LETHAL AND SUB-LETHAL TOXICITY OF CHLORPYRIFOS ON FISH ROHU (*Labeo rohita* Hamilton, 1822)

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DECLARATION

I hereby declare that the work presented in this thesis entitled "Lethal and Sub-lethal toxicity of Chlorpyrifos on fish Rohu *Labeo rohita* Hamilton, 1822" has been done by myself, and has not been submitted elsewhere for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

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RECOMMENDATIONS

This is to recommend that the thesis dissertation entitled "Lethal and Sub-lethal toxicity of Chlorpyrifos on fish Rohu (*Labeo rohita* Hamilton, 1822)" has been carried out by Ms. Samiksha Badu for the partial fulfilment of Master's Degree of Science in Zoology with special paper "Fish biology and aquaculture". This is her original work and has been carried out under my supervision. To the best of my knowledge, this thesis work has not been submitted for any other degree in any institutions.

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CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Ms. Samiksha Badu, entitled "Lethal and Sub-lethal toxicity of Chlorpyrifos on freshwater fish Rohu (*Labeo rohita* Hamilton, 1822)" has been accepted as partial fulfillment for the requirements of Master's Degree of Science in Zoology with special paper Fish Biology and Aquaculture.

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LIST OF ABBREVIATIONS

Abbreviated form	Details of abbreviations
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BCG	Bromocerol reagent
BW	Body weight
CBS	Central Bureau of Statistics
CFPCC	Central Fisheries Promotion and Conservation Centre
CPF	Chlorpyrifos
DDT	Dichlorodiphenyltrichloroethane
DDVP	2, 2-Dichlorovinyl dimethyl phosphate
DO	Dissolved Oxygen
EC	Emulsifiable Concentrate
EDTA	Ethylene Diamine Tetra Acetic acid
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
Hb	Haemoglobin
IPM	Integrated Pest Management
LC	Lethal Concentration
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular volume
OP	Organophosphate Pesticide

PCV	Packed cells volume
Ppm	Parts per million
RBC	Red Blood Cells
SPSS	Statistical Package for Social Science
WBCC	White Blood Cells Count

Abstract

Chlorpyrifos is an organophosphate insecticide which is commercially used to control insect pest. It is second largest selling organophosphate and found to be more toxic to fish. Present study was performed to determine the acute toxicity of Chlorpyrifos (LC_{50}), their behavioural changes after exposure to pesticide and also haematological study of Rohu (*Labeo rohita).* Acute toxicity test was accompanied following the standard method (OECD 203 testing guidelines, 1992). In treated fish alterations in various behavioural patterns respiratory metabolism, opercular beat rate and blood parameters were examined for sub-lethal end-points following 1 h, 24 h, 48 h, 72 h, and 96 h exposure. The LC_{50} value of Chlorpyrifos was found to be 0.362 mg/L. Behavioural changes were also observed in control and exposed fish. Schooling behaviour was observed to be disrupted in first day after exposure to pesticide. High opercular beat rate ($p < 0.01$) at 24 h and ($p < 0.001$) from 48-96 h and oxygen consumption rate was recorded in pesticide-exposed groups in comparison to control. The pesticide stress caused a significant elevation in haemoglobin ($p < 0.01$). Glucose and protein content was found to be increased ($p > 0.05$) in the pesticide group, compared to the control. Some general behavioural changes such as erratic movement, gulping, schooling, mucus secretion, equilibrium, aggregating behaviour, and paleness in the body were observed in pesticide-exposed fish. The current study revealed that CPF was found to be highly toxic pesticide. Hence, its discriminate use should be avoided as it can contribute to decline of Rohu in natural habitat.

1. INTRODUCTION

1.1 General background

Water quality is important for all forms of life and nobody can survive without water. Change in the quality of water directly affects all the community found in that area. Various types of substances coming from home and industries are mixed with water which changes its quality. Other than this, agriculture run off, different kinds of pesticides, different fertilizers etc. cause water pollution and affect water bodies (Misha et al. 2014). These chemicals enter into the body either directly or via food chain and cause serious problem in most aquatic fauna and flora (Avoaja et al. 1997).

Pesticides are pest destruction agents. The most frequent used pesticides are insecticides, herbicides and fungicides. Pesticides are two types, naturally occurring and synthetic pesticide. Synthetic pesticides include chlorinate, organophosphate and carbonate. Dichlorodiphenyltrichloroethane (DDT) and other organic chlorine chemical may have an influence on endocrinal system. Beside this, DDT is one of the most commonly used across globe which is highly stable chlorinated hydrocarbon or extremely low degradability. DDT has been declared illegal by almost all countries (Napit 2013). On the other hand, commonly used organophosphate pesticides are most toxic to vertebrate and few of them have already been prohibited for their application.

Insecticides are extensively used in residential and agricultural areas to control household and crop pests. Pesticides are important and useful tools in agriculture and forestry but their contribution to gradual degradation of aquatic ecosystem cannot be ignored (Konar 1977). The prevalence of genotoxic pollutants in the aquatic environment is one of the major concerns in the area of environmental sciences and this has necessitated the need to develop sensitive methods to monitor the genotoxic potentials of these chemicals in aquatic organisms.

The adverse effects of toxicants become significant when they transfer through food chain from one organism to other or human beings, producing stress conditions either in the form of physiological and biochemical damage to the vital organs or even in the form of death of living organisms of the terrestrial and aquatic environment (Lazher and Ricardo 2012). Widely used all over the world for pest control in agriculture and fish farming, pesticides ultimately find their way into aquatic ecosystems, thus posing risk to economically important non-target species (Avoaja et al. 1997).The toxic effects of pesticides may range from alterations in a

single cell to changes in whole organisms or even populations (Giari et al. 2008). Fish are suitable bio-indicators of aquatic environmental pollution since they are exposed to the chemicals resulting from agricultural production either directly or indirectly of their ecosystem.

Many documents have proved the toxic effects of pesticides which pollutants to aquatic environment and their presence in surface waters was reported in Europe and North America since 50 years (Tarkhani et al. 2012). There is a growing concern over aquatic pollution because of its detrimental effects on biological life including human beings. Chemical pesticides with persistent molecules (long half-life periods) pose threat to aquatic life and also to the human population which consume the affected fish.

In case of developing countries, indiscriminate use of different pesticides especially in agricultural crops in increasing (Muthukumarravel et al. 2013). These pesticides via surface run off reach various unrestricted areas like ponds and rivers and directly affect the physico-chemical properties of water affecting aquatic organisms (Kamble and Muley 2000).

1.1.1 Biology of Rohu (*Labeo Rohita*)

Labeo rohita (**Hamilton, 1822)** is a species of fish of the carp family. Rohu is the most important among the three Indian major carp species used in carp polyculture systems. Beside this, due to its high growth potential coupled with high consumer preference, have established Rohu as most important freshwater species cultured in India, Bangladesh and other adjacent countries in the region. When cultured, it does not breed in lake ecosystems, so induced spanning is necessary. The rohu is also prized as game fish. It is a large omnivore and extensively used in aquaculture. Rohu is the most abundant among the species of Genus *Labeo*, a commercially important species and preferred as a food. Rohu is found in tropical and temperate region. Rohu is essentially an herbivorous column feeder, and prefers algae and submerged vegetation (Barbieri 2009).

1.1.2 Organophosphate pesticides

Organophosphorus (OP) insecticides are the most broadly used synthetic chemicals for controlling the pests having negative effects on aquatic organisms especially fish (Ismail et al. 2009). Organophosphorus pesticides (OPs) are widely used in agriculture, and the aquatic environment near to fields is under influence of OPs.

Chlorpyrifos, being an OP insecticide, is used worldwide for the control of agricultural and non-agricultural insect pests (Lemus and Abdelghani 2000). Furthermore, Chlorpyrifos is a broad-spectrum organophosphate insecticide (OP) which is commercially used for more than a decade ago so as to control foliar insects that affect agricultural crops and subterranean termites (Rusnyiak and Nanagas 2004). Chlorpyrifos is an acetylcholinesterase (AChE) inhibitor, which damages the nervous system and accumulates in aquatic organisms (Sun and Chen 2008). Chlorpyrifos is a commonly used organophosphate insecticide that causes toxicological effects in aquatic organisms especially in fish (Ismail et al. 2017). The highest reported environmental concentration of chlorpyrifos is about 300 mg/L in the surface water in the United States and it is one of the most important pesticides detected in the fishery products, as well (Hutchington et al. 1998). Half-life of chlorpyrifos in water (pH 7.0) is 25.6 days (Shi et al. 2000) and its occurrence in freshwater bodies is harmful to the fish and other nontarget aquatic organisms, which make it a strong candidate for toxicity studies.

Among the many forms of chemical pesticides, chlorpyrifos is considered to be one of the most hazardous environmental pollutants since they are very persistent, non-biodegradable and biaccumulative (Barbieri 2009, Kumar et al. 2011). Thus, contamination by pesticides is a serious water pollution issue, which may cause an environmental imbalance and an increase in poisoning of fish and other aquatic species. In recent years, selected insecticides/herbicides have been intensively and widely used in the Galician area (AEPLA 2012), and all selected pesticides, except diazinon and pirimiphos-methyl, are on the list of priority substances.

