Lambda cyhalothrin

4.7.2 Retention time of Lambda cyhalothrin

The instrument was optimized after trial and error. Standard solutions were injected. The retention time calculated for Lambda cyhalothrin was 22.8min.

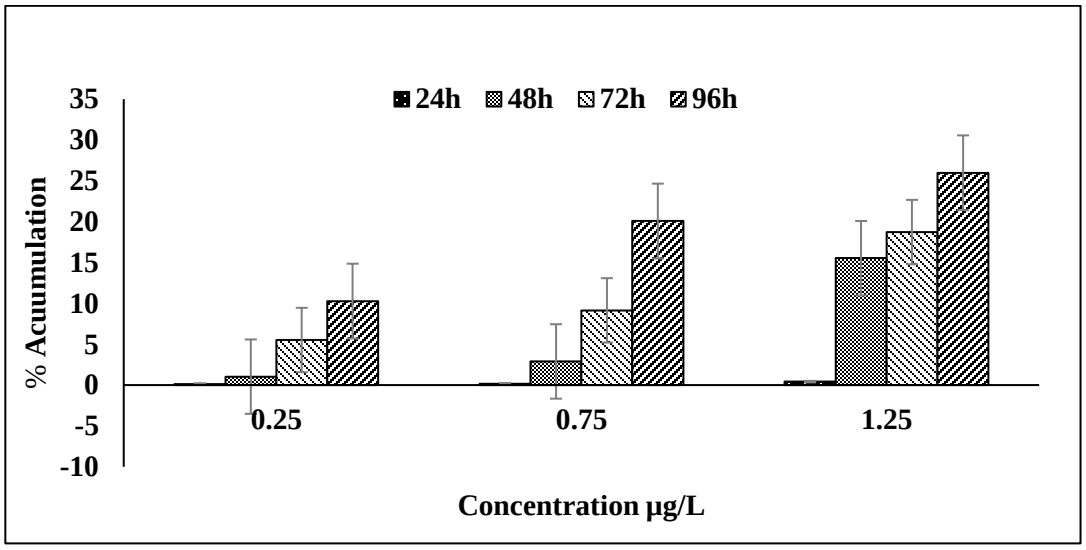


Figure 4.25 Bioaccumulation of Lambda cyhalothrin

The fish taken from control tank showed no accumulation in their body as no visible peak was observed but treated group showed a visible peak of Lambda cyhalothrin. The percentage accumulation of Lambda cyhalothrin was found 0.13,0.15 and 0.42 % for 0.25, 0.75 and 1.25µg/L respectively after 24 hours of exposure and 1.0, 2.8, and 15.53% for 0.25, 0.75 and 1.25µg/L respectively after 48 hours of treatment. For 72 hours 5.5, 9.1 and 18.72% were observed for 0.25, 0.75 and 1.25µg/L respectively whereas after 96 hours of exposure the bioaccumulation was increased gradually to 10.2, 20.0, and 25.98% 0.25, 0.75 and 1.25µg/L respectively. The gradual increase was shown both for time and concentration in the exposed group. Research studies have shown that Lambda cyhalothrin has the potential to bio accumulate in fish body (WHO, 1990). Residues of Lambda cyhalothrin have been reported in fish specimens collected from rivers (Corcellas et al., 2015). The levels of pesticides in the muscles of carp specimens were monitored. The insecticide Lambda cyhalothrin and the fungicide penbutaconazole showed evidence of bioaccumulation in the muscles of the fish. The concentration range

of cyfluthrin is 13.4 to 18.1 µg/kg, and the range of penbutaconazole is 23.8 to 39.9 µg/kg (Clasen et al., 2018). Another study showed the presence of Lambda cyhalothrin residues in muscle and liver tissues of camel carcass (Meligy et al., 2019).

4.8 Physical and 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 grass carp after lambda cyhalothrin 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. The fish moved to the corner of the experimental tanks. Stress was observed in the fish before death. They are drowsy and restless and secreted too much mucus all over the body. Similar results have been reported in other research studies by (Sharbidre et al., 2011), (Nobonita & Suchismita, 2013) and (Halappa & David, 2009).

