TOXICITY STUDY OF ENDOCRINE DISRUPTING CHEMICALS (EDCs) ON FRESHWATER FISH *Cyprinus carpio*

Thesis

Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I hereby *declare* that the Research Thesis entitled **TOXICITY STUDY OF ENDOCRINE DISRUPTING CHEMICALS (EDCs) ON FRESHWATER FISH** *Cyprinus carpio* which is being submitted to the National Institute of Technology Karnataka, Surathkal in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in **ENVIRONMENTAL ENGINEERING** is a *bonafide report of the research work carried out by me.* The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to *certify* that the Research Thesis entitled **TOXICITY STUDY OF ENDOCRINE DISRUPTING CHEMICALS (EDCs) ON FRESHWATER FISH** *Cyprinus carpio* submitted by **REKHA RAO**, (Register Number: **123001CV12F06**) as the record of the research work carried out by her, *is accepted as the Research Thesis submission* in Partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy.**

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To GOD

…without Your Grace, I wouldn't have survived the frenemies. THANK YOU, for leading me by Hand, protecting me and being with me through-out…

To my Mother

I am highly indebted to you for your everlasting faith in me which has been the backbone of my hard work and patience. THANK YOU!

TRIBUTE
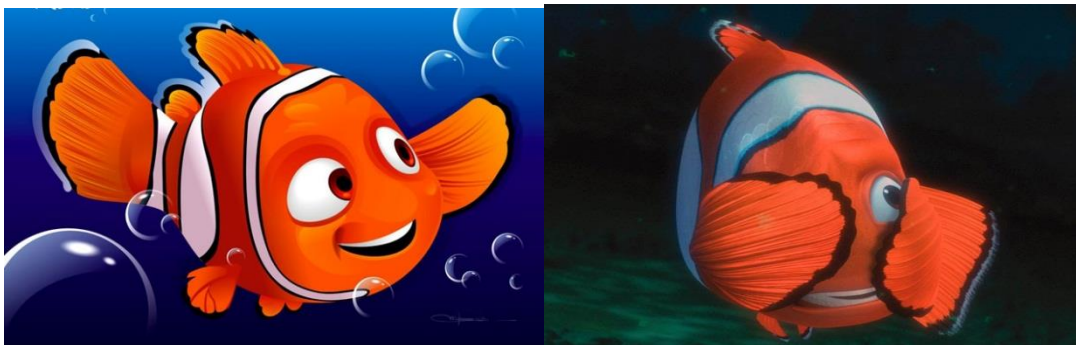
My sincere apologies to all the fishes that suffered the torture of dissection and the ones that survived the toxic doses.

I pay a heartfelt homage to all the fishes that were sacrificed during the project work.

I also pay an honor to all the fishes that died due to some technical problems.

ABSTRACT

Water. It is one of the basic needs of all living creatures; be it man, plant or animal. The pharmaceutical waste dumping in the freshwater bodies in one of the main problems. These wastes contain many chemicals, antibiotics and other substances that degrade the water quality; and make it unworthy of human use and/or aquatic life. This work focuses on one of the main pollutants: Endocrine Disrupting Chemicals (EDCs). Some main examples are: Amoxicillin, Paracetamol and Diclofenac. These are Antibiotics and thus more commonly used. The chosen freshwater fish species for this study was Cyprinus carpio at fingerlings, juveniles and adult life-stages. This species is moderately tolerant to adverse conditions, hence an ideal choice. Also, it is very commonly found in the freshwaters of South Asian regions. The study comprised of two sets of experiments: Enzyme Assays and Predictive Toxicological Analysis. The Enzyme Assays comprise of (a) studying the Behavioral, Physical and Biochemical Responses induced by the pollutant exposure and (b) understanding the effects of presence and/or absence of water plants. The Predictive Toxicological Analysis gives an estimation of the impact of the same pollutants on some other freshwater species.

From our experiments (Enzyme Assays), we have come to believe that, the newborn fishes are more susceptible to the pollutants than the adults. Also, one more interesting phenomenon was observed. The juvenile fishes showed more resistance to toxicity than both new-born and adult fishes. Literature says that, the enzymes and their activity at this growth stage is so active that it manages to get acclimatized with the toxic conditions. So, the situation imitates a vaccination procedure where the subject is given lower doses of pathogens (toxins, in this case) to help develop immunity to the higher loads of same pathogens (toxins, in this case). The Amoxicillin affected the liver most, whereas Paracetamol and Diclofenac paralyzed the muscle and brain tissues. The Predictive Toxicological Analysis provided that the genetically closer species (to C. carpio) were more endangered by these pollutants than the taxonomically related ones. The farther the relation from C. carpio, lesser the chances of almost same reactions and toxic effects.

Keywords: *Amoxicillin, Paracetamol, Diclofenac, Endocrine Disrupting Chemicals (EDCs), Enzyme Assays, Predictive Toxicological Analysis, Cyprinus carpio, Behavioral Responses.*

NOMENCLATURE

Endocrine Disrupting Chemicals	*EDCs*
Amoxicillin	*AMX*
Paracetamol	*PCM*
Diclofenac	*DCF*
Pharmaceutically Active Compounds	*PhACs*
Wastewater Treatment Plants	*WWTPs*
Reactive Oxygen Species	*ROS*
Hydrogen Peroxide	H_2O_2
Superoxide Anion	O_2^-
Hydroxyl Radical	OH^-
Lipid Peroxidation	*LPO*
Deoxyribose nucleic acid	*DNA*
Lowest Observed Adverse Effect Level	*LOAEL*
Chlorpyrifos	*CHL*
Real-Time Polymerase Chain Reaction	*RT-PCR*
Cytochrome P450	*CYP450*
Cytochrome P1A	*CYP1A*
Cytochrome P3A	*CYP3A*
Cytochrome P2M	*CYP2M*
N-acetyl-p-benzoquinone imine	*NAPQI*
Acetylcholine esterase	*AchE*
Lactate dehydrogenase	*LDH*
Acid phosphatase	*ACP*
Alkaline phosphatase	*ALP*
Alanine aminotransferase	*ALT/ GPT*
Aspartate aminotransferase	*AST/ GOT*
Nonsteroidal Anti-Inflammatory Drugs	*NSAIDs*
Cyclooxygenases	*COXs*
Cyclooxygenase 1	*COX1*

Cyclooxygenase 2	*COX2*
Dihydropteroate Synthase	*DHPS*
Inhibitor Concentration where response is reduced by 50%	*IC50*
Enzyme Commission number	*EC number*
Glutathione	*GSH*
Acetylcholine	*AChol*
Interleukin-1	*IL-1*
Interleukins 1A	*IL-1A*
Interleukins 1B	*IL-1B*
Histamine H1 receptor	*H1R*
Acetylcholine Receptor	*AChR*
Nicotine ACh Receptor	*nAChR*
Sodium ion	*Na+*
Potassium ion	*K+*
Muscarinic Acetylcholine Receptor	*mAChR*

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CHAPTER 1

INTRODUCTION

1.1. CURRENT SCENARIO

Many pharmaceutical compounds persist through the human body and are resistant to conventional waste water treatment practices. They are often detected in aquatic environments such as lakes, rivers and ground water, which can receive direct inputs of treated waste water. Data collected by the U.S. Geological Survey and by individual municipal water utilities strongly suggest that pharmaceuticals are entering the environment and bypassing current treatment processes. It is known with certainty that the most important sources of these pharmaceuticals include those intentionally disposed into the sewer system, discharged or released from livestock farms, and that are excreted with human waste. The lifecycle of pharmaceuticals - from production, to use, to excretion and disposal - generates significant excess that ends up as waste.

Some of the main pharmaceutical pollutants are the diclofenac, paracetamol and amoxicillin. These chemicals or pollutants tend to bio-accumulate and/or bio-magnify. Since they are very commonly found in most of the medications used for humans and animals, their disposal into the environment is very frequent. They are found to be present in the local water bodies and adversely affecting the health of the aquatic life within. The harm caused to the edible aquatic life is carried forward and thus can have a negative impact on the animals and humans consuming them.

One of the most frequently detected pharmaceuticals in environmental water samples is the anti-rheumatic drug, diclofenac. Despite its increasing environmental significance, investigations concerning the effects of this drug on the early developmental stages of aquatic species are lacking up to now. The impact of diclofenac on river biofilm communities was also investigated. The overall eco-toxicological effect of pharmaceutically active compounds (PhACs) detected in the effluents of wastewater treatment plants (WWTPs) was studied using different bioassays. It has been observed

that diclofenac is persistent in wastewaters and a considerable amount reaches the river streams.

The toxicity of any substance is nothing but the potential harmful effects of that substance over any life-form. The level of toxicity depends on different factors like the organism, age of the organism, extent of exposure to the toxicant, nature of the toxicant (mode of action and medium), environmental factors and health factors. There are many different techniques to assess the toxicity. The methods used, generally are based on the organism to be tested for toxic effects; on the nature of the toxicant; the medium of exposure; mode of action of the toxicant. Latest techniques facilitate estimation and evaluation of the bioaccumulation and biomagnification levels of the organism. The toxicity analysis will be categorized as acute and chronic toxicity. Acute toxicity is the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance. Chronic toxicity is the adverse effects of a substance that result either from a prolonged exposure to a substance or prolonged internal exposure because a substance remains in the body for a long time. To be described as chronic toxicity, the exposure of the substance should be 30-180 days.

The predictive toxicity for different species of freshwater fishes clubbed with the lab results give us a more relative scenario of the "Affected" and "Would-be-Affected". Thus, it is important, that we understand the impacts of the same pollutants over different species of freshwater fish- either genetically related or just taxonomically.

1.2. PROBLEM IDENTIFICATION

From an extensive literature survey, it was observed that the Indian contribution in research conducted for the toxicity studies on freshwater fishes is minimal. Research papers were found on toxicity studies of EDCs, antibiotics, pesticides and some other harmful compounds on freshwater fishes. But most of them either focus on the eggs, larvae or adults, while a few experimented-on fingerlings or juveniles. Aforesaid researches based their study on the concentrations of pollutants usually found in the aquatic environment; thereby discovered disruptions in the reproductive, vascular,

histopathological systems leading to severe health problems in the target freshwater fish species.

This study differentiates itself by analyzing (observing and comparing) the toxic effects of EDCs (AMX, PCM, DCF) on 3 life-stages (fingerlings, juveniles, adults) of a freshwater fish species (common carp). It also studies the influence caused by inclusion and/or exclusion of aeration and aquatic plants on the toxicity levels. The Predictive Analysis gives an estimation of other freshwater fish species that can be affected similarly.

1.3. OBJECTIVES

The following are the objectives of the present study,

1. To analyze the toxicity of the individual EDCs on the freshwater fish by evaluation of the degree of damage to the different vital organs of the fish and the consequent physiological, biochemical and behavioral changes.

2. To understand and estimate the influence and/or interference of water plants (hydrilla) and aeration on the pollutant concentration in the observation tank.

3. To predict possible toxicological outcomes for some other freshwater species.

1.4. SCOPE OF THE WORK

This study is meant to *draw a line* for pollutant disposal and concentrations levels of chosen compounds in the freshwater sources. Pollution would dwindle the abundance of reserves of freshwater. It is also a common threat to *all* the life forms using the said water as habitat or for day-to-day purposes.

The safety of aquatic lifeforms in a freshwater source is questionable due to the presence of pharmaceutical wastes and drug remnants. The Pisces are the most common choice to evaluate the impact in an aquatic environment. They possess both adaptability and vulnerability required to be the choicest target species. Their similarities with the other aquatic life forms with respect to physiological, biological, habitual, responsive, dietary attributes is an additional advantage to the test. The work proposed would estimate the risks and levels of the pharmaceutical compounds that are tolerable and lethal to the concerned species.

Determination of the toxicity is essential for evaluating the degree of damage to the target organs and the consequent physiological, biochemical and behavioral disorders. Also, analysis of the impact in a predictive way using the bioinformatics advances would save lot of fish being sacrificed for experimental purposes; provide insight into the possibility of level of toxicity among genetically or taxonomically related (or otherwise) species.

1.5. ORGANISATION OF THESIS

In this study, the entire work is divided into five chapters. The essence of each chapter is as mentioned below.

Chapter 1 focuses on provision of a Background to understand the relevance and the significance of this topic.

Chapter 2 gives an astute reflection of the Literature that has already hit the mark. It presents extracts and reviews from the work of other researchers who have worked in the similar areas. It also explains the reason for choosing pollutants for this study. The physico-chemical properties of the chemicals and pollutants used, the characteristics of the fish involved are detailed in here.

Chapter 3 is all about the Materials and Methods adopted in this study. The instruments used, their specifications, chemicals used are mentioned in here. This chapter also gives a detailed account of all the procedures involved to attain reliable and accurate results.

Chapter 4 is dedicated to the Results and Discussions of the same. The focus is on the explanation of observations and results and justification for the accuracy of the results. This encompasses the fulfillment of all objectives set.

The last chapter- Chapter 5 is allotted for Summary and Conclusions. The results of the entire work are presented concisely herein. All the Observations, Justifications, Explanations, Predictions and Probabilities boil down to this chapter.

CHAPTER 2

LITERATURE REVIEW

The freshwater sources are easily accessible, more locally available: Unlike the marine water samples, that are available only on the coast, these are spread far & wide and often found near human habitats/colonies and industrial establishments. We do not have to travel a lot to find a freshwater source) and thus more frequently polluted. Pharmaceutical wastes are one of the main hazards. These get into the waters from various point and non-point sources.

Toxicity to fish varies considerably and depends on many external and internal factors. Among the most important ones are water quality (e.g. pH, temperature, cation, anion and oxygen concentration), duration of exposure, fish species, fish size and age, and individual fish susceptibility. The exposure of fish to the toxicants during the different phases of growth have different impacts on the fish health (Hong, *et al.,* 2007; Holmstrup, *et al.,* 2010; Islas-Flores, *et al.,* 2013, 2014; Jeon, *et al.,* 2016). Some of the toxic materials retard the growth of organs and systems while some other impair their movements.

2.1. FRESHWATER FISHES

Fish can serve as bio-indicators of environmental pollution and can play significant roles in assessing potential risk associated with contamination in aquatic environment since they are directly exposed to chemicals resulting from agricultural production via surface run-off or indirectly through food chain of ecosystem. Fish are endowed with defensive mechanisms to counteract the impact of Reactive Oxygen Species (ROS) resulting from metabolism of various chemicals or xenobiotics. Oxidative stress develops when there is an imbalance between pro-oxidants and antioxidants ratio, leading to the generation of ROS. Environmental contaminants such as herbicides, heavy metals and insecticides are known to modulate antioxidant defensive systems and to cause oxidative damage in aquatic organisms by ROS production. ROS such as

hydrogen peroxide (H_2O_2), superoxide anion O_2^- and hydroxyl radical (OH^-) at supranormal levels can react with biological macromolecules potentially leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and even cell death but at low concentrations their effects are less pronounced.

Several eco toxicological characteristics of C. carpio such as wide distribution in the freshwater environment, availability throughout the year, easy acclimatization to laboratory conditions and commercial importance make this species an excellent test species for toxicity and biochemical studies. Determination of the toxicity is essential for determining sensitivity of the animals to the toxicants and also useful for evaluating the degree of damage to the target organs and the consequent physiological, biochemical and behavioral disorders.

The fish species chosen was *Cyprinus carpio.* Its commonly known as the common carp. *Cyprinus* is Greek and *carpio* is Latin; both words mean carp. The common carp is a widespread freshwater fish of eutrophic waters in lakes and large rivers in Europe and Asia. The wild populations are considered vulnerable to extinction, but the species has also been domesticated and introduced into environments worldwide, and is often considered an invasive species.

Table 2.1: Scientific classification of common carp

SCIENTIFIC CLASSIFICATION	
Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebreta
Superclass	Gnathostomata
Class	Actinopterygii
Order	Cypriniformes
Family	Cyprinidae
Genus	Cyprinus
Species	Cyprinus carpio
Subspecies	Cyprinus carpio carpio

It is related to the common goldfish (*Carassius auratus*), with which it is capable of interbreeding. Common carp is in the family Cyprinidae (minnow and carp family). *Cyprinus carpio* is easily identified by two pairs of barbells on each side of the upper jaw. These silver-grey fish have serrated dorsal and anal fin spines. Common carp is one of the largest members of the minnow family. The common carp have large scales, a long dorsal fin base, and two pairs of long barbells (whiskers) in its upper jaw. Wild common carp are typically slimmer than domesticated forms; with body length about four times body height, red flesh, and a forward-protruding mouth. Their average growth rate by weight is about half the growth rate of domesticated carp.

Although tolerant of most conditions, common carp prefer large bodies of slow or standing water and soft, vegetative sediments. As schooling fish, they prefer to be in groups of five or more. They naturally live in temperate climates in fresh or slightly brackish water with a pH of 6.5–9.0 and salinity upto about 0.5%, and temperatures of 3 to 35°C. The ideal temperature is 23 to 30°C, with spawning beginning at 17–18°C; they easily survive winter in a frozen-over pond, as long as some free water remains below the ice. Carp are able to tolerate water with very low oxygen levels, by gulping air at the surface.

Cyprinus carpio is the number one fish of aquaculture. The annual tonnage of common carp, not to mention the other cyprinids, produced in China alone exceeds the weight of all other fish, such as trout and salmon, produced by aquaculture worldwide. Roughly three million tons are produced annually, accounting for 14% of all farmed freshwater fish in 2002. China is by far the largest commercial producer, accounting for about 70% of carp production. Carp is eaten in many parts of the world both when caught from the wild and raised in aquaculture.

2.2. THE PHYSIOLOGY OF FISHES

Freshwaters and Marines are two broadly classified sources of water on this planet. The characteristics of the freshwater fish differ with that of the marine fish. The physiology varies as much as it should; considering the two entirely different habitats. The composition and general characteristics of these two are remarkably different. Hence the significant difference in the species living in these two sub-environments.

Evans, *et al.,* (2013) in their book "The Physiology of Fishes" explain that the physiology of the fish is such that it allows for a certain degree of modification and alteration when it is in a non-ideal environment/habitat. This is generally the overall body going through the modifications in the case of the fingerings. The juveniles, however, have less scope for the modification; whereas the adults have them the least. These changes in the tissues are a way of blocking/ coping with the external pollutants present in the immediate environment. Literature also ascertains that the fish immune system grows stronger over time. The fingerlings, by far have the least immunity as compared to the juvenile and the adult. Also, the adult has the advantage of a stronger physique and highly developed, more complex exterior that reduce the damage caused to some extent.

The muscles, brain and liver all have their own way of fighting off the pollutants' effects. All along, so many kinds of reactions are triggered. Some reactions and pathways are blocked while some are enhanced. Some enzymes are inhibited while others are expressed more than usual and normal amounts. All these details when matched with the physical appearances of the fishes undergoing toxicity give us an almost complete picture of the toxicity effects.

As per the literature, the study of pollutants affecting the aquatic life is an intricate web of many careful observations and detailed analysis. The dosage of the pollutants in the water, the health and life-stage of fish being exposed to the pollutants all matter alike. The physiological-behavioral changes occurring all-along provide the proof of the damage caused by the pollutants. It is catalogued in graphs and tables as a part of the interpretations of observations done during study. All the above has been carried out thoroughly in this study. By this, we aim to recognize the range of specific pollutants that can trigger a negative response. These responses include a spectrum from inconvenience to mortality. We also hold interest in coming up with a suggestive toxicological prediction that can predict the toxic range and the response associated with the similar freshwater fish species.

Some of the literature that highlight and support the key points of the conclusion are discussed in this chapter. Literature study reveals that, the scales first begin to form during the alevin phase of the physical development. At this stage the oxygen intake through the cutaneous membranes is higher than in other stages of development. Since

the surface area to total mass ratio is too high at this stage, comparatively, the rate of oxygen diffusion to the internal tissues through the skin is also higher than in adults (Atwood, *et al.*, 2001; Ramesh, *et al.*, 2014). This means that, in the newborns, the skin acts as a semi-permeable barrier that allows for exchange of ions and compounds between the aquatic environment and the fish internal tissues. Thus, because the newborns are, as it is more susceptible to the pollutants due to lack of proper immunity, this semi-permeability of the skin acts as an additional passage/intake of pollutants into the body. Whereas in adults the skin is hardly semi-permeable, thereby reducing simultaneous intake level of pollutant. Also, adults have the advantage of a developed defense system.

There are pollutants that are blood-poisons. These highly rely on the oxygen-carrying capacity of the blood to induce negative effects. The fingerlings have proven to have less of that when compared to the adults. Therefore, it makes sense that adults are more prone to such pollutants than the fingerlings are (Bergman, *et al.*, 1996). The oxygen-carrying capacity of blood is a vital factor. Since the juvenile and new-born are devoid of this dependability, they tend to survive longer even when the blood is infected. Also, Atwood et al. (2001) found out that the tolerance of *Nile tilapia* (Oreochromis niloticus) to nitrite is dependent on the fish size i.e., the life stage of the target fish. The difference in age and sexual development between both target groups was not mentioned.

From Ichthyology, we also understand that, the fish gill is a multipurpose organ that, in addition to providing for aquatic gas exchange, plays dominant roles in osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes. Thus, even though all fish groups have functional kidneys, the gill epithelium is the site of many processes that are mediated by renal epithelia in terrestrial vertebrates. Indeed, many of the pathways that mediate these processes in mammalian renal epithelial are expressed in the gill, and many of the extrinsic and intrinsic modulators of these processes are also found in fish endocrine tissues and the gill itself. The basic patterns of gill physiology were outlined over a half century ago, but modern immunological and molecular techniques are bringing new insights into this complicated system. Nevertheless, substantial questions about the evolution of these mechanisms and control remain unanswered. It is also observed that, more pharmaceuticals were detected at higher concentrations and with greater frequency in liver than in fillet tissues.