1.1.3 Acute toxicity and sub-lethal effects

Aquatic ecosystems around the world face serious threats from anthropogenic contaminations such as increasingly used pesticides (Eder et al. 2009). Acute toxicity of a pesticide refers to the chemical's ability to cause damage to an animal from a single exposure, generally of short duration (Tarkhani et al. 2012). Acute toxicity tests of pesticides have been commonly performed on fish to acquire rapid estimates of the concentrations that lead to direct, irreversible harm to the tested organisms. The acute toxicity data provide information which is useful to identify the mode of action of a substance and also help to comparison of dose response among various chemical substances. The 96-h LC_{50} tests are conducted to assess the vulnerability and survival potential of organisms to particular toxic chemical substances and chemical agents with lower LC_{50} values are more toxic because their lower concentrations result 50% of mortality in organisms (Tarkhani et al. 2012). The contamination by pesticides

can affect diverse non-target organisms, (Pesando et al. 2004) or fish, from early embryos to adult animals, which are unable to produce normal gametes and show morphological abnormalities and high mortality.

1.2 Objectives of the study

General objective

Assessment of lethal and sub-lethal toxicity of chlorpyrifos on freshwater fish Rohu (*Labeo rohita*, Hamilton, 1822) was the general objective of this study.

Specific objectives

- To estimate the LC_{50} value of chlorpyrifos on Rohu.
- To examine the general behaviours, respiratory metabolism, and opercular beat rate of fish after exposure to chlorpyrifos.
- To measure the blood parameters (haemoglobin, glucose, albumin, globulin and total protein) of fish exposed to chlorpyrifos.

1.3 Rationale of the study

The bio-accumulative and non-biodegradable nature of pesticides are serious threats to aquatic organisms, but pesticides are utilized because they are considered an integral part of modern agricultural systems. Agriculture production can be improved by the use of these pesticides, but unfortunately, they may reach nontargeted areas and can impact non-target organisms, especially aquatic species and their environments. Environmental toxicology studies have confirmed that pesticides affect non-target species in the environment because these substances are not fully selective and they are applied to crops in large amounts, only a small portion of which reaches the target and the remaining portion affects non-targeted areas such as wildlife, birds, and aquatic organisms (particularly fish) – causing effects like; reducing biodiversity, altering reproductive and behavioural responses, altering physical and chemical composition of aquatic bodies. Which in turn alter the haematological and biochemical parameters of fish, increasing disease susceptibility, and accumulating toxic substances that can reach humans though the food chain, thus affecting humans as well (Khan et al. 2016). Hence, in order to assess the safety level of any poisonous chemical for higher animals, the first task is to determine the acute toxic level LC_{50} value, which is an expression of degree of toxicity that can be understood by toxicologists (Doubois and Geiling 1959). In spite of increment in use of

pesticide in fishes and assessment of toxicity in fishes, the vital information regarding effect of pesticides on these major carps are scarce. However, some studies have been conducted to assess the sub lethal effects on behavioural patterns of *L. rohita* (Ismail et al. 2014, Koprucu et al. 2006) haematological and biochemical changes (Saxena and Seth 2002, Mukherjee 2003, Borges et al. 2007).

This study has been designed so as to evaluate the acute toxicity of frequently used chlorpyrifos as well as its sub lethal effects on the fish behaviour, aerobic energy metabolism, opercular movement and haematological parameters of *L. rohita* under laboratory condition. The final result obtained from this research may be helpful and could be useful for management and monitoring organophosphate pesticide chlorpyrifos contamination in the environment. Beside this, this study will be helpful for environment protection authorities in order to access the risk possessed by chlorpyrifos on the aquatic ecosystem and so as to minimize the threats to freshwater fish Rohu. Furthermore, the information extracted from this study will play vital role in planning and formulating guidelines of proper pesticide use regarding welfare of aquatic organisms and aquatic ecosystem.

2. LITERATURE REVIEW

2.1 Pesticide toxicity in fishes

Pesticides are generally routinely used in the integrated farming that protect various crops from varieties of insects, weeds and diseases that are posing great danger to aquatic environment, persistent residue in air and water (Sharma and Prakash 2005). Fishes are suitable bio-indicators of aquatic environment pollution as they are exposed to chemicals coming from agricultural production (Lakra and Nagpure 2009). The early life stages of fishes are generally regarded as life history stages as they are most sensitive to toxic agents (Hutchington et al. 1998). During early ontogenesis, critical development of tissue and organ takes place which can be easily disrupted by unfavourable environmental conditions caused by exposure to toxic compounds (Foekema et al. 2008, Kammann et al. 2009). These pesticides are readily absorbed via skin and are highly toxic by all routes of exposure (Malla 2009). When inhaled, the effects could range from priliminary effects of respiratory problems to bloody or runing nose, chest discomfort, involuntary muscles contraction, paralysis of body, irregular heart beats, unconciousness, coma to death (Misha et al. 2014).

The presence of pesticides due to huge consumption for agriculture purpose is very prevalent in surface waters which could finally accumulate in aquatic ecosystems and have been found to be have caused toxic effects to aquatic animals (Tarkhani et al. 2012). Pesticide could affect from early embryos to adult animals that are unable to produce normal gametes and show morphological abnormalities and high mortality. Beside this, fish embryo-larvae toxic assays using lethal and sublethal endpoints are useful because they can provide consistent information on the short-term toxicity of chemicals (Lazher and Ricardo 2012).

The increasing awareness of aquatic pollution demands toxicity tests to assess the efficacy of contamination and extrapolate their safety levels permissible in the environment. In assessing the safety level of any poisonous chemical for higher animals, the first task is to determine the acute toxic level LC_{50} value, which is an expression of degree of toxicity that can be understood by toxicologists (Doubois and Geiling 1959). Koprucu et al. (2006) conducted study so as to determine the acute toxicity of this organophosphorus pesticide, contaminating aquatic ecosystems as a pollutant, and its effects on behaviour, and some haematological parameters

of fingerling European catfish, *Silurus glanis*. Beside this, Lazher and Ricardo (2012) evaluated the short-term toxicity of seven selected pesticides: four kinds of insecticides (chlorpyrifos, dieldrin, diazinon and pirimiphosmethyl) and other thee herbicides (diuron, alachlor and atrazine) and as per the toxic data, chlorpyrifos was highly toxic pesticide on both turbot embryos and larvae. Furthermore, Muthukumarravel et al. (2013) found that monocrotophos caused 100% mortality of *L. rohita* at 0.0044 ppm and 50% mortality at 0.0036 ppm. As per LC_{50} value, lambda cyhalothrin showed higher toxicity than monocrotophos. Kiran and Jha (2009) exhibited increased opercular movements in fish *Labeo rohita* when exposed in herbicide, herboclin. Similarly, Shivakumar and David (2004) found irregular, erratic darting movements with imbalanced swimming activity along with fish *L. rohita* trying to jump out of toxic medium when exposed to endosulfan.

Similarly, Sekhara and Jammu (2016) studied acute toxicity of chlorpyrifos in different fish species and exhibited that sub-lethal toxicity of chlorpyrifos in aquatic environments can induce morphological, neurobehavioral, oxidative, biochemical, histopathological, haematological, developmental alteration etc. causing mass mortalities in non-target organisms and fishes. Tarkhani et al. (2012) conducted a research with an objective to determine the acute toxicity of diazinon and deltamethrin as potential dangerous organic pesticides to assess mortality effects of these chemical agents to the zebra fish, Danio rerio, Golestan province of Iran. Under methodology applied for this, fish samples were exposed to different concentrations of Diazinon (60%) (0, 1, 2.5, 5, 10, 12, 20, 35, and 50 ppm) and Deltamethrin (2.5%) (0, 0.04, 0.05, 0.06, and 0.07 ppm) for 96 h within the 120 L glass aquaria and cumulative mortality of Zebra fish was obtained with 24 h interval. According to World Health Organization (WHO) chlorpyrifos is moderately hazardous and degrades more rapidly in alkaline pH. Furthermore, according to their acute toxicity, it was exhibited that the insecticides were more toxic than the herbicides. In addition to this, all insecticides and herbicides appear to be teratogenic to turbot ELS (Lazher and Ricardo 2012).

