ASSESSMENT OF ORGANOPHOSPHATE TOXICITY IN ECONOMICALLY IMPORTANT FISH SPECIES OF NEPAL

A THESIS SUBMITTED TO THE
CENTRAL DEPARTMENT OF ZOOLOGY
INSTITUTE OF SCIENCE AND TECHNOLOGY
TRIBHUVAN UNIVERSITY
NEPAL

FOR THE AWARD OF
DOCTOR OF PHILOSOPHY
IN ZOOLOGY

BY
PRABESH SINGH KUNWAR
DECEMBER 2022

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Institute of Science and Technology

DEAN'S OFFICE

Kirtipur, Kathmandu, Nepal

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EXTERNAL EXAMINERS

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DECLARATION

Thesis entitled **"Assessment of organophosphate toxicity in economically important fish species of Nepal"** which is being submitted to the Central Department of Zoology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Kumar Sapkota of the Central Department of Zoology, Tribhuvan University and co-supervised by Prof. Dr. Gudrun De Boeck of the Department of Biology, University of Antwerp, Belgium and Associate Prof. Dr. Amit Kumar Sinha of the Department of Aquaculture and Fisheries, University of Arkansas, USA.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

Prabesh Singh Kunwar

RECOMMENDATION

This is to recommend that **Prabesh Singh Kunwar** has carried out research entitled **"Assessment of organophosphate toxicity in economically important fish species of Nepal"** for the award of Doctor of Philosophy (Ph.D.) in **Zoology- Fish and Fisheries** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

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LETTER OF APPROVAL

Date: 29/12/2022

On the recommendation of Prof. Dr. Kumar Sapkota, Prof. Dr. Gudrun De Boeck, and Assoc. Prof. Dr. Amit Kumar Sinha, this Ph.D. thesis submitted by **Prabesh Singh Kunwar,** entitled **"Assessment of organophosphate toxicity in economically important fish species of Nepal"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U..

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……..

Prabesh Singh Kunwar

December 2022

ABSTRACT

Pesticides are harmful chemical compounds intended to kill pests. Pesticides are used to protect crops and improve production. Because of this property, pesticides are emerging as one of the necessary items in agriculture sector. The applied pesticides reach to aquatic environment through different routes. Hence, aquatic life including fish are under a serious threat with increasing pesticide application globally. This study was conducted to assess chlorpyrifos, dichlorvos and their mixture toxicity to fish. Although individual effects of these pesticides on fish are known, their joint effects are still unexplored. For this study, healthy and clinically active juveniles of the economically important freshwater fish species *Tor putitora* (golden mahseer), *Cyprinus carpio* (common carp) and *Cirrhinus mrigala* (mrigal) were exposed to individual pesticides and their mixtures. The experiment was accomplished in three phases. In the first phase, acute toxicity test was conducted to estimate lethal concentrations of pesticides. During this experiment general fish behavior was also observed simultaneously. The second phase of the experiment was designed to quantify effect of pesticides on specific fish behavior and aerobic respiratory metabolism. The third phase of the experiment was conducted to examine the effects of pesticides on fish blood biochemical parameters. In the first experimental phase, all three species were used. For second and third phase of the experiments, only golden mahseer and common carp were selected. The species screening was based on the results obtained from the first experiment, one representing antagonistic and another representing synergistic interaction of the pesticide mixture by the end of the experiment. At 96 h median lethal concentration of chlorpyrifos and dichlorvos to golden mahseer, common carp and mrigal were estimated to be 0.753 (0.616-0.931) and 12.964 (10.866-15.515), 0.440 (0.373-0.504) and 15.705 (14.385-16.963), and 0.380 (0.319-0.450) and 11.367 (9.496-13.536) mg/L, respectively. The results showed that for all the three species tested, chlorpyrifos is more toxic than dichlorvos. The joint actions of these organophosphates were found to be antagonistic in golden mahseer and mrigal whereas they were synergistic in common carp, indicating species-specific action of pesticides. General behavioral manifestation in pesticide exposed fishes included hypo excitement, loss of equilibrium and schooling behavior, surfacing and gulping air occasionally, clumping at the corners of the aquaria, color changes, excess mucus production, and abrupt swimming in spirals before death.

Significant elevation of opercular movements and feeding depression were also observed in all pesticide treatments and exposure time. However, in common carp the elevation was not significant at the first exposure hour. The aerobic respiratory metabolism was always in increasing trend in common carp, but in golden mahseer, both increasing and decreasing trends were recorded. Gill lesions and excessive mucus secretion could be the possible reasons for low oxygen consumption in golden mahseer with individual pesticide treatments. In golden mahseer, the compiled data analysis showed significant elevation of glucose, alanine aminotransferase (ALT), alkaline phosphatase (ALP), increasing trends of urea, creatinine, aspartate aminotransferase (AST) and decreasing trends of protein, albumin, and globulin. However, the compiled data analysis in common carp revealed a significant increase in glucose, protein, albumin, urea, AST, ALT, ALP and a rising trend of globulin and creatinine. In both fish species, triglyceride levels were significantly reduced in pesticide exposed groups. The results based on various biochemical parameters indicate that vital organs such as fish kidneys and liver were affected by both organophosphates. The kidney was mainly affected by the pesticide mixture treatment in golden mahseer, but it was affected by all pesticide treatments in common carp. The liver was more sensitive to the individual pesticides (chlorpyrifos and dichlorvos) in golden mahseer, but in common carp, it was more sensitive to chlorpyrifos and pesticide mixture treatments. Effects of organophosphates on vital organs of common carp were visible from the beginning of the exposure while it was observed only in the later phase of the exposure in golden mahseer. Consequences of pesticides on fish physiological and biochemical parameters can influence animals' overall performance leading to destruction of aquatic biodiversity in the long run. The majority of the deviating biochemical indicators tended to settle down during the one week depuration period and exhibited recovery signs from pesticide effects. To strengthen the fundamental factors responsible for antagonistic or synergistic effects of chlorpyrifos and dichlorvos toxicity, estimation of key enzymes (glutathione-S-transferase, acetylcholinesterase, carboxylesterases, and cytochrome P450) and histopathological analysis of gills, kidney and liver are recommended in future studies. The study also recommends application of bio-pesticides and implementation of integrated pest management programs for the protection of aquatic resources.

LIST OF ACRONYMS AND ABBREVIATIONS

ACh	: Acetylcholine
AChE	: Acetylcholinesterase
ADP	: Adenosine Diphosphate
AGREAD	: Animal Genetic Resources and Economic Analysis Division
AI	: Additive Index
ALP/ALKP	: Alkaline Phosphatase
ALT/ALAT	: Alanine Aminotransferase
AST/ASAT	: Aspartate Aminotransferase
ATP	: Adenosine Triphosphate
BAF	: Bioaccumulation Factor
BCF	: Bioconcentration Factor
BCG	: Bromocresol Green
BW	: Body Weight
CaEs	: Carboxylesterases
CBS	: Central Bureau of Statistics
CFP	: Chlorpyrifos
CFPCC	: Central Fisheries Promotion and Conservation Centre
CYP	: Cypermethrin
DDT	: Dichlorodiphenyltrichloroethane
DDVP	: Dichlorvos
FAO	: Food and Agriculture Organization
FPBCPC	: Fish Pure line Breed Conservation and Promotion Centre
GLDH	: Glutamate Dehydrogenase
GOD	: Glucose Oxidase
GPO	: Glycerophosphate-Oxidase
GST	: Glutathione-S-transferase
IPM	: Integrated Pest Management
IUCN	: International Union for Conservation of Nature
LC	: Lethal Concentration
LD	: Lethal Dose
MDH	: Malate Dehydrogenase

NA	: Not Applicable
NAD	: Nicotinamide Adenine Dinucleotide
NADH	: Nicotinamide Adenine Dinucleotide + Hydrogen
NCBI	: National Center for Biotechnology Information
NHRC	: Nepal Health Research Council
OBR	: Opercular Beat Rate
OECD	: Organisation for Economic Co-operation and Development
POD	: Peroxidase
ppm	: Parts Per Million
PQPMC	: Plant Quarantine and Pesticide Management Centre
rpm	: Revolutions Per Minute
SD	: Standard Deviation
SGOT	: Serum Glutamic Oxaloacetic Transaminase
SGPT	: Serum Glutamic Pyruvic Transaminase
USD	: United States Dollar
WHO	: World Health Organization
WR	: Week Recovery

LIST OF SYMBOLS

α	: Alfa
ΔA	: Change in absorbance
•	: Dark Circle
≥	: Equal or greater than
≤	: Equal or less than
IU/L	: International Units Per Liter
µg	: Microgram
µl	: Microliter
ng	: Nanogram
nm	: Nanometer
n	: Number
±	: Plus minus
o	: White circle

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CHAPTER 1

1. INTRODUCTION

1.1. General background

Pesticides are substances that are primarily used to control crop-damaging pests. They are, however, not confined to agriculture; they are also employed in households and the public health to eradicate disease-causing organisms. The United Nations Food and Agriculture Organization (FAO, 2002) defines pesticides as "any substance or mixture of substances designed to prevent, destroy, or control any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transportation, or marketing of food, agriculture, or other products". As the human population is ever-increasing, pressure on agriculture to apply more pesticides for productivity enhancement is unavoidable. Therefore, substances like pesticides are highly concerned chemical in toxicology.

The application of pesticides against crop-damaging pests has a very long history (Tudi *et al.*, 2021) and bio-pesticide science is as old as human civilization (Rao *et al.*, 2007). Dichlorodiphenyltrichloroethane (DDT), one of the first synthetic chemicals, was created by Austrian chemist Othmar Zeidler in 1874, and its insecticidal properties were documented by Swiss chemist Paul Hermann Müller in 1939 (Garcia *et al.,* 2012). During World War II, DDT was first used to prevent the spread of malaria and typhus among civilians and military. For this discovery, Müller was awarded a Nobel Prize in 1948 (Metcalf, 1973). With the emergence of synthetic pesticides, the application of pesticides was intensified globally. Asia, specially India and China, is the dominating continent in terms of pesticide consumption (FAOSTAT, 2021). In 2018, global pesticide trade reached nearly 5.9 million tones equivalent to USD 37.6 billion. During the period of 2010 - 2018, over 80 percent of global pesticides trade was from Asia and Europe. China and India were responsible for a major share of pesticide export from Asia. In 2018, China, Germany, the United States of America, France, and India were the top five pesticide exporters. In the same year, the top five exporters of hazardous pesticides were Thailand, South Africa, the United States of America, Malaysia, and Nigeria. In 2018, the top five pesticides importers were Brazil, France, Germany, Canada, and the United States of

America and the top five importers of hazardous pesticides were Myanmar, Malaysia, Philippines, Thailand, and Costa Rica (FAO, 2020a).

In Nepal, pesticide (DDT) was imported for the first time to eradicate malaria during the 1950s. For the same reason, various insecticides such as Gammexene and nicotine sulfates were introduced later. Gradually, different groups of pesticides were also imported (Dhital *et al.*, 2015). During the last three decades (1990 - 2018), pesticide application increased from 60 to 574 tonnes in Nepal (FAOSTAT, 2021). Among the various pesticides used in the country, insecticide (a group of pesticides intended to kill insects) alone occupied 23.81%; of the total pesticide consumption (PQPMC, 2021). It is reported that 98.6% of the total insecticide was used only in the agriculture sector and the remaining very small fraction- 1.4%, in households and the public health sector in Nepal (PQPMC, 2019). Currently, there are 10 different types of pesticides registered under 164 common names and 3,839 trade names (PQPMC, 2021). Nepal government has already banned 24 different pesticides for their import, formulation, and application (PQPMC, 2021).

There are different ways of classification of pesticides. These classifications can be based on their toxicity level, target organisms, chemical composition, mode of action, formulation, source of origin, and so on (Akashe *et al.*, 2018). Classifying pesticides based on chemical composition is the most common and useful method (Akashe *et al.*, 2018). According to this method, pesticides are broadly classified into organophosphate, organochlorine, carbamate, and pyrethroid groups (Garcia *et al.*, 2012). Organophosphate is the group of pesticides this study has focused on because of their wide application not only in Nepal but also globally.

1.1.1. Organophosphate pesticides

Organophosphates are usually "esters, amides, or thiol derivatives of phosphoric, phosphonic, or phosphinic acids with the general structure formula $O=P(OR)_3$". They were invented during the early 19^{th} century, but their effect on insects was discovered only in 1932. Some of the organophosphates are very toxic which were also used in World War II (Marrazza, 2014).

Organophosphate pesticides are broad-spectrum pesticides that control wide range of pests. Organophosphate is the most commonly used pesticide in the agriculture and aquaculture sector worldwide (El Nahas *et al.*, 2017; Rao *et al.*, 2017; Rodrigues *et*

al., 2001). It is also one of the most widely used pesticide groups in Nepal (Aryal *et al.*, 2016; Kafle *et al.*, 2015). Organophosphate was the major contributor (35.25%) of the total insecticide consumption of the country in the fiscal year 2017/2018 (PQPMC, 2019). Organophosphates are not target-specific pesticides, therefore non-target terrestrial and aquatic species can be severely affected by such pesticides.

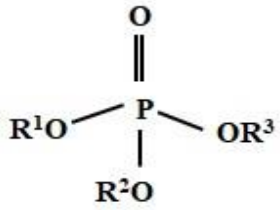

Figure 1: Chemical structure of organophosphates.

The inhibition of acetylcholinesterase (AChE) is the mechanism of organophosphate toxicity (LeBlanc *et al.*, 2012) and hence often called anticholinesterases. AChE is an enzyme that breaks down neurotransmitter acetylcholine (ACh) into acetic acid and choline. When this enzyme (AChE) is inhibited, ACh accumulates in synapses and neuromuscular junctions, causing nervous system dysfunction and, eventually, death (Jokanović, 2009; Stepić *et al.*, 2013). Organophosphates are irreversible inhibitors of AChE. This is because the time required to release the enzyme from inhibition may be longer than the time required to liberate the enzyme (Wang *et al.*, 2015). Among the various commonly used organophosphate pesticides, chlorpyrifos and dichlorvos are the pesticides of interest for this study.

1.1.1.1. Chlorpyrifos (CPF)

"Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a synthetic, non-systemic, wide-spectrum organophosphate pesticide" (Deb & Das, 2013; Halappa & David, 2009) . It has a molecular formula $C_9H_{11}Cl_3NO_3PS$ and a molecular weight of 350.6 (NCBI, 2021a). The World Health Organization has classified chlorpyrifos as a moderately hazardous pesticide (class II) (WHO, 2020).

Chlorpyrifos was first introduced in 1965 (Deb & Das, 2013) and since then, it has been widely used for agricultural and domestic purposes (Ali *et al.*, 2009; Halappa & David, 2009; Li & Tan, 2011; Sun *et al.*, 2015). It is one of the most commonly used insecticides in Nepal as well (Aryal *et al.*, 2016; Diwakar *et al.*, 2008).

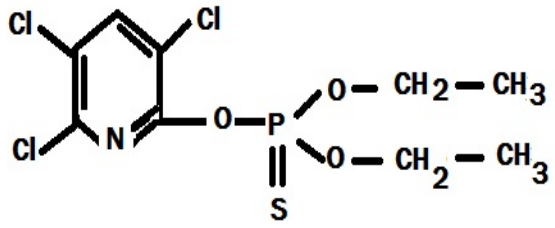

Figure 2: Chemical structure of chlorpyrifos.

In Taiwan, average chlorpyrifos levels reported in natural and cultured fish were 25 ng/gm and 17 ng/gm, respectively (Sun & Chen, 2008). In the river Deomoni, West Bengal, chlorpyrifos levels in the water, sediment, and fish were found to be 0.0091 ± 0.0020 mg/L, 0.0513 ± 0.0085 µg/g, and 5.0371 ± 1.4236 µg/g, respectively (Singh *et al.,* 2015). Fish sampled from the Tono reservoir, Ghana were detected to contain chlorpyrifos residue upto 0.20 µg/gm (Akoto *et al.,* 2016). Similarly, water and fish sampled in the Chilika lake, India contained chlorpyrifos residue 0.019 - 2.73 µg/L and 0.053 µg/gm, respectively (Nag *et al.,* 2020).

1.1.1.2. Dichlorvos (DDVP)

Dichlorvos [2, 2-dichlorovinyl dimethyl phosphate] belongs to the synthetic, non-systemic, wide range organophosphate pesticide group. Commercial production of the pesticide began from 1961 (Mennear, 1998). It has a molecular formula $C_4 H_7 Cl_2 O_4 P$ and a molecular weight of 220.97 (NCBI, 2021b). World Health Organization has classified dichlorvos as a highly hazardous pesticide (class Ib) (WHO, 2020).

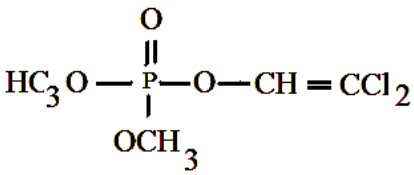

Figure 3: Chemical structure of dichlorvos.

Dichlorvos is a commonly used pesticide all over the world (Das, 2013; Mennear, 1998; Sun *et al.*, 2015; Tak *et al.*, 2014; Ural & Çalta, 2005). It is also in common use in Nepal (Aryal *et al.,* 2016; Diwakar *et al.*, 2008). Dichlorvos is most commonly used pesticide against fish parasites (Varó *et al.*, 2007) specially used in the treatment of sea lice (*Lepophtheiru ssalmonis* and *Caligus elongatus*) on commercial salmon farms (Das, 2013). Dichlorvos (0.647 µg/L) was found in the water sampled from

Chilika Lake, India (Nag *et al.*, 2020). Kafle *et al.* (2015) also documented 390 µg dichlorvos per kilogram of the soil sample from an agriculture intensified area of Nepal. The United States Environmental Protection Agency banned dichlorvos in 1981 (Laxmi *et al.*, 2019) and it is also prohibited in Nepal from 2021. However, this pesticide is still available in many parts of the world and is applied as a common insecticide.

1.1.2. Pesticide toxicity

The degree to which any chemical can harm organisms is referred to as toxicity. The most common method of reporting chemical toxicity is the median lethal concentration (LC_{50}) at which half of the organisms are dead within the exposed time period which is generally expressed between 24 h to 96 h of the exposure period. Sabra and Mehana (2015) have categorized pesticides toxicity response based on LC_{50} values. Similarly, the World Health Organization has provided a classification based on acute oral and dermal toxicity (LD_{50}, lethal dose) to the rat (WHO, 2020).

Pesticides are deleterious compounds and even small amount of pesticides can be lethal (Jokanović, 2009). Exposure to pesticides at various stages, i.e., development, production, or use can have adverse effects which are not always seen immediately but can be noticed in long term after years. Pesticides are also harmful to beneficial microbes, insects, and aquatic organisms including fish. There are several incidences of pesticide poisonings and fish kills in natural waters throughout the world (Polidoro & Morra, 2016; Sabra & Mehana, 2015). Toxic effect of pesticide on fish was reported around four decades before (Rath & Mishra, 1981). Since then, many studies have been conducted worldwide to elucidate pesticide toxicity in fish and the importance of such studies for the aquatic ecosystem.

1.1.3. Pesticide uptake and depuration

Toxic chemicals reach aquatic organisms through three different routes: absorption through the skin, transport through the respiratory surface, and uptake through contaminated diet (MacKay & Fraser, 2000). The accumulation of substances in an organism by any means is known as bioaccumulation which can be expressed by bioaccumulation factor (BAF) or bioconcentration factor (BCF) (El-Amrani *et al.*, 2012). Authors reported bioaccumulation of pesticides in fish through pesticide contaminated food (Pucher *et al.*, 2014) or through pesticide spiked water (Hanson *et*

al., 2007; Iturburu *et al*., 2016; Sancho *et al*., 1998; Welling & De Vries, 1992). There are various factors like pesticide properties, types of organism, physiological and environmental conditions which are responsible for bioaccumulation of organophosphates in fish tissues (Sancho *et al*., 1998). Pesticides such as chlorpyrifos with poor water solubility have a tendency to accumulate in living tissues (Varó *et al*., 2002). Pesticide accumulation is higher in the fish species that have a higher fat content (Sancho *et al*., 1998). Generally, the toxicants reach to important tissues and organs of fish (Iturburu *et al*., 2016) and generate toxic effects on them.

Pesticides can accumulate in human body through the food chain, and are highly dangerous for our health. Fortunately, fish are capable to eliminate the pesticides by excretion or biotransformation (Kwong *et al*., 2008; Welling & De Vries, 1992). Welling and De Vries (1992) concluded that elimination of chlorpyrifos was dominated by metabolic degradation in guppies (*Poecilia reticulata*). Among the various organs responsible for the removal of pesticides from fish body, gills are one of the efficient organs. Kwong *et al.* (2008) also documented that the removal of DDTs was faster from gills than from other routes. Fish can remove the contaminants more quickly when they are placed in pesticide free water. Within 120 h, about 62% of the pesticide was eliminated by zebrafish (*Danio rerio*; Toledo & Jonsson, 1992). After 24 h of recovery, pesticide was not detected in European eel (*Anguilla anguilla*) muscle treated with 0.02 mg/L fenitrothion, whereas 91% recovery was found in the same species treated with 0.04 mg/L (Sancho *et al*., 1998). The time required to get rid of pesticides may depend on fish species, nature of pesticides, pesticide concentration in the body and environmental factors. The pesticide elimination process might be an adaptive mechanism that enables fish to survive under unfavorable environmental conditions.

1.1.4. Aquaculture in Nepal

Nepal is one of the richest countries in freshwater, with around 2.27 % of the world's water resources (CBS, 2021). The major water resources of Nepal are rivers, lakes, reservoirs, ponds, low land paddy fields, swamps, canals, and ditches. They all together occupy 828,898 ha water surface area (CFPCC, 2019; AGREAD, 2021), which is about 5.6 % of the total area of the country.

In Nepal, aquaculture is one of the fastest-growing subsectors (Kunwar & Adhikari, 2016 & 2017). Animal Genetic Resources and Economic Analysis Division reported 97,271 tonnes of fish production that came from both aquaculture (76,271 tonnes) and capture fisheries (21,000 tonnes). Pond aquaculture was the dominating fish farming activity in Nepal with an 87.7 % contribution of the total aquaculture production. Similarly, river fishing was one of the major contributors (33.85 %) to capture fisheries (AGREAD, 2021). It is reported that 143,241 people are directly engaged in aquaculture- related works (CFPCC, 2020). From ancient time, fishing is a traditional activity and reliable income source for the communities inhabiting along the riverside, and 421,354 people (CFPCC, 2020) from 24 different ethnic communities (Husen *et al*., 2019) are engaged, in capture fisheries of Nepal. This implies that both aquaculture and capture fisheries have a significant role in employment generation and livelihood support. The fisheries sub-sector has also contributed to some extent in the reduction of youth migration in search of jobs (Mishra & Kunwar, 2014).

Nepal is a land-locked country, and the aquatic resources are solely based on inland freshwater. However, as the country is being privileged with diverse geography, aquatic habitats, and climatic conditions, it is possible for both warm and coldwater species to thrive. The distribution of the indigenous species is also diverse ranging from the lowest altitude of 60 m to 3323 m from the sea level (Rajbanshi, 2012). Fishes of Nepal have tremendous socio-economic importance because of their food and nutritional value, ornamental value, and game fish (sports) value (Rajbanshi, 2002). In Nepal, the most common fishes under cultivation are indigenous and exotic carps, pangas catfish (*Pangasianodon hypophthalmus*) and rainbow trout (*Oncorhynchus mykiss*; Kunwar & Adhikari, 2016 & 2017). According to Adhikari *et al.* (2018) these potential aquatic resources, including habitats and fish species, might be considered as opportunities for the country's fisheries and aquaculture development; but unplanned and haphazard developmental works, industrialization, and urbanization leads to aquatic habitat degradation, migratory obstruction and water pollution related threats to fisheries (Gurung, 2012). This compromises the potential of sustainable aquatic resource harnessing and thousands of people from rural fisher communities are under threat to lose their employment and income.

1.1.5. Economically important key fish species of Nepal

Nepal harbors 252 fish species belonging to 16 exotic and 236 indigenous species (Shrestha, 2019). Among these fish species, golden mahseer, common carp and mrigal are extremely important species because of their contribution in aquaculture production as well as in capture fisheries. Golden mahseer is a flagship species of Nepal whereas common carp and mrigal are the first and second major contributors in aquaculture production of the country that plays significant role in rural economy especially for water dependent communities.

1.1.5.1. Golden mahseer (*Tor putitora*)

Golden mahseer, also locally known as Sahar in Nepal, belongs to Kingdom- Animalia, Phylum- Chordata, Super-class- Pisces, Class- Teleostomi, Order- Cypriniformes, Family- Cyprinidae, Genus- *Tor* and Species- *putitora* (Pandit, 2015). Their natural habitats include Nepal, Pakistan, India, and Myanmar in the trans-Himalayan region, as well as Thailand, Lao PDR, Cambodia, Vietnam, southern China, Peninsular Malaysia, Borneo, Sumatra, and Java in Southeast Asia (Ingram *et al*., 2005).

Figure 4: Golden mahseer

Golden mahseer is categorized as one of the most beautiful food and game fish (Shrestha, 2019). Their structure and appearance are similar to a typical carp and it is one of the largest species of the family cyprinidae (Shrestha & Pandit, 2012). Their body is elongated and slightly compressed. The sides of the body are greenish silver and their belly is silvery to white. The mouth is small and the upper jaw is a little longer than the lower jaw. They have thick and fleshy lips. The head contains two pairs of barbells. Eyes are rounded, large, and located more towards the dorsal side. The whole body except the head and fins is covered with large scales (Shrestha & Pandit, 2012). Scales are golden with dark bases. Pectoral, pelvic and anal fins are reddish yellowish in color (Shrestha, 2019).

Golden mahseer is an important species for both commercial and recreational fishing (Ingram *et al.*, 2005). They are highly valued freshwater cyprinid fish in Nepal (Rajbanshi, 2002; Shrestha & Pandit, 2012; Swar, 2002). Anthropogenic threats to this species include pollution from both urban and agricultural sources. Yousafzai *et al.* (2008) documented that pollution might be one of the major reasons for rapid decline of *Tor* population. The population of this species is estimated to have decreased by more than half in the last two decades (Jha *et al.,* 2018).

1.1.5.2. Common carp (*Cyprinus carpio*)

Common carp is systematically classified to Kingdom- Animalia, Phylum- Chordata, Super-class- Pisces, Class- Teleostomi, Order- Cypriniformes, Family- Cyprinidae, Genus- *Cyprinus* and Species- *carpio.* Common carp is native to Europe and Asia and is now introduced globally (FishBase, 2021).

The common carp is characterized by an elongated to the deep oval body. Color varies from gray through silver to bronze with a yellowish or reddish belly (FAO, 2021a). The head is small, the mouth is protractile, two pairs of maxillary barbells are present, and the dorsal fin is long containing a sharp spine. They are bottom dwellers, omnivorous, and propagate easily in ponds without artificial breeding. They have good growth; and can attain 1-3 kg in the first year (Shrestha & Pandit, 2012). The optimum water temperature for breeding and growth is 20–25 °C (FAO, 2021a).

Figure 5: Common carp

Common carp is one of the world's most widely cultivated species (Shrestha & Pandit, 2012) contributing the fourth position (7.7 %) of the global finfish production (FAO, 2020b). In Nepal, it was introduced in 1956 and 1960 from India and Israel, respectively (Shrestha & Pandit, 2012) for rearing purposes and its successful artificial breeding was achieved in the mid-1960s (Singh & Yadav, 1997) which was

a great leap for aquaculture development in Nepal. This is the main cultivable species in Nepalese aquaculture. Fish production of Nepal is dominated by common carp occupying 31% of total aquaculture production (Mishra, 2015). There are two varieties of common carp in Nepal; one is German carp or scale carp (*Cyprinus carpio var. communis*) and the other is Israeli carp or mirror carp (*Cyprinus carpio var. specularis*). The German carps are uniformly covered with golden scales whereas the Israeli carps are unevenly covered with few large shiny scales (Shrestha & Pandit, 2012).

1.1.5.3. Mrigal/Naini (*Cirrhinus mrigala*)

Mrigal, also locally known as Naini in Nepal, belongs to Kingdom- Animalia, Phylum- Chordata, Super-class- Pisces, Class- Teleostomi, Order- Cypriniformes, Family- Cyprinidae, Genus- *Cirrhinus* and Species- *mrigala* (Pandit, 2015). The species is distributed in Pakistan, India, Nepal, and Bangladesh (FishBase, 2021).

Figure 6: Mrigal (Naini)

Mrigal is an important species for culture. They have an elongated and cylindrical body. The head is small and the mouth is sub-terminal with thin non-fringed lips. A single pair of barbells is present. The upper jaw is longer than the lower jaw (Shrestha & Pandit, 2012). The backside of the body is usually dark grey while the sides and belly are silvery in color. Fins are grayish and during the breeding season, tips of the pelvic, anal, and lower lobe of caudal are stained orange (FAO, 2021b). It is a bottom feeder and omnivorous species. Compared to common carp, their growth is relatively slow in the first year.

Mrigal is also known as indigenous major carp and their induced breeding was implemented successfully in the late 1970s which further accelerated the aquaculture development pace in Nepal by initiating a polyculture fish farming system (Singh & Yadav, 1997). Production of finger size mrigal fish, known as Chhadi, is a highly

productive culture system (Kunwar & Adhikari, 2016 & 2017; Mishra & Kunwar, 2014). In Nepal, Mrigal is the second main species having around 18% contribution in fish production after common carp (Mishra, 2015).

1.2. Rationale of the study

Pesticides are among the most hazardous chemicals. As a result, increased pesticide use poses a significant threat to human health and biodiversity. In some situations, pesticide application might be the only practical method (Dogan & Can, 2011) but the benefits of pesticides come with some negative consequences that cannot be ignored. Additionally, Nepal is a small country that lies between two huge countries China and India. Since both of these countries are using an enormous amount of pesticides (FAOSTAT, 2021), this tiny nation is always threatened by its harmful effects, therefore pesticides are chemicals of interest in toxicity studies especially for a country like Nepal.

The greater portion of the pesticides used for various purposes are discharged into the environment (Tišler *et al.*, 2009). According to Pimentel (1995), just 0.1% of pesticides sprayed reach their intended pests, with the great portion (>99.9%) remaining in the environment. Pesticides can enter the aquatic system by agricultural runoff, industrial effluent, leaching, precipitation, spray drift and improper disposal (Adhikari *et al.*, 2004; Sunanda *et al.*, 2016; Wang *et al.*, 2013; Xing *et al.*, 2012a). Chlorpyrifos and dichlorvos are regularly used organophosphate pesticides all over the world including in Nepal, therefore, their residues have already been detected in soil, water, sediments, and fish and fisheries products (Akoto *et al.*, 2016; Ensminger *et al.*, 2011; Kafle *et al.*, 2015; Nag *et al.*, 2020; Phillips *et al.*, 2007; Singh *et al.*, 2015; Sun *et al.*, 2006; Sun & Chen, 2008; USEPA, 1999; Zahran *et al.*, 2018). Due to their ubiquity in aquatic systems, chlorpyrifos and dichlorvos pesticides are priority substances to examine.

Generally, toxicity assessments are based on single pesticide tests. In aquatic habitat, pesticides are accumulated from several sources and catchment areas (Wang *et al.*, 2013) and organisms thriving there are always exposed to pesticide mixtures. Therefore, such individual pesticide assessment studies cannot reflect the actual hazards posed to aquatic organisms and these studies can be insufficient or even misleading. Pesticide mixture toxicity can be additive, synergistic, or antagonistic

depending on nature of pesticides, their combination and target species (Chen *et al.*, 2014; Wang *et al.*, 2015; Wang *et al.*, 2017; Wu *et al.*, 2018). Though toxicity of many individual pesticides and a combination of few pesticides to some fish species has been evaluated, there is still no information on the combined effect of chlorpyrifos and dichlorvos on fish.

Pesticides are one of the potential sources of contamination in the aquatic system, which has posed a threat to the fisheries sector. Water pollution changes the structure of fish communities by encouraging the spread of invasive species (Gomes-Silva *et al.*, 2020). Pesticides have also been linked to decreased pond fish productivity (Sweilum, 2006) and a decline in the population of wild fish (Klemick & Lichtenberg, 2008). As a result, monitoring pesticides' effects on aquatic organisms is critical, especially when the country is home to 16 indigenous fish species (Rajbanshi, 2012). Fish is an ideal animal for study because they are easily available, widely distributed in aquatic systems, and also a good indicator organism for water quality evaluation. For this study, three fish species (golden mahseer, common carp, and mrigal) were used as test animals because they are economically important key fish species of Nepal. Golden mahseer is a flagship species of Nepal and well recognized for its delicacy and sports fishery (Swar, 2002). The International Union for Conservation of Nature documented golden mahseer in endangered species category. Common carp and mrigal are dominating candidates in the aquaculture sector of Nepal occupying the first and second position, respectively in terms of total fish production. These three selected species are representative of both fisheries and aquaculture sector of Nepal. Therefore, conservation and protection of such species from harmful chemicals like pesticides is critical for both aquaculture and fisheries development of the nation.

Pesticides are toxic compounds and are fatal to fish. It is essential to know the toxicity level of pesticides for effective monitoring and protection of fish diversity in any water body. Therefore, this study attempted to calculate 24 h to 96 h median lethal toxicity (LC_{50}) of the selected organophosphate pesticides (chlorpyrifos, dichlorvos and their mixture) on the target fish species. In general, fish mortalities are easily noticed but pesticides at lower concentrations are also able to produce sub-lethal effects. These effects are generally neglected but are also equally important for the well-being of fish and their population. Important sub-lethal effects considered in

this study are general fish behavior, feeding response, opercular beatings, and aerobic respiratory metabolism of fish. These are non-invasive tools for toxicity evaluation, therefore these parameters were incorporated in this study.

Since fish come into direct contact with water, any type of pollution in the environment is clearly reflected in their blood (Ismail *et al.*, 2017). As a result, blood parameters can be used as a biomarker for both aquatic pollution and animal health. Authors (Öner *et al*., 2008; Roche & Boge, 1996; Singh & Srivastava, 2010; Subhashini *et al*., 2018; Witeska, 2013) suggested blood parameters could be used as important and sensitive biomarkers in eco-toxicological studies. Important biochemical parameters such as blood glucose, total protein, albumin, globulin, triglycerides, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured in this study because they are key indicators of stress, energy metabolism, and kidney and liver function in animals.

The outcomes of this study will provide baseline information to extrapolate their safe levels permissible in the environment. The joint toxicity of chlorpyrifos and dichlorvos being novel research, it is believed to make a significant contribution in the area of aquatic toxicology.

1.3. Objectives of the study

The primary objective of this research is to investigate the toxicity of organophosphate pesticide to economically important fish species (golden mahseer, common carp, and mrigal) of Nepal. To meet this broad objective, the following specific objectives were set:

- To estimate the lethal toxicity of chlorpyrifos, dichlorvos, and their mixture and assess general fish behavior against pesticide treatments.
- To quantify the effects of chlorpyrifos, dichlorvos, and their mixture on specific behavioral responses and aerobic respiratory metabolism of fish
- To examine the blood biochemical parameters of fish exposed to chlorpyrifos, dichlorvos, and their mixture.

CHAPTER 2

2. LITERATURE REVIEW

The relevant literature was reviewed considering the specific objectives set by the study.

2.1. Lethal toxicity of pesticides

Lethal toxicity of any compound is evaluated based on the concentration required to kill half of the population within the given time period. The lower the required concentration, the higher is the toxicity of the compound. The general trend of expressing lethal toxicity is between 24 h to 96 h LC_{50}. There are various factors that determine the toxicity of any chemical substance, i.e., species, exposure hour, concentration (Kunwar *et al.*, 2021a, 2021b, 2022a).

The lethal toxicity of chlorpyrifos to different fish species has been reported by various authors. In common carp, 96 h median lethal concentrations were estimated to be 0.16 (Halappa & David, 2009), 0.582, (Xing *et al.*, 2012b; Xing *et al.*, 2015), and 0.20 mg/L (Banaee *et al.*, 2013). In mrigal, 96 h-LC_{50} value was 0.44 mg/L (Bhatnagar *et al.*, 2017). In Nile tilapia (*Oreochromis niloticus*), 96 h LC_{50} value was found to be 1.57 mg/L (Gül, 2005). Similarly, Oruç (2010) reported 96 h LC_{50} values in juvenile and adult Nile tilapia as 98.67 and 154.01 µg/L, respectively. In African catfish (*Clarias gariepinus*), 24 h and 96 h LC_{50} values of chlorpyrifos (Termifos) were 1.662 and 0.861 mg/L, respectively (Nwani *et al.*, 2013). In mosquitofish (*Gambusia affinis*), 96 h LC_{50} was 297 µg/L (Kavitha & Rao, 2008). Toxicity test in zebrafish revealed 48 h and 96 h LC_{50} values to embryo (30 minutes post-spawning) were 119.7 (68.07 -554.6) and 13.03 (7.54 -19.71) mg/L, larvae (72 h post-hatching) were 0.39 (0.24 - 0.51) and 0.28 (0.13 - 0.38) mg/L and juvenile (1 month) were 1.85 (1.31 - 2.72) and 1.32 (0.81 - 1.75) mg/L. This study reported fish embryos were the most resistant while larvae were the most sensitive stage for chlorpyrifos toxicity (Wang *et al.*, 2017).

Several studies conducted in various parts of the world have documented the lethal toxicity of dichlorvos to fish species. In common carp, 96 h median lethal concentration was 21.1 mg/L (Laxmi *et al.*, 2019) and 9.4 mg/L (Ural & Çalta, 2005). In mrigal, 96 h LC_{50} was reported to be 9.1 mg/L (Velmurugan *et al.*, 2009). Similarly, the median lethal concentration of dichlorvos to the same species was

20.72 - 21.02 mg/L (Srivastava *et al.,* 2012). In rohu (*Labeo rohita*), 96 h LC_{50} was reported as 7.5 mg/L (Satyavani *et al.,* 2011) and 16.7 mg/L (Bhat *et al.,* 2012). In grass carp (*Ctenopharyngodon idella*), 96 h median lethal concentration was 6.5-7.5 mg/L under different exposure conditions (Tilak & Kumari, 2009).

Both organophosphates- chlorpyrifos and dichlorvos are very toxic pesticides and their lethal concentration to fish varies from µg/L to mg/L. The toxicity of chlorpyrifos and dichlorvos has been studied using several fish species as a model organism but their toxicity level in golden mahseer remains unknown. In toxicology, golden mahseer is one of the least explored fish species and only one study on lethal toxicity of cypermethrin in this species has been reported (Ullah *et al.,* 2015) so far.

2.2. Joint toxicity of pesticides

Evaluation of pesticide toxicity remains inadequate unless their action in pesticide mixture is well understood. A pesticide that is less toxic in individual exposure may turn out to be highly toxic in mixture form (Laetz *et al.,* 2009), therefore joint toxicity assessment is important and great caution is needed when recommending water quality standards and preparing such guidelines. Depending on the combination of pesticide mixture, their joint effects can be additive, synergistic (more than additive), or antagonistic.

In Pacific salmon (*Oncorhynchus kisutch*), Laetz *et al.* (2009) reported additive or more than additive action of pesticides (diazinon, malathion, chlorpyrifos, carbaryl, and carbofuran) in all possible combinations. In common carp, more than additive effect of chlorpyrifos and malathion was observed at the 50:50 % effect mixture (Chen *et al.,* 2014). Wang *et al.* (2015) documented the synergistic effects of malathion and triazophos and carbofuran and triazophos to common carp. Synergistic effect in zebrafish was also reported when they were exposed to pesticide mixture (Wang *et al.,* 2017; Wu *et al.,* 2018).

In common carp, antagonistic effect of carbosulfan and chlorpyrifos was observed at 50:50 % effect mixture (Chen *et al.,* 2014). A research on common carp documented an antagonistic action when fenobucarb was combined with triazophos or malathion (Wang *et al.,* 2015). In zebrafish, Wang *et al.* (2017) reported the antagonistic action of chlorpyrifos- phoxim in a binary mixture, phoxim-atrazine-chlorpyrifos in the ternary mixture (96 h), and phoxim-atrazine-chlorpyrifos-butachlor in quaternary

mixtures. In a mixture, the toxicokinetic of the individual compounds is changed which can affect the joint actions of pesticides (Hernández *et al.,* 2013). Imam *et al.* (2018) also suggested chemical interaction between two pesticides may influence the toxicity of pesticide mixture.

There are very limited studies on the joint effect of pesticides, although individual pesticides have been widely studied. Moreover, no published information on the combined action of chlorpyrifos and dichlorvos on any fish species was documented which also justifies studying the joint effects of chlorpyrifos and dichlorvos to fish.

2.3. General fish behavior

Fish exposed to pesticides can cause behavioral impairments. Fish behavior reflects the condition of the aquatic environments; therefore it is considered a promising tool in toxicity evaluation (Chebbi & David, 2010; Kesharwani *et al.*, 2018; Little & Finger, 1990). Authors reported chlorpyrifos-mediated behavioral changes in common carp (Xing *et al.,* 2015). Halappa and David (2009) described disrupted schooling behavior, fish positioning on the bottom and corner of the aquaria, loss of equilibrium with a vertical position in the water column, excess mucus secretion, and gulping air by common carp after exposure to chlorpyrifos. Behavioral studies in mrigal in response to chlorpyrifos were elucidated (Cheema *et al.,* 2018). Chlorpyrifos-mediated behavioral changes were also observed in mosquitofish (Kavitha & Rao, 2008) and African catfish (Nwani *et al.,* 2013). Similarly, dichlorvos mediated behavioral alterations were also reported in common carp (Gunde & Yerli, 2012; Ural & Çalta, 2005), mrigal (Srivastava *et al.,* 2012), rohu (Kesharwani *et al.,* 2018), African catfish (Ezike *et al.,* 2017) and guppy (*Poecilia reticulate*; Gunde & Yerli, 2012).

Behavioral abnormalities in fish might be due to inhibition of acetylcholinesterase (AChE) at cholinergic synapses accumulating acetylcholine (Ach) and causing overstimulation (Halappa & David, 2009).

Loss of equilibrium of the fish exposed to higher concentrations is probably due to the dysfunction of the central nervous system (Sikka & Gurbuz, 2006, as cited in Saha, *et al.,* 2016). The excess mucous secretion by fish exposed to pesticides was probably due to the dysfunction of the regulatory mechanism of the pituitary gland (*Saha et al.,* 2016). Another reason for excess mucus secretion could be to avoid

contact with toxic compounds (Halappa & David, 2009). The gasping of air shown by fish might be to overcome respiratory stress as well as avoid irritating toxicants (Halappa & David, 2009).

Limited studies on chlorpyrifos and dichlorvos-mediated behavioral changes are available in common carp and mrigal, but no such study is reported in golden mahseer. There was only one pesticide-behavior study found in this species where cypermethrin was used as an exposure chemical and the exposed fish exhibited some behavioral changes like hyperactivity, jumping, abrupt swimming, surfacing, aggregating in corners, balance loss, and lying vertically in aquarium (Ullah *et al.*, 2014). Although toxicologists incorporated fish behavior in their study, the effects of pesticide mixture on fish behavior are still unknown.

2.4. Feeding behavior of fish

Feeding is an important behavior to evaluate the comforts of animals in the growing environment. Impaired feeding leads to poor fish growth that has negative consequences on the overall fitness of the animal (Floyd *et al.,* 2008). The authors reported a significant reduction in feeding attempts among herbicide-exposed walking catfish (*Clarias batrachus;* Soni & Verma, 2018). The growth rate of the African catfish was significantly affected by endosulfan associated with the poor feed utilization, low protein efficiency, and the exhaustion of energy reserve (Agbohessi *et al.,* 2014). Pyrethroid exposed fathead minnow (*Pimephales promelas*) also showed impaired feeding (Floyd *et al*., 2008).

Halappa and David (2009) documented the loss of feeding in common carp after the exposure to chlorpyrifos. According to Padmanabha *et al.* (2015) tilapia (*Oreochromis mossambicus*) when exposed to lethal and sub-lethal concentrations of chlorpyrifos exhibited impaired feeding. Loss of feeding in chlorpyrifos exposed mosquitofish was found (Kavitha & Rao, 2008). A study conducted on common carp documented negative impact of dichlorvos on food consumption (Laxmi *et al.,* 2019). Loss of feeding in dichlorvos exposed bream (*Abramis brama*) was also documented (Pavlov *et al.*, 1992).

According to Halappa and David (2009), loss of feeding may be a mechanism used by fish to reduce the energetic expenditures of digestion when they are stressed. Such decreased feeding attempts may be due to the loss of gustatory sensitivity of fish to

pesticides (Ishida & Kobayashi, 1995). Although information pertaining to the feeding behavior of common carp in response to pesticide exposure is available, such information on mrigal and golden mahseer is still lacking.

2.5. Opercular beat rate (OBR) and aerobic respiratory metabolism

The opercular beat rate of the fish is directly linked to the oxygen demand. A higher opercular beat rate signifies the animal's struggle to meet the oxygen requirement. There are several studies that show elevated opercular beat rate in fish during the chemical stress. Nwani *et al.* (2013) found faster opercular movements in African catfish when exposed to chlorpyrifos-based pesticide Termifos. Likewise, lethal and sub-lethal levels of chlorpyrifos increased opercular movements in tilapia (Padmanabha *et al.*, 2015). The elevated opercular beat rate was noticed in African catfish exposed to dichlorvos (Ezike *et al.*, 2017). Rapid opercular movements were also reported in mrigal exposed to malathion (Rauf, 2015), rohu exposed to phenthoate (Tripathi & Yadav, 2015), tilapia exposed to mancozeb (Saha *et al.*, 2016), and walking catfish (*Clarias batrachus*) exposed to herbicide pretilachlor (Soni & Verma, 2018). A study in triazophos exposed common carp exhibited elevated opercular movements at lower pesticide concentration and suppressed opercular movements with increasing pesticide concentration compared to the control levels at different sampling intervals (Sarkar *et al.*, 2016). Opercular movements were elevated by triazophos but not by deltamethrin (Singh *et al.*, 2018). High ventilation rate is often associated with toxic exposure (Omoregie *et al.*, 2009) and is considered a stress response in fish (Sprague, 1971).

In aerobic organisms, respiration is directly linked with the oxidation of food substrate and energy release. A change in oxygen consumption is one of the most common physiological reactions to toxicants. This is simple to quantify and often employed globally in toxicology to assess metabolic alteration in response to adverse environment (Patil & David, 2008). Fish gills are in close contact with toxicants dissolved in water; therefore, toxicants have a greater impact on respiration (Padmanabha *et al.*, 2015). The aerobic respiratory metabolism of tilapia was higher than control when exposed to chlorpyrifos (Padmanabha *et al.*, 2015). In contrast, a reduction in oxygen consumption was reported by Tilak and Kumari (2009) when grass carp were exposed to dichlorvos.

Pesticides are responsible to cause respiratory distress or even failure by affecting the respiratory center of the brain or the tissue involved in breathing (Padmanabha *et al.*, 2015). Close contact of the respiratory surface with contaminants can incite a lesion on its surface, leading to a depression in the oxygen uptake (Barbieri & Ferreira, 2011; Jothinarendiran, 2012; Tilak & Kumari, 2009). Increased mucus secretion during stress might have also created a physical barrier for oxygen diffusion leading to reduced oxygen uptake. The stimulation of specialized protein synthesis or increased enzymatic activity to detoxify the toxicant could explain an increase in respiration (oxygen consumption) rate measured during pesticide exposure (Connell *et al.,* 1999, as cited in Patil & David, 2008). From this intensive literature review, it can be conclude that no study is available on the effects of chlorpyrifos or dichlorvos on the aerobic respiratory metabolism of common carp, mrigal, and golden mahseer.

2.6. Blood biochemical parameters

The presence of toxic compounds in the aquatic systems produces effects at the cellular or molecular level which cause significant deviation in biochemical parameters (Kavitha *et al.,* 2010). Any change in blood parameters implies alteration in animals' health; therefore these measures are considered as significant indicators in toxicity studies. For environmental risk assessment, bio-monitoring methods are more advantageous than chemical monitoring methods (Van der Oost *et al.,* 2003).

2.6.1. Glucose

Blood glucose is a widely studied biochemical parameter. It is considered one of the most sensitive stress indicators (Vosylienė, 1999). In common carp, glucose level was elevated by chlorpyrifos exposure (Banaee *et al.,* 2013; Hatami, *et al.,* 2019; Ramesh & Saravanan, 2008). Increased level of glucose was found in mrigal exposed to sub-lethal concentrations of chlorpyrifos (Bhatnagar *et al.,* 2017). Chlorpyrifos-induced hyperglycemia was also reported in African catfish, (Nwani *et al.,* 2013). The condition of hyperglycemia was reported in common carp exposed to an acute concentration of lindane (Saravanan *et al.,* 2011), mrigal exposed to ibuprofen (Saravanan *et al.,* 2012), rohu exposed to cypermethrin (Das & Mukherjee, 2003), and rainbow trout exposed to dimethoate (Dogan & Can, 2011).

Due to high energy demand, the gluconeogenesis process is accelerated that results in elevated glucose levels in the blood (Ramesh & Saravanan, 2008; Saravanan *et al.*,

2011; Bhatnagar *et al.,* 2017). Another mechanism for high glucose levels is due to accelerated glycogenolysis to fulfill the energy requirement (Ezike *et al.,* 2017). Vijayan *et al.* (1997) also suggested an immediate rise in glucose in stressed tilapia was due to glycogenolysis, whereas the long-term maintenance of glucose was due to gluconeogenesis from substrates, including lactate and amino acids. According to Firat *et al.* (2011), excessive glucose levels are caused by high glucose 6-phosphatase activity in the liver, increased breakdown of liver glycogen, or glucose synthesis from proteins and amino acids. Glucose levels may rise to cope with the elevated energy requirement during pesticide-induced stress, which is a key step in the stress recovery process (Fırat *et al.,* 2011).

In contrast to these observations, glucose level was decreased by sub-lethal treatment of dichlorvos to catla (*Catla catla*; Medda, 1993) and snakehead (*Channa gachua* ; Koul *et al.,* 2007). The hypoglycemic condition could have been caused by enhanced utilization of glucose as an instant source of energy during highly stressful conditions. Low serum glucose can also result from undernourished conditions and liver failure (Yang & Chen, 2003).

2.6.2. Protein (total protein, albumin, and globulin)

The blood protein of fish has several important functions such as compound transportation, immune function, and osmotic pressure control (Ghelichpour *et al.,* 2017). Evaluation of protein content is a diagnostic method to get insights into the physiological condition of cells (Dogan & Can, 2011) and the general health condition of the organism (Saravanan *et al.,* 2011). Since the bulk of proteins are generated in the liver, serum protein content is utilized as a measure of liver health (Yang & Chen, 2003).

Effects of chlorpyrifos on plasma protein content were examined in common carp (Ramesh & Saravanan, 2008; Banaee *et al.,* 2013) and mrigal (Bhatnagar *et al.,* 2017), wherein protein levels were reduced. Similar to chlorpyrifos, dichlorvos treatments reduced plasma protein in snakehead (Koul *et al.,* 2007), catla (Medda, 1993), and singi (*Heteropneustes fossilis*; Ahmad & Gautam, 2014).

Saravanan *et al.* (2011) reported decreased plasma protein level in common carp during sub-lethal treatment of lindane but the protein level was increased in an acute concentration of the same pesticide. Ghelichpour *et al.* (2017) documented an

increase in plasma protein in common carp after 7 days in sub-lethal exposure of indoxacarb but it began to decrease during prolonged (after 14 days) exposure. Reduction of blood protein was also reported in ibuprofen exposed mrigal (Saravanan *et al.,* 2012). Similar results were obtained during treatments of triazophos (Ghaffar *et al.,* 2015) and cypermethrin (Das & Mukherjee, 2003) in rohu and dimethoate in rainbow trout (Dogan & Can, 2011). But, there was no significant effect on blood protein level in cadmium exposed common carp (De Smet & Blust, 2001), cypermethrin exposed rainbow trout (Velisek *et al.,* 2006) and benomyl exposed Nile tilapia (Min & Kang, 2008).

Albumin and globulins are main components of total protein. Banaee *et al.* (2013) reported decreased plasma albumin and globulin in common carp during sub-lethal exposure to chlorpyrifos. Mrigal subjected to sub-lethal chlorpyrifos had lower blood albumin and globulin levels (Bhatnagar *et al.,* 2017). A mixed trend of globulin content (initially slightly increment after 7 days and decline after 14 days onwards) was reported, but no significant change in albumin content was observed in common carp exposed to a sub-lethal dose of indoxacarb (Ghelichpour *et al.,* 2017). An insignificant effect on albumin and globulin was also documented in cypermethrin exposed rainbow trout (Velisek *et al.,* 2006). Similarly, there was no significant change in albumin content in benomyl exposed Nile tilapia (Min & Kang, 2008).

During stressful conditions, organisms enhance proteolytic activity and produce energy by oxidation of amino acids to counter the stress effect of the pesticide (Dogan & Can, 2011; Narra *et al.,* 2015; Ramesh & Saravanan, 2008). The utilization of protein in metabolic purposes leads to plasma protein reduction (Saravanan *et al.,* 2012). Reduction of proteins might also be due to interruption of an amino acid (Bhatnagar *et al.,* 2017) and protein synthesis (Bhatnagar *et al.,* 2017; Dogan & Can, 2011). In addition to liver disorder and impaired protein synthesis, reduced plasma protein level might have also occurred due to kidney disorder and loss of protein from the body (Saravanan *et al.,* 2011). Contrarily, the high plasma protein level observed might be due to hepatocellular damage caused by pesticide intoxication (Saravanan *et al.,* 2011). Increased protein level under stress is an adaptive strategy to bind toxic compounds in plasma (Remyla *et al.,* 2008).

2.6.3. Triglycerides

Triglycerides fuel cellular energy and are used as an indication of the nutritional status of an animal (Öner *et al.,* 2008; Patriche *et al.,* 2011). Plasma triglyceride level was decreased in chlorpyrifos exposed common carp (Hatami *et al.,* 2019). However, it was found to be increased in the previous experiments in the same pesticide exposure to the same fish species (Banaee *et al.,* 2013). Increased level of triglycerides was also documented in sub-lethal exposure of dichlorvos to snakehead (Koul *et al.,* 2007). Experiments have also shown no significant change in triglycerides content in benomyl exposed Nile tilapia (Min & Kang, 2008).

Liver dysfunction and disorder in triglyceride uptake by adipose tissue might be the reason for the high triglyceride level in the blood (Banaee *et al.,* 2013). Low triglyceride is due to malnutrition, lower absorption in the intestine, and liver disorder for their synthesis (Hatami *et al.,* 2019).

2.6.4. Urea and creatinine

Urea and creatinine are frequently used as kidney function tests. Urea is generated as the main end product of protein metabolism while creatinine is an anhydride of creatine found in muscles (Jyothi & Narayan, 2000). Creatinine is a more persistent excretory substance than any other, making it a more accurate and powerful indicator for evaluating kidney function (Jyothi & Narayan, 2000).

Plasma urea and creatinine were reported to increase by sub-lethal exposure of chlorpyrifos to common carp (Jaffer *et al.,* 2017) and dichlorvos to singi (Shaikh & Gautum, 2014). But plasma creatinine level was reduced in snakeheads in sub-lethal exposure to dichlorvos (Koul *et al.,* 2007). These parameters rise when kidneys do not work properly (Ajeniyi & Solomon, 2014). The elevated urea and creatine were attributed to low filtration capacity and protein catabolic rates, compromising the ability of the kidney to remove from the body (Abdel-Daim *et al.,* 2020). Ahmad and Gautam (2014) also suggested high blood urea in organophosphate treated fish was because of the poor urea filtration owing to damaged kidney.

2.6.5. Blood enzymes (AST, ALT, and ALP)

AST, ALT, and ALP are the enzymes used to access the status of the liver. Plasma AST was elevated in chlorpyrifos (sub-lethal) treated common carp (Banaee *et al.,* 2013; Jaffer *et al.,* 2017). A similar response was observed in sub-lethal exposure of

dichlorvos to catla (Medda, 1993) and snakehead (Koul *et al.,* 2007). A significant effect of a sub-lethal dose of indoxacarb to common carp was observed during the early exposure phase (7 days) where AST was elevated (Ghelichpour *et al.,* 2017). Elevation of plasma AST was also reported in mrigal after treatment of ibuprofen (Saravanan *et al.,* 2012), in rohu after sub-lethal treatment of triazophos (Ghaffar *et al.,* 2015), and in Nile tilapia after copper, lead and cypermethrin treatment (Fırat *et al.,* 2011).