The Fingerlings will not have the scales fully developed. This results in the absorbance of the pollutants through the muscle tissue in addition to the general mode of contact. The Plasticity is at its best, in the juvenile. The pros being well adapted to already-adverse conditions and cons are the non-recognition of the hazardous materials around them and thus failure at self-defense as Adult or even when the concentration of pollutants increase in the Aquatic environment. Other than muscles, Plasticity is also observed for various other systems including the cardiovascular system. The normal functioning of these systems is dependent on the availability of Oxygen in water. Hypoxia and Normoxia both, affect the growth, swimming ability, speed and formation & development of muscles. Thus, Hypoxia and Normoxia affect the fingerlings and juvenile more than the adult. The abundant Oxygen levels along with moderate temperatures (for acclimatization) in water not only encourage regulated gene expression & cell cycles, they also steady metabolism rate, angiogenesis, cell stress and apoptosis. The EDCs are studies more rigorously over the last couple of decades, but the beginning of the impact goes way back to 1970s. The EDCs are observed to target the brain, thyroid, liver, and gonad by hiking the stress levels near the cerebral region and clearance of steroid hormones by liver. The Conventional Regulatory Toxicology strongly adheres to tests like High-Dose Testing and Lowest Observed Adverse Effect Level (LOAEL). These phenomena and scenarios is extensively illustrated by Evans, *et al.,* (2005).

A major proportion of energy production in fish is inclusive of the catabolism and oxidation of proteins and amino acids. The major end product of nitrogen metabolism in most fish is ammonia (Anderson, 1995). When this ammonia is not released out from the body regularly, it accumulates in the mitochondria, impacts liver efficiency, increases the pressure on the brain and nerves. Loss of appetite are the initial symptoms; liver failure: one of the various possible end results.

Jeon, *et al.,* 2016 worked on different life-stages of zebrafish (adults and larvae). The chlorpyrifos (CHL), an insecticide used extensively in agricultural fields was tested for toxicity. They found that "Relative gene expression analyses using real-time polymerase chain reaction (RT-PCR) of DNA from zebrafish embryos revealed that different subtypes of cytochrome P450 (CYP450), such as cytochrome P1A (CYP1A)

and cytochrome P3A (CYP3A), were significantly up-regulated" even when the concentrations of pollutant were in μg/L concentrations. The AChE levels dipped but "no acute toxicity was detected in the embryo toxicity test; malformation of zebrafish larvae was observed, with many individuals harboring curved spines."

From Literature Study of Recent Research in Aquatic Toxicology (Ariens, 1984; Bodiwala, 1988; Anderson, 1995; Almar, *et al.,* 1998; Atwood, *et al.,* 2001; Brian, *et al.,* 2005; Hayes, 2007; Andersen, *et al.,* 2009; Martin, *et al.,* 2010; Bell, *et al.,* 2011; Saravanan, *et al.,* 2012; Ambili, *et al.,* 2013; Banaee, *et al.,* 2014; Burkina, *et al.,* 2016; Xiong, *et al.,* 2017; etc.), we found, fishes generally used include *Cirrhinus mrigala*, *Labeo rohita*, Rainbow Trout, Zebrafish, Nile tilapia, etc. Some of the studies have experimented and compared the results for more than one growth-stage using various standard procedures for on-site and off-site samples. Some of the studies (Newman, 2012; Walker, *et al.,* 2012) on Amoxicillin and Diclofenac toxicity have reported major retardations in vital systems and induced impotency in reproductive glands of both male and female fish species.

To analyze the impacts on freshwater fishes, the researchers have selected paracetamol as a model-toxin, since it is known to be bio-activated by 3-methylcholanthrene inducible cytochromes P450 presumably to *N*-acetyl-*p*-benzoquinone imine (NAPQI), a reactive metabolite which upon over-dosage causes protein- and non-protein thiol-depletion, lipid peroxidation and cytotoxicity measured as LDH-leakage. They found that the product is fatal, but the effects are unknown, when it is combined with other pharmaceuticals present alongside.

Though the structure and activity of the amoxicillin are very well known, little is known about its reaction on the freshwater fish systems. The amoxicillin enters the freshwater streams by bypassing the treatment techniques. This drug component is one of the most commonly used drugs. Thus, the entry into the freshwater systems can also be possible through general disposal of these medicines. The intentional disposal into the drains is one of the main sources. Especially the disposal of outdated medicines poses the threat of unknown reactions of the amoxicillin with other pharmaceuticals and pollutants present in the wastewaters.

From all the above excerpts, we can understand that the gills, liver and muscle are some of the vital organs of the fish system and that the impact of pollutants onto these have a

direct implication on the overall fish health. Not much literature was available on the impacts of the toxins onto the brain of the freshwater fish. Thus, we have attempted to understand and analyze the impacts on the brain in the results and discussion chapter. More focus has been given to the symptoms, causes, abnormalities caused, and intensity of brain tissue damage and its impacts on the overall health and mortality of the fish.

In this study, we decided to carry out toxicity tests to the extent of mortality for Amoxicillin, Paracetamol and Diclofenac on 3 stages of growth (fingerlings, juvenile, adult) for *C. carpio*. The reason for picking these 3 stages is that, they are easily available, abundantly found in South Asia and considerably tolerant and adaptive to adverse conditions. The embryo and larvae are too sensitive comparatively and the survival rates are usually low in carp fishes. The fingerlings are sometimes, a meal to the other adults in the school. Also, the physiology and the attributes related to growth are almost common among these stages than the embryo and larvae.

2.3. NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, naproxen, ibuprofen, ketoprofen, and indomethacin, are human pharmaceuticals that are regularly detected in both WWTW effluent and surface waters at concentrations in the μg/L range (Minguez, *et al.*, 2016). The therapeutic role of these pharmaceuticals is to reduce inflammation and pain; these drugs work by inhibiting cyclooxygenases (COXs), enzymes that catalyze the synthesis of prostaglandins, via the oxidation of arachidonic acid.

There are two isozymes: COX1 is responsible for the baseline levels of prostaglandins, and COX2 produces prostaglandins in response to stimulation (i.e., it is an inducible enzyme) at the site of inflammation. NSAIDs can be COX1 or COX2 selective, or can inhibit both isozymes equally. This drug target is conserved across vertebrates (Han, *et al.,* 2010), and both COX1 and COX2 isozymes have been characterized in several fish species (Zhou et al., 2013). Most NSAIDs inhibit both COX1 and COX2 isoforms, and as such, result in the nonspecific inhibition of prostaglandins. This, in turn, means there is the potential for effects on any of the normal physiological functions mediated by prostaglandins, which are diverse (Fernandes, *et al.,* 2014).

In fish, prostaglandins are found in numerous cells and tissues, including red blood cells, macrophages, and oocytes, and their key roles include in reproduction, where they have a paracrine role in the ovary, stimulating ovulation and oestradiol production (Agus, *et al.*, 2015; Berntssen, *et al.*, 2016); eliciting female sexual behavior through effects on the brain; and as a sex pheromone, stimulating male sexual behavior. Consistent with the likelihood that NSAID can affect reproduction in fish, indomethacin has been shown to disrupt the process of oocyte maturation and ovulation in zebrafish at a concentration of $100mgL^{-1}$ (Ambili, *et al.*, 2013) and ibuprofen has been shown to alter the pattern of spawning in Japanese medaka at concentrations of μgL^{-1} (Flippin *et al.*, 2007).

These reported effects, however, exceed those reported in any aquatic environment. COX-synthesized prostaglandins are also known to be important in cortisol biosynthesis in fish and accordingly it has been demonstrated that NSAIDs can disrupt cortisol production in trout (Hartung, 2009; Bell, *et al.*, 2011). Cortisol is known to play an important part in osmoregulation in fish, specifically the development and proliferation of chloride cells in the gills and stimulation of Na^+, K^+, -ATPase activity for seawater adaptation. Exposure of rainbow trout to ibuprofen at a concentration of $1mgL^{-1}$ has been shown to impair ion regulation and so the hypo-osmoregulatory capacity in seawater (Corcoran *et al.*, 2012). NSAID exposure has also been linked to cardiac abnormalities and lowered heart rate, depletion of glycogen in the liver, teratogenicity in zebrafish embryos, disruption of the heat shock response in rainbow trout, and inhibition of CYP2M activity in carp (Flippin, *et al.*, 2007; Barney, *et al.*, 2008; Banaee, *et al.*, 2014).

2.3.1. Antibiotics

Antibiotics are a wide-ranging group of compounds of which there are several classes with different mechanisms of action. The antibiotics include sulfonamides, penicillins, and tetracyclins, many examples of which have been detected in WWTW effluents and surface waters at nanogram to microgram level concentrations (Bergman, *et al.*, 1996; Barney, *et al.*, 2008). There are also reports of much higher concentrations at point discharge sources; for example, sulfonamide concentrations of $5mgL^{-1}$ were detected downstream of a landfill used for pharmaceutical production waste disposal (Brausch,

et al., 2012; Burkina, *et al.,* 2016) and oxytetracyclin was found to be present at concentrations of up to 50mgL^{-1} in effluent from a production facility in China (Gan, 2010; Fu, *et al.,* 2011; Gao, *et al.,* 2012).

Antibiotics are a commonly used and important group of pharmaceuticals in both human and veterinary medicine, used to combat bacterial infection. Their modes of action vary according to type: penicillins impede synthesis of the bacterial cell wall; tetracyclines bind to ribosomes and impair protein manufacture; and sulfonamides competitively inhibit bacterial enzyme dihydropteroate synthase (DHPS). Despite the different modes of actions, however, the ultimate effect is the suppression of bacterial growth, and so they are used therapeutically to prevent and treat bacterial infections, as well as growth promoters in farming and aquaculture. The presence of antibiotics in the aquatic environment has generally been investigated in terms of the development of bacterial resistance (Carlsson, *et al.,* 2009; Cuklev, *et al.,* 2011; Darwano, *et al.,* 2014; Chen, *et al.,* 2015), and knock on effects regarding human health by the transfer of resistance to human pathogens, rather than for any concern for possible toxicity to aquatic organisms.

Unlike most pharmaceuticals, antibiotics are specifically aimed at bacterial targets avoiding possible toxicity to the infected human or animal. Some classes of antibiotics are used to combat bacterial infections in fish farms. Despite the frequent occurrence of various antibiotics in the aquatic environment, there is almost nothing in the literature reporting toxic effects of these drugs in fish (Ghorpade, *et al.,* 2002; Gerhardt, 2007; Gholami-Seyedkolaei, 2013; González-González, *et al.,* 2014; Guiloski, *et al.,* 2015). Sulphonamides, however, are acutely toxic to fish medaka, *Thamnocephalus platyurus*, *Oryzias latipes* (Kim *et al.,* 2007, 2009), at high exposure concentrations (>100mgL^{-1}), but these are found rarely in the aquatic environment. In rainbow trout, harmful effects of diclofenac, including the hypertrophy of epithelial chloride cells in gills. It is not known, however, whether the gill effects are associated with prostaglandin inhibition, or through another mechanism (Fu, *et al.,* 2011). In contrast, no pathological damage to the gills was detected on exposure of Japanese medaka. This may be due differences in the relative potencies of these drugs: diclofenac COX-2 IC50 is 0.06 μM, whereas the IC50 for ibuprofen is 19 μM (Carballeira, *et al.,* 2012). Some of the biochemical and genetic changes caused by the diclofenac intake by the freshwater species are as

follows: In Brown trout, DCF hinders prostaglandin synthesis and damages the gill, liver, and kidney. In Rainbow trout, histological alterations in gills and kidney; cytological alterations in liver, kidney, gills are caused by DCF exposure. Inhibition of CYP2M in Carp is also resulted by the DCF. But these research articles have, generally, chosen the adult fish for the experimentation. The literature on the fingerlings and the juvenile fish are hardly available for AMX, PCM and DCF.

2.4. ENZYMES

There are many kinds of protein, which can basically be split into two groups. The first group covers the structural proteins, which are the main constituents of our bodies. Well-known structural proteins are collagen, which is the protein of bones, tendons and ligaments, and keratin, the protein of nails, hair and feathers. The second large group of proteins covers the biologically active proteins. Most of these catalyze biochemical reactions in cells. All known enzymes are proteins and can occur in the body in very small amounts. All the same, enzymes catalyze all processes in the body, enabling organisms to build up chemical substances such as other proteins, carbohydrates or fats that are necessary for life. In short, all enzymes are proteins, but not all proteins are enzymes. If a protein can catalyze a biochemical reaction, it is an enzyme (Eckstein, *et al.,* 2011; Das, *et al.,* 2013; Diniz, *et al.,* 2015).

The enzyme activity occurring in the cells of organisms, plays an important part in determining the toxicity of any compound. Enzymes are proteins or catalysts in any reaction. These are triggered into action or lulled to sleep during various complex pathways. They are promptly and adequately generated in case of emergencies. Toxicity is one such emergency. When an organism is living in an environment that is not perfectly comfortable for its survival and sustenance, it does express new and modified set of proteins that can help self-preservance. These disturbing components are named as pollutants. The pollutants can be of varied nature. They may be harmful in the long run; if not in the short run. These can have a range of effects from physical, behavioral, cellular, genetic etc. The degree of harm caused to the life system in the environment, determines the intensity of the problem. The body's natural way of addressing this anomaly is by enzyme production. Hence, the study of the enzyme levels of the test organism while subjected to the pollutants gives a clear image of the toxicity scenario.

2.5. ENZYME ASSAY

The enzymes concerned in this test are the following: Alkaline phosphatase (ALP), Acid phosphatase (ACP), Aspartate aminotransferase (AST/ GOT), Alanine aminotransferase (ALT/ GPT), Lactate dehydrogenase (LDH), Acetylcholine esterase (AchE). See Appendix A for specifications on enzymes and their reactions. The AST and ALT enzymes are shot up if there is any harm caused to liver. The ACP, ALP levels shoot up if there is damage to liver as well as muscle tissues. The AchE levels are associated with the normal functioning of the brain and the muscle while LDH are only concerned with the muscle tissues. Normal LDH levels assure the regular and the normal functioning of the muscle tissues. Unlike other enzymes, when brain and muscle tissues get negatively affected by the pollutants, AchE levels drop significantly. This drop, in levels cause inhibition of activity in brain and muscle tissues. The AchE enzyme is mainly responsible for the signal transfer and communication from brain to all other parts and within muscles.

The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme. The Appendix B mentions about the top-level EC numbers as well as the EC numbers of the enzymes monitored in the current study. The enzymes belonging to the EC4, 5 and 6 are the ones that are affected by the rise and fall of the enzyme levels of the EC1, 2 and 3 in the fish biological system. These enzymes help the building and repair of different cells and/or organs. They play a crucial role in the survival of the species.

2.5.1. Functions of the enzymes

The pollutant that affects the liver (amoxicillin, in this case) gets conjugated or chelated to glutathione and enters by bile. Therefore, the GSH levels rise. The ACP and ALP enzyme levels are membrane damage indicators. Therefore, it is obvious that their receptors lie on the membrane surface and not on any organelle within the cells. ACP and ALP levels are basic, and first step of damage caused. The AST and ALT are the final step of damage. They confirm the lethality inflicted on the liver. Liver failure occurs soon thereafter and leads to the mortality of the concerned fish. The AST and

ALT are responsible protein metabolism during the regular, non-toxified state of the 0fishes. They also maintain amino acid turnover levels in the cells. These are generally produced in low levels as to suffice the maintenance of the cells and its regular activities (Landis, *et al.* 2011; Walker, *et al.* 2012; Jia, *et al.* 2014; Jeon, *et al.* 2016).

During the pollutant infusion, the normal or regular cell cycles and pathways are disturbed. The amino acids that are functional and responsible for cell damage repair and healing are rushed to the damaged or infiltrated sites. So, when the damage caused is more intense, more amino acids are required. The AST/ALT levels start soaring so as to facilitate higher amino acid turnover. Thus, that leads to higher protein levels in the cells. The AST and ALT also help in post-translational modifications of the proteins. Therefore, there would be steep rise in AST and ALT when compared to ACP and ALP levels in the liver, during liver damage (Landis, *et al.* 2011; Walker, *et al.* 2012; Jia, *et al.* 2014; Jeon, *et al.* 2016).

It inhibits the actions of acetyl cholinesterase (AChE) enzyme which in turn inactivates a "neurotransmitter", acetylcholine (AChol). The neurotransmitter is present and necessary in various parts of the nervous system to enable transmission of stimulation either between nerves, or between nerves and various organs. Normally AChE catalyzes the hydrolysis of the acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation. In the absence of acetyl cholinesterase, acetylcholine level increased resulting in the failure of transmission of stimuli to the nerves or organs. This leads to the abnormal functioning of the body including loss of balance, moving in circular form (convulsions) and at higher concentrations of insecticides resulting in death of the organism. Inactivation of AChE activity results in excess accumulation of acetylcholine in cholinergic synapses leading to hyper stimulation and cessation of neuronal transmission i.e., paralysis (Fierstine, *et al.,* 1968; Landis, *et al.,* 2011; Walker, *et al.,* 2012; Lee, *et al.,* 2014).

2.5.2. *Receptor and its position*

The pollutants act differently on different organs. It also depends on whether the receptor is on the surface membrane of the cell or on any intracellular organelle. There can be promoters other than inhibitors and enhancers for enzymes. Every organism has

some genes that are induced only during inflammation or any other abnormalities (Ambili, *et al.* 2013; Jeon, *et al.* 2016).

The Interleukin-1 family (IL-1 family) is a group of 11 cytokines, which plays a central role in the regulation of immune and inflammatory responses to infections. The Interleukins 1A and 1B act as receptors for enzymes ACP and ALP respectively (Karaca, *et al*. 2014). They are found in the blood serum. The Histamine H1 receptor (H1R) is responsible for the AST and ALT expression. H1R is induced during cardiovascular and liver abnormalities. The extent of expression varies with the species as well as the health of the organism (Goksoyr, *et al.* 1992; Jia, *et al.* 2014; Karaca, *et al*. 2014). An acetylcholine receptor (AChR) is an integral membrane protein that responds to the binding of acetylcholine, a neurotransmitter. Like other transmembrane receptors, acetylcholine receptors are classified according to their "pharmacology," or according to their relative affinities and sensitivities to different molecules. Although all acetylcholine receptors, by definition, respond to acetylcholine, they respond to other molecules as well. Nicotinic and muscarinic are two main kinds of "cholinergic" receptors. The nicotine ACh receptor (*nAChR)* is also a Na+ and K+ ion channel along with nicotine. Muscarinic acetylcholine receptors (*mAChR*) are particularly responsive to muscarine (Itakura, *et al.* 2005; Yan, *et al.* 2012)

2.5.3.   Effect on regular enzyme pathways

From literature review, we already know that, NSAIDs can be COX1 or COX2 selective, or can inhibit both isozymes equally (Goksoyr, *et al.* 1992; Das, *et al.* 2013). Most NSAIDs inhibit both COX1 and COX2 isoforms, and as such, result in the nonspecific inhibition of prostaglandins. In fish, prostaglandins are found in numerous cells and tissues, including red blood cells, (Das, *et al.* 2004; Corcoran, *et al.* 2012; Burkina, *et al.* 2016) thereby affecting the system negatively. Both COX1 and COX2 isozymes have been characterized in the above-mentioned choice of fish species (Das, *et al.* 2004; Das, *et al.* 2013; Diniz, *et al.* 2015). NSAID exposure has also been linked to cardiac abnormalities and lowered heart rate, depletion of glycogen in the liver (Karaca, *et al*. 2014) and inhibition of CYP2M activity in carp (Kaminishi, *et al.* 2007; Barney, *et al.* 2008; Blaauboer, *et al.* 2012; Banaee, *et al.* 2014; Agus, *et al.* 2015).

2.6. PREDICTIVE TOXICOLOGICAL ANALYSIS

The pathway of the pollutants in the freshwater fish must be traced. The enzymes in the target organs and/or tissues have either increased or decreased in the wake of the pollutant infusion in the fish body. So, the possibility of involvement of some enhancers or inhibitors is very likely. That is saying, the pollutants act like enhancers or inhibitors during the enzyme release pathway.

Each enzyme is the end product of a series of processes in the cell. These processes may/may not change during pollutant invasion. The changes can be due to interruptions in the transcription, translation or the post-translation modifications. The operon models must be compared with the basic enzyme output mechanism. That would explain whether the pollutant is directly or indirectly responsible. Only then will the gene for these expressions can be identified.

The researches and advances in Bioinformatics provide us with softwares and databases that allow us to visualize the both virtual and hypothetical possibilities in most of the processes at the genetic level. Ensembl (www.ensembl.org) is a joint scientific project between the European Bioinformatics Institute and the Wellcome Trust Sanger Institute, which was launched in 1999 in response to the imminent completion of the Human Genome Project with scientists in the United States, the international consortium comprised geneticists in China, France, Germany, and the United Kingdom. Its aim is to provide a centralized resource for geneticists, molecular biologists and other researchers studying the genomes of our own species and other vertebrates. Ensembl is one of several locations for the retrieval of genomic information. Similar databases and browsers are found at NCBI and the University of California, Santa Cruz (UCSC). NCBI website (www.ncbi.nlm.nih.gov) is a website that acts a database for the genomes of almost all organisms varying across all phyla and taxa. The organisms are deeply classified and placed in accurate hierarchy; their species variations and evolution also considered.

2.6.1. Genome matching

There are two sets of DNA found in each cell. One is nuclear set, found in the nucleus in cell. Mitochondria are intracellular organelles containing non-nuclear DNA. These are relatively easy to amplify because of high number of mitochondria in a cell as

opposed to the single nucleus present in each cell. The circular set of mitochondrial DNA remains stable post-extraction while the nuclear DNA is prone to degradation, being more sensitive to heat and other variations (Fange, 1983; Ariens, 1984; Avise, 1994; Andersen, *et al.,* 2009). Researchers have attempted and succeeded at mapping the entire genomes of organisms. This usually refers to the nuclear set. The mitochondrial set is also mapped but available as a second set. The only drawback of using mitochondrial gene set is that, as these are inherited from the maternal side of the organism, (unlike the nuclear that are inherited from the paternal side) the identification of the species becomes complicated if it is a hybrid (a cross between two species).

In the last decade, genetic identifications have focused on using the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene as a global identification system for animals because several studies across a range of taxa have found that intraspecific variation in COX1 is very low, whereas variation among species, even closely related species, is relatively high (Hebert *et al*. 2003; Ku, *et al.* 2014). Other genes are preferred for some animal groups (for example, the mitochondrial cytochrome oxidase b gene for fish, although the availability of fish COX1 sequences is increasing). (Hayes, 2007).