2.2 96 h LC_{50} values of different pesticides on different fishes

Acute toxicity of a pesticide is the ability of chemical to cause the effects on a particular species. The 96h - LC_{50} tests are done to assess the vulnerability of organism to certain toxic chemical substance. F.A. Malla (2009) analysed toxicity of chlorpyrifos on fish *Channa*

punctatus and found that toxicity of chemicals depends upon exposure time. Beside this, Bhatnagar and Cheema (2016), after studying genotoxic effects of chlorpyrifos in fresh water fish *Cirrhinus mrigala* using micronucleus and the LC_{50} values for fingerlings were found to be 0.44mg/L. Beside this, Lazher and Ricardo (2012) conducted a study where they studied the median lethal concentration of the selected pesticides during a 48-h and a 96-h exposure for turbot embryos and larvae were, respectively (in micrograms per litre): chlorpyrifos, 116.6 and 94.65; dieldrin, 146 and 97; pirimiphos-methyl, 560 and 452; diazinon, 1,837 and 1230; alachlor, 2177 and 2233; diuron, 10,076 and 7826; and atrazine, 11,873 and 9,957. On the other hand, the study conducted by Tarkhani et al. (2012) revealed low LC_{50}s values were obtained for Deltamethrin (0.05 ± 0.027 ppm) compared to Diazinon (17.5 ± 1.32 ppm) indicated that Deltamethrin had more toxicity compared to the other in Zebra fish (Tarkhani et al. 2012). In conclusion, it was found that both Deltamethrin and Diazinon has toxic effect on Zebra fish and related mortality rates were increased with increasing concentration as per time. Beside this, Zebra fish was more sensitive to lower values of Deltamethrin compared to Diazinon. Diazinon was applied at concentrations of 1, 2, 4, 8, 16, 32, and 64 mg/L. The water temperature in the experimental units was kept at 16±1 °C. The number of dead fishes significantly increased in response to diazinon concentrations 2–64 mg/ L ($p < 0.05$). With increasing diazinon concentrations, the fishes exposed duration 1– 96 h significantly increased the number of dead fishes ($p < 0.05$ for each case). The 1, 24, 48, 72, and 96 h LC_{50} values (with 95% confidence limits) of diazinon for fingerling European catfish were estimated as 14.597 (12.985–16.340), 12.487 (11.079–14.471), 8.932 (7.907–10.348), 6.326 (no data because of $p > 0.05$), and 4.142 (no data because of $p > 0.05$) mg/L, respectively.

Similarly, the 96h LC_{50} for fathead minnow was 180.0 ppm (Pimentel 1971). Similarly, Koprucu et al. (2006) found 96h LC_{50} value of diazinon for fingerling European catfish to be 4.142 mg/L. However, the technical grade CPF was found to be mutagenic and genotoxic to fishes even at nonlethal concentration (i.e., $1/12^{th}$ of LC_{50} =~68.0mg/L), which indicated apprehension about the potential hazards of CPF to aquatic organisms (Ali et al. 2008). Beside this, the 96h LC_{50} value of endosulfan was reported to be 41mg/L (Pandey et al. 2006). Similarly, the 96 h LC_{50} value of chlorpyrifos, estimated by Trimmed Spearman-Karber (TSK) in static bioassay, was found to be 442.8 mg/L (Ismail et al. 2014).

2.3 Behavioural study of fishes after pesticide exposure

After exposing fishes to 297 mg/L for 96h, LC_{50} of chlorpyrifos, the fishes under pesticide stress showed behavioural symptoms of dullness, loss of equilibrium, loss of feeding and erratic swimming (Kavita and Rao 2007). Furthermore, similar behavioural changes were also seen previously in the fish exposed to OP pesticides (Machado and Fanta 2003). Behavioural abnormalities like cancer, haematological and biochemical change have been observed in fish exposed in pesticide (Napit 2013).

Furthermore, Fish exposed to sublethal concentrations of pesticides showed irregular, erratic and darting movement were observed in fish trout and *L. rohita* when exposed to fenvelrate (Murthy 1987). Furthermore, Santhakumar and Balaji (2000) found similar kind of erratic swimming, imbalance in posture, increased surface activity with gradual decrease in opercular movement with loss in equilibrium and excess of mucus all over body followed by sluggishness and death of a fish after exposure to monocryptophos. Beside this, after exposure of *C. punctatus* to organophosphorus, the change in the behaviour were considered directly related to complex physiological responses and was used as a sensitive indicator of stress (Little and Finger1990). Apart from this, the abnormal behavioural responses were observed at all concentration higher than 2 mg/L which included less activity, loss of equilibrium, erratic swimming, rapid gill movement, hanging vertically in the water and staying motionless in the aquarium bottom (Koprucu et al. 2006).

In addition to this, Siang et al. (2007) determined the acute toxicity of endosulfan and its effect on the behaviour of the fish. After exposure, fish experienced series of abnormal behaviour, which include imbalanced position, restlessness, lethargy, flashing, erratic swimming and tremor. Furthermore, Ismail et al. (2014) found that fish exposed to different concentrations of chlorpyrifos showed different neurotoxic behavioural responses. It was concluded that chlorpyrifos is a genotoxic and neurotoxic insecticide causing DNA damage and neurotoxic effects in *L. rohita*. In another study, using higher concentration of chlorpyrifos, alterations in physiological and behavioural responses especially erratic swimming, gulping, mucus secretion, increased opercular movement and profuse emission of mucus all above the body were observed during the primary stages of contact after which it became occasional (Zahan et al. 2019).

2.4 Oxygen uptake and opercular beat rate

One of the most important physiological parameters to assess the toxic stress is the respiratory potential or oxygen consumption of an animal. As aquatic organisms have their outer bodies and important organs such as gills almost entirely exposed to water, hence, the effect of toxicants on the respiration is more pronounced. Pesticides enter into the fish mainly though gills and with the onset of symptoms of poisoning, the rate of oxygen consumption increases. It was observed that one of the earliest symptoms of acute pesticide poisoning is respiratory distress (Holden 1973). Similarly, Halappa and David (2009) revealed that chlorpyrifos (20% EC) was highly toxic and had a profound impact on the oxygen consumption and food consumption of *Oreochomis mossambicus* exposed to lethal and sublethal concentrations.

Beside this, Hartl et al. (2001) exhibited that if gills or membrane functions are destroyed due to xenobiotic chemicals or the membrane functions are disturbed by a change in permeability the oxygen uptake rate would rapidly decrease. Likewise, Napit (2013) exhibited that Dimecron exposed *Heteropneustes fossils* showed significant decrease O2 carrying capacity of blood, decrease in Hb% and lower red blood cells numbers. On the other hand, Peter et al. (2018) revealed hyper-excitability of *Oreochomis mossambicus* fish at higher concentration during 24h of dichlorvos exposure. Beside this, significant increase in opercular movement of fish was noted over the control with the increasing concentration of toxicant. A similar to this result was observed by Padmanabha et al. (2015) when *Oreochromis mossaambicus* was exposed to lethal dose of CPF. It caused gulping of air, rapid opercular movement, excess secretion of mucus along with body colour change into dark before death. Furthermore, it was revealed from the study that exposure to dichlorvos can alter oxygen consumption rate in *Ctenopharyngodon Idella* (Tilak and Kumari 2009) and also cause histopathical changes in Rohu (Kesharwani et al. 2018).

2.5 Alteration on haematological and biochemical parameters

The micronucleus assay is one of the most important genotoxic endpoints used to monitor the an eugenic and clastogenic effects both in vivo and in vitro studies. Because the fish blood in gills is in direct contact with water medium, any adverse change in the aquatic environment could be revealed in the circulatory system. Therefore, fish blood has been widely used in

toxicological and environmental monitoring as a possible indicator of physiological changes in fisheries. So, the haematological studies of fish could be used to indicate the health status of fish and water quality. A number of studies were conducted to evaluate the effects of different pesticides on fish blood. Also, acute toxicity and genotoxicity of chlorpyrifos have been evaluated in freshwater fish *L. rohita* (Tavares et al. 1999, Svoboda et al. 2001, Saxena and Seth 2002, Das and Mukherjee 2003 and Borges et al. 2007). Beside this, Napit (2013) found that exposure of Dimecrom 0.0068 ppm to *Gambusia affinis* caused histopathological changes such as hepatic lesion with necrosis pyconic nuclei, vasculation damaged blood vessel in alimentary canal, liver, kidney and gill.

Exposure of fish to toxic agent such as pesticides causes histological alterations at the level of tubular epithelium and glomerulus. Beside this, dilation of tubules and necrotic changes characterized by karyorrhexis and karyolysis at the nuclei of affected cells of *Labeo rohita* exposed to hexachlorocyclohexane were reported (Tilak et al. 2001). Similarly, Napit (2013) found that endosulfan exposed *Oreochromis mossambicus* showed dysfunction of osmoregulation processes resulting in alteration of ionic composition of blood. Similarly, compared to the control specimens, fish after an acute exposure to diazinon was significantly lower erythrocyte, leukocyte, haemoglobin, haematocrit, MCV, MCH, and MCHC values ($p < 0.05$). In addition, it was also showed a significantly negative correlation between these haematological parameters and exposure times of diazinon ($p < 0.01$) (Koprucu et al. 2006).