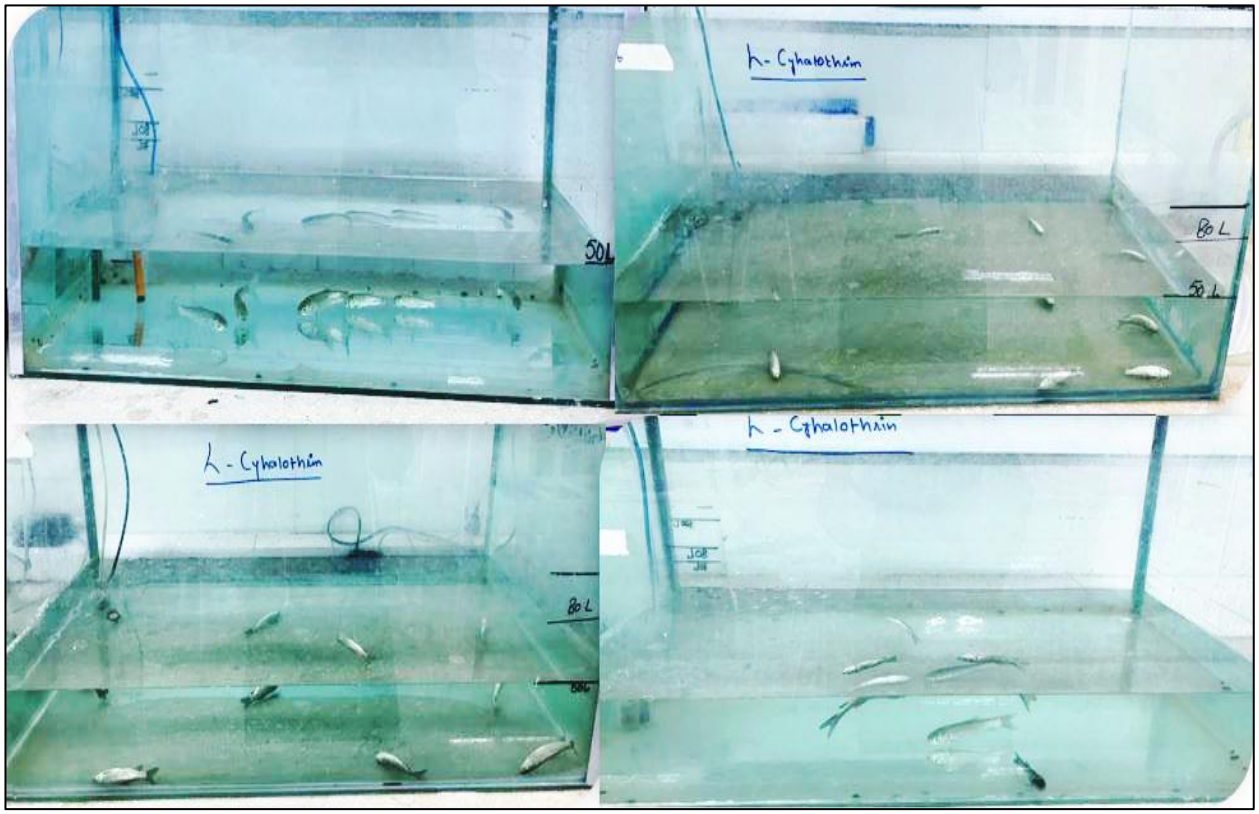


Figure 4.26 Behavioral patterns exhibited by fish in response to Lambda cyhalothrin exposure

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Agricultural runoff and industrial effluents are directly contributing to the surface water contamination. These chemicals represent a substantial damage to living organisms as damage to reproductive, immune, endocrine and nervous systems. It has been concluded that LD-50 of Lambda cyhalothrin for fish changes with the temperature variation as toxicity of pesticide applied increased with the increase in temperature as LD-50 observed at 27 and 13ºC was 1.175 and 1.5µg/L respectively. Its exposure induced oxidative stress in gills and brain and maximum values of ROS reported after 96 hours were 88.7,148.6, 180.7, 199.5, 214.67 and 275.6 and 128, 212.61, 322.33, 361.67,384.60 for gills and brain respectively. It also caused respiratory burst activity in the fish blood as values for exposed group were significantly higher than the control group as observed values at different concentrations were 0.433, 0.85, 0.89, 1.22, 1.39, 1.40. In gills and liver of exposed fish Lambda cyhalothrin caused several deformations and abnormalities as necrosis, vacuolization and loss of hepatic and gill architecture. Severe histopathological damages indicated that the fish were responding to the direct effects of pesticide. There were significant fluctuations in blood glucose lever were also observed in exposed as compared with control. Pesticides cause hematological changes in fish i.e. protein level, ALT and triglycerides levels. Genotoxicity was observed in

terms of DNA damage, tail DNA and tail length via comet assay. Altered behavioral patterns were exhibited by fish in the experimental tanks due to pesticide exposure.

5.2 RECOMMENDATIONS

The following potential recommendations were forwarded, based on the study findings: This study has been determined the toxic effects of pesticide on fish organs for the period of 96 hours so it is recommended that the chronic effects of Lambda cyhalothrin on fish organs should be assessed in order to monitor the long term impacts of contaminants.

This study mainly focused on the temperature based acute toxicity of Lambda cyhalothrin hence it is recommended that the impact of temperature on sub-acute toxicity should be determined using different biomarkers.

Further researches can be conducted out on the determination of seasonal environmental concentration of Lambda cyhalothrin in surface water bodies.

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