Predictive toxicological analysis comprised of identification of genes responsible for the over-expression/inhibition of certain key enzymes; the contribution of pollutant in affecting their level rise/fall; as well as comparison of the genetic composition of the *Cyprinus carpio* with other freshwater fishes: *Cirrhinus mrigala* (mrigal carp), *Salmo gairdneri* (rainbow trout), *Carassius cuvieri* (Japanese crucian carp), *Carassius auratus* (goldfish) and a goldfish- common carp hybrid. The choice of other species was based on the most commonly used species for Bioassays. In this case, the impacts of amoxicillin, paracetamol and diclofenac are known on the freshwater species *Cyprinus carpio*. So, by predictive modelling, an attempt at an educated guess on the impacts of the same pollutants onto different other freshwater species.

CHAPTER 3

MATERIALS AND METHODS

The analysis and evaluation of toxic effects would be done by two techniques. The first one will establish the toxic levels while the second will facilitate predictive conclusions. The two techniques employed in this study are:

1. *Enzyme Assays*

This is one of the standard methods for analyzing the negative effects caused by the pollutants on the target species. The changes in levels of enzymes within the organs of the target species is understood via in-vitro analytical procedures for qualitative conclusions. The organs are extracted from the subjects and samples are readied for the Enzyme Assays. The variations in levels are identified by comparing them with the control group subjects. This is one of the most effective methods to understand the effects caused by pollutants on different organs of the same species. This method holds good for all 3 stages of fish growth (fingerlings, juveniles, adults).

2. *Predictive Toxicological Analysis*

Though the lab results are more reliable in their own way; but occasionally such methods mean using up lot of live resources for experimentation purposes. This method was taken up as supplementary to the lab experiments. Both the methods have their own set of pros and cons. The Predictive Analytical method would give an estimate of the effects on the related species without literally sacrificing them.

3.1. MATERIALS

3.1.1. *Enzyme Assays:*

* **Freshwater fish**

The freshwater fish species, *Cyprinus carpio* (common carp) was selected. They were bought from the College of Fisheries, Mangalore, India. The fish of different age group:

fingerlings, juvenile and adult were tested separately. The fishes obtained were of approximately same length and weight (to be considered in the same age group).

- **Observation tanks (Glass Aquaria)**

The *C. carpio* fish were first transferred into a glass aquarium of 48×24×24inches. This 'control' tank was filled with dechlorinated water to a predetermined level of 150liters. Once in every 14days, 75liters of water in the tank was replaced by fresh 75liters of dechlorinated water. The fishes were then divided into three groups of six each and placed in glass aquaria measuring 24×12×12inches. These 'test' tanks were filled with dechlorinated water to a predetermined level of 50liters. After each run of pollutant dosage, the water was replaced by fresh dechlorinated water in all the tanks.

The control tank was supplied with adequate aeration with two-way high-performance air pump (Champion RS-0088 brand; output of 3.5L/min; AC 220/240- 50Hz; 5W), a light source (12W Essential Energy Saver CFL Lamp -Cool daylight model- Philips brand- AC 220/240- 50Hz) and hydrilla plants (75g/ 150L water). Each of the test tanks were supplied with adequate aeration with one-way high-performance air pump (Champion RS-0078 brand; output of 2.5L/min; AC 220/240- 50Hz; 3W), a 5W Essential Energy Saver CFL Lamp (Cool daylight model- Philips brand- AC 220/240- 50Hz) and hydrilla plants (25g/ 50L water).

The air pump operated all hours relentlessly. The plants played dual role of food as well as oxygen supply back-up system. These plants are functional for the Interference Analysis. The tanks were cleaned regularly to avoid any infections or algae build up in the tanks. A detachable submersible filter pump (model SP- 1000A; 20W; output of 27.5L/min) was used to clean debris and excreta- fortnightly in the control tank, after every run in the test tanks.

- **Feed**

Fishes were regularly fed high quality floating food pellets consisting of fish meal, wheat flour, soybean meal, corn meal, yeast, vitamins and minerals. According to proximate analysis, it has crude protein, min. 32%; crude fat, min. 4%; crude fiber, max. 5% and moisture max. 10%.

- **Dissection instruments**

The fish were dissected using sterile instruments. Standard dissection set is to be used for the same. The glass homogenizer is used to macerate the tissue samples. The tissue samples were pooled on an aluminum foil that is placed on an ice block to maintain very low temperatures. The aluminum foil was 11micron thick and bacteria-build up-resistant.

- **Chemicals used**

All the chemicals and reagents used in this study were of analytical grade of 99.99% purity and were obtained from E. Merck, Hi-Media, CDH, SRL and Nice Chemicals. Amoxicillin, Diclofenac and Paracetamol are the pollutants chosen. They were procured from E. Merck (99.99% pure, HPLC grade). They were stored at room temperature in the lab in amber bottles.

3.1.2. Predictive Toxicological Analysis:

The following are a must to proceed with the Predictive Toxicological Analysis and achieve a probable impact estimation for the chosen species.

1. Identify the Functions of the enzymes affected (level rise or fall)
2. Identify their Receptor and its Position
3. Regular mechanisms or pathways in which these enzymes are involved; their roles in the same
4. Choose freshwater species for comparison and prediction
5. Obtain Genbank Accession Numbers for the chosen freshwater species
6. Use BLAST tool and compare the entire genome of common carp with the other chosen species
7. Based on the BLAST results, draw predictive conclusions about the impacts on the other species.

3.2. POLLUTANT EXPOSURE (for Enzyme Assays)

They are added in different concentrations in 3 different observation tanks. The pollutant concentrations would range from 1 to 80mg/L. The exposure time is set to

96hrs (4days) for acute toxicity test. The fish are to be dissected on the fifth day and their liver, muscle and brain samples will be subject to Enzyme Assay procedures.

3.3. METHODOLOGY

3.3.1. Enzyme Assays:

To observe the effect of exposure of EDCs at different concentrations on the activities of some key enzymes of metabolic importance in vital tissues of the *C. carpio* fish, such as the liver, muscle and brain, the following experiments are to be carried out. The details of the procedure adopted, results obtained, and the significance of the results are presented in the ensuing section.

Table 3.1: Specifications of test organisms and pollutant concentrations.

Life-stage	Size (inch)	Weight (mg)	Pollutant Conc. (mg/L)		
			AMX	PCM	DCF
Fingerlings	1-1.5	~1.2	2, 5, 8, 10, 11, 13, 15	10, 20, 21, 25, 30, 40	2, 5, 8, 10, 14
Juvenile	2-2.5	~2.8	10, 15, 25, 35, 37	20, 25, 40, 48, 50	5, 10, 45, 17
Adult	3.5-4	~4.2	25, 40, 70, 80	30, 35, 45, 50	10, 14, 15, 18

The run was conducted using 6 fishes for each concentration of the pollutant. According to literature, the duration for a short-term analysis must be from 4 to 14 days. Each run for all AMX, PCM and DCF was scheduled for 4 days (96 hrs.) initially. But then the DCF behaved differently and the fishes displayed a delayed response. Hence the study for DCF was extended to 14 days. The first run was conducted on 6 fish (fingerlings) / tank / concentration. The second run was carried out with 10 fish (fingerlings) / tank / concentration. The double run provided us with confirmation of results of the first run. The samples of corresponding organs and concentrations were pooled to suffice for analysis.

The *C. carpio* fish were exposed to different concentrations of EDCs. After the respective run, fish from both control and EDC-exposed groups, are cold-stunned and

dissected to obtain liver, muscle and brain samples. The samples were analyzed, and the optical density readings were noted using a UV-VIS Spectrophotometer and tabulated. The graphs were then plotted for these readings as compared to the control readings. The statistical analysis of the results was done by using Newman-Keul's test and Duncan's multiple-range test. Values are expressed as mean $\pm$ SD, with the significance at $P \leq 0.05$ (Ghorpade *et al*., 2002).

3.3.2. Interference Analysis:

The Interference Analysis was done to check the effects of the aeration and the water plants instilled in the tanks. The hydrilla plants were used for these tests. The hydrilla plants were chosen because- usually found in natural freshwater bodies; they provide natural aeration in the tank; the fish can feed on them for a natural diet. These plants were added as 25g/50L in the tanks i.e. 1g/2L in conical flasks and the aeration was run continuously. The effects of presence and/or absence of both plants and aeration was tested. Four cases were designed: 1) Without aeration, With plants; 2) With aeration, With plants; 3) With aeration, Without plants; 4) Without aeration, Without plants.

3.3.3. Predictive Toxicological Analysis:

This is inclusive of extensive literature review for the data collection that includes studying vital pathways, obtaining accession numbers, identifying the common functional genes responsible for the basic functions like breathing, locomotion, signal transport and barriers. The websites like Ensembl and other tools from NCBI websites, researches on these aspects by various similar Organizations helped compile and study the relevant data. Though, some chosen species did not have a completed genome mapping, relevant and vital portions along with the other available data were used to compare.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. ENZYME ASSAY FOR AMX:

It was observed that as the concentrations significantly increased, the fish were observed to be less active gradually. At the higher concentrations ($\geq$ 10mg/L) the fish would be very passive for about 5-6hrs. They also schooled themselves in a lower posterior corner of the tank. No free movement within the tank was observed. They took a long time to swim to the surface even to feed. They sometimes drifted to some other corner, but hardly came to the surfaces. They lay low and motionless. The physical impacts were in the form of change of fish scale color from silvery to greyish. (refer Appendix C)

During the experiments, all the fingerlings exposed to concentrations in the range 2-5mg/L survived. But at 8mg/L concentration, mortality was observed. Hence 8mg/L is the LOEC (Lowest Observed Effect Concentration). 50% mortality was observed at 11mg/L in 36hrs after the addition of AMX to the water and the remaining fish continued to survive. 100% mortality was observed for 15mg/L in 45hrs. Thus, it was observed that 11mg/L is LC-50 concentration at 36hrs while 15mg/L proved to be lethal for them.

Fig. 4.1: ACP levels in Liver and Muscle in Fingerlings C. carpio due to AMX intake; significance given at mean $\pm$ SD where *P $\leq$ 0.05*

The juveniles, apparently, had a higher resistance to AMX. The LOEC and LC-50 are the 10mg/L and 25mg/L dosages at 96 and 84hrs respectively. 100% mortality was noted at 37mg/L at 48hrs. The adults dealt it better having 25mg/L as the LOEC after 96hrs. The 40mg/L and 80mg/L were the 50% and 100% mortality rates at 72 and 48hrs respectively. The Table 4.1 details the physical and behavioral changes noted during the exposure of fingerlings, juvenile and the adult fish to different concentrations of the AMX.

The ACP levels were estimated in both liver and muscle samples- Fig. 4.1, 4.2, 4.3 for fingerlings, juvenile and adult respectively. The impact is not distinctively noticeable in the range 2-5mg/L concentrations for fingerlings and no mortality observed for those dosages. But there was enough rise in the levels in the all age groups where mortality was observed. Then it noticeably increased in dose-dependent manner. This confirms the rise is apparently due to less extensive harm, but significant rises are indicative for extensive tissue damage leading to death.

Fig. 4.2: ACP levels in Liver and Muscle in Juvenile C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

The change in behavior was as described earlier. It also showed less appetite compared to fish in the control tank. The significant increase gradually had a toll on the fish behavior as well as its health. All the values are directly proportional to damage inflicted.

Fig. 4.3: ACP levels in Liver and Muscle in Adult C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

The ALP levels were estimated in both liver and muscle samples- Fig. 4.4, 4.5, 4.6 for fingerlings, juvenile and adult respectively. Although the impacts were not visibly strong at 2 and 5mg/L, it had negative effects on the fingerlings overall health. The impacts, physical or behavioral, were fatal from 8mg/L concentration onwards for fingerlings. For juvenile and adult, it was 10 and 25mg/L respectively. The differences in ALP levels in the test fish when compared to the control fish indicate severe anomalies in the fish biological systems.

Fig. 4.4: ALP levels in Liver and Muscle in Fingerlings C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

Fig. 4.5: ALP levels in Liver and Muscle in Juvenile C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$
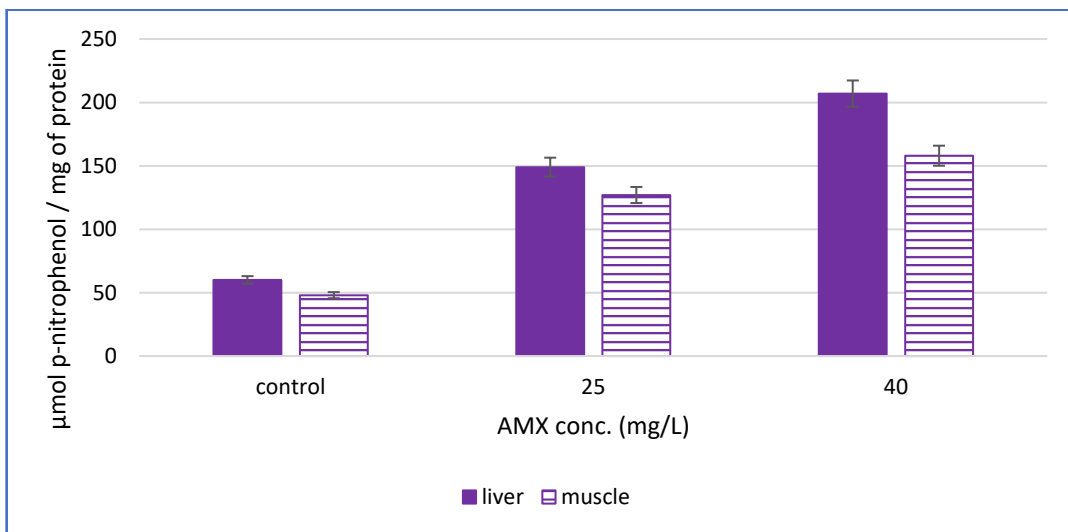
Fig. 4.6: ALP levels in Liver and Muscle in Adult C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

Similar to others, AST levels in liver- Fig. 4.7, 4.8, 4.9 for fingerlings, juvenile and adult respectively rose significantly at 8, 10, 25mg/L respectively. Even in the case of ALT of liver (Fig. 4.10, 4.11, 4.12), the considerable rise was seen at all LOEC. Even though there was almost double rise in ALT level since the first concentration tested, no death occurred in fingerlings. But, the overall health of the fish had undergone significant changes on the negative side.
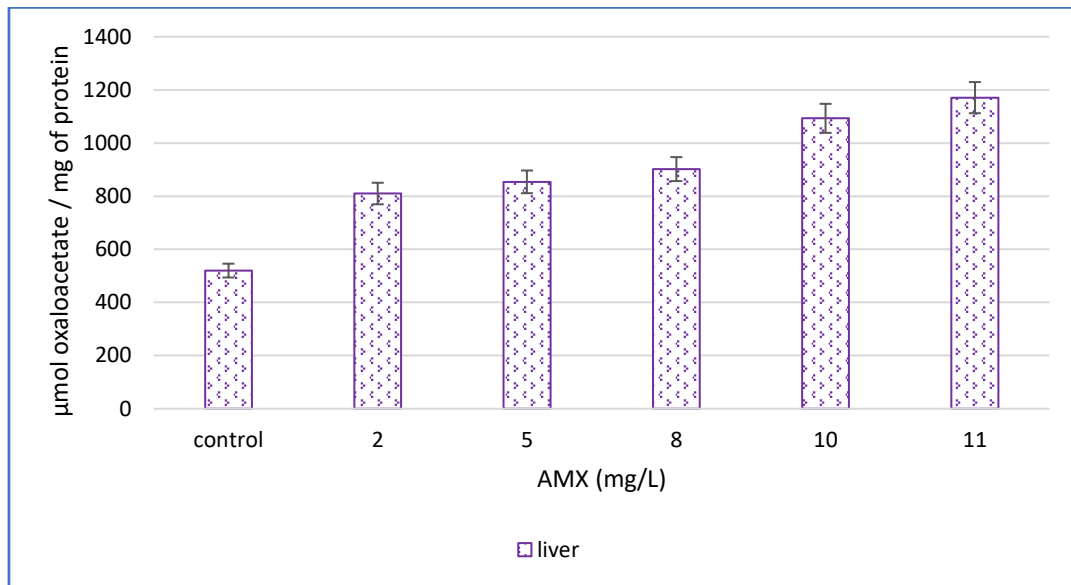
Fig. 4.7: AST levels in Liver in Fingerlings C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*
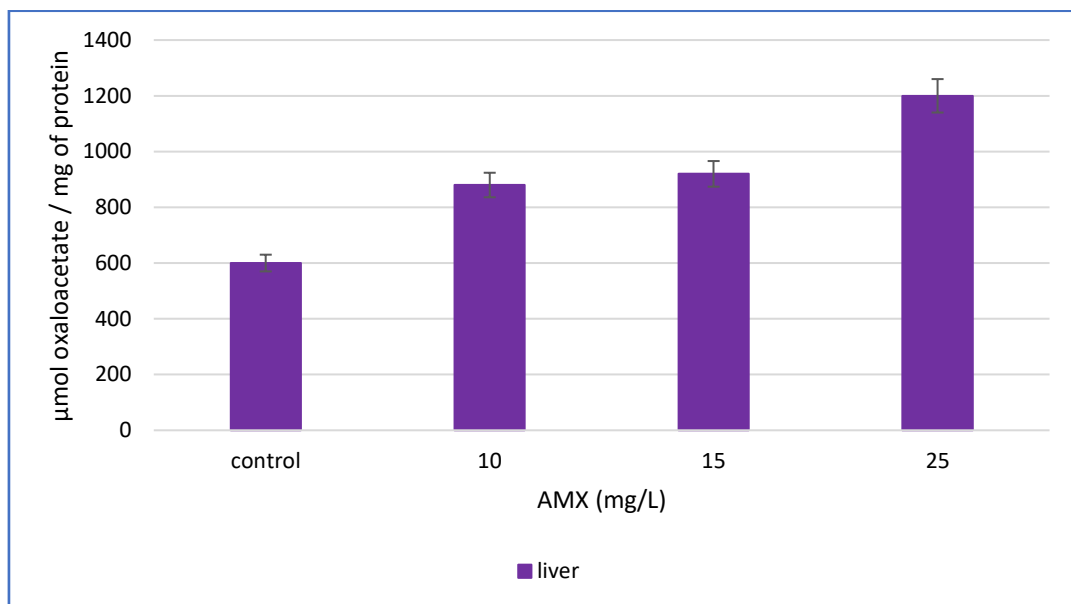
Fig. 4.8: AST levels in Liver in Juvenile C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*

The AST and ALT are conducted only on the liver since the enzymes affected are found only in the liver of the fish system. The liver is one of the important organs for survival and the AST tests act as the confirmation of the severity of the damage.
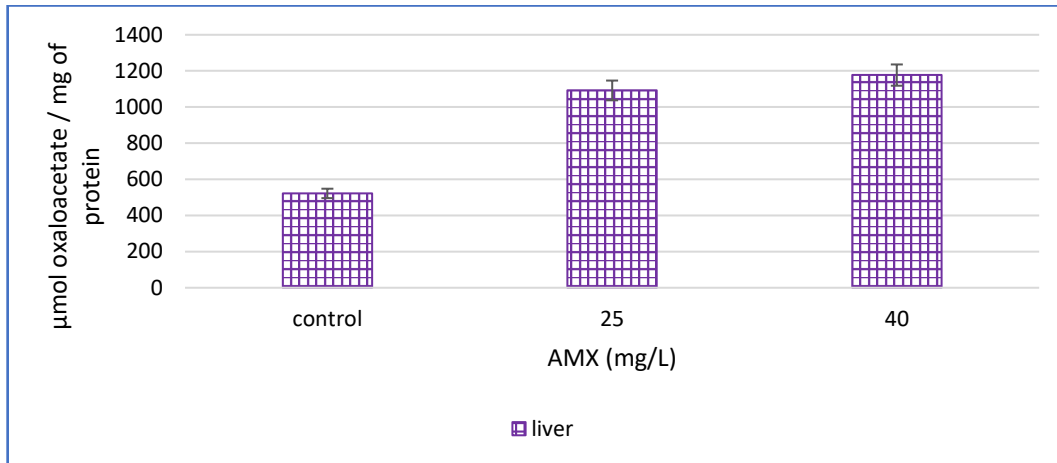
Fig. 4.9: AST levels in Liver in Adult C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*

Fig. 4.10: ALT levels in Liver in Fingerlings C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*

Fig. 4.11: ALT levels in Liver in Juvenile C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*
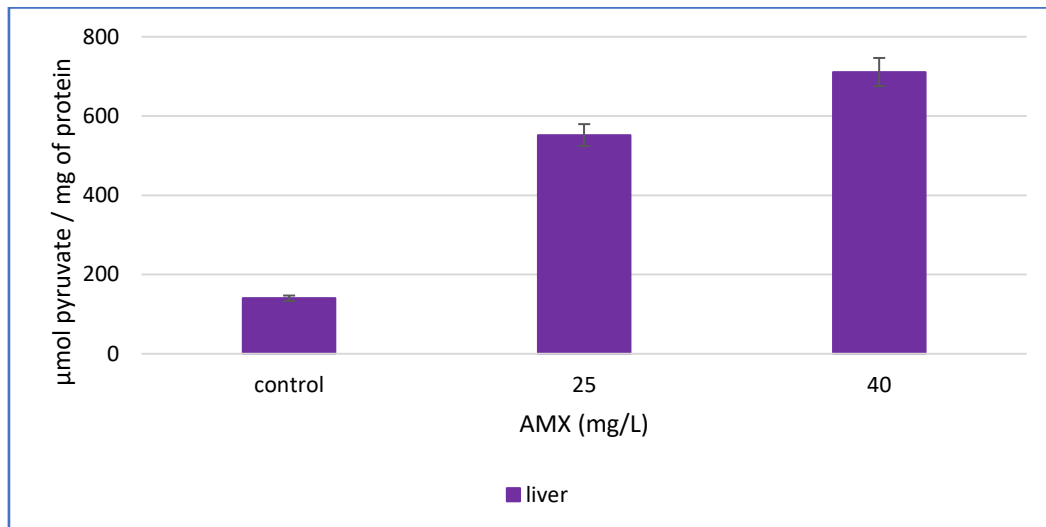
Fig. 4.12: ALT levels in Liver in Adult C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*