Plasma ALT was elevated in chlorpyrifos (sub-lethal) treated common carp (Jaffer *et al.,* 2017). A similar response was observed in sub-lethal exposure of catla (Medda, 1993) and snakehead (Koul *et al.,* 2007) to dichlorvos. Sub-lethal treatment of indoxacarb to common carp caused high ALT activity after 7 days of exposure (Ghelichpour *et al.,* 2017). Elevation of plasma ALT was also reported in mrigal after treatment of ibuprofen (Saravanan *et al.,* 2012), in rohu after sub-lethal treatment of triazophos (Ghaffar *et al.,* 2015), and in Nile tilapia after treatment of copper, lead and cypermethrin (Fırat *et al.,* 2011).

Plasma ALP was increased by sub-lethal exposure of dichlorvos to snakehead (Koul *et al.,* 2007). It was also elevated in rohu treated with triazophos (Ghaffar *et al.,* 2015). In contrast, plasma ALP activity was reduced after 7 days when common carp were exposed to a sub-lethal dose of indoxacarb but it came close to the control level from 14 days onwards (Ghelichpour *et al.,* 2017).

Higher plasma AST and ALT activities indicate some sort of disorder in Kreb's cycle (Saravanan *et al.,* 2012). Increases in such enzyme activities in serum are mainly due to the leakage of enzymes especially from hepatic cell damage (Banaee *et al.,* 2013; Deka & Mahanta, 2015; Jaffer *et al.,* 2017; Ghelichpour *et al.,* 2017) that shows the hepatotoxic effects of the toxicants (Fırat *et al.,* 2011). Although blood biochemical parameters of common carp and mrigal have been assessed, no information on these parameters of golden mahseer is available. In addition, a comparative study of individual and joint toxicity of chlorpyrifos and dichlorvos on such biomarkers is still lacking in fish.

CHAPTER 3

3. MATERIALS AND METHODS

From December 2019 to July 2021, the study was carried out at the Central Fisheries Promotion and Conservation Centre (CFPCC), Balaju, Kathmandu, Nepal. This proposal (Reg. no. 725/2019) received ethical approval from Nepal Health Research Council on 18th September 2019 (Ref. no. 1215).

3.1. Acquisition of animals and their husbandry

Three economically important fish species- golden mahseer, common carp, and mrigal were selected for the toxicity assessment. Golden mahseer used in the lethal toxicity, behavior, and aerobic respiratory metabolism study were collected from Fisheries Research Centre, Begnas, Kaski, Nepal. For blood biochemical parameters, fish (golden mahseer) were collected from Fisheries Development Centre, Markhu, Makawanpur, Nepal. Common carp used in the tests were bred and raised at CFPCC and Mrigal was collected from the Fish Pure line Breed Conservation and Promotion Centre (FPBCPC), Bhairahawa, Rupandehi, Nepal. Fish were packed in oxygenated polythene bags and safely transported to CFPCC, Balaju. For at least two weeks, these fish were acclimatized in a laboratory setting. Aquaria of 350 liters were employed for acclimatization. Individual water recirculation and filter systems were installed in each aquarium. The aeration was done with a common compressor. The fish were fed ad libitum with 32% protein feed (Sreema feed Pvt. Ltd., India). To avoid debris in the aquarium water, unconsumed feed and excrement were removed using a scoop net and siphon. Regular cleaning of water filters, pumps, pipelines, air stones, and compressors was conducted. To ensure optimal water quality, around 50% of the aquarium's water was replaced with clean water every day. Water temperate, dissolved oxygen, pH, total ammonia all fell between 23.5 - 25.7 ºC, 5.7 - 6.7 mg/L, 7.5 - 7.8, 0.18 - 0.26 mg/L, respectively.

3.2. Test chemicals (Pesticides)

Pesticides of commercial grade were chosen due to their widespread use in agricultural crops. For chlorpyrifos, Dursban manufactured by Dow Agro Sciences Pvt. Ltd., India and for dichlorvos, G-VAN manufactured by Greenriver Industry Co., Ltd., China were used in the experiments.

3.3. Toxicity experiments

The experiment was organized in three phases- one lethal exposure and two sub-lethal exposures to achieve the specific objectives set by the research.

3.3.1. Lethal exposure

Lethal pesticide exposure was the first stage of the experiment designed to estimate the lethal toxicity of the pesticides along with the assessment of the general behavior of fish. This was the first specific objective of the research.

The lethal toxicity tests were carried out as per the testing guidelines of the Organization for Economic Co-operation and Development (OECD, 1992). The experiment was conducted in 35-L rectangular glass chambers in semi-static settings. Fish were weighed and transferred from the acclimating aquarium to test aquaria and left overnight to acclimatize in the changing environment. Average weights of golden mahseer, common carp, and mrigal were 4.6 ± 0.9, 3.16 ± 0.54, and 5.52 ± 0.91 gm (mean ± SD), respectively.

The feeding was stopped one day before the trial. On the day of exposure, any remaining dirt in the test chamber was siphoned. The pesticide concentrations were selected based on the range finding test. To achieve the desired pesticide concentrations, the stock solution of pesticides was prepared in distilled water and the desired amount was added to the aquaria. For common carp the concentration were 0.25, 0.5, 0.75, 1.0 and 1.25 mg/L and 10, 15, 20, 25 and 30 mg/L for chlorpyrifos and dichlorvos, respectively. For mrigal concentrations of chlorpyrifos were 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L and of dichlorvos were 4, 8, 16, 32 and 64 mg/L, and for golden mahseer concentrations of chlorpyrifos were 0.2, 0.4, 0.8, 1.6 and 3.2 mg/L and of dichlorvos were 2, 4, 8, 16, and 32 mg/L, respectively. During each exposure experiment, a control was also maintained parallel. All of the exposure groups and controls were tested in triplicate. Aquaria were cleaned on a daily basis, and one-fourth of the water was exchanged with pesticide-containing water.

Fish mortality was monitored on a regular basis, and all dead fish were removed from the aquaria as quickly as possible. Fish mortality data was presented after 24 h, 48 h, 72 h, and 96 h of exposure. In the control group, no fish mortality was found.

Following the estimation of 96 h-LC_{50} of chlorpyrifos and dichlorvos, LC_{50} of the pesticide mixture was assessed. For this, five separate doses of pesticide mixtures

were made by combining them in equal fraction i.e. 12.5% to 200% 96 h-LC_{50} of both pesticides. Fish mortality was noted as described previously. The program used for the calculation of lethal concentration of individual pesticides and their mixture is provided in the section statistical analysis.

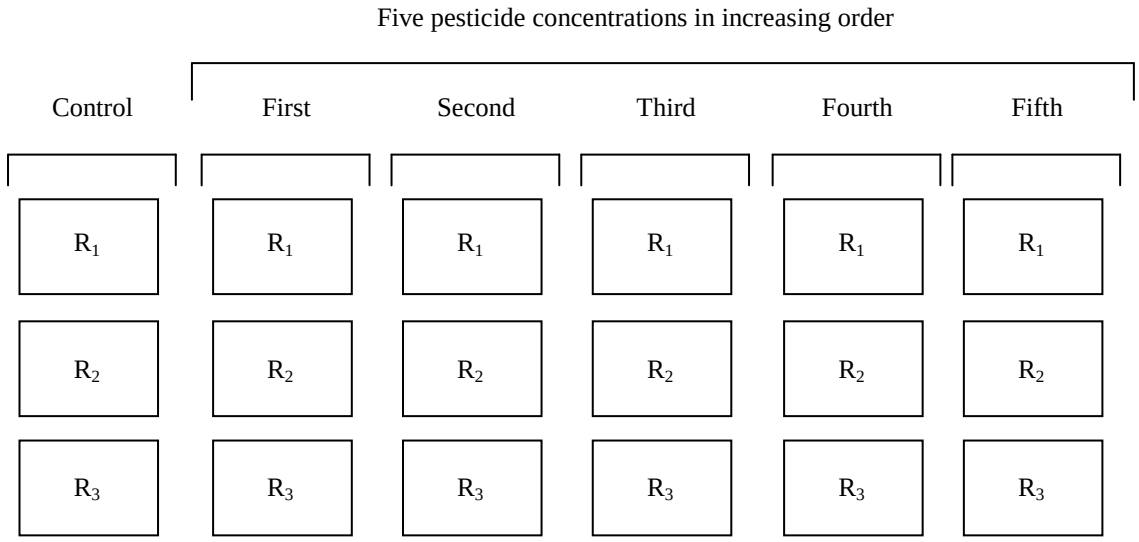

Figure 7: Research design for lethal toxicity exposures (R_1, R_2 and R_3 represent replications of each treatment).

General fish behavior

During the 96-hour fatal exposure experiment, general fish behavior such as hypo-activity, equilibrium loss, color change, aggregating at the corners of the aquarium, avoiding schooling behavior were also recorded. The observation was done very cautiously to avoid any human interference in the fish behavior study. The description of the fish behaviors recorded during the experiment is provided below:

- **Hypo-activity:** Fish becoming slow and sluggish exhibiting slow body movements.
- **Equilibrium loss:** Unable to balance body while swimming and hanging in water column vertically with head downwards.
- **Color change:** Body surface and fins and tail region becoming reddish in color.
- **Aggregating at the corners of the aquarium:** Fish tended to accumulate at the corner of the test chambers.

- **Avoiding schooling behavior:** Fish not swimming in a group and scattered throughout the test chamber.

Fish behavioral intensity (absent, mild, moderate and strong) of the pesticide exposed fish compared to the control group was recorded based on observer's evaluation.

Evaluation of joint toxicity

The joint action of the pesticides was estimated according to the additive index (AI) which was determined based on Marking (1985).

$AI = (1/S) - 1$ when $S \leq 1$ and,

$AI = 1 - S$ when $S > 1$

$S = (A_m/A_i) + (B_m/B_i)$

where, AI represents additive index, S represents the sum of biological activity, A and B represent two different pesticides, 'm' represents LC_{50} of pesticides in a mixture, 'i' represents LC_{50} of individual pesticides. The AI value equal, less, or greater than zero indicates additive, antagonistic, or synergistic action, respectively.

- Additive: this is the condition where the effect generated by pesticide mixture is equivalent to the sum of its individual pesticide's effects.
- Antagonistic (less than additive): this is the condition where the effect generated by pesticide mixture is lower compared to the sum of its individual pesticide's effect.
- Synergistic (more than additive): this is the condition where the effect generated by pesticide mixture is higher compared to the sum of its individual pesticide's effect.

3.3.2. Sub-lethal exposure for specific behavior and aerobic respiratory metabolism

Two fish species, one showing synergistic action and the other showing antagonistic action during the first phase of the experiment were selected for the sub-lethal pesticide exposure. While selecting the fish species, it was also considered that one represents aquaculture and other represents fisheries sector of Nepal. According to these selection criteria, two fish species i.e. common carp and golden mahseer were taken for further experiment.

This experiment was designed to quantify the specific behavioral response- feeding attempt, opercular beat rate, and aerobic respiratory metabolism of fish in response to pesticides. This was the second objective of the research. This experiment was conducted in 35-L rectangular glass aquaria. Two concentrations (10% and 50% 96 h-LC_{50}) of chlorpyrifos and dichlorvos and its mixture in equal proportion were used for this exposure. 96 h-LC_{50} values of chlorpyrifos and dichlorvos to golden mahseer were 0.753 (0.616-0.931) mg/L and 12.964 (10.866-15.515) mg/L, respectively and to common carp these were 0.440 (0.373-0.504) mg/L and 15.705 (14.385-16.963) mg/L, respectively. The stock solution of the pesticides was freshly prepared in distilled water. For each exposure experiment, control was maintained simultaneously. Fish were placed in aquaria for overnight acclimation after being weighed. There were 6 replicates for each treatment with one fish in each aquarium. The weight (mean ± SD) of golden mahseer and common carp were 4.5 ± 0.8 gm and 9.97 ± 2.19 gm, respectively. The same fish were used in feeding behavior, opercular beat rate and aerobic respiratory metabolism. The observation was conducted very cautiously to avoid any effect of human activities on the measured parameters.

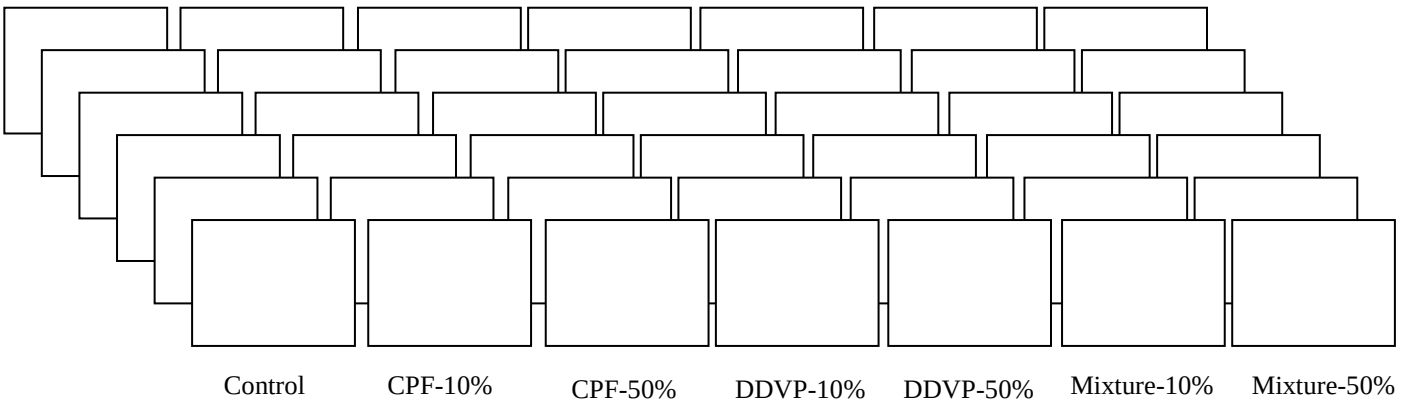

Figure 8: Research design for feeding behavior, opercular beat rate and respiration (six replicates for each treatment).

3.3.2.1. Feeding behavior

For the study of fish feeding response, twenty tiny feed pellets were spread over the aquaria and monitored carefully for five minutes. A manual tally counter was used to count the number of fish feeding attempts after 1 h, 24 h, 48 h, 72 h, and 96 h of exposure. At each sampling hour, feeding attempt of each fish was counted only once. To keep the spiking water clean, remaining food was removed promptly by scooping.

Due to the low feeding response, data is presented as average feeding attempts per five minutes.

3.3.2.2. Opercular beat rate

Opercular movements of the fish were carefully observed and counted for five minutes using a hand tally counter. Each fish was counted thrice so that a mean value could be used in the analysis for better precision. Such counting were done after 1 h, 24 h, 48 h, 72 h, and 96 h of treatment, and data is reported as mean opercular beatings per minute.

3.3.2.3. Aerobic respiratory metabolism

After accomplishing feeding and opercular counting, oxygen measurement was started. Each tank had a screened water pump that generated a mild water velocity, allowing the fish to swim gently against the current. The aquaria were sealed airtight after the aeration was turned off and the initial oxygen concentration of the water was taken with a Milwaukee MW600 PRO Portable Dissolved Oxygen meter. The following day the final oxygen content (mg/L) was measured and the process was repeated until the end of the experiment (96 h). The time interval between the initial and final oxygen measurement was 19-20 h. To avoid hypoxic conditions for fish, each aquarium was aerated at least for 3 hours before taking initial oxygen concentration. The oxygen consumption rate of fish was calculated using the following formulae,

Oxygen consumption rate of fish (mg/g/h) = $(O2i - O2f) \times V \times (1/BW) \times (1/T)$

where, O2i is initial oxygen concentration (mg/L) and O2f is final oxygen concentration (mg/L); V is total water volume (L); BW is body weight (gm) and T is time interval (h).

3.3.3. Sub-lethal exposure for biochemical analysis

The fish species were the same as in the previous sub-lethal exposure experiments. In this phase, the third objective of the research was achieved, which was to examine blood biochemical parameters in pesticide exposed fish. The acclimated fish were weighed and placed in tiny glass aquaria having 35-L capacity, where they stayed whole night. Only one fish was accommodated in each test chamber.

The fish tested in this study had average weights of 53.1 ± 4.1 gm (mean ± SD) for golden mahseer and 57.8 ± 5.3 gm for common carp. Pesticide exposure doses were based on previously estimated 96 h-LC_{50} values of chlorpyrifos and dichlorvos to golden mahseer and common carp (please refer section 3.3.2). The working pesticide solution was made in distilled water on the day of exposure, and fish were exposed to 10% 96 h-LC_{50} of each pesticide and their mixture in equal ratio. There were seven replicates for all treatments and control. After accomplishing the sub-lethal exposure experiment (96 h), fish were left in pesticide free water for 1-week of depuration.

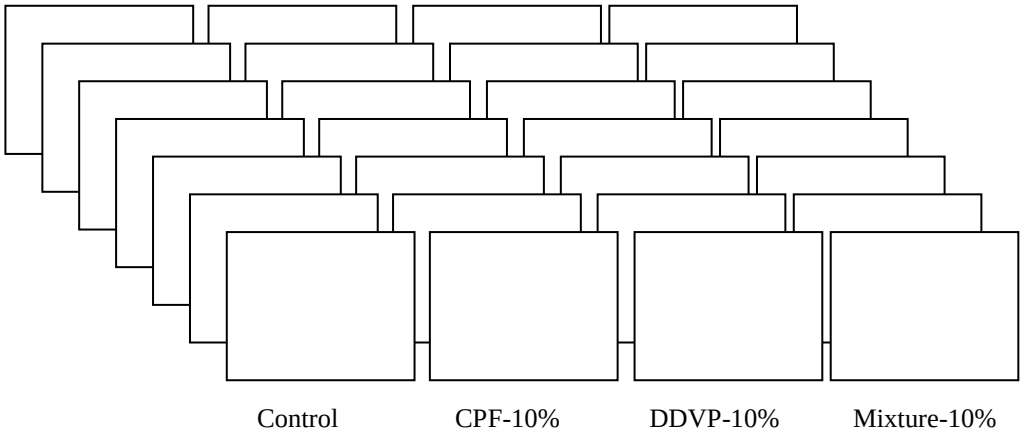

Figure 9: Research design for biochemical analysis (seven replicates for each treatment).

For biochemical analysis, blood samples were taken three times: after 24 hours, 96 hours, and one week of depuration. The fish were carefully taken from the aquarium and anesthetized with clove oil. Blood was drawn from the caudal vein of the sedated fish using a 3 ml syringe. For optimal coagulation, the blood was left for half an hour (Tuck *et al.,* 2009). The serum was then separated from the blood cells by centrifugation for 10 minutes at 3000 rpm (Zahran *et al.,* 2018) and stored at -30° C for biochemical analyses later. Samples were collected from the freezer and defrosted at room temperature on the day of measurement. Biochemical tests were performed on properly thawed samples.

3.3.3.1. Glucose

The glucose oxidase (GOD) and peroxidase (POD) methods were used to determine serum glucose using a glucose kit (Accurex Biomedical Pvt. Ltd., India). In this process, GOD transforms glucose to gluconic acid. In the presence of POD, the hydrogen peroxide produced combines with 4-aminoantipyrine and phenol to

generate red color. The color complex's intensity is correlated to the amount of glucose in the serum.

$$\text{B-D Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red dye} + H_2O$$

For glucose measurement, 1 ml of working solution was filled in several glass tubes. Ten µl of distilled water was added in one tube and marked as blank, 10 µl of known concentration of glucose (100 mg/dl) was added in another tube and marked as standard and 10 µl of serum was added in all remaining tubes as test samples.

Table 1: Sample preparation for glucose test.

Glucose test	**Blank**	**Standard**	**Test**
Working Solution	1 ml	1 ml	1 ml
Distilled water	10 µl	-	-
Glucose standard (100 mg/dl)	-	10 µl	-
Sample (serum)	-	-	10 µl

The assay mixture was incubated for 15 minutes at 37°C. After incubation, the absorbance of the standard and samples were measured against blank at 505 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China) and glucose was calculated as follows:

$$\text{Glucose (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

3.3.3.2. Total protein

The Biuret method was used to determine total protein (total protein kit, Tulip Diagnostics Pvt., Ltd., India). In an alkaline medium, proteins react with the cupric ions to generate a blue-violet color. The color intensity is directly proportional to the amount of proteins present in the sample.

$$\text{Proteins} + Cu^{++} \longrightarrow \text{Blue-violet colored complex}$$

For the measurement, 1 ml of Biuret reagent was taken in several glass tubes. Twenty µl of distilled water was added in one tube as a blank, 20 µl of known concentration of protein (8g/dl) was added in another tube as a standard and 20 µl of serum was added in all remaining tubes as test samples.

Table 2: Sample preparation for protein test.

Total protein test	**Blank**	**Standard**	**Test**
Biuret Reagent	1 ml	1 ml	1 ml
Distilled water	20 µl	-	-
Protein standard (8 g/dl)	-	20 µl	-
Sample (serum)	-	-	20 µl

The solution was mixed well and incubated at 37°C for 10 minutes. After incubation, standard and samples absorbance was measured against blank at 550 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China) and total protein was calculated as follows:

$$\text{Total protein (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \text{ X } 8$$

3.3.3.3. Albumin

The Bromocresol Green (BCG) method was used to determine albumin using an albumin kit (Tulip Diagnostics Pvt., Ltd., India). In a buffered media, albumin interacts with the dye Bromocresol Green to generate a green color. The amount of albumin in the sample is directly related to the intensity of the color generated.

Albumin + Bromocresol Green $\longrightarrow$ Green albumin BCG complex

During measurement 1 ml of BCG reagent was taken in several glass tubes. Ten microliter of distilled water was added in one tube as a blank, 10 µl of known concentration of albumin (4g/dl) was added in another tube as a standard and 10 µl of serum was added in all remaining tubes as test samples.

Table 3: Sample preparation for albumin test.

Albumin test	**Blank**	**Standard**	**Test**
BCG Reagent	1 ml	1 ml	1 ml
Distilled water	10 µl	-	-
Albumin standard (4 g/dl)	-	10 µl	-
Sample (serum)	-	-	10 µl

The solution was mixed well and incubated at room temperature for 5 minutes. After incubation, standard and samples absorbance was measured against blank at 630 nm

(UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China) and serum albumin was calculated as follows:

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

3.3.3.4. Globulin

Albumin was subtracted from total protein to calculate globulin (Qureshi *et al.*, 2016).

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

3.3.3.5. Triglyceride

Glycerophosphate-Oxidase (GPO)/PAP technique was used to quantify triglycerides (triglycerides kit, Tulip Diagnostics Pvt., Ltd., India). Triglycerides are broken down by lipoprotein lipase into glycerol and free fatty acids. In the presence of glycerol kinase, glycerol is converted to glycerol 3 phosphate, which is then oxidized to hydrogen peroxide by the enzyme glycerol phosphate oxidase. The hydrogen peroxide further reacts with 4- aminoantipyrine in presence of peroxidase to form a red colored complex. The amount of triglycerides present in the sample determines the intensity of the color produced.

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol + Free fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol 3 Phosphate + ADP}$$

$$\text{Glycerol 3 Phosphate + } O_2 \xrightarrow{\text{Glycerol 3 PO}} \text{Dihydroxyacetone phos. + } H_2O_2$$

$$H_2O_2 + 4\ \text{Aminoantipyrine} + \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye + } H_2O$$

Table 4: Sample preparation for triglycerides test.

Triglycerides test	**Blank**	**Standard**	**Test**
Working Reagent	1 ml	1 ml	1 ml
Distilled water	10 µl	-	-
Triglycerides standard (200 mg/dl)	-	10 µl	-
Sample (serum)	-	-	10 µl

During measurement, 1 ml of working reagent was taken in glass tubes. Ten µl of distilled water was added in one tube as a blank, 10 µl of known concentration of triglycerides (200 mg/dl) was added in another tube as a standard and 10 µl of serum was added in other tubes as test samples.

The solution was mixed well and incubated at 37°C for 5 minutes. After incubation, standard and samples absorbance was measured against blank at 505 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd., China) and blood triglyceride was calculated as follows:

$$\text{Triglyceride (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

3.3.3.6. Urea

The Glutamate Dehydrogenase (GLDH) Kinetic technique was used to determine urea using a urea kit (Tulip Diagnostics Pvt., Ltd., India). Urease is an enzyme that hydrolyzes urea into ammonia and carbon dioxide. Glutamate and NAD are generated when ammonia is combined with α ketoglutarate and NADH. The urea concentration in the sample is proportional to the rate of oxidation of NADH to NAD. This is measured by a drop in absorbance over a set period of time.

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\alpha\text{Ketoglutarate} + 2\text{NADH} \xrightarrow{\text{GLDH}} 2\text{ L-glutamate} + 2NAD^+ + 2\,H_2O$$

Table 5: Sample preparation for urea test.

Urea test	**Standard**	**Test**
Enzyme Reagent (L1)	0.8 ml	0.8 ml
Urea standard (40 mg/dl)	10 µl	-
Sample (serum)	-	10 µl
Starter reagent (L2)	20 µl	20 µl

The test was done according to the substrate start assay. For this 0.8 ml of enzyme reagent (L_1) was filled in a glass tube labeled S (standard) and T (test). Ten µl of known concentration of urea (40 mg/dl) was added in the standard and 10 µl of sample was added in the test glass tubes. The solution was incubated at 37 ºC for 5 minutes then 0.2 ml of starter reagent (L_2) was added in the tubes and mixed well.

The spectrophotometer (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China) used in this measurement was set zero at 340 nm against distilled water. Initial absorbance (A_1) was measured after 30 seconds then final absorbance (A_2) was measured after 60 seconds. Urea concentration in serum was calculated as follows:

$$\text{Urea (mg/dl)} = \frac{\text{Change in absorbance } (\Delta A) \text{ of the test}}{\text{Change in absorbance } (\Delta A) \text{ of standard}} \text{ X } 40$$

3.3.3.7. Creatinine

A creatinine kit (Tulip Diagnostics Pvt., Ltd., India) was used to determine creatinine using a modified Jaffe's Kinetic technique. Picric acid interacts with creatinine in an alkaline media to generate an orange-colored complex with the alkaline picrate. The amount of creatinine in the sample is proportional to the color intensity created during the set time.

Creatinine + Alkaline picrate $\longrightarrow$ Orange colored complex

For this 0.5 ml of picric acid reagent (L_1) was taken in glass tubes where 0.5 ml of buffer reagent (L_2) was added. The mixture was incubated to bring the reagents to assay temperature 37 ºC. In test-tube labeled S (standard), 10 µl of known

concentration of creatinine (2 mg/dl) was added. Similarly, in test tubes labeled T (test), 10 µl of serum was added.

Table 6: Sample preparation for creatinine test.

Creatinine test	**Standard**	**Test**
Picric acid reagent (L1)	0.5 ml	0.5 ml
Buffer reagent (L2)	0.5 ml	0.5 ml
Creatinine standard (2 mg/dl)	10 µl	-
Sample (serum)	-	10 µl

The solution was mixed well. Initial absorbance (A_1) was measured after 30 seconds then final absorbance (A_2) was measured after 60 seconds. The spectrophotometer (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China) used in this measurement was already set zero at 520 nm against distilled water. Creatinine concentration was calculated as follows:

$$\text{Creatinine (mg/dl)} = \frac{\text{Change in absorbance } (\Delta A) \text{ of the test}}{\text{Change in absorbance } (\Delta A) \text{ of standard}} \times 2$$

3.3.3.8. Aspartate aminotransferase (AST)

The activity of aspartate aminotransferase (AST or ASAT) was determined using a calkine serum glutamic oxaloacetic transaminase (SGOT)/ASAT kit (Tulip Diagnostics Pvt., Ltd., India) by a modified IFCC method. The transfer of amino group between L-Aspartate and α-Ketoglutarate is catalyzed by SGOT which produces Oxaloacetate and Glutamate. In the presence of Malate Dehydrogenase, the Oxaloacetate produced interacts with NADH to create NAD. The rate of NADH oxidation to NAD is assessed as a drop in absorbance proportional to the sample's SGOT (ASAT) activity.

$$\text{L-Aspartate} + \alpha\text{ Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

The test was done according to the sample start assay. One ml of working reagent was taken in a glass tube and incubated in water bath to maintain 37°C. Hundred µl of sample was added and mixed well.

Table 7: Sample preparation for AST test.

AST test	Addition sequence
Working reagent	1 ml
Sample	100 µl

The initial absorbance (A_0) was recorded after 1 minute and repeated the measurement three times (A_1, A_2, and A_3) at every one minute interval at 340 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China). AST activity was calculated as follows:

AST activity (U/L) = Average change in absorbance (ΔA) per minute X 1746

3.3.3.9. Alanine aminotransferase (ALT)

The activity of alanine aminotransferase (ALT or ALAT) was measured using a calkine serum glutamic-pyruvic transaminase (SGPT)/ALAT kit (Tulip Diagnostics Pvt., Ltd., India) by a modified IFCC method. SGPT (ALAT) catalyzes the transfer of amino group between L- Alanine and α Ketoglutarate to produce Pyruvate and Glutamate. In the presence of Lactate Dehydrogenase, the Pyruvate produced interacts with NADH to create NAD. A decline in absorbance proportionate to the sample's SGPT (ALAT) activity is used to determine the rate of oxidation of NADH to NAD.

$$\text{L-Alanine} + \alpha\text{ Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^{+}$$

The test was done according to the sample start assay. One ml of working reagent was taken in glass tube and incubated in a water bath to maintain 37°C. Hundred µl of the sample was added and mixed well.

Table 8: Sample preparation for ALT test.

ALT test	Addition sequence
Working reagent	1 ml
Sample	100 µl

The initial absorbance (A_0) was recorded after one minute and repeated the measurement three times (A_1, A_2, and A_3) at every one-minute interval at 340 nm

(UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China). ALT activity was calculated as follows:

ALT activity (U/L) = Average change in absorbance (ΔA) per minute X 1746

3.3.3.10. Alkaline phosphatase (ALP)

The activity of alkaline phosphatase (ALP or ALKP) was measured using a calkine Alkaline phosphatase kit using the p-Nitrophenylphosphate (pNPP) kinetic technique (Tulip Diagnostics Pvt., Ltd., India). ALP hydrolyzes p-Nitrophenylphosphate to generate p-Nitrophenol and Phosphate at an alkaline pH. The rate of p-Nitrophenol synthesis is measured by a rise in absorbance proportionate to the ALP activity present in the sample.

$$\text{p-Nitrophenylphosphate} \xrightarrow{\text{ALP}} \text{p-Nitrophenol + Phosphate}$$

For the test, 1 ml of working reagent was taken in glass tubes and incubated in the water bath to maintain 37°C. Twenty µl of the sample was added and mixed well.

Table 9: Sample preparation for ALP test.

ALP test	Addition sequence
Working reagent	1 ml
Sample (serum)	20 µl

The initial absorbance (A_0) was measured after 30 seconds and repeated the measurement three times (A_1, A_2, and A_3) at the interval of 1 minute at 405 nm (UV/VIS Spectrophotometer, 1000 series, Shanghai Yoke Instrument Co., Ltd, China). ALP activity was calculated as follows:

ALP activity (U/L) = Average change in absorbance (ΔA) per minute X 275

3.4. Statistical analysis

In this study, the lethal concentrations of pesticides (CPF & DDVP) in individual treatments as well as in mixture treatments was estimated by a log probit analysis program and presented as 24 h to 96 h-LC_{10} - LC_{90} with a 95% confidence interval, where LC_{10} represents 10% mortality and LC_{90} represents 90% mortality of fish.

For the comparison of means among the various pesticide treatments both parametric and non-parametric tests were applied. The appropriate tests were selected based on the distribution and variance of the data. Normality of the data was determined by Shapiro-Wilk test and homogeneity of variances was assessed by Levene's test. One-way ANOVA followed by Post Hoc tests were used to perform a parametric test. Whenever the data did not satisfy the requirement of the parametric test, a nonparametric Kruskal-Wallis test with multiple pair comparisons was used. The statistical analyses were performed in SPSS.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Results

4.1.1. Lethal toxicity and general fish behavior

4.1.1.1. Lethal toxicity of chlorpyrifos

For the estimation of chlorpyrifos toxicity, golden mahseer, common carp, and mrigal were subjected to five different doses of chlorpyrifos in increasing order. In golden mahseer, no fish mortality was observed at the lowest concentration (0.2 mg/L) of the chlorpyrifos. When the pesticide concentration was gradually increased up to 1.6 mg/L and 3.2 mg/L 100% fish mortality was recorded (Fig. 10).

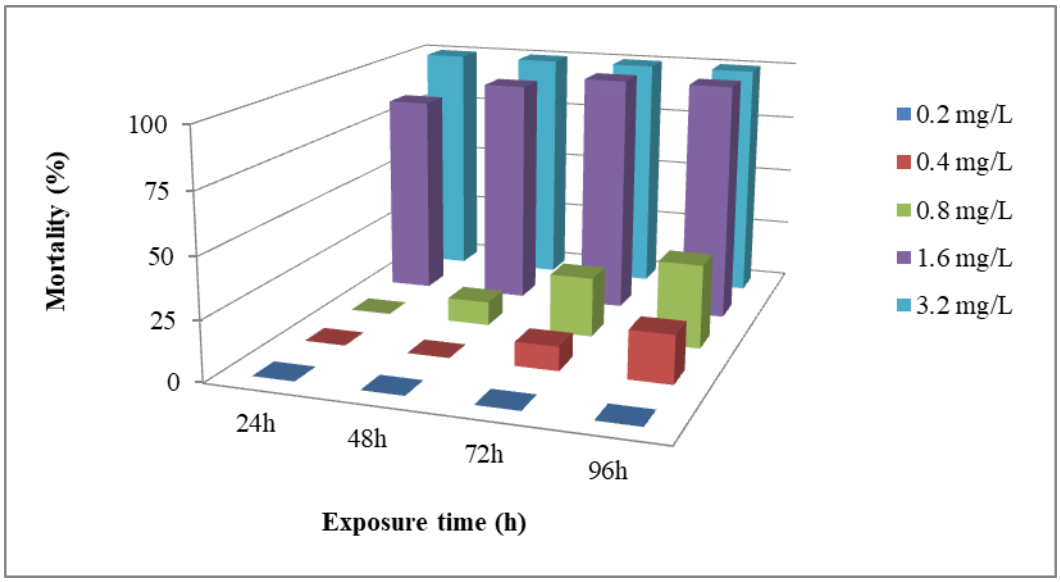

Figure 10: Mortality of golden mahseer in various concentrations of chlorpyrifos at different exposure time.

In common carp, all five concentrations of chlorpyrifos were fatal to fish. The lowest fish mortality (17%) was recorded at 0.25 mg/L and the highest mortality (100%) at 1.25 mg/L (Fig. 11). Mrigal fish were also sensitive to very low dose of chlorpyrifos-0.25 mg/L. The higher concentrations of chlorpyrifos (1 mg/L, 2 mg/L, and 4 mg/L) were highly toxic to fish resulting in 100% mortality within the short exposure period (Fig. 12).

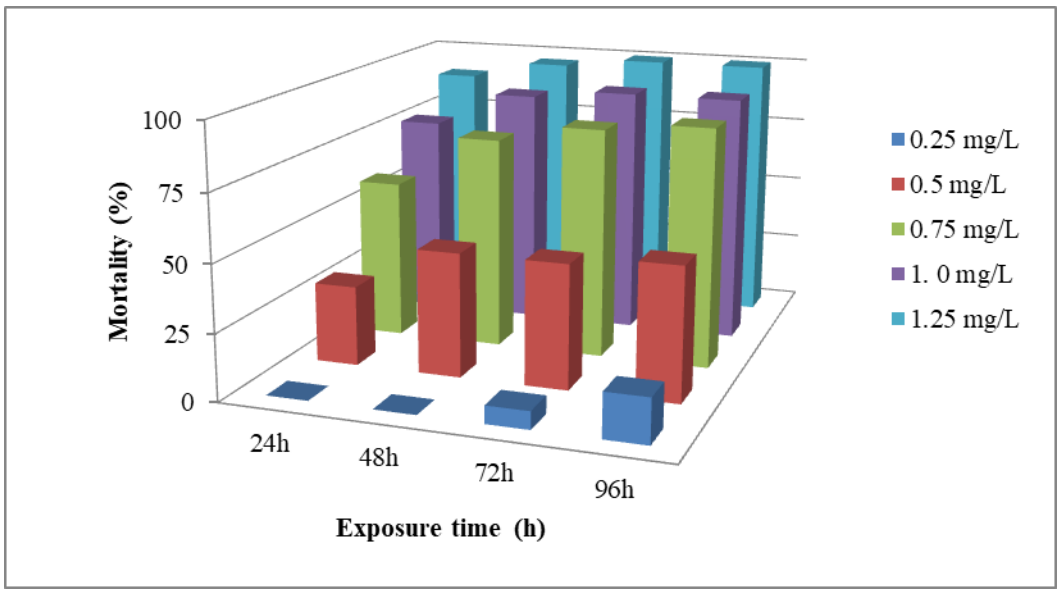

Figure 11: Mortality of common carp in various concentrations of chlorpyrifos at different exposure time.

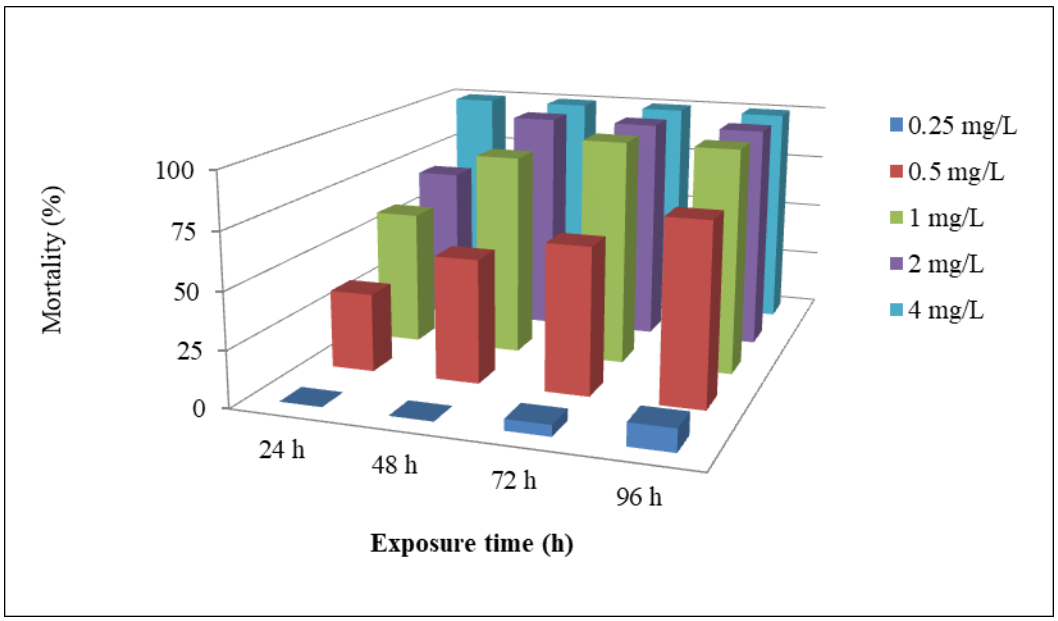

Figure 12: Mortality of mrigal in various concentrations of chlorpyrifos at different exposure time.

Based on the mortality response of fish, lethal concentrations of chlorpyrifos to all three species were estimated. The median lethal concentrations of chlorpyrifos (LC_{50}) with 95% confidence limit ranged between 0.753 (0.616-0.931) mg/L to 1.298 (0.945-1.471) in golden mahseer (Table 10); 0.440 (0.373-0.504) to 0.678 (0.594-0.762) mg/L in common carp (Table 11) and 0.380 (0.319-0.450) to 0.906 (0.689-1.179) mg/L in mrigal (Table 12).

Table 10: Toxicity of chlorpyrifos (mg/L) to golden mahseer at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	1.018 (0.509-1.220)	0.801 (0.621-0.928)	0.501 (0.349-0.615)	0.393 (0.262-0.496)
LC_{20}	1.106 (0.633-1.294)	0.889 (0.724-1.021)	0.603 (0.456-0.723)	0.491 (0.360-0.602)
LC_{30}	1.175 (0.739-1.353)	0.959 (0.804-1.102)	0.688 (0.547-0.822)	0.577 (0.447-0.700)
LC_{40}	1.237 (0.841-1.410)	1.022 (0.873-1.182)	0.771 (0.632-0.927)	0.662 (0.531-0.806)
LC_{50}	1.298 (0.945-1.471)	1.085 (0.938-1.270)	0.858 (0.715-1.049)	0.753 (0.616-0.931)
LC_{60}	1.362 (1.056-1.544)	1.152 (1.002-1.372)	0.954 (0.800-1.201)	0.856 (0.706-1.090)
LC_{70}	1.434 (1.176-1.643)	1.229 (1.070-1.498)	1.069 (0.893-1.401)	0.982 (0.807-1.306)
LC_{80}	1.524 (1.309-1.802)	1.324 (1.149-1.671)	1.222 (1.006-1.695)	1.153 (0.932-1.633)
LC_{90}	1.656 (1.461-2.129)	1.470 (1.258-1.957)	1.469 (1.174-2.231)	1.441 (1.124-2.255)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2021b).

Table 11: Toxicity of chlorpyrifos (mg/L) to common carp at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	0.371 (0.270-0.448)	0.326 (0.243-0.390)	0.288 (0.217-0.345)	0.232 (0.164-0.287)
LC_{20}	0.457 (0.358-0.532)	0.390 (0.309- 0.454)	0.347 (0.276-0.403)	0.289 (0.220-0.345)
LC_{30}	0.530 (0.436-0.604)	0.445 (0.366-0.507)	0.396 (0.327-0.453)	0.338 (0.269-0.396)
LC_{40}	0.602 (0.514-0.678)	0.497 (0.422- 0.560)	0.444 (0.377-0.502)	0.388 (0.320-0.447)
LC_{50}	0.678 (0.594-0.762)	0.551 (0.479-0.617)	0.494 (0.428-0.556)	0.440 (0.373-0.504)
LC_{60}	0.764 (0.679-0.865)	0.611 (0.540-0.684)	0.549 (0.483-0.619)	0.499 (0.431-0.572)
LC_{70}	0.868 (0.773-1.003)	0.683 (0.610-0.771)	0.615 (0.546-0.70)	0.572 (0.499-0.661)
LC_{80}	1.008 (0.888-1.209)	0.778 (0.694-0.897)	0.703 (0.624-0.816)	0.670 (0.585-0.794)
LC_{90}	1.239 (1.062-1.589)	0.931 (0.819-1.122)	0.846 (0.739-1.024)	0.835 (0.715-1.042)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2021a)

Table 12: Toxicity of chlorpyrifos (mg/L) to mrigal at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	0.312 (0.167-0.445)	0.311 (0.203-0.388)	0.287 (0.195-0.345)	0.251(0.172-0.302)
LC_{20}	0.450 (0.280-0.603)	0.373 (0.268-0.451)	0.331 (0.246-0.389)	0.289 (0.217-0.340)
LC_{30}	0.586 (0.401-0.762)	0.425 (0.325-0.507)	0.367 (0.289-0.428)	0.320 (0.253-0.374)
LC_{40}	0.734 (0.536-0.945)	0.475 (0.379-0.566)	0.400 (0.329-0.469)	0.350 (0.287-0.409)
LC_{50}	0.906 (0.689-1.179)	0.527 (0.433-0.633)	0.435 (0.366-0.517)	0.380 (0.319-0.450)
LC_{60}	1.118 (0.867-1.503)	0.585 (0.489-0.718)	0.472 (0.403-0.576)	0.412 (0.352-0.500)
LC_{70}	1.401 (1.083-1.994)	0.654 (0.550-0.832)	0.515 (0.442-0.654)	0.450 (0.385-0.565)
LC_{80}	1.824 (1.376-2.838)	0.746 (0.622-1.001)	0.571 (0.486-0.768)	0.498 (0.424-0.660)
LC_{90}	2.630 (1.873-4.737)	0.894 (0.727-1.316)	0.659 (0.547-0.973)	0.575 (0.479-0.829)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2022a).

4.1.1.2. Lethal toxicity of dichlorvos

Golden mahseer, common carp, and mrigal fish were subjected to five different dichlorvos concentrations. In golden mahseer, no mortality occurred at lower concentrations. Mortality was visible only from 8 mg/L of dichlorvos concentration. The highest concentration 32 mg/L killed 100% of fish within 72 h of exposure (Fig. 13).

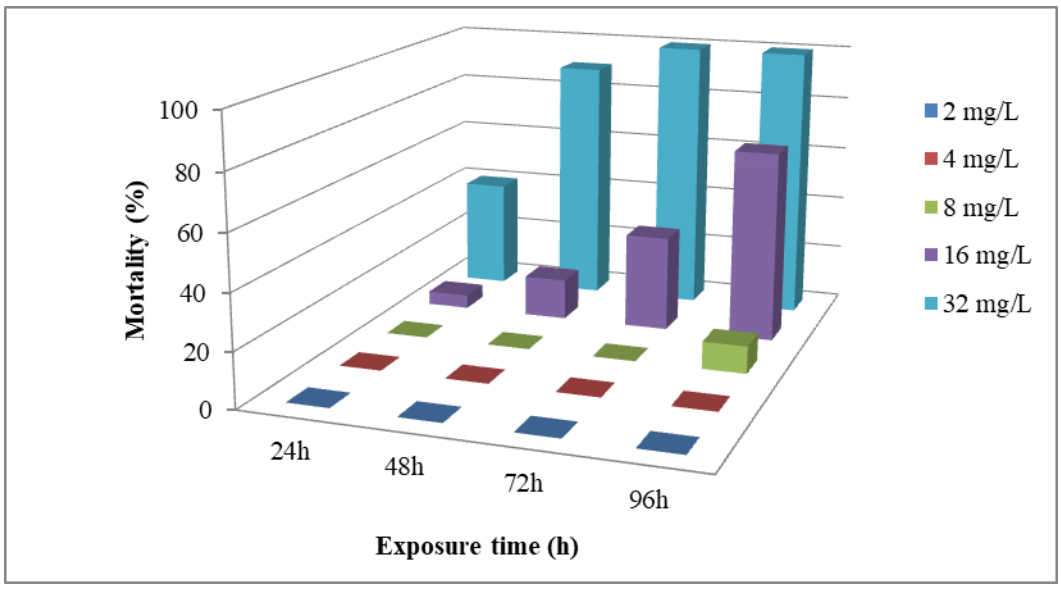

Figure 13: Mortality of golden mahseer in various concentrations of dichlorvos at different exposure time.

For common carp, all five concentrations of dichlorvos were fatal resulting in 7% mortality at the lowest concentration- 10 mg/L and 100% mortality at the highest concentration- 30 mg/L (Fig. 14). Mortality response of mrigal fish was very similar to golden mahseer where no mortality took place at the lower doses of 4 mg/L and 8 mg/L. But, the higher concentrations (16 mg/L, 32 mg/L, and 64 mg/L) were very toxic and killed all exposed fish within 96 h, 48 h, and 24 h of exposure, respectively (Fig. 15).

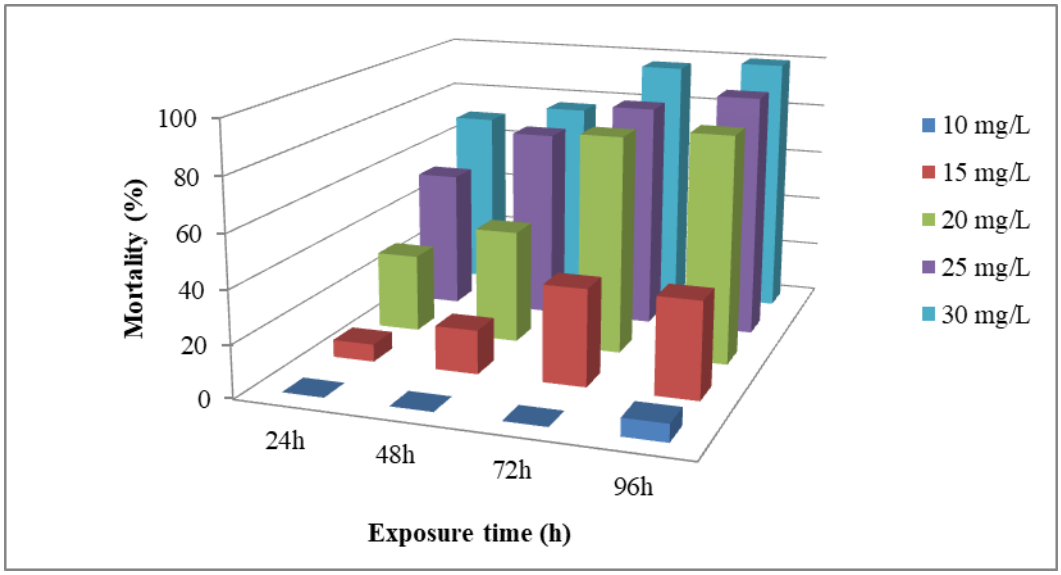

Figure 14: Mortality of common carp in various concentrations of dichlorvos at different exposure time.

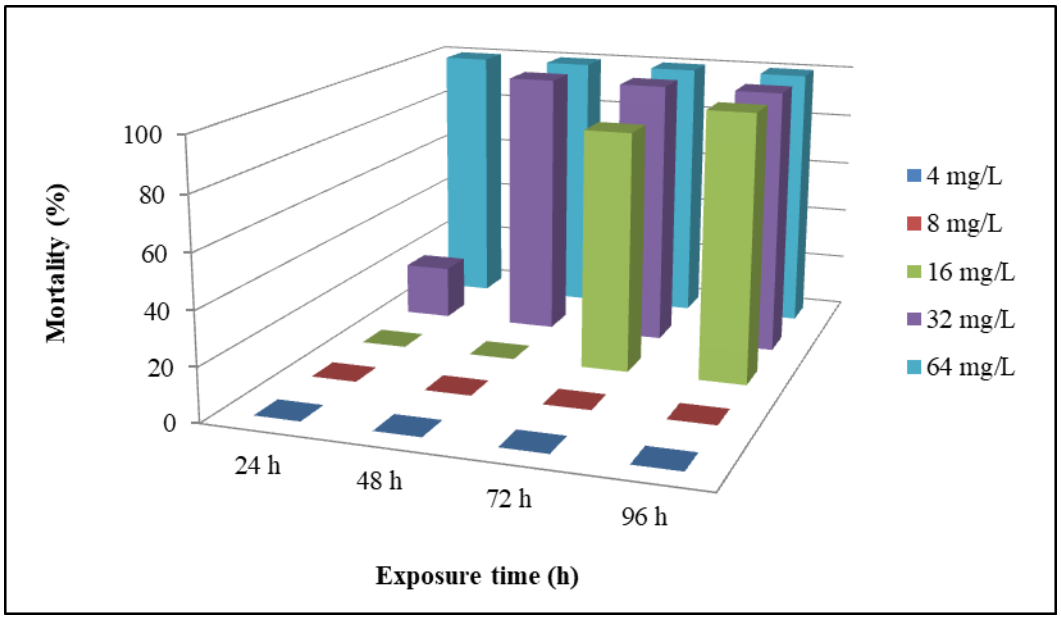

Figure 15: Mortality of mrigal in various concentrations of dichlorvos at different exposure time.

According to the mortality data of the dichlrovos exposed fishes, pesticide toxicity to the selected species was calculated. The median lethal concentrations (LC_{50}) of dichlorvos to golden mahseer ranged between 12.964 (10.866-15.515) to 36.501 (28.410-90.712) mg/L (Table 13). Similarly, the estimated lethal concentrations of dichlorvos to common carp varied between 15.705 (14.385-16.963) to 24.540 (22.561-27.217) mg/L (Table 14); and for mrigal, these concentrations were 11.367 (9.496-13.536) to 38.432 (33.625-47.866) (Table 15).

4.1.1.3. Joint toxicity of pesticides (chlorpyrifos and dichlorvos)

The mortality responses of fish against pesticide mixture (chlorpyrifos and dichlorvos) were observed for 96 h. The mixtures with lower pesticide concentrations (12.5% and 25%) were non-fatal to golden mahseer. In 50% and 100% mixture, only 5% and 80% fish were dead. But 200% mixture was highly toxic resulting in 100% fish mortality (Fig. 16). In common carp, all mixtures (lowest to highest mixture concentrations) were fatal resulting in 37%, 70%, 87%, and 100% mortality by the end of the exposure in 25%, 50%, 75%, and 100% pesticide mixture, respectively. But, in the 125% mixture, complete mortality occurred within 72 h of exposure (Fig. 17). In mrigal, no fish mortality occurred at the lowest concentration- 12.5% mixture, whereas 20%, 40%, and 70% of the fish population were killed in 25%, 50%, and 100% of pesticide mixture, respectively. The highest concentration- 200% mixture killed all exposed fish within 48 h (Fig. 18).

Table 13: Toxicity of dichlorvos (mg/L) to golden mahseer at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	18.846 (7.989-24.129)	14.756 (10.479- 17.563)	12.122 (7.740- 14.403)	8.183 (5.589- 9.937)
LC_{20}	23.647 (15.097- 31.096)	16.860 (12.939- 19.693)	13.746 (9.929- 15.982)	9.583 (7.157- 11.360)
LC_{30}	27.851 (20.975- 42.525)	18.561 (14.937- 21.570)	15.051 (11.741- 17.435)	10.739 (8.473-12.631)
LC_{40}	32.030 (25.120- 61.448)	20.150 (16.743- 23.517)	16.263 (13.359- 19.048)	11.837 (9.690- 13.969)
LC_{50}	36.501 (28.410- 90.712)	21.758 (18.459- 25.727)	17.485 (14.829- 21.029)	12.964 (10.866- 15.515)
LC_{60}	41.597 (31.496- 136.614)	23.495 (20.160- 28.412)	18.798 (16.192- 23.603)	14.199 (12.046- 17.429)
LC_{70}	47.838 (34.795- 213.996)	25.506 (21.945- 31.895)	20.312 (17.530- 27.100)	15.650 (13.300- 19.965)
LC_{80}	56.343 (38.816- 364.472)	28.079 (24.005- 36.868)	22.240 (18.998- 32.258)	17.538 (14.769- 23.667)
LC_{90}	70.694 (44.871- 767.851)	32.084 (26.888- 45.575)	25.220 (20.983- 41.576)	20.539 (16.866- 30.339)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.,* 2021b).

Table 14: Toxicity of dichlorvos (mg/L) to common carp at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	15.904 (12.896-17.881)	13.655 (11.007-15.512)	11.757 (9.784-13.206)	11.094 (9.328-12.405)
LC_{20}	18.458 (15.935-20.254)	15.948 (13.602-17.658)	13.300 (11.493-14.656)	12.500 (10.884-13.734)
LC_{30}	20.550 (18.397-22.356)	17.838 (15.757-19.496)	14.537 (12.875-15.840)	13.623 (12.132-14.821)
LC_{40}	22.524 (20.570-24.597)	19.628 (17.742-21.366)	15.684 (14.147-16.975)	14.662 (13.272-15.864)
LC_{50}	24.540 (22.561-27.217)	21.464 (19.652-23.479)	16.838 (15.395-18.173)	15.705 (14.385-16.963)
LC_{60}	26.737 (24.494-30.425)	23.471(21.560- 26.048)	18.07 (16.679-19.542)	16.821 (15.526-18.214)
LC_{70}	29.306 (26.548-34.532)	25.827 (23.598-29.368)	19.504 (18.070-21.240)	18.104 (16.761-19.756)
LC_{80}	32.627 (29.012-40.269)	28.886 (26.031-34.051)	21.317 (19.710-23.577)	19.731 (18.221-21.859)
LC_{90}	37.865 (32.651-50.080)	33.737 (29.614-42.106)	24.114 (22.037-27.490)	22.230 (20.300-25.348)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.,* 2021a).

Table 15: Toxicity of dichlorvos (mg/L) to mrigal at different time intervals.

Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	28.710 (20.917-32.898)	17.453 (13.322-20.394)	9.837 (6.003-11.713)	9.068 (6.821-10.655)
LC_{20}	31.733 (25.407-36.257)	19.037 (15.208-22.140)	10.663 (7.093-12.449)	9.800 (7.704-11.473)
LC_{30}	34.109 (28.721-39.581)	20.267 (16.638-23.622)	11.302 (7.980-13.041)	10.363 (8.371-12.159)
LC_{40}	36.279 (31.377-43.364)	21.381 (17.882-25.083)	11.877 (8.803-13.604)	10.871 (8.952-12.828)
LC_{50}	38.432 (33.625-47.866)	22.477 (19.047-26.646)	12.442 (9.619-14.196)	11.367 (9.496-13.536)
LC_{60}	40.713 (35.671-53.374)	23.630 (20.200-28.427)	13.033 (10.466-14.876)	11.887 (10.037-14.335)
LC_{70}	43.304 (37.710-60.428)	24.929 (21.415-30.603)	13.697 (11.384-15.738)	12.469 (10.606-15.304)
LC_{80}	46.546 (39.997-70.309	26.540 (22.812-33.534)	14.517 (12.430-16.988)	13.186 (11.260-16.602)
LC_{90}	51.447 (43.129-87.287)	28.948 (24.723-38.344)	15.736 (13.759-19.275)	14.250 (12.146-18.718)

Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2022a).

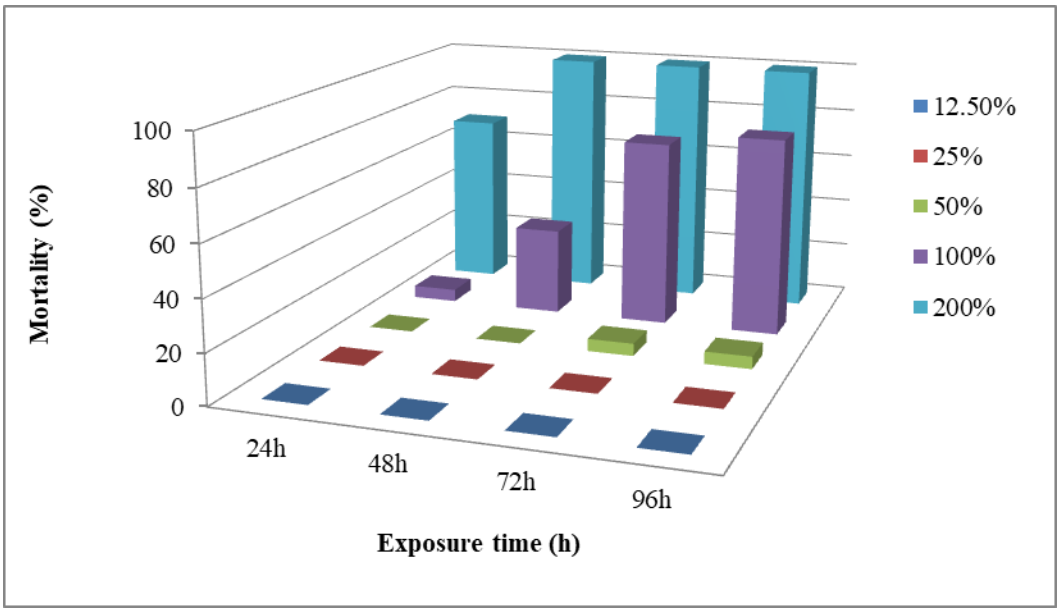

Figure 16: Mortality of golden mahseer in various concentrations of pesticide mixture at different exposure time.

The pesticide mixtures were 12.5% to 200% of 96 h LC_{50} of chlorpyrifos and dichlorvos.

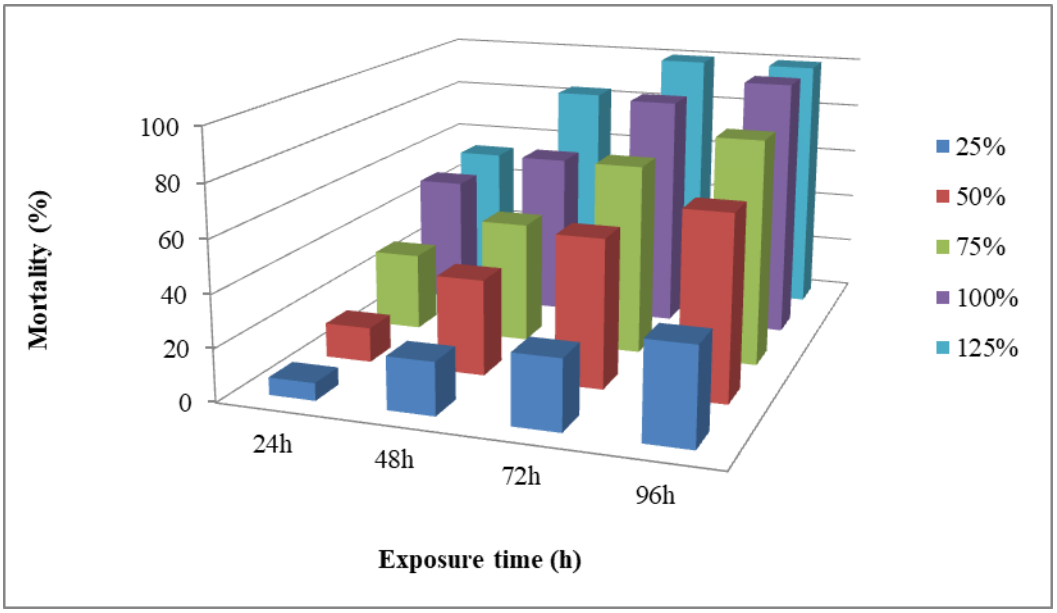

Figure 17: Mortality of common carp in various concentrations of pesticide mixture at different exposure time.

The pesticide mixtures were 25% to 125% of 96 h LC_{50} of chlorpyrifos and dichlorvos.

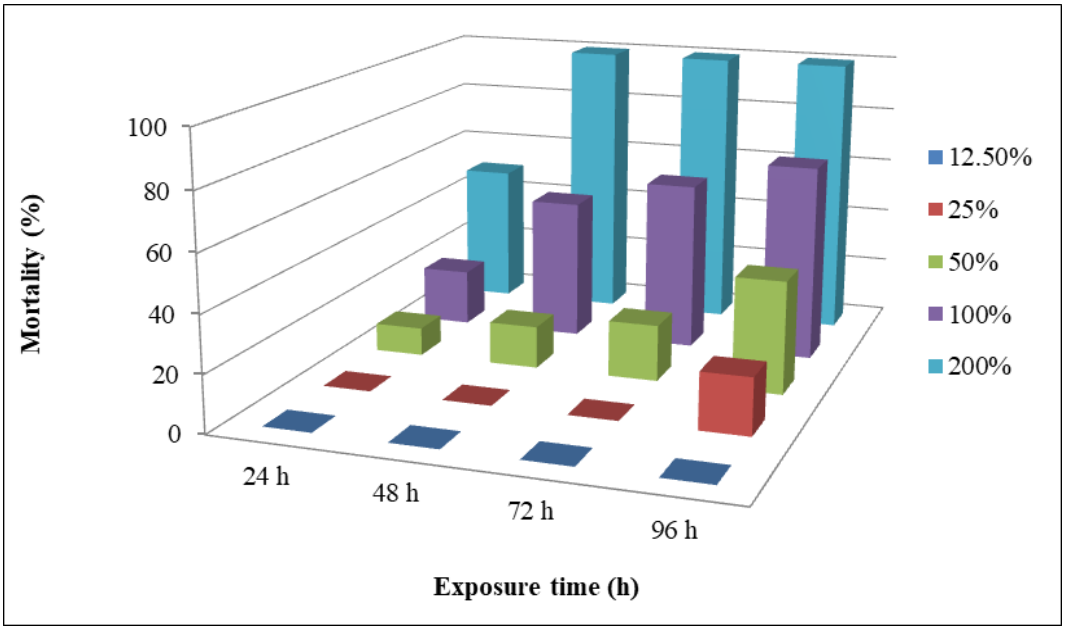

Figure 18: Mortality of mrigal in various concentrations of pesticide mixture at different exposure time. The pesticide mixtures were 12.5% to 200% of 96 h LC_{50} of chlorpyrifos and dichlorvos.

The estimated median lethal concentrations of chlorpyrifos in pesticide mixture with 95% confidence limit were ranged between 0.595 (0.504-0.694) to 1.272 (1.070-1.540) mg/L in golden mahseer (Table 16); 0.145 (0.113-0.173) to 0.499 (0.403-0.733) mg/L in common carp (Table 17), and 0.217 (0.170-0.279) to 0.761 (0.535-1.665) mg/L in mrigal (Table 18). Similarly, for dichlorvos it was calculated to be 10.242 (8.676-11.955) to 21.898 (18.429-26.510) mg/L in golden mahseer (Table 16); 5.180 (4.021-6.169) to 17.804 (14.376-26.158) mg/L in common carp (Table 17), and 6.484 (5.089-8.333) to 22.774 (16.010-49.793) mg/L in mrigal (Table 18).

Table 16: Toxicity of pesticide mixture (mg/L) to golden mahseer at different time intervals.

Lethal concentrations	**24 h (CPF and DDVP)**	**48 h (CPF and DDVP)**	**72 h (CPF and DDVP)**	**96 h (CPF and DDVP)**
LC_{10}	0.844 and 14.522	0.577 and 9.942	0.421 and 7.252	0.417 and 7.171
	(0.54 -1.015, 9.377-17.475)	(0.364- 0.683, 6.272- 11.766)	(0.292- 0.502, 5.022- 8.647)	(0.294- 0.494, 5.063- 8.504)
LC_{20}	0.971 and 16.721	0.652 and 11.231	0.480 and 8.257	0.471 and 8.105
	(0.705-1.142, 12.129-19.657)	(0.469- 0.756, 8.072- 13.013)	(0.361- 0.560, 6.220- 9.641)	(0.359- 0.548, 6.176- 9.427)
LC_{30}	1.075 and 18.511	0.712 and 12.264	0.527 and 9.066	0.514 and 8.852
	(0.838- 1.258, 14.427-21.658)	(0.555- 0.823, 9.561- 14.171)	(0.418- 0.610, 7.201- 10.510)	(0.411- 0.594, 7.078- 10.226)
LC_{40}	1.173 and 20.191	0.768 and 13.221	0.570 and 9.821	0.554 and 9.545
	(0.959-1.386, 16.503-23.854)	(0.632- 0.899, 10.882-15.477)	(0.470- 0.663, 8.094- 11.409)	(0.459- 0.641, 7.896- 11.040)
LC_{50}	1.272 and 21.898	0.824 and 14.182	0.615 and 10.583	0.595 and 10.242
	(1.070- 1.540, 18.429-26.510)	(0.701- 0.993, 12.066-17.104)	(0.520- 0.722, 8.945- 12.433)	(0.504- 0.694, 8.676- 11.955)
LC_{60}	1.380 and 23.751	0.884 and 15.214	0.662 and 11.404	0.638 and 10.989
	(1.177- 1.738, 20.265-29.920)	(0.764- 1.117, 13.146-19.238)	(0.568- 0.795, 9.785- 13.687)	(0.549- 0.759, 9.451- 13.060)
LC_{70}	1.505 and 25.906	0.953 and 16.402	0.718 and 12.353	0.688 and 11.849
	(1.285- 2.006, 22.121-34.535)	(0.824- 1.287, 14.192-22.150)	(0.619- 0.890, 10.659-15.330)	(0.596- 0.842, 10.258-14.490)
LC_{80}	1.666 and 28.679	1.040 and 17.909	0.788 and 13.564	0.752 and 12.942
	(1.406- 2.402, 24.210-41.352)	(0.890- 1.536, 15.330-26.451)	(0.677- 1.028, 11.654-17.697)	(0.649- 0.960, 11.178-16.532)
LC_{90}	1.918 and 33.021	1.175 and 20.232	0.897 and 15.444	0.850 and 14.626
	(1.574- 3.122, 27.101-53.750)	(0.979- 1.988, 16.860-34.233)	(0.756- 1.270, 13.024-21.869)	(0.723- 1.167, 12.440-20.090)

CPF and DDVP signify pesticides chlorpyrifos and dichlorvos. Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.,* 2021b).

Table 17: Toxicity of pesticide mixture (mg/L) to common carp at different time intervals.

Lethal concentrations	24 h (CPF and DDVP)	48 h (CPF and DDVP)	72 h (CPF and DDVP)	96 h (CPF and DDVP)
LC_{10}	0.158 and 5.657 (0.085- 0.211, 3.018-7.535)	0.083 and 2.953 (0.035-0.123, 1.252-4.403)	0.077 and 2.760 (0.045-0.105, 1.604-3.751)	0.067 and 2.385 (0.038-0.091, 1.369-3.243)
LC_{20}	0.235 and 8.385 (0.161-0.291, 5.745-10.369)	0.127 and 4.543 (0.070-0.171, 2.491-6.117)	0.104 and 3.718 (0.068-0.134, 2.433-4.765)	0.087 and 3.112 (0.056-0.112, 1.998-4.011)
LC_{30}	0.312 and 11.137 (0.244-0.384, 8.702-13.711)	0.174 and 6.198 (0.113-0.220, 4.036-7.861)	0.129 and 4.608 (0.092-0.159, 3.271-5.689)	0.106 and 3.771 (0.073-0.131, 2.614-4.693)
LC_{40}	0.398 and 14.193 (0.325-0.522, 11.593-18.629)	0.226 and 8.083 (0.167-0.279, 5.958-9.966)	0.155 and 5.536 (0.117-0.186, 4.188-6.657)	0.124 and 4.443 (0.092-0.151, 3.276-5.389)
LC_{50}	0.499 and 17.804 (0.403-0.733, 14.376-26.158)	0.290 and 10.359 (0.231-0.363, 8.235-12.951)	0.184 and 6.572 (0.147-0.218, 5.234-7.772)	0.145 and 5.180 (0.113-0.173, 4.021-6.169)
LC_{60}	0.626 and 22.333 (0.486-1.057, 17.350-37.742)	0.372 and 13.277 (0.302-0.498, 10.779-17.774)	0.219 and 7.801 (0.181-0.257, 6.459-9.189)	0.169 and 6.038 (0.137-0.200, 4.893-7.126)
LC_{70}	0.797 and 28.463 (0.586-1.587, 20.927-56.640)	0.485 and 17.314 (0.384-0.732, 13.723-26.123)	0.263 and 9.372 (0.222-0.314, 7.936-11.205)	0.199 and 7.114 (0.167-0.236, 5.949-8.434)
LC_{80}	1.059 and 37.804 (0.724-2.573, 25.843-91.848)	0.662 and 23.623 (0.495-1.183, 17.673-42.233)	0.325 and 11.618 (0.276-0.406, 9.836-14.509)	0.242 and 8.620 (0.205-0.295, 7.307-10.512)
LC_{90}	1.570 and 56.036 (0.963-5.066, 34.390-180.823)	1.018 and 36.346 (0.689-2.352, 24.583-83.951)	0.438 and 15.648 (0.359-0.601, 12.827-21.439)	0.315 and 11.250 (0.263-0.414, 9.386-14.775)

CPF and DDVP signify pesticides chlorpyrifos and dichlorvos. Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2021a).

Table 18: Toxicity of pesticide mixture (mg/L) to mrigal at different time intervals.

Lethal concentrations	24 h (CPF and DDVP)	48 h (CPF and DDVP)	72 h (CPF and DDVP)	96 h (CPF and DDVP)
LC_{10}	0.236 and 7.052 (0.113-0.332, 3.387-9.945)	0.190 and 5.671 (0.126-0.236, 3.781-7.063)	0.170 and 5.072 (0.112-0.212, 3.348-6.353)	0.086 and 2.563 (0.051-0.116, 1.537-3.465)
LC_{20}	0.353 and 10.546 (0.225-0.495, 6.740-14.807	0.231 and 6.911 (0.170-0.280, 5.075-8.364	0.208 and 6.212 (0.151-0.252, 4.522-7.547)	0.118 and 3.525 (0.080-0.152, 2.388-4.548)
LC_{30}	0.471 and 14.097 (0.334-0.730, 10.005-21.829)	0.266 and 7.970 (0.207-0.320, 6.202-9.558)	0.240 and 7.189 (0.186-0.289, 5.555-8.641)	0.148 and 4.435 (0.108-0.188, 3.233-5.613)
LC_{40}	0.604 and 18.063 (0.435-1.095, 13.020-32.756)	0.301 and 9.002 (0.243-0.362, 7.272-10.843	0.272 and 8.146 (0.219-0.328, 6.545-9.816)	0.180 and 5.397 (0.138-0.228, 4.123-6.825)
LC_{50}	0.761 and 22.774 (0.535-1.665, 16.010-49.793)	0.337 and 10.088 (0.279-0.413, 8.332-12.358)	0.306 and 9.155 (0.252-0.374, 7.533-11.200)	0.217 and 6.484 (0.170-0.279, 5.089-8.333)
LC_{60}	0.960 and 28.714 (0.645-2.583, 19.285-77.269)	0.378 and 11.304 (0.315-0.477, 9.423-14.268)	0.344 and 10.288 (0.286-0.433, 8.557-12.947)	0.260 and 7.790 (0.206-0.346, 6.171-10.355)
LC_{70}	1.230 and 36.794 (0.777-4.185, 23.245-125.176)	0.427 and 12.768 (0.355-0.563, 10.617-16.846)	0.390 and 11.657 (0.324-0.512, 9.682-15.315)	0.317 and 9.480 (0.249-0.444, 7.458-13.287)
LC_{80}	1.644 and 49.182 (0.958-7.425, 28.668-222.120)	0.492 and 14.724 (0.403-0.692, 12.066-20.702)	0.451 and 13.491 (0.369-0.631, 11.050-18.872)	0.399 and 11.928 (0.306-0.604, 9.162-18.075)
LC_{90}	2.459 and 73.550 (1.271-16.591, 38.018-496.275)	0.600 and 17.942 (0.476-0.933, 14.225-27.906	0.552 and 16.523 (0.438-0.854, 13.095-25.558)	0.548 and 16.403 (0.401-0.942, 11.981-28.175

CPF and DDVP signify pesticides chlorpyrifos and dichlorvos. Data in parentheses represent minimum and maximum values with 95% confidence interval (Kunwar *et al.*, 2022a).

By the end of 96 h exposure experiments, the joint actions of chlorpyrifos and dichlorvos were antagonistic in golden mahseer where AI value ranged from -0.937 to -0.302 (Table 19). In contrast, it was synergistic in common carp with AI value varied from 0.989 to 0.132 (Table 20). But in mrigal, there was a mixture of synergistic and antagonistic action but mostly dominated by antagonistic effects where the AI was calculated to be 0.37 to -1.10 (Table 21).

Table 19: Joint toxicity of pesticide mixture to golden mahseer.

	AI (Additive Index)			
Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	-0.600	-0.394	-0.439	-0.937
LC_{20}	-0.585	-0.400	-0.397	-0.805
LC_{30}	-0.580	-0.406	-0.368	-0.715
LC_{40}	-0.579	-0.408	-0.343	-0.643
LC_{50}	-0.580	-0.411	-0.322	-0.580
LC_{60}	-0.584	-0.415	-0.301	-0.519
LC_{70}	-0.591	-0.418	-0.280	-0.458
LC_{80}	-0.602	-0.423	-0.255	-0.390
LC_{90}	-0.625	-0.430	-0.223	-0.302

Additive index equal, higher or lower than zero implies additive, synergistic, or antagonistic action, respectively (Kunwar *et al.,* 2021b).

Table 20: Joint toxicity of pesticide mixture to common carp.

	Additive Index (AI)			
Lethal concentrations	24 h	48 h	72 h	96 h
LC_{10}	0.279	1.130	0.989	0.989
LC_{20}	0.033	0.638	0.725	0.817
LC_{30}	-0.130	0.356	0.556	0.699
LC_{40}	-0.290	0.153	0.424	0.603
LC_{50}	-0.460	-0.009	0.311	0.517
LC_{60}	-0.653	-0.174	0.206	0.434
LC_{70}	-0.889	-0.380	0.103	0.349
LC_{80}	-1.209	-0.668	-0.008	0.255
LC_{90}	-1.746	-1.170	-0.167	0.132

Additive index equal, higher or lower than zero implies additive, synergistic, or antagonistic action, respectively (Kunwar *et al.,* 2021a).

Table 21: Joint toxicity of pesticide mixture to mrigal.

Lethal concentrations	Additive Index (AI)			
	24 h	48 h	72 h	96 h
LC_{10}	0.00	0.06	-0.11	0.37
LC_{20}	-0.12	0.02	-0.21	0.23
LC_{30}	-0.22	-0.02	-0.29	0.11
LC_{40}	-0.32	-0.05	-0.37	-0.01
LC_{50}	-0.43	-0.09	-0.44	-0.14
LC_{60}	-0.56	-0.12	-0.52	-0.29
LC_{70}	-0.73	-0.17	-0.61	-0.46
LC_{80}	-0.96	-0.21	-0.72	-0.71
LC_{90}	-1.36	-0.29	-0.89	-1.10

Additive index equal, higher or lower than zero implies additive, synergistic, or antagonistic action, respectively (Kunwar *et al.,* 2022a).

4.1.1.4. General fish behavior

Golden mahseer became slow, sluggish, and calm after exposure to all pesticides. The calmness of the fish was most observed at the highest pesticide concentration. All pesticide doses resulted in a slight loss of equilibrium, however this was only noticeable at higher concentrations. Throughout the trial, the pesticides had an effect on fish schooling behavior, which became more apparent as pesticide concentrations increased. The fish became disoriented and dispersed across the aquaria. This behavior became prominent in the mixture treatment, where the fish avoided swimming in groups and congregated at the corners of the aquarium. With increased pesticide concentrations, the fish's caudal fin became reddish. In higher concentrations of chlorpyrifos and the mixed pesticide solution, caudal fins were found to be degenerated. When exposed to chlorpyrifos, fish tended to swim towards the bottom of the aquarium, but when exposed to dichlorvos, fish preferred to swim above the midline of the tank, and the surfacing of fish was exacerbated with higher dichlorvos concentration. At lower concentrations of pesticide mixture, fish were scattered throughout the tank, but at the highest mixed concentration, they remained almost at the bottom. Fish that had been exposed to chlorpyrifos were occasionally seen gulping air. Before death, fish became hyperactive

and displayed strong swimming and then became abruptly quiet. The deceased fish were loaded with mucus around the gill surfaces (Table 22).

During lethal toxicity assessment of common carp, fish were carefully observed to record their behavior. Significant behavioral changes included rapid opercular movements, aggregation at the aquaria's corners, loss of equilibrium and hanging vertically with the head upwards or downwards in the water column, loss of schooling behaviour, abrupt hyperactivity with fast spiral movements, excess mucus secretion, and dull and faded body color. The fish remained motionless on the bottom of the test chamber during the later stage of lethal toxicity experiment. For all pesticides, the severity of behavioral effects was dose-dependent (Table 23).

Pesticide mixture treated mrigal fish became hypo-active compared to individual pesticide treatments. With increasing pesticide concentrations, such fish behavior became more pronounced. Opercular movements of fish were increased in all pesticide treatments. At higher pesticide concentrations, fish were unable to maintain physical equilibrium. All pesticides impacted fish schooling behavior, and changes were more prominent as pesticide doses increased, where swimming coordination among fish was lost, and they were scattered around the aquaria, occupying more territory. Fish were frequently gathered in the corners of the test chambers. Fish showed slight color changes, with the fins becoming reddish and the body becoming pale. Before dying, some fish became very excited and started making rapid jerks in all directions. In higher pesticide concentrations, the deceased fish gills were heavily loaded with mucus (Table 24).

4.1.2. Specific behavioral response and aerobic respiratory metabolism

4.1.2.1. Feeding behavior

Feeding behavior of fish was diminished by pesticide exposure. In golden mahseer, the most significant reduction was observed in chlorpyrifos and pesticide mixture treatments. But some feeding response was still observed in dichlorvos treated fish, though it was significantly reduced in dichlorvos -50% treatment after 96 h ($P < 0.001$) (Table 25). In common carp, the effects of pesticide exposure on feeding were more prominent during the early phase of the exposure. However, the reduction in feeding was also observed during the late exposure hours (Table 26). The compiled data (1 h, 24 h, 48 h, 72 h, and

96 h) analysis also revealed all pesticide treatments (chlorpyrifos- 10% and 50%, dichlorvos- 10% and 50%, and mixture- 10% and 50%) were able to produce a significant loss in fish feeding ($P < 0.001$) (Table 25 and 26).

The average feeding attempts at different sampling intervals in both fish species were analyzed and it was found that the treated fish were significantly lower ($P< 0.001$) compared to the control. In golden mahseer, the lowest feeding was observed after 1 h of exposure, and in common carp, it was noticed after 24 h of exposure. No significant difference was noticed in pair-wise comparison neither among the exposed nor among the control groups in both fish species (Figs. 19 and 20).

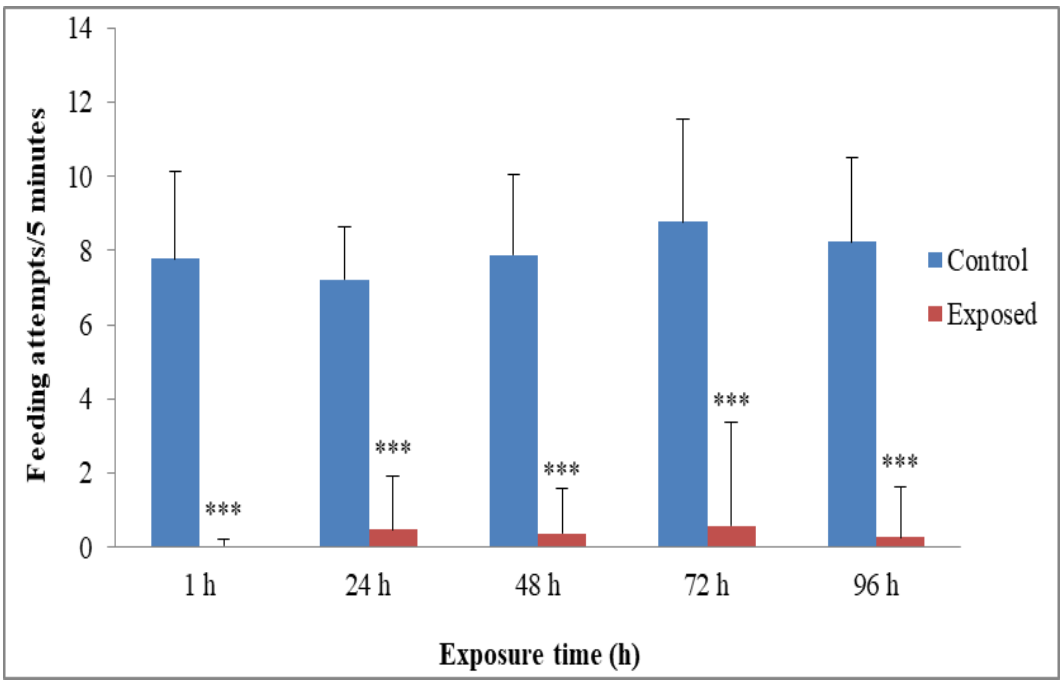

Figure 19: Feeding attempts by the control and pesticide exposed golden mahseer at different exposure time.

Values are mean ± SD (n = 5 for control and n= 30 for pesticide exposed groups). Asterisk denotes significant difference between the control and pesticide exposed group at various sampling intervals ($^{***}P< 0.001$).

Table 22: Behavioral expressions exhibited by golden mahseer during pesticide exposure.

Fish behavior	CPF (mg/L)				DDVP (mg/L)				Pesticide mixtures (mg/L)			
	0.1	0.2	0.4	0.8	2	4	8	16	CPF-0.094 DDVP-1.620	CPF-0.188 DDVP-3.241	CPF-0.376 DDVP-6.482	CPF-0.753 DDVP-12.964
Hypo-activity	+	+	+	++	_	+	+	++	+	+	+	++
Equilibrium loss	_	_	_	+	_	_	_	+	_	_	_	+
Color change	_	_	+	+	_	_	+	+	_	_	_	_
Aggregating at corners of the aquarium	_	_	_	_	_	_	_	_	++	+	+	+
Avoiding schooling behavior	+	+	+	++	_	+	+	++	+	++	++	+++

Chlorpyrifos (CPF) and dichlorvos (DDVP) pesticide mixtures were the combinations of 12.5%, 25%, 50%, and 100% 96 h-LC_{50} of the individual pesticides. The symbol -, +, ++ and +++ represent absent, mild, moderate, and strong behavior, respectively(Kunwar *et al.*, 2021b).

Table 23: Behavioral expressions exhibited by common carp during pesticide exposure.

Fish behavior	CPF (mg/L)				DDVP (mg/L)				Pesticide mixtures (mg/L)			
	0.25	0.5	0.75	1.0	10	15	20	25	CPF- 0.11 DDVP-3.93	CPF- 0.22 DDVP-7.875	CPF-0.33 DDVP-11.812	CPF- 0.44 DDVP-15.75
Hypo-activity	+	+	+	++	_	_	+	+	+	+	++	++
Equilibrium loss	_	_	+	++	_	_	_	++	_	+	++	+++
Color change	_	_	+	+	_	_	_	+	_	_	+	++
Aggregating at corners of the aquarium	_	_	+	++	_	_	+	+	_	+	++	+++
Avoiding schooling behavior	+	+	++	++	+	+	++	++	+	++	+++	+++

Chlorpyrifos (CPF) and dichlorvos (DDVP) pesticide mixtures were the combinations of 12.5%, 25%, 50%, and 100% 96 h-LC_{50} of the individual pesticides. The symbol -, +, ++ and +++ represent absent, mild, moderate, and strong behavior, respectively.

Table 24: Behavioral expressions exhibited by mrigal during pesticide exposure.

Fish behavior	CPF (mg/L)				DDVP (mg/L)				Pesticide mixtures (mg/L)			
	0.25	0.5	1	2	4	8	16	32	CPF-0.047 DDVP-1.420	CPF-0.095 DDVP-2.841	CPF-0.190 DDVP-5.683	CPF-0.380 DDVP-11.367
Hypo-activity	_	_	_	NA	_	_	_	NA	+	+	++	++
Equilibrium loss	+	++	++	NA	_	_	_	NA	_	_	_	_
Color change	_	_	+	NA	_	_	+	NA	_	_	+	+
Aggregating at corners of the aquarium	+	+	++	NA	+	+	++	NA	+	+	+	+
Avoiding schooling behavior	_	+	++	NA	_	+	+	NA	++	+	+	+

Chlorpyrifos (CPF) and dichlorvos (DDVP) pesticide mixtures were the combinations of 12.5%, 25%, 50%, and 100% 96 h-LC_{50} of the individual pesticides. The symbol -, +, ++ and +++ represent absent, mild, moderate, and strong behavior, respectively(Kunwar *et al.,* 2022a).

Table 25: Feeding attempts (number/5 minutes) by golden mahseer during pesticide exposure.

Time intervals	Control	CPF-10%	CPF-50%	DDVP-10%	DDVP-50%	Mixture-10%	Mixture-50%
1 h	7.78 ± 2.33	0.00*	0.00*	0.20 ± 0.45	0.00	0.00*	0.00*
24 h	7.22 ± 1.39	0.00	0.00	2.60 ± 2.88	0.20 ± 0.45	0.00	0.00
48 h	7.89 ± 2.15	0.00*	0.00*	1.60 ± 2.51	0.60 ± 1.34	0.00*	0.00*
72 h	8.78 ± 2.77	0.20 ± 0.45	0.00*	3.00 ± 6.71	0.20 ± 0.45	0.00	0.00*
96 h	8.22 ± 2.28	0.00*	0.00	1.40 ± 3.13	0.00*	0.00	0.00
Total	7.98 ± 2.19	0.04 ± 0.20***	0.00***	1.76 ± 3.55***	0.21 ± 0.66***	0.00***	0.00***

Pesticide treatments were 10% and 50% 96 h-LC_{50} of the pesticides (chlorpyrifos- CPF, dichlorvos- DDVP, and their mixture). Values are mean ± SD (n = 6). Asterisk denotes the significant differences between the control and other exposure groups ($^{*}P< 0.05$; $^{***}P< 0.001$) (Kunwar *et al.*, 2021b).

Table 26: Feeding attempts (number/5 minutes) by common carp during pesticide exposure.

Time intervals	Control	CPF-10%	CPF-50%	DDVP-10%	DDVP-50%	Mixture-10%	Mixture-50%
1 h	10.40 ± 1.52	0.00*	0.5 ± 0.84	0.00*	1.00 ± 2.00	0.17 ± 0.41	0.00*
24 h	9.17 ± 2.32	0.00*	0.00*	0.00*	0.17 ± 0.41	0.00*	0.00*
48 h	8.67 ± 1.37	0.17 ± 0.41	0.17 ± 0.41	1.50 ± 1.64	1.50 ± 2.51	0.67 ± 1.21	0.00*
72 h	7.67 ± 2.25	0.33 ± 0.52	0.17 ± 0.41	2.83 ± 3.25	1.17 ± 1.47	0.33 ± 0.82	0.67 ± 0.82
96 h	8.67 ± 1.37	0.50 ± 0.84	0.20 ± 0.45	1.83 ± 2.71	3.00 ± 4.29	0.17 ± 0.41	0.50 ± 0.84
Total	8.86 ± 1.90	0.20 ± 0.48***	0.21 ± 0.49***	1.23 ± 2.19***	1.37 ± 2.50***	0.27 ± 0.69***	0.23 ± 0.57***

Pesticide treatments were 10% and 50% 96 h-LC_{50} of the pesticides (chlorpyrifos- CPF, dichlorvos- DDVP, and their mixture). Values are mean ± SD (n = 6). Asterisk denotes the significant differences between the control and other exposure groups ($^{*}P< 0.05$; $^{***}P< 0.001$)(Kunwar *et al.*, 2021a).

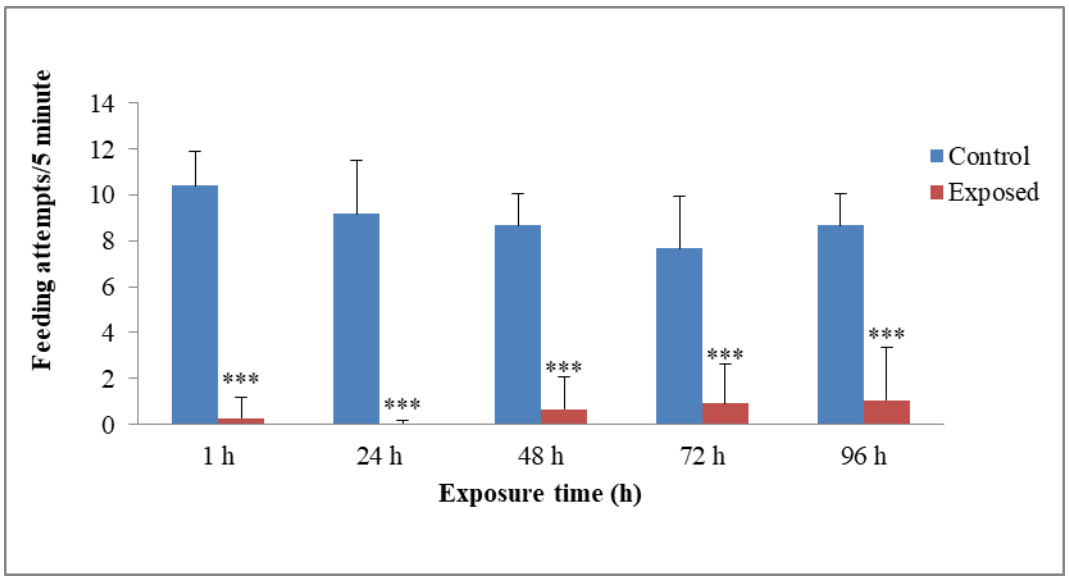

Figure 20: Feeding attempts by the control and pesticide exposed common carp at different exposure time.

Values are mean ± SD (n = 6 for control and n= 36 for pesticide exposed groups). Asterisk denotes significant difference between the control and pesticide exposed group at various sampling intervals ($^{***}P< 0.001$) (Kunwar *et al.*, 2021a).

4.1.2.2. Opercular beat rate

The opercular beat rate was accelerated after pesticide exposure in both fish species but the elevation was not always significant. In golden mahseer, all pesticide treatments except chlorpyrifos-10% caused significant elevation ($P< 0.001$) after 1 h of exposure (Fig. 21). In contrast, none of these elevations were significant in common carp during the same exposure period (Fig. 22). After 24 h of exposure, all pesticide treatments except dichlorvos- 10% in golden mahseer and chlorpyrifos-10% and dichlorvos-10% in common carp caused significant elevation ($P< 0.05$ and 0.001) of the opercular beat rate. After 48 h of exposure, all elevations, except dichlorvos-10% in golden mahseer and chlorpyrifos-10% in common carp, were significantly high ($P< 0.05$, 0.01 and 0.001). After 72 h of exposure, again a similar trend was followed in golden mahseer where all exposures, except dichlorvos-10%, were significantly high ($P< 0.05$, 0.01 and 0.001). During the same exposure period, common carp accelerated a higher opercular beat rate compared to control ($P< 0.01$ and 0.001) in all treatments except in mixture-10%. After 96 h of exposure, only chlorpyrifos treated golden mahseer exhibited a significantly higher opercular beat

rate than control (P< 0.001) but all pesticide treatments were significantly high (P< 0.01 and 0.001) in common carp during this exposure period (Fig. 21 and 22). When the opercular beat rate in all treatments from 24 h to 96 h was compared to the 1 h of observation, a significant difference (P< 0.05) was noted only between 1 h and 48 h in dichlorvos-10% treated group in golden mahseer (Fig. 21). In contrast, most of the observations recorded at the later phase were significantly different than the 1 h of observation (P< 0.05, 0.01 and 0.001) in common carp. Between the low and high concentrations, a significant difference was noticed only after 96 h in chlorpyrifos treated common carp (Fig. 22).

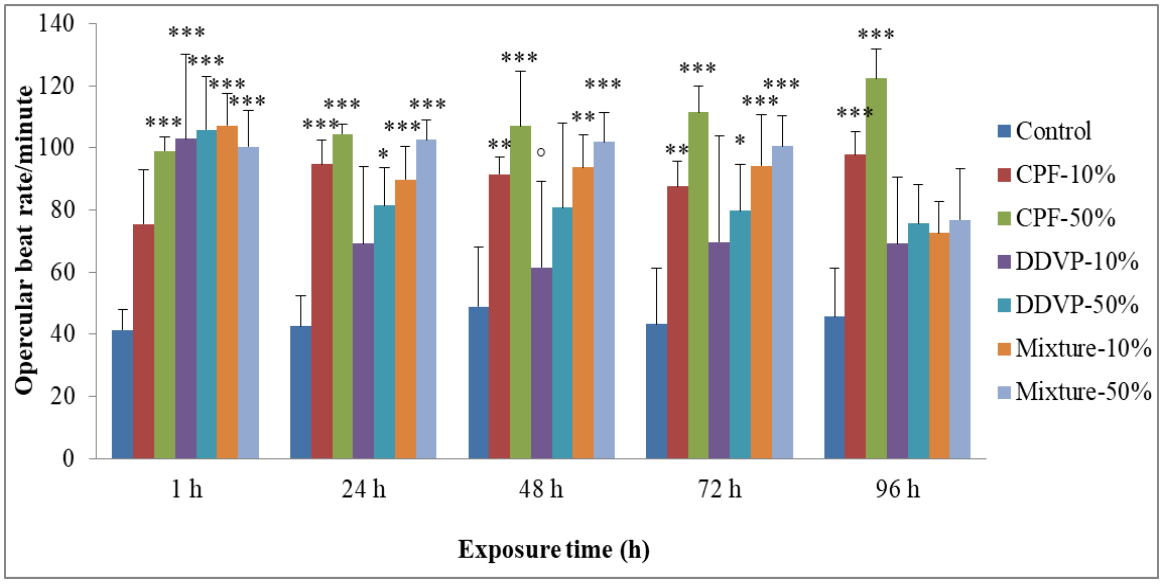

Figure 21: Opercular beat rate of golden mahseer in various treatments and exposure time.

Pesticide concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Values are mean ± SD (n= 5). Asterisk denotes significant difference between the control and pesticide exposed groups during the same sampling interval ($^{*}P$ < 0.05; $^{**}P$ < 0.01; $^{***}P$< 0.001); and white circle denotes significant difference in the same treatment groups during different sampling intervals compared to 1 h of exposure ($^{\circ}P$< 0.05) (Kunwar *et al.*, 2021b).

The average opercular movements of golden mahseer and common carp over the whole exposure period (compiled observation of 1 h, 24 h, 48 h, 72 h, and 96 h) were significantly higher (P< 0.001) than that of control in all treatment groups (Figs. 23 and 24). The highest opercular movements were exhibited by chlorpyrifos-50% in golden mahseer (Fig. 23) and dichlorvos-50% in common carp (Fig. 24). Comparing low and high doses of the same pesticide treatment, only a significant difference (P< 0.001) was observed in chlorpyrifos treated groups in golden mahseer (Fig. 23). But in common carp; compared to the low dose, the high dose always exhibited higher

buccal movements in all pesticide groups- chlorpyrifos ($P< 0.05$), dichlorvos ($P< 0.01$), and mixture ($P< 0.05$) (Fig. 24).

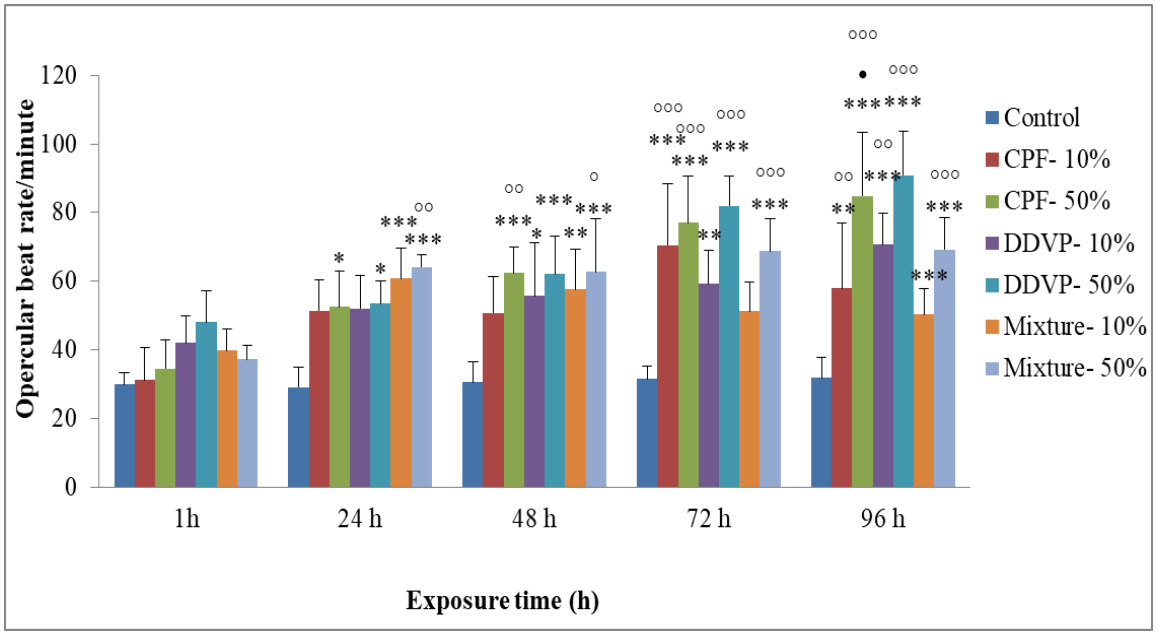

Figure 22: Opercular beat rate of common carp in various treatments and exposure time.

Pesticide concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Values are mean ± SD (n = 6). Asterisk denotes significant difference between the control and pesticide exposed groups during the same sampling interval ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P< 0.001$); white circle denotes significant difference in the same treatment groups during different sampling intervals compared to 1 h of exposure ($^{\circ}P< 0.05$; $^{\circ\circ}P< 0.01$; $^{\circ\circ\circ}P< 0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment at the same sampling interval ($^{\bullet}P < 0.05$) (Kunwar *et al.*, 2021a).

When the average buccal movements of all pesticide exposed golden mahseer and common carp at different time points were compiled and compared to their respective controls, significantly higher movements ($P< 0.001$) were observed throughout the experiment except at 1 h of measurement in common carp (Fig. 25 and 26). In golden mahseer no significant differences were observed either among the controls or among the pesticide exposed groups (Fig. 25); but in common carp, pesticide treated groups exhibited significant differences when compared among the different sampling intervals (Fig. 26). The opercular beat rate of the pesticide exposed group was more or less stable in golden mahseer but in common carp, there was an increasing trend from the beginning until the end of the experiment (Figs. 25 and 26).

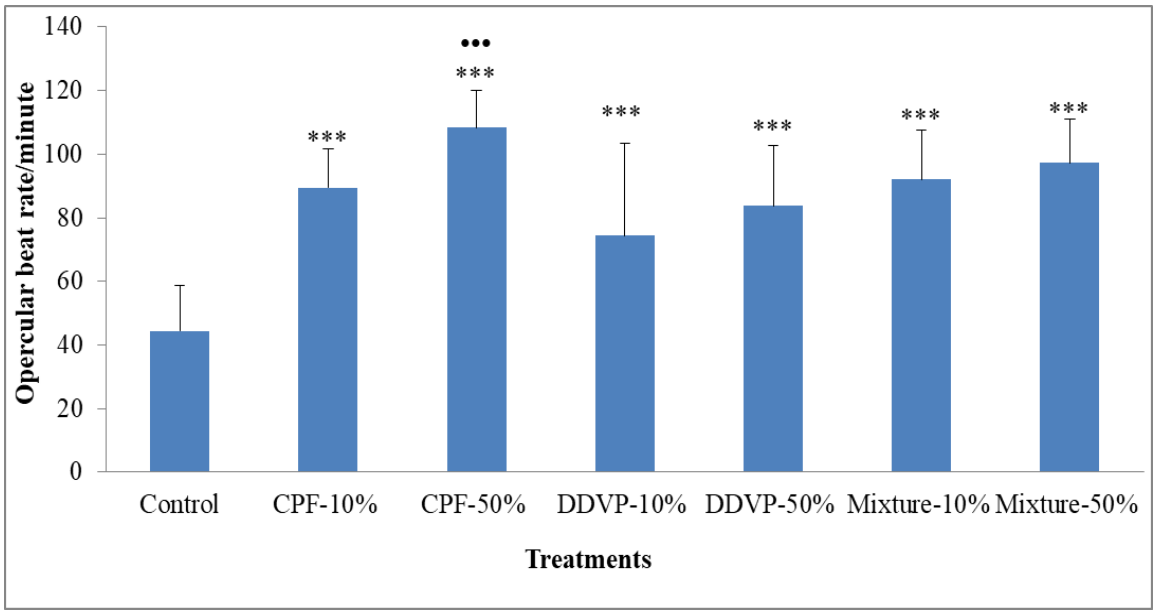

Figure 23: Opercular beat rate of golden mahseer in the control and various pesticide treatments.

Values are mean ± SD (n = 25). Exposure concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Asterisk denotes significant difference between the control and pesticide treated groups ($^{***}P < 0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment ($^{\bullet\bullet\bullet}P < 0.001$) (Kunwar *et al.*, 2021b).

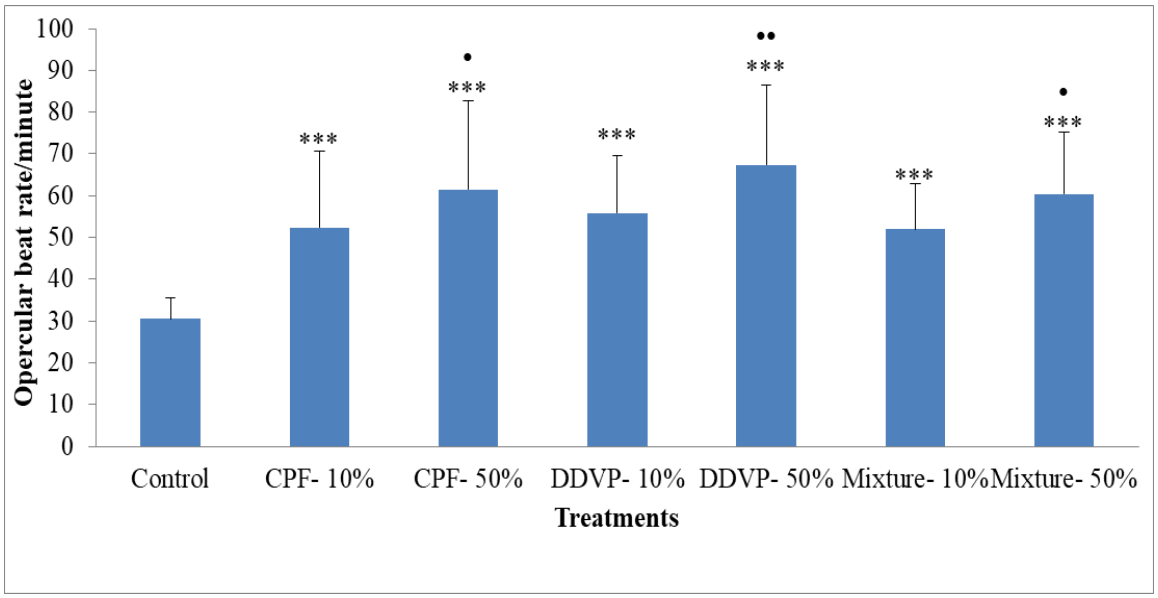

Figure 24: Opercular beat rate of common carp in the control and various pesticide treatments.

Values are mean ± SD (n = 30). Exposure concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Asterisk denotes significant difference between the control and pesticide treated groups ($^{***}P<0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment ($^{\bullet}P< 0.05$; $^{\bullet\bullet}P < 0.01$) (Kunwar *et al.*, 2021a).

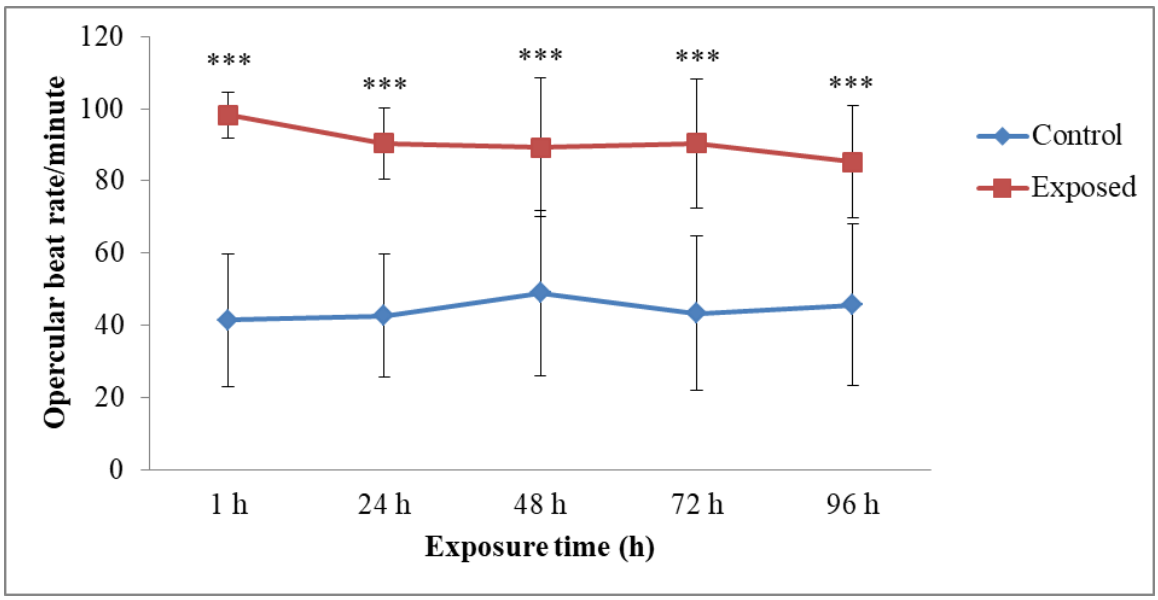

Figure 25: Opercular beat rate of golden mahseer at different exposure time.

Values are mean ± SD (n = 5 for control and n = 30 for pesticide exposed groups). Asterisk denotes significant difference between the control and pesticide exposed group at various sampling intervals (***P < 0.001).

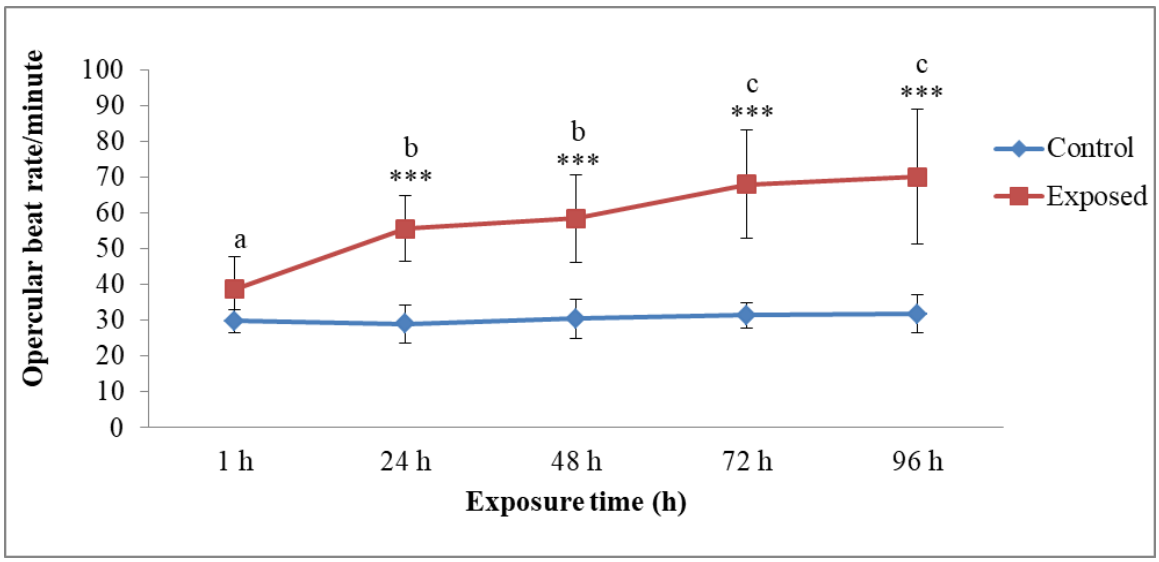

Figure 26: Opercular beat rate of common carp at different exposure time.

Values are mean ± SD (n = 6 for control and n = 36 for pesticide exposed groups). Asterisk denotes significant difference between the control and pesticide exposed group at various sampling intervals (***P< 0.001). The same letters (a, b, c) represent no significant difference and different letters represent significant differences among the pesticide exposed groups (Kunwar *et al.*, 2021a).

4.1.2.3. Aerobic respiratory metabolism

The respiratory metabolism of fish was affected by pesticide exposure in both species. In golden mahseer, there were both increasing and decreasing trends of oxygen consumption (Fig. 27) but in common carp, there was always an increasing trend of oxygen consumption after pesticide exposure compared to control (Fig. 28). During 0-24 h, there was no significant difference in oxygen consumption between control and pesticide exposed groups in golden mahseer; but in common carp, pesticide mixture treatments (10% and 50%) significantly elevated ($P< 0.05$ and 0.001) oxygen consumption rate. During 24-48 h, chlorpyrifos-10% caused significantly lower ($P< 0.05$) oxygen consumption while mixture-50% caused significantly higher ($P< 0.001$) oxygen consumption compared to control in golden mahseer. During this period, high concentrations (50%) of dichlorvos and pesticide mixture significantly elevated ($P< 0.001$) oxygen consumption rate in common carp. During 48-72 h, mixture-50% ($P< 0.001$) in golden mahseer and dichlorvos-50% ($P < 0.05$) and mixture-50% ($P < 0.001$) in common carp significantly elevated the oxygen consumption rate. During 72-96 h, mixture low ($P< 0.01$) and high concentration ($P< 0.05$) in golden mahseer and dichlorvos high ($P< 0.01$) and mixture high ($P< 0.001$) concentration in common carp caused significantly higher oxygen uptake compared to the control (Fig. 27 and 28). In golden mahseer, among the same pesticide treatments substantially higher ($P< 0.001$) oxygen uptake was observed during 24-48 h and 48-72 h compared to 0-24 h of measurement in mixture 50% treated fish. The elevated oxygen consumption was stabilized during 72-96 h but oxygen uptake in mixture 10% group was still higher ($P< 0.001$) than its initial (0-24 h) measurement (Fig. 27) but such differences among the same pesticide treatments were not observed in common carp. Most of the time, fish exposed to 50% pesticide doses consumed more oxygen than fish exposed to 10% pesticide doses, but the difference was significant only between both mixture groups at 24-48 h and 48-72 h of exposure in golden mahseer ($P< 0.01$) (Fig. 27) and dichlorvos and mixture treatment groups at 48-72 h in common carp ($P< 0.05$) (Fig. 28).