Furthermore, pesticide exposed fish showed haematological changes like decrease of erythrocyte and leucocytes count in *C. carpio* (Napit 2013). Similarly, in *Catla catla, Nandus nandus and L. rohita* significant histological and biochemical alteration in gill and fin region were observed due to effect of endosulfan, diazinon and chlorpyrifos. As per the turbot ELS test results, it was observed that chlorpyrifos was most toxic pesticides tested for both embryos and larvae and was followed in order of decreasing toxicity by dieldrin, pirimiphos-methyl, diazinon, alachlor, atrazine and diuron. It was found that larvae were more sensitive than compared to embryos to the seven different pesticides (Lazher and Ricardo 2012).

3. MATERIALS AND METHODS

3.1 Experimental site

The whole experiment was carried out in Central Fishery Promotion and Conservation Centre (CFPCC), Balaju, Kathmandu. The fishes required for experiment were brought from Bhairahawa.

3.2 Materials

3.2.1. Experimental set up apparatus/materials

a) Air-pump
b) Air-stones
c) Aluminium foil
d) Aquarium (35 L×12 and 3_{50} L×2)
e) Aquarium filter
f) Aquarium cover
g) Aquarium heater
h) Buckets
i) Extension cord
j) Filter tube
k) Scoop net
l) Fish feed
m) Sealing tape
n) Scissors
o) Siphoning tube
p) Sponge
q) Volumetric flask

3.2.2. Instrumental apparatus

a) Cuvette
b) Refrigerator
c) Weighing balance
d) Multiparameter water quality analyser (Hanna, Hl98194)
e) Spectrophotometer (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China).

3.2.3. Analytical apparatus

a) Beaker
b) Bullet tubes
c) Dropper
d) Eppendorf tube
e) Ethylene Diamine Tetra Acetic acid (EDTA) vials
f) Gloves
g) Ice box
h) Manual counter
i) Aquarium filter
j) Aquarium cover
k) Aquarium heater
l) Buckets
m) Extension cord
n) Filter tube
o) Scoop net
p) Fish feed
q) Sealing tape
r) Scissors
s) Siphoning tube
t) Sponge
u) Volumetric flask

3.3 Chemical used

For acute toxicity

Table 1: Specification of chemicals

Chemical	*CAS R. No*	*Producer*	*Chemical Name*	*Trade name*
Chlorpyrifos	2921-89-2	Dow agro sciences. Pvt. Ltd. India	3,5,6-trichloro-2-pyridinyl	Dubsan

For haematological and biochemical parameters

- Eco-pak glucose reagent (glucose test) and standard
- Biuret reagent (protein test) and standard
- Bromocerol Green reagent (albumin test) and standard
- HEMOCHORD-D (haemoglobin reagent) and standard

3.4 Methodology

The experiment was conducted from September to November 2019. The experiment was performed to estimate the median lethal concentration (24, 48, 72, and 96 h) of the chlorpyrifos to the fingerlings of Rohu, as well as to examine the behavioural changes, opercular beat rate, respiratory metabolism and blood parameters of the pesticide exposed fish.

3.4.1 Fish collection and acclimatization:

Fish were transported safely from Bhairahawa and stocked in hapas at CFPCC, Balaju for two weeks. Fishes were acclimatized to laboratory condition for 15 days in a large aquarium containing 300 L of water. Fishes were fed with commercial pellet feed during acclimating. Water was renewed on alternate days to remove faeces and food remnants. The freshwater healthy fingerlings of Rohu with length 6±2 cm and weight 10±2 grams were selected for experiment. Water temperature and DO was maintained using a heater

and aerator. But they were not fed during last 24 h before starting the test and throughout the test.

Table 2: Physicochemical properties of test water

Parameter	Value
Temperature (℃)	24.96±0.25
Dissolved oxygen (ppm)	5±0.45
pH	7±0.2
Ammonia (Mg/L)	0.18-0.23
Hardness of water (**Mg $CaCO_3$**/L)	53-59

3.4.2 Acute toxicity test

To know the lethal concentrations of mixed pesticides with the definitive test in semi- static condition in laboratory the acute toxicity test was accompanied following the standard method (OECD 203 testing guidelines, 1992).

Table 3: Experimental design for acute toxicity test

Pesticide = Chlorpyrifos				**General fish behaviour**	**Water quality parameters**
Concentration (mg/L)	Total Replication	No of fish	Mortality		
Control=0	4	20		Colour change Avoiding schooling behaviour Mucus secretion Hypo excitement Equilibrium loss Fish position in aquarium	DO Ammonia Hardness of water pH Temperature
C1=0.5	4	20			
C2=1	4	20			
C3=2	4	20			
C4=4	4	20			
C5=8	4	20			

To determine the acute toxicity, following fishes were sorted out and distributed in each aquarium. Before starting the test, all experimental aquaria were cleaned and filled with 25 L of water in each aquarium. Stock solution of chlorpyrifos was prepared and five different concentration (0.5, 1, 2, 4, 8) mg/L of chlorpyrifos and control unit were used in basic test. Five fish specimens were used for every concentration and also in control. Five fishes were transferred to each aquarium containing 25 L of water in 24 aquaria with four replications. The aquarium water was changed every day and oxygenated using an air pump. Opercular movement of fish was counted using manual counter. Mortality was assessed at 1, 24, 48,

72 and 96 h after start and dead fishes were removed immediately. Fish were considered dead when they did not respond to probing with a glass rod. The dead fishes were discarded from the tank immediately to avoid water fouling. The water in aquarium tank was renewed periodically to maintain water quality temperature=24.96±0.5 °C, pH=7±0.2, dissolved oxygen≥5mg/L, parameters as per the standard quality recommended by American Public Health Association (1998). Replicates were used for calculation of mean values. Data obtained were statistically analysed.

Statistical Analysis: Percent mortality was calculated and probit analysis method was used to calculate the 96-hLC_{50} with SPSS Statistical Software. The analysed result was then presented as 24 h to 96 h- LC_{10}-LC_{90} with a 95% confidence limit, where LC_{10} and LC_{90} represent the 10% and 99% fish mortality at given concentrations.

3.4.2.1 General behavioural changes

During acute toxicity test, general behavioural responses of fish such as erratic movements and abnormal swimming, gradual loss of equilibrium and drowning were also triggered by the toxicant. Agitated movement was observed at all the concentrations. Loss of equilibrium, mucus secretion, change in body colour, swimming pattern, schooling behaviour was observed at a particular time and results were recorded in order to minimize the potential error. Human flow around the aquarium was avoided.

3.4.3 Experimental design for advance behaviour

The experimental set up was designed to investigate the specific behaviour of fish after exposure of pesticide i.e., opercular beat rate and respiratory metabolism of Rohu to Chlorpyrifos. Fishes of similar size and weight were distributed to each aquarium having equal level of fresh filtered water. The experimental set up had 4 replications for sub lethal concentration and also for control. Fishes were exposed to only one sub lethal concentration of test chemical, i.e., 50% of 96h LC_{50} of CPF (0.181mg/L).

Observation was made at time interval from 1 hour to 96 hours. After managing proper dose of pesticide and appropriate temperature (23±2°C) and DO (≥5 mg/l) by using heater and aerator, behaviour changes were started to record mainly focusing on opercular beat rate and oxygen consumption.

3.4.3.1 Opercular beat rate

Opercular movement was recorded for up to 5 minutes at different time interval (1-96h) with the help of hand tally counter. Opercular beat rate was counted 3 times. Human and external interventions were avoided during the data collection.

3.4.3.2 Respiratory metabolism:

The media was left aerated before exposure of pesticide so that oxygen level reach up to 5-6 ppm and pH and temperature also maintained constant. Once the aerator was switched off initial dissolved oxygen was measured quickly by using MilwaukeeMW600 PRO portable dissolved oxygen meter, then aquaria were sealed tightly with a glass lid and duct tape to avoid the mixing of external air in water. After 24 hours, the aquaria were unsealed without disturbing fish, and at the same time, the final DO, pH, and temperature were recorded. In addition, the process was repeated in the same way every 24 hours for four days. Based on the difference between the initial and the final DO, the oxygen consumption rate (mg/g/h) of fish

was measured using the following formulae,

$(O_2i-O_2f)\times V\times(1/BW)\times(1/T)$

Where, O_2i is initial oxygen concentration (mg/L) and O_2f is the final oxygen concentration(mg/L); V is total water volume (L), BW is body weight (gm) and T is the time interval (h).

3.4.4 Haematological parameters

Two concentrations of pesticide were designed for this study,

1. Chlorpyrifos low dose: 0.0362 mg/L (1/10 of 96 h- LC_{50})
2. Chlorpyrifos high dose: 0.181mg/L (1/2 of 96h-LC_{50})

The experiment was run parallel in three groups (control, CPF low dose, and CPF high dose). Each group had three replications each having six fish. Glass aquaria were filled with water up to the appropriate level (25 L), aeration was regulated, and the temperature was maintained. Altogether 18 fishes have experimented. After the 96h of exposure, fishes from each concentrated group were taken out and anesthetized in clove oil. Fresh blood was collected from the caudal vein puncture utilizing a 1ml heparinized plastic syringe and

immediately transferred into sterile ethylene diamine tetra-acetic acid (EDTA) vials to assess haemoglobin and in Eppendorf tubes to determine blood glucose, albumin and total protein.