Unlike other enzymes, in the AchE tests, the drop in AchE levels of brain and muscle (Fig. 4.13, 4.14, 4.15) point at the damage inflicted. A gradual drop does not indicate as much harm as much a steep drop does. Thus, it can be said that AchE level difference in muscle is more noticeable than in the brain. Since then, the drop was observed regarding the fish health too.
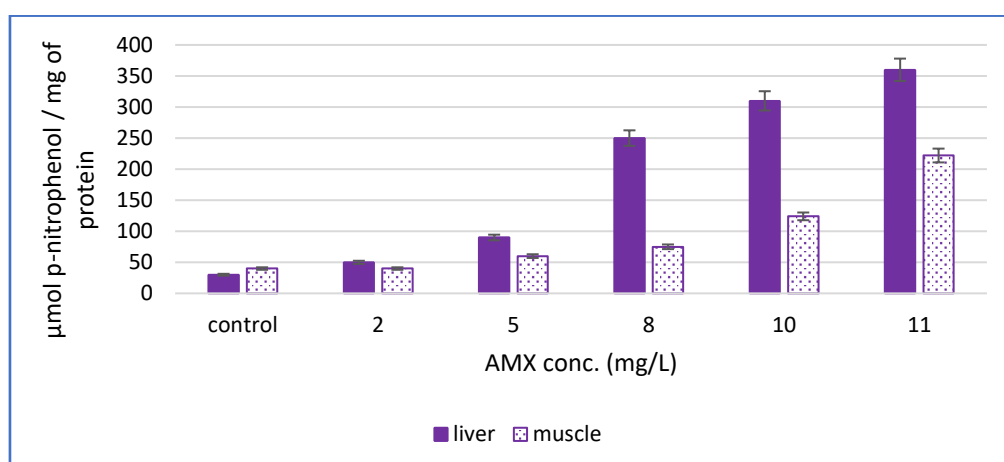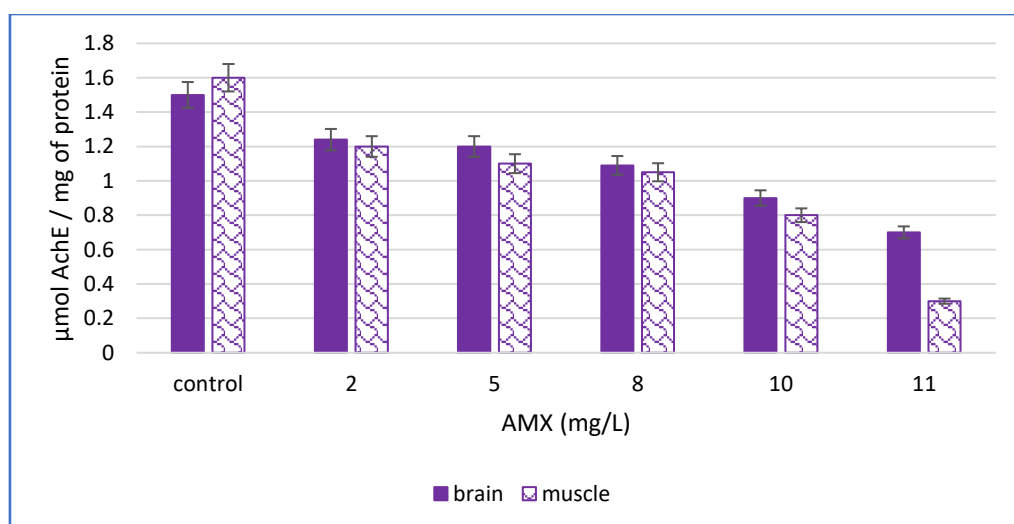

Fig. 4.13: AchE levels in Brain and Muscle in Fingerlings C. carpio due to AMX intake; significance given at mean ± SD where *P ≤ 0.05*

Fig. 4.14: AchE levels in Brain and Muscle in Juvenile C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

Fig. 4.15: AchE levels in Brain and Muscle in Adult C. carpio due to AMX intake; significance given at mean ± SD where $P \leq 0.05$

Unlike AchE, there was a considerable rise in the LDH levels in the muscle (Fig. 4.16, 4.17, 4.18). Variation in LDH levels affects the movement and its feed pattern. Increased LDH levels in target fish represents the toxicity of amoxicillin. Most enzymes showed dose-dependent increase in levels except AchE, which showed dose dependent decrease.
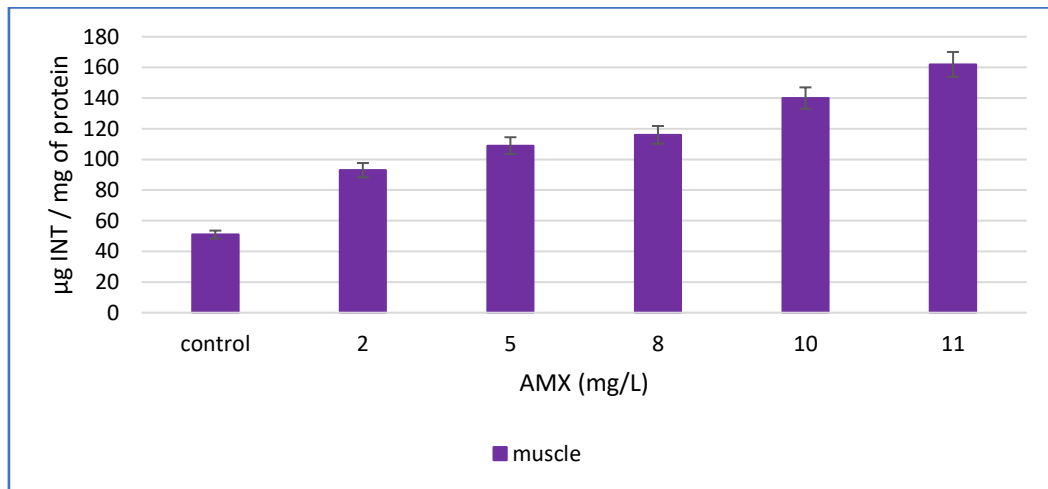
Fig. 4.16: LDH levels in Muscle in Fingerlings C. carpio due to AMX intake;

significance given at mean ± SD where $P \leq 0.05$
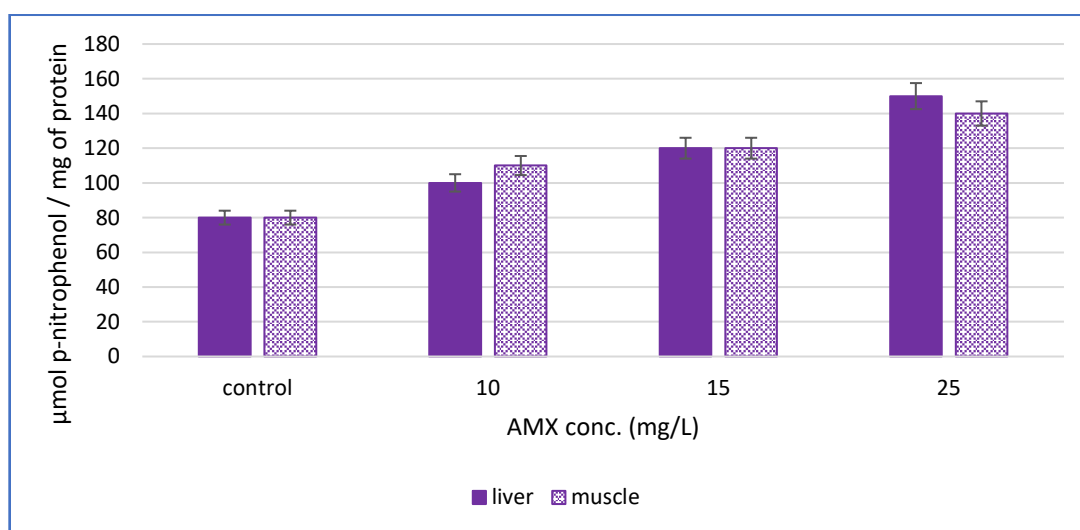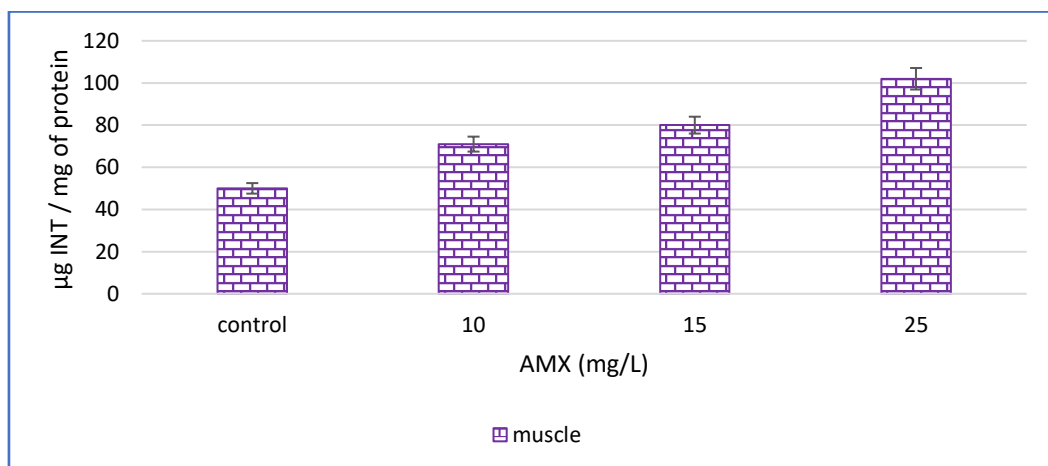

Fig. 4.17: LDH levels in Muscle in Juvenile C. carpio due to AMX intake;
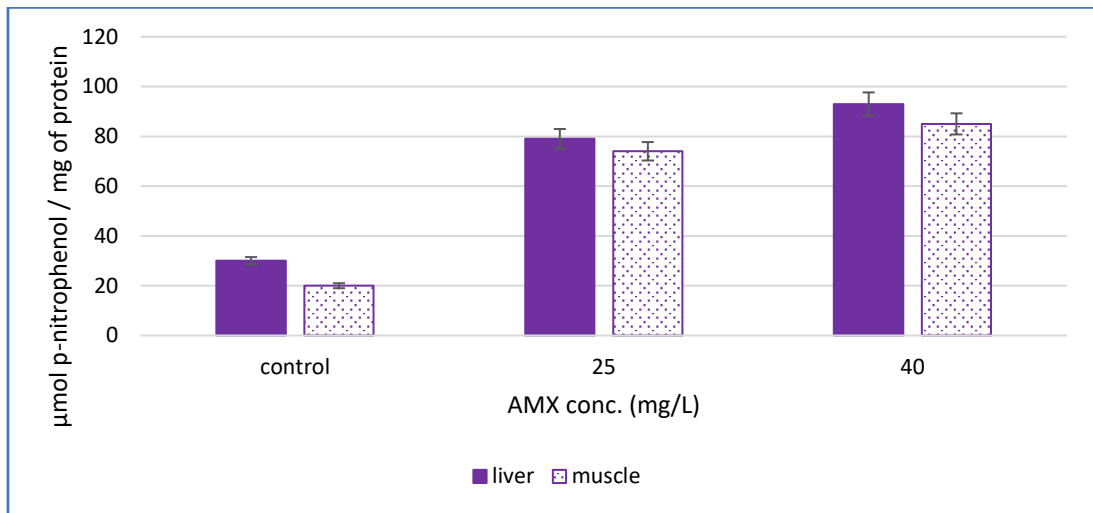
significance given at mean ± SD where $P \leq 0.05$

Fig. 4.18: LDH levels in Muscle in Adult C. carpio due to AMX intake; significance

given at mean ± SD where $P \leq 0.05$

The protein levels were estimated for both control and target fish. Unnatural and adverse conditions initiate/deter some protein expression. It was found that protein levels were found to be increased in liver, muscle and brain samples for all the tested concentrations of AMX. The liver samples show much deviation from a std. linear graph; which implies that the liver tissues suffered maximum damage when compared to muscle and brain tissues.

4.2. ENZYME ASSAY FOR PCM:

It was observed that as the concentrations significantly increased, the fish were observed to be less active gradually. At the higher concentrations (>20mg/L) the fish would be very passive for many hours each day. They showed similar responses of schooling themselves; no free movement in the observation tank; lay low and motionless. The physical impacts were also seen. For the concentrations 10 and 20mg/L respectively, the gills, the insides of the mouth and muscle below the stomach of fingerlings had reddened. The reddening of the same parts was seen for juveniles and adult at 20 and 30mg/L respectively. (refer Appendix D)

During the experiments, all the fingerlings exposed to 10 and 20mg/L survived. But at 21mg/L concentration, mortality was observed. Hence 21mg/L is the LOEC (Lowest Observed Effect Concentration). 50% mortality was observed at 25mg/L in 60hrs after the addition of PCM to the water and the remaining fish continued to survive. 100% mortality was observed for 40mg/L in 72hrs. Thus, it was observed that 25mg/L is LC-50 concentration at 60hrs while 40mg/L proved to be lethal for them. Similarly, LOEC for juvenile and adult was 25 and 35mg/L respectively. The LC-50 for juveniles was at 40mg/L at 84hrs; LC-50 for adults was 45mg/L at 24hrs. The 50mg/L concentration was responsible for 100% mortality in both juvenile and adults at 24hrs each. The Table 4.2 details the physical and behavioral changes noted during the exposure of fingerlings, juvenile and the adult fish to different concentrations of the PCM.

The ACP levels for liver and muscle samples as shown in Fig. 4.19, 4.20, 4.21 for fingerlings, juvenile, adult respectively, prove that the impact is not distinctively noticeable up to 20mg/L concentrations.
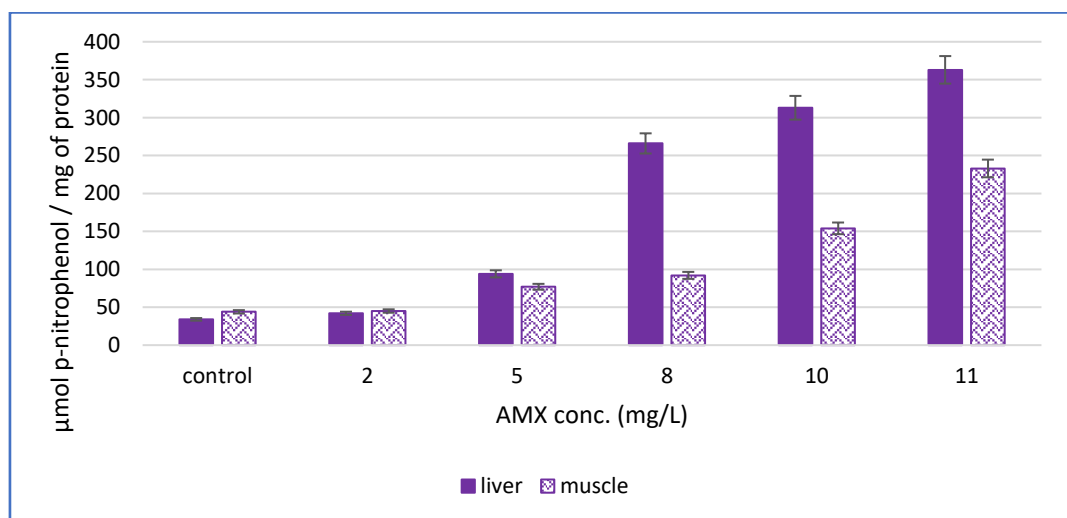
Fig. 4.19: ACP levels in Liver and Muscle in Fingerlings C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*
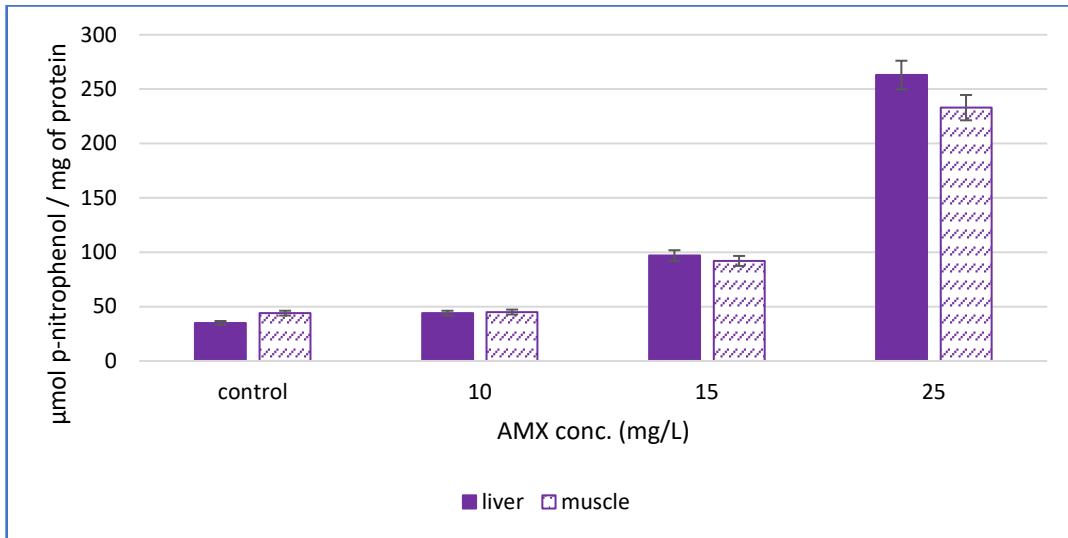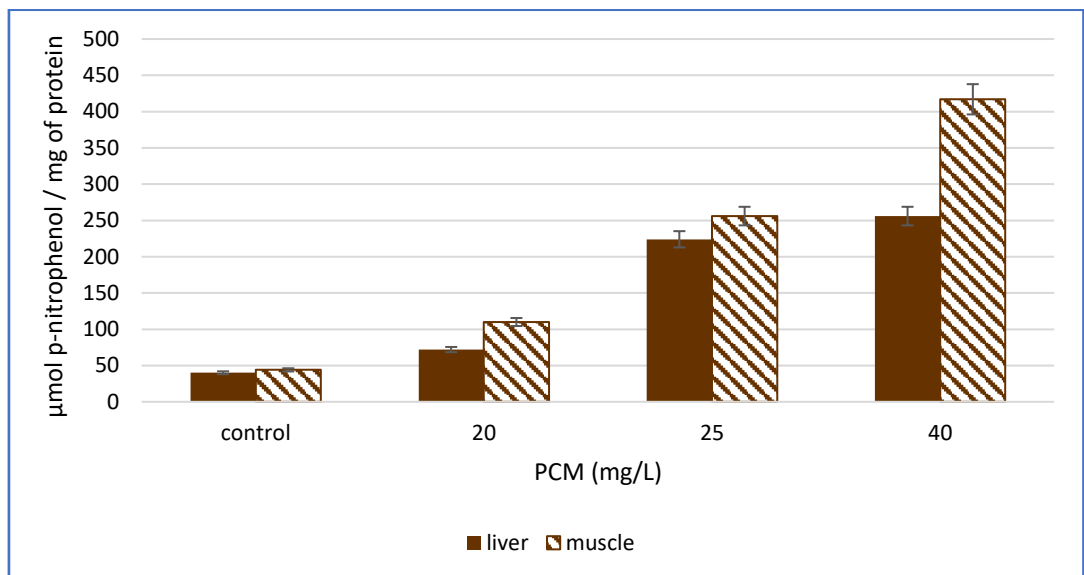

Fig. 4.20: ACP levels in Liver and Muscle in Juvenile C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*

After 20mg/L run, it noticeably hikes in dose-dependent manner. The difference in readings is comparatively steep for the muscle samples. All the values are directly proportional to damage inflicted in terms of toxicity. The change in behavior was as described earlier. It also showed less appetite compared to fish in the control tank.

Fig. 4.21: ACP levels in Liver and Muscle in Adult C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$

The ALP levels were estimated in both liver and muscle samples for all age groups (Fig. 4.22, 4.23, 4.24). For both the liver and muscle samples, the considerable difference noted was at 21mg/L for fingerlings. Although the effects were more pronounced in the muscle samples than the liver samples, the impacts were loud enough to cause toxicity. Just as ACP test results for liver, muscle samples, ALP showed marked increase in the values confirming the lethal effects.

Fig. 4.22: ALP levels in Liver and Muscle in Fingerlings C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$
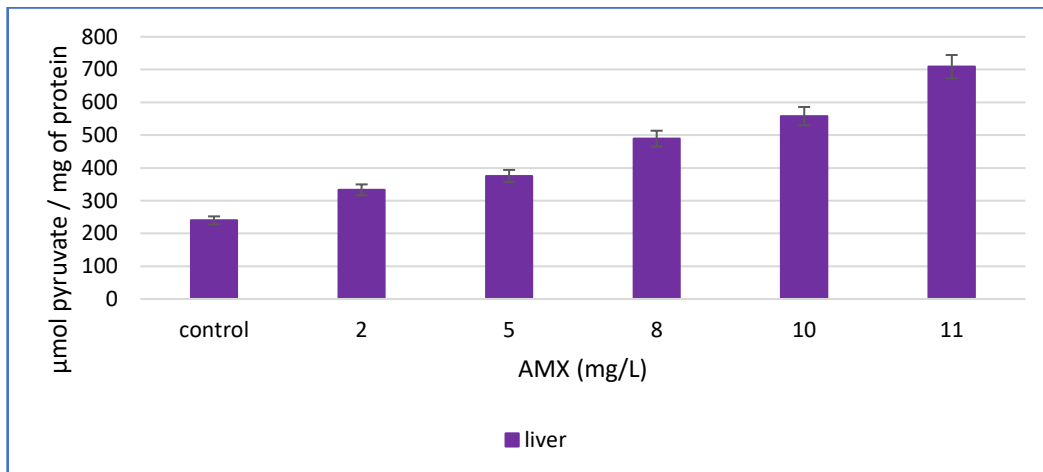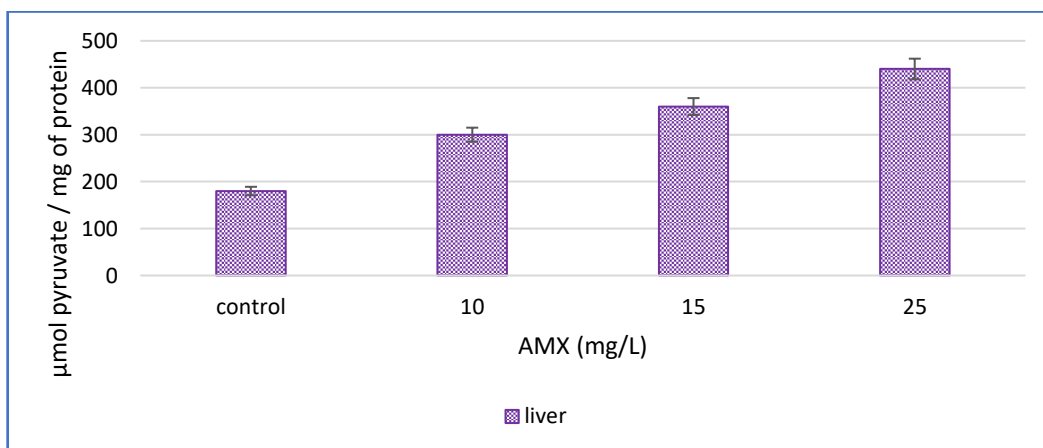
60

Fig. 4.23: ALP levels in Liver and Muscle in Juvenile C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$

Fig. 4.24: ALP levels in Liver and Muscle in Adult C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$

For fingerlings, though the sample vs control reading was almost double in AST levels in liver (Fig. 4.25) right from the first test concentration of 10mg/L, there was no toxic effect observed.