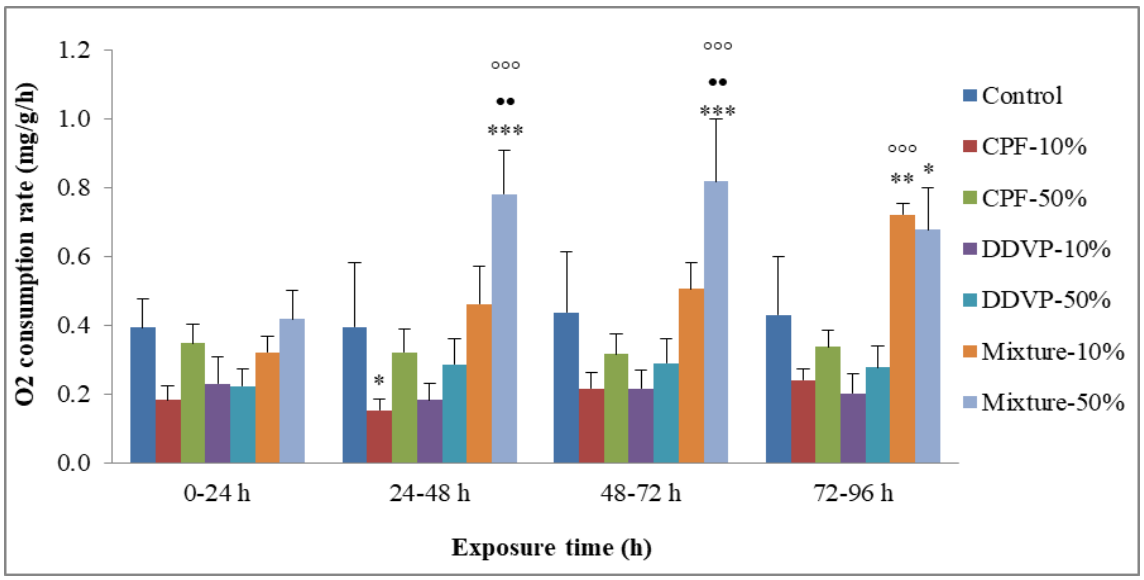

Figure 27: Oxygen consumption rate of golden mahseer in various treatments and exposure time.

Pesticide concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Values are mean ± SD (n = 5). Asterisk denotes significant difference between the control and pesticide treated groups during the same sampling interval ($^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P < 0.001$); white circle denotes significant difference in the same treatment groups during different sampling intervals compared to 0-24 h of exposure ($^{\circ\circ\circ}P< 0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment at the same sampling interval ($^{\bullet\bullet}P< 0.01$)(Kunwar *et al.*, 2021b).

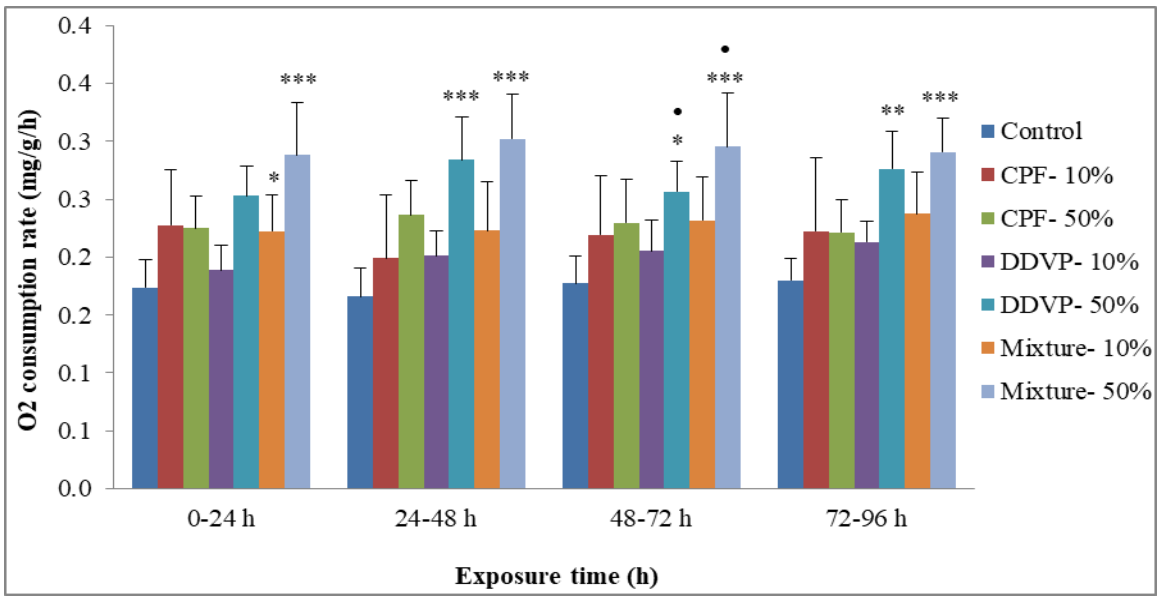

Figure 28: Oxygen consumption rate of common carp in various treatments and exposure time.

Pesticide concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Values are mean ± SD (n = 6). Asterisk denotes significant difference between the control and pesticide treated groups during the same sampling interval ($^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$); and

dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment at the same sampling interval ($^{\bullet}P< 0.05$) (Kunwar *et al.*, 2021a).

The patterns became clearer when the average oxygen consumption over the whole experimental period was examined (Figs. 29 and 30). Statistical analysis revealed a significantly reduced oxygen consumption ($P<0.001$) compared to control in chlorpyrifos-10%, dichlorvos-10% and 50% but significantly high ($P<0.001$) consumption in mixture-50% in golden mahseer (Fig. 29). But in common carp, all pesticides elevated ($P< 0.01$-0.001) oxygen consumption (Fig. 30). In both fish species, the highest oxygen uptake was noticed in mixture- 50% treatments. Between the low and high concentrations of the same pesticide treatment, significant differences were noted in chlorpyrifos ($P< 0.01$) and in the pesticide mixture ($P< 0.001$) treated groups in golden mahseer (Fig. 29) and in dichlorvos and in mixture pesticide treatment groups in common carp ($P< 0.001$) (Fig. 30).

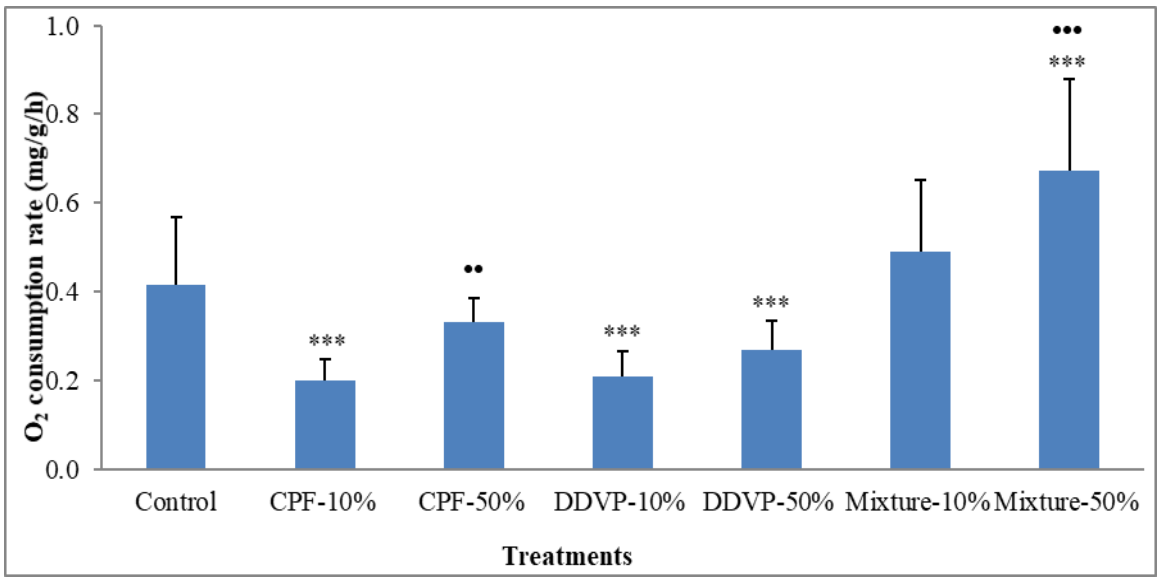

Figure 29: Oxygen consumption rate of golden mahseer in the control and various pesticide treatments.

Values are mean ± SD (n = 20). Exposure concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Asterisk denotes significant difference between the control and pesticide treated groups ($^{***}P < 0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment ($^{\bullet\bullet}P< 0.01$; $^{\bullet\bullet\bullet}P < 0.001$) (Kunwar *et al.*, 2021b).

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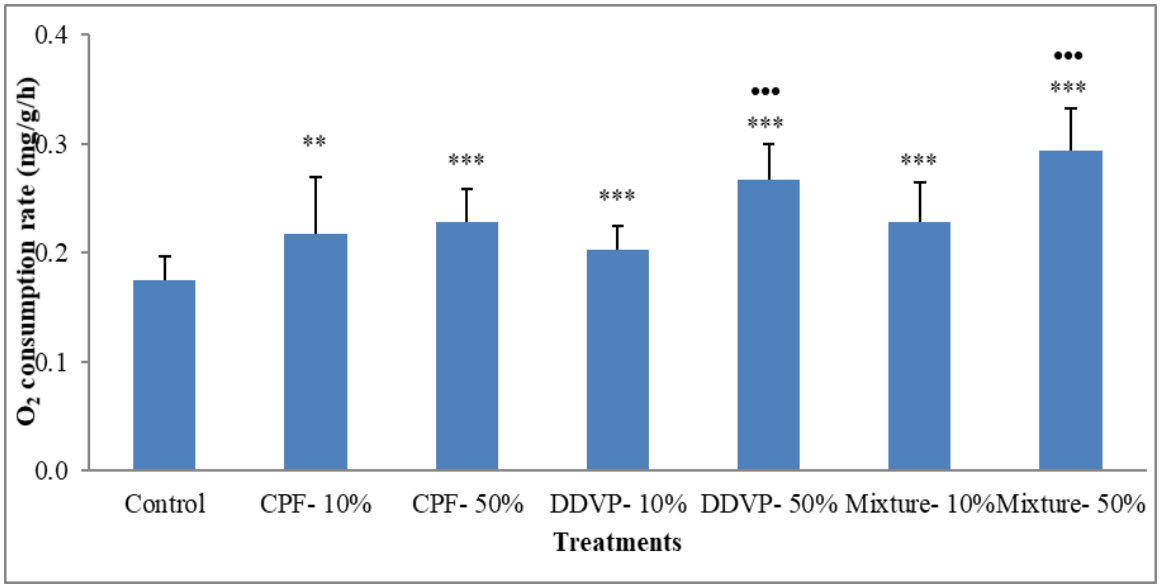

Figure 30: Oxygen consumption rate of common carp in the control and various pesticide treatments.

Values are mean ± SD (n = 24). Exposure concentrations were 10% and 50% 96 h-LC_{50} values of chlorpyrifos (CPF) and dichlorvos (DDVP). Asterisk denotes significant difference between the control and pesticide treated groups ($^{**}P< 0.01$; $^{***}P< 0.001$); and dark circle denotes significant difference between the low and high concentrations of the same pesticide treatment ($^{•••}P < 0.001$) (Kunwar *et al.*, 2021a).

The compiled data analysis of golden mahseer at different sampling intervals showed a decreasing trend of oxygen consumption in the exposed group compared to the control, however, the differences were not significant (Fig. 31). In contrast to this result, pesticide-exposed common carp always consumed more oxygen than their respective controls, although the difference was only significant during the 24-48 h ($P< 0.01$) and 48-72 h ($P< 0.05$) sampling intervals (Fig. 32). In both fish species, the oxygen uptake was constant either within the controls or within the exposed groups over the whole experimental period (Figs. 31 and 32)

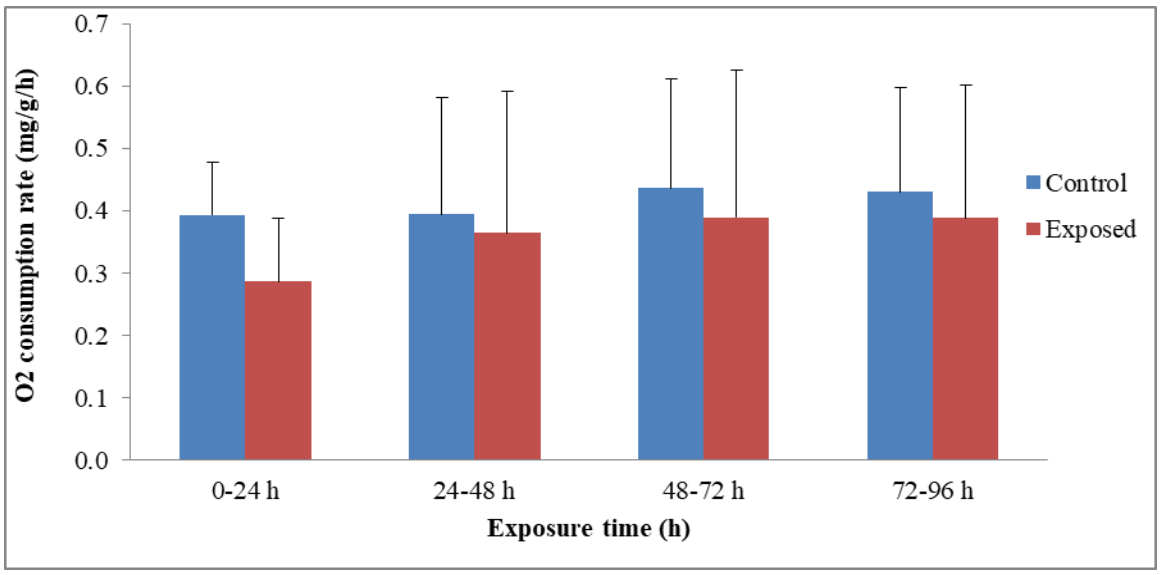

Figure 31: Oxygen consumption rate of golden mahseer at different exposure time.

Values are mean ± SD (n = 5 for control and n = 30 for pesticide exposed groups).

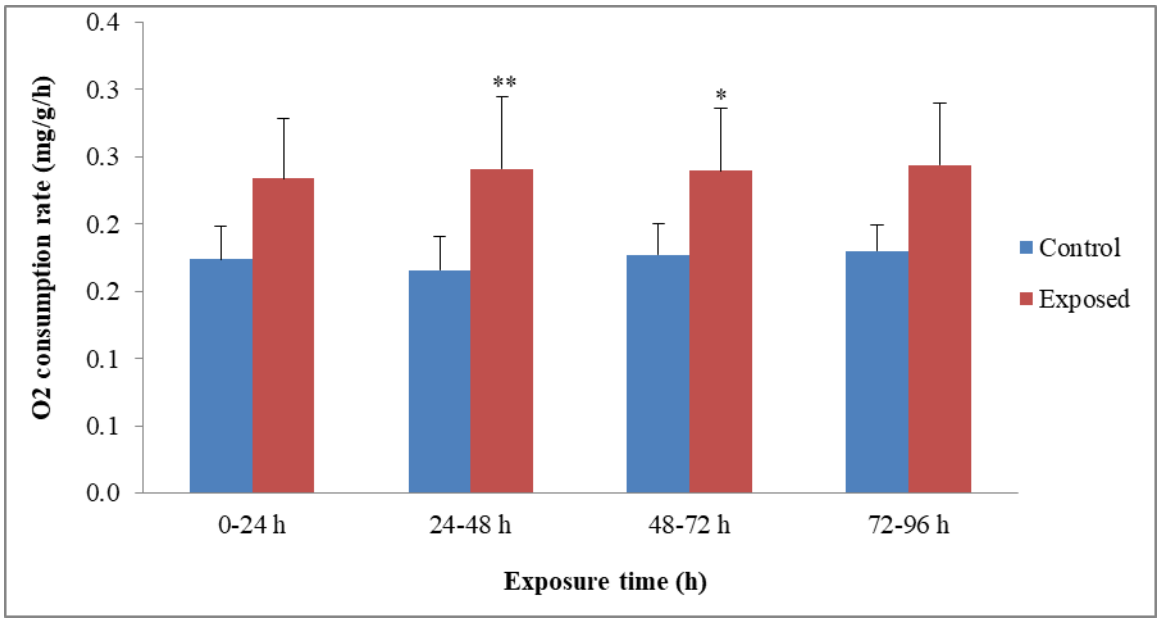

Figure 32: Oxygen consumption rate of common carp at different exposure time.

Values are mean ± SD (n = 6 for control and n = 36 for pesticide exposed groups). Asterisk denotes significant difference between the control and pesticide exposed group at various sampling intervals ($^{*}P< 0.05$; $^{**}P< 0.01$)(Kunwar *et al.*, 2021a).

4.1.3. Blood biochemical parameters

4.1.3.1. Glucose

In general, a rising trend in blood glucose levels was observed after pesticide exposure in both fish species (Fig. 33 and 34), but the elevation was only significant after 24 h of exposure in dichlorvos treated golden mahseer (P < 0.001) and chlorpyrifos and pesticide mixture treated common carp (P< 0.001). When the glucose levels of the identical pesticide-treated fish groups were compared during different sampling intervals, a significant recovery (P< 0.001) of glucose level was observed after 96 h and 1 week recovery (WR) period compared to the 24 h measurement in dichlorvos treated golden mahseer (Fig. 33). Compared to the 24 h measurement, similar recovery was observed in common carp after 1 week recovery period in chlorpyrifos treated fish (P< 0.001) and after 96 h (P< 0.05) and 1 WR (P< 0.001) in pesticide mixture treated fish (Fig. 34).

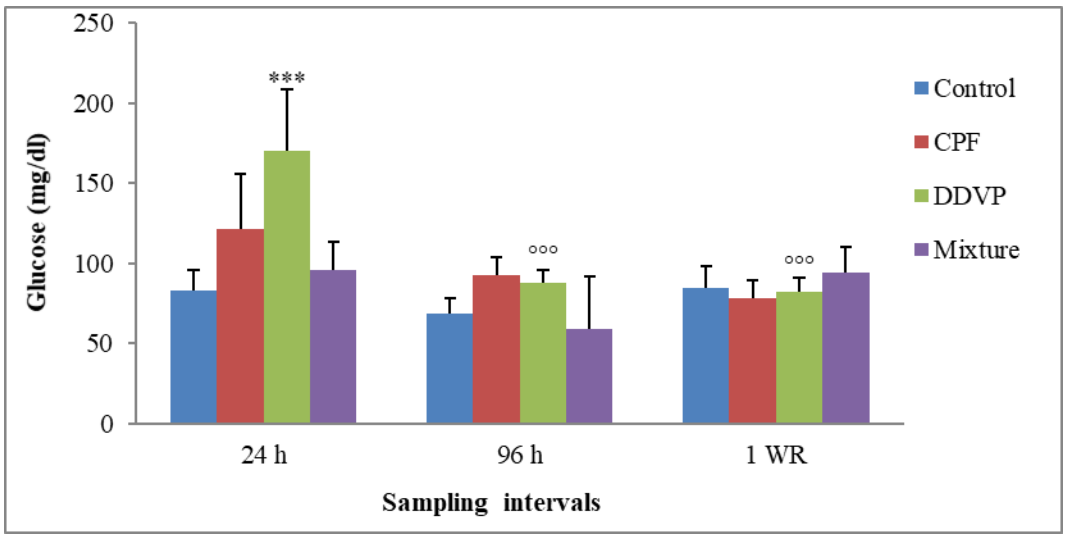

Figure 33: Blood glucose (mg/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{***}P$< 0.001); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{°°°}P$< 0.001) (Kunwar *et al.*, 2022b).

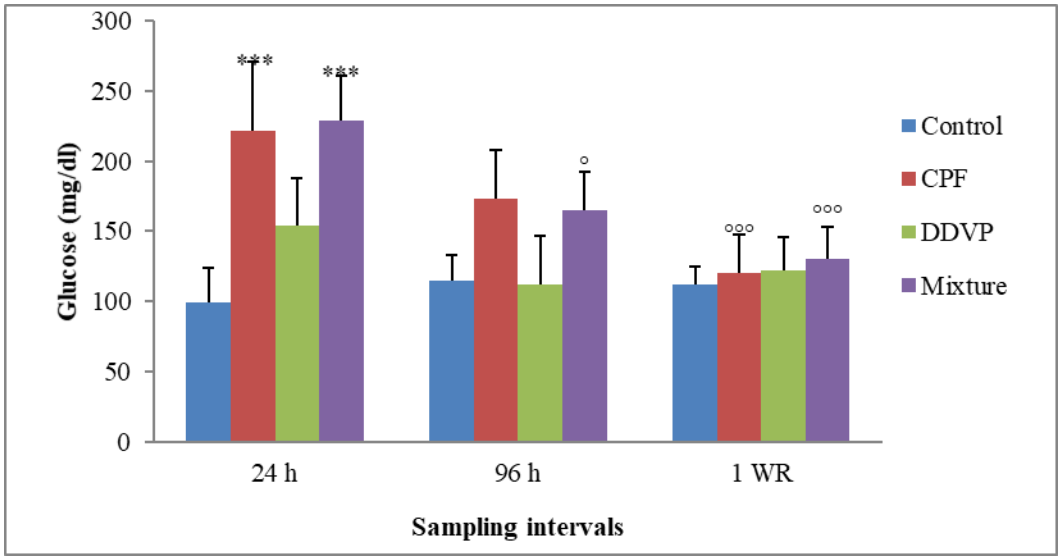

Figure 34: Blood glucose (mg/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{***}P< 0.001$); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ\circ\circ}P< 0.001$).

4.1.3.2. Total protein

Regardless of the pesticide treatments, exposure time, or depuration phase, no significant effect on serum total protein was found in golden mahseer. However, the mixture showed a decreasing tendency, especially after 96 hours, which restored after one week of depuration (Fig. 35). In contrast, there was an increasing trend of serum total protein in common carp which was significantly higher ($P< 0.001$) in the chlorpyrifos fish group compared to control after 24 h of exposure. This rise was again settled ($P< 0.001$) by the one week depuration period (Fig. 36).

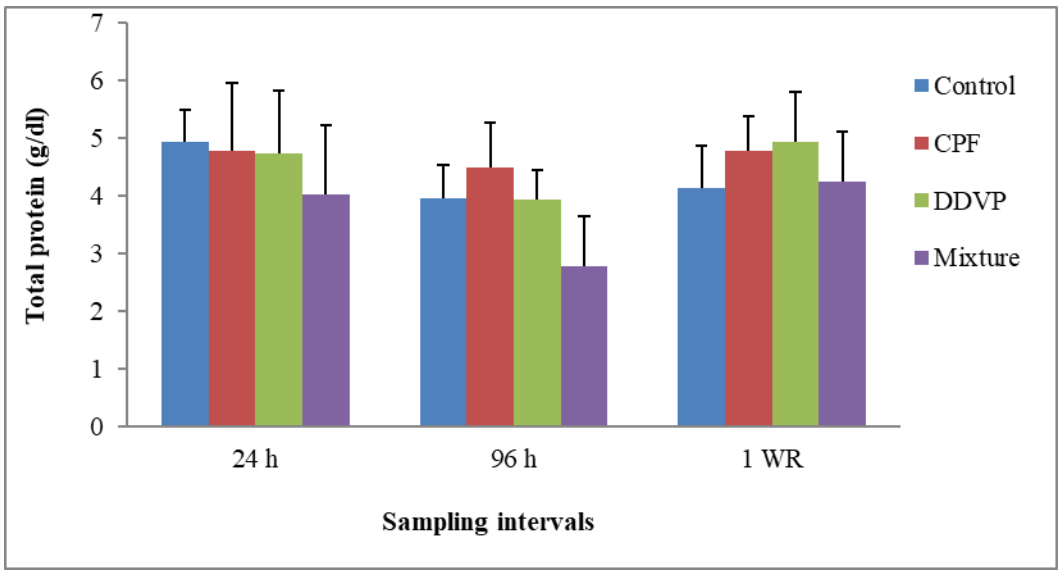

Figure 35: Blood total protein (g/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7) (Kunwar *et al.*, 2022b).

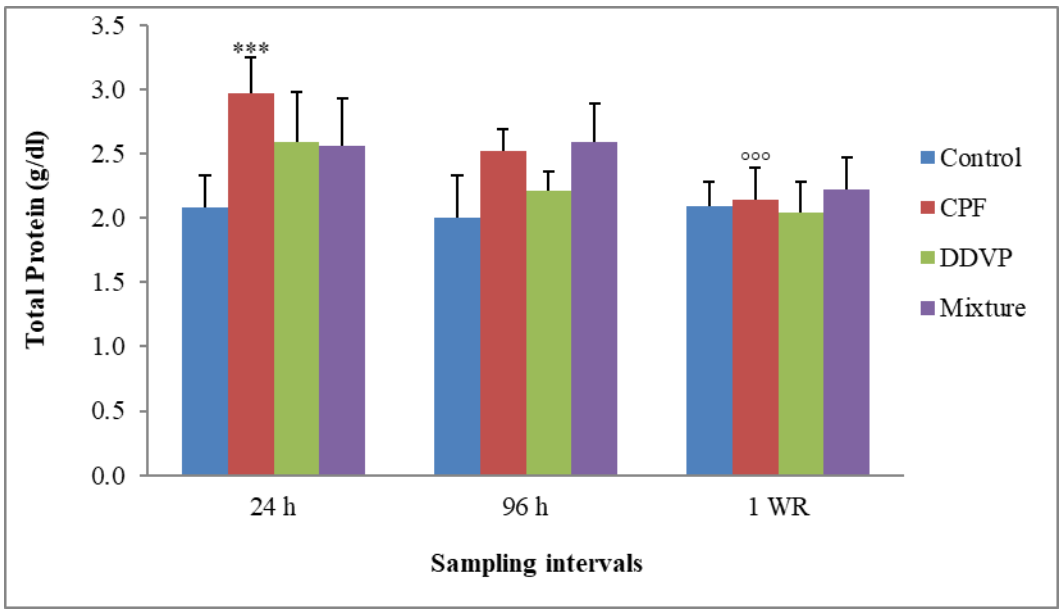

Figure 36: Blood total protein (g/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{***}P< 0.001$); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{°°°}P< 0.001$).

4.1.3.3. Albumin and globulin

In general, golden mahseer showed a declining trend of the serum albumin with pesticide exposure but the difference was significant ($P< 0.01$) only at 24 h of exposure in the mixture group when compared to the control level. The diminished albumin at 96 h was significantly recovered ($P< 0.05$) in the mixture group during the depuration period (Fig. 37). In contrast, there was an increasing trend of serum albumin in common carp where significant rise compared to control level was observed in chlorpyrifos ($P< 0.01$) and pesticide mixture ($P< 0.05$) fish groups after 24 h of exposure (Fig. 38). These increments were restored after 96 h ($P< 0.05$) and 1 WR ($P< 0.01$) in the chlorpyrifos group and after 96 h ($P< 0.05$) in the mixture group (Fig. 37). In the case of globulin, no significant differences were observed in both fish species regardless of the pesticide treatments, exposure time, or depuration phases (Fig. 39 and 40).

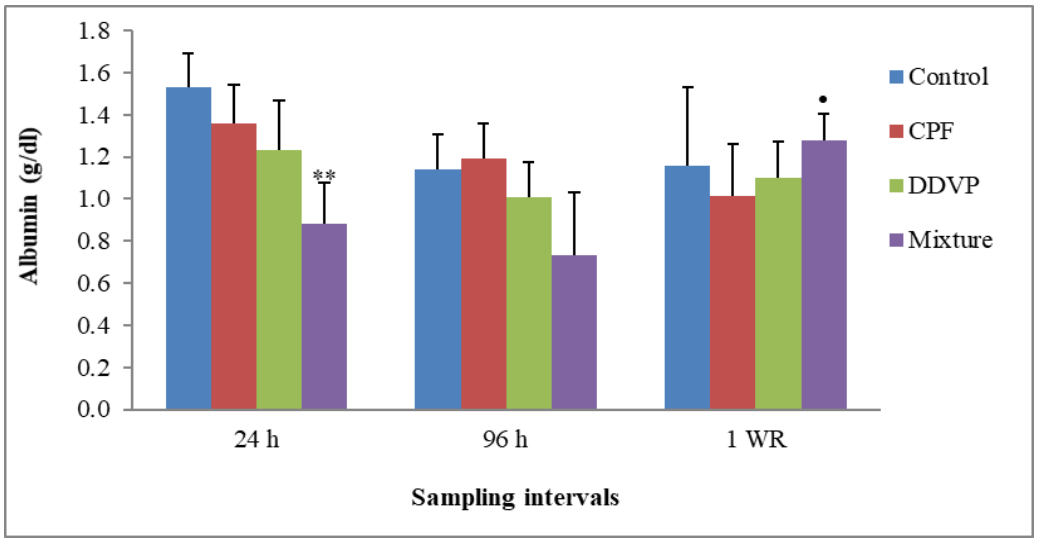

Figure 37: Blood albumin (g/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide treated groups at the same sampling interval ($^{**}P< 0.01$); and dark circle denotes significant difference in the same groups between 96 h and one week recovery (1 WR) ($^{\bullet}P< 0.05$) (Kunwar *et al.*, 2022b).

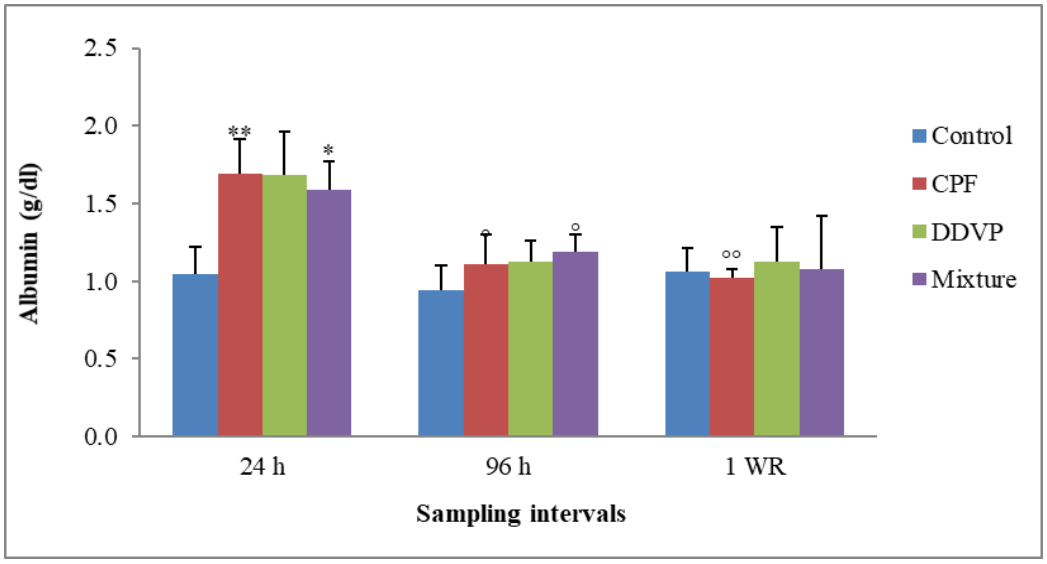

Figure 38: Blood albumin (g/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{*}P< 0.05$; $^{**}P< 0.01$); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ}P< 0.05$; $^{\circ\circ}P< 0.01$).

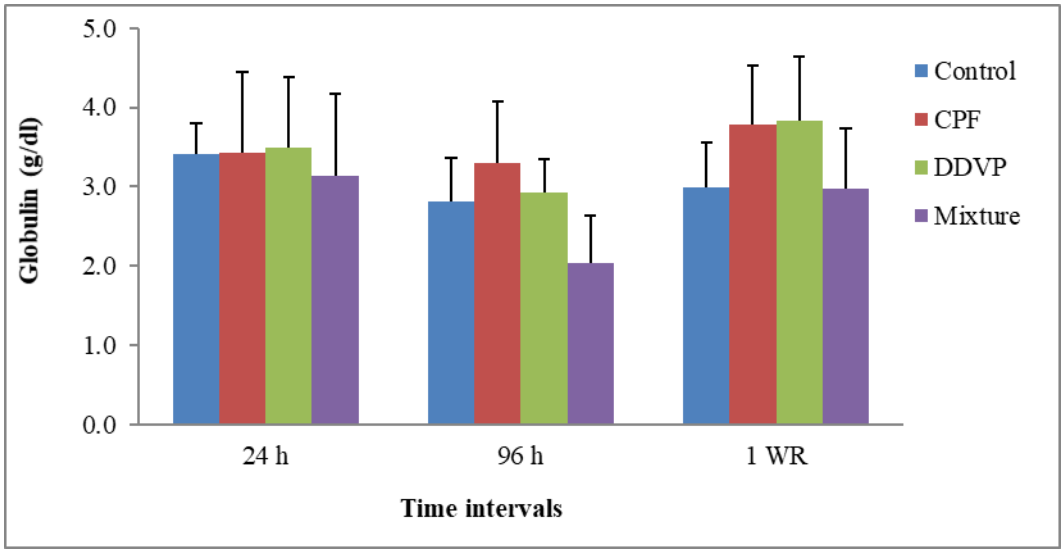

Figure 39: Blood globulin (g/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7) (Kunwar *et al.*, 2022b).

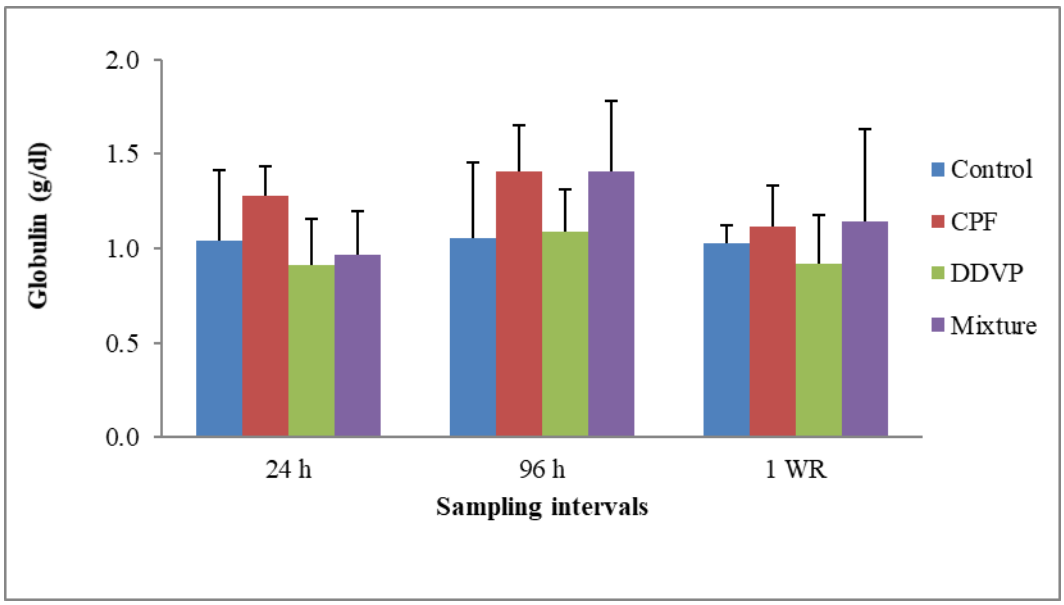

Figure 40: Blood globulin (g/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7).

4.1.3.4. Triglycerides

In both fish species, there was a distinct decreasing tendency of serum triglycerides in all pesticide treatments compared to control but these differences were not always significant. In golden mahseer, a sharp significant drop ($P< 0.001$) was noticed only in the pesticide mixture group after 96 h of treatment which was also significantly lower ($P< 0.05$) compared to the same pesticide treatment at 24 h (Fig. 41). In common carp, a significant drop of triglycerides was also observed in the chlorpyrifos treated group ($P< 0.05$) after 24 h of exposure and chlorpyrifos ($P< 0.05$) and pesticide mixture ($P< 0.01$) treated group after 96 h of exposure. When the triglyceride levels of the identical pesticide treatment groups were analyzed, a significant difference ($P< 0.01$) was seen between the 24 h and 96 h chlorpyrifos treated fish (Fig. 42).

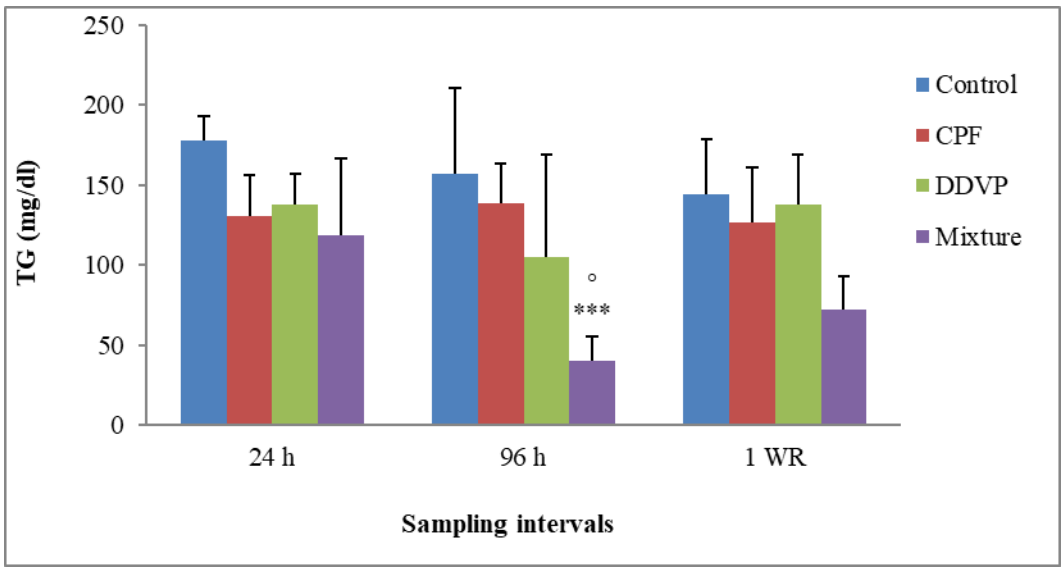

Figure 41: Blood triglycerides (mg/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L).Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval (****P*< 0.001); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h (°*P*< 0.05)(Kunwar *et al.*, 2022b).

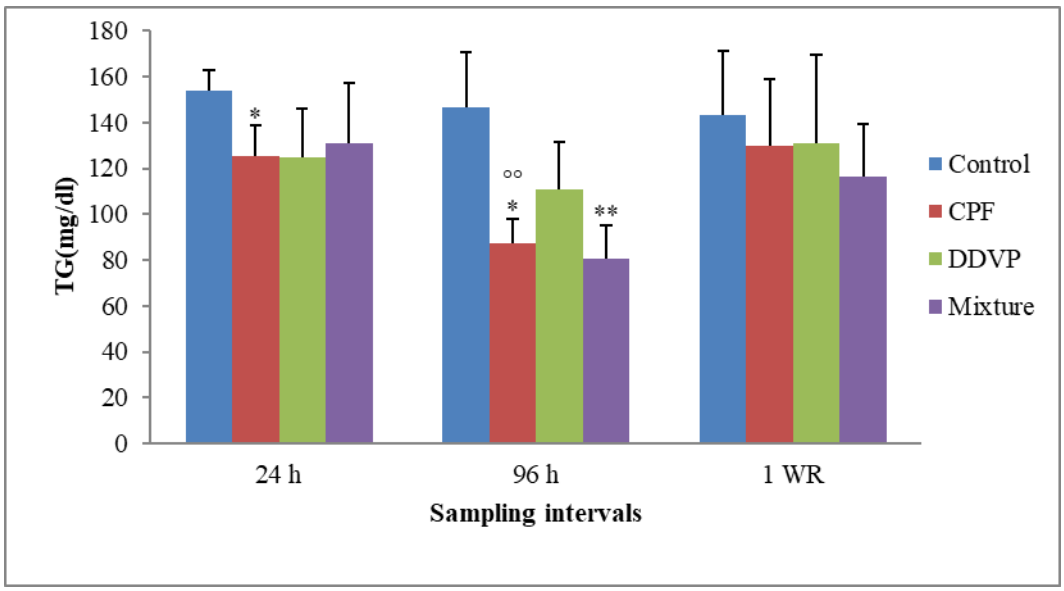

Figure 42: Blood triglycerides (mg/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval (**P*< 0.05; ***P*< 0.01); and white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h (°°*P*< 0.01).

4.1.3.5. Urea and creatinine

In golden mahseer, blood urea levels were steady after exposure to chlorpyrifos. However, they were in increasing trends in dichlorvos and pesticide mixture treatments, but a significant rise ($P< 0.01$) was noticed only after 96 h in the pesticide mixture group (Fig. 43). But, in common carp, there was a distinct elevation of serum urea with all pesticide treatments after 24 h ($P< 0.05–0.001$) and 96 h ($P< 0.01–0.001$) of exposure. During a one-week depuration interval, the increased urea was considerably recovered in both the chlorpyrifos ($P< 0.01$) and mixture pesticide ($P< 0.001$) treatment groups (Fig. 44). The pesticide treatments noted a growing trend for serum creatinine in both fish species, however these increments were not significant. During a one-week depuration interval, the numerically increased creatinine dropped to the control level (Figs. 45 and 46). Over the whole exposure period, statistical analysis indicated no significant differences in both parameters (urea and creatinine) among the identical pesticide treatments (Figs. 43 to 46).

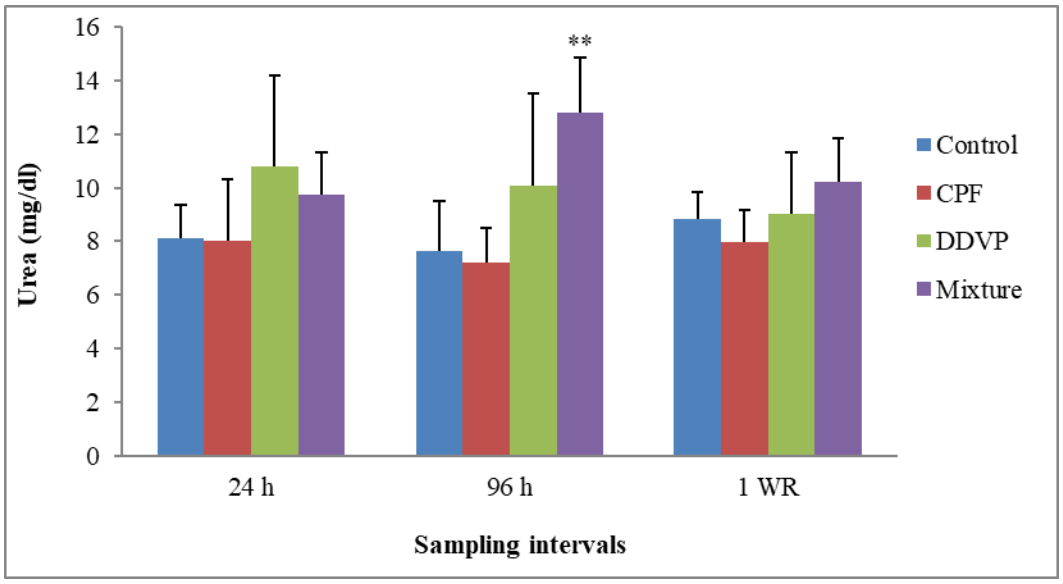

Figure 43: Blood urea (mg/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval (**$P< 0.01$) (Kunwar *et al.*, 2022b).

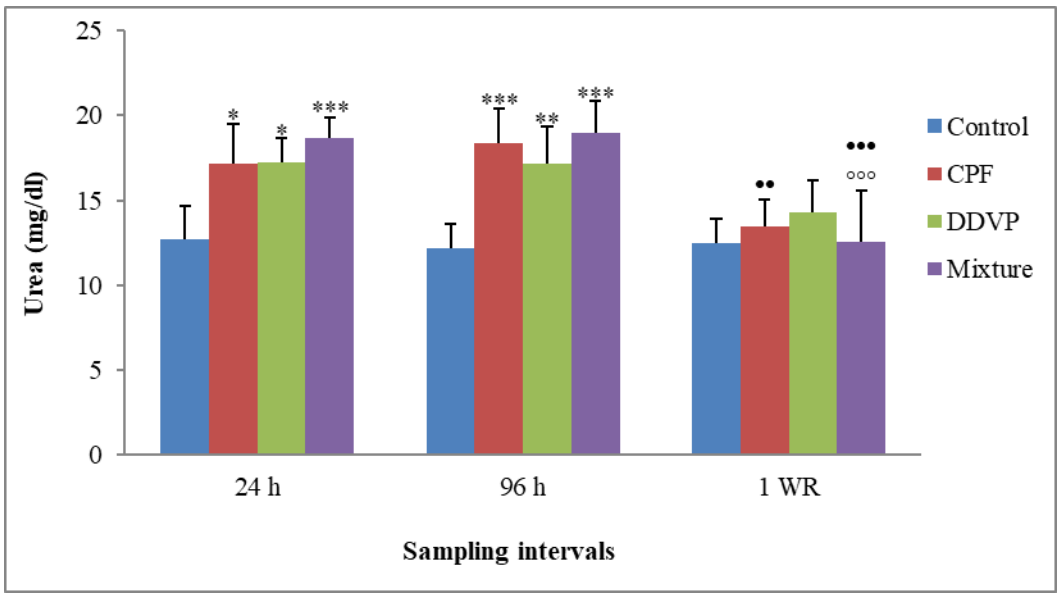

Figure 44: Blood urea (mg/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$); white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ\circ\circ}P< 0.001$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet\bullet}P< 0.01$; $^{\bullet\bullet\bullet}P< 0.001$).

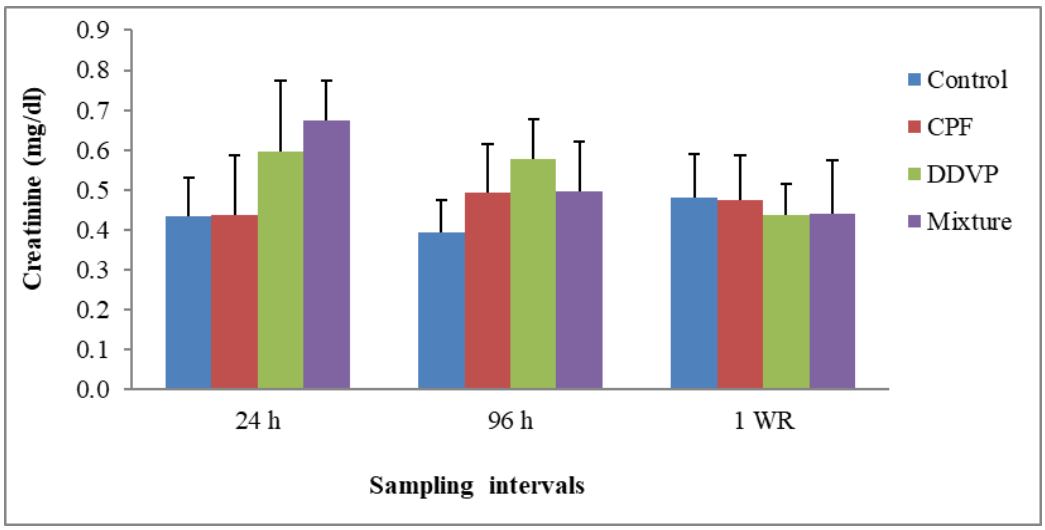

Figure 45: Blood creatinine (mg/dl) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7) (Kunwar *et al.*, 2022b).

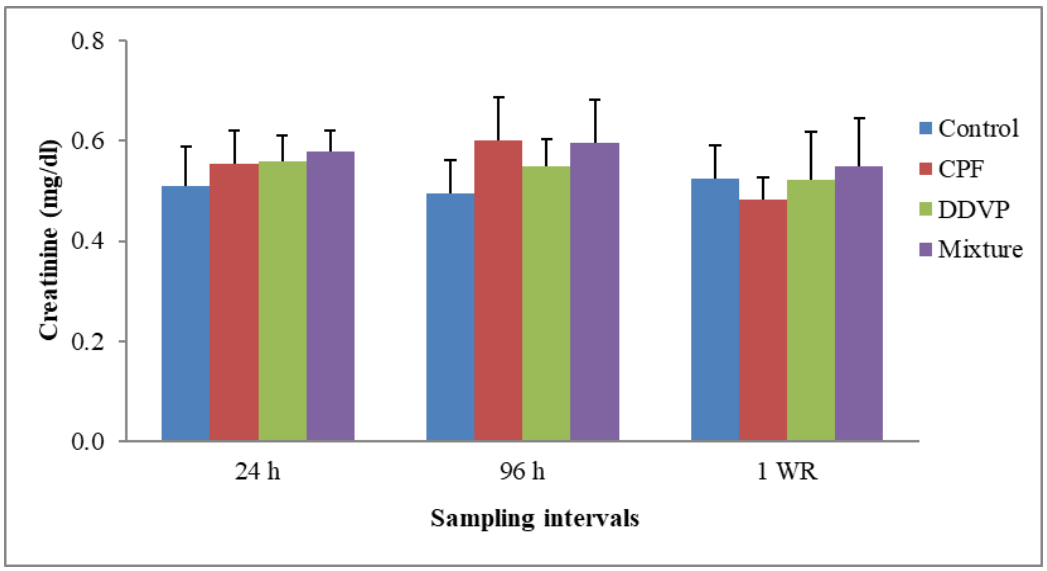

Figure 46: Blood creatinine (mg/dl) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7).

4.1.3.6. Serum enzymes (AST, ALT, and ALP)

Aspartate aminotransferase (AST)

Blood AST activity tended to be increased by all pesticide treatments in both fish species; but in golden mahseer, significantly higher AST activities compared to respective controls were observed only in pesticide mixture treatment after 24 h ($P< 0.01$) and 96 h ($P< 0.001$) of exposure (Fig. 47). In common carp, all pesticide treatments significantly elevated AST activities after 24 h ($P< 0.01–0.001$) and 96 h ($P< 0.05–0.01$) of exposure (Fig. 48). Among the identical pesticide treatments, significantly higher AST activity was observed in the mixture group after 96 h ($P< 0.01$) compared to the 24 h which was significantly recovered ($P< 0.001$) after 1 week recovery period in golden mahseer (Fig. 47). In common carp, AST activities were also significantly restored both in chlorpyrifos ($P< 0.01$) and pesticide mixture ($P< 0.05$) exposures compared to its 24 h and 96 h elevations (Fig. 48).

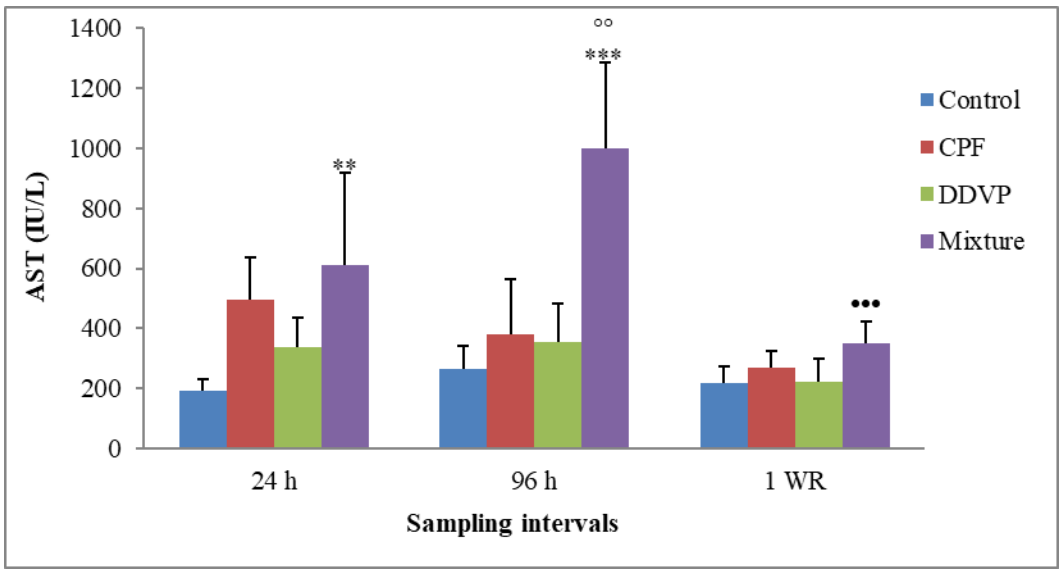

Figure 47: Blood AST (IU/L) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{**}P< 0.01$; $^{***}P< 0.001$); white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ\circ}P< 0.01$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet\bullet\bullet}P< 0.001$) (Kunwar *et al.*, 2022b).

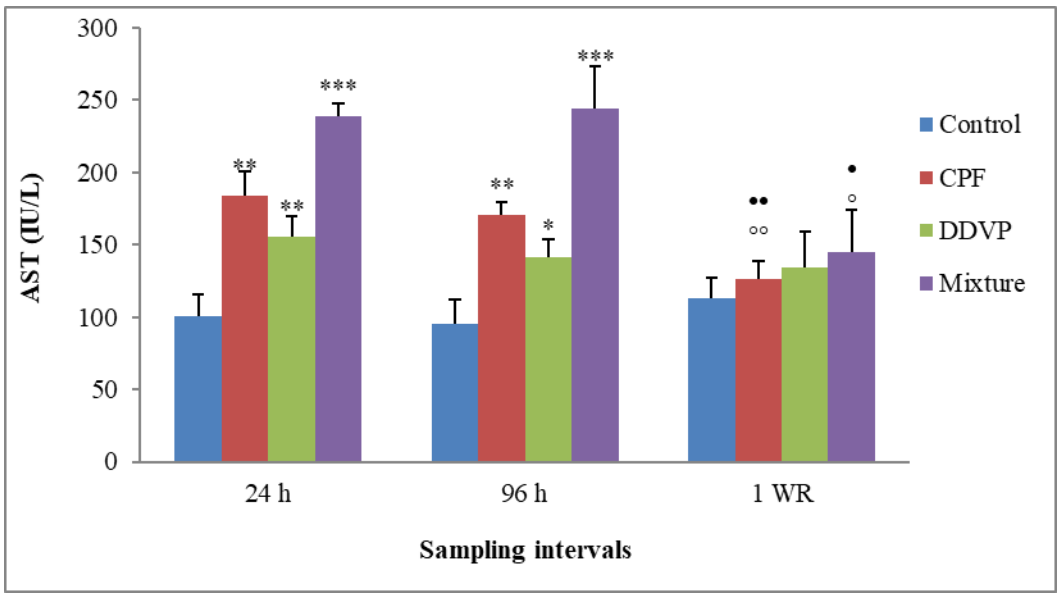

Figure 48: Blood AST (IU/L) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$); white circle denotes significant difference between the

same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ}P< 0.05$; $^{\circ\circ}P< 0.01$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet}P< 0.05$; $^{\bullet\bullet}P< 0.01$).