I. Haemoglobin test

Hemocor D as Hb test kit is used for the determination of haemoglobin in whole blood by using internationally recommended Cyanmethemoglobin method. Three test tubes marked as 'Blank', 'Standard' and 'Test solution' 5 ml of Drabkin's solution was taken. 20 µl of distilled water was added to the 'Blank' and 20 µl of haemoglobin standard (60 mg/dl) was added to the 'Standard'.

20 µl Blood was drawn from the posterior caudal vein in vial by using syringe containing EDTA. Pipette the recommended solution inside the vials as per separated by blank (b) and test (t). The blood was expelled to the test tube marked 'Test solution'. After mixing thoroughly, the samples were left undisturbed for 15 minutes at room temperature. After this the absorbance was measured in spectrophotometer at wavelength 540 nm. Haemoglobin (Hb) concentration was calculated using the following formula.

$$\text{Hb (g/dl)} = \frac{absorbance\ test}{absorbance\ standard} . \times \frac{251}{1000} \times 60$$

Where,

251 is the dilution factor i.e., total reagent volume (5.02 ml)/ sample volume (0.02 ml).

1000 is the Multiplication Factor to convert mgs. to grams.

60 is the Concentration of the HEMOCOR Haemoglobin Standard in mg/dl.

II. Protein

The total seral protein was measured by the standard Biuret method using total protein kit (Coral Clinical systems, Tulip Diagnostics Pvt. Ltd. India) as described by Lawrence (1986), which is based on the principle that the reaction between peptide bond of protein and Cu++ (from the copper Sulphate solution) that produces a blue-violet colour complex in alkaline solution. In this method, after adding 1ml of burette reagent, 20 µl distilled water was then added to the 'Blank', 20 µl of protein standard (8 g/dl) was added to the 'Standard' and 20 µl of serum was added to the remaining test tubes named 'Test' solution.

The solution was mixed well and incubated at 37 ℃ for 10 minutes. When slight colour change was noticed, the absorbance of standard and test solution were measured against the blank in the spectrophotometer at 540 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co. Ltd. China).

The total protein was calculated using following formula

Total protein (g/dl) $= \frac{absorbance\ test}{absorbance\ standard} \times 8$

III. Glucose

The glucose test was done by Eco-Pak Glucose method using the Glucose test kit (Accurex Biomedical Pvt. Ltd.) Mumbai, India.

The Eppendorf tube containing the blood sample was subjected to the centrifugation process at 3000 rpm to obtain the serum. The obtained serum was then extracted to another fresh Eppendorf tube. 1 ml Eco-Pak glucose reagent (working solution) was taken into three test-tubes named 'Blank', 'Standard' and 'Test'. After that 10 µl distilled water, 10 µl of known concentration of glucose (100 mg/dl) and 10 µl serum was added to the test tube named 'Blank', 'Standard' and 'Test', respectively. Then the assay solutions were incubated at 37℃ for 15 minutes. After the completion of the incubation period, the absorbance of testing and standard solutions were measured against blank solution at 505 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China).

The glucose was then calculated by using the following formula,

Glucose in mg/dl $= \frac{absirbance\ of\ sample}{absorbance\ of\ standard} \times 100.$

IV. Albumin

Albumin kit is used for the determination of Albumin in serum or plasma using BCG (Bromocresol green) method .

1 ml of BCG reagent were taken into several test tubes and named as 'Blank', 'Standard' and 'Test'. 10 µl of distilled water was added to the 'Blank' test tube, 10 µl of standard solution of known concentration was added to the 'Standard' and 10 µl of blood plasma was added to the test tube named 'Test'. The assay mixtures were blended well and incubated at room temperature for 5 min. Then the absorbance of the test and blank

solutions were measured against the blank in the spectrometer at 630 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China).

Albumin was calculated using following formula

$$\text{Albumin (g/dl)} = \frac{Absorbance\ of\ Te}{Absorbance\ of\ standard} \times 4$$

V. Globulin

Globulin was estimated by subtracting an albumin value from the total protein as mentioned below:

Globulin (g/dl) = Total protein (g/dl) – Albumin (g/dl)

3.5 Data Analysis

All the data were normally distributed. Opercular movement and oxygen consumption data were analysed by t-test whereas the haematological and biochemical parameters were analysed by one-way ANOVA followed by TUKEY-HSD post hoc test. The Probit analysis method was used to determine the LC_{50} value of CPF. For this analysis, a statistical software package (SPSS-version 20) was used.

4. RESULTS

4.1 Acute toxicity

During this experiment, no fish mortalities were recorded in control groups. By the 24 h of exposure, mortality started to begin from 0.5 mg/L. During the entire 96 h experimental period, 100% of fishes were found to be dead at 4 and 8 mg/L concentration within a very short exposure period (24 h). No fish were able to survive up to 96 h at concentrations from 0.5 to 8 mg/L (Table 4).

Table 4: Cumulative mortality of fingerling of Rohu in 4 replications

Concentration	**Fish exposed**	**24h**	**48h**	**72h**	**96h**
0 mg/L	20	0	0	0	0
0.5 mg/L	20	11	20	20	20
1 mg/L	20	14	20	20	20
2 mg/L	20	14	20	20	20
4 mg/L	20	20	20	20	20
8 mg/L	20	20	20	20	20

The LC_{50} value of Chlorpyrifos to *L. rohita* in at 24, 48, 72, and 96 h with a 95% confidence limit was recorded as 0.521 mg/L, 0.446 mg/L, 0.405 mg/L, and 0.362 mg/L, respectively. The inverse relation between lethal concentration values and exposure period was seen, i.e., the lethal concentration of pesticide depends on time. As the time duration increases the concentration of pesticides goes in decreasing order.

Table 5: LC (50-90)% of Chlorpyrifos to Rohu in different exposure hours

Species	Pesticide	LC(50-90)%	Hours			
			24	48	72	96
Labeo rohita	Chlorpyrifos	50	0.521	0.446	0.405	0.362
		60	0.72	0.484	0.442	0.396
		70	1.018	0.529	0.486	0.436
		80	1.528	0.586	0.542	0.488
		90	2.68	0.677	0.632	0.571

4.2 General behavioural responses of fish

The observation was done from the first hour of pesticide exposure. The alterations in general behaviour exhibited by Rohu have been provided in detail in table 6. The fish in the control group exhibited normal behaviour. Pesticide exposed fishes exhibited hyper-exciting movements, change in colour, avoiding schooling behaviour, aggregating at the corner of the aquarium, water surfacing, increased air gulping, abrupt swimming, sluggishness or motionlessness, adopting vertical positions and internal haemorrhage in Rohu.

Table 6: General behaviour changes in fish (alive) in different concentrations. (-: absent, +: mild, ++: moderate, +++: high).

Fish behaviour	**Concentration of CPF (mg/l)**					
	0	0.5	1	2	4	8
Mucus secretion	-	-	+	++	+++	+++
Hyper excitement	-	++	++	++	+++	+++
Equilibrium loss	-	+	+	++	+++	+++
Change in body colour	-	+	+	++	+++	+++
Avoiding schooling behaviour	-	++	++	++	+++	+++
Aggregating at the corner	-	++	++	++	+++	+++

Hyper excitement, equilibrium loss started from the first hour of the exposure. Mucus secretion and mortality on fish started from 48 h of exposure in 1 mg/L concentration. Fishes started to avoid schooling and aggregating at the corner from 72 h of first exposure. As all the fishes were dead within 24 h of the exposure at the high concentrated group, no more particular behaviour changes could be recorded but some abnormal behaviour changes such as rapid operculum movements, erratic movements, gulping at water surface with change in body colour were observed before death.

4.3 Sub-lethal exposure

4.3.1 Opercular beat rate

The fishes exposed to the chlorpyrifos always exhibited significantly higher ($p < 0.01$-0.001) opercular movements as compared to control. The highest opercular movements were observed after 96 h of exposure (Fig. 1).