Fig. 4.25: AST levels in Liver in Fingerlings C. carpio due to PCM intake;
significance given at mean ± SD where *P ≤ 0.05*

Similarly, for the test results of ALT of liver (Fig. 4.26), even though the levels were more than double right from the initial run, no death of the target species was observed. The rise in the level indicates the intensity of the impact. The juvenile and adult liver sample analysis too, showed considerable increase in AST levels (Fig. 4.27, 4.28) and ALT levels (Fig. 4.29, 4.30).

Fig. 4.26: ALT levels in Liver in Fingerlings C. carpio due to PCM intake;
significance given at mean ± SD where *P ≤ 0.05*

Fig. 4.27: AST levels in Liver in Juvenile C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*

Fig. 4.28: AST levels in Liver in Adult C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*

From the Fig. 4.25 to 4.30, we witness that the rise in the AST and ALT levels is not as steep as that during the AMX exposure. It points out that the AMX is more hostile towards the liver than PCM is.
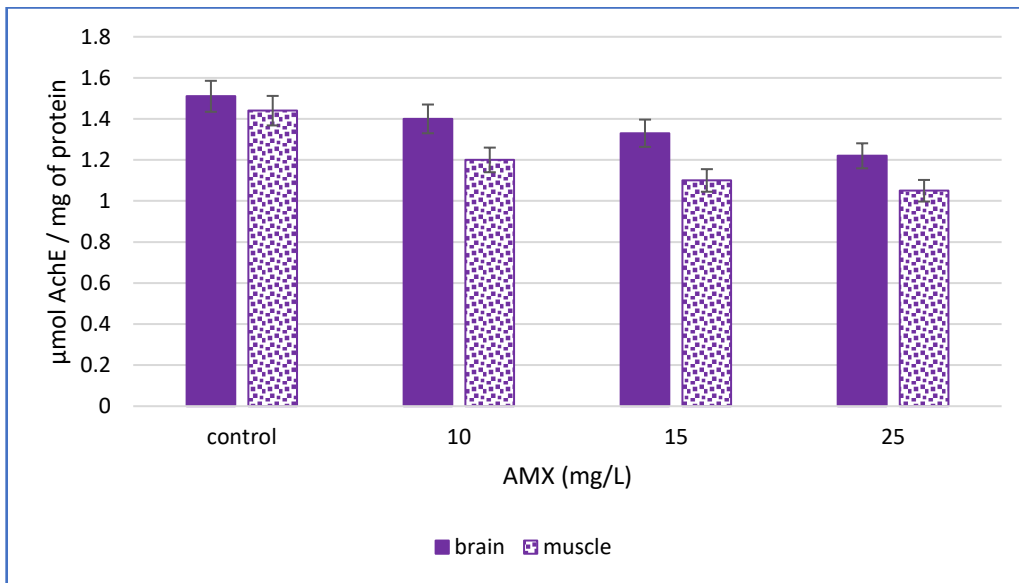
Fig. 4.29: ALT levels in Liver in Juvenile C. carpio due to PCM intake; significance given at mean $\pm$ SD where $P \leq 0.05$
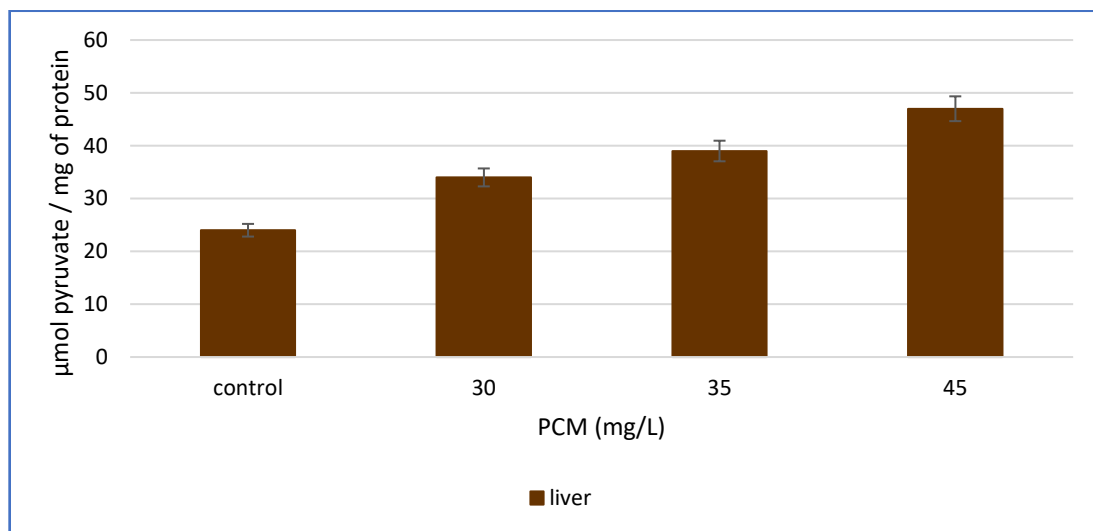
Fig. 4.30: ALT levels in Liver in Adult C. carpio due to PCM intake; significance given at mean $\pm$ SD where $P \leq 0.05$

Unlike other enzymes, in the AchE tests, the drop in AchE levels of brain and muscle (Fig. 4.31, 4.32, 4.33) point at the damage inflicted. A gradual drop indicates the harm is slow and steady. In accordance to this, drop was observed regarding the fish health too. For fingerlings, at 21mg/L the AchE levels in muscle have gone very low. The sluggish behavior of fish, non-motile nature at the succeeding concentrations of PCM

significantly correlates with drop in AchE levels in both brain and muscle in dose dependent manner. Same goes true for the juveniles and adults tested.

Fig. 4.31: AchE levels in Brain and Muscle in Fingerlings C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$

Fig. 4.32: AchE levels in Brain and Muscle in Juvenile C. carpio due to PCM intake; significance given at mean ± SD where $P \leq 0.05$
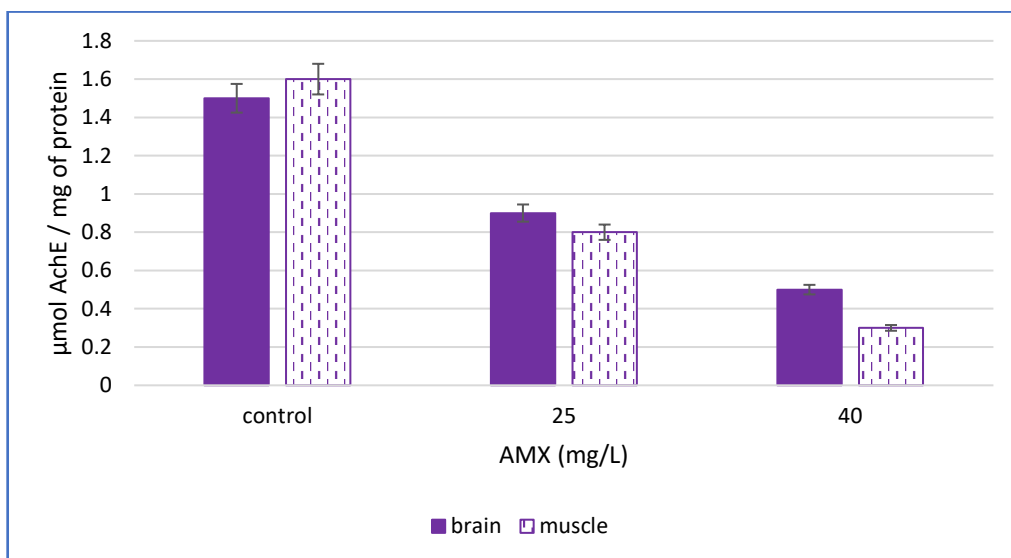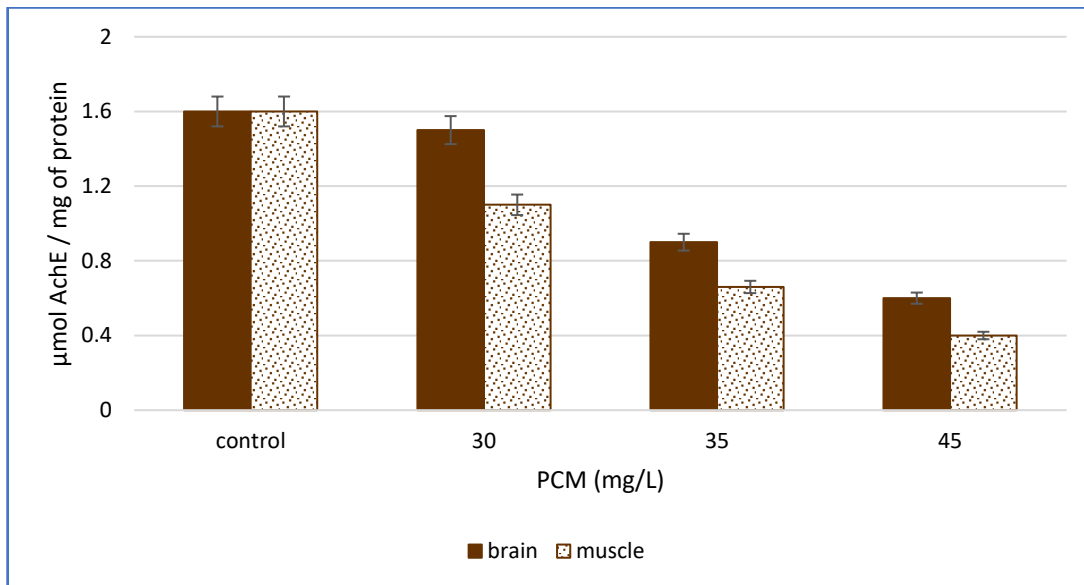
Fig. 4.33: AchE levels in Brain and Muscle in Adult C. carpio due to PCM intake; significance given at mean $\pm$ SD where *P ≤ 0.05*

Unlike AchE, there was a steep rise in the LDH levels in the muscle (Fig. 4.34, 4.35, 4.36). Variation in LDH levels affects the glycolytic pathway, its movement and also its feed pattern. Increased LDH levels in dose dependent manner significantly correlates with sluggish behavior and non-motile nature of fish with increased concentration of PCM. The protein levels were test also estimated in all the samples of fish.

Fig. 4.34: LDH levels in Muscle in Fingerlings C. carpio due to PCM intake; significance given at mean $\pm$ SD where *P ≤ 0.05*
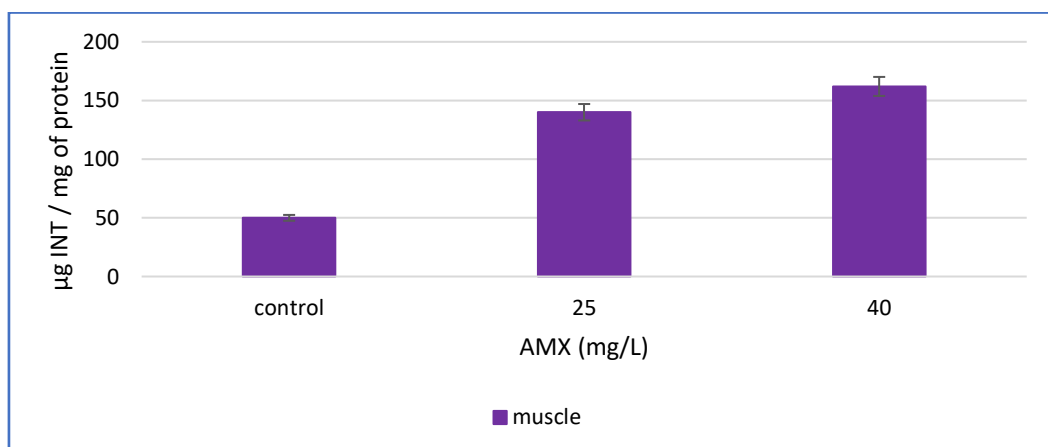
Fig. 4.35: LDH levels in Muscle in Juvenile C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*

The protein levels were estimated in fish tissue samples present a drastic variation in values due to change in physical and behavioral patterns that were observed at respective concentrations. The rise in protein levels in liver, muscle and brain samples for all the test concentrations of PCM are nothing but obvious.
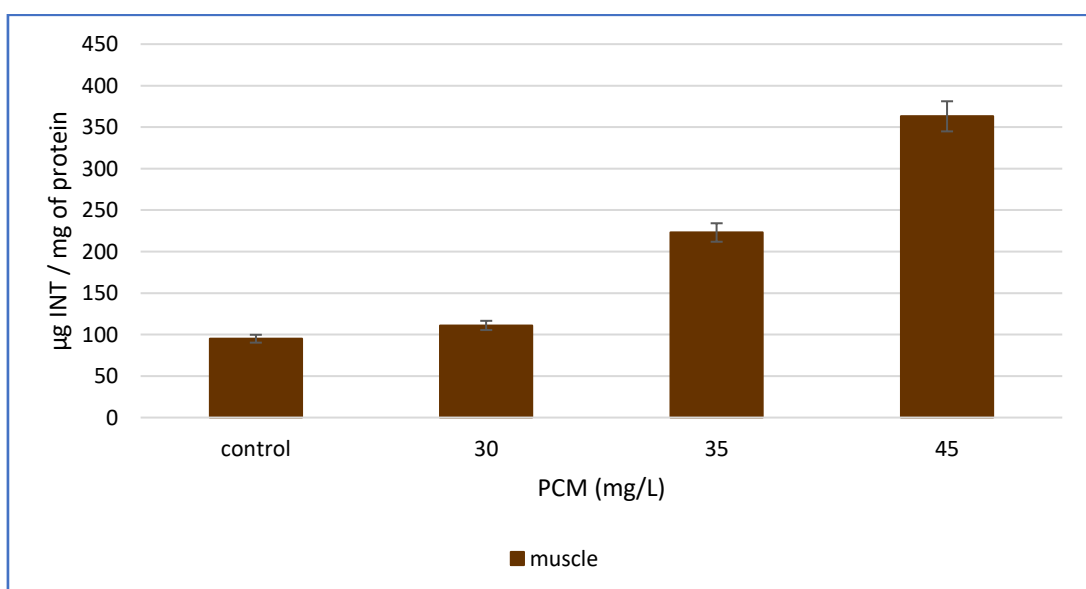
Fig. 4.36: LDH levels in Muscle in Adult C. carpio due to PCM intake; significance given at mean ± SD where *P ≤ 0.05*

4.3. ENZYME ASSAY FOR DCF:

It was observed that as the concentrations significantly increased, the fish were observed to be less active gradually. At the higher concentrations, the fish would be very passive for almost entire day. The symptoms of mortality were extended from a few hours to few days span. The fish moved about in a random involuntary way. Occasionally, it would be able to use its fins for a slight detour. (refer Appendix E)

During the experiments, all the fingerlings exposed to 2mg/L survived. But at 5mg/L concentration, mortality was observed. Hence 5mg/L is the LOEC (Lowest Observed Effect Concentration). 50% mortality was observed at 8mg/L in 10days after the addition of DCF to the water and the remaining fish continued to survive. 100% mortality was observed for 14mg/L in 16days. Thus, it was observed that 8mg/L is LC-50 concentration at 10days while 14mg/L proved to be lethal for them. Similarly, the juveniles and the adults had 5 and 10mg/L as the LOEC. The LC-50 for juveniles and adults were 10mg/L at 8days and 15mg/L at 11days respectively. 100% mortality was observed at 17 and 18mg/L at 14 and 13days respectively. The Table 4.3 details the physical and behavioral changes noted during the exposure of fingerlings, juvenile and the adult fish to different concentrations of the DCF.
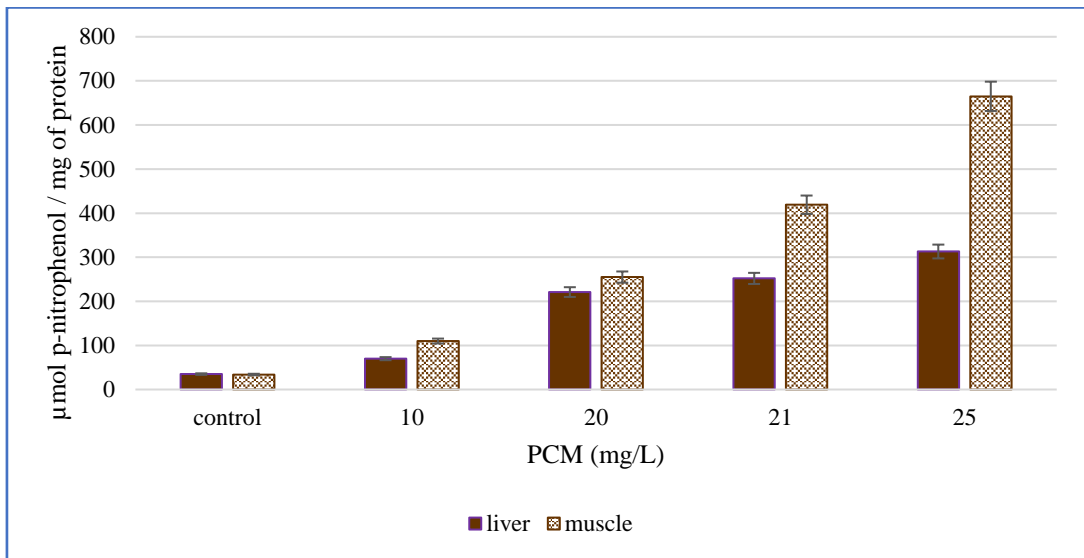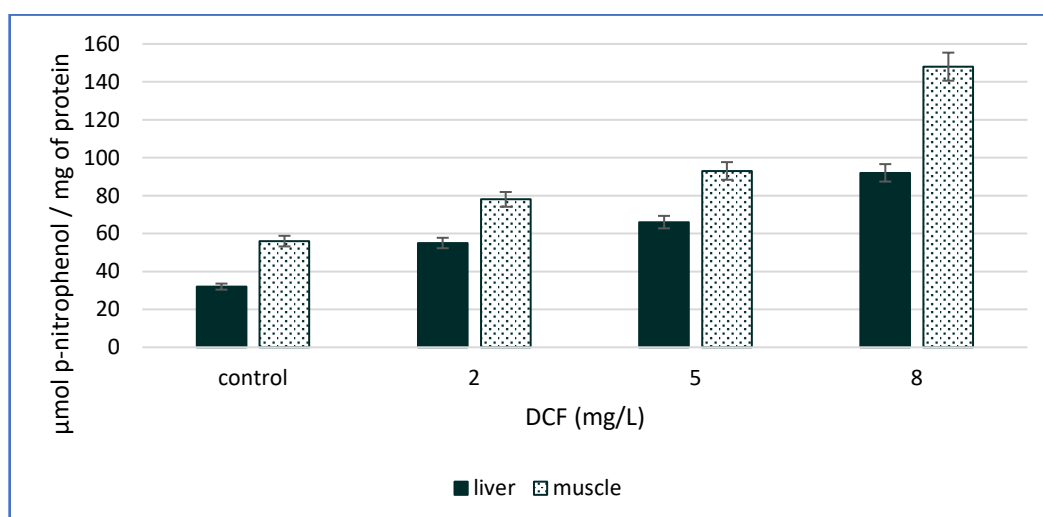

Fig. 4.37: ACP levels in Liver and Muscle in Fingerlings C. carpio due to DCF intake; significance given at mean $\pm$ SD where $P \leq 0.05$