Alanine aminotransferase (ALT)

In both fish species, there was an elevating trend of serum ALT activities in response to pesticide treatments (Figs. 49 and 50). In golden mahseer, the ALT activity was significantly increased than the control after 96 h of exposure ($P< 0.01$) in both chlorpyrifos and dichlorvos treated fish which was significantly restored after 1 week of the recovery period ($P< 0.05$-0.01) (Fig. 49). In common carp, the increased ALT level compared to control was noticed in pesticide mixture at 24 h ($P< 0.01$) and 96 h ($P< 0.001$), and chlorpyrifos treatment after 96 h ($P< 0.05$). The elevated ALT activities were completely recovered during one week depuration phase, where ALT activities in chlorpyrifos and mixture groups were significantly lower ($P< 0.01$-0.001) than its 24 h and 96 h observations. Similarly, the ALT activities after the depuration period in the dichlorvos treated fish was also lower ($P< 0.05$) compared to its 96 h measurement (Fig. 50).

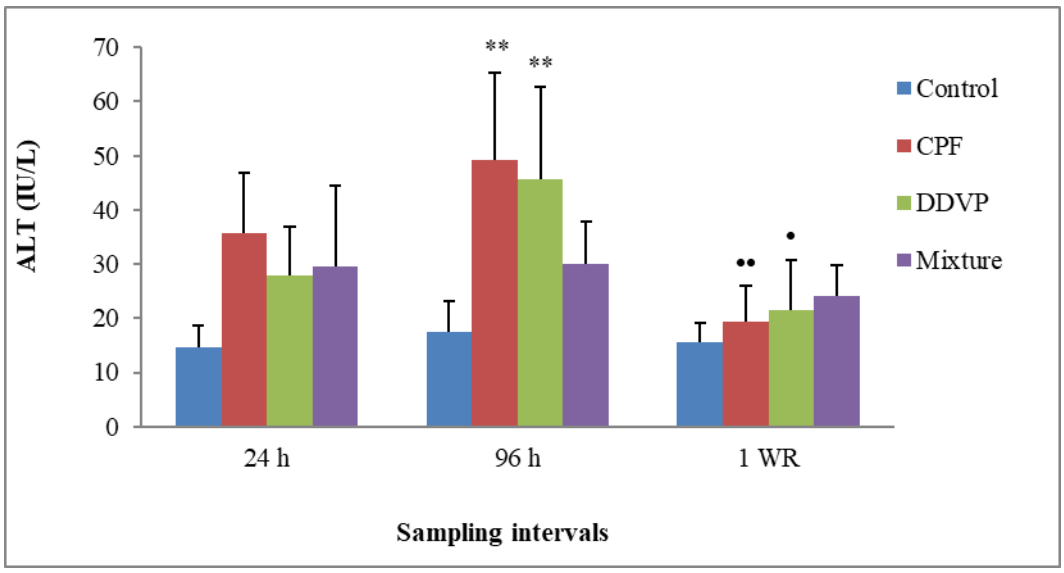

Figure 49: Blood ALT (IU/L) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{**}P< 0.01$); and dark circle denotes significant difference in the same groups between 96 h and one week recovery (1 WR) ($^{\bullet}P< 0.05$; $^{\bullet\bullet}P< 0.01$) (Kunwar *et al.*, 2022b).

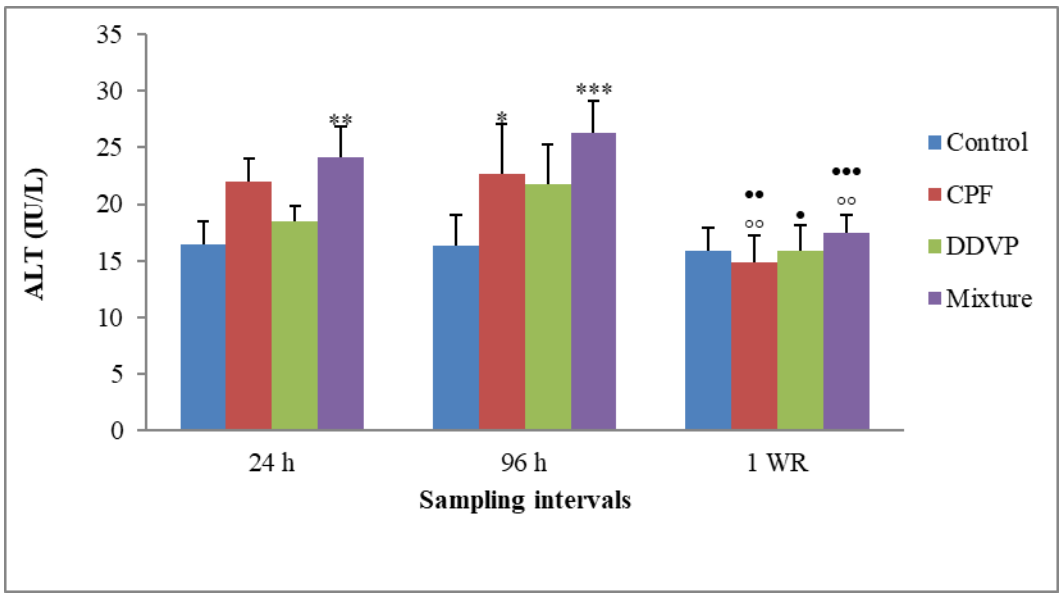

Figure 50: Blood ALT (IU/L) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling intervals ($^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$); white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ\circ}P< 0.01$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet}P< 0.05$; $^{\bullet\bullet}P< 0.01$; $^{\bullet\bullet\bullet}P< 0.001$).

Alkaline phosphatase (ALP)

Similar to AST and ALT activities, serum ALP activity also showed a rising trend in both fish species (Figs. 51 and 52). In golden mahseer, the significant elevation compared to control was noted after 96 h of exposure in chlorpyrifos ($P< 0.001$) and dichlorvos ($P< 0.01$) pesticide groups. Among the same pesticide treatments, chlorpyrifos treated fish exhibited significantly higher ALP at 96 h ($P< 0.05$) compared to 24 h observation. These 96 h elevated ALP values dropped again in chlorpyrifos ($P< 0.001$) and dichlorvos ($P< 0.01$) treated fish after 1 week depuration (Fig. 51). In common carp, significant rise in ALP activities were found in the pesticide mixture group ($P< 0.01$) after 24 h, and chlorpyrifos and pesticide mixture group after 96 h of treatments ($P< 0.001$). The 96 h elevated ALP in the mixture treatment was significantly recovered ($P< 0.01$) during 1 week depuration phase (Fig. 52).

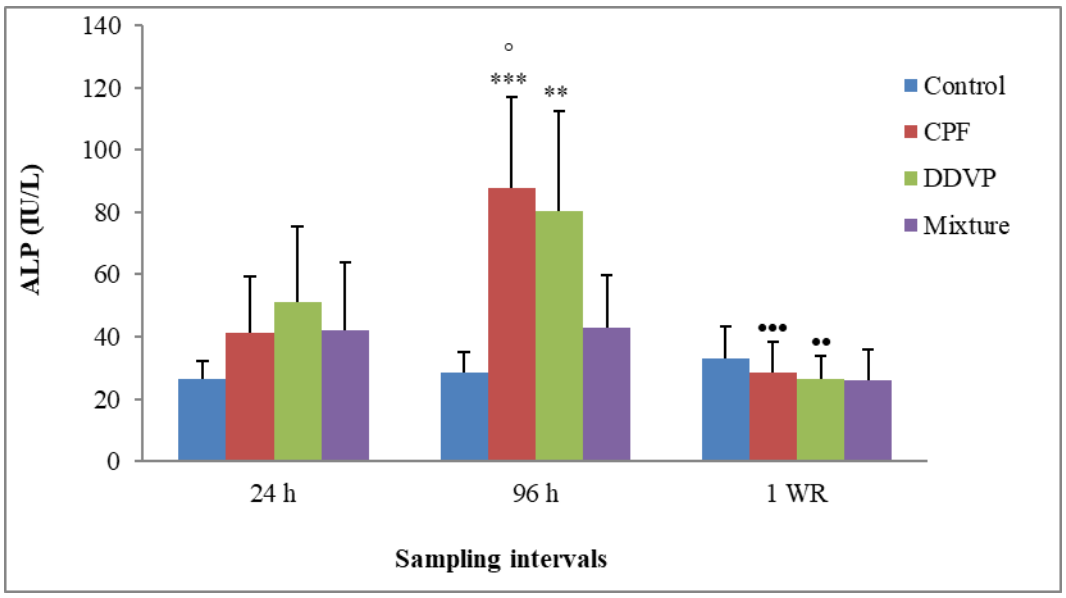

Figure 51: Blood ALP (IU/L) in the control and pesticide treated golden mahseer.

The pesticides concentrations were chlorpyrifos (CPF)- 0.075 mg/L, dichlorvos (DDVP)- 1.29 mg/L and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling interval ($^{**}P< 0.01$; $^{***}P< 0.001$); white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ}P< 0.5$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet\bullet}P< 0.01$; $^{\bullet\bullet\bullet}P< 0.001$) (Kunwar *et al.*, 2022b).

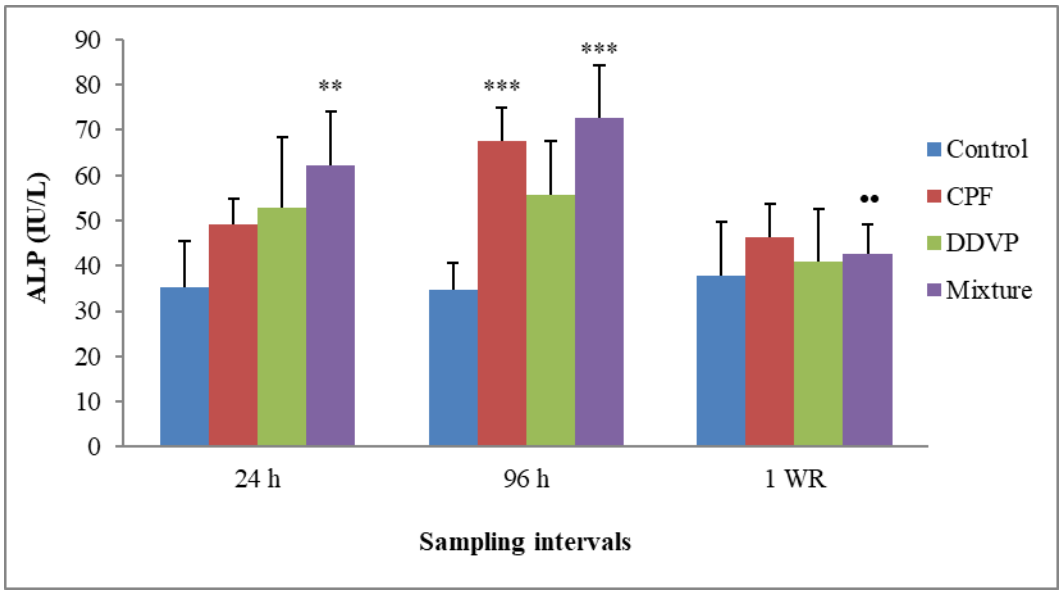

Figure 52: Blood ALP (IU/L) in the control and pesticide treated common carp.

The pesticides concentrations were chlorpyrifos (CPF)- 0.044 mg/L, dichlorvos (DDVP)- 1.57 mg/L and mixture (CPF- 0.044 mg/L and DDVP- 1.57 mg/L). Values are mean ± SD (n = 5-7). Asterisk denotes significant difference between the control and pesticide exposed groups at the same sampling

interval ($^{**}P< 0.01$; $^{***}P< 0.001$); and dark circle denotes significant difference in the same groups between 96 h and one week recovery (1 WR) ($^{\bullet\bullet}P< 0.01$).

In golden mahseer, the compiled data analysis for all biochemical parameters showed significant elevation of glucose after 24 h ($P< 0.05$) ALT and ALP after 96 h ($P< 0.01$) and significant drop of triglycerides after 96 h ($P< 0.05$) of exposure in pesticide exposed groups compared to their respective controls (Table 27). The 24 h elevated glucose in the exposed group was significantly dropped after 96 h and 1 WR ($P< 0.01$). In pesticide exposed fish, the ALP level recorded in 96 h was also greater ($P< 0.05$) than its 24 h of observation. The high AST, ALT, and ALP values recorded in 96 h exposed groups were also significantly restored ($P< 0.01$-0.001) during one week depuration (Table 27). In common carp, the compiled data analysis revealed significantly higher ($P< 0.05$-0.001) glucose, protein, albumin, urea, AST, ALT, and ALP, as well as significantly lower ($P< 0.01$) triglycerides in the pesticide treated fish compared to their respective controls after 24 h of exposure. Similarly, after 96 h of exposure, serum urea, AST, ALT, and ALP levels were elevated ($P< 0.05$–0.001), while triglyceride levels were decreased ($P< 0.05$) in pesticide exposed fish (Table 28). Among the pesticide exposed groups, significant differences ($P< 0.05$ and 0.001) were observed between 24 h and 96 h in glucose, albumin, and triglycerides; between 24 h and 1 WR ($P< 0.05$-0.001) in glucose, protein, albumin, urea, AST, ALT, and ALP; and between 96 h and 1 WR ($P< 0.05$-0.001) in triglycerides, urea, AST, ALT and ALP measurements (Table 28).

Table 27: Blood biochemical parameters of golden mahseer at various sampling intervals.

Parameters	24 h		96 h		1 WR	
	Control	**Exposed**	**Control**	**Exposed**	**Control**	**Exposed**
Glucose (mg/dl)	83.08±12.44	129.18±43.20*	69.04±9.42	79.89±24.40$^{\circ\circ}$	84.86±13.11	84.78±13.40 $^{\circ\circ}$
Protein (g/dl)	4.94±0.54	4.50±1.13	3.96±0.59	3.73±1.01	4.14±0.73	4.66±0.78
Albumin (g/dl)	1.53±0.16	1.15±0.28	1.14±0.17	0.98±0.28	1.16±0.37	1.13±0.21
Globulin (g/dl)	3.42±0.38	3.35±0.93	2.81±0.55	2.75±0.79	2.99±0.57	3.53±0.82
TG (mg/dl)	178.26±15.18	129.05±32.75	156.97±53.93	94.53±56.49 *	144.14±34.50	112.22±40.30
Urea (mg/dl)	8.12±1.25	9.61±2.71	7.64±1.88	10.03±3.27	8.85±0.98	9.07±1.88
Creatinine (mg/dl)	0.43±0.10	0.58±0.17	0.39±0.08	0.52±0.11	0.48±0.11	0.45±0.10
AST (IU/L)	191.40±40.24	480.00±226.97	267.20±73.85	577.40±363.72	220.40±53.51	281.33±84.47 $^{\bullet\bullet}$
ALT (IU/L)	14.60±4.16	30.88±11.59	17.40±5.73	41.60±15.85 **	15.60±3.51	21.60±7.08 $^{\bullet\bullet\bullet}$
ALP (IU/L)	26.30±5.92	45.00±20.93	28.30±6.67	70.30±32.23$^{**,\circ}$	33.10±10.14	27.13±8.36 $^{\bullet\bullet\bullet}$

Values are mean ± SD. Asterisk denotes significant difference between the control and exposed groups at the same sampling interval (*P < 0.05; **P < 0.01); white circle denotes significant difference between the same groups of 96 h and one week recovery (1WR) compared to 24 h ($^{\circ}P$< 0.5); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet\bullet}P$< 0.01; $^{\bullet\bullet\bullet}P$< 0.001)(Kunwar *et al.,* 2022b).

Table 28: Blood biochemical parameters of common carp at various sampling intervals.

Parameters	24h		96h		1 WR	
	Control	**Exposed**	**Control**	**Exposed**	**Control**	**Exposed**
Glucose (mg/dl)	99.41 ±24.09	202.65±50.26***	114.79±18.46	151.01±40.42°	111.67±13.42	123.67±23.46$^{\circ\circ\circ}$
Protein (g/dl)	2.09±0.25	2.73±0.38**	2.00±0.34	2.44±0.26	2.09±0.19	2.13±0.24$^{\circ\circ\circ}$
Albumin (g/dl)	1.04±0.17	1.65±0.22***	0.94±0.16	1.14±0.14$^{\circ\circ\circ}$	1.06±0.15	1.08±0.23$^{\circ\circ\circ}$
Globulin (g/dl)	1.04±0.37	1.08±0.26	1.06±0.40	1.30±0.31	1.03±0.10	1.05±0.33
Triglycerides (mg/dl)	153.81±8.82	126.97±20.04**	146.78±23.85	93.87±20.27$^{*,\circ\circ\circ}$	143.17±27.82	125.49±30.06$^{\bullet}$
Urea (mg/dl)	12.72±1.96	17.71±1.73***	12.20±1.40	18.17±2.06***	12.47±1.41	13.47±2.17$^{\circ\circ\circ,\bullet\bullet\bullet}$
Creatinine (mg/dl)	0.51±0.08	0.56±0.05	0.50±0.07	0.58±0.08	0.52±0.07	0.52±0.08
AST (IU/L)	100.64±14.76	192.57±38.05***	95.53±16.63	188.92±49.16***	113.40±14.01	135.44±22.70$^{\circ\circ,\bullet\bullet}$
ALT (IU/L)	16.49±1.99	21.72±3.14*	16.29±2.78	23.59±3.94*	15.85±2.04	16.07±2.25$^{\circ\circ\circ,\bullet\bullet\bullet}$
ALP (IU/L)	35.20±10.27	54.66±12.51**	34.68±6.07	64.77±12.35***	37.84±11.79	43.22±8.62$^{\circ,\bullet\bullet\bullet}$

Values are mean ± SD. Asterisk denotes significant difference between the control and exposed groups at the same sampling interval ($^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$); white circle denotes significant difference between the same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ}P < 0.05$; $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$); and dark circle denotes significant difference in the same groups between 96 h and 1 WR ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$).

4.2. Discussion

4.2.1. Lethal toxicity and general fish behavior

4.2.1.1. Toxicity of individual pesticides

The first step in evaluating a chemical's safety threshold is to determine its lethal toxicity. Lethal toxicity of chlorpyrifos and dichlorvos was estimated based on the mortality responses of the target fish species against these toxicants. In this study, the 96 h-LC_{50} of chlorpyrifos to golden mahseer, common carp, and mrigal were reported to be 0.753 (0.616-0.931) mg/L, 0.440 (0.373-0.504) mg/L, and 0.380 (0.319-0.450) mg/L, respectively. These results corroborate with the chlorpyrifos toxicity range documented globally, where 96 h-LC_{50} of chlorpyrifos in common carp were 0.16, 0.20, and 0.58 mg/L, according to Halappa and David (2009), Banaee *et al.* (2013), and Xing *et al.* (2015), respectively. Similarly, in mrigal, it was reported to be 0.44 mg/L (Bhatnagar *et al.,* 2016).

In this study, the 96 h median lethal concentrations of dichlorvos were calculated to be 12.964 (10.866-15.515) mg/L, 15.705 (14.385-16.963) mg/L, and 11.367 (9.496-13.536) mg/L to golden mahseer, common carp, and mrigal, respectively. These results are also comparable to other publications, wherein 96 h median lethal concentrations of dichlorvos to common carp were reported to be 9.41 mg/L (Ural & Çalta, 2005) and 21.11 mg/L (Laxmi *et al.,* 2019). Similarly, it was 9.1 mg/L in mrigal (Velmurugan *et al*., 2009). These findings suggest that among the three fish species golden mahseer was the least sensitive and mrigal was the most sensitive in terms of chlorpyrifos toxicity whereas mrigal was the most sensitive and common carp was the most resistant species for the dichlorvos toxicity.

The World Health Organization has classified chlorpyrifos as a moderately hazardous pesticide (class II) and dichlorvos as a highly hazardous pesticide (class Ib) (WHO, 2020). However, the acute toxicity results are just the opposite, revealing chlorpyrifos is more toxic than dichlorvos. Such difference in pesticide toxicity is due to the fact that WHO provided pesticide classification based on acute oral and dermal toxicity (LD_{50}) to the rat whereas the test species in this experiment was aquatic animal (fish). To the best of my knowledge, this is a novle work to report the lethal toxicity of chlorpyrifos and dichlorvos to golden mahseer.

4.2.1.2. Joint toxicity of pesticides

This study documented the joint effect of chlorpyrifos and dichlorvos to fish. Because the aquatic environment is a sink of multiple pollutants including pesticides; the risk assessments of a single pesticide can be misleading. As such the combined action of pesticides was evaluated and found to be antagonistic in golden mahseer and mrigal, whereas it was synergistic in common carp. The antagonistic effect exemplifies that the pesticide mixture was less harmful compared to the total of the individual pesticides tested. The synergistic action is just the opposite, which represents the mixture had higher toxicity compared to the toxicity of the sum of the individual pesticides tested. Chlorpyrifos and carbosulfan (Chen *et al.,* 2014) and fenobucarb with triazophos or malathion (Wang *et al.,* 2015) were found to have antagonistic effects in common carp, similar to the present findings in golden mahseer and mrigal. The antagonistic effect of chlorpyrifos and other pesticides mixtures on zebrafish was also reported by Wang *et al.* (2017). Investigators have proposed a number of theories to explain the antagonistic impact. According to Hernández *et al.* (2013), the pesticide combination modifies the toxicokinetic of the individual chemicals, therefore altering their toxicity. As a result, the antagonistic outcome might be the consequence of a chemical interaction between two insecticides (Imam *et al.,* 2018). Similarly, other authors (Stepić *et al.,* 2013; Wang *et al.,* 2017) claimed that increased metabolization processes led to quicker metabolite elimination, resulting in lower pesticide toxicity. Although not evaluated in this work, the increased activity of carboxylesterases (CaEs) enzymes under the binary action might be another potential explanation for such an antagonistic finding. Many pesticides, including organophosphate, have been documented to be hydrolyzed using this enzyme (Jokanovic, 2001; Wheelock *et al.,* 2005). Furthermore, it is thought that this enzyme protects AChE against pesticide toxicity through direct binding and sequestration (Maxwell, 1992). The findings suggest that a combination of chlorpyrifos and dichlorvos might trigger CaEs activation. The activity dynamics of glutathione-S-transferase (GST) might also play a role in antagonistic relationships. GST is present in a variety of fish tissues and is involved in the cellular detoxification of xenobiotics like pesticides (Jin-Clark *et al.,* 2002). When malathion and pirimiphos-methyl insecticides were administered together, GST activity was shown to be considerably higher than when they were applied separately (Stepić *et al.,* 2013). Although GST

activity was not examined in the current investigation, it is plausible to hypothesize, based on the existing information (Stepić *et al.,* 2013), that a possible increase in GST activity under chlorpyrifos and dichlorvos combined exposure might promote efficient detoxification of these metabolites and therefore minimize toxicity.

Aquatic organisms are frequently exposed to combination of various pesticides, therefore knowledge on pesticide mixture toxicity becomes critical, especially when the joint actions turn out to be synergistic. Similar to the present observation with common carp, Wang *et al.* (2015) documented a synergistic action of triazophos and malathion as well as triazophos and carbofuran to common carp. Additive or more than additive toxicity of pesticide mixtures was reported in Pacific salmon (Laetz *et al.,* 2009) and zebrafish (Wang *et al.,* 2017; Wu *et al.,* 2018). Chlorpyrifos and dichlorvos had a synergistic impact in this experiment, this was most likely due to the fact that both of these pesticides have same mode of action as AChE inhibitor (LeBlanc *et al.,* 2012). Inhibition of this enzyme is dose-dependent (Singh *et al.,* 2018; Stepić *et al.,* 2013). The synergistic impact of endosulfan and temephos also manifested a higher inhibition of the AChE activity in comparison to the degree of suppression incited by these individual insecticides (Stepić *et al.,* 2013). However, it should be considered that the pesticide concentrations in the mixture treatments were twice that of the individual pesticide exposures, therefore high suppression of the enzyme was expected, and this caused a high fatality rate in pesticide mixture exposures. Pesticide detoxification processes mediated by carboxylesterases (CaEs) might explain the synergistic impact of the pesticide combination seen in this investigation. CaEs plays a protective role against pesticide (Jokanovic, 2001; Maxwell, 1992; Wheelock *et al.,* 2005). CaEs suppression may have happened in fish treated with a mixture of pesticides; therefore, CaEs were unable to play a protective role against a xenobiotic threat, ultimately resulting synergistic impact in pesticide co-exposure. Similarly, Barata *et al.* (2004) also reported high pesticide toxicity due to suppression of the CaEs enzyme. Enhanced activity of cytochrome P450 enzymes in fish tissue following exposure to the pesticide mixture might be another underlying mechanism for the synergistic effect. This enzyme is known to speed up the conversion of organophosphate to a more toxic metabolite with high AChE inhibitory properties. Wang *et al.* (2017) reported a similar toxicity mechanism when phoxim and atrazine were co-administered.

4.2.1.3. General fish behavior

Behavior changes are one of the most sensitive indications of potential toxic effects in animals. As such, behavioral studies are being incorporated in toxicity assessment. Overall fish behavioral responses in this study were hypo excitement, loss of balance, loss of coordination, aggregating at the corner of the aquaria, sitting on the bottom of the aquaria, surfacing activity, intermittent air gulping, excessive mucus secretion, becoming pale and abrupt swimming in spiral fashion before death which are consistent with observations made by other authors after pesticide exposure (Nwani *et al.,* 2013; Padmanabha *et al.,* 2015; Saha *et al.,* 2016; Soni & Verma, 2018; Ullah *et al.,* 2014).

These behavioral manifestations in this study might be related to AChE inhibition, resulting in acetylcholine (ACh) buildup in cholinergic synapses and overstimulation (Halappa & David, 2009). Excess mucus production might be a fish's first defense strategy to avoid contact with the harmful chemical or to remove it by epidermal mucus shedding (Halappa & David, 2009; Patil & David, 2008). The air gasping exhibited by fish in response to pesticides exposure might be a compensatory mechanism for respiratory stress (Halappa & David, 2009). Such a series of behavioral endpoints are simple, non-invasive, and can be used as a biomonitoring tool to assess the impacts of pollutants during acute and chronic exposure. While comparing fish behavior against different pesticide treatments, the intensity of behavioral changes in golden mahseer and mrigal were not remarkably different in chlorpyrifos, dichlorvos, and pesticide mixture treatments but in common carp, the intensity of behavioral expressions were more pronounced in the pesticide mixture compared to individual pesticide treatments. Such behavioral observation also aligns with the mortality outcomes and hence the joint action of the pesticides. In this exposure experiment, fish mortality occurred most probably by a neurological disorder. It is claimed on the basis of clear erratic swimming of fish before death indicating AChE mediated behavior.

4.2.2. Specific behavioral response and aerobic respiratory metabolism

4.2.2.1. Feeding behavior

In this experiment, reduction or ceasing food intake in response to pesticide treatments is a general response of fish to stress. Authors also observed such effects

in chlorpyrifos or dichlorvos treated fish (Halappa & David, 2009; Kavitha & Rao, 2008; Padmanabha *et al.,* 2015; Pavlov *et al.,* 1992). Though all pesticide treatments were significantly effective in overall feeding depression, the effect was more pronounced in chlorpyrifos and pesticide mixture treatments in both fish species, namely golden mahseer, and common carp. During pesticide exposure, significant feeding depression could be a strategy of fish to minimize the energetic cost for digestion (Halappa & David, 2009) and channel energy towards the pesticide detoxification processes. Moreover, the low or no feeding may be related to unpleasant pesticide smells and gustatory sensitivity of fish to the tested pesticides. Although not much known about this aspect, previous research on common carp reported that benthiocarb, isoprothiolane, and fenitrothion can influence nerve innervating terminal buds sensitivity in the lip region (Ishida & Kobayashi, 1995). Fish foraging depends on olfactory and gustatory sense and food intake is affected if these sense receptors are affected by chemicals (Olse´n, 2011). Tierney *et al.* (2010) also reported fish olfaction is essential for locating food and this ability is lost by exposure to contaminants like pesticides.

4.2.2.2. Opercular beat rate

The elevated opercular beat rate in golden mahseer and common carp in response to pesticides in this experiment corroborates the other findings (Saha *et al.,* 2016; Soni & Verma, 2018). Similar findings were observed in African catfish (Nwani *et al*., 2013) and tilapia (Padmanabha *et al.,* 2015) after exposure to chlorpyrifos-based pesticides. The increased opercular beat rate can be considered as a sign of stress in fish. Significant elevation in opercular beat rate in golden mahseer and insignificant elevation in common carp at 1 h of pesticides exposure reveals golden mahseer was under stress from the environmental change immediately, but not the common carp. Opercular motions are linked to respiratory movements, and the significant increase implies that acute treatment of pesticides alone and in combination can affect fish respiration processes.

4.2.2.3. Aerobic respiratory metabolism

Since fish gills are directly exposed to water, aquatic toxicants have a greater impact on respiratory processes (Padmanabha *et al.,* 2015). One of the most typical physiological reactions to toxicants is an increase in oxygen consumption, which is

simple to quantify and often employed in toxicity studies to assess metabolic alterations under stressful environmental conditions (Patil & David, 2008).

In this experiment, both increasing and decreasing trends of oxygen consumption was observed in golden mahseer but it was always an increasing trend in common carp. Pesticides have been shown to have variable effects on fish aerobic respiratory metabolism, with both increases and decreases in oxygen consumption rate recorded in various investigations (Barbieri *et al.,* 2018; Barbieri & Ferreira, 2011; Basha *et al.,* 1984; Jothinarendiran, 2012; Padmanabha *et al.,* 2015; Patil & David, 2008; Rao *et al.,* 1981; Tilak & Kumari, 2009).

In all pesticide treatments in both fish species, a high opercular beat rate was found; therefore, high oxygen uptake by fish is obvious with increased ventilation rate. But in golden mahseer, the oxygen uptake was suppressed in individual pesticide treatments despite their accelerated opercular movements. This situation clearly demonstrates that individual pesticides may have damaged fish gills, and excessive mucus formation may have increased diffusion distance, affecting oxygen consumption in those species. It is a well-established fact that the quicker the rate of metabolism, the faster harmful compounds are eliminated (Stepić *et al.,* 2013; Wang *et al.,* 2017). Faster removal of harmful compounds in mixed pesticide treatments compared to separate pesticide treatments may have been the other explanation for the antagonistic impact of joint pesticide in golden mahseer.

The same pesticides in individual treatments (chlorpyrifos and dichlorvos- 10% and 50%) suppressed oxygen uptake in golden mahseer but accelerated the uptake in common carp. Different toxicity mechanisms of the same pesticide on different fish species could explain such differences in respiration. To support this position, cabofuran-mediated respiratory toxicity in different species (Barbieri *et al.,* 2018; Campos-garcia *et al.,* 2015) can be compared. Despite the fact that both trials employed the identical carbofuran concentration (0.5 mg/L), there was no influence on the oxygen consumption rate in Nile tilapia (Campos-garcia *et al.,* 2015), however there was a substantial reduction in oxygen consumption in A*styanax ribeirae* (Barbieri *et al.,* 2018).

From the lethal toxicity experiment, it is concluded that chlorpyrifos and dichlorvos were antagonistic to golden mahseer and mrigal but synergistic to common carp in

combined exposure. Therefore, the sub-lethal effects of the pesticide mixture (chlorpyrifos and dichlorvos) on feeding behavior, opercular beat rate, and aerobic respiratory metabolism were also anticipated to be higher than individual pesticide treatments in common carp; however, the results of sub-lethal exposure was different than assumption. Fish treated with chlorpyrifos had the strongest feeding suppression, dichlorvos-treated fish had the greatest buccal movements, and fish treated with pesticide mixtures had the highest oxygen uptake. These results show that, despite their synergistic impact in lethal exposure, chlorpyrifos and dichlorvos were not additive/synergistic under the provided experimental circumstances at sub-lethal exposure. Imam *et al.* (2018) reported similar results in a mammalian model, finding that the combined impacts of chlorpyrifos and dichlorvos were not substantially higher than the impacts of the individual pesticides. They also documented that several responses in the combined treatment were even lower than the individual pesticide treatments.

4.2.3. Blood biochemical parameters

4.2.3.1. Glucose

Serum glucose, along with plasma cortisol, has been extensively researched as a biomarker of environmental stress (Banaee *et al.,* 2013; Bhatnagar *et al.,* 2017; Dogan & Can, 2011; Koul *et al.,* 2007; Medda, 1993; Ramesh & Saravanan, 2008; Saravanan *et al.,* 2011). In this study, pesticide exposure raised glucose levels in both fish species but this was only significant for dichlorvos in golden mahseer, and chlorpyrifos and pesticide mixture in common carp. Chlorpyrifos (Banaee *et al*., 2013; Hatami *et al.,* 2019; Ramesh & Saravanan, 2008) and lindane (Saravanan *et al*., 2011) exposure to common carp, chlorpyrifos exposure to mrigal (Bhatnagar *et al*., 2017), and dimethoate exposure to rainbow trout (Dogan & Can, 2011) have all been linked to increased glucose levels. Significant increase in blood glucose level is because of gluconeogenesis which provides energy for the elevated metabolic needs in response to stressors such as pesticides (Bhatnagar *et al.,* 2017; Ramesh & Saravanan, 2008; Saravanan *et al.,* 2011). Furthermore, in previous investigations, depletion of liver glycogen in response to pesticide exposure was associated with a rise in blood glucose levels, implying that increased glycogenolysis could be used to meet energy demands (Ezike *et al.,* 2017; Narra *et al.,* 2015). It is believed that the high glucose levels reported in this experiment were caused not only by increased

glycogenolysis but also by elevated gluconeogenesis. The reduction of triglycerides in the experiments also supports the speculation that it might have fueled glucose in blood circulation through gluconeogenesis.

4.2.3.2. Total protein

Protein is an important biochemical parameter used to know the health and metabolism status of an animal (Saravanan *et al.,* 2011). In the case of total protein, there was just the opposite observation in two fish species in this experiment. In golden mahseer, total protein was in decreasing trend but still insignificant indicating that pesticide exposure had no significant impact on protein metabolism. Similarly, there was no effect of chemical stress on the blood protein of common carp and rainbow trout (De Smet & Blust, 2001; Velisek *et al.,* 2006). The sub-lethal concentrations applied in this study may have been too low to have a substantial impact on protein catabolism of golden mahseer.

After 24 hours of exposure to chlorpyrifos, total protein levels in common carp increased substantially. Saravanan *et al.* (2011) found a similar result in sub-lethal lindane exposure in common carp. The possible reason for increased serum protein could be due to liver cell damage and the release of protein in circulation. Hepatocellular damage was also described as a reason for increased plasma protein in lindane-exposed common carp (Saravanan *et al.,* 2011). As an adaptive response to chemical stress, pesticide-exposed fish may have enhanced protein synthesis. Similarly, Remyla *et al.* (2008) argued that high plasma protein levels in freshwater fish under chemical stress were attributable to an accelerated rate of protein synthesis, which was a general adaptation strategy to bind the toxicant in the blood.

4.2.3.3. Albumin and globulin

Total protein is composed primarily of albumin and globulin. Serum albumin contents in two experimented fish species showed different trends. In golden mahseer, albumin showed a clear declining trend, however it was only significant in the mixed group. But, in common carp, albumin levels in chlorpyrifos and pesticide mixture treated fish were clearly rising. However, the trends for globulin content in both fish species were not distinct. In sub-lethal chlorpyrifos exposure, blood albumin and globulin levels decreased in mrigal (Bhatnagar *et al.,* 2017) and common carp (Banaee *et al.,* 2013). But in rainbow trout, insignificant effects on these proteins were documented

(Velisek *et al.,* 2006). Albumin followed a similar pattern to that seen in total protein but the pattern was not followed by the globulin. This indicates that albumin, one of the main constituents of protein, is mostly influenced by pesticide exposure but globulin remains more stable. Albumin is synthesized by the liver, while globulins are synthesized by the immune system and the liver. As a result, the shift in albumin in this study could be linked to liver damage, also supported by high AST, ALT, and ALP in the experimental animal. Impaired liver function in golden mahseer resulted in insufficient albumin production whereas high albumin content in common carp could be due to hepatic cell damage and increased albumin synthesis as an adaptive syndrome against stress.

4.2.3.4. Triglyceride

The decreasing trend in triglyceride levels in pesticide-exposed fish, with a significant impact in the mixture group in golden mahseer and chlorpyrifos, and the mixture group in common carp, could be another indication for the switch to a different metabolic pathway (gluconeogenesis) that is activated to provide energy during these exposures. Hatami *et al.* (2019) found lower triglyceride levels in chlorpyrifos-exposed fish, which is consistent with this finding.

4.2.3.5. Urea and creatinine

Blood urea and creatinine levels are commonly used as markers of kidney function. Creatinine is an anhydride of creatine found in muscles, while urea is the main end product of protein metabolism (Jyothi & Narayan, 2000). Creatinine production is more consistent than that of any other excretory product, making it a more trustworthy and effective biomarker for evaluating kidney function (Jyothi & Narayan, 2000). The elevating trend of the urea level was significant only in the mixture group in golden mahseer but in all pesticide treatments in common carp. Furthermore, the rising trend in creatinine found in this study agrees with observations in singi (Shaikh & Gautum, 2014) and common carp (Jaffer *et al.,* 2017) subjected to dichlorvos and chlorpyrifos, respectively. When the kidneys are unable to function effectively, excessive levels of urea and creatinine build up in the blood, as seen in golden mahseer and common carp during this study. The supposition is further complemented by histopathological alterations in fish kidney under chemical stress (Shirdel *et al.,* 2020*;* Xing *et al.,* 2012c). The kidney was mainly affected by

the pesticide mixture in golden mahseer but it was affected by all pesticide treatments (chlorpyrifos, dichlorvos, and mixture) in common carp. The influence of the pesticide mixture on renal function of golden mahseer can also be illustrated by the significantly elevated AST level by the pesticide mixture but not by individual pesticides treatments. AST is not a liver specific enzyme (Ghelichpour *et al.,* 2017); and substantial AST activity was found in the kidney of common carp (De Smet & Blust, 2001) which also supports this notion.

4.2.3.6. Serum enzymes (AST, ALT, and ALP)

The AST, ALT, and ALP enzyme profiles were examined to assess the liver health of animals. Pesticide exposure increased the activity of these enzymes in both tested species- golden mahseer and common carp. Other fish species also showed increased blood AST, ALT, and ALP activities under pesticide treatments (Banaee *et al.,* 2013; Ghaffar *et al.,* 2015; Jaffer *et al.,* 2017; Koul *et al.,* 2007; Medda, 1993). The elevated levels of these enzymes found in the fish could be attributed to liver cell injury, as previously documented in many investigations (Banaee *et al.,* 2013; Deka & Mahanta, 2015; Ghelichpour *et al.,* 2017; Jaffer *et al.,* 2017). The liver tissue of nonylphenol-exposed fish was shown to have histological lesions (Shirdel *et al.,* 2020).

ALT is a liver-specific enzyme, and a spike in ALP activity occurs when there are hepatobiliary issues (Ghelichpour *et al.,* 2017). In golden mahseer, significantly high ALT and ALP were noted in chlorpyrifos and dichlorvos whereas it was not significant in the joint treatment, indicating that liver cells were more susceptible to the single pesticides (chlorpyrifos and dichlorvos) than the pesticide mixture. In common carp, liver-specific enzymes ALT and ALP were elevated only in chlorpyrifos and pesticide mixture treatments but not in the dichlorvos treatment, which shows destructive effects of these pesticides (chlorpyrifos and mixture) in common carp liver.

The time series-wise data analysis shows that the fish which were initially stressed by pesticide exposure tend to adapt to the changing environmental condition with the progression of the exposure period. Triglycerides, which are used as an alternate source of energy by fish during stress, become more prominent at 96 h of exposure. The highest impact on renal function of golden mahseer occurs after 96 h of exposure

in the pesticide mixture group. Blood glucose, total protein, albumin, globulin, and triglycerides were the lowest for the entire trial period at that time and pesticide treatment, although only the triglyceride reduction was significant. Despite the fact that the stress indicator glucose was expected to be higher under pesticide treatment throughout the exposure, it remained lower than the control at this point. This group also had the highest levels of blood urea and AST. This obviously suggests that the fish kidneys were considerably affected at that particular sampling interval (96 h). The loss of most of the observed biochemical markers could have been owing to the damaged kidneys, whilst the highest urea level was possibly caused by the compromised filtration capacity of the kidney. Similarly, renal cell damage was most likely the cause of the increased blood AST.

In common carp, the scenario was slightly different, where all pesticides treatments (chlorpyrifos, dichlorvos, and mixture) enhanced urea levels from early exposure hour and a similar trend was followed by serum AST. This indicates that, unlike golden mahseer, the functional status of the kidney was affected by all pesticide treatments from 24 h onwards and continued until 96 h.

The effect of pesticide on the fish liver was pronounced only at 96 h in golden mahseer but in common carp, it was noticed both at 24 h and 96 h. The results showed that the vital organs (kidney and liver) were affected in golden mahseer at the later phase of the pesticide exposure but in common carp, these organs were affected from the beginning of the pesticide exposure. During a one-week depuration interval, most of the deviating biochemical measures stabilized, indicating that pesticide effects were being recovered. This signifies that pesticide detoxifying enzymes became active in the tested organisms resulting pesticide removal from the body. Furthermore,the stimulation of the detoxifying enzyme and depuration were documented to be proportionate to the toxicant concentrations (Ikpesu, 2013). Adhikari *et al.* (2004) also reported recovery of blood parameters in fish when they were kept in pesticide free water.

In this study, fish showed metabolic modification even at sub-lethal pesticide exposures. This allows us to speculate on the potential impact of such chemicals at higher concentrations, which could result in mass fish mortality in nature (Polidoro & Morra, 2016; Sabra & Mehana, 2015).

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

The lethal toxicity study on fish suggests that organophosphate pesticide chlorpyrifos is more deleterious than dichlorvos. Among the tested freshwater fish species, mrigal was the most sensitive species to both chlorpyrifos and dichlorvos pesticides. This study is probably the first to report the joint action of chlorpyrifos and dichlorvos in fish. These pesticides in joint action showed antagonistic effects in golden mahseer and mrigal whereas the effect was synergistic in common carp. This suggests that pesticides having the same mode of action are not necessarily synergistic and their action can be species-specific.

Organophosphate pesticides at the sub-lethal levels are not fatal but they are still stressful to fish leading to abnormal behavior, feeding depression, and respiratory distress which may cause serious health hazards. This is definitely not an ideal environmental condition for a prosperous aquatic life. Therefore, sub-lethal effects of organophosphate pesticides can be used as a non-invasive technique for water quality evaluation and animals' health assessment. In spite of the synergistic or antagonistic action demonstrated by the fish in lethal toxicity test, the effects were not similar in sub-lethal toxicity assessments.

Sub-lethal effects of organophosphate pesticides were also noticeable at the biochemical level. The examined blood biochemical parameters clearly showed that the fish were definitely stressed and the energy metabolism was disrupted. The vital organs (liver and kidney) of fish were also affected by pesticides exposures. In golden mahseer, the renal function was compromised by the mixture of pesticides and hepatic function by individual pesticide (chlorpyrifos and dichlorvos) treatments. In common carp, all pesticide treatments (chlorpyrifos, dichlorvos, and mixture) were equally effective for kidney dysfunction and liver function was affected by chlorpyrifos and pesticide mixture treatments. Such effects were prominent only at the later exposure phase (96 h) in golden mahseer but it was distinct from the beginning (24 h) of exposure in common carp. Most of the biochemical parameters were stabilized after one week of the depuration period, which shows recovery signs from deleterious effects of pesticides. Any divergence in biochemical parameters

indicates a disturbance in the animal's homeostasis, which could lead to health problems.

This study revealed important information on the effects of single and pesticide mixture exposures in 3 fish species: golden mahseer (*Tor putitora*), common carp (*Cyprinus carpio*), and mrigal (*Cirrhinus mrigala*). Nevertheless, further research is needed to fully understand the mode of action of these chemicals. Therefore, the recommendations made by the study are as follows:

- To strengthen the biological principle responsible for antagonistic or synergistic effects of chlorpyrifos and dichlorvos toxicity, estimation of key enzymes-GST, AChE, CaEs, and cytochrome P450 are required.
- Kidney and liver function tests coupled with histopathological analyses are recommended for a clear understanding of pesticide toxicity.
- Pesticide action in discrete exposures or in combined mixtures can be different; therefore, greater caution is required while predicting pesticide exposure risks, and setting water quality standards and guidelines. When the combined toxicity of pesticides is taken into account, environmental risk assessment becomes more realistic.
- To protect precocious fish diversity from extinction, toxic pesticide applications must be closely regulated, and the use of bio-pesticides and integrated pest management programs should be promoted.

CHAPTER 6

6. SUMMARY

Pesticides are hazardous chemicals that are employed to manage crop-damaging pests and disease-carrying vectors in agriculture, households, and public health. The application of pesticides is a traditional practice which is also mentioned in Rigveda, a Hindu religious epic. In Nepal, pesticides were imported in the 1950s to control malaria. The development and synthesis of chemical pesticides during the 20th century intensified their application globally. Pesticides can be classified broadly into four main groups- organochlorines, organophosphates, carbamates, and pyrethroids. Organophosphates are the most commonly used pesticide worldwide including in Nepal. They are broad-spectrum pesticides that lack target specificity, hence non-target organisms are also affected by such chemicals. The mode of action of organophosphate is acetylcholinesterase (AChE) inhibition, hence called anticholinesterases. Chlorpyrifos and dichlorvos, organophosphate pesticide group, are widely applied in agriculture and aquaculture.

The majorities of the pesticides used are discharged into the environment, eventually ending up in the aquatic system and poses a serious threat to aquatic biodiversity. Detection of chlorpyrifos and dichlorvos pesticides in aquatic systems makes them priority compounds for research. The global fisheries sector, a huge industry for food production as well as income and employment generation, is threatened by pesticide pollution. Therefore, assessment of pesticide toxicity on fish is essential as they are also important indicator organisms for water quality evaluation. Three fish species (golden mahseer, common carp, and mrigal) were selected for this toxicity research because they are economically important key fish species of Nepal.

Most of the toxicity studies are based on a single pesticide assessment, but in reality, pesticides are generally present in mixture form in nature, therefore single pesticide risk assessments can be insufficient or even misleading. Therefore toxicity assessment study was conducted not only individually but also in mixture. There was no information on the joint toxicity of chlorpyrifos and dichlorvos on fish.

The major objective of this study was to investigate the organophosphate toxicity to economically important fish species (golden mahseer, common carp, and mrigal) of Nepal with the following specific objectives:

- To estimate the lethal toxicity of chlorpyrifos, dichlorvos, and their mixture and assess general fish behavior against pesticide treatments.
- To quantify the effects of chlorpyrifos, dichlorvos, and their mixture on specific behavioral responses and aerobic respiratory metabolism of fish
- To examine the blood biochemical parameters of fish exposed to chlorpyrifos, dichlorvos, and their mixture.

To achieve these objectives, the laboratory experiments were conducted at the Central Fisheries Promotion and Conservation Centre, Balaju, Kathmandu, Nepal. Three economically important freshwater fish species- golden mahseer, common carp, and mrigal were selected for the toxicity research. The pesticides used in this study were commercial-grade pesticides- Dursban (chlorpyrifos) manufactured by Dow Agro Sciences Pvt. Ltd., India and G-VAN (dichlorvos) manufactured by Greenriver Industry Co., Ltd., ShenZhen, China.

The experiments were conducted in three phases. In the first phase, lethal pesticide exposure was done following acute toxicity testing guidelines to estimate the toxicity of the pesticides to the tested organisms. During this exposure, general fish behavior was assessed simultaneously. In the second phase, fish were exposed to two sub-lethal doses (10% and 50% of the 96 h-LC_{50}) of chlorpyrifos and dichlorvos and their mixture in equal proportion, and their feeding behavior, opercular beat rate, and aerobic respiratory metabolism were quantified. Feeding attempts and opercular movements were counted using a hand tally counter whereas the oxygen concentration in aquaria water was measured using dissolved oxygen meter. The third phase of the experiment was designed to examine blood biochemical parameters. For this, fish were exposed to 10% 96 h-LC_{50} of the respective pesticide and their mixture in equal proportion. The pesticide exposed fish were placed in clean water for one week depuration. Blood samples were collected at 24 h and 96 h of exposure followed by one week recovery. Standard techniques were used to examine blood biochemical parameters such as glucose, total protein, albumin, globulin, triglyceride, urea, creatinine, AST, ALT, and ALP.

The results showed 96 h-LC_{50} of chlorpyrifos in golden mahseer, common carp and mrigal were 0.753 (0.616-0.931), 0.440 (0.373-0.504) and 0.380 (0.319-0.450) mg/L, respectively. Similarly, the 96 h-LC_{50} of dichlorvos to golden mahseer, common carp,

and mrigal were calculated to be 12.964 (10.866-15.515), 15.705 (14.385-16.963), and 11.367 (9.496-13.536) mg/L. The lethal toxicity range of chlorpyrifos and dichlorvos found in the experiments corroborate findings in fish elsewhere. The findings revealed that among the three tested species, mrigal was the most sensitive, and golden mahseer were the most resistant species in terms of chlorpyrifos toxicity. But for dichlorvos, mrigal was the most sensitive, and common carp was the most resistant species. According to the World Health Organization chlorpyrifos is a moderately hazardous pesticide (class II) and dichlorvos is a highly hazardous pesticide (class Ib). But, the results obtained from this study showed chlorpyrifos pesticides were more toxic than dichlorvos. Such a difference in pesticide toxicity is because of the difference in test species. The World Health Organization provided pesticide classification based on toxicity to the rats whereas in this study test species were aquatic animals.

The joint actions of chlorpyrifos and dichlorvos, by the end of the experiment, were antagonistic in golden mahseer where additive index (AI) value ranged from -0.937 to -0.302, but it was synergistic in common carp with AI values after 96 h varying from 0.132 to 0.989. In mrigal, there was a mixture of synergistic and antagonistic action but mostly dominated by antagonistic effects where the AI value was calculated to be 0.37 to -1.10 by the end of the experiment. The antagonistic effects could be linked to elevated carboxylesterases (CaEs) and glutathione-S-transferase (GST) enzymes under the joint action of chlorpyrifos and dichlorvos. These enzymes are supposed to play a protective role against pesticides. The synergistic action of chlorpyrifos and dichlorvos in this study could be attributed to the suppression of these protective enzymes (CaEs and GST) in a binary mixture in common carp. In addition, the cytochrome P450 enzyme activity might have enhanced following exposure to the pesticide mixture. This enzyme is known to accelerate the conversion of organophosphate to a more toxic metabolite with a strong AChE inhibitory effect.

Important behavioral changes observed in pesticide exposed fish might be due to inhibition of AChE. Excess mucus production may be a first line of defense for fish to avoid contact with the harmful chemical. Fish gulping air in reaction to pesticide exposure might be a way for them to adjust for respiratory stress. Feeding depression was observed in pesticide exposed fish. The reduction or complete cessation of food intake in relation to pesticide exposure is a common stress response in fish.

Significant reduction of feeding during pesticide exposure could be a strategy to reduce the metabolic expense for digestion and to channel energy for the pesticide detoxification processes. Furthermore, reduced feeding may be connected to unpleasant chemical odors and fish gustatory sensitivity to the tested pesticides.

The opercular beat rate of the fish was elevated by pesticide treatments. The increased opercular beat rate can be considered as a sign of stress in fish. Opercular motions are linked to respiratory movements, and the significant increase shows that acute exposure to both pesticides individually and in combination can affect respiration processes. High oxygen uptake by fish is obvious with increased ventilation rate. But in golden mahseer, the oxygen uptake was suppressed in individual pesticide treatments despite their accelerated opercular movements. This certainly suggests that fish gills may have been injured, and that excessive mucus deposition may have increased diffusion distance, thus leaving oxygen consumption in those fish groups at risk.

In pesticide exposed fish, the biochemical parameters of blood deviated. It is speculated that increased glycogenolysis together with gluconeogenesis caused the high glucose levels in the experimental animals. The significant reduction of triglyceride levels in pesticide exposed fish could be another sign of a switch to a different metabolic process (gluconeogenesis) that is triggered to generate energy during these exposures. In golden mahseer, the total protein metabolism was not severely influenced by pesticide exposure. But, in common carp, total protein levels were in increasing trend with significant effects in some treatments. The possible reason for increased serum protein could be due to liver cell damage and the release of protein in circulation. Pesticide exposed fish might have also increased protein synthesis as an adaptive syndrome against chemical stress. Albumin and globulin are the main constituents of total protein. Albumin followed a similar trend as observed in total protein but the trend was not followed by the globulin. This indicates that albumin, one of the main constituents of protein, is mostly influenced by pesticide exposure but globulin remains more stable. The liver produces albumin, while the liver and the immune system both make globulins. Therefore, the change in albumin in this study could be linked solely to liver dysfunction. The levels of blood urea and creatinine are typical indicators of renal function. When the kidneys don't work properly, substantial levels of urea and creatinine build up in the blood which might

have happened in the tested organisms. The kidney was mainly affected by the pesticide mixture in golden mahseer but it was affected by all pesticide treatments (chlorpyrifos, dichlorvos, and mixture) in common carp. To assess the liver health, the enzyme profiles of AST, ALT, and ALP were examined. After pesticide exposure, the activity of these enzymes increased. Hepatic cell injury may be the reason for the elevated levels of these enzymes found in the fish. The hepatic cells were more susceptible to the individual pesticides (chlorpyrifos and dichlorvos) in golden mahseer, but in common carp, they were more sensitive to chlorpyrifos and pesticide mixtures.

The time series-wise data analysis shows that the fish which were initially stressed by pesticide exposure tended to adapt to the changing environmental condition with the progression of the exposure period. The vital organs (kidney and liver) were affected in golden mahseer at a later phase of the pesticide exposure but in common carp, these organs were affected from the beginning of the pesticide exposure. The majority of the deviated biochemical indicators tended to be resumed during a one week freshwater treatment.

In conclusion, the lethal toxicity study on fish suggests that chlorpyrifos is more deleterious than dichlorvos. The pesticides having the same mode of action are not necessarily synergistic and their action can be species-specific. Organophosphate pesticides at the sub-lethal levels are stressful to fish leading to abnormal behavior, feeding depression, and respiratory distress. The vital organs (liver and kidney) of fish are also affected by sub-lethal pesticides exposures. Most of the biochemical parameters stabilized after one week of depuration period, which shows recovery signs from deleterious effects of pesticides.

To strengthen the biological principle responsible for antagonistic or synergistic effects of chlorpyrifos and dichlorvos toxicity, estimation of key enzymes and histopathological analysis are recommended. The application of toxic pesticides must be monitored. It is also suggested considering joint toxicity assessments while anticipating pesticide exposure threats, developing water quality standards and guidelines. The government should also enhance pesticide awareness programs and effectively implement an integrated pest management approach.

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APPENDIX- SCIENTIFIC PUBLICATIONS

Paper 1

Kunwar, P. S., Parajuli, K., Badu, S., Sapkota, B., Sinha, A. K., De Boeck, G., & Sapkota, K. (2021). Mixed toxicity of chlorpyrifos and dichlorvos show antagonistic effects in the endangered fish species golden mahseer (*Tor putitora*). *Comparative Biochemistry and Physiology Part- C, Toxicology and Pharmacology*, **240**(108923): 1–9. https://doi.org/10.1016/j.cbpc.2020.108923

Paper 2

Kunwar, P. S., Basaula, R., Sinha, A. K., De Boeck, G., & Sapkota, K. (2021). Joint toxicity assessment reveals synergistic effect of chlorpyrifos and dichlorvos to common carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology Part- C, Toxicology and Pharmacology*, **246**(108975): 1–10. https://doi.org/10.1016/j.cbpc.2021.108975

Paper 3

Kunwar, P. S., Sapkota, B., Badu, S., Parajuli, K., Sinha, A. K., De Boeck, G., & Sapkota, K. (2022). Chlorpyrifos and Dichlorvos in Combined Exposure Reveals Antagonistic Interaction to Freshwater Fish Mrigal, *Cirrhinus mrigala*. *Ecotoxicology*, **31**(4): 657-666. https://doi.org/10.1007/s10646-022-02534-6

Paper 4

Kunwar, P. S., Sinha, A. K., De Boeck, G., & Sapkota, K. (2022). Modulations of blood biochemical parameters of golden mahseer, *Tor putitora* following exposures to single and mixed organophosphate. *Comparative Biochemistry and Physiology Part- C, Toxicology and Pharmacology*, **251**(109207): 1–8. https://doi.org/10.1016/j.cbpc.2021.109207

Manuscript 5

Kunwar, P. S., Sinha, A. K., De Boeck, G., & Sapkota, K. Organophosphate mediated physiological distress compromises fish health and production in common carp, *Cyprinus carpio*.