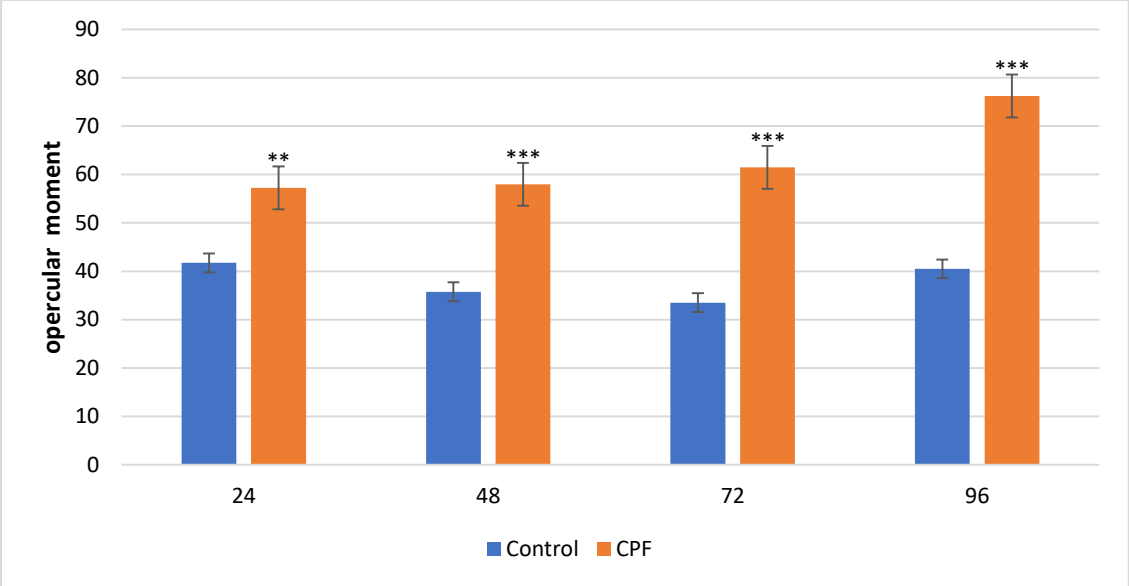

Figure 1: Opercular movement by Rohu during different exposure periods. Data are expressed as mean ± SD (n=4). Asterisk (*) represent the significant difference between the control and pesticides exposed groups (**$p < 0.01$, ***$p < 0.001$).

4.3.2 Oxygen consumption

Exposed fish consumed more oxygen compared to control. After 24 h there was an increasing trend of oxygen consumption with the exposure time and the highest oxygen consumption was observed during 72-96 h of exposure. However, these increments were insignificant in statistical analysis (Fig. 2).

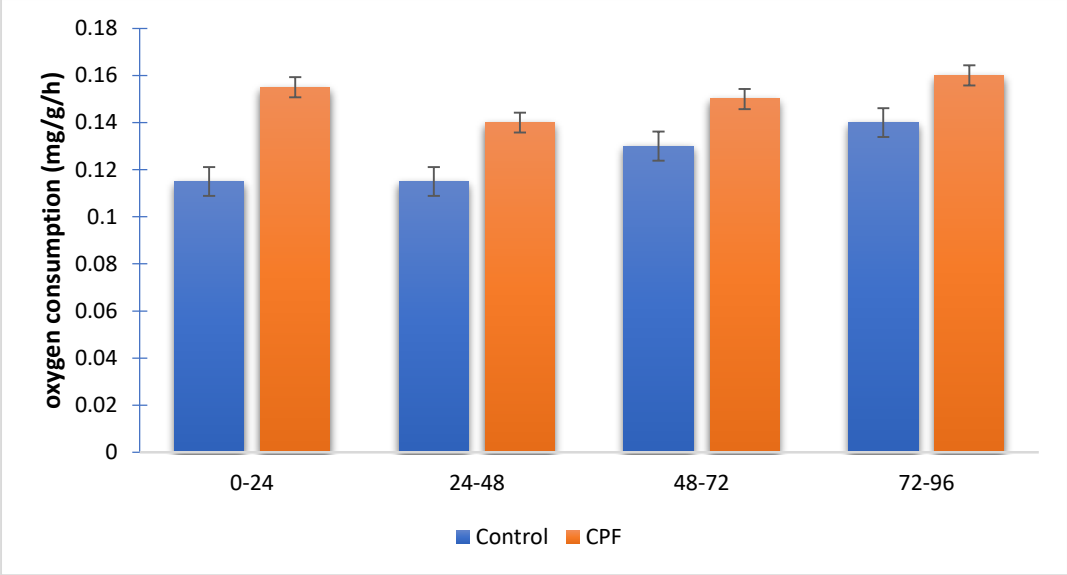

Figure 2: Bar represents the mean oxygen consumption rate of fish *Labeo rohita* in control and sub-lethal concentration of chlorpyrifos (0.1mg/L). Data are expressed as mean ± SD (n=4).

4.3.3 Haematological and biochemical Parameters

A. Haemoglobin

After analysis of haemoglobin content among three experimental groups, after 96 h of exposure, it was found that both low and high CPF dose exposed fish exhibited higher haemoglobin content compared to control ($p < 0.01$). Furthermore, the haemoglobin of fish exposed to CPF high dose was slightly higher than that of CPF low dose exposed fish. Hence it was statistically significant which indicated effect of CPF dose on haemoglobin (Fig. 3).

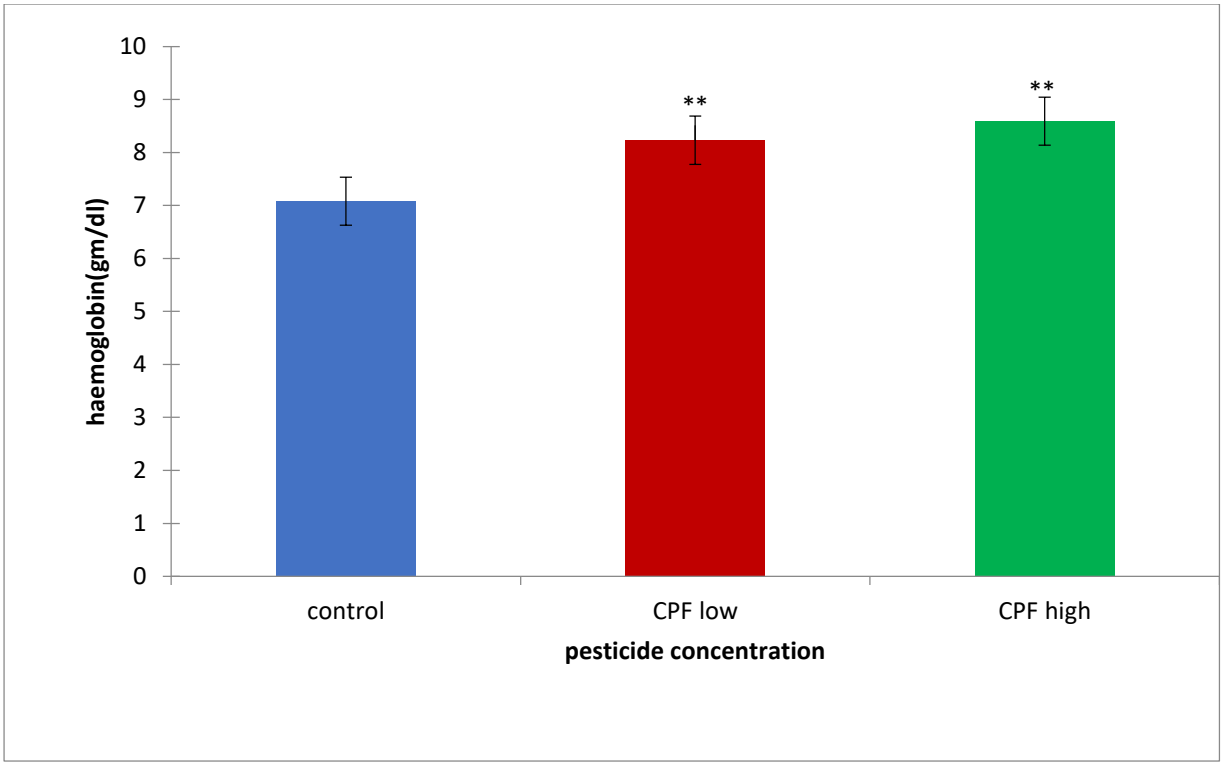

Figure 3: Sub-lethal effect of CPF on haemoglobin content in the blood of *Labeo rohita*. Data are expressed as mean ± SD (n=6). Asterisk (*) represent the significant difference between the control and pesticides exposed groups (**$P < 0.01$)

B. Glucose

After 96-h treatment, the sub-lethal concentration caused the rising of glucose level in blood serum of pesticide-exposed fishes but statistical analysis showed no significant difference between control and low chlorpyrifos exposed groups whereas significant difference was found between high chlorpyrifos dose and control group ($p < 0.05$). High dose showed higher glucose level than that of its low dose but the difference was again insignificant (Fig. 4).