The ACP levels for fingerlings, as shown in Fig. 4.37 for liver and muscle sample, prove that the impact is not distinctively noticeable in 2mg/L concentration. After 2mg/L run, it noticeably hikes for the rest of the concentrations. All the values are directly proportional to damage inflicted in terms of toxicity. The muscle samples show intense effects compared to liver samples. The change in behavior was as described earlier. It also showed less appetite compared to fish in the control tank. The Fig. 4.38, 4.39 show the ACP levels in the juvenile and the adults. It is evident that a similar reaction pattern entailed in these too.
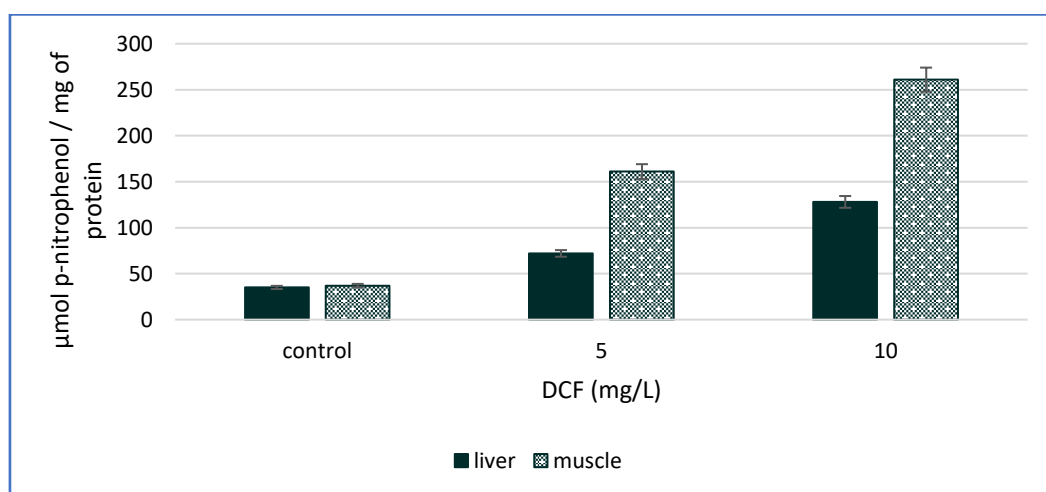
Fig. 4.38: ACP levels in Liver and Muscle in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$
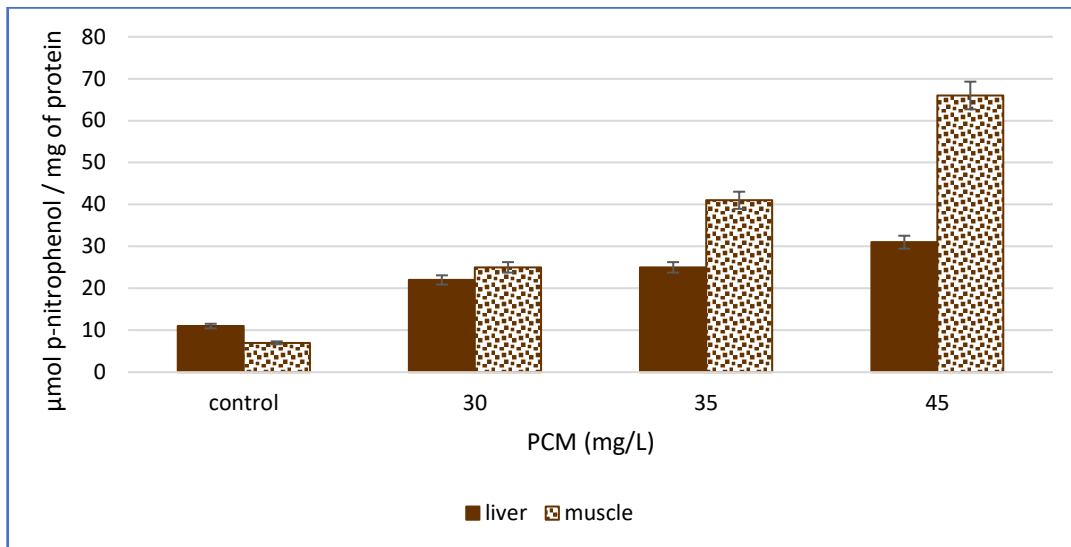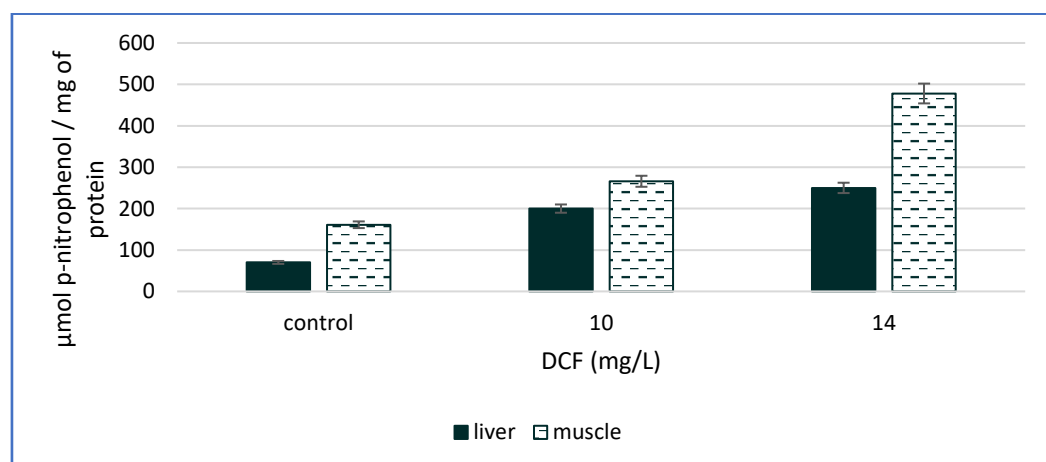

Fig. 4.39: ACP levels in Liver and Muscle in Adult C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$

The ALP levels were estimated in both liver and muscle samples of all age groups (Fig. 4.40, 4.41, 4.42). For both the liver and muscle samples, the considerable difference noted was at 5mg/L in fingerlings. Although the effects were more pronounced in the muscle samples than the liver samples, the impacts were loud enough to cause toxicity.
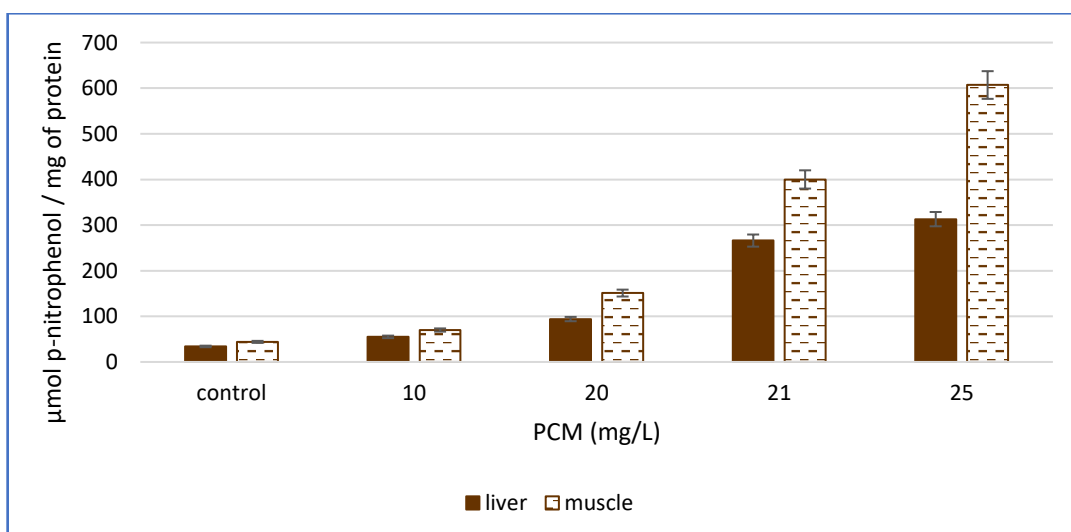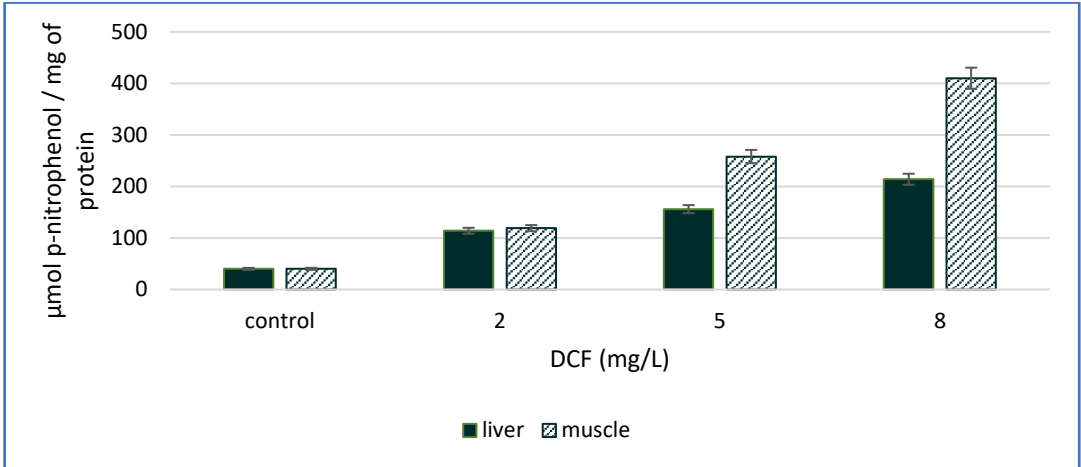

Fig. 4.40: ALP levels in Liver and Muscle in Fingerlings C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*

Just as ACP test results for liver and muscle samples, ALP showed marked increase in the values confirming the lethal effects. It has to be noted that the DCF showed more intense impacts on muscle tissues than the liver in all groups for both ACP and ALP.
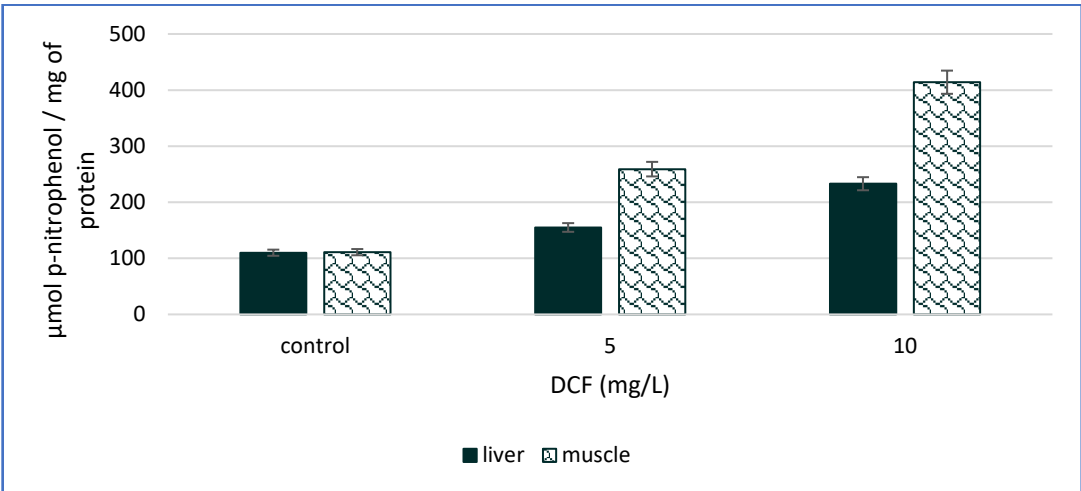
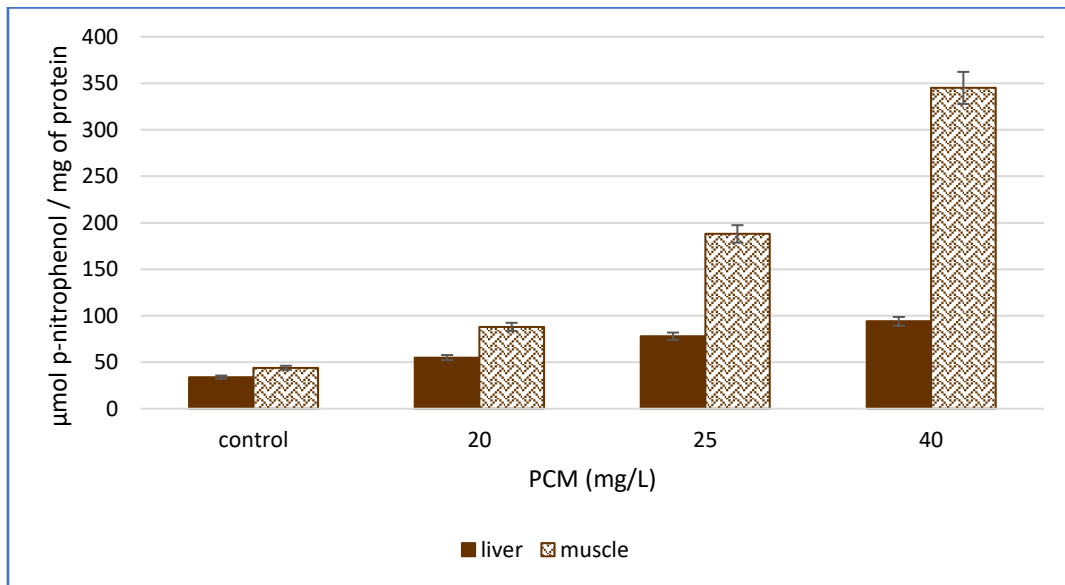
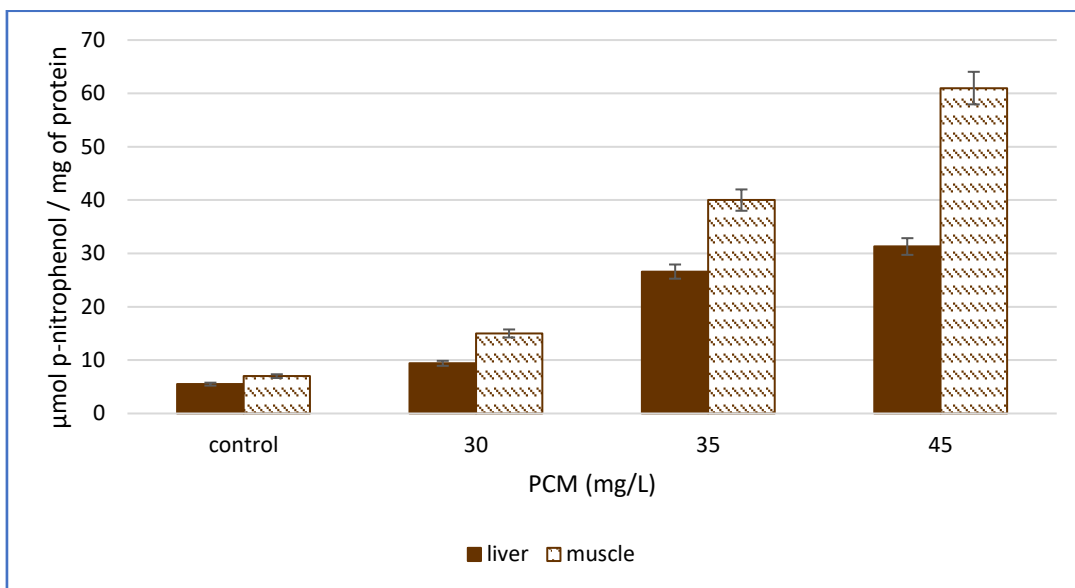Fig. 4.41: ALP levels in Liver and Muscle in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
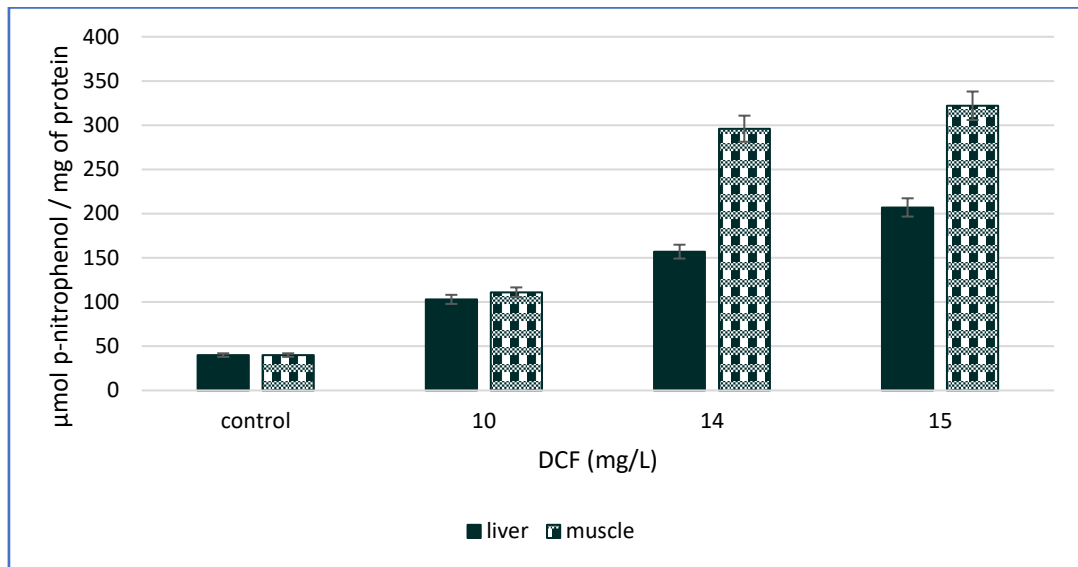
Fig. 4.42: ALP levels in Liver and Muscle in Adult C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$

AST levels are analyzed for liver samples only for all fingerlings, juvenile and adult (Fig. 4.43, 4.44, 4.45). Similarly, ALT levels of liver were analyzed (Fig. 4.46, 4.47, 4.48). Even though the levels were more than double right from the initial run, no death of the target species was observed in fingerlings. The rise in the level indicates the intensity of the impact. The increase in ALT levels at the LOEC concentration of 5mg/L is more than that of the AST.
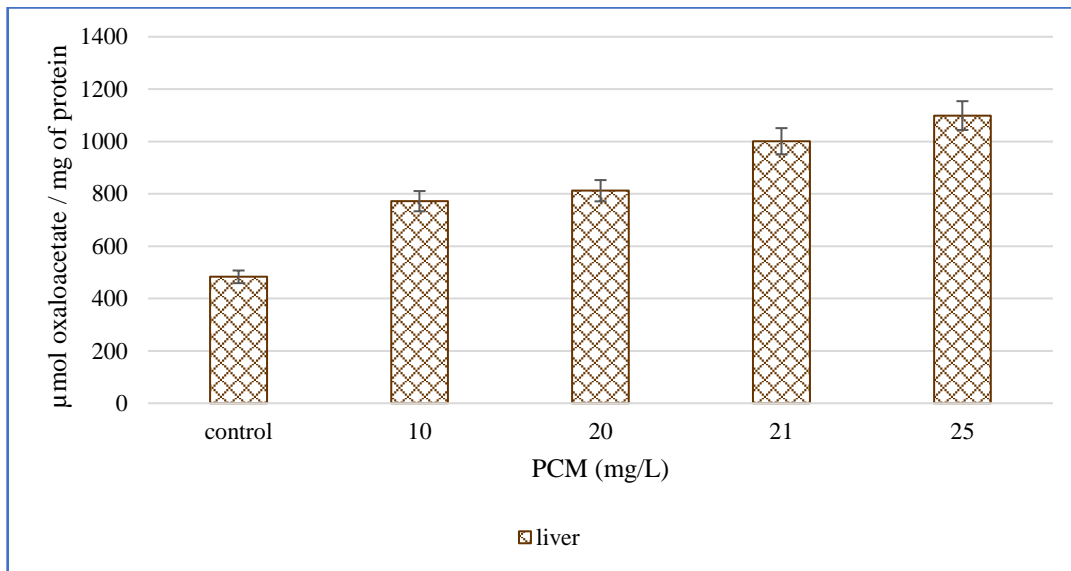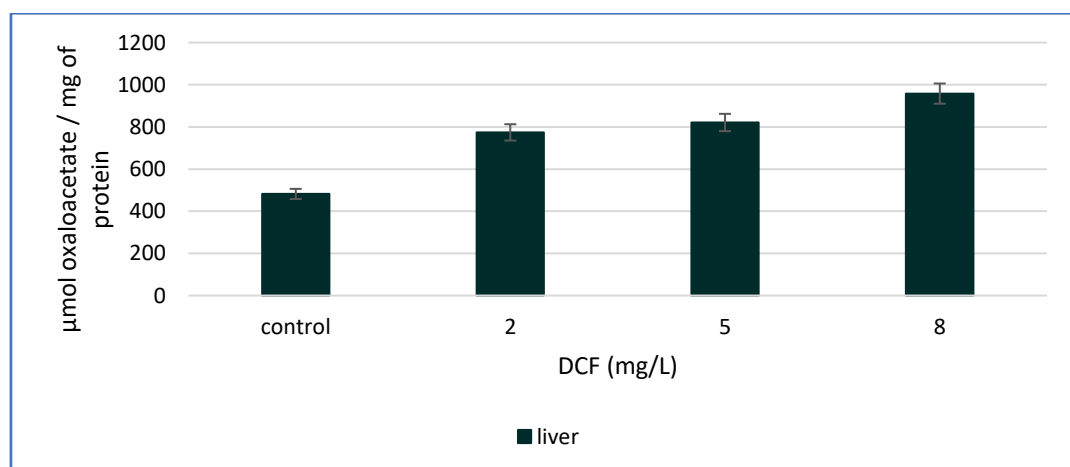