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Mixed toxicity of chlorpyrifos and dichlorvos show antagonistic effects in the endangered fish species golden mahseer (*Tor putitora*)

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ABSTRACT

Golden mahseer (*Tor putitora*) is an economically important but endangered fish species in many countries. Increasing pesticide application can possess a threat to this species but their sensitivity to pesticides, typically chlorpyrifos and dichlorvos, is unknown. We determined 96 h-LC_{50} of chlorpyrifos and dichlorvos to be 0.753 mg/L and 12.964 mg/L, respectively, indicating higher toxicity of chlorpyrifos than dichlorvos. Despite the same mode of action, their joint effect was antagonistic, with an additive index value of - 0.58 at 96 h-LC_{50}. Moreover, to get insights in the temporal sub-lethal effects, fish were exposed to 10% and 50% of the 96 h-LC_{50} values of the respective pesticides. Aerobic metabolism, opercular movements, and feeding behavior were examined for sub-lethal end-points following 24 h, 48 h, 72 h and 96 h exposure. Both chlorpyrifos and dichlorvos in single exposures induced a significant drop in oxygen consumption rate; while it was significantly elevated in the mixed pesticide exposure. Accelerated opercular movements were observed in all pesticide treatment groups but were more persistent in chlorpyrifos treatments. Reduced feeding attempts were more pronounced in chlorpyrifos and mixture treatments wherein feeding attempts dropped to zero. Overall, the acute toxicity data reported in the present study can be used to assess the maximum tolerance level of golden mahseer to chlorpyrifos and dichlorvos, and their mixture. Furthermore, the sub-lethal end point responses can be applied in monitoring the environmental risk posed by these waterborne pesticides either individually or in combination to the aquatic life.

1. Introduction

Pesticides are substances used in agriculture and in the public health sector to control crop damaging pests and disease causing vectors. Application of pesticides is increasing worldwide and during the period of 1996–2016, the global pesticide use has increased by 46% (WHO and FAO, 2019). Plant Quarantine and Pesticides Management Centre, Nepal (2019) has also reported increasing trend of pesticide use within the country. With the increasing human population, pressure on agriculture is obvious which compels to use more and more pesticides to enhance production per unit area. These pesticides not only contaminate soils but also the aquatic environment, mainly through agricultural runoff and irrigation waters (Wang et al., 2013). This threatens aquatic life thriving there, including fish. Pesticides can enter the fish's body through different routes i.e. dermal absorption, across the respiratory surface (gills) or via food ingestion (MacKay and Fraser, 2000) and interfere with normal functioning of the organism. Such deleterious effects of pesticides on fish have already been documented almost four decades ago by Rath and Misra (1981).

Organophosphate compounds are the most widely used pesticides in agriculture and aquaculture (Rodrigues et al., 2001). Chlorpyrifos and dichlorvos belong to the organophosphate group, and are extensively used pest control products in most parts of the world and in Asia (Sun et al., 2015). Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a synthetic, non-systemic, wide-spectrum pesticide. Its commercial production started in 1969, and since then it is being used for various purposes (Halappa and David, 2009). This is also one of the most common pesticides in aquatic systems and its residues

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have been detected in water (Nag et al., 2020; Singh et al., 2015), sediments (Singh et al., 2015) and both wild (Akoto et al., 2016; Nag et al., 2020; Singh et al., 2015) and cultured fish (Sun and Chen, 2008). Several studies had explored chlorpyrifos mediated behavioral changes in common carp (*Cyprinus carpio*; Halappa and David, 2009; Xing et al., 2015), African catfish (*Clarias gariepinus*; Nwani et al., 2013) and tilapia (*Oreochromis mossambicus*; Padmanabha et al., 2015). Among others, impaired feeding and respiration rate were observed in tilapia (Padmanabha et al., 2015). Chlorpyrifos had also been documented to disrupt the haematological and biochemical parameters of common carp (Ramesh and Saravanan, 2008), African catfish (Nwani et al., 2013), Asian catfish (*Clarias batrachus*; Narra et al., 2015) and mrigal (*Cirrhinus mrigala*; Bhatnagar et al., 2017). It is also immunotoxic to common carp (Xing et al., 2015) and Nile tilapia (*Oreochromis niloticus*; Zahran et al., 2018).

Dichlorvos (2, 2-dichlorovinyl dimethyl phosphate) is another extensively used organophosphate pesticide. Their residues in soil (Kafle et al., 2015) and water samples (Nag et al., 2020) have also been reported. It is extremely toxic to non-target organisms like fish (Das, 2013). Rath and Misra (1981) reported inhibitory effect of dichlorvos on Acetylcholinesterase (AChE) activity in Mozambique tilapia (*Tilapia mossambica*). Adverse effects of dichlorvos on behavior and haematological changes in rohu (*Labeo rohita*; Kesharwani et al., 2018), behavioral changes of guppy (*Poecilia reticulate*; Gunde and Yerli, 2012) and changes in energy metabolism of zebrafish (*Danio rerio*; Bui-Nguyen et al., 2015) were also documented. Similarly, there is also the evidence that exposure to dichlorvos can alter oxygen consumption in grass carp (*Ctenopharyngodon idella*; Tilak and Kumari, 2009), and cause histopathological changes in mrigal (Velmurugan et al., 2009) and rohu (Kesharwani et al., 2018). Several studies performed on common carp reported adverse effect of dichlorvos on metabolism (Demael et al., 1990), immune response (Dunier et al., 1991), behavior (Gunde and Yerli, 2012; Ural and Çalta, 2005), food consumption and ammonia excretion rate (Laxmi et al., 2019).

Despite extensive studies done worldwide on the effect of chlorpyrifos and dichlorvos in a number of fish species; no study, to the best of our knowledge, has been done to elucidate the toxicity of these two pesticides on golden mahseer (*Tor putitora*). It is a widely distributed fish species in south and Southeast Asia. However, it is under serious anthropogenic threats including pollution from both urban and agro-based sources. It is estimated that populations of this species have declined by more than 50% in the past 21 years (Jha et al., 2018). Golden mahseer is considered as a potential candidate for cultivation as well as for sports fisheries (Ingram et al., 2005). This species is also listed as endangered species by the International Union for Conservation of Nature (IUCN). Therefore, conservation and protection of this species from deleterious compounds such as pesticides is vital.

In the aquatic environments pesticides are accumulated from different sources and catchment areas, therefore pesticides are always present in mixture form. Toxicity also depends on whether the pesticides act individually or in combination. Synergistic effects of pesticides to zebrafish have been reported (Wang et al., 2017; Wu et al., 2018). Another study on common carp reported synergistic effect when triazophos was combined with malathion or carbofuran but an antagonistic effect was found when fenobucarb was used in combination with triazophos or malathion (Wang et al., 2015). Similar mixed results, both synergistic and slightly antagonistic, were obtained when common carp were exposed to a mixture of chlorpyrifos with other pesticides (Chen et al., 2014). Though different combinations of pesticide toxicity to fish have been assessed, information on joint effect of chlorpyrifos and dichlorvos is still scarce.

To fill this knowledge gap, this study was designed to evaluate toxicity of chlorpyrifos and dichlorvos not only individually but also in combination to golden mahseer. Acute toxicity of these pesticides as well as their sub-lethal effects on general fish behavior, aerobic energy metabolism, opercular movements and feeding attempts were explored. Findings of this study will offer baseline information for environmental protection authorities to formulate water quality guidelines of these two pesticides and assessing their risk for the welfare of golden mahseer. In terms of unexplored species and pesticides selection for mixed toxicity, we expect this study to be a significant contribution in the arena of aquatic toxicity.

2. Materials and methods

2.1. Experimental animal

Healthy golden mahseer juveniles (Cypriniformes, Cyprinidae; 3.5–5.0 g; exact weight mentioned in respective test sections) were purchased from Fisheries Research Centre, Pokhara and brought to Central Fisheries Promotion and Conservation Centre, Balaju, Kathmandu, Nepal. These fish were stocked in 3 nylon net cages installed in an earthen pond for one week. The required number of fish for the phase-wise experiment was transferred from these cages to 350 L indoor glass aquaria, where they were acclimatized at least for 2 weeks before commencing the experiment. Fish were fed ad libitum with commercial feed, 32% protein (Sreema feed Pvt. Ltd., India) and water quality of the aquarium was maintained by cleaning and exchanging water on daily basis. Water temperate, pH, dissolved oxygen, total ammonia, hardness, Na^+, K^+ and Cl^- were 24.6–25.4 °C, 7.6–7.8, 5.72–6.56 mg/L, 0.18–0.22 mg/L, 50–55 mg $CaCO_3$/L, 1.1–1.3 mmol/L, 0.07–0.1 mmol/L and 0.4–0.6 mmol/L respectively. This research was approved by the ethical review board of Nepal Health Research Council (Ref. No. 1215), Government of Nepal.

2.2. Pesticides tested

We selected commercial pesticides that are actually being used in the field, and are available under different trade names. For dichlorvos, G-VAN (80%; Greenriver Industry Co., Ltd., ShenZhen, China) and for chlorpyrifos, Dursban (20%; Dow Agro Sciences Pvt. Ltd., India) was used in the present work.

2.3. Acute lethal toxicity test

Acute toxicity tests were conducted as per OECD 203 testing guidelines (OECD, 1992). This test was performed in 35 L glass aquaria in semi static conditions. The average weight of the fish used in this experiment was 4.6 ± 0.9 g (mean ± SD). Dissolved oxygen of water always remained above 5.5 mg/L, temperature ranged between 24.76 and 25.2 °C, and pH between 7.6 and 7.8. Feeding was suspended 24 h prior to the experiment. Freshly prepared stock solution in distilled water was used to prepare different concentrations of pesticide. For determination of the LC_{50} values, following a range finding test, fish were exposed to five different concentrations of dichlorvos and chlorpyrifos in geometric series. For dichlorvos, concentrations of 2, 4, 8, 16, and 32 mg/L were used; whereas 0.2, 0.4, 0.8, 1.6 and 3.2 mg/L were tested for chlorpyrifos. All exposure groups and controls were conducted in triplicate. Feces and other waste residue were removed daily, and consequently 25–30% of the water in the aquaria was replaced with water containing the respective amount of pesticides. Fish mortality was observed regularly and all dead fish were immediately removed from the aquaria. Mortality was recorded after 3 h, 12 h, 24 h, 48 h, 72 h and 96 h of exposure. No fish mortality was observed in control. After determination of 96 h median lethal concentrations (96 h-LC_{50}) of both chlorpyrifos and dichlorvos, lethal toxicity of their mixture was evaluated. For this, 5 different concentrations of mixed pesticides were prepared by mixing them in equal proportion i.e. 12.5%, 25%, 50%, 100% and 200% of the 96 h-LC_{50} values of both pesticides and mortality was recorded as mentioned before. Toxicity of chlorpyrifos, dichlorvos, and their mixture were calculated (by using a log probit analysis program- SPSS ver. 20) and presented as 24 h to 96 h-LC_{10} - LC_{90} with 95% confidence

limit where, LC_{10} indicates 10% mortality and LC_{90} indicates 90% mortality of fish at given concentrations.

Joint toxicity of the pesticides was evaluated based on the additive index (AI) which was calculated according to Marking (1985).

$$AI = (1/S) - 1 \text{ for } S \leq 1 \text{ and,}$$

$$AI = 1 - S \text{ for } S > 1$$

where, AI represents additive index and S represents sum of biological activity

$$S = (A_m/A_i) + (B_m/B_i)$$

where, A and B represents two different pesticides, 'm' represents LC_{50} of pesticides in mixture, 'i' represents LC_{50} of individual pesticides. The AI value- less, equal or greater than zero indicates antagonistic, additive or synergistic action, respectively.

During acute toxicity assessment, general fish behavior like body movements, color change, swimming pattern and schooling behavior were also observed and recorded simultaneously after 1 h, 24 h, 48 h, 72 h and 96 h of pesticides exposure. Behavior examination was started with five concentrations of each pesticide (listed above) but due to complete mortality of fish in higher concentration before accomplishing the study, their behavior data could not be included in our results.

2.4. Sub-lethal exposures

Oxygen consumption was measured in 35 L rectangular glass aquaria (water volume set to 25 L) with airtight glass lids. Each aquarium was also equipped with a screened water pump to create a water velocity (~ 5–10 cm/s). The pumps were installed on the short side of the aquaria which prompted the fish to swim gently against the water current. Fish were exposed to 10% and 50% of the 96 h-LC_{50} values of the respective pesticides individually and in mixture for a period of 24 h, 48 h, 72 h and 96 h. The average weight of the fish used in this experiment was 4.5 ± 0.8 g. The test was conducted in five replicates with one fish in each aquarium. After each pesticide exposure period, initial oxygen concentration (mg/L) in water was measured using a Milwaukee MW600 PRO Portable Dissolved Oxygen meter, and then aquaria were made air tight by sealing the aquarium with glass lids and duct tape, and the air-stone were removed. The fish were left overnight and next day final dissolve oxygen concentration was measured. Thereafter, water was aerated for at least 3 h before commencing for the subsequent exposure trail, and same procedure was followed till the end of the experimentation.

Oxygen consumption rate (mg/g/h) was measured using following formulae

$$(\Delta O_2 i - O_2 f) \times V \times (1/BW) \times (1/T)$$

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

The experimental setup and fish used for counting opercular movements, feeding attempts and oxygen consumption were the same. Opercular movements of fish were counted for 5 min after 1 h, 24 h, 48 h, 72 h and 96 h of pesticide exposure. There were five replicates for each test solution and each fish was counted three times to use an average value for more accuracy. After counting the opercular movements, fish were left undisturbed for 2 h and then feeding attempts by fish were counted for 5 min by offering 20 small floating feed pellets (1.5 mm). All counting was done manually with the help of hand tally counter. At the end of the experiment, uneaten food was completely removed by scoop net.

2.5. Statistical analysis

Normality of the data was assessed by Shapiro-Wilk test, and homogeneity of variance was tested using Levene's test. Mean difference among various treatment groups was analyzed by one-way ANOVA. In case of significant difference, Tukey-HSD post Hoc tests was conducted. Whenever data did not satisfy the assumptions for parametric test, non-parametric Kruskal-Wallis with multiple pair comparison was performed. All analyses were done using statistical program SPSS version 20.

3. Results

3.1. Acute toxicity

3.1.1. Chlorpyrifos toxicity

As illustrated in Section 2.3, the acute toxicity test of golden mahseer to chlorpyrifos was conducted in five different concentrations. During the entire exposure period no fish mortality was observed at 0.2 mg/L. Mortality of fish at 0.4 mg/L and 0.8 mg/L was observed only starting at 72 h and 48 h of exposure, respectively. But at 1.6 mg/L and 3.2 mg/L fish mortality was already observed within 3 h of exposure. 100% of fish died at higher concentrations, within 72 h and 24 h at 1.6 mg/L and 3.2 mg/L, respectively. The median lethal concentrations (LC_{50}) with 95% confidence limit at 24 h, 48 h, 72 h and 96 h were 1.298 (0.945–1.471), 1.085 (0.938–1.270), 0.858 (0.715–1.049) and 0.753 (0.616–0.931) mg/L, respectively showing a decreasing trend of lethal pesticide concentration with increasing time of exposure (Table 1).

3.1.2. Dichlorvos toxicity

No fish mortalities were recorded at the lower concentrations of 2 mg/L and 4 mg/L dichlorvos during the 96 h exposure period. Mortality was only visible from 8 mg/L and above. During the entire 96 h experimental period only 10% of the fish died at 8 mg/L while 70% of fish died at 16 mg/L and 100% of fish were found dead at the highest concentration of 32 mg/L. The median lethal concentrations (LC_{50}) of dichlorvos to golden mahseer with 95% confidence limit were calculated to be 36.501 (28.410–90.712), 21.758 (18.459–25.727), 17.485 (14.829–21.029) and 12.964 (10.866–15.515) mg/L at 24 h, 48 h, 72 h and 96 h, respectively showing a decreasing trend of lethal pesticide concentration with increasing time of exposure (Table 2).

3.1.3. Mixture toxicity

No fish mortality was observed in 12.5% and 25% pesticide mixture whereas only 5% fish died in 50% mixture. In 100% mixture 80% fish had died by the end of the experiment but in the 200% mixture all fish were dead within 48 h of exposure period. The LC_{50} values of chlorpyrifos in mixture with 95% confidence limit were 1.272 (1.070–1.540),

Table 1
24 h - 96 h lethal concentrations (LC_{10}- LC_{90}) of chlorpyrifos to golden mahseer.

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	1.018 (0.509–1.220)	0.801 (0.621–0.928)	0.501 (0.349–0.615)	0.393 (0.262–0.496)
LC_{20}	1.106 (0.633–1.294)	0.889 (0.724–1.021)	0.603 (0.456–0.723)	0.491 (0.360–0.602)
LC_{30}	1.175 (0.739–1.353)	0.959 (0.804–1.102)	0.688 (0.547–0.822)	0.577 (0.447–0.700)
LC_{40}	1.237 (0.841–1.410)	1.022 (0.873–1.182)	0.771 (0.632–0.927)	0.662 (0.531–0.806)
LC_{50}	1.298 (0.945–1.471)	1.085 (0.938–1.270)	0.858 (0.715–1.049)	0.753 (0.616–0.931)
LC_{60}	1.362 (1.056–1.544)	1.152 (1.002–1.372)	0.954 (0.800–1.201)	0.856 (0.706–1.090)
LC_{70}	1.434 (1.176–1.643)	1.229 (1.070–1.498)	1.069 (0.893–1.401)	0.982 (0.807–1.306)
LC_{80}	1.524 (1.309–1.802)	1.324 (1.149–1.671)	1.222 (1.006–1.695)	1.153 (0.932–1.633)
LC_{90}	1.656 (1.461–2.129)	1.470 (1.258–1.957)	1.469 (1.174–2.231)	1.441 (1.124–2.255)

Values in parentheses are lower and upper bound at 95% confidence limit.

Table 2
24 h - 96 h lethal concentrations (LC_{10} - LC_{90}) of dichlorvos to golden mahseer.

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	18.846 (7.989–24.129)	14.756 (10.479–17.563)	12.122 (7.740–14.403)	8.183 (5.589–9.937)
LC_{20}	23.647 (15.097–31.096)	16.860 (12.939–19.693)	13.746 (9.929–15.982)	9.583 (7.157–11.360)
LC_{30}	27.851 (20.975–42.525)	18.561 (14.937–21.570)	15.051 (11.741–17.435)	10.739 (8.47312.631-)
LC_{40}	32.030 (25.120–61.448)	20.150 (16.743–23.517)	16.263 (13.359–19.048)	11.837 (9.690–13.969)
LC_{50}	36.501 (28.410–90.712)	21.758 (18.459–25.727)	17.485 (14.829–21.029)	12.964 (10.866–15.515)
LC_{60}	41.597 (31.496–136.614)	23.495 (20.160–28.412)	18.798 (16.192–23.603)	14.199 (12.046–17.429)
LC_{70}	47.838 (34.795–213.996)	25.506 (21.945–31.895)	20.312 (17.530–27.100)	15.650 (13.300–19.965)
LC_{80}	56.343 (38.816–364.472)	28.079 (24.005–36.868)	22.240 (18.998–32.258)	17.538 (14.769–23.667)
LC_{90}	70.694 (44.871–767.851)	32.084 (26.888–45.575)	25.220 (20.983–41.576)	20.539 (16.866–30.339)

Values in parentheses are lower and upper bound at 95% confidence limit.

0.824 (0.701–0.993), 0.615 (0.520–0.722) and 0.595 (0.504–0.694) mg/L at 24 h, 48 h, 72 h and 96 h, respectively. Similarly, LC_{50} values of dichlorvos in mixture with 95% confidence limit at 24 h, 48 h, 72 h and 96 h were 21.898 (18.429–26.510), 14.182 (12.066–17.104), 10.583 (8.945–12.433) and 10.242 (8.676–11.955) mg/L, respectively (Table 3).

The LC_{50} values of both pesticides when added together in a mixture were only slightly lower than when acting individually (Tables 1, 2 and 3). Therefore AI values calculated for both pesticides were negative throughout the experimental period (Table 4). The negative AI value indicates the antagonistic effect of these pesticides in mixture. The highest antagonistic effect (−0.937) was observed at 96 h-LC_{10} whereas the lowest antagonistic effect (−0.223) was found at 72 h-LC_{90} (Table 4).

Table 4
Joint toxicity of chlorpyrifos and dichlorvos to golden mahseer.

Toxicity	AI (additive index) value			
	24 h	48 h	72 h	96 h
LC_{10}	−0.600	−0.394	−0.439	−0.937
LC_{20}	−0.585	−0.400	−0.397	−0.805
LC_{30}	−0.580	−0.406	−0.368	−0.715
LC_{40}	−0.579	−0.408	−0.343	−0.643
LC_{50}	−0.580	−0.411	−0.322	−0.580
LC_{60}	−0.584	−0.415	−0.301	−0.519
LC_{70}	−0.591	−0.418	−0.280	−0.458
LC_{80}	−0.602	−0.423	−0.255	−0.390
LC_{90}	−0.625	−0.430	−0.223	−0.302

AI value less than zero indicates antagonistic effect.

3.2. General fish behavior in acute exposures

Behavioral changes exhibited by golden mahseer in the acute exposure scenario are provided in detail in Table 5. Fish became slow, sluggish and calm after exposure to chlorpyrifos. Such behavior was observed only by the end of the experiment, after 72 h, at lower concentrations while it was observed from the first hour until the end of the experiment at the higher concentrations of 0.8 mg/L. A similar effect was also noticed with dichlorvos from the beginning until the end of the experiment from 4 to 16 mg/L, but it was visible at 2 mg/L. Fish also exhibited such response in mixed pesticides exposure throughout the experimental period. The calmness was most pronounced at the highest concentration of the pesticide treatments. Mild loss of equilibrium was noticed in all pesticide exposures, but only at higher concentrations. Schooling behavior of fish was also affected by the pesticides throughout the experiment, and that behavior became more distinct with increasing pesticide concentrations. Fish lost coordination towards each other and spread all over the aquarium. This behavior was more distinct in the

Table 3
24 h - 96 h lethal concentrations (LC_{10}- LC_{90}) in mg/L of mixed pesticides (chlorpyrifos 'CPF' and dichlorvos 'DDVP') to golden mahseer.

Toxicity	24 h (CPF and DDVP)	48 h (CPF and DDVP)	72 h (CPF and DDVP)	96 h (CPF and DDVP)
LC_{10}	0.844 and 14.522 (0.54–1.015, 9.377–17.475)	0.577 and 9.942 (0.364–0.683, 6.272–11.766)	0.421 and 7.252 (0.292–0.502, 5.022–8.647)	0.417 and 7.171 (0.294–0.494, 5.063–8.504)
LC_{20}	0.971 and 16.721 (0.705–1.142, 12.129–19.657)	0.652 and 11.231 (0.469–0.756, 8.072–13.013)	0.480 and 8.257 (0.361–0.560, 6.220–9.641)	0.471 and 8.105 (0.359–0.548, 6.176–9.427)
LC_{30}	1.075 and 18.511 (0.838–1.258, 14.427–21.658)	0.712 and 12.264 (0.555–0.823, 9.561–14.171)	0.527 and 9.066 (0.418–0.610, 7.201–10.510)	0.514 and 8.852 (0.411–0.594, 7.078–10.226)
LC_{40}	1.173 and 20.191 (0.959–1.386, 16.503–23.854)	0.768 and 13.221 (0.632–0.899, 10.882–15.477)	0.570 and 9.821 (0.470–0.663, 8.094–11.409)	0.554 and 9.545 (0.459–0.641, 7.896–11.040)
LC_{50}	1.272 and 21.898 (1.070–1.540, 18.429–26.510)	0.824 and 14.182 (0.701–0.993, 12.066–17.104)	0.615 and 10.583 (0.520–0.722, 8.945–12.433)	0.595 and 10.242 (0.504–0.694, 8.676–11.955)
LC_{60}	1.380 and 23.751 (1.177–1.738, 20.265–29.920)	0.884 and 15.214 (0.764–1.117, 13.146–19.238)	0.662 and 11.404 (0.568–0.795, 9.785–13.687)	0.638 and 10.989 (0.549–0.759, 9.451–13.060)
LC_{70}	1.505 and 25.906 (1.285–2.006, 22.121–34.535)	0.953 and 16.402 (0.824–1.287, 14.192–22.150)	0.718 and 12.353 (0.619–0.890, 10.659–15.330)	0.688 and 11.849 (0.596–0.842, 10.258–14.490)
LC_{80}	1.666 and 28.679 (1.406–2.402, 24.210–41.352)	1.040 and 17.909 (0.890–1.536, 15.330–26.451)	0.788 and 13.564 (0.677–1.028, 11.654–17.697)	0.752 and 12.942 (0.649–0.960, 11.178–16.532)
LC_{90}	1.918 and 33.021 (1.574–3.122, 27.101–53.750)	1.175 and 20.232 (0.979–1.988, 16.860–34.233)	0.897 and 15.444 (0.756–1.270, 13.024–21.869)	0.850 and 14.626 (0.723–1.167, 12.440–20.090)

Values in parentheses are lower and upper bound at 95% confidence limit.

Table 5
Behavioral changes shown by golden mahseer during 96 h acute exposure of chlorpyrifos (CPF), dichlorvos (DDVP) and their mixture.

Fish behavior	CPF (mg/L)				DDVP (mg/L)				Mixed pesticides (mg/L)			
	0.1	0.2	0.4	0.8	2	4	8	16	CPF-0.094, DDVP-1.620	CPF-0.188, DDVP-3.241	CPF-0.376, DDVP-6.482	CPF-0.753, DDVP-12.964
Hypo-activity	+	+	+	++	_	+	+	++	+	+	+	++
Equilibrium loss	_	_	_	+	_	_	_	+	_	_	_	+
Color change	_	_	+	+	_	_	+	+	_	_	_	_
Aggregating at corners of aquarium	_	_	_	_	_	_	_	_	++	+	+	+
Avoiding schooling behavior	+	+	+	++	_	+	+	++	+	++	++	+++

Mixed concentrations were prepared as 12.5%, 25%, 50% and 100% of 96 h-LC_{50} values of the respective pesticides. 96 h-LC_{50} of CPF- 0.753 mg/L and DDVP- 12.964 mg/L (−: absent; +: mild; ++: moderate; +++: strong).

mixed pesticide solution where fish not only avoided swimming in group but also aggregated at corners of the aquarium. Caudal fin of the fish became reddish with increasing pesticide concentrations. Caudal fins were found to be fragile and degraded in the higher concentrations of chlorpyrifos and the mixed pesticide solution. Fish tended to swim towards the bottom of the aquarium when exposed to chlorpyrifos but in contrast, fish tended to swim above the mid line of the aquarium when exposed to dichlorvos and surfacing of fish was intensified with increased dichlorvos concentration. In lower concentrations of the mixed pesticides, fish were found to be distributed everywhere in the aquarium but they remained almost on the bottom at the highest mixed concentration. Gulping of air was observed occasionally with chlorpyrifos exposed fish. Fish became over active and showed vigorous swimming and then became abruptly silent, both in swimming behavior and opercular movements, before death. The dead fish were found to be loaded with mucus around their gill surface.

3.3. Aerobic energy metabolism

Analysis of oxygen consumption showed no significant difference between control and pesticides exposed groups at 0–24 h of exposure (Fig. 1). Significant differences were noticed at 24–48 h of exposure between control and 10% chlorpyrifos treated group ($P < 0.05$) with lower oxygen consumption rates and control and 50% mixture group ($P < 0.001$) with higher oxygen consumption rates. Similarly, significantly higher ($P < 0.001$) levels of oxygen were consumed by the 50% mixture exposed fish group at 48–72 h exposure, and the 10% ($P < 0.01$) and 50% mixture ($P < 0.05$) groups at 72–96 h of exposure compared to their respective controls (Fig. 1). In brief, elevated oxygen consumption rates compared to control was only recorded in mixed pesticide treatments. In contrast to this observation, oxygen consumption tended to be suppressed at individual pesticide treatments; however the trend was not always significant. Most of the time, fish confronted with 50% pesticide doses demanded higher oxygen consumption use than their respective 10% pesticide doses, but the difference was significant ($P < 0.01$) only between both mixture treatment groups at 24–48 and 48–72 h of exposure. Among the same pesticide treatments significantly higher ($P < 0.001$) oxygen uptake was observed in mixture 50% treatment group during 24–48 h and 48–72 h compared to 0–24 h of measurement. This elevated oxygen uptake was stabilized during 72–96 h but oxygen uptake in mixture 10% treatment was still higher than its initial (0–24 h) measurement (Fig. 1).

The trends were clearer when looking at the average oxygen consumption over the whole 96 h period (compiled measurement of 24 h, 48 h, 72 h and 96 h) (Fig. 2). Statistical analysis revealed a significantly reduced oxygen consumption ($P < 0.001$) in 10% chlorpyrifos, 10% and 50% dichlorvos, and significantly high ($P < 0.001$) consumption in 50% mixture compared to control fish (Fig. 2). Between the low and high doses of the same pesticide treatment, significant differences were noted for chlorpyrifos ($P < 0.01$) and for the mixture ($P < 0.001$) fish groups (Fig. 2).

3.4. Opercular movements

Opercular movements in pesticide exposed fish were generally faster than in their respective controls, even though it was not always significant. After 1 h of exposure all pesticide treated fish, except in the 10% chlorpyrifos, showed significantly higher ($P < 0.001$) opercular movements (Fig. 3). All pesticide treated fish groups also elevated opercular movements compared to control after 24 h, 48 h and 72 h except in the dichlorvos treatment. After 96 h of exposure only the chlorpyrifos treatment still exhibited significantly higher ($P < 0.001$) opercular movements than control (Fig. 3). No significant difference was observed between low and high doses of the same pesticide treated groups at the

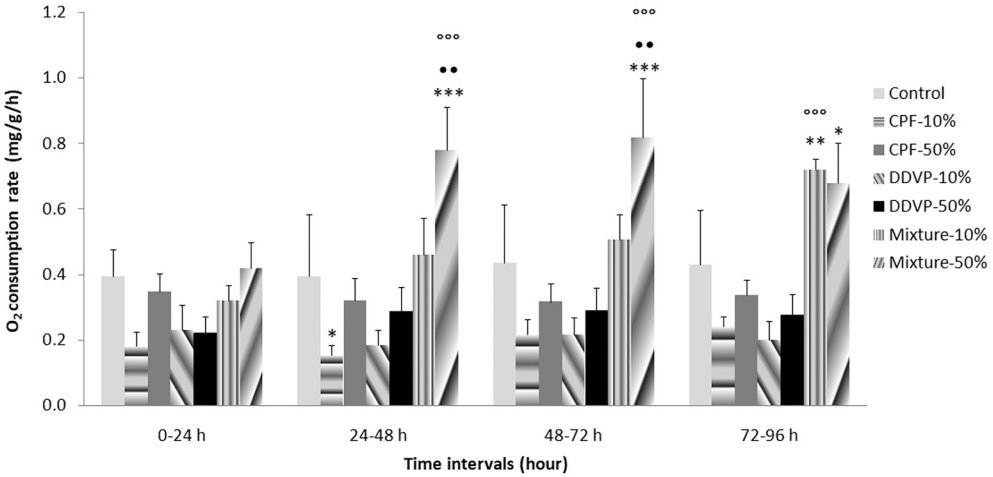

Fig. 1. Oxygen consumption rate by golden mahseer during different exposure periods. Exposure concentrations were 10% and 50% 96 h-LC_{50} values of the respective pesticides. Values are mean ± SD ($n = 5$). Asterisk denotes significant difference between control and other treatment groups during the same exposure period ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$); dark circle denotes significant difference between 10% and 50% concentrations of the same pesticide treatment within same exposure period ($^{\bullet\bullet}P < 0.01$); and white circle denotes significant difference in the same treatment groups during different exposure periods compared to 0–24 h of exposure ($^{\circ\circ\circ}P < 0.001$).

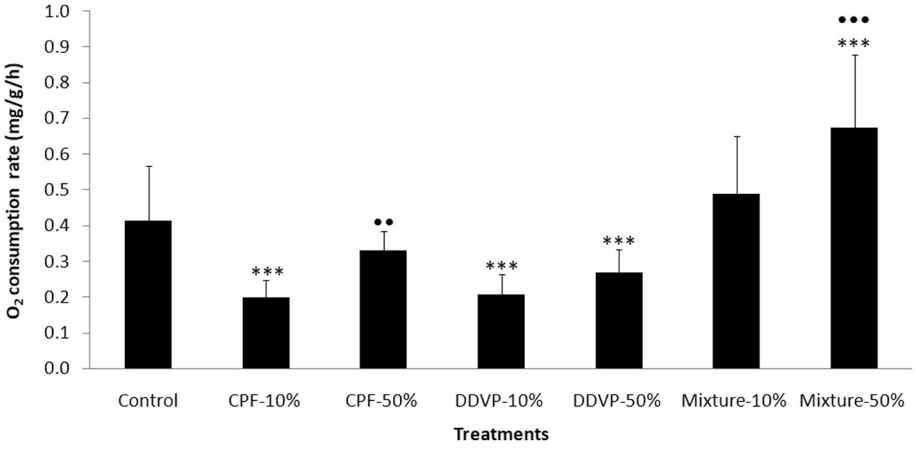

Fig. 2. Oxygen consumption rate by golden mahseer during 96 h exposure to different pesticides. Exposure concentrations were 10% and 50% 96 h-LC_{50} values of the respective pesticides. Values are mean ± SD ($n = 20$). Asterisk denotes significant difference between control and other treatment groups (***$P < 0.001$); dark circle denotes significant difference between 10% and 50% concentration of the same pesticide treatment (●●$P < 0.01$; ●●●$P < 0.001$).

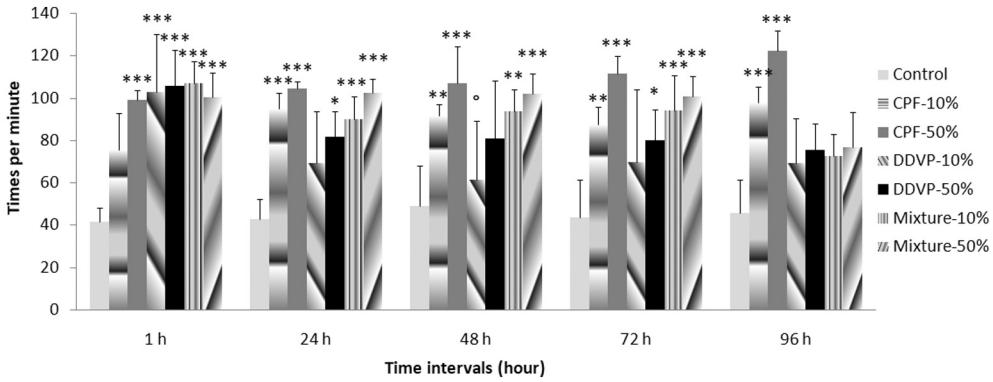

Fig. 3. Opercular movements by golden mahseer during different exposure periods. Exposure concentrations were 10% and 50% 96 h-LC_{50} values of the respective pesticides. Values are mean ± SD (n = 5). Asterisk denotes significant difference between control and other treatment groups during the same exposure period (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); white circle denotes significant difference in the same treatment groups during different exposure periods compared to 1 h of exposure (°$P < 0.05$). No significant difference was observed between 10% and 50% concentration of the same pesticide treatment at same exposure period.

same time interval. Comparing the opercular movements in the same treatment group over time showed only significantly low opercular movements in 10% dichlorvos group after 48 h compared to 1 h of exposure (Fig. 3).

The average opercular movements over the whole exposure period (compiled observation of 1 h, 24 h, 48 h, 72 h and 96 h) in all treatment groups were significantly higher ($P < 0.001$) than that of control fish (Fig. 4). Among the pesticide exposed groups the highest opercular movements were exhibited by 50% chlorpyrifos and the lowest by 10% dichlorvos fish groups. Between low and high doses of the same pesticide treatment only significant difference ($P < 0.001$) was observed in chlorpyrifos treated groups (Fig. 4).

3.5. Feeding attempts

Fish from control group exhibited feeding attempts at every time interval whenever food was offered to them. Chlorpyrifos and mixed pesticides exposed fish displayed almost no feeding attempt except for one fish at 72 h in 10% chlorpyrifos treatment group. Dichlorvos exposed fish exhibited some feeding attempts except after 1 h and 96 h

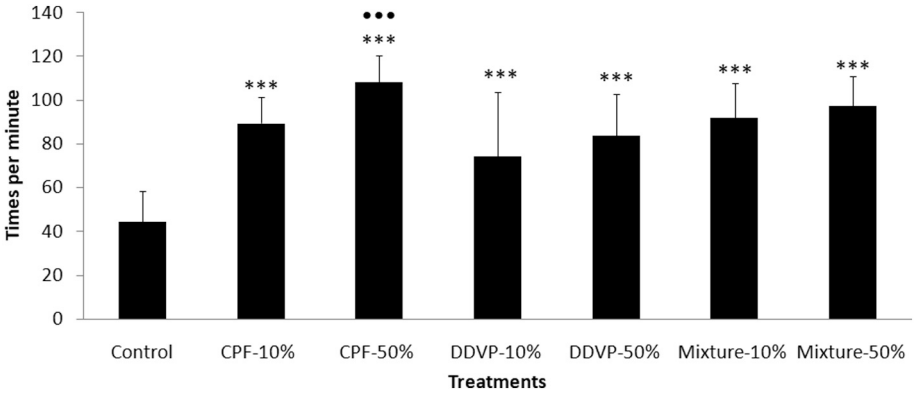

Fig. 4. Opercular movements by golden mahseer during 96 h exposure to different pesticides. Exposure concentrations were 10% and 50% 96 h-LC_{50} values of the respective pesticides. Values are mean ± SD ($n = 25$). Asterisk denotes significant difference between control and other treatment groups (***$P < 0.001$); dark circle denotes significant difference between 10% and 50% concentration of the same pesticide treatment (●●●$P < 0.001$).

in high exposure dose where no feeding attempt was shown at all (Table 6). In total, the average feeding attempts by control fish was 7.98 ± 2.19 times which were significantly higher ($P < 0.001$) than all pesticide exposed counterparts (Table 6). This result clearly indicates that feeding behavior of golden mahseer was notably affected by pesticides.

4. Discussion

The findings of the acute toxicity assessment (96 h-LC_{50}) suggest that chlorpyrifos can be categorized as highly toxic and dichlorvos as a moderately toxic pesticide to the endangered freshwater fish species golden mahseer. As far as we know, this study is first to report acute toxicity of chlorpyrifos and dichlorvos to golden mahseer. The 96 h median lethal concentration of chlorpyrifos (0.753 mg/L) and dichlorvos (12.964 mg/L) obtained in this study for golden mahseer juveniles is comparable to the values that had been published for other freshwater fish species, wherein 96 h-LC_{50} value of chlorpyrifos varied between 0.16 mg/L to 1.57 mg/L (Banaee et al., 2013; Bhatnagar et al., 2016; Gül, 2005; Halappa and David, 2009; Nwani et al., 2013; Xing et al., 2015) and dichlorvos ranged between 6.5 mg/L to 9.41 mg/L (Satyavani et al., 2011; Tilak and Kumari, 2009; Ural and Çalta, 2005; Velmurugan et al., 2009).

The fact that aquatic environment is a sink of multiple pollutants including pesticides; the risk assessments of single pesticide can be misleading. As such the joint toxic effect of pesticides was evaluated, and surprisingly the antagonistic effect of binary mixture of chlorpyrifos and dichlorvos to golden mahseer was revealed. This exemplifies that the mixture had a lower toxicity compared to the toxicity of the sum of the individual pesticides tested.

We also provided the first report on the joint effect of chlorpyrifos and dichlorvos to fish. A possible reason for such an antagonistic observation, although not measured in our study, could be attributed to an elevated activity of carboxylesterases (CaEs) enzymes under the binary action of these two pesticides. This enzyme has been reported to play a role in the detoxification of many pesticides, including organophosphate, via hydrolysis (Jokanovic, 2001; Wheelock et al., 2005). In addition, this enzyme is also believed to protect AChE from pesticide toxicity by direct binding and sequestration (Jokanovic, 2001; Maxwell, 1992). The findings of our study suggest that chlorpyrifos and dichlorvos in combination may act as a cue for triggering CaEs activity. Another justification for the occurrence of antagonistic interactions could potentially be related to the Glutathione-S-transferase (GST) activity dynamics. GST is present in different tissues of fish, and promotes cellular detoxification of xenobiotics including pesticides (Booth et al., 1998; Jin-Clark et al., 2002). The activity of GST was reported to be significantly higher when malathion and pirimiphos-methyl pesticides were applied in combined form, compared to the individual exposure (Stepić et al., 2013). Although GST activity was not measured in the present study, based on the existing literature (Stepić et al., 2013) it is reasonable to speculate that a possible upsurge in GST activity under chlorpyrifos and dichlorvos combined exposure would facilitate an efficient detoxification of these metabolites, and therefore reduced the toxicity. Furthermore, a recent study done by Imam et al. (2018) on rats revealed that chlorpyrifos and dichlorvos in combination (double dose) did not elicit significantly greater adverse effects in comparison to the individual pesticide. They further reported that some of the responses were even less affected in joint pesticides compared to individual pesticide treatment, and proposed a chemical interaction between chlorpyrifos and dichlorvos might be the reason for such antagonistic outcomes (Imam et al., 2018). Nevertheless, studies involving measurements of these factors are needed to understand the underlying mechanism for the antagonistic interaction of chlorpyrifos and dichlorvos observed in the present study.

Changes in behavior are considered as one of the most sensitive and early indicators of potential toxic effect in fish even at sub-lethal levels. As such, behavioral studies are gaining popularity in toxicity assessment and our overall observations in this study were loss of balance and schooling behavior, aggregating at corner and bottom of the test chamber, surfacing activity, occasionally air gulping and excess mucus secretation which corroborates to observations documented by other authors (Halappa and David, 2009; Kavitha and Rao, 2008; Padmanabha et al., 2015; Ullah et al., 2014). Such series of behavioral endpoints associated with equilibrium, grouping, swimming reactions, opercular movements and feeding attempts are non-invasive and easily accessible, and can easily be applied as the potential biomonitoring tool in evaluating the sub-lethal effects of contaminants during acute as well as chronic exposure. In the present case, these behavioral changes may be due to inhibition of AChE leading to accumulation of acetylcholine (Ach) in cholinergic synapses and overstimulation (Halappa and David, 2009). Excess mucus secretion could be an initial defense mechanism of fish to minimize contact with the toxic compound or to eliminate it through epidermal mucus (Halappa and David, 2009; Patil and David, 2008). Gulping of air displayed by fish in response to pesticides exposure might be to compensate for respiratory stress (Halappa and David, 2009). Likewise, the opercular movements are directly associated with respiratory movements, and the remarkable increment suggests that acute exposure to both pesticides in discrete as well as their combined exposure can negatively influence respiration processes in fish. Similar observations have also been reported following chlorpyrifos-based pesticide exposure in African catfish (Nwani et al., 2013) and tilapia (Padmanabha et al., 2015).

One of the common physiological responses to toxicants is alteration in oxygen consumption which is easy to measure and used worldwide in toxicity study to evaluate the changes in metabolism under stressful environmental conditions (Patil and David, 2008). Respiratory organs of fish (gills) are directly exposed to water, therefore effect of toxicants on the respiratory processes is more pronounced. There are several studies elucidated the effect of pesticides on respiratory metabolism of fish, and mixed responses (both increase and decrease) have been documented in terms of oxygen consumption rate (Barbieri et al., 2018; Barbieri and Ferreira, 2011; Basha et al., 1984; Jothinarendiran, 2012; Padmanabha et al., 2015; Patil and David, 2008; Rao et al., 1981). Similar mixed response in aerobic metabolism was obtained in our study wherein it was suppressed by individual pesticide exposure but elevated in pesticide mixture, despite accelerated opercular movements in all pesticide treatments. Higher opercular movements should facilitate higher oxygen uptake but this was not the case in our individual pesticide

Table 6
Feeding attempts (number/5 min) by golden mahseer during different exposure periods.

Time intervals	Control	CPF-10%	CPF-50%	DDVP-10%	DDVP-50%	Mixture-10%	Mixture-50%
1 h	7.78 ± 2.33	0.00*	0.00*	0.20 ± 0.45	0.00	0.00*	0.00*
24 h	7.22 ± 1.39	0.00	0.00	2.60 ± 2.88	0.20 ± 0.45	0.00	0.00
48 h	7.89 ± 2.15	0.00*	0.00*	1.60 ± 2.51	0.60 ± 1.34	0.00*	0.00*
72 h	8.78 ± 2.77	0.20 ± 0.45	0.00*	3.00 ± 6.71	0.20 ± 0.45	0.00	0.00*
96 h	8.22 ± 2.28	0.00*	0.00	1.40 ± 3.13	0.00*	0.00	0.00
Total	7.98 ± 2.19	0.04 ± 0.20***	0.00***	1.76 ± 3.55***	0.21 ± 0.66***	0.00***	0.00***

Exposure concentrations were 10% and 50% 96 h-LC_{50} values of the respective pesticides. Values are mean ± SD (n = 5). Asterisk denotes the significant differences between control and other treatment groups during same exposure period (*$P < 0.05$; ***$P < 0.001$).

treatment groups. This scenario clearly indicates that fish gills might have been damaged by the individual pesticides and also excess mucus production could have increased diffusion distance, thus compromising oxygen uptake by the fish. Nevertheless, in the pesticide mixture such effect was not severe enough to compromise oxygen uptake.

It is a well known fact that the higher the rate of metabolism, the faster will be the elimination of toxic metabolites (Stepić et al., 2013; Wang et al., 2017). Faster elimination of toxic chemical from mixture pesticide treated fish as a consequence of accelerated metabolic rate might have been another reason for such antagonistic effect to them.

In our study, retarding or ceasing food uptake in response to pesticide is a general response of fish to stress. Similar observations by pesticide exposed fish have been described by many authors (Halappa and David, 2009; Kavitha and Rao, 2008; Padmanabha et al., 2015; Pavlov et al., 1992). In pesticide mixtures, complete cessation of feeding might be a strategy of fish to minimize the energetic cost for digestion (Halappa and David, 2009) and channelize energy towards pesticide detoxification processes. In addition, the reduction in feeding may be linked to altered gustatory sensitivity of golden mahseer to the tested pesticides. Though not much known on this aspect, a previous study on common carp documented that the nerve innervating terminal buds sensitivity in the lip region can be influenced following exposure to herbicides (benthiocarb and isoprothiolane) and insecticide (fenitrothion) (Ishida and Kobayashi, 1995).

While closely analyzing behavioral response of golden mahseer in individual and mixture pesticide treatments, fish tended to swim on the bottom of the aquaria with increasing pesticide concentration both in chlorpyrifos and mixture group, but in contrast, surfacing activity was prominent with dichlorvos toxicity. Likewise, complete cessation of feeding was also a similar behavioral response shown by fish in chlorpyrifos and mixture pesticide treatments. From these observation we can conclude that chlorpyrifos is the dominating pesticide in binary mixture.

5. Conclusion

This study confirms that chlorpyrifos is relatively more toxic to golden mahseer compared to dichlorvos. The most interesting finding of this study is that these pesticides in mixture produce antagonistic effect in spite of having same mode of action. A possible reason for this antagonistic effect could be that their respiratory organs were less affected by the pesticide mixture. This hypothesis is also supported by the elevated oxygen consumption by fish exposed to the pesticide mixture. Increased oxygen supply might have been helpful for fish to detoxify toxic compounds. In essence, histopathological analysis of fish gills from individual and mixture pesticide treatments is recommended. Moreover, for understanding the underlying mechanism responsible for the antagonistic interaction of chlorpyrifos and dichlorvos, future studies are warranted to evaluate the protective role of CaEs and GST enzymes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Joint toxicity assessment reveals synergistic effect of chlorpyrifos and dichlorvos to common carp (*Cyprinus carpio*)

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ABSTRACT

Common carp (*Cyprinus carpio*) is an important aquaculture species. However, their production and health is sometimes threatened by pesticides. In common carp, extensive studies have been done for exposures of single pesticides, but effects of mixtures such as those of the commonly used chlorpyrifos and dichlorvos, are still unknown for this species. In the first phase of this work, an acute lethal exposure experiment was conducted to estimate 24 h to 96 h lethal concentrations ($LC_{10–90}$) of chlorpyrifos, dichlorvos and their mixture. Compared to dichlorvos, chlorpyrifos was found to be highly toxic to the tested species. Joint toxicity assessment of these pesticides in binary mixtures was dominated by synergism. In the second experimental phase, common carp were exposed to sub-lethal concentrations (LD-10% and HD-50% 96 h-LC_{50}) of individual pesticides and their mixture. General fish behaviors, buccal movements and feeding attempts by fish were recorded after 1 h, 24 h, 48 h, 72 h and 96 h whereas aerobic metabolism of fish was recorded for 0–24 h, 24–48 h 48–72 h and 72–96 h of exposure. All pesticide treatments elevated buccal movements and oxygen uptake in a dose dependent manner. Feeding depression was also observed by pesticide exposure. The augmented deleterious effect of these pesticides in a mixture suggests that joint toxicity assessment is critical to develop more realistic water quality standards and monitoring guidelines.

1. Introduction

Chlorpyrifos and dichlorvos are organophosphates, whose mode of action is the inhibition of acetylcholinesterase (AChE) (LeBlanc et al., 2012), and this group is one of the most commonly used pesticides globally (El Nahas et al., 2017; Kafle et al., 2015; Rao et al., 2017). AChE is an enzyme responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) to acetic acid and choline. Inhibition of this enzyme (AChE) leads to accumulation of ACh in synapses and neuromuscular junctions that cause uninterrupted stimulation of cholinergic fibers throughout the nervous systems resulting overstimulation and death (Jokanović, 2009; Stepić et al., 2013). Exposure to even small amounts of organophosphates can be fatal (Jokanović, 2009).

Application of pesticides is becoming an integral part of agriculture to enhance production and profitability. Pesticide use is not only limited to agriculture but it is also intensively used in the public health sector to control disease causing vectors. According to the Food and Agriculture Organization of the United Nations database (FAOSTAT, 2020), the pesticide use in Nepal has increased from 60 t in 1990 to 574 t in 2018. Likewise, the global pesticide application has increased from 2,299,979 t in 1990 to 4,122,334 t in 2018 (FAOSTAT, 2020). In addition, it was reported that only a small fraction of the applied pesticides reaches the target organisms (Tišler et al., 2009). According to Pimentel (1995) less than 0.1% of the applied pesticide reaches their target pests and more than 99.9% of pesticides are released into the environment. As such, pesticides can harm beneficial organisms on land and in water including different species of fish.

Sun and Chen (2008) reported chlorpyrifos residue in wild and farmed fish in Taiwan were 25 ± 23 ng/g and 17 ± 47 ng/g, respectively. Singh et al. (2015) documented chlorpyrifos level in water,

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sediment and fish sampled from the river Deomoni, West Bengal as 0.0091 ± 0.0020 ppm, 0.0513 ± 0.0085 ppm and 5.0371 ± 1.4236 ppm, respectively. Nag et al. (2020) recorded chlorpyrifos (0.019–2.73 μg/L) and dichlorvos (0.647 μg/L) in the water samples and 0.053 μg/g chlorpyrifos in the fish sample from the Chilika lake, India. Chlorpyrifos residue (up to 0.20 μg/g) was detected in fish sampled from the Tono reservoir, Ghana (Akoto et al., 2016). A study in agriculture intensified area of Nepal also reported 390 μg dichlorvos per kg of soil sample (Kafle et al., 2015). Occurrence of these pesticides' residue in water, sediments, soil and fish makes them potential pesticides to study their toxicity mechanisms in aquatic organisms.

We chose fish as a candidate species to study the potential toxicity of pesticides in aquatic environment due to fish's socio-economic importance as well as wide distribution in aquatic systems. For this study, the freshwater fish common carp (*Cyprinus carpio*) was used because they are one of the dominant species in the aquaculture industry of Asia, including Nepal. Also, they inhabit both polluted and non-polluted water sources. Consequently, this is considered one of the sentinel species for the biomonitoring of contaminants including pesticides in the freshwater ecosystem.

Existing reports suggest that exposure to chlorpyrifos can incite behavioral changes in common carp (Halappa and David, 2009; Xing et al., 2015). Altered behavioral expressions were also observed when common carp were exposed to Triazophos (Sarkar et al., 2016). Similarly, various studies on dichlorvos toxicity elucidated effects on behavior (Gunde and Yerli, 2012; Ural and Çalta, 2005), food consumption (Laxmi et al., 2019) and metabolism (Demael et al., 1990) of common carp.

In natural scenarios, aquatic organisms are often exposed to pesticide mixtures. The situation can aggravate when such mixtures turn out to be synergistic and toxicity becomes several times higher than individual pesticide toxicity. Laetz et al. (2009) reported that mixtures of organophosphates can be lethal at concentrations that were sub-lethal in single pesticide trials. Therefore, while defining water quality standards and preparing environmental risk assessment protocols, joint effect of chemicals need to be considered (Wang et al., 2017). Stepić et al. (2013) also reported environmental risk assessment based on single pesticide toxicity could be inaccurate, therefore both individual and mixture toxicity should be taken in account for such assessments. Realizing the severity of mixture toxicity, several studies have been carried out to elucidate joint toxicity of pesticides (Chen et al., 2014; Kunwar et al., 2021; Laetz et al., 2009; Wang et al., 2017, 2015; Wu et al., 2018). However, till date the effect of chlorpyrifos and dichlorvos mixtures in common carp is still unknown.

With this background, the main purpose of the present study was to obtain insights in the lethal and sub-lethal toxicity of chlorpyrifos, dichlorvos and their mixtures to common carp. For sub-lethal effects, we examined general fish behaviors, buccal movements, feeding attempts and aerobic metabolism. These responses are non-invasive, less explored and equally important indicators of stress. Prevalence of chlorpyrifos and dichlorvos in aquatic systems makes them important toxicants to be studied. Common carp being a key candidate in the aquaculture production and widely distributed throughout the world, thus represents an ideal bio-model species for this study. Chlorpyrifos and dichlorvos have same mode of action, therefore their joint effect is hypothesised to be additive in exposed fish. We expect the findings of this study will be helpful to enrich the existing knowledge from the facet of joint toxicity of chlorpyrifos and dichlorvos to economically important freshwater aquaculture species common carp.

2. Materials and methods

2.1. Animal husbandry

Breeding of common carp fish was done in Central Fisheries Promotion and Conservation Centre (CFPCC), Balaju, Kathmandu, Nepal. Common carp juveniles (exact weight is mentioned in specific test section, refer below) were transferred from outdoor ponds to 350 L glass aquaria located at in-door CFPCC research facility. Fish were fed ad libitum with commercial pellet feed- 32% protein (Sreema feed Pvt. Ltd., India) every day around 10 am. Remaining food and faecal matters were siphoned and 50% of water was changed on daily basis to maintain good water quality. The experimental animals were acclimatized for at least 2 weeks before starting the experiment. Water temperature, pH, dissolved oxygen, total ammonia, hardness, Na^+, K^+ and Cl^- were ranged between 24.32 and 25.78 °C, 7.59 and 7.83, 5.82 and 6.78 mg/L and 0.19 and 0.24 mg/L, 52 and 58 mg $CaCO_3$/L, 1.12 and 1.28 mmol/L, 0.07 and 0.11 mmol/L and 0.48 and 0.66 mmol/L respectively. This research was approved by the ethical review board of Nepal Health Research Council (Ref. No. 1215), Government of Nepal.

2.2. Pesticides

Commercial formulations of chlorpyrifos and dichlorvos pesticides were used for this study. For dichlorvos G-VAN (80%; Greenriver Industry Co., Ltd., ShenZhen, China) and for chlorpyrifos Dursban (20%; Dow AgroSciences Pvt. Ltd. India) were used.