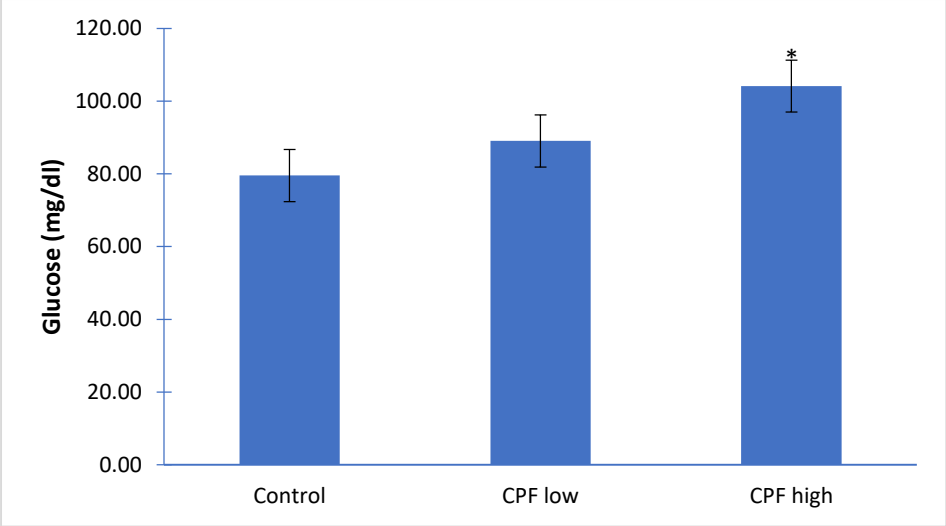

Figure 4: Sublethal effect of CPF in glucose content among three groups in 96-h exposure duration. Asterisk represent the significant difference between the control and exposed groups (*$P < 0.05$).

C. Albumin, globulin and Total Protein.

With compared to control, the globulin content in fish was found to be decreased in pesticide exposed groups and the nearly same result was found in the analysis of albumin content. Reduction in albumin and globulin decreased the total protein content in a similar pattern but the difference were not significant (Fig.5).

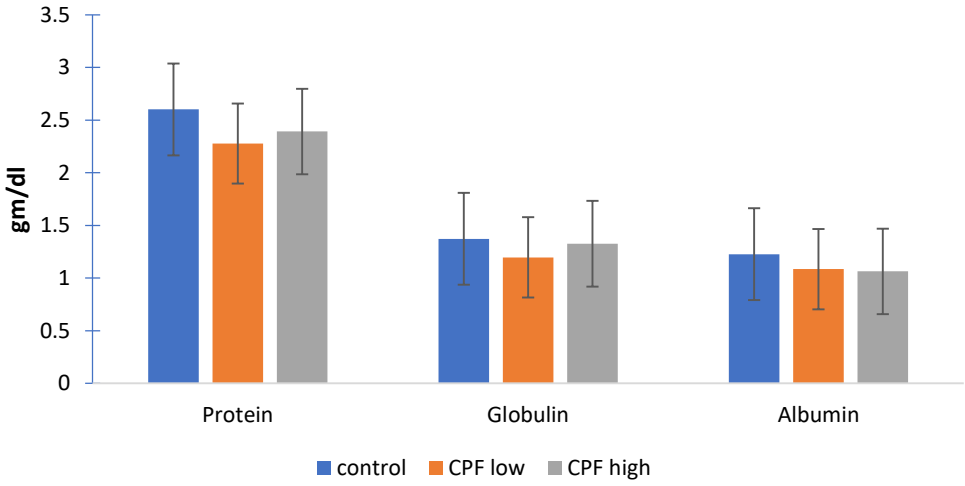

Figure 5: Albumin, globulin, and total protein content in three different groups after exposure to sub-lethal concentration of CPF.

5. DISCUSSION

The study of acute toxicity of various pesticides to fish for different exposure period reveals wide differences in the LC_{50} value as it varies with duration of exposure and kind of fish (Ramasamy et al. 2007). The 96 h LC_{50} tests are conducted to assess the vulnerability and survival potential of organisms to particular toxic chemical substances and chemical agents with lower LC_{50} values are more toxic because their lower concentrations result 50% of mortality in organisms (Tarkhani et al. 2012).

Median lethal dose (LC_{50})

LC_{50} value is the measure of concentration of the chemical that kills 50% of test animals during the observation period. It is used to find the acute toxicity of pesticides. The present finding demonstrates LC_{50} value to be 0.362 mg/L after 96 h of treatment. Beside this, the 96 h LC_{50} of nonylphenol (NP) for *Labeo rohita* was estimated to be 0.548 mg /L (Karmakar et al. 2020). In addition to this, Binukumari and Basanthi (2013) exhibited LC_{50} value as 0.398 ppm for 96 h upon exposure of pesticide dimethoate on fresh water fish *Labeo rohita.* Shobana et al. (2020) revealed that the value of 96 h LC_{50} was 0.035 mg /L upon exposure of fish *L. rohita* to silver nitrate. Isamail et al. (2014) did a study and 96 h LC_{50} value of chlorpyrifos was estimated to be 442.8 mg/L.

In another study, four fish species namely, stinging catfish (*Heteropneustes fossilis*), spotted snakehead (*Channa punctatus*), climbing perch (*Anabas testudines*) and tangra (*Batasio tengana*) were exposed to various concentrations of chlorpyrifos to investigate the mortality rate of fish species and the toxicity level of the pesticide. The LC_{50} values of chlorpyrifos on these fish species were 23.10, 20.32, 16.61 and 13.94 ppm, respectively at 96 h of exposure.

Furthermore, Lazher and Ricardo (2012) evaluated the short-term toxicity of seven selected pesticides: four kinds of insecticides (chlorpyrifos, dieldrin, diazinon and pirimiphosmethyl) and other three herbicides (diuron, alachlor and atrazine). It was observed that chlorpyrifos was most toxic pesticides tested for both embryos and larvae and median lethal concentration of CPF during a 48-h and a 96-h exposure was 116.6 mg/L and 94.65 mg/L. Among these results, it is clear that the lethal concentration is varying species to species because their strengths are different (Zahan et al. 2019). Laboratory setups, fish size, weight, physiochemical parameters, fish handling methods etc. impact the

value of LC_{50} of pesticide to a particular fish, which might be the reason behind in variation in LC_{50} value of same pesticide to similar fish (Omoniyi et al. 2013).

Respiration

One of the most important physiological parameters to assess the toxic stress is the respiratory potential or oxygen consumption of an animal. As aquatic organisms have their outer bodies and important organs such as gills almost entirely exposed to water, hence, the effect of toxicants on the respiration is more pronounced (Holden 1973). Halappa and David (2009) revealed that chlorpyrifos (20% EC) was highly toxic and had a profound impact on the oxygen consumption and food consumption of *Oreochromis mossambicus* exposed to lethal and sublethal concentrations. Beside this, Hartl et al. (2001) exhibited that if gills or membrane functions are destroyed due to xenobiotic chemicals or the membrane functions are disturbed by a change in permeability the oxygen uptake rate would rapidly decrease. However, in the present study, although elevation of oxygen consumption rate in comparison to control was found, the result was not significant. It may be due to increase in metabolic activity of fish to reduce toxic effect (Chang et al. 2020).

Behaviour

It has been found that, upon pesticide exposure, fishes show various change in their behaviour patterns like erratic swimming, loss in equilibrium, surfacing phenomenon and many more. One of the major symbolic behaviours shown by fishes was avoidance of schooling. During this study, similar kind of behaviour as that of control was observed in lower concentration of CPF. However, in higher concentration of CPF, change in behavioural patterns such as erratic swimming, loss of equilibrium, mucus secretion, imbalanced posture, surfacing, schooling behaviour, rapid operculum beat rate, low feeding rate etc. were pronounced. The reason for change in behaviour shown by fish may be its response to avoid contact of pesticide. Various studies have shown that decreased Ache activities due to organophosphate exposure can disturb various behaviours in fishes such as swimming, feeding etc. (Scholz et al. 2000). Similarly, Kalavathy et al. (2001) found that surfacing phenomenon in exposed group might be due to high oxygen demand during the experimental period. Similar result to this study was found when fishes were exposed to 297 μg/L for 96h, LC_{50} of chlorpyrifos, the fishes under pesticide stress showed behavioural symptoms of dullness, loss of equilibrium, loss of feeding and erratic swimming (Kavita and Rao 2007). Furthermore, similar behavioural changes were also

seen previously in the fish exposed to OP pesticides (Machado and Fanta 2003). Acute toxicity of NP induced several behavioural alternations like erratic swimming, avoiding schooling behaviour, loss of feeding etc. in fish *Labeo rohita* upon exposure to nonylphenol (NP) (Karmakar et al. 2020). Similarly, Ismail et al. (2014) found that fish exposed to different concentrations of chlorpyrifos showed different neurotoxic behavioural responses. It was concluded that chlorpyrifos is a genotoxic and neurotoxic insecticide causing DNA damage and neurotoxic effects in *L. rohita*. In addition to this, in another study, using higher concentration of chlorpyrifos, alterations in physiological and behavioural responses especially erratic swimming, gulping, mucus secretion, increased opercular movement and profuse emission of mucus all above the body were observed during the primary stages of contact after which it became occasional (Zahan et al. 2019).