Fig. 4.43: AST levels in Liver in Fingerlings C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$
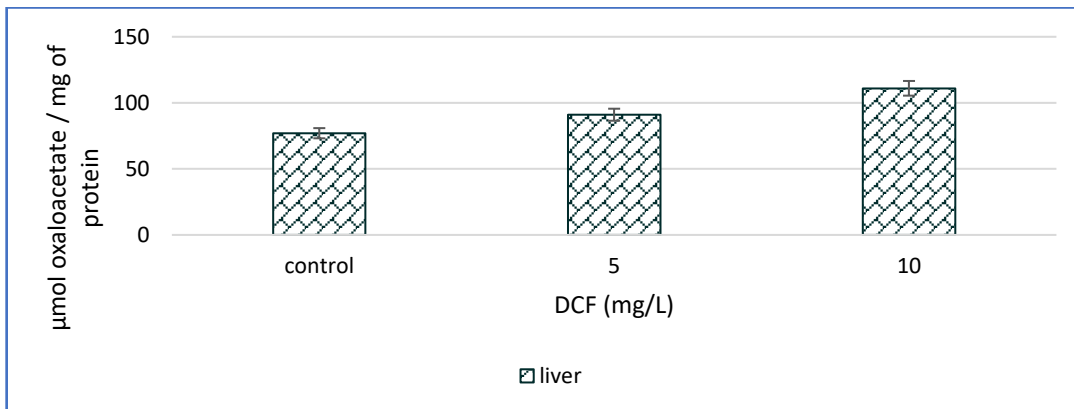
Fig. 4.44: AST levels in Liver in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
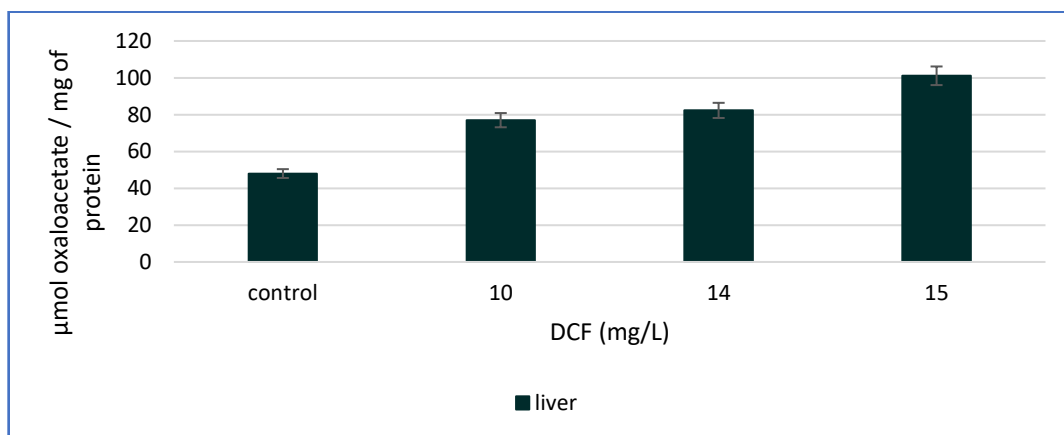
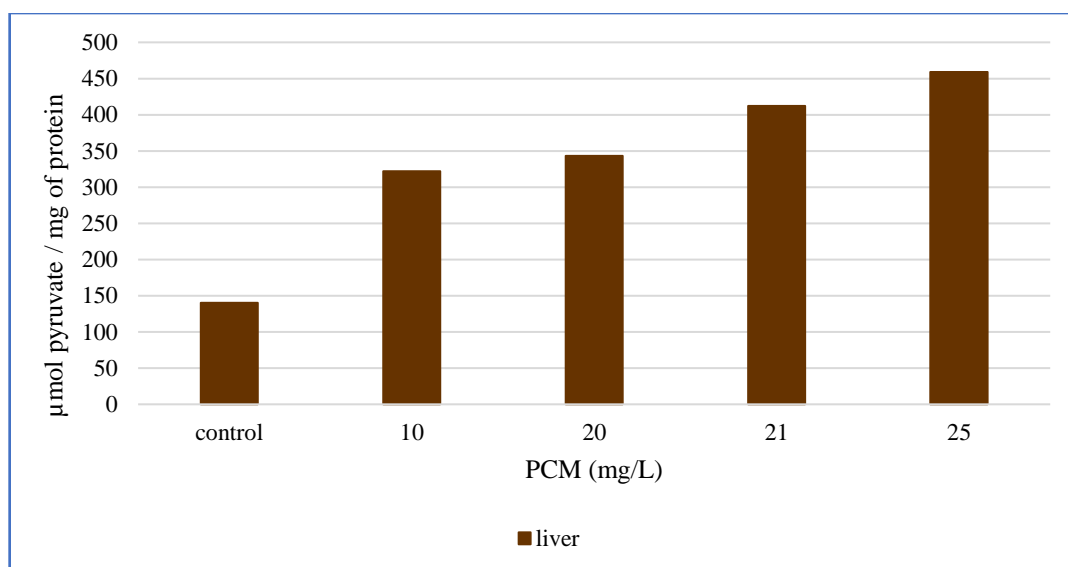Fig. 4.45: AST levels in Liver in Adult C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
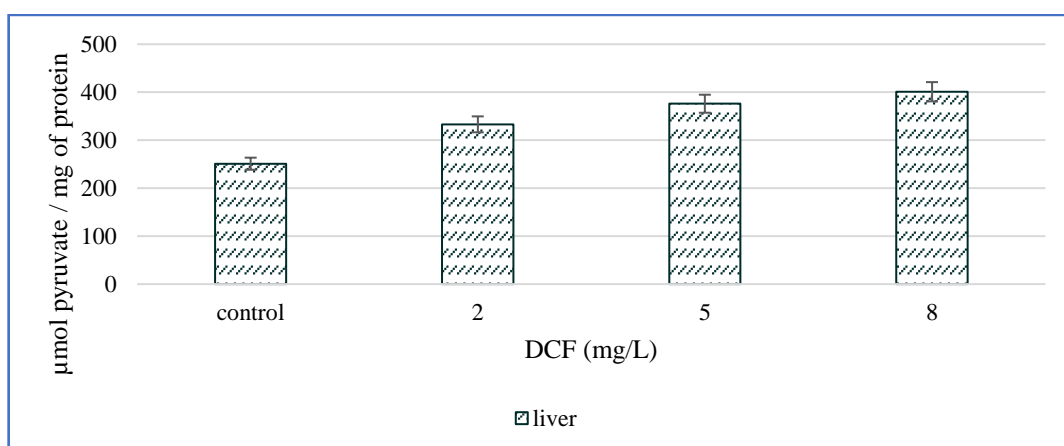
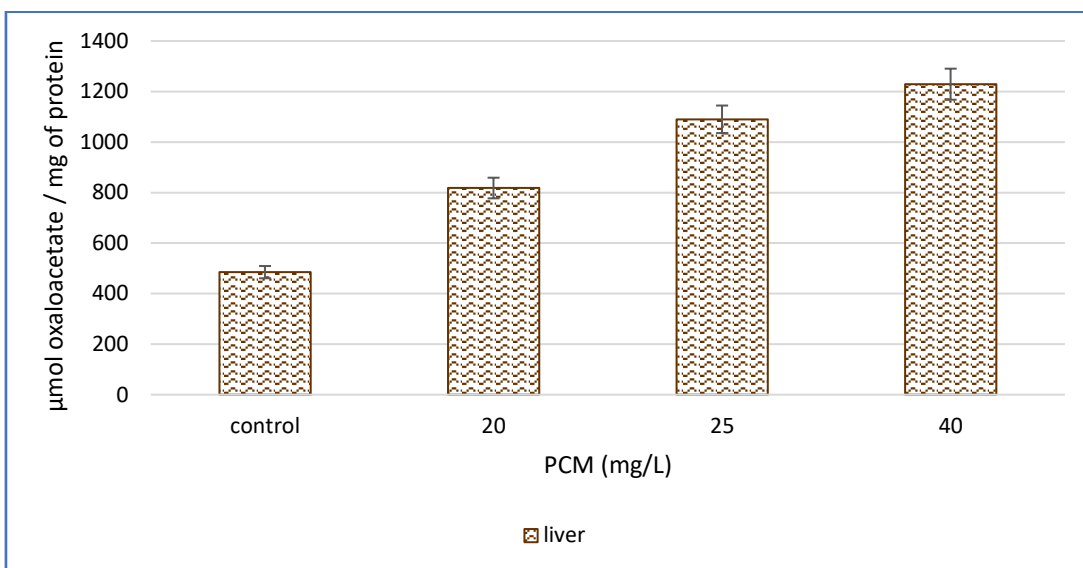Fig. 4.46: ALT levels in Liver in Fingerlings C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
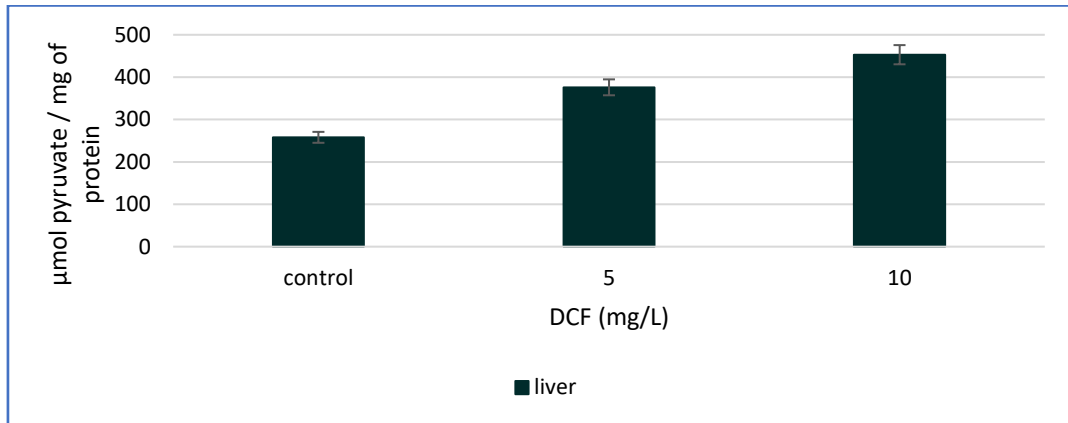
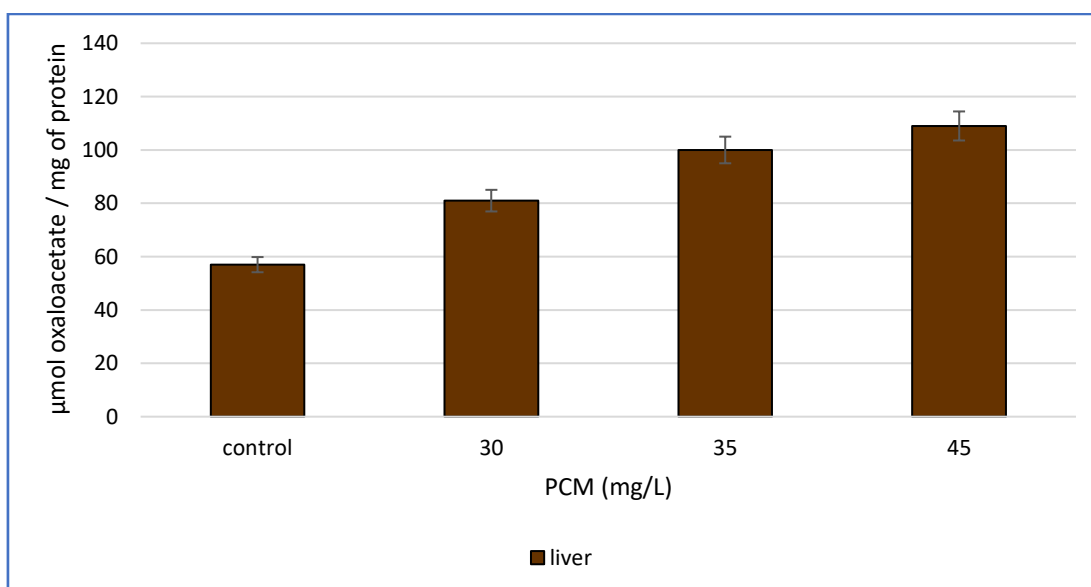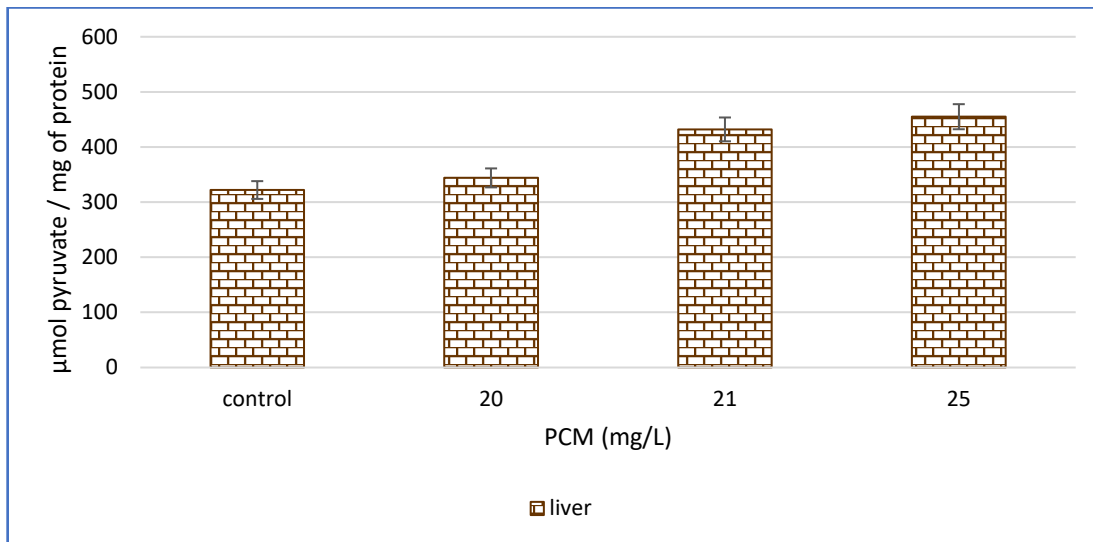Fig. 4.47: ALT levels in Liver in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
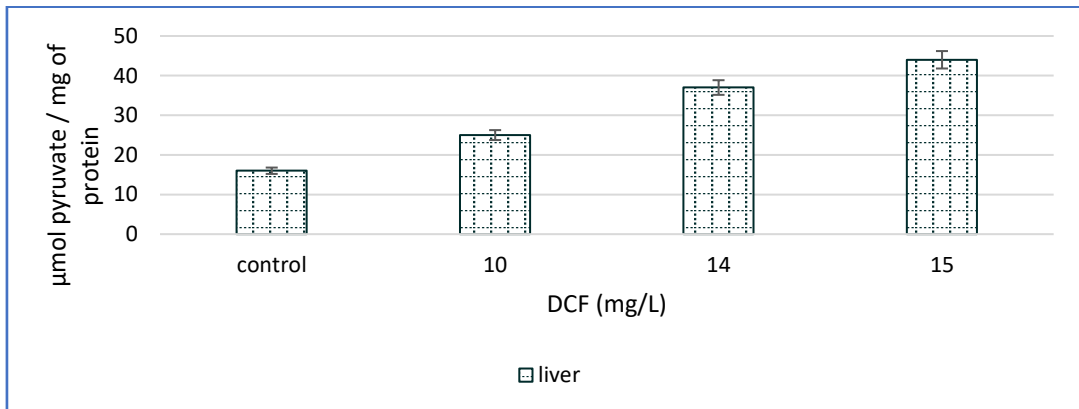
Fig. 4.48: ALT levels in Liver in Adult C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
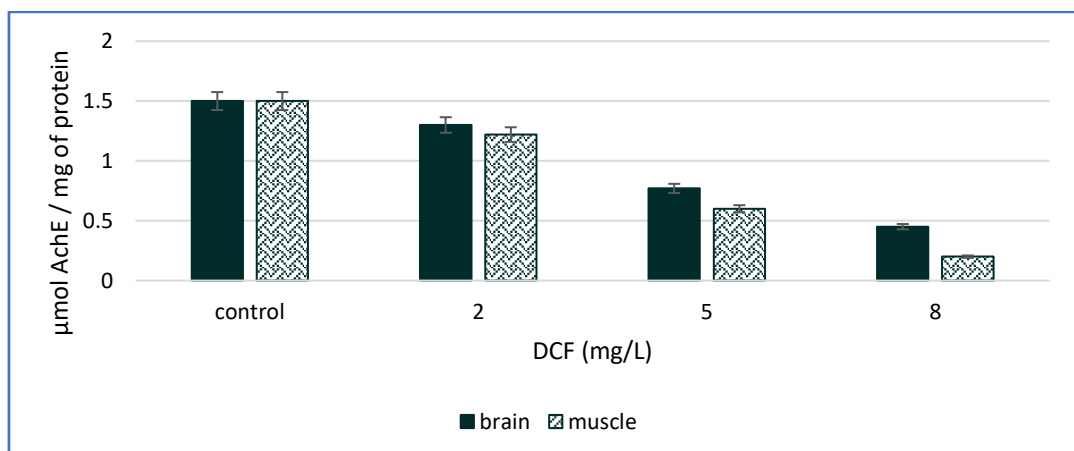
Fig. 4.49: AchE levels in Brain and Muscle in Fingerlings C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*

Unlike other enzymes, in the AchE tests, the drop in AchE levels of brain and muscle (Fig. 4.49, 4.50, 4.51) point at the damage inflicted. A blunt/shallow drop indicates the harm is of low impact. In accordance to this, the mortality rate and the already-prolonged duration of death symptoms grew consistently. The sluggish behavior of fish, non-motile nature at the succeeding concentrations of DCF significantly correlates with drop in AchE levels in both brain and muscle in terms of toxicity.
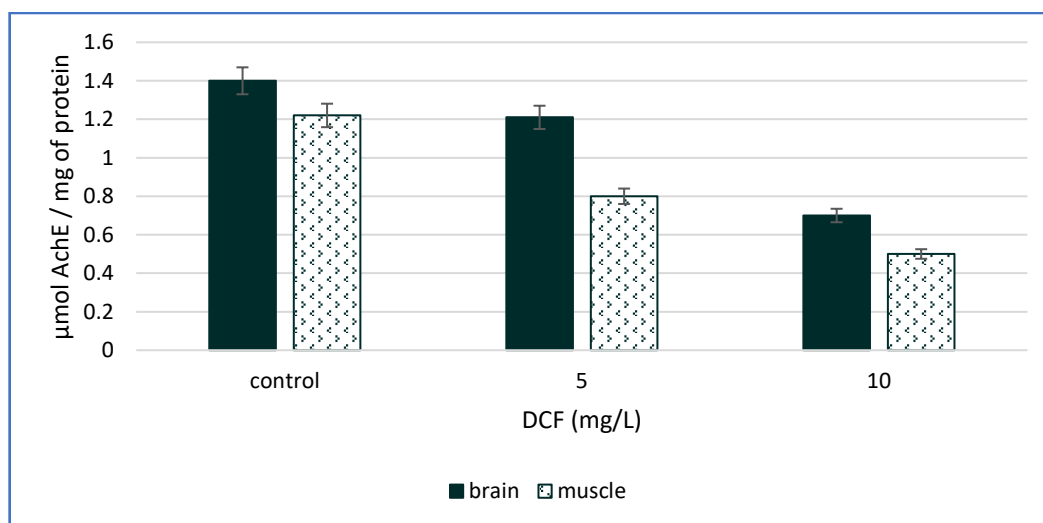
Fig. 4.50: AchE levels in Brain and Muscle in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$
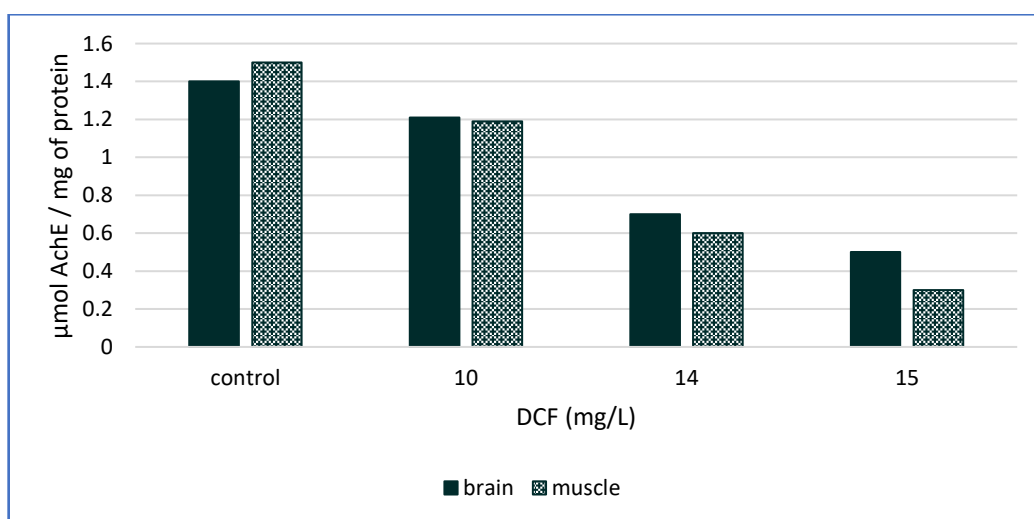
Fig. 4.51: AchE levels in Brain and Muscle in Adult C. carpio due to DCF intake; significance given at mean ± SD where $P \leq 0.05$

The LDH levels, unlike AchE, saw a considerable rise in the muscle samples of the fingerlings. (Fig. 4.52, 4.53, 4.54). Variation in LDH levels affects the glycolytic pathway, its movement and its feed pattern. Increased LDH levels due to increased concentration of DCF correlates with a direct, intense impact on the muscular composition of the fish. These readings prove that the DCF targets muscle and brain tissues more than the liver tissues.
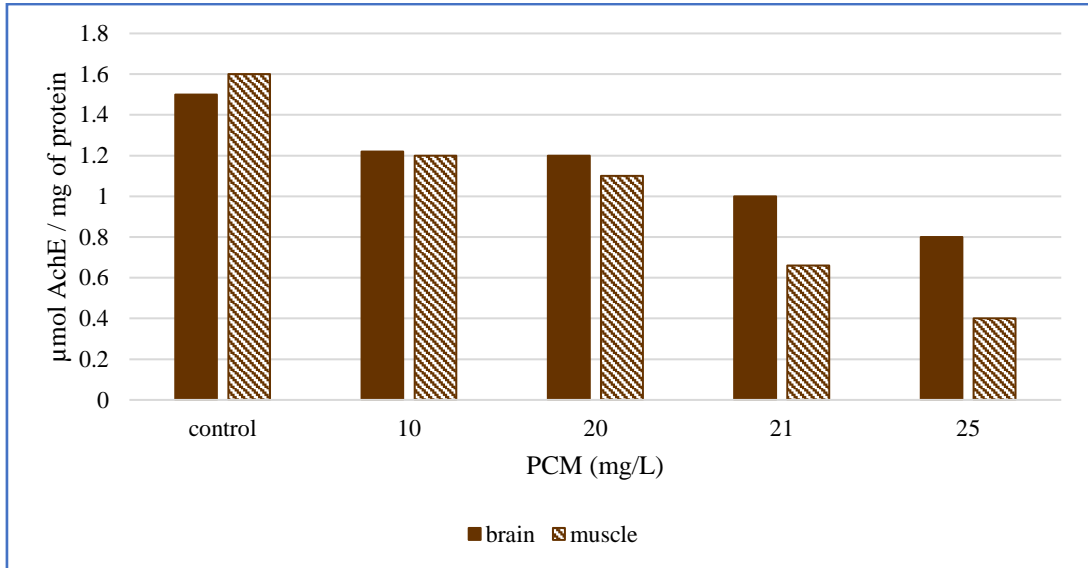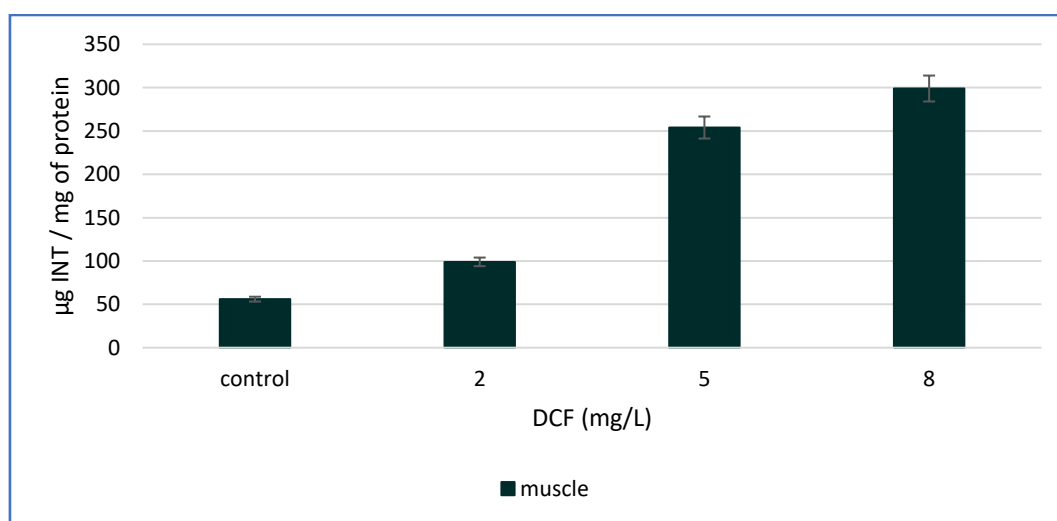