2.3. Lethal exposures

Lethal toxicity tests were conducted in triplicate (10 fishes in each replicate) under semi-static condition as per the standard guidelines (OECD, 1992). The average weight of the fish was 3.16 ± 0.54 g (mean ± SD). Feeding was stopped 24 h prior to the exposure experiment. Stock solution was freshly prepared in distilled water and a calculated amount of the solution was added in test chamber (rectangular glass aquaria - 35 L filled up to 25 L) to maintain five different pesticide concentrations. The concentrations for chlorpyrifos were 0.25, 0.5, 0.75, 1.0 and 1.25 mg/L, and the concentrations of dichlorvos were 10, 15, 20, 25 and 30 mg/L. For acute toxicity assessment of mixture pesticides, chlorpyrifos and dichlorvos were mixed in equi-toxic concentrations on the percentage basis of 96 h-LC_{50} values of the respective pesticides (25%: 0.11 and 3.92; 50%: 0.22 and 7.85; 75%: 0.33 and11.77; 100%: 0.44 and 15.705; 125%: 0.55 mg/L and 19.63 mg/L). Mortality of fish was regularly monitored and recorded. Dead fish were immediately removed from the aquarium; no fish mortality was observed in control (no pesticides added; performed in triplicate). Based on the fish mortality response, 96 h-LC_{10} - LC_{90} (with 95% confidence limit) of both pesticides and their mixture was calculated by using a log probit analysis program (SPSS version 20).

Joint toxicity of pesticide was assessed by using the additive index (AI) which was calculated according to Marking (1985).

$$AI = (1/S) - 1 \text{ for } S \leq 1$$

and,

$$AI = 1 - S \text{ for } S > 1$$

where, AI represents additive index and S represents sum of biological activity

$$S = (A_m/A_i) + (B_m/B_i)$$

where, A and B represents two different pesticides, 'm' represents LC_{50} of pesticides in mixture, 'i' represents LC_{50} of individual pesticides. The AI value-equal, greater or less than zero indicates additive, synergistic or antagonistic effect, respectively.

2.4. Sub-lethal exposures

The sub-lethal exposure experiment was conducted under semi-static condition. The average weight of the fish used in this experiment was

9.97 ± 2.19 g (mean ± SD). The fish used in the measurement of buccal movements, feeding trial and aerobic metabolism were the same. The sub-lethal exposure concentrations were designed based on 96 h-LC_{50} values of chlorpyrifos (0.440 mg/L) and dichlorvos (15.705 mg/L) obtained from the lethal toxicity experiment (refer Section 3.1.1). The 10% 96 h-LC_{50} value was designated as the low dose (LD) and the 50% 96 h-LC_{50} value as the high dose (HD) of pesticide treatment. There were all together 6 pesticide treatments CPF-LD (0.044 mg/L), CPF-HD (0.220 mg/L), DDVP-LD (1.570 mg/L), DDVP-HD (7.853 mg/L), Mixture-LD (CPF-0.044 mg/L and DDVP-1.570 mg/L) and Mixture-HD (CPF-0.220 mg/L and DDVP-7.853 mg/L).

2.4.1. Buccal movements

This experiment (6 treatments and 1 control) was conducted in 6 replicates in 35 L rectangular glass aquaria. Water was filled up to the top to avoid any air space in the container. After weighing, fish were placed one in each test chamber, for the overnight acclimatization. The next day, fish were exposed to different pesticide treatments (concentrations mentioned above). Buccal movements were counted for 5 min using a hand tally counter. Each fish was counted three times to use an average value in analysis for more accuracy. The counting was performed after 1 h, 24 h, 48 h, 72 h and 96 h of exposure and the data is presented as average buccal movements per minute.

2.4.2. Feeding trial

The study on feeding behavior was started after counting buccal movements. For this study, 20 small floating feed pellets were offered in the test chambers and fish were observed for 5 min. Feeding attempts by the fish were counted after 1 h, 24 h, 48 h, 72 h and 96 h of the exposure with the help of a hand tally counter. Uneaten feed was immediately removed by a scoop net to keep the spiked water clean.

2.4.3. Aerobic metabolism

After accomplishing the feeding behavior study, measurements for oxygen consumption rates were initiated. Each aquarium was equipped with a screened water pump to create a water velocity (~5–10 cm/s) which prompted the fish to swim gently against the water current. Aeration was switched off and initial oxygen concentration (mg/L) in water was measured using a Milwaukee MW600 PRO Portable Dissolved Oxygen meter, and then aquaria were made air tight by sealing the aquarium with glass lids and duct tape. The next day the final oxygen concentration (mg/L) was recorded after 19 h interval. Water was aerated for at least 4 h before initiating the next trial, and the same procedure was followed till the end of the experimentation. Oxygen consumption rate of fish (mg/g/h) at different time intervals (0–24 h, 24–48 h, 48–72 h and 72–96 h) was calculated as

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

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

2.5. Data analysis

Shapiro-Wilk test was applied to check the normality of data whereas Levene's test was used to check homogeneity of variances. Mean difference among various treatment groups was analyzed by one-way ANOVA followed by Tukey-HSD post Hoc tests. If data did not satisfy the assumptions for parametric test, Kruskal-Wallis with multiple pair comparison was performed. All analyses were done using statistical program SPSS version 20.

3. Results

3.1. Lethal toxicity

3.1.1. Individual pesticide toxicity

24 h, 48 h, 72 h and 96 h median lethal concentrations (LC_{50}) of chlorpyrifos with 95% confidence intervals were calculated to be 0.678 (0.594–0.762), 0.551 (0.479–0.617), 0.494 (0.428–0.556) and 0.440 (0.373–0.504) mg/L (Table 1). Similarly, for dichlorvos the median lethal concentrations (LC_{50}) were 24.540 (22.561–27.217), 21.464 (19.652–23.479), 16.838 (15.395–18.173) and 15.705 (14.385–16.963) mg/L at 24 h, 48 h, 72 h and 96 h, respectively (Table 2).

3.1.2. Joint pesticide toxicity

In the pesticide mixture, LC_{50} values with 95% confidence limit for chlorpyrifos were 0.499 (0.403–0.733), 0.290 (0.231–0.363), 0.184 (0.147–0.218) and 0.145 (0.113–0.173) mg/L and for dichlorvos LC_{50} values were 17.804 (14.376–26.158), 10.359 (8.235–12.951), 6.572 (5.234–7.772) and 5.180 (4.021–6.169) mg/L at 24 h, 48 h, 72 h and 96 h, respectively (Table 3).

Combined effects of chlorpyrifos and dichlorvos were estimated according to the AI value. Antagonistic action was observed during the early exposure period which then gradually shifted to additive or synergistic effects with the advancement of exposure period. Ultimately, the joint effect became synergistic by the end of the experiment (Table 4).

3.2. Sub-lethal effects

3.2.1. Buccal movements

Buccal movements of fish were found to be accelerated with all pesticide treatments from the beginning until the end of the experiment. Significantly higher buccal movements were observed for chlorpyrifos low dose at 72 h ($P < 0.001$) and 96 h ($P < 0.01$); dichlorvos low dose at 48 h ($P < 0.05$), 72 h ($P < 0.01$) and 96 h ($P < 0.001$) and mixture low dose at 24 h ($P < 0.001$), 48 h ($P < 0.01$) and 96 h ($P < 0.001$) of exposure. The high dose of all pesticide treatments were significantly effective in elevating buccal movements from 24 h to 96 h of exposure ($P < 0.05$–0.001) (Fig. 1). Except at the first hour of observation, buccal movements in the high pesticide dose were always higher compared to the low dose of the same pesticide treatment at the same time of observation but the difference was significant only with the chlorpyrifos low and high dose after 96 h of exposure ($P < 0.05$). Within each treatment group, when buccal movements from 24 h - 96 h were compared to its 1 h of observation, chlorpyrifos low dose was significantly higher at 72 h ($P < 0.001$) and 96 h ($P < 0.01$); chlorpyrifos high

Table 1
24 h–96 h lethal concentrations (LC_{10}- LC_{90}) of chlorpyrifos to common carp.

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	0.371 (0.270–0.448)	0.326 (0.243–0.390)	0.288 (0.217–0.345)	0.232 (0.164–0.287)
LC_{20}	0.457 (0.358–0.532)	0.390 (0.309–0.454)	0.347 (0.276–0.403)	0.289 (0.220–0.345)
LC_{30}	0.530 (0.436–0.604)	0.445 (0.366–0.507)	0.396 (0.327–0.453)	0.338 (0.269–0.396)
LC_{40}	0.602 (0.514–0.678)	0.497 (0.422–0.560)	0.444 (0.377–0.502)	0.388 (0.320–0.447)
LC_{50}	0.678 (0.594–0.762)	0.551 (0.479–0.617)	0.494 (0.428–0.556)	0.440 (0.373–0.504)
LC_{60}	0.764 (0.679–0.865)	0.611 (0.540–0.684)	0.549 (0.483–0.619)	0.499 (0.431–0.572)
LC_{70}	0.868 (0.773–1.003)	0.683 (0.610–0.771)	0.615 (0.546–0.70)	0.572 (0.499–0.661)
LC_{80}	1.008 (0.888–1.209)	0.778 (0.694–0.897)	0.703 (0.624–0.816)	0.670 (0.585–0.794)
LC_{90}	1.239 (1.062–1.589)	0.931 (0.819–1.122)	0.846 (0.739–1.024)	0.835 (0.715–1.042)

Values in parentheses are lower and upper bound at 95% confidence limit.

Table 2
24 h–96 h lethal concentrations (LC_{10}–LC_{90}) of dichlorvos to common carp.

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	15.904 (12.896–17.881)	13.655 (11.007–15.512)	11.757 (9.784–13.206)	11.094 (9.328–12.405)
LC_{20}	18.458 (15.935–20.254)	15.948 (13.602–17.658)	13.300 (11.493–14.656)	12.500 (10.884–13.734)
LC_{30}	20.550 (18.397–22.356)	17.838 (15.757–19.496)	14.537 (12.875–15.840)	13.623 (12.132–14.821)
LC_{40}	22.524 (20.570–24.597)	19.628 (17.742–21.366)	15.684 (14.147–16.975)	14.662 (13.272–15.864)
LC_{50}	24.540 (22.561–27.217)	21.464 (19.652–23.479)	16.838 (15.395–18.173)	15.705 (14.385–16.963)
LC_{60}	26.737 (24.494–30.425)	23.471(21.560–26.048)	18.07 (16.679–19.542)	16.821 (15.526–18.214)
LC_{70}	29.306 (26.548–34.532)	25.827 (23.598–29.368)	19.504 (18.070–21.240)	18.104 (16.761–19.756)
LC_{80}	32.627 (29.012–40.269)	28.886 (26.031–34.051)	21.317 (19.710–23.577)	19.731 (18.221–21.859)
LC_{90}	37.865 (32.651–50.080)	33.737 (29.614–42.106)	24.114 (22.037–27.490)	22.230 (20.300–25.348)

Values in parentheses are lower and upper bound at 95% confidence limit.

Table 3
24 h–96 h lethal concentrations (LC_{10}–LC_{90}) in mg/L of mixed pesticides (chlorpyrifos 'CPF' and dichlorvos 'DDVP') to common carp.

Toxicity	24 h (CPF and DDVP)	48 h (CPF and DDVP)	72 h (CPF and DDVP)	96 h (CPF and DDVP)
LC_{10}	0.158 and 5.657 (0.085–0.211, 3.018–7.535)	0.083 and 2.953 (0.035–0.123, 1.252–4.403)	0.077 and 2.760 (0.045–0.105, 1.604–3.751)	0.067 and 2.385 (0.038–0.091, 1.369–3.243)
LC_{20}	0.235 and 8.385 (0.161–0.291, 5.745–10.369)	0.127 and 4.543 (0.070–0.171, 2.491–6.117)	0.104 and 3.718 (0.068–0.134, 2.433–4.765)	0.087 and 3.112 (0.056–0.112, 1.998–4.011)
LC_{30}	0.312 and 11.137 (0.244–0.384, 8.702–13.711)	0.174 and 6.198 (0.113–0.220, 4.036–7.861)	0.129 and 4.608 (0.092–0.159, 3.271–5.689)	0.106 and 3.771 (0.073–0.131, 2.614–4.693)
LC_{40}	0.398 and 14.193 (0.325–0.522, 11.593–18.629)	0.226 and 8.083 (0.167–0.279, 5.958–9.966)	0.155 and 5.536 (0.117–0.186, 4.188–6.657)	0.124 and 4.443 (0.092–0.151, 3.276–5.389)
LC_{50}	0.499 and 17.804 (0.403–0.733, 14.376–26.158)	0.290 and 10.359 (0.231–0.363, 8.235–12.951)	0.184 and 6.572 (0.147–0.218, 5.234–7.772)	0.145 and 5.180 (0.113–0.173, 4.021–6.169)
LC_{60}	0.626 and 22.333 (0.486–1.057, 17.350–37.742)	0.372 and 13.277 (0.302–0.498, 10.779–17.774)	0.219 and 7.801 (0.181–0.257, 6.459–9.189)	0.169 and 6.038 (0.137–0.200, 4.893–7.126)
LC_{70}	0.797 and 28.463 (0.586–1.587, 20.927–56.640)	0.485 and 17.314 (0.384–0.732, 13.723–26.123)	0.263 and 9.372 (0.222–0.314, 7.936–11.205)	0.199 and 7.114 (0.167–0.236, 5.949–8.434)
LC_{80}	1.059 and 37.804 (0.724–2.573, 25.843–91.848)	0.662 and 23.623 (0.495–1.183, 17.673–42.233)	0.325 and 11.618 (0.276–0.406, 9.836–14.509)	0.242 and 8.620 (0.205–0.295, 7.307–10.512)
LC_{90}	1.570 and 56.036 (0.963–5.066, 34.390–180.823)	1.018 and 36.346 (0.689–2.352, 24.583–83.951)	0.438 and 15.648 (0.359–0.601, 12.827–21.439)	0.315 and 11.250 (0.263–0.414, 9.386–14.775)

Values in parentheses are lower and upper bound at 95% confidence limit.

Table 4
Joint toxicity of chlorpyrifos and dichlorvos to common carp.

Toxicity	Additive index (AI)			
	24 h	48 h	72 h	96 h
LC_{10}	0.279	1.130	0.989	0.989
LC_{20}	0.033	0.638	0.725	0.817
LC_{30}	−0.130	0.356	0.556	0.699
LC_{40}	−0.290	0.153	0.424	0.603
LC_{50}	−0.460	−0.009	0.311	0.517
LC_{60}	−0.653	−0.174	0.206	0.434
LC_{70}	−0.889	−0.380	0.103	0.349
LC_{80}	−1.209	−0.668	−0.008	0.255
LC_{90}	−1.746	−1.170	−0.167	0.132

AI value equal, less or greater than zero indicates additive, antagonistic or synergistic effect, respectively.

dose at 48 h ($P < 0.01$), 72 h and 96 h ($P < 0.001$); dichlorvos low dose at 96 h ($P < 0.01$) and dichlorvos high dose at 72 h and 96 h ($P < 0.001$) of exposure. No significant difference from its 1 h observation was found in mixture low dose, but in contrast it was significantly higher ($P <$ 0.05–0.001) in mixture high dose treated fish over the entire exposure period (Fig. 1).

The average buccal movements over the whole experimental period (1 h to 96 h) clearly showed significant elevated buccal movements in all pesticide treatments ($P < 0.001$), where the highest movements were record in the dichlorvos high dose treatment (Fig. 2). Compared to the low dose, the high dose always exhibited higher buccal movements in all pesticide groups- chlorpyrifos ($P < 0.05$), dichlorvos ($P < 0.01$) and mixture ($P < 0.05$) (Fig. 2).

When average buccal movements of all pesticide exposed fish at different time points were compiled and compared to their controls at the same time point, significantly higher movements ($P < 0.001$) were observed throughout the experimental period except at 1 h of exposure (Fig. 3). Average buccal movements recorded in pesticide exposed fish at different time points (24 h, 48 h, 72 h and 96 h) were also significantly higher ($P < 0.001$) compared to the 1 h observation. Similarly, buccal movements recorded at later phase of the experiment (72 h and 96 h) were significantly higher than the initial phase (24 h and 48 h) ($P <$ 0.05–0.001) but statistically no difference was observed either between 24 h and 48 h or between 72 h and 96 h of observations. In control fish, the buccal movements were constant and very stable throughout the whole experimental period (Fig. 3).

3.2.2. Feeding trial

Control fish exhibited feeding attempts at every observation time that ranged between 7.67 ± 2.25 and 10.40 ± 1.52 times per 5 min. After pesticide exposure, feeding attempt by fish was either reduced or completely ceased. Among the pesticide exposed fish the highest feeding attempt was observed in the dichlorvos high dose fish at 96 h which was 3.00 ± 4.29, but this was still very low compared to the control range (Table 5).

The average feeding data (compiled from 1 h to 96 h) clearly exhibited that all pesticides used in this experiment significantly impaired ($P < 0.001$) feeding behavior of fish but no difference between low and high dose of the same pesticide was seen (Fig. 4). The time series data analysis showed the average feeding attempts by pesticide exposed fish was significantly lower ($P < 0.001$) compared to the control fish at each time point of observation, but again no difference was noticed in pair wise comparison neither among the exposed nor among the control groups (Fig. 5).

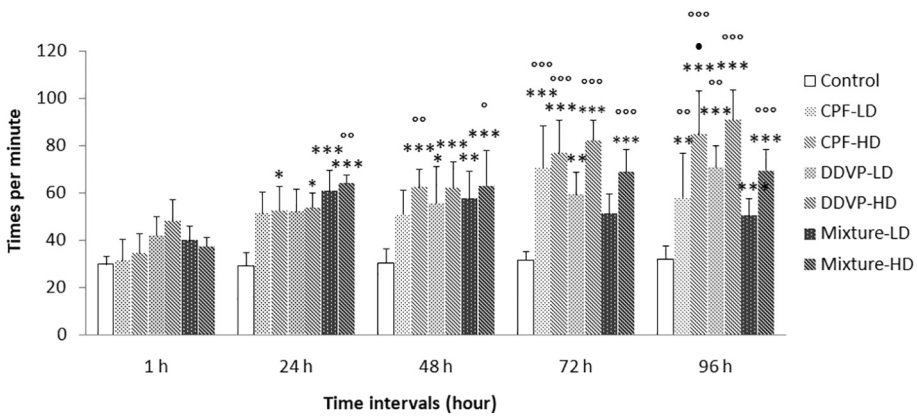

Fig. 1. Buccal movements by common carp at different time intervals. LD = 10% and HD = 50% of the 96 h-LC_{50} of the respective pesticides. Values are mean ± SD (n = 6). Asterisk denotes significant difference between control and other treatment groups during the same exposure period (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); white circle denotes significant difference in the same treatment groups during different exposure periods compared to 1 h of exposure (°$P < 0.05$; °°$P < 0.01$; °°°$P < 0.001$); dark circle denotes significant difference between low and high dose of the same pesticide treatment and exposure period (●$P < 0.05$).

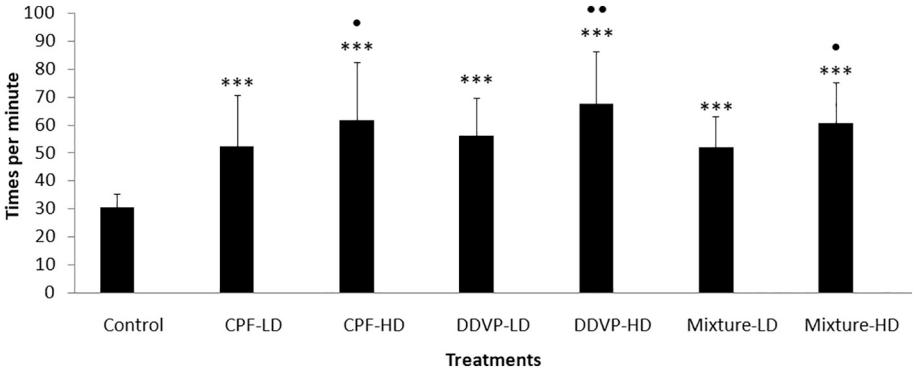

Fig. 2. Buccal movements by common carp during 96 h exposure to tested pesticides. LD = 10% and HD = 50% of the 96 h-LC_{50} of the respective pesticides. Values are mean ± SD (n = 30). Asterisk denotes significant difference between control and other treatment groups (***$P < 0.001$); dark circle denotes significant difference between low and high dose of the same pesticide treatment (●$P < 0.05$; ●●$P < 0.01$).

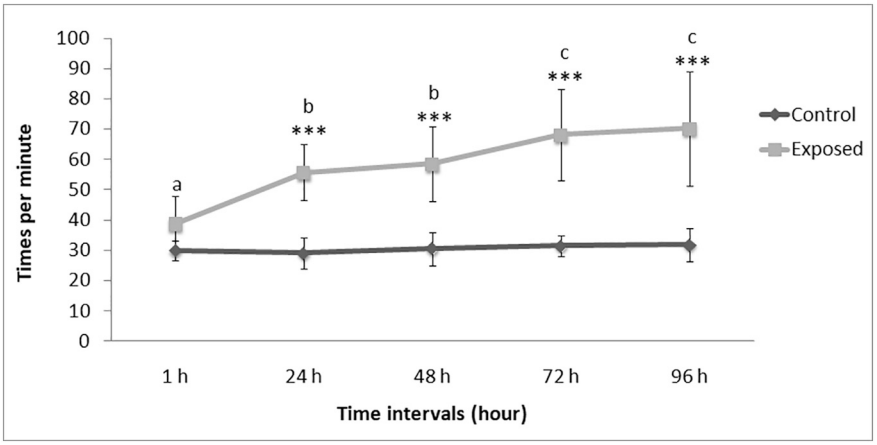

Fig. 3. Buccal movements in pesticides exposed common carp at different time intervals. Values are mean ± SD (n = 6 for control and n = 36 for pesticide exposed groups). Asterisk denotes significant difference between control and exposed group at each time period (***$P < 0.001$). Different letters (a, b, c) represent significant difference among the exposed groups from 1 to 96 h of exposure.

3.2.3. *Aerobic metabolism*

Oxygen consumption rate of fish increased to some extent throughout the experimental period following all pesticide exposures, although not always remarkable. Significantly higher oxygen consumption was observed in the mixture low dose ($P < 0.05$) and high dose ($P < 0.001$) during 0–24 h of exposure. From 24 h onwards, significant elevation in oxygen consumption was found only in the high pesticide dose of dichlorvos and mixture treatments ($P < 0.05$–0.001) (Fig. 6).

Statistical analysis of the compiled data of the whole experimental period reveals that all pesticides were effective in elevating oxygen consumption rate of fish ($P < 0.01$–0.001) and the highest oxygen uptake was noticed in high dose of mixture pesticide treated fish. Comparing low and high dose of the same pesticide, a difference ($P < 0.001$) was noticed only between dichlorvos and between mixed pesticide treatment groups (Fig. 7).

Compiled data analysis at different time intervals clearly indicates that pesticide exposed fish always consumed higher oxygen levels compared to their respective controls, however the difference was

Table 5
Feeding attempts (number/5 min) by common carp at different exposure periods.

Time intervals	Control	CPF-LD (0.044 mg/L)	CPF-HD (0.220 mg/L)	DDVP-LD (1.570 mg/L)	DDVP-HD (7.853 mg/L)	Mixture-LD (CPF- 0.044 mg/L, DDVP- 1.570 mg/L)	Mixture-HD (CPF- 0.220 mg/L, DDVP- 7.853 mg/L)
1 h	10.40 ± 1.52	0.00*	0.5 ± 0.84	0.00*	1.00 ± 2.00	0.17 ± 0.41	0.00*
24 h	9.17 ± 2.32	0.00*	0.00*	0.00*	0.17 ± 0.41	0.00*	0.00*
48 h	8.67 ± 1.37	0.17 ± 0.41	0.17 ± 0.41	1.50 ± 1.64	1.50 ± 2.51	0.67 ± 1.21	0.00*
72 h	7.67 ± 2.25	0.33 ± 0.52	0.17 ± 0.41	2.83 ± 3.25	1.17 ± 1.47	0.33 ± 0.82	0.67 ± 0.82
96 h	8.67 ± 1.37	0.50 ± 0.84	0.20 ± 0.45	1.83 ± 2.71	3.00 ± 4.29	0.17 ± 0.41	0.50 ± 0.84

Values are mean ± SD (n = 6).
* Significant differences between control and other treatment groups during same exposure period ($P < 0.05$).

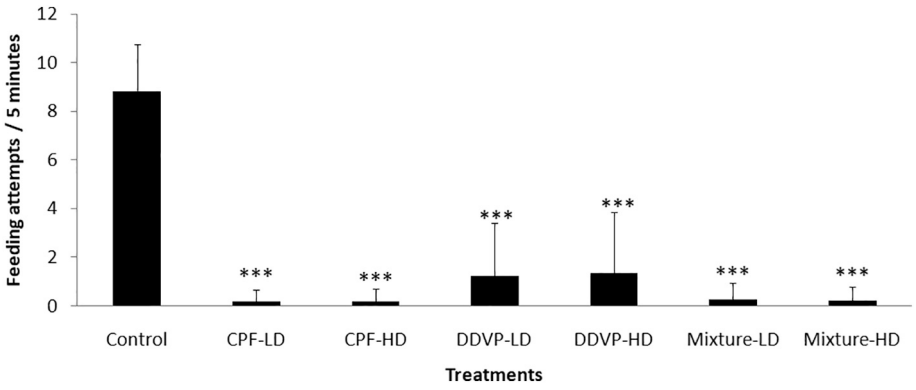

Fig. 4. Feed intake by common carp during 96 h exposure to tested pesticides. LD = 10% and HD = 50% of the 96 h-LC_{50} of the respective pesticides. Values are mean ± SD (n = 30). Asterisk denotes significant difference between control and other treatment groups (***$P < 0.001$). No significant difference was observed between low and high dose of the same pesticide treatments.

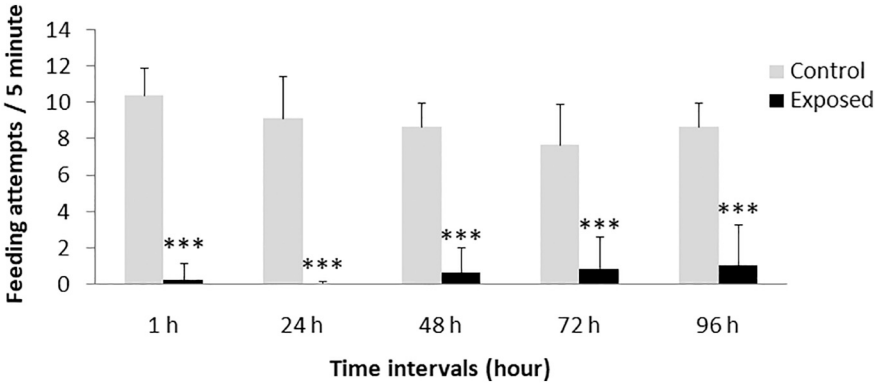

Fig. 5. Feeding attempt by pesticides exposed common carp at different time intervals. Values are mean ± SD (n = 6 for control and n = 36 for pesticide exposed groups). Asterisk denotes significant difference between control and exposed group at each time period (***$P < 0.001$). No significant difference was observed either among control or exposed groups from 1 to 96 h of exposure.

significant only during 24–48 h ($P < 0.01$) and 48–72 h ($P < 0.05$). Within the controls or exposed groups, the oxygen consumption was stable over the entire exposure period (Fig. 8).

3.2.4. Fish behavior

During lethal toxicity assessment, fish were closely monitored to document their behavior. Important behavioral changes were rapid buccal movements, aggregating at the corners of the test chamber, loss of equilibrium and hanging vertical with the head upwards or downwards in the water column, loss of schooling behavior, abrupt hyperactivity showing fast movements in spiral fashion, excess mucus secretion, dull and faded body color. During the later phase of lethal toxicity exposure, fish were lying motionless on the bottom of the test chamber. Intensity of behavioral changes was dose-dependent for all pesticides.

4. Discussion

The results obtained from the lethal toxicity experiments reveal that chlorpyrifos is a highly toxic and dichlorvos is a moderately toxic pesticide to common carp. Similar observations were documented in golden mahseer (*Tor putitora*; Kunwar et al., 2021) and mrigal (*Cirrhinus*

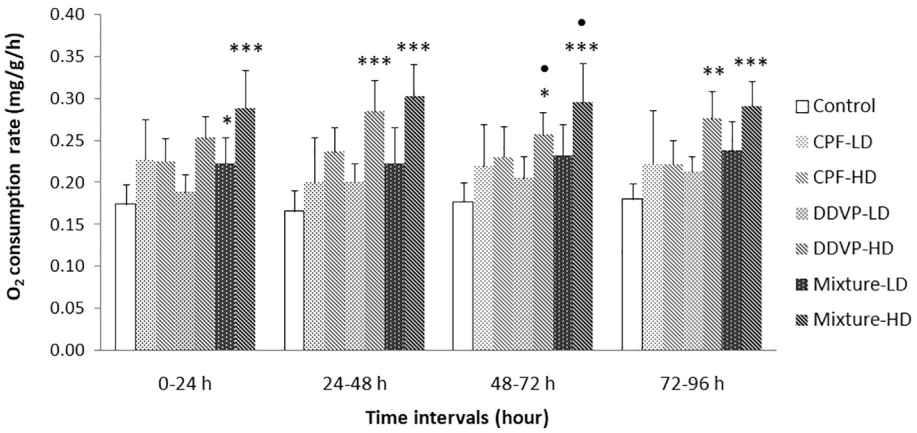

Fig. 6. Oxygen consumption by common carp at different time intervals. LD = 10% and HD = 50% of the 96 h-LC_{50} of the respective pesticides. Values are mean ± SD, n = 6. Asterisk denotes significant difference between control and other treatment groups during the same exposure period (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$); dark circle denotes significant difference between low and high dose of the same pesticide treatment and exposure period ($^{\bullet}P < 0.05$). No significant difference was observed among the same treatment groups during different exposure periods.

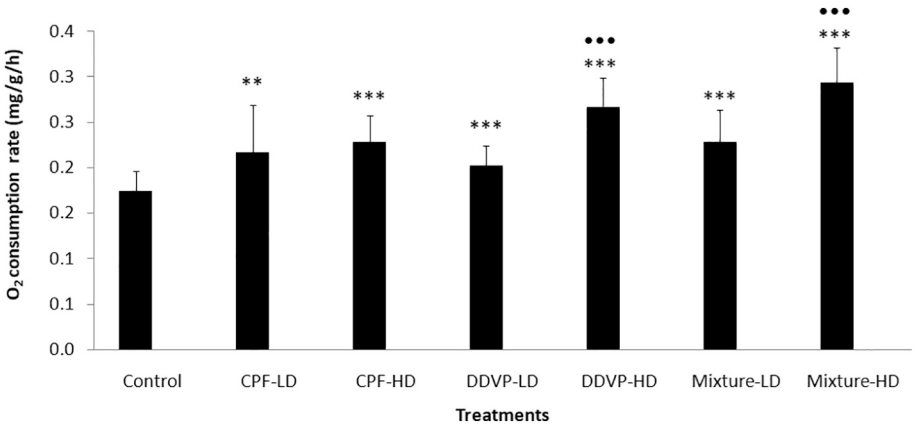

Fig. 7. Oxygen consumption by common carp during 96 h exposure to tested pesticides. LD = 10% and HD = 50% of the 96 h-LC_{50} of the respective pesticides. Values are mean ± SD (n = 24). Asterisk denotes significant difference between control and other treatment groups (**$P < 0.01$; ***$P < 0.001$); dark circle denotes significant difference between low and high dose of the same pesticide treatment ($^{\bullet\bullet\bullet}P < 0.001$).

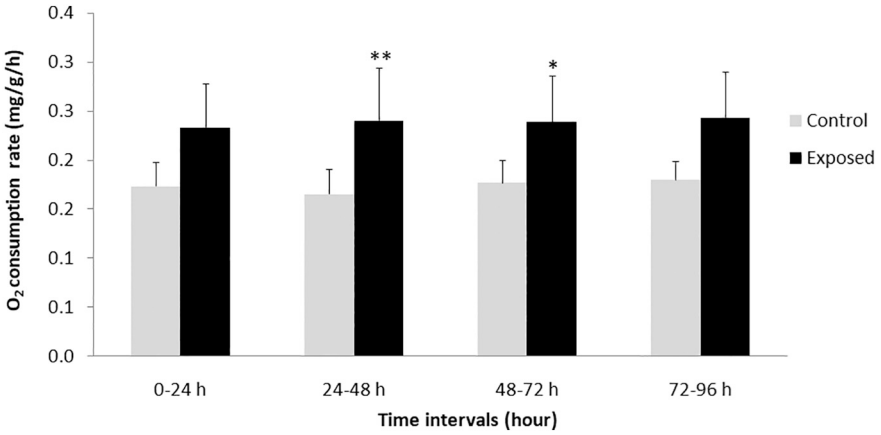

Fig. 8. Oxygen consumption by common carp following exposure to pesticides at different time intervals. Values are mean ± SD (n = 6 for control and n = 36 for pesticide exposed groups). Asterisk denotes significant difference between control and exposed group at each time period (*$P < 0.05$; **$P < 0.01$). No significant difference was observed among controls as well as exposed groups from beginning till end of the experiment.

mrigala; unpublished result). Halappa and David (2009), Banaee et al. (2013) and Xing et al. (2015) reported 96 h median lethal concentrations of chlorpyrifos to common carp as 0.16, 0.20 and 0.58 mg/L, respectively. Similarly, 96 h median lethal concentrations of dichlorvos to common carp were reported to be 9.41 mg/L (Ural and Çalta, 2005) and 21.11 mg/L (Laxmi et al., 2019). These findings corroborate to our estimated toxicity range of chlorpyrifos and dichlorvos to common carp.

Aquatic organisms are often exposed to mixtures of different pesticides, therefore findings on combined pesticide toxicity become crucial, particularly when the combined effects turn out to be synergistic. Wang et al. (2015) reported synergistic effect of triazophos and malathion as well as triazophos and carbofuran to common carp. Additive or more than additive toxicity of pesticide mixtures was found in Pacific salmon (*Oncorhynchus kisutch*; Laetz et al., 2009) and zebrafish (*Danio rerio*;

Wang et al., 2017; Wu et al., 2018). Although individual toxicity of chlorpyrifos and dichlorvos has already been assessed, their combined effect to common carp was still unknown; this study is the first to elucidate synergistic effect of chlorpyrifos and dichlorvos co-exposure to common carp.

The synergistic effect of chlorpyrifos and dichlorvos found in this experiment was probably due to the same mode of action as AChE inhibitors (LeBlanc et al., 2012). Inhibition of this enzyme is dose dependent (Singh et al., 2018; Stepić et al., 2013). The synergistic effect of endosulfan and temephos also manifested a greater inhibition of the AChE activity compared to the degree of inhibition incited by these individual insecticides (Stepić et al., 2013). However, we have to take into account that pesticide doses in our mixture treatments were twice than that of the individual pesticide treatments, therefore high inhibition of the enzyme was obvious which caused a high mortality rate in mixture pesticide treatments.

A possible explanation for the synergistic effect of the pesticide mixture noted in the present study could be linked to carboxylesterases (CaEs) mediated pesticide detoxification processes. CaEs can detoxify pesticide by hydrolysis (Jokanovic, 2001; Wheelock et al., 2005), direct binding and sequestration (Jokanovic, 2001; Maxwell, 1992). Suppression of CaEs, although not measured in our experiment, might have occurred in mixture pesticides treated fish. Due to inhibition of CaEs, they could not play protective role against xenobiotic threat, eventually leading to synergistic outcome in pesticide co-exposure. Likewise, Barata et al. (2004) also documented elevated pesticide toxicity due to inhibition of the CaEs enzyme. Another underlying mechanism responsible for the synergistic effect might be associated with the elevated activity of cytochrome P450 enzymes in the fish tissue following exposure to the mixture pesticide. This enzyme is known to enhance the transformation of organophosphate to more toxic metabolite, which has high AChE inhibiting property. A similar toxicity mechanism was also described by Wang et al. (2017) during co-exposure of phoxim and atrazine.

Fish behavior is a highly sensitive and non-invasive tool for toxicity assessment, therefore there is increasing trend of incorporating behavioral responses in the toxicological studies. During our lethal exposure experiment, common carp exhibited behavioral changes that corroborate with the earlier studies in common carp exposed with chlorpyrifos (Halappa and David, 2009) and dichlorvos (Gunde and Yerli, 2012; Ural and Çalta, 2005). Elevated buccal movements in common carp in response to pesticides was identical to our previous experiment with golden mahseer (Kunwar et al., 2021) and also corroborates to the findings of other researchers (Saha et al., 2016; Soni and Verma, 2018). Buccal movements were gradually increased from the beginning until the end of the experiment. This clearly indicates that fish were in respiratory distress and put more effort to maintain a high oxygen demand, despite their lower body movement and food ingestion when treated with pesticides. Increased buccal movements by chlorpyrifos have also been reported in African catfish (*Clarias gariepinus*; Nwani et al., 2013) and tilapia (*Oreochromis mossambicus*; Padmanabha et al., 2015). Food uptake by common carp was severely compromised during pesticide exposure. This phenomenon was also observed in chlorpyrifos or dichlorvos treated fish (Halappa and David, 2009; Kavitha and Rao, 2008; Padmanabha et al., 2015; Pavlov et al., 1992). Significant reduction of feeding during pesticide exposure might be a strategy to reduce energy requirement for digestion (Halappa and David, 2009) and utilize energy for chemical detoxification. Moreover, the diminished feeding may be linked to unpleasant pesticide smells and altered gustatory sensitivity of common carp to chlorpyrifos, dichlorvos and their mixture. A study on common carp documented that benthiocarb, isoprothiolane and fenitrothion can negatively affect nerve innervating terminal buds sensitivity in the lip region (Ishida and Kobayashi, 1995).

Fish gills are directly exposed to water and any contamination in medium is immediately reflected by altering oxygen uptake (Padmanabha et al., 2015) which is used as an important indicator for stress evaluation (Patil and David, 2008). In our study, all pesticide treatments significantly increased oxygen consumption. There are number of studies that documented the effect of pesticides on fish respiration. The aerobic metabolism of tilapia was increased with increasing concentration of chlorpyrifos (Padmanabha et al., 2015). Similarly, rohu (*Labeo rohita*) exposed to malathion organophosphate were recorded up to 80.5% increment in oxygen uptake (Patil and David, 2008). In contrast to our study, reduction in oxygen consumption was reported in dichlorvos exposed grass carp (*Ctenopharyngodon idella*; Tilak and Kumari, 2009). Such discrepancies in oxygen uptake might be due to different toxicity mechanism of dichlorvos on different fish species. Cabofuran mediated respiratory toxicity to Nile tilapia (*Oreochromis niloticus*; Campos-garcia et al., 2015) and *Astyanax ribeirae* (Barbieri et al., 2018) was different though same concentration of carbofuran (0.5 mg/L) was used in both experiments. In Nile tilapia there was no effect on oxygen consumption rate but in *A. ribeirae* a significant drop in oxygen consumption was documented. The time series-wise data analysis reveals that oxygen uptake was elevated in the beginning of the pesticide exposure and subsequently remained stable over the whole experimental period; but buccal movements continued to gradually increase from beginning until end of the experiment. With increase in the ventilation rate, oxygen consumption rate is also supposed to rise, but that did not happen in our experimental animal. This scenario indicates that the efficiency of the respiratory organ was compromised by pesticide exposure. Most probably, the excess mucus production during pesticide exposure maximized the diffusion distance that affected respiratory gas exchange between blood and water, eventually limiting the oxygen consumption by fish despite of elevated ventilation rate.

Similar to the synergistic findings in the lethal toxicity experiment, sub-lethal effects of the pesticide mixtures were also expected to be higher than individual pesticide treatments; but the responses recorded under sub-lethal exposure did not support our prediction. The highest feeding suppression was found in chlorpyrifos treated fish, the highest buccal movements were observed in dichlorvos treated fish and the highest oxygen uptake was found in mixture pesticide treated fish. These responses exemplify that under our experimental condition, chlorpyrifos and dichlorvos mixture was not additive at sub-lethal levels despite their synergistic effect in lethal exposure. Similar results were documented by Imam et al. (2018) on a mammalian model where effects produced by chlorpyrifos and dichlorvos in combination were not significantly greater than the effects caused by individual pesticide. They further reported that some of the responses in the combined treatment were even lower compared to the individual pesticide treatments. Our results indicate that respiratory failure most likely was not the primary cause of death but that it was probably related to neurological disorder. We base this conclusion on the fact that despite increased buccal movements, no significant effect on respiratory efficiency of fish was observed. On the other hand, they exhibited clear erratic swimming before death indicating AChE mediated behavior.

5. Conclusion

Our findings suggest that toxicity of chlorpyrifos was comparatively higher than that of dichlorvos. These organophosphate pesticides produced synergistic effect in co-exposure at lethal levels, therefore more caution is needed while anticipating pesticide exposure threats, developing water quality standards and guidelines for the aquatic organisms. Environmental risk assessment becomes more realistic when mixture toxicity of pesticide is given due consideration. Despite synergistic result in lethal exposure, non-additive sub-lethal effects were observed; therefore, we conclude that mortality of fish was probably not due to respiratory distress but might be related to biochemical or neurological disorders that need further investigations.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chlorpyrifos and dichlorvos in combined exposure reveals antagonistic interaction to the freshwater fish Mrigal, *Cirrhinus mrigala*

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Abstract

Toxicity imposed by organophosphate pesticides to the freshwater cultivable fish species mrigal (*Cirrhinus mrigala*) was assessed under laboratory conditions. Healthy juveniles were exposed to chlorpyrifos, dichlorvos, and their equitoxic mixture in geometric series. Median lethal concentrations of chlorpyrifos were found to be 0.906 (0.689–1.179), 0.527 (0.433–0.633), 0.435 (0.366–0.517) and 0.380 (0.319–0.450) mg/L and dichlorvos were found to be 38.432 (33.625–47.866), 22.477 (19.047–26.646), 12.442 (9.619–14.196) and 11.367 (9.496–13.536) mg/L after 24 h, 48 h, 72 h and 96 h of exposure respectively. Surprisingly, the joint toxicity of these organophosphates in the binary mixture was less than additive during most of the exposure periods. Behavioral changes exhibited by individual as well as mixture pesticide treatments were loss of schooling behavior, aggregating at corners of the test chamber, elevated opercular beatings, surplus mucus secretion, slight color changes and sudden and rapid body movements before death. Loss of fish equilibrium was noticed only in chlorpyrifos treated fish, whereas sluggish behavior was noticed only in mixture pesticide treatment. Such behavioral studies can be applied as a non-invasive bio-monitoring tool for water quality assessment for fish growth and development. Despite the same mode of action of both pesticides, the antagonistic action in the binary mixture is an interesting outcome of this research that requires further investigation for a lucid understanding of the joint toxicity mechanism of such pesticides.

Keywords: Additive index · Fish behavior · Growth · Organophosphate · Pesticide mixture

Introduction

Pesticides are toxic compounds and their capacity to harm fish and aquatic animals depends on exposure time, concentration, and persistence in the environment (Sabra and Mehana 2015). Pesticides can be classified as minimal, slight, moderate, high, extreme and super-toxic compounds based on their LC_{50} values (Sabra and Mehana 2015). There are a number of studies that estimated median lethal concentration (LC_{50}) of chlorpyrifos (Bhatnagar et al. 2017), dichlorvos (Velmurugan et al. 2009, Srivastava et al. 2012), carbaryl, carbofuran, profenfos and triazophos (Mahboob et al. 2015), endosulfan (Ilyas and Javed 2013) and malathion (Rauf 2015) in mrigal (*Cirrhinus mrigala*).

Pesticides are one of the most potentially harmful chemicals and even small amounts of pesticides can be fatal (Jokanović, 2009), therefore increasing pesticide application is a serious threat to human health and biodiversity.

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Chlorpyrifos is a broad-spectrum organophosphate pesticide. It is extensively used for pest control throughout the world for agriculture and domestic purposes (Ali et al. 2009, Sun et al. 2015). Dichlorvos is another commonly used organophosphate pesticide (Ural and Çalta 2005, Sun et al. 2015). It is one of the main chemical agents used in bath treatment against fish ectoparasites (Varó et al. 2007). A small fraction of the applied pesticide reaches the target pests and the majority of it is released into the environment (Tišler et al. 2009). During the rainy season, the pesticides from agricultural fields are flushed away and drained into the aquatic system (Adhikari et al. 2004, Ramesh and Saravanan 2008) that alter physico-chemical parameters of water and ultimately affect the performance of aquatic organisms inhabiting there (Muthukumaravel et al. 2013). Since the final destination of all applied pesticides is the water bodies, organisms thriving there are always threatened by the mixture of various pesticides which can be more hazardous than single pesticide exposure. Laetz et al. (2009) also reported that pesticide mixtures can be much more toxic compared to single pesticide exposure.

Pesticides become fatal to fish at higher concentrations but even at lower concentrations they are able to generate biochemical modifications without any fatality (Kunwar et al. 2022). Such sub-lethal effects are generally ignored and receive less attention since no direct mortality is observed, but these effects ultimately determine the overall success of any species and their population. Behavioral change is one of the sub-lethal effects which is an important indicator of water pollution and stress (Chebbi and David 2010, Kesharwani et al. 2018). In recent studies, behavioral observations have gained popularity because they are noticed at low chemical concentrations and are non-invasive.

The global fisheries sector is threatened by aquatic pollution and pesticides are one of the serious sources of pollution. In this context, we selected mrigal as a model organism to evaluate the toxicity of pesticides. This is an important aquaculture candidate species in Nepal which is successfully cultivated under single stocking and multiple harvesting techniques. This farming technique, locally called Chhadi farming, is gaining popularity in Nepal (Mishra and Kunwar 2014, Kunwar and Adhikari 2016 and 2017, Adhikari et al. 2018). Chlorpyrifos and dichlorvos were selected for a toxicity assessment because they are commonly used in many countries (Sun et al. 2015) and their traces were detected in nature, fish and fisheries products (Kafle et al. 2015, Singh et al. 2015, Akoto et al. 2016, Zahran et al. 2018, Nag et al. 2020). Chlorpyrifos residue was reported to be 0.0091 ± 0.0020 mg/L in the river Deomoni, West Bengal, (Singh et al. 2015). Similarly, water sampled from the Chilika lake, India contained chlorpyrifos with concentration ranging 0.019–2.73 µg/L (Nag et al. 2020).

Individual effects of chlorpyrifos and dichlorvos on mrigal had already been documented but their joint toxic effect on this species is still lacking. Pesticides in a mixture can interact with each other (additive or competitive) to modulate the overall resultant toxicity effect, therefore water quality assessment based on single pesticide toxicity can be misleading. Wang et al. (2015) had also highlighted that single pesticide risk assessments are more likely to underestimate the impacts of these pesticides to aquatic organisms. Therefore, the present study was designed with the aim to elucidate lethal toxicity as well as behavioral manifestation of mrigal in response to chlorpyrifos and dichlorvos not only individually but also in combination. The results obtained from this study are expected to enrich the existing knowledge on joint pesticide toxicity.

Materials and methods

Fish acclimatization

Mrigal hatchlings (one week after hatching) were purchased from Fish Pure-line Breed Conservation and Promotion Centre, Bhairahawa, Rupandehi, Nepal and transported in oxygen-packed polythene bags to Central Fisheries Promotion and Conservation Centre (CFPCC) Balaju, Kathmandu, Nepal. Hatchlings were grown in an earthen pond for two months until they reached finger size. Healthy fingerlings with uniform size (exact weight provided below) were transferred to a 350-L indoor glass aquarium of CFPCC for acclimatization. The aquarium was fitted with a water filter and aeration system. Fish were regularly fed ad libitum with commercial pellet feed having 32% protein (Sreema feed Pvt. Ltd., India). Uneaten food and fecal matter were removed with the help of a scoop net and siphon. Everyday approximately half of the aquarium water was exchanged with freshwater to maintain optimum water quality. Water pumps, air stones, pipes and filters were cleaned twice a week. Water temperature, pH, dissolved oxygen and total ammonia ranged between 23.97–24.62 °C, 7.68–7.85, 5.80–6.74 mg/L and 0.20–0.23 mg/L, respectively. Fish were acclimatized for 15 days before using them for the lethal toxicity experiment.

Pesticides selection

Two pesticides-chlorpyrifos and dichlorvos were selected for the present toxicity experiment. These are commonly applied organophosphate insecticides in crops for pest control. Dichlorvos (G-VAN-80%) Greenriver Industry Co., Ltd., ShenZhen, China and chlorpyrifos (Dursban-20%) Dow AgroSciences Pvt. Ltd., India were the commercial-grade pesticides used in this study.

Lethal toxicity test

Lethal toxicity tests were conducted according to the standard guidelines (OECD 1992) in semi-static conditions. Well cleaned glass aquaria (35-L capacity) with 25-L water volume were used for the test. The average weight of the fish used in this experiment was 5.52 ± 0.91 g (mean ± SD). Feeding was ceased 24 hours prior to the exposure experiments to avoid any interference of waste with pesticides. Stock solutions were freshly prepared in distilled water and calculated amounts of the solution were added in the aquaria to obtain five different pesticide concentrations in geometric series (chlorpyrifos: 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L; dichlorvos: 4.0, 8.0, 16.0, 32.0 and 64.0 mg/L). The exposure range was determined by performing a range finding test for the pesticides chlorpyrifos and dichlorvos. The actual concentration of the pesticide in the water was not measured but the water was renewed on a daily basis to maintain the desired pesticide concentration at the same level (Nwani et al. 2013). There were four replicates for each concentration with 5 five fish in each group. In total 200 fish were used for the lethal toxicity tests. Exposed fish were regularly monitored and dead fish were immediately removed from the aquaria; no fish died in the control. Mortality was recorded after 24 h, 48 h, 72 h and 96 h of exposure. The mortality data obtained from chlorpyrifos and dichlorvos treatments were analyzed to estimate lethal concentrations (24 h to 96 h-$LC_{10–90}$) of both pesticides by using a log probit analysis program.

After estimation of individual pesticide toxicity, their joint toxicity was assessed. For this, five geometric series of pesticides mixtures were prepared by adding both pesticides in equitoxic concentration i.e. 12.5%, 25%, 50%, 100% and 200% 96 h-LC_{50} of chlorpyrifos and dichlorvos. Fish mortality was recorded as described above. As before, no fish mortality was observed in the control. Similar to individual pesticides, 24 h to 96 h-LC_{10} to LC_{90} of the mixture pesticide toxicity was also calculated; where LC_{10} and LC_{90} indicate pesticide concentrations required to kill 10% and 90% of the fish population, respectively.

Joint toxicity assessment

The joint toxicity of pesticide was assessed by the additive index (AI). This was calculated according to Marking (1985).

$$AI = (1/S) - 1 \text{ for } S \leq 1 \text{ and,}$$

$$AI = 1 - S \text{ for } S > 1$$

where, AI represents additive index and S represents the sum of biological activity

$$S = (A_m/A_i) + (B_m/B_i)$$

where, A and B represent two different pesticides, 'm' represents LC_{50} of pesticides in mixture, 'i' represents LC_{50} of individual pesticides.

AI values equal, greater or less than zero indicates additive, synergistic or antagonistic action of the pesticide mixture respectively.

Fish behavior

Fish exposed to chlorpyrifos, dichlorvos and mixture pesticides in geometric series (doses mentioned above) for lethal toxicity assessment were carefully observed during the whole experimental period and their behavior like body movements, operculum movements, color change, swimming pattern, schooling behavior, mucus secretion was recorded. Although the behavior study was started with five concentrations of each pesticide, data from all treatments could not be presented due to fish mortality in higher pesticide concentrations.

Data presentation and analysis

The average weight, standard deviation and mortality percentage of fish were calculated in Microsoft Excel. The diagrams were also prepared in Microsoft Excel. The lethal concentrations (24 h, 48 h, 72 h and 96 h LC_{10} to LC_{90}) of individual and mixture pesticides were calculated by log probit analysis using statistical program SPSS version 20. The lethal concentration data are presented with their upper and lower limit at a 95% confidence interval. The joint toxicity of the pesticides was analyzed in Microsoft Excel based on formulae described by Marking (1985).

Results

Lethal toxicity of chlorpyrifos

Lethal toxicity testing of chlorpyrifos was conducted in five different concentrations ranging from 0.25 mg/L to 4 mg/L. Chlorpyrifos at a low dose (0.25 mg/L) was slightly toxic to fish where mortality started at a later phase (after 72 h) where only 10% of the fish population died by the end of the experiment (96 h). In contrast, chlorpyrifos concentrations 1 mg/L, 2 mg/L and 4 mg/L caused 100% fish mortality after 72 h, 48 h and 24 h of exposure periods, respectively (Fig. 1).

The median lethal concentrations (LC_{50}) of chlorpyrifos with their 95% confidence limit were found to be 0.906 (0.689-1.179), 0.527 (0.433–0.633), 0.435 (0.366–0.517) and 0.380 (0.319–0.450) mg/L at 24 h, 48 h, 72 h and 96 h, respectively (Table 1). The range of LC_{10} to LC_{90} at 24 h,

48 h, 72 h and 96 h were 0.312 (0.167–0.445) to 2.630 (1.873–4.737), 0.311 (0.203–0.388) to 0.894 (0.727–1.316), 0.287 (0.195–0.345) to 0.659 (0.547–0.973) and 0.251 (0.172–0.302) to 0.575 (0.479–0.829) mg/L, respectively, showing a decreasing trend of lethal pesticide concentration with increasing time of exposure (Table 1).

Lethal toxicity of Dichlorvos

Lethal toxicity of dichlorvos to mrigal was assessed by exposing fish to five different concentrations ranging from 4 mg/L to 64 mg/L. The lower dichlorvos concentrations (4 mg/L and 8 mg/L) exhibited no fish mortality during 96 h exposure. Other pesticide concentrations viz. 16 mg/L, 32 mg/L and 64 mg/L killed 100% of stocked fish after 96 h, 48 h and 24 h of exposure, respectively (Fig. 2).

The median lethal concentrations (LC_{50}) at 24 h, 48 h, 72 h and 96 h with 95% confidence limits were 38.432 (33.625–47.866), 22.477 (19.047–26.646), 12.442 (9.619–14.196) and 11.367 (9.496–13.536) mg/L, respectively (Table 2). The range of LC_{10} to LC_{90} at 24 h, 48 h, 72 h and 96 h were calculated to be 28.710 (20.917–32.898) to 51.447 (43.129–87.287), 17.453 (13.322–20.394) to 28.948 (24.723–38.344), 9.837 (6.003–11.713) to 15.736 (13.759–19.275) and 9.068 (6.821–10.655) to 14.250 (12.146–18.718) mg/L, respectively, showing a decreasing trend of lethal pesticide concentration with increasing time of exposure (Table 2). The acute toxicity results of both pesticides clearly indicate that chlorpyrifos is more toxic than dichlorvos to freshwater fish such as mrigal (Tables 1 and 2).