Blood parameters

The fish blood in gills is in direct contact with water medium, any adverse change in the aquatic environment could be revealed in the circulatory system. Because of these reasons, fish blood has been widely used in toxicological and environmental monitoring as a possible indicator of physiological changes in fisheries. So, the haematological studies of fish could be used to indicate the health status of fish and water quality (Borges et al. 2007). Behavioural abnormalities and haematological and biochemical change have been observed in fish exposed in pesticide (Napit 2013). In context of present study, it was found that haemoglobin content significantly increased with increase in concentration of pesticide CPF. The reason for this might be due to increment of metabolic activity to eliminate toxic elements. As the metabolic rate increases, the rate of elimination of the toxic substances off the body also increases (Wang et al. 2017). Similarly, Lavanya et al. (2010) and Sarvanan et al. (2012) also found significant rise in haemoglobin content in fish blood after exposure to pesticide, the reason for which they explained was to supply enough oxygen to the tissue due to toxic effect. However, Ismail et al. (2017) conducted a study and effects of chlorpyrifos on the haematological parameters of the fish were observed. During the experimental period, haematological parameter like haemoglobin (Hb) decreased due to chlorpyrifos in freshwater fish, *L. rohita*. Similar result was found by Ramesh et al. (2014) and Priya et al. (2015), where haematological parameter like haemoglobin decreased in fish *L. rohita* when exposed to sublethal concentration of Na_2SeO_3 (sodium selenite). The alteration in these parameters were found to be dependent on dose and exposure period. Also, the results of present study indicate that the alteration

of these parameters may relate due to physiological stress system. The haematological characteristics of fishes are an integral part of evaluating their health status. However, the diet composition, metabolic adaptation and variation in fish activity are the main factors responsible for the change in haematological parameters of fish (Binukumari 2013).

Beside this, study conducted by Kavita et al. (2010) on Indian major carp *Catla catla* upon exposure to arsenate concluded increment in plasma glucose levels throughout the exposure period. In the present study, glucose level increased. The reason might be due to use of protein to produce glucose to meet energy requirement. Similar study was found by Shobana et al. (2020) where increased level of glucose was observed in fish *L. rohita* upon exposure to silver nitrate. It has been found that stress stimuli elicit the rapid secretion of Glucocorticoids and catecholamine hormones that are responsible for production of hyperglycema in animals (Sharmin et al. 2016). Hence, in present study, the increment in the level of glucose may be due to increased glucogenesis response of stressed fish to meet their energy requirements.

In the present study, it was found that protein level declined compared to fish kept under control environment. The reason for this could be due to mobilization of protein to produce glucose to meet extra energy requirement. Similar result was found in a study conducted by Siddique et al. (2020) where comparison among different concentrations indicated that total protein contents in all selected tissues of CPF exposed fish Rohu were decreased with the passage of time. Similarly, Ramesh et al. (2014) recorded a decrease in protein level in fish *Labeo rohita* when exposed to sublethal concentration of Na_2SeO_3 (sodium selenite). Furthermore, Lavanya et al. (2010) also found a decrease in protein level in *Catla catla*, an Indian major carp, upon exposure to arsenic trioxide. Beside this, glucose is synthesized by the metabolic consumption of keto acids which ultimately result in the depletion of protein contents (Muley et al. 2007). Also, the study by Rani et al. (2008) reported a very significant decrement in protein and lipids in *L. rohita* due to nuvan toxicity. Similarly, study conducted by Karmakar et al. (2020) showed that among the biochemical parameters, blood glucose level increased in fish *L. rohita* however, significant decrease in total serum protein, albumin and globulin level was noticed upon exposure to nonylphenol (NP). In summary, in the presence of OP pesticide, protein content is reduced either due to the inhibition of protein synthesis or increased degradation/oxidation of proteins by ROS (Tilak et al. 2005).

6. CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The study confirms that chlorpyrifos is highly toxic to *L. rohita*. Chlorpyrifos brought in behavioural changes, enhanced operculum beat rate, and oxygen consumption rate. Beside this, research also showed the increment in haemoglobin as well as glucose level but depletion of albumin, globulin and total protein content under sublethal toxicity of chlorpyrifos. After the overall analysis, it is summarized that pesticide chlorpyrifos shows a serious effect on the physiological, behavioural, metabolic, and biochemical aspects of *L. rohita*. Hence, it is expected that the use of this pesticide is eventually becoming an environmental hazard to non-target organisms thus causing serious threats to aquatic biodiversity until and unless protective measures are taken.

6.2 Recommendations

Pesticide's unplanned and improper use causes significant deleterious effects on aquatic life; hence, they must be carefully used. Therefore, my recommendations for future researchers as follows.

- To conduct more research to test whether these effects are detrimental to other fish species in the field.
- To aware people about both negative consequences of pesticides and promote biological measures for pest control. The governments should emphasize and encourage in conducting more research in this field and must give continuity of IPM programme for minimizing the use of chemical pesticides.

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APPENDICES

Photo plates

Photo 1- Experimental setup for Lc50 test

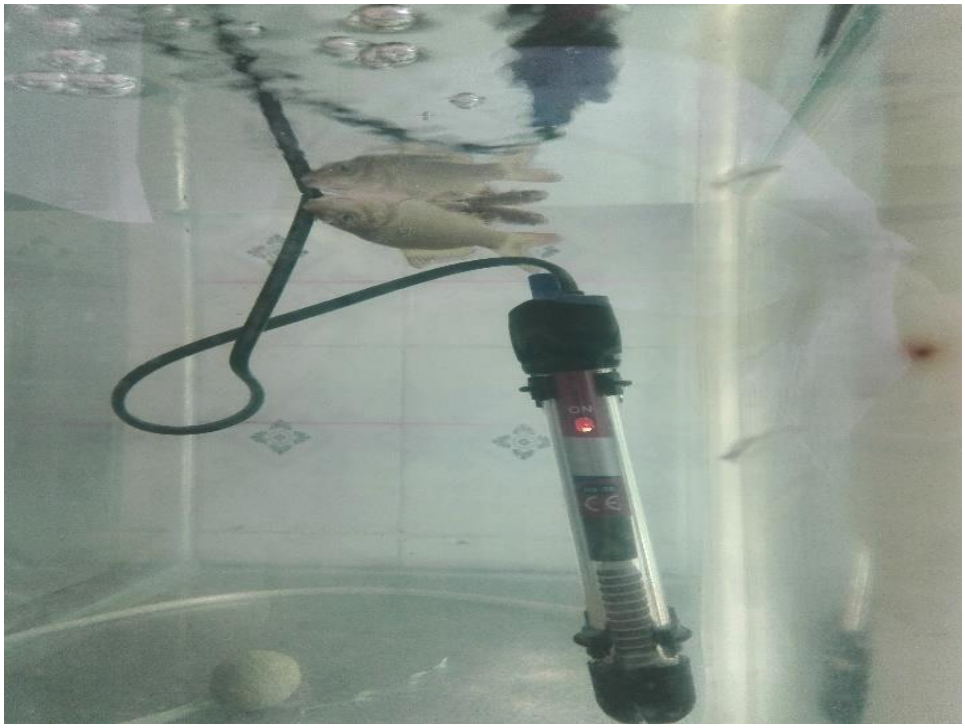

Photo 2- Inspecting equilibrium loss by fish

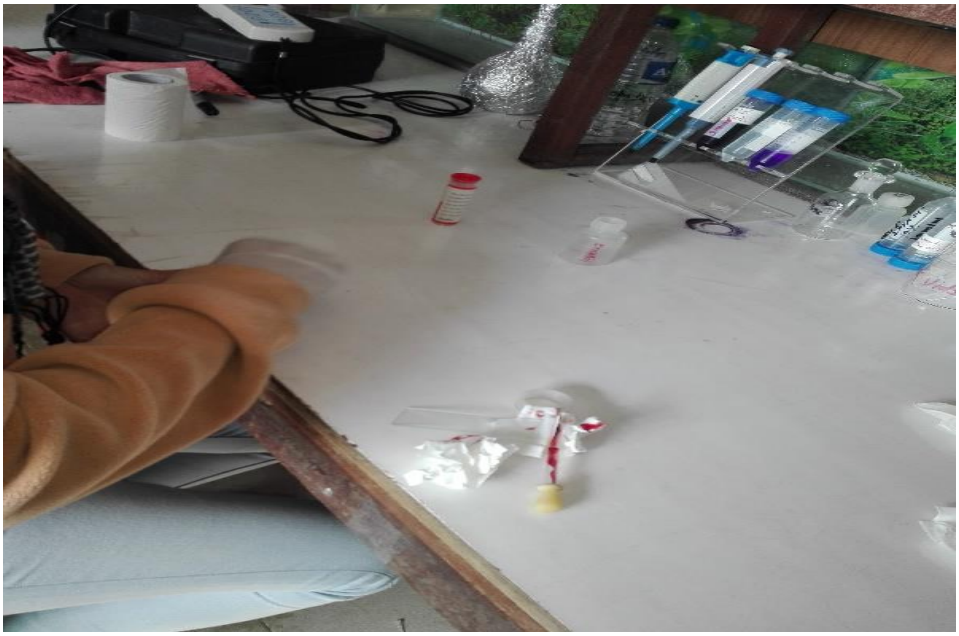

Photo 3- Collection of blood using a syringe and capillary tube for haematological analysis

Photo 4- Observing fish behaviour