Fig. 4.52: LDH levels in Muscle in Fingerlings C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*
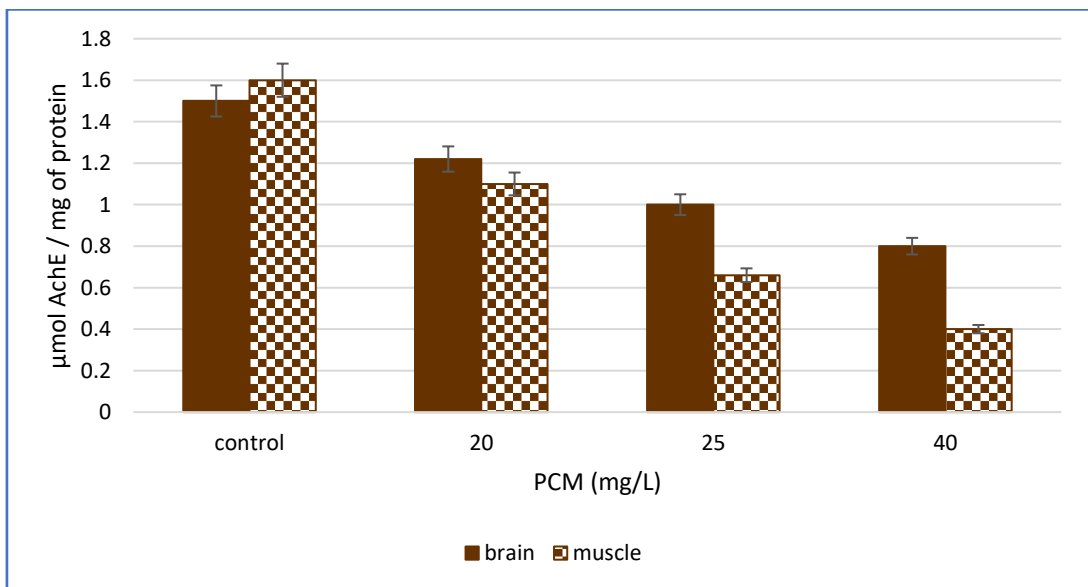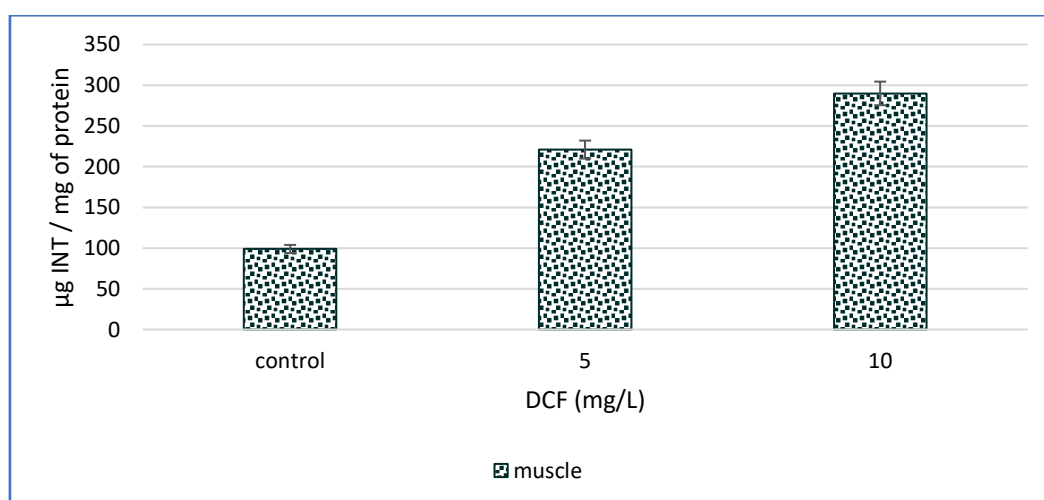

Fig. 4.53: LDH levels in Muscle in Juvenile C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*

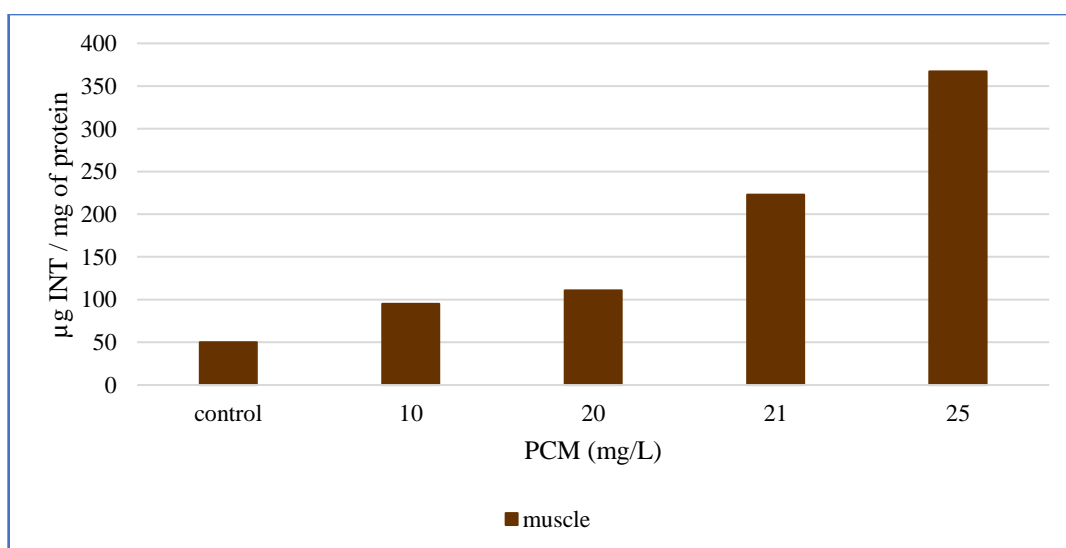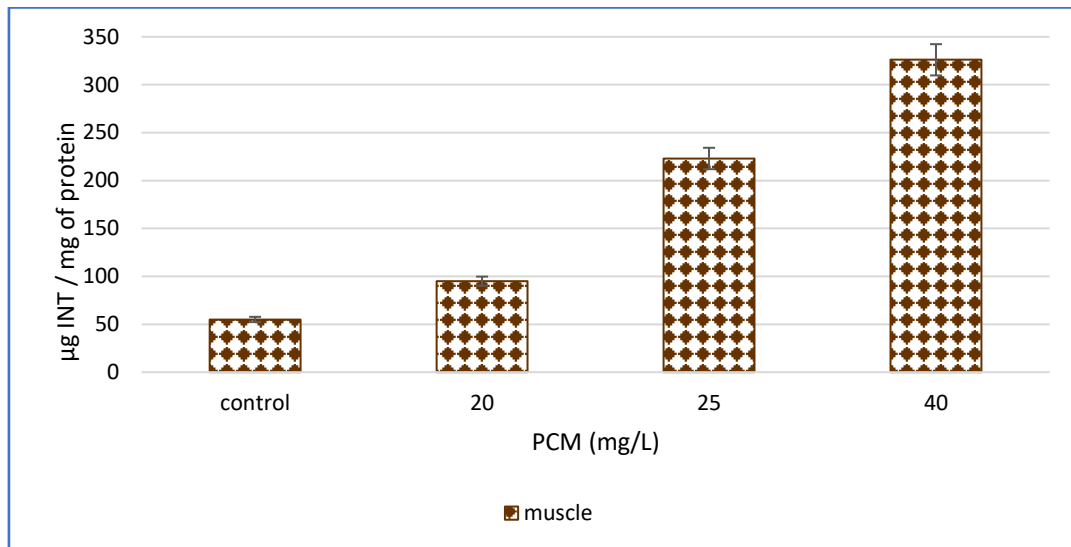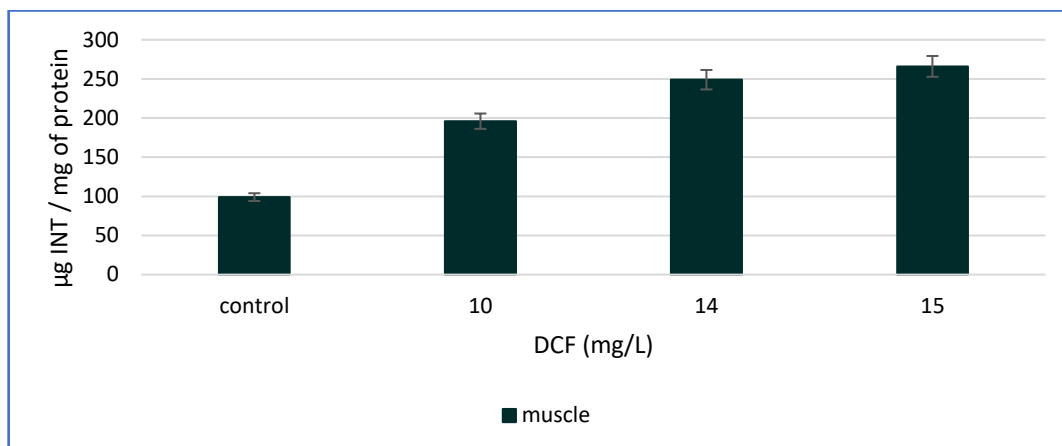Fig. 4.54: LDH levels in Muscle in Adult C. carpio due to DCF intake; significance given at mean ± SD where *P ≤ 0.05*

The protein levels were also estimated in all the samples of fish. The protein levels were estimated in fish tissue samples present a variation in values due to change in physical and behavioral patterns that were observed at respective concentrations. The rise in protein levels in liver, muscle and brain samples for all the test concentrations of DCF are nothing but obvious. The proteins that were either not-routinely-expressed or not-at-all-expressed, got released in the target tissues resulting in variation of protein levels.

4.4. INTERFERENCE ANALYSIS

Four cases were designed: 1) Without aeration, With plants; 2) With aeration, With plants; 3) With aeration, Without plants; 4) Without aeration, Without plants. The following tabulations (Table 4.1, 4.2, 4.3) display the decrease in concentrations.

4.5. PREDICTIVE TOXICOLOGICAL ANALYSIS:

The BLAST search included: *Cirrhinus mrigala* (mrigal carp), *Salmo gairdneri* (rainbow trout), *Carassius cuvieri* (Japanese crucian carp), *Carassius auratus* (goldfish) and a goldfish- common carp hybrid. These were compared with the species of first choice *Cyprinus carpio* (common carp). The BLAST search for goldfish, *Carassius auratus* (Genbank accession numbers AB379915 – AB379921) were found to be 88% similar. The highest match of 99% was with the goldfish- common carp hybrid

(AY694420), 92% match with Japanese crucian carp (AB045144), 90% match with *Cirrhinus mrigala,* 78% match with rainbow trout.

The genes mainly found similar were the COX1, COX2, CYP4501A, CYP4502M, IL-1, IL-2, some more that are active in the energy as well as metabolism cycles (ATP syntheses and cell signaling). (Goksoyr, *et al.* 1992; El-Kady, *et al.* 2004; Itakura, *et al.* 2005; Fisher, *et al.* 2006; Kaminishi, *et al.* 2007; Barney, *et al.* 2008; Martin, *et al.* 2010; Tang, *et al.* 2010; Landis, *et al.* 2011; Liu, *et al.* 2012; Tan, *et al.* 2012; Rhee, *et al.* 2013; Yuan, *et al.* 2013; Karaca, *et al*. 2014; Ku, *et al.* 2014; Lee, *et al.* 2015; Liu, *et al.* 2015; Li, *et al.* 2015 (a); Li, *et al.* 2015 (b); Agus, *et al.* 2015; Jeon, *et al.* 2016). The nucleotide sequences of these genes across all species considered here gave a match similarity of 90% (on an average).

Table 4.1: Reduction in AMX concentration due to various factors

Set	Condition maintained	**Day 1** Initial conc. (mg/L)	**Day 2** (mg/L)	**Day 3** (mg/L)	**Day 4** Final conc. (mg/L)	Conc. reduced (mg/L)	% Reduction
1	Without aeration; With plants	2	1.81	1.62	1.41	0.59	29.50
		5	4.51	4.02	3.53	1.47	29.40
		8	7.25	6.46	5.69	2.31	28.87
		10	9.04	8.08	7.11	2.89	28.90
		11	9.95	8.88	7.82	3.18	28.91
		13	11.76	10.6	9.25	3.75	28.84
		15	13.55	12.11	10.67	4.33	28.86
2	With aeration; With plants	2	1.96	1.92	1.88	0.12	6.00
		5	4.96	4.92	4.70	0.30	6.00
		8	7.95	7.91	7.88	0.12	1.50
		10	9.92	9.82	9.6	0.40	4.00
		11	10.98	10.72	10.50	0.50	4.54
		13	12.74	12.48	12.22	0.78	6.00
		15	14.70	14.40	14.10	0.90	6.00

Set	Condition maintained						
3	With aeration; Without plants	2	1.91	1.82	1.76	0.24	12.00
		5	4.77	4.54	4.39	0.61	12.02
		8	7.70	7.50	7.08	0.92	11.50
		10	9.63	9.26	8.88	1.12	11.20
		11	10.78	10.42	10.01	0.99	9.00
		13	12.59	12.18	11.77	1.23	9.46
		15	14.54	14.03	13.56	1.45	9.69
4	Without aeration; Without plants	2	1.98	1.96	1.93	0.07	3.50
		5	4.97	4.94	4.90	0.10	2.00
		8	7.98	7.95	7.93	0.07	0.80
		10	9.94	9.91	9.85	0.15	1.50
		11	10.94	10.88	10.82	0.18	1.80
		13	12.95	12.92	12.87	0.13	1.30
		15	14.97	14.92	14.88	0.12	1.20

Table 4.2: Reduction in PCM concentration due to various factors

Set	Condition maintained	Day 1 Initial conc. (mg/L)	Day 2 (mg/L)	Day 3 (mg/L)	Day 4 Final conc. (mg/L)	Conc. reduced (mg/L)	% Reduction
1	Without aeration; With plants	10	8.98	7.96	6.94	3.06	30.60
		20	17.97	15.95	13.91	6.09	30.45
		21	18.89	16.77	14.65	6.35	30.23
		25	22.48	19.99	17.44	7.56	30.24
		30	26.94	23.33	20.82	9.18	30.60
		40	35.22	31.84	27.76	12.24	30.60
2	With aeration; With plants	10	9.73	9.46	9.19	0.81	8.10
		20	19.44	18.93	18.88	1.12	5.60
		21	20.48	19.88	19.29	1.71	8.14
		25	24.83	23.68	22.99	2.01	8.04

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Set	Condition						
		30	29.18	28.83	27.59	2.41	8.03
		40	38.92	37.84	36.76	3.24	8.10
3	With aeration; Without plants	10	9.63	9.26	8.88	1.12	11.20
		20	19.65	19.25	18.88	1.12	5.60
		21	20.64	20.28	19.92	1.08	5.14
		25	24.62	24.24	23.86	1.14	4.56
		30	29.64	29.28	28.92	1.08	3.60
		40	39.64	39.28	38.92	1.08	2.70
4	Without aeration; Without plants	10	9.94	9.91	9.85	0.15	1.50
		20	19.95	19.91	19.40	0.60	3.00
		21	20.96	20.91	20.87	0.13	0.61
		25	24.95	24.89	24.84	0.16	0.64
		30	29.96	29.92	29.88	0.12	0.40
		40	39.94	39.88	39.82	0.18	0.45

Table 4.3: Reduction in DCF concentration due to various factors

Set	1					2				
Condition maintained	Without aeration; With plants					With aeration; With plants				
Day 1 Initial conc. (mg/L)	2	5	8	10	14	2	5	8	10	14
Day 2	1.88	4.76	7.64	9.55	13.07	1.88	4.93	7.95	9.80	13.52
Day 3	1.80	4.60	7.28	9.19	12.12	1.76	4.84	7.79	9.61	13.24
Day 4	1.75	4.44	6.92	8.65	11.21	1.66	4.76	7.63	9.40	12.86
Day 5	1.60	4.00	6.56	8.22	10.28	1.53	4.68	7.47	9.25	12.48

Day 6	1.54	3.75	6.2	7.75	9.33	1.42	4.60	7.31	9.11	12.20
Day 7	1.42	3.51	5.84	7.31	8.42	1.28	4.52	7.15	8.90	11.72
Day 8	1.31	3.26	5.48	6.85	7.47	1.18	4.44	6.99	8.71	11.33
Day 9	1.22	3.00	5.12	6.42	6.56	1.04	4.36	6.88	8.52	10.96
Day 10	1.10	1.26	4.76	5.95	5.63	0.95	4.28	6.61	8.40	10.55
Day 11	0.98	2.74	4.40	5.50	4.70	0.81	4.22	6.53	8.20	10.22
Day 12	0.95	2.49	4.04	5.05	3.77	0.68	4.12	6.36	8.01	9.82
Day 13	0.80	2.23	3.68	4.60	2.82	0.56	4.04	6.19	7.70	9.44
Day 14 Final conc. (mg/L)	0.70	1.97	3.33	4.16	1.91	0.52	3.96	6.03	7.52	9.06
% Reduction	65.00	60.60	58.37	58.40	86.35	74.00	20.80	24.62	24.80	35.28

(Table 4.3 is split for convenience of presentation)

Set	3					4				
Condition maintained	Without aeration; Without plants					With aeration; Without plants				
Day 1 Initial conc. (mg/L)	2	5	8	10	14	2	5	8	10	14
Day 2	1.98	4.98	7.98	9.99	13.97	2.00	5.00	8.00	10.00	14.00
Day 3	1.96	4.97	7.97	9.97	13.95	2.00	5.00	8.00	10.00	14.00
Day 4	1.94	4.96	7.95	9.93	13.90	2.00	5.00	10.00	10.00	14.00

Day 5	1.94	4.95	7.94	9.93	13.90	2.00	4.98	7.99	9.99	13.98
Day 6	1.93	4.94	7.92	9.91	13.87	1.99	4.98	7.99	9.99	13.98
Day 7	1.93	4.92	7.90	9.90	13.84	1.97	4.98	7.98	9.98	13.97
Day 8	1.91	4.91	7.87	9.88	13.81	1.97	4.97	7.98	9.98	13.97
Day 9	1.88	4.88	7.85	9.86	13.79	1.96	4.97	7.97	9.98	13.96
Day 10	1.86	4.86	7.84	9.84	13.77	1.95	4.97	7.97	9.97	13.96
Day 11	1.84	4.85	7.82	9.82	13.75	1.95	4.97	7.97	9.97	13.95
Day 12	1.82	4.82	7.80	9.80	13.73	1.95	4.96	7.96	9.97	13.95
Day 13	1.80	4.80	7.77	9.79	13.71	1.94	4.96	7.96	9.96	13.94
Day 14 Final conc. (mg/L)	1.77	4.78	7.75	9.77	13.70	1.94	4.96	7.96	9.96	13.94
% Reduction	11.50	4.40	3.13	2.30	2.14	3.00	0.80	0.20	0.40	0.40

4.6. DISCUSSION

4.6.1. Enzyme Assays:

The Enzyme Assays done, produced a set of data that had to be understood on a biochemical basis. The observations regarding the behavior of the fish, their physical appearances and their dietary abilities was done on an hourly basis. The symptoms of disorders were noted. The literature review on the basic chemistry, biochemical reactions, and enzyme pathways and physiology of freshwater fish were done. It helped in analyzing the symptoms and understanding the causes of the same.

The AMX, PCM and DCF concentrations added to the freshwaters, had lethal impacts on the fish at varied concentrations. The combinatorial effect of these pollutants is yet

to be analyzed. The ratios of pollutant concentrations in the combinatorial trials is to be chosen based on the results of the individual trials.

A perfect set of results for Enzyme Assay should show dose-dependent increase in all enzyme levels except AchE; AchE is supposed to show dose dependent decrease. The levels vary (increase/decrease) drastically from LOEC. The succeeding readings are expected to follow the same pattern. In the assay analysis of AMX, PCM and DCF toxicity, the results considerably matched the standard linear graph.

The following graphs are the amalgamation of the results mentioned above, drawn for comparing all the results simultaneously for a better understanding of the impacts. From the comparison graphs (Fig. 4.55, 4.56, 4.57), it is evident that liver and muscle ACP levels were significantly increased in the antibiotic-treated fish. This increase is probably due to increased lysosomal activity in the liver and muscle. It is apparent that the antibiotics AMX, PCM and DCF increased ACP activity in the liver by interacting with lysosomes.