Lethal toxicity of mixture pesticides

To assess the lethal toxicity of chlorpyrifos and dichlorvos mixture, fish were exposed to 5 different pesticide mixtures

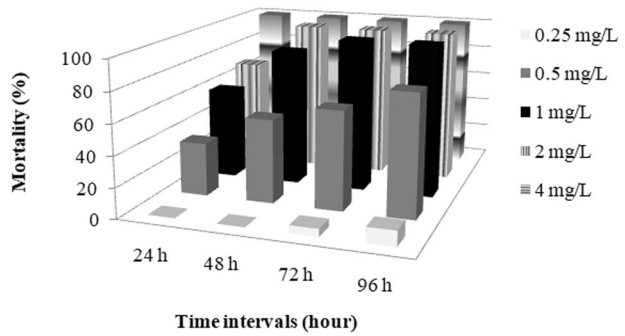

Fig. 1 Mortality of mrigal at different concentrations of chlorpyrifos (CPF)

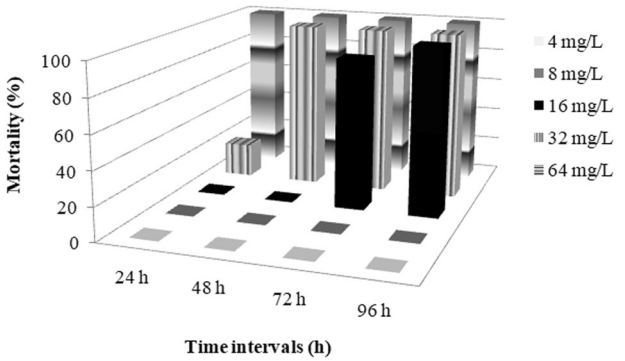

Fig. 2 Mortality of mrigal at different concentrations of dichlorvos (DDVP)

Table 1 24–96 h lethal concentrations (LC_{10}- LC_{90}) of chlorpyrifos to mrigal

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	0.312 (0.167–0.445)	0.311 (0.203–0.388)	0.287 (0.195–0.345)	0.251 (0.172–0.302)
LC_{20}	0.450 (0.280–0.603)	0.373 (0.268–0.451)	0.331 (0.246–0.389)	0.289 (0.217–0.340)
LC_{30}	0.586 (0.401–0.762)	0.425 (0.325–0.507)	0.367 (0.289–0.428)	0.320 (0.253–0.374)
LC_{40}	0.734 (0.536–0.945)	0.475 (0.379–0.566)	0.400 (0.329–0.469)	0.350 (0.287–0.409)
LC_{50}	0.906 (0.689–1.179)	0.527 (0.433–0.633)	0.435 (0.366–0.517)	0.380 (0.319–0.450)
LC_{60}	1.118 (0.867–1.503)	0.585 (0.489–0.718)	0.472 (0.403–0.576)	0.412 (0.352–0.500)
LC_{70}	1.401 (1.083–1.994)	0.654 (0.550–0.832)	0.515 (0.442–0.654)	0.450 (0.385–0.565)
LC_{80}	1.824 (1.376–2.838)	0.746 (0.622–1.001)	0.571 (0.486–0.768)	0.498 (0.424–0.660)
LC_{90}	2.630 (1.873–4.737)	0.894 (0.727–1.316)	0.659 (0.547–0.973)	0.575 (0.479–0.829)

Values in parentheses are lower and upper bound at 95% confidence limit

Table 2 24 h–96 h lethal concentrations (LC_{10} - LC_{90}) of dichlorvos to mrigal

Toxicity	24 h (mg/L)	48 h (mg/L)	72 h (mg/L)	96 h (mg/L)
LC_{10}	28.710 (20.917–32.898)	17.453 (13.322–20.394)	9.837 (6.003–11.713)	9.068 (6.821–10.655)
LC_{20}	31.733 (25.407–36.257)	19.037 (15.208–22.140)	10.663 (7.093–12.449)	9.800 (7.704–11.473)
LC_{30}	34.109 (28.721–39.581)	20.267 (16.638–23.622)	11.302 (7.980–13.041)	10.363 (8.371–12.159)
LC_{40}	36.279 (31.377–43.364)	21.381 (17.882–25.083)	11.877 (8.803–13.604)	10.871 (8.952–12.828)
LC_{50}	38.432 (33.625–47.866)	22.477 (19.047–26.646)	12.442 (9.619–14.196)	11.367 (9.496–13.536)
LC_{60}	40.713 (35.671–53.374)	23.630 (20.200–28.427)	13.033 (10.466–14.876)	11.887 (10.037–14.335)
LC_{70}	43.304 (37.710–60.428)	24.929 (21.415–30.603)	13.697 (11.384–15.738)	12.469 (10.606–15.304)
LC_{80}	46.546 (39.997–70.309	26.540 (22.812–33.534)	14.517 (12.430–16.988)	13.186 (11.260–16.602)
LC_{90}	51.447 (43.129–87.287)	28.948 (24.723–38.344)	15.736 (13.759–19.275)	14.250 (12.146–18.718)

Values in parentheses are lower and upper bound at 95% confidence limit

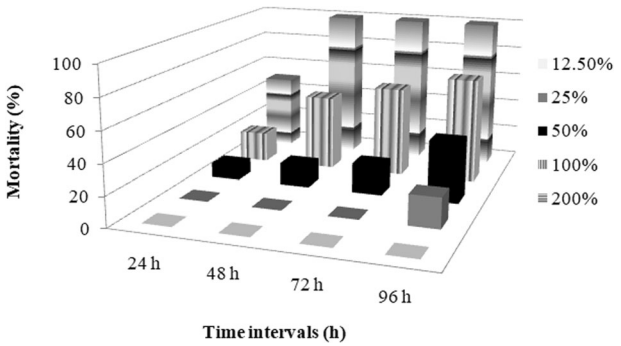

Fig. 3 Mortality of mrigal at different concentrations (12.5% to 200% of 96 h LC_{50}) of pesticide mixture (CPF and DDVP) where 96 h-LC_{50} of CPF- 0.380 mg/L and DDVP-11.367 mg/L

prepared in an equitoxic concentration ranging from 12.5% to 200% 96 h-LC_{50} values of the respective pesticide. 12.5% pesticide mixture caused no fish mortality whereas 25%, 50% and 100% of pesticide mixture killed 20%, 40% and 70% of the fish population respectively during 96 h of exposure. 200% pesticide mixture was highly toxic and killed all fish within 48 h of exposure (Fig. 3).

In the pesticides mixture, the LC_{50} values of chlorpyrifos with 95% confidence limit were 0.761 (0.535–1.665), 0.337 (0.279–0.413), 0.306 (0.252–0.374) and 0.217 (0.170–0.279) mg/L and dichlorvos were 22.774 (16.010–49.793), 10.088 (8.332–12.358), 9.155 (7.533–11.200) and 6.484 (5.089–8.333) mg/L at 24 h, 48 h, 72 h and 96 h, respectively (Table 3). In joint toxicity assessment, the dominating action of chlorpyrifos and dichlorvos mixture was found to be antagonistic (Table 4).

Fish behavior

In terms of body movement, fish treated with the pesticide mixture became hypo-active compared to chlorpyrifos and dichlorvos exposed fish. Such fish behavior was more intense with increasing concentrations of the pesticide mixture. Fish were unable to balance their body in chlorpyrifos treatments but such loss of equilibrium was not noticed in other pesticide treatments. Slight color changes were observed in fish; caudal and pectoral fins were reddish and the body became pale in higher dose of all treatment groups. Fish schooling behavior was influenced by chlorpyrifos, dichlorvos and the pesticide mixtures and changes became more distinct with increasing pesticide concentrations where swimming coordination among fish was lost and fish were scattered everywhere in the test chamber occupying a greater space than control fish. Frequently, fish were also aggregated at the corners of the test chambers in all pesticide treatments. In each treatment group, fish were overexcited and suddenly showed vigorous movements in different directions before death. The dead fish were found to be loaded with mucus around their respiratory organs in higher pesticide concentrations. Opercular movements of fish were also elevated after pesticide exposure in all treatment groups (Table 5).

Discussion

The first step in determining a chemical's safety threshold in the aquatic environment is to ascertain its lethal toxicity.

Table 3 24–96 h lethal concentrations (LC_{10}–LC_{90}) in mg/L of pesticide mixture (chlorpyrifos 'CPF' and dichlorvos 'DDVP') to mrigal

Toxicity	24 h (CPF and DDVP)	48 h (CPF and DDVP)	72 h (CPF and DDVP)	96 h (CPF and DDVP)
LC_{10}	0.236 and 7.052 (0.113–0.332, 3.387–9.945)	0.190 and 5.671 (0.126–0.236, 3.781–7.063)	0.170 and 5.072 (0.112–0.212, 3.348–6.353)	0.086 and 2.563 (0.051–0.116, 1.537–3.465)
LC_{20}	0.353 and 10.546 (0.225–0.495, 6.740–14.807	0.231 and 6.911 (0.170–0.280, 5.075–8.364	0.208 and 6.212 (0.151–0.252, 4.522–7.547)	0.118 and 3.525 (0.080–0.152, 2.388–4.548)
LC_{30}	0.471 and 14.097 (0.334–0.730, 10.005–21.829)	0.266 and 7.970 (0.207–0.320, 6.202–9.558)	0.240 and 7.189 (0.186–0.289, 5.555–8.641)	0.148 and 4.435 (0.108–0.188, 3.233–5.613)
LC_{40}	0.604 and 18.063 (0.435–1.095, 13.020–32.756)	0.301 and 9.002 (0.243–0.362, 7.272–10.843	0.272 and 8.146 (0.219–0.328, 6.545–9.816)	0.180 and 5.397 (0.138–0.228, 4.123–6.825)
LC_{50}	0.761 and 22.774 (0.535–1.665, 16.010–49.793)	0.337 and 10.088 (0.279–0.413, 8.332–12.358)	0.306 and 9.155 (0.252–0.374, 7.533–11.200)	0.217 and 6.484 (0.170–0.279, 5.089–8.333)
LC_{60}	0.960 and 28.714 (0.645–2.583, 19.285–77.269)	0.378 and 11.304 (0.315–0.477, 9.423–14.268)	0.344 and 10.288 (0.286–0.433, 8.557–12.947)	0.260 and 7.790 (0.206–0.346, 6.171–10.355)
LC_{70}	1.230 and 36.794 (0.777–4.185, 23.245–125.176)	0.427 and 12.768 (0.355–0.563, 10.617–16.846)	0.390 and 11.657 (0.324–0.512, 9.682–15.315)	0.317 and 9.480 (0.249–0.444, 7.458–13.287)
LC_{80}	1.644 and 49.182 (0.958–7.425, 28.668–222.120)	0.492 and 14.724 (0.403–0.692, 12.066–20.702)	0.451 and 13.491 (0.369–0.631, 11.050–18.872)	0.399 and 11.928 (0.306–0.604, 9.162–18.075)
LC_{90}	2.459 and 73.550 (1.271–16.591, 38.018–496.275)	0.600 and 17.942 (0.476–0.933, 14.225–27.906	0.552 and 16.523 (0.438–0.854, 13.095–25.558)	0.548 and 16.403 (0.401–0.942, 11.981–28.175

Values in parentheses are lower and upper bound at 95% confidence limit

Table 4 Joint toxicity of chlorpyrifos and dichlorvos to mrigal

Toxicity	Additive Index (AI) value			
	24 h	48 h	72 h	96 h
LC_{10}	0.00	0.06	-0.11	0.37
LC_{20}	−0.12	0.02	−0.21	0.23
LC_{30}	−0.22	−0.02	−0.29	0.11
LC_{40}	−0.32	−0.05	−0.37	−0.01
LC_{50}	−0.43	−0.09	−0.44	−0.14
LC_{60}	−0.56	−0.12	−0.52	−0.29
LC_{70}	−0.73	−0.17	−0.61	−0.46
LC_{80}	−0.96	−0.21	−0.72	−0.71
LC_{90}	−1.36	−0.29	−0.89	−1.10

In our study 96 h-LC_{50} value of chlorpyrifos to mrigal was found to be 0.380 (0.319-0.450) mg/L. Our recent work on common carp (*Cyprinus carpio*) and golden mahseer (*Tor putitora*) reported 96 h median lethal concentration of chlorpyrifos to be 0.44 and 0.753 mg/L respectively (Kunwar et al. 2021a, b). Similarly, 96 h-LC_{50} values of chlorpyrifos were reported to be 0.44 mg/L in mrigal (Bhatnagar et al. 2017) and 0.58 mg/L in common carp (Xing et al. 2015). In this experiment, we found 96 h-LC_{50} of dichlorvos was 11.367 (9.496-13.536) mg/L. The present finding corroborates to other experiments that documented 96 h median lethal concentration of dichlorvos to be 9.1 mg/L in mrigal (Velmurugan et al. 2009), 9.41 mg/L (Ural and Çalta 2005) or 15.705 mg/L in common carp (Kunwar et al. 2021a) and 12.964 mg/L in golden mahseer (Kunwar et al. 2021b). Our results distinctly reveal chlorpyrifos is relatively more toxic than dichlorvos for mrigal.

In general, toxicity evaluations are based on single pesticide assessments but these compounds are oftentimes found as complex mixtures in nature; therefore such assessment studies on individual pesticides cannot represent the actual threats posed to aquatic organisms. The combined pesticide toxicity can be additive (an effect produced by mixture pesticides is exactly equal to the sum of individual pesticide's effects), synergistic (an effect caused by mixture pesticides is higher than the sum of its individual pesticide's effect) or antagonistic (an effect caused by mixture pesticides is less than the sum of its individual pesticide's effect). In our study, the joint action of chlorpyrifos and dichlorvos to mrigal was observed to be antagonistic. An antagonistic effect of these two pesticides was also recorded in our recent experiment with golden mahseer (Kunwar et al. 2021b). Wang et al. (2017) documented antagonistic effect of chlorpyrifos and other pesticides mixture on zebrafish (*Danio rerio*). Antagonistic effects of chlorpyrifos and carbosulfan (Chen et al. 2014) and fenobucarb with triazophos or malathion (Wang et al. 2015) were also observed in common carp.

Chlorpyrifos and dichlorvos belong to the organophosphate group that have the same mode of action (MOA). Pesticides having the same MOA are not necessarily

Table 5 Behavioral changes shown by mrigal during 96 h acute exposure of chlorpyrifos (CPF), dichlorvos (DDVP) and their mixture

Fish behavior	CPF (mg/L)				DDVP (mg/L)				Mixed pesticides (mg/L)			
	0.25	0.5	1	2	4	8	16	32	CPF-0.047 DDVP-1.420	CPF-0.095 DDVP-2.841	CPF-0.190 DDVP-5.683	CPF-0.380 DDVP-11.367
Hypo-activity	−	−	−	NA	−	−	−	NA	+	+	++	++
Equilibrium loss	+	++	++	NA	−	−	−	NA	−	−	−	−
Color change	−	−	+	NA	−	−	+	NA	−	−	+	+
Aggregating at corners of the aquarium	+	+	++	NA	+	+	++	NA	+	+	+	+
Avoiding schooling behavior	−	+	++	NA	−	+	+	NA	++	+	+	+

Mixed concentrations were prepared as 12.5%, 25%, 50%, 100% and 200% of 96 h-LC_{50} values of the respective pesticides. 96 h-LC_{50} of CPF-0.380 mg/L and DDVP-11.367 mg/L (−: absent; +: mild; ++: moderate; +++: strong)

synergistic (LeBlanc et al. 2012, Wang et al. 2017). To the best of our knowledge, this is the first study to show that chlorpyrifos and dichlorvos have antagonistic effects in the freshwater fish mrigal. There are various explanations put forward by investigators for the antagonistic effect. Hernández et al. (2013) described that the pesticide mixture changes the toxicokinetics of the individual compounds, thus modifying their toxicity. Therefore, chemical interaction between two pesticides might be the reason for the antagonistic result (Imam et al. 2018). Likewise, authors (Stepić et al. 2013, Wang et al. 2017) stated enhanced metabolization processes leading to faster excretion of metabolites, eventually resulting in decreased pesticide toxicity. A potential basis for such an antagonistic outcome could be the elevation of carboxylesterases (CaEs) activity. CaEs can detoxify pesticides by hydrolysis (Jokanović 2001, Wheelock et al. 2005). Moreover, this enzyme is also believed to protect AChE from pesticide toxicity by direct binding and sequestration (Jokanović 2001, Maxwell 1992). Therefore, the observed outcome in this experiment might be due to the protective role of CaEs induced by the chlorpyrifos and dichlorvos mixture in mrigal. Another possible interpretation for antagonistic interaction could be Glutathione-S-transferase (GST) mediated pesticide detoxification. Available reports suggest that GST promotes cellular detoxification of xenobiotics including pesticides (Booth et al. 1998, Jin-Clark et al. 2002). In response to pesticide co-exposure, GST activity was reported to be significantly higher compared to individual pesticide exposure (Stepić et al. 2013). Based on this, we hypothesized that combined treatment of chlorpyrifos and dichlorvos might have elevated GST activity in our experimental animal which played a role in efficient detoxification of metabolites leading to reduced toxicity.

Fish behavior studies are an important non-invasive tool for toxicity assessment. Pesticide exposed fish exhibited loss of coordination with each other, residing at corners of the test chamber, excess mucus secretion, becoming pale, rapid opercular movements and abrupt swimming before death. Alike behavioral expressions in response to pesticide were exhibited by common carp and golden mahseer (Kunwar et al. 2021a, b) under similar exposure environments. Many other authors (Kavitha and Rao 2008, Halappa and David 2009, Nwani et al. 2013, Ullah et al. 2014, Padmanabha et al. 2015, Saha et al. 2016, Soni and Verma, 2018) have also documented comparable behavioral changes in fish after pesticides exposure. Inhibition of acetylcholinesterase (AChE) leads to accumulation of acetylcholine (ACh) in cholinergic synapses and overstimulation resulting behavioral changes in fish (Halappa and David 2009). The excess mucus secretion by pesticide exposed fish would protect them by avoiding contact with toxicants or by getting rid of it through the shedding of the mucus layer (Patil and David 2008, Halappa and David 2009). Fish are stressed when exposed to pesticides and their oxygen demand becomes high during such circumstances (Schmidt et al. 2005). Therefore rapid opercular movements after pesticide exposure in mrigal would have facilitated in supplying high oxygen to detoxify pesticides and protect them from deleterious effects. Most of the behavioral symptoms were similar in all pesticide treatments but only chlorpyrifos treated fish exhibited equilibrium loss. This behavioral difference might be due to the severe inhibition of chlorpyrifos on AChE leading to high accumulation of ACh in these fish groups compared to other pesticide treated groups.

Conclusion

Compared to dichlorvos, chlorpyrifos is highly toxic to fish. Despite the same mode of action, the majority of the binary mixture effects were antagonistic in the present study. This requires in-depth investigation such as measurement of ACh, AChE, CaE and GST to explore the toxicity mechanism of these pesticides in co-exposure. Behavioral manifestations detected even at low pesticide concentration suggest that such observations should be incorporated in

toxicity studies because it is a highly sensitive and non-invasive bio-monitoring tool. Such assessments can be correlated to the health status and growth performance of fish under the available rearing condition. To sum up, the application of toxic pesticides should be regulated and the use of bio-pesticides and integrated pest management programs should be promoted to save precocious aquatic flora and fauna from pesticide threats.

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Author contributions PSK designed this research, monitored the lab works and prepared the draft version of the manuscript. BS and SB conducted the exposure experiments and collected fish mortality and behavior data. KP compiled and analyzed the data. AKS and GDeB reviewed, corrected and improved the manuscript. KS approved the research design, validated the data and reviewed the manuscript for publication.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethics approval Ethical approval for the study (Ref no. 1215) was received on 18 September 2019 from the Ethical Review Board of Nepal Health Research Council, Government of Nepal.

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Research article

Modulations of blood biochemical parameters of golden mahseer, *Tor putitora* following exposures to single and mixed organophosphate

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ABSTRACT

Increasing pesticide application is a serious threat to human health and biodiversity. In nature, pesticides prevail in mixtures; therefore the joint effects of pesticides should be taken into consideration due to their priority in toxicity research when aiming at realistic evaluations. In this study, individual and mixture toxicity of the commonly used organophosphate insecticides- chlorpyrifos and dichlorvos was explored. Healthy and clinically active juveniles of golden mahseer (*Tor putitora*) were exposed to sub-lethal doses (10% of the 96 h-LC_{50}) of the chlorpyrifos, dichlorvos, and their mixture. Blood sampling was conducted after 24 h and 96 h of exposure, followed by a 1 week recovery period. Among the examined biochemical parameters; blood glucose in dichlorvos treatment; alanine aminotransferase and alkaline phosphatase in chlorpyrifos and dichlorvos treatments; and aspartate aminotransferase and urea in mixture pesticide treatments were elevated. In contrast, blood albumin and triglycerides were diminished in mixture pesticide treatments. Vital organs like kidney and liver of the tested animals were compromised to different magnitudes in different pesticide treatments. Kidney was found to be more sensitive than liver in terms of pesticide toxicity during this short exposure experiment. This study revealed that most of the biomarkers were mainly affected at a later exposure phase (after 96 h) and steadily recovered during the depuration period.

1. Introduction

The use of pesticides began a long time ago (before 2000 BCE) to protect crops from pests. However, their application was intensified since 1940 with the emergence of synthetic pesticides (Garcia et al., 2012). From 1990 to 2018, the global pesticide application raised from 2,299,979 t to 4,122,334 t. During the same period, it was increased from 60 t to 574 t in Nepal (FAOSTAT, 2021). The government authority of Nepal reported insecticides as the second major group of pesticides imported and formulated in Nepal, after fungicide. But, in terms of monetary value insecticide is the major group possessing 51.65% of the total national investment in pesticides (PQPMC, 2019).

Despite the pesticide's role in disease control and production enhancement of agro crops, the harmful effects of pesticides to the environment, non-targeted biota and human health cannot be ignored. Dogan and Can (2011) also stated that in some situations, it is a practical method, however the benefits of pesticides are not derived without their negative consequences. It is unfortunate that only a small portion (<0.1%) of the applied pesticide reach their target pests while the majority (>99.9%) of it remains in the environment, contaminating the atmosphere, soil, and water (Pimentel, 1995). In this way, non-target aquatic organisms, which are of great economic importance to humans, are distressed by the haphazard application of pesticides and their discharge into the aquatic systems through leaching, agricultural runoff, precipitation, spray drift, improper disposal, irrigation waters, and industrial effluent (Adhikari et al., 2004; Sunanda et al., 2016; Wang et al., 2013).

Golden mahseer (*Tor putitora*) are important freshwater cyprinid fishes, which are naturally distributed in the trans-Himalayan region of Nepal, Pakistan, India, Myanmar and South-east Asia including Thailand, Lao PDR, Cambodia, Vietnam, southern China, Peninsular Malaysia, Borneo, Sumatra and Java (Ingram et al., 2005). They are

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highly valuable species both for game and food fisheries (Ingram et al., 2005). Because of the delicacy and sports value, golden mahseer is a highly prioritized freshwater cyprinid fish in Nepal, and it is also considered as a flagship species of this country (Swar, 2002). In general, fish are a good indicator for water quality assessment (Velmurugan et al., 2007); therefore, many studies have been performed worldwide addressing pesticide toxicity and their potential impact on fish, but golden mahseer has received little attention. There is an alarming decline of *Tor* populations from the natural environment due to various causes like environmental degradation, pollution and overfishing. However, one of the major causes of their population decline is water pollution (Yousafzai et al., 2008). Jha et al. (2018) estimated more than 50% of their population decline in the last 21 years. Water pollution not only alters fish community structure but also facilitates the establishment of invasive species (Gomes-Silva et al., 2020).

Among the different groups of pesticides, organophosphate-based formulations are the most commonly used pesticides for crop treatment and fish farming operations (El Nahas et al., 2017; Kafle et al., 2015; Rao et al., 2017; Varó et al., 2007). Chlorpyrifos and dichlorvos are widely used organophosphate pesticides worldwide (Ali et al., 2009; Sun et al., 2015; Ural and Çalta, 2005), evident by traces of their residues in water, soil, sediments, and in wild and cultured fish (Akoto et al., 2016; Kafle et al., 2015; Nag et al., 2020; Singh et al., 2015; Sun and Chen, 2008). Therefore, they are a serious source of water pollution, and their toxicity mechanisms on important species must be elucidated if targeted conservation measures are to be taken.

The toxic effects of pesticides vary when treated individually or in combination. The combined effects of pesticide toxicity can be additive (an effect produced by mixture pesticides is exactly equal to the sum of individual pesticide's effects), synergistic (an effect caused by mixture pesticides is higher than the sum of its individual pesticide's effect), or antagonistic (an effect caused by mixture pesticides is less than the sum of its individual pesticide's effect). The joint effects of the same pesticide combination can also be different indicating species specific action of pesticide in fish (Kunwar et al., 2021a, 2021b). In the aquatic system, pesticides arise from various sources and are oftentimes present in the mixtures; therefore evaluation of the joint effects of pesticides becomes more realistic than the individual pesticide toxicity.

In aquatic animals, including fish, any kind of waterborne pollution is easily reflected in the circulatory system (Ismail et al., 2017); therefore, blood parameters can serve as important bioindicators of aquatic pollution as well as of the health status of the fish (Bhatnagar et al., 2017; Öner et al., 2008; Saravanan et al., 2011; Vosylienė, 1999). In one of the few studies on blood parameters of golden mahseer, the effects of synthetic pyrethroid- cypermethrin on RBC and WBC counts were documented (Ullah et al., 2015). Yousafzai et al. (2008) elucidated changes in haemoglobin (Hb), packed cell volume (PCV), blood cells count, blood total protein, cholesterol, glucose, glutamate oxaloacetate transaminase (GOT), glutamate oxaloacetate transaminase (GPT) and creatine phosphokinase (CPK) in golden mahseer sampled from the polluted natural waters but no literature on blood biochemical effects induced by organophosphate pesticides could be found for this species. Therefore this research aims to understand the toxic effect of the representative organophosphate insecticide chlorpyrifos, dichlorvos and their mixture on biochemical parameters like blood glucose, total protein, albumin, globulin, triglycerides, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) of golden mahseer. These are key parameters for the assessment of stress, energy metabolism as well as functional status of kidney and liver in animals.

2. Materials and methods

2.1. Acclimatization of the test animals

Healthy juveniles of golden mahseer (exact weight mentioned in Section 2.2) were collected from the Fisheries Development Centre, Kulekhani, Makawanpur, Nepal and transported in oxygen packed plastic bags to Central Fisheries Promotion and Conservation Centre, Kathmandu, Nepal. Fish were stocked in 350 L indoor glass aquaria equipped with individual water recirculation and aeration systems. Fish were regularly fed ad libitum with commercial pellet feed (Sreema feed Pvt. Ltd., India) containing 32% protein. Uneaten food and fecal matters were removed with the help of a scoop net and siphon. Water filters, pumps, pipes and air stones were regularly cleaned. Everyday approximately half of the aquarium water was exchanged with freshwater to maintain optimum water quality. Water temperature, pH, dissolved oxygen, total ammonia, hardness, Na^+, K^+ and Cl^- ranged between 23.5 and 25.2 °C, 7.6–7.8, 5.7–6.6 mg/L, 0.20–0.26 mg/L, 51–58 mg $CaCO_3$/L, 1.13–1.25 mmol/L, 0.06–0.10 mmol/L and 0.52–0.64 mmol/L, respectively. Fish were acclimatized for two weeks in these conditions before using them for the experiment. This research followed ethical guidelines as approved by the ethical review board of Nepal Health Research Council (Ref. No. 1215), Government of Nepal.

2.2. Pesticide exposure and depuration

Acclimatized fish were weighed and transferred to 35 L small glass aquaria. Each aquarium accommodated only one fish at a time. The average weight of the fish used in this experiment was 53.12 ± 4.13 g (mean ± SD). Fish were left undisturbed overnight with continuous aeration. Commercial grade pesticides were selected for exposure. For dichlorvos, G-VAN (80%; Greenriver Industry Co., Ltd., ShenZhen, China) and for chlorpyrifos, Dursban (20%; Dow Agro Sciences Pvt. Ltd., India) were used. These products are legally registered by the Plant Quarantine and Pesticide Management Centre, Government of Nepal for marketing and application.

In total, 24 aquaria of 35 L capacity each were used for this sub-lethal exposure. Each aquarium accommodated three fish for the test. On the day of exposure, working solutions of pesticides were freshly prepared in distilled water and the calculated volume of the stock solution was added to reach the sub-lethal concentrations of chlorpyrifos- 0.075 mg/L, dichlorvos- 1.29 mg/L and their mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L) in the test chamber. The selected concentrations represent 10% of the determined 96 h-LC_{50} of chlorpyrifos and dichlorvos to golden mahseer (Kunwar et al., 2021b). There were 6 replicates for each pesticide treatment and for the control. The test was conducted in semi-static condition and the test solution was renewed periodically (Shirdel et al., 2020). After accomplishing the 96 h exposure, the fish were left undisturbed in the same aquaria for one further week in the pesticide free water to evaluate their recovery from the pesticide effects.

2.3. Serum collection

Blood sampling was done three times- after 24 h and 96 h of pesticide exposure, and 1 week of depuration period. Fish were gently removed from aquaria and anesthetized in clove oil. The anesthetized fish were wrapped with a wet towel and blood was drawn from the caudal vein using a 3 mL syringe. The blood collected in eppendorf tubes was left for 30 min to clot properly (Tuck et al., 2009). Later, it was centrifuged for 10 min at 3000 rpm to separate serum from blood cells (Zahran et al., 2018). Transparent serum was transferred to clean eppendorf tubes and stored at −30 °C for later biochemical measurements.

2.4. Biochemical measurements

Prior to the biochemical tests, serum samples were properly thawed at room temperature. Glucose was determined by the oxidase (GOD) and peroxidase (POD) method (Eco-pak glucose kit, Accurex Biomedical Pvt. Ltd., India). Total protein was determined by the Biuret method using a total protein kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India). Albumin was determined by the Bromocresol Green (BCG)

method using an albumin kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India). Globulin was estimated by subtracting albumin from total protein (Qureshi et al., 2016). Urea was determined by the Glutamate Dehydrogenase (GLDH) Kinetic method using a urea kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India). Creatinine was estimated by a modified Jaffe's Kinetic method using a creatinine kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India). Triglycerides were measured by the Glycerophosphate-Oxidase (GPO)/PAP method using a triglycerides kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India). Alanine aminotransferase (ALT or ALAT) activity was assayed by a modified IFCC method using a calkine serum glutamic-pyruvic transaminase (SGPT)/ALAT kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India); Aspartate aminotransferase (AST or ASAT) activity was quantified by a modified IFCC method using a calkine serum glutamic oxaloacetic transaminase (SGOT)/ASAT kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India) and alkaline phosphatase (ALP or ALKP) activity was determined by the *p*- Nitrophenylphosphate (pNPP) kinetic method using a calkine ALP kit (Coral Clinical systems, Tulip Diagnostics Pvt., Ltd., India).

2.5. Statistical analysis

The normality of the data and homogeneity of variances was assessed by Shapiro-Wilk and Levene's test, respectively. Mean difference among the various treatment groups was analyzed by one-way ANOVA followed by Post Hoc tests. Whenever the data were not normally distributed, nonparametric Kruskal-Wallis test with multiple pair comparison was applied. All analyses were done using SPSS version 20.

3. Results

3.1. Blood glucose

In general, there was a numerically increasing trend of blood glucose in single pesticide treatments but not in mixture groups. However, the elevation was significant ($P < 0.001$) only in dichlorvos treated fish after 24 h of exposure. When the glucose level was compared among the same pesticide treated fish during different time intervals, a significant recovery ($P < 0.001$) of glucose level was observed after 96 h and 1 week recovery (WR) compared to the 24 h measurement in dichlorvos treated fish (Fig.1). The compiled data (all pesticide treatments as an exposed group) analysis revealed that although blood glucose level was increased by pesticide exposure, significant elevation ($P < 0.05$) compared to control was observed only during the early sampling time (24 h). The

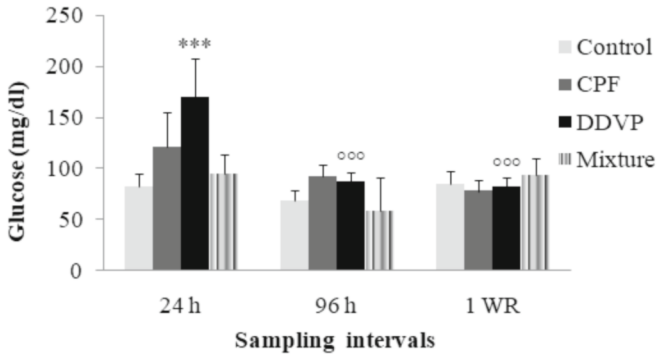

Fig. 1. Blood glucose (mg/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD ($n = 5–7$). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (***$P < 0.001$) and white circle denotes significant difference between same groups of 96 h and 1 week recovery (1 WR) compared to 24 h ($^{°°°}P < 0.001$). No significant difference was observed in same groups between 96 h and 1 WR.

elevated blood glucose significantly recovered ($P < 0.01$) at successive sampling intervals- 96 h and 1 WR (Table 1).

3.2. Blood proteins

Regardless of the pesticide treatments, exposure time or depuration phase, no significant effect on blood total protein and globulin was found (Figs. 2 and 4). However, there was a declining trend for the mixture, especially at 96 h, which recovered after 1 week of depuration. In general, there was also a declining trend of the blood albumin with pesticide exposure but the difference was significant ($P < 0.01$) only at 24 h of exposure compared to its control level. The diminished albumin at 96 h in mixture group was significantly recovered ($P < 0.05$) during the depuration period (Fig. 3).

Data for total protein, albumin and globulin were also analyzed by compiling different pesticide treatments as exposed group and compared with their respective controls but the differences always remained insignificant. However, the decreasing trend was again observed in pesticide exposed fish at both 24 h and 96 h of exposure and recovered during the depuration phase (Table 1).

3.3. Blood triglycerides

There was a clear declining trend of blood triglycerides in all pesticide treatments compared to control, but a sharp significant drop ($P < 0.001$) was observed only in mixture pesticide group after 96 h of exposure. When the triglyceride level was compared among the same pesticide treated fish during different time intervals, a significantly lower ($P < 0.05$) level was observed after 96 h in mixture pesticide treated fish compared to its 24 h of measurement (Fig. 5). The compiled data analysis revealed that pesticide exposure reduces the triglycerides level significantly ($P < 0.05$) at the later phase of the exposure i.e. 96 h (Table 1).

3.4. Blood urea and creatinine

Blood urea was stable in response to chlorpyrifos exposure. However, there was an increasing trend of urea levels in dichlorvos and mixture pesticide exposure, but a significant elevation ($P < 0.01$) was found only at 96 h in the mixture pesticide treated fish. The increasing trend for blood creatinine was also noticed by the pesticide treatments but all these increments were insignificant. The elevated urea and creatinine dropped to the control level during one week depuration period (Figs. 6 and 7). Statistical analysis also revealed no significant difference in both parameters among the same pesticide treatments over the whole experimental period (Figs. 6 and 7). The same was true for compiled data analysis, although exposed groups showed numerically higher urea and creatinine compared to their respective controls (Table 1).

3.5. Alanine aminotransferase (ALT)

Blood ALT level increased significantly compared to the control after 96 h of exposure ($P < 0.01$) in both chlorpyrifos and dichlorvos treated fish. The elevated ALT levels restored after 1 week of recovery period showing significantly lower ALT levels in chlorpyrifos ($P < 0.01$) and dichlorvos ($P < 0.05$) treated fish after 1 week of recovery period compared to their 96 h counterparts (Fig. 8). The compiled data analysis also revealed increased ALT levels in exposed group after 96 h ($P < 0.01$) which significantly dropped ($P < 0.001$) after 1 week of recovery period (Table 1).

3.6. Aspartate aminotransferase (AST)

Blood AST level tended to be increased by all pesticide treatments but significantly higher AST levels compared to respective controls were observed only in mixture pesticide treatment after 24 h ($P < 0.01$) and

Table 1
Blood parameters of golden mahseer at different sampling intervals.

Parameters	24 h		96 h		1 WR	
	Control	Exposed	Control	Exposed	Control	Exposed
Glucose (mg/dl)	83.08 ± 12.44	129.18 ± 43.20*	69.04 ± 9.42	79.89 ± 24.40°°	84.86 ± 13.11	84.78 ± 13.40 °°
Protein (g/dl)	4.94 ± 0.54	4.50 ± 1.13	3.96 ± 0.59	3.73 ± 1.01	4.14 ± 0.73	4.66 ± 0.78
Albumin (g/dl)	1.53 ± 0.16	1.15 ± 0.28	1.14 ± 0.17	0.98 ± 0.28	1.16 ± 0.37	1.13 ± 0.21
Globulin (g/dl)	3.42 ± 0.38	3.35 ± 0.93	2.81 ± 0.55	2.75 ± 0.79	2.99 ± 0.57	3.53 ± 0.82
TG (mg/dl)	178.26 ± 15.18	129.05 ± 32.75	156.97 ± 53.93	94.53 ± 56.49 *	144.14 ± 34.50	112.22 ± 40.30
Urea (mg/dl)	8.12 ± 1.25	9.61 ± 2.71	7.64 ± 1.88	10.03 ± 3.27	8.85 ± 0.98	9.07 ± 1.88
Creatinine (mg/dl)	0.43 ± 0.10	0.58 ± 0.17	0.39 ± 0.08	0.52 ± 0.11	0.48 ± 0.11	0.45 ± 0.10
ALT (IU/L)	14.60 ± 4.16	30.88 ± 11.59	17.40 ± 5.73	41.60 ± 15.85 **	15.60 ± 3.51	21.60 ± 7.08 ●●●
AST (IU/L)	191.40 ± 40.24	480.00 ± 226.97	267.20 ± 73.85	577.40 ± 363.72	220.40 ± 53.51	281.33 ± 84.47 ●●
ALP (IU/L)	26.30 ± 5.92	45.00 ± 20.93	28.30 ± 6.67	70.30 ± 32.23**,°	33.10 ± 10.14	27.13 ± 8.36 ●●●

Values are mean ± SD. Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (*P < 0.05; **P < 0.01); white circle denotes significant difference between same groups of 96 h and 1 WR compared to 24 h (°$P < 0.5$) and dark circle denotes significant difference in same groups between 96 h and 1 WR (●●$P < 0.01$; ●●●$P < 0.001$).

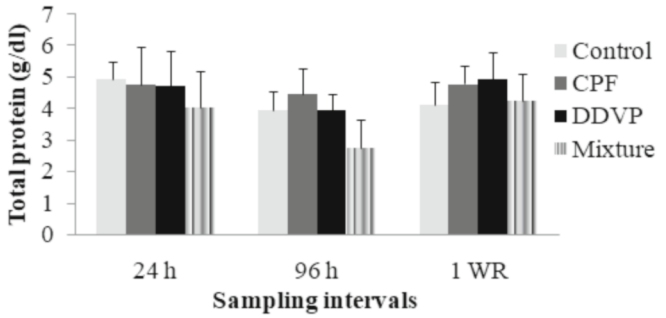

Fig. 2. Blood total protein (g/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). No significant difference was observed between control and pesticide treated groups at same sampling intervals; between same groups of 96 h and one week recovery (1 WR) compared to 24 h and in same groups between 96 h and 1 WR.

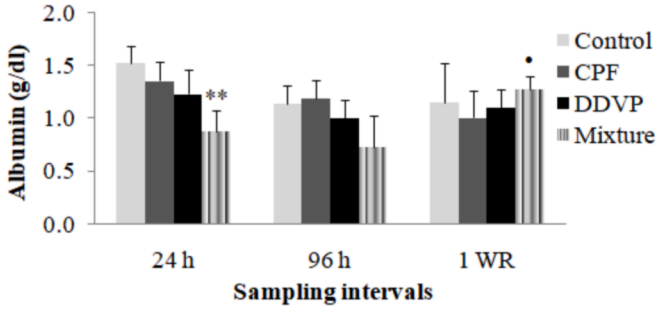

Fig. 3. Blood albumin (g/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (**$P < 0.01$) and dark circle denotes significant difference in same groups between 96 h and one week recovery (1 WR) (●$P < 0.05$). No significant difference was observed between same groups of 96 h and 1 WR compared to 24 h.

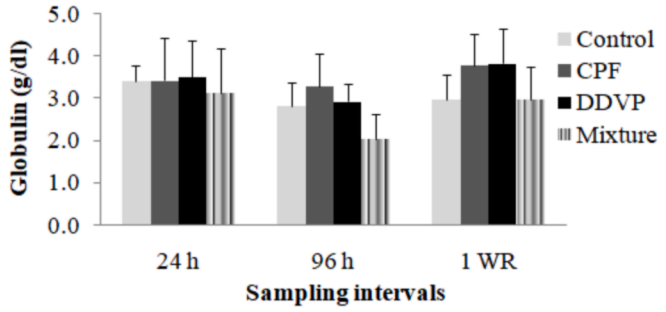

Fig. 4. Blood globulin (g/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). No significant difference was observed between control and pesticide treated groups at same sampling intervals; between same groups of 96 h and one week recovery (1 WR) compared to 24 h and in same groups between 96 h and 1 WR.

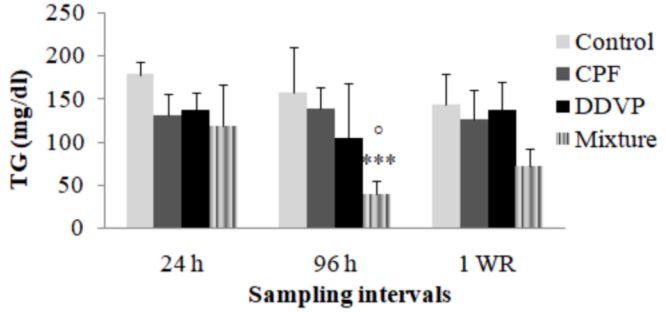

Fig. 5. Blood triglycerides (mg/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (***$P < 0.001$) and white circle denotes significant difference between same groups of 96 h and one week recovery (1 WR) compared to 24 h (°$P < 0.05$). No significant difference was observed in same groups between 96 h and 1 WR.

96 h ($P < 0.001$) of exposure. Among the same pesticide treatments over the whole experimental period, significantly elevated ($P < 0.01$) AST was observed in mixture group after 96 h compared to the 24 h, and significantly lower ($P < 0.001$) AST was observed after 1 week recovery compared to 96 h observation (Fig. 9). The average blood AST in exposed group (compiled) were not significant from their control levels; but after 1 week of recovery period, blood AST in exposed group was significantly lower ($P < 0.01$) compared to 96 h of observation in the same treatment group (Table 1).

3.7. Alkaline phosphatase (ALP)

Blood ALP was elevated significantly compared to control values after 96 h of exposure in chlorpyrifos ($P < 0.001$) and dichlorvos ($P < 0.01$) pesticide groups. These elevations sharply declined again to values close to the control level after 1 week of recovery period. Among the

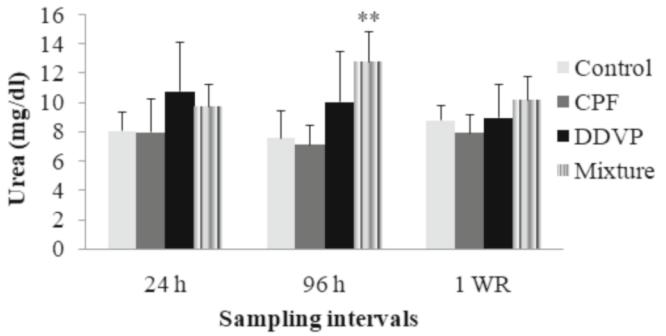

Fig. 6. Blood urea (mg/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (**$P < 0.01$). No significant difference was observed between same groups of 96 h and one week recovery (1 WR) compared to 24 h and in same groups between 96 h and 1 WR.

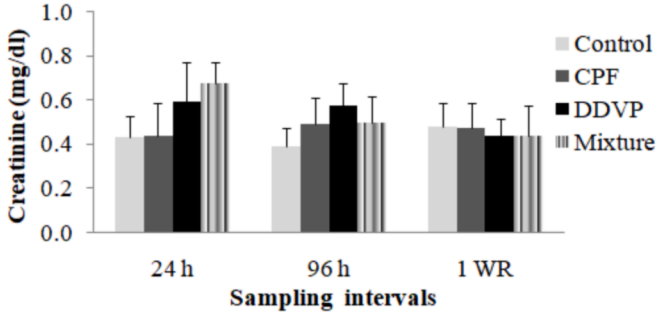

Fig. 7. Blood creatinine (mg/dl) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). No significant difference was observed between control and pesticide treated groups at same sampling intervals; between same groups of 96 h and one week recovery (1 WR) compared to 24 h and in same groups between 96 h and 1 WR.

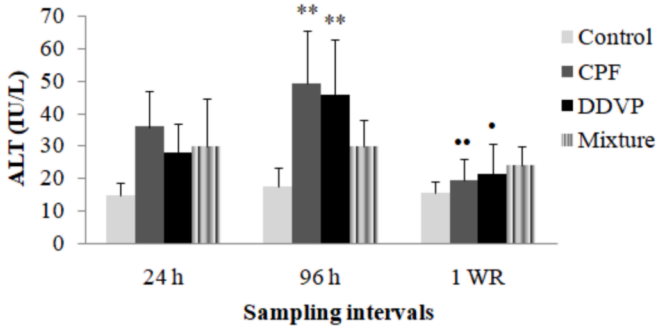

Fig. 8. Blood ALT (IU/L) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (**$P < 0.01$) and dark circle denotes significant difference in same groups between 96 h and one week recovery (1 WR) ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$). No significant difference was observed between same groups of 96 h and 1 WR compared to 24 h.

same pesticide treatments, chlorpyrifos treated fish exhibited significantly higher ALP at 96 h ($P < 0.05$) compared to 24 h values which became significantly lower again compared to this 96 h value in chlorpyrifos ($P < 0.001$) and dichlorvos ($P < 0.01$) treated fish after 1 week of

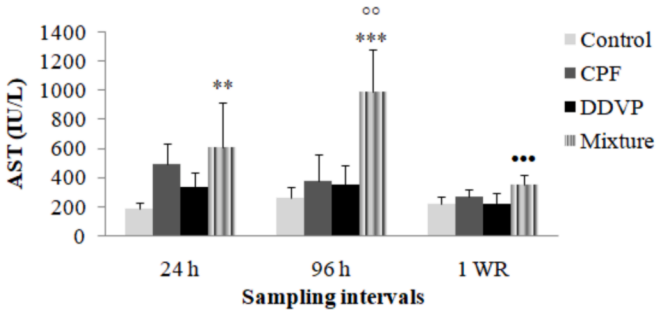

Fig. 9. Blood AST (IU/L) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (**$P < 0.01$; ***$P < 0.001$); white circle denotes significant difference between same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ\circ}P < 0.01$) and dark circle denotes significant difference in same groups between 96 h and 1 WR ($^{\bullet\bullet\bullet}P < 0.001$).

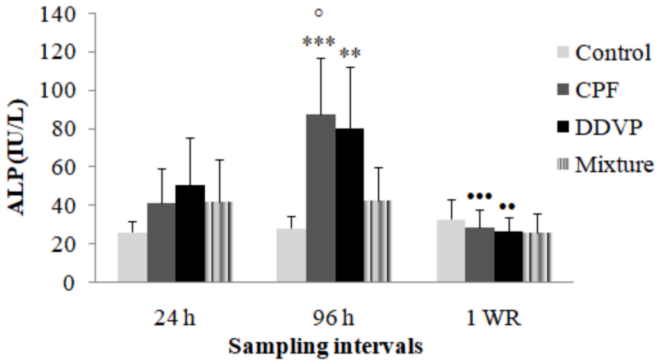

Fig. 10. Blood ALP (IU/L) of golden mahseer exposed to chlorpyrifos (CPF- 0.075 mg/L), dichlorvos (DDVP- 1.29 mg/L) and mixture (CPF- 0.075 mg/L and DDVP- 1.29 mg/L). Values are mean ± SD (n = 5–7). Asterisk denotes significant difference between control and pesticide treated groups at same sampling intervals (**$P < 0.01$; ***$P < 0.001$); white circle denotes significant difference between same groups of 96 h and one week recovery (1 WR) compared to 24 h ($^{\circ}P < 0.5$) and dark circle denotes significant difference in same groups between 96 h and 1 WR ($^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$).

recovery (Fig. 10). The average blood ALP (compiled) data analysis showed significant elevation ($P < 0.01$) by pesticide exposure compared to control after 96 h which was also significantly higher ($P < 0.05$) than 24 h ALP measurement of the same exposed group. This elevated ALP significantly dropped again ($P < 0.001$) reaching control levels after one week of recovery period (Table 1).

4. Discussion

Chlorpyrifos is a pesticide extensively applied in agriculture and domestic operations (Ali et al., 2009; Halappa and David, 2009; Sun et al., 2015). Similarly, dichlorvos is also a commonly used pesticide (Das, 2013; Mennear, 1998; Sun et al., 2015; Ural and Çalta, 2005). It is one of the main chemical agents used against fish ectoparasites (Das, 2013; Varó et al., 2007). The majority of the applied pesticides are released into the environments that ultimately reach the aquatic systems which can alter the biochemical parameters of the aquatic animals.

Biochemical parameters are used as important biomarkers in toxicity research. Any deviation in biochemical parameters indicates some sort of disturbance in the animal's homeostasis and could potentially deteriorate its health. Blood glucose is one of the bioindicators that, together with plasma cortisol, is extensively studied as environmental stress indicator (Banaee et al., 2013; Bhatnagar et al., 2017; Dogan and Can,

2011; Koul et al., 2007; Medda, 1993; Ramesh and Saravanan, 2008; Saravanan et al., 2011). In common carp (*Cyprinus carpio*), Hatami et al. (2019) reported an increment in the glucose level in response to pesticide mediated stress. Significant elevation of blood glucose is due to gluconeogenesis which fuels the energy for the increased metabolic demands to cope with stressors including pesticides (Bhatnagar et al., 2017; Ramesh and Saravanan, 2008; Saravanan et al., 2011). Moreover, in previous studies depletion of liver glycogen in response to pesticide exposure was coupled with increased glucose in circulation, suggesting enhanced glycogenolysis as a strategy to meet the energy requirements (Ezike et al., 2017; Narra et al., 2015). In our experiment, glucose levels tended to be elevated by both single pesticide exposures, even though this was only significant for dichlorvos. Increased glucose levels due to pesticide exposures have been observed before, e.g. due to exposure of chlorpyrifos (Banaee et al., 2013; Ramesh and Saravanan, 2008) and lindane (Saravanan et al., 2011) to common carp, chlorpyrifos to mrigal (*Cirrhinus mrigala*; Bhatnagar et al., 2017), and dimethoate to rainbow trout (*Oncorhynchus mykiss*; Dogan and Can, 2011). We speculate that the high glucose levels observed in our experimental animals was due to elevated glycogenolysis together with gluconeogenesis. The reducing trend of triglyceride levels in pesticide exposed golden mahseer, with a significant impact in the mixture group, might offer an additional indication for the switch towards another metabolic pathway (gluconeogenesis) which is activated to provide energy during these exposures. Similar to our observation, Hatami et al. (2019) reported a decreased level of triglycerides in chlorpyrifos exposed common carp. In our experimental organism, there seemed no considerable contribution of protein in energy production. No reduction of overall protein in pesticide exposed fish indicates that protein metabolism was not severely affected by pesticide exposure. Likewise, no effect of chemical stress was documented on the blood protein of common carp and rainbow trout (De Smet and Blust, 2001; Velisek et al., 2006). Animals prioritize oxidation of carbohydrates and fat for energy production, therefore utilizing protein as an energy source comes only at critical conditions. Probably such situation did not prevail in the present case during the short term observation period (96 h). Moreover, the sub-lethal doses used in our experiment might have been too low to cause a significant effect in protein catabolism.

Enzymatic profiles of ALT, AST and ALP were measured to evaluate the liver health of animals. These enzyme activities were elevated after pesticides exposure. Increased blood ALT, AST and ALP were also found in other fish species in response to pesticides (Banaee et al., 2013; Ghaffar et al., 2015; Jaffer et al., 2017; Koul et al., 2007; Medda, 1993). The high level of these three enzymes observed in golden mahseer could be due to hepatic cell damage as described in other studies on fish (Banaee et al., 2013; Deka and Mahanta, 2015; Ghelichpour et al., 2017; Jaffer et al., 2017). The histopathological lesions such as congestion, cytoplasmic degeneration, hypertrophy, necrosis, nuclear degeneration, pyknosis, sinusoids dilation and congestion, and vacuolar degeneration were reported in the liver tissue of nonylphenol exposed fish (Shirdel et al., 2020).

Albumin and globulins are major components of total protein. There was a clear decreasing trend of albumin, although it was significant only in the mixture group at 24 h. There was no distinct trend for globulin. Decreasing blood albumin and globulin was reported in sub-lethal exposure of chlorpyrifos to mrigal (Bhatnagar et al., 2017) but insignificant effects on these proteins were also reported in rainbow trout (Velisek et al., 2006). Albumin is synthesized by the liver while globulins are produced both by the liver and immune system (plasma cells). Therefore, reduction in albumin in our experiment could be attributed to the liver impairment, also supported by high ALT, AST and ALP, leading to insufficient albumin production.

Blood urea and creatinine levels are commonly used signs for kidney function. Urea is the chief end product of protein metabolism while creatinine is an anhydride of creatine present in muscles (Jyothi and Narayan, 2000). Production of creatinine is generally more stable than any other excretory product which makes it a more reliable and strong biomarker for accessing renal function (Jyothi and Narayan, 2000). The observed increasing trend of urea, although significant only in mixture group at 96 h, and the similar trend of creatinine observed in our study corroborates with the findings in Singhi (*Heteropneustes fossilis*; Shaikh and Gautum, 2014) and common carp (Jaffer et al., 2017) exposed to dichlorvos and chlorpyrifos, respectively. When the excretory organs (kidney) cannot function properly, high urea and creatinine build up in the blood which might have occurred in golden mahseer during this experiment. Our postulation is also supported by histopathological alteration like Bowman's space increase, congestion, glomerular degeneration, increment of tubule diameter, melanomacrophages centers, necrosis, and tubule degeneration of kidney tissue in nonylphenol-exposed fish (Shirdel et al., 2020).

The highest impact on renal function seems to be in the mixture pesticide group after 96 h of exposure. At that sampling time and treatment group blood glucose, total protein, albumin, globulin and triglycerides were noted the lowest over the whole experimental period, but the reduction was significant only for the triglycerides. The stress indicator glucose, which is expected to be higher during pesticide treatment throughout the exposure, surprisingly remained lower than the control at this point. Blood urea and AST were also highest in this group. This clearly indicates that fish kidney was significantly affected by chlorpyrifos and dichlorvos mixture at this particular sampling time (96 h). Most of the measured biochemical parameters could have been diminished due to their loss from the damaged kidneys whereas the highest urea level could have been due to the compromised filtration capacity of the excretory organ (kidney). Similarly, the highest blood AST was contributed most probably to renal cell damage. AST is not a specific hepatic enzyme (Ghelichpour et al., 2017); and high AST activity in common carp kidney was reported by De Smet and Blust (2001) which also supports this notion.

Elevation of blood ALP occurs when there are hepatobiliary problems (Ghelichpour et al., 2017) and ALT is also a liver specific enzyme. Significantly high ALP and ALT were found after 96 h with individual pesticide exposures whereas it was not significant in the mixture, indicating that hepatic cells were more sensitive to the individual pesticides compared to the mixture pesticides treatment. Blood AST was significantly elevated in the mixture pesticide group but not in individual pesticide groups which indicates renal cells were more sensitive to mixture pesticides compared to individual pesticide treatments. Significant elevation of urea and AST, significant reduction of triglycerides, and elevating trend of creatinine signifies renal cells were highly affected by the pesticides. At the same time, despite high AST, ALT and ALP levels, the protein metabolism of the fish remained unchanged which indicate liver tissues of the fish were less affected by such treatments.

In this study, the biochemical modulation exhibited by fish was noticeable even at sub-lethal concentrations of pesticides. From this, we can envisage the potential impact of such chemicals at higher concentrations that can be linked to mass mortalities of fish in natural water systems (Polidoro and Morra, 2016; Sabra and Mehana, 2015).

The time-wise data analysis revealed pesticide exposure was more stressful to fish in the beginning and gradually dropped, and the animals adapted to the changing environmental condition. However, the effects of pesticides on the liver and kidney were more prominent at the later phase of exposure (96 h) indicating that it might have taken some time to inflict such impairment through the circulatory system. Adhikari et al. (2004) documented that blood parameters in rohu (*Labeo rohita*) were improved by slowly eliminating pesticides when they were transferred to freshwater. Similar results were found after one week depuration period in our experimental animals, where most of the deviated biochemical parameters tended to be stabilized exhibiting recovery signs from pesticide effects. This indicates pesticide detoxifying enzymes were induced in our test species causing the elimination of pesticides from their body. In addition, the induction of the detoxifying enzyme

and depuration was found proportional to the concentration of the toxicants (Ikpesu, 2013).

5. Conclusion

Biochemical parameters of golden mahseer were affected by sub-lethal exposure of chlorpyrifos, dichlorvos and their mixture. Fish exhibited stress response in the early exposure phase but the effects on vital organs were prominent at the later phase of the experiment. The liver was found to be more sensitive to individual pesticides, and kidney to mixture pesticide treatments. Detrimental effects of pesticides were more severe on the kidney than the liver during this short term sub-lethal exposure. However, the fish recovered from such toxic effects during the one week depuration period.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Wish you all the best

5th - 6th February 2021

Hon. Sudarshan Baral
Chief Guest
Minister, Ministry of Social Development, Lumbini Province

Prof. Dr. Mahendra Maharjan
President
Nepal Zoological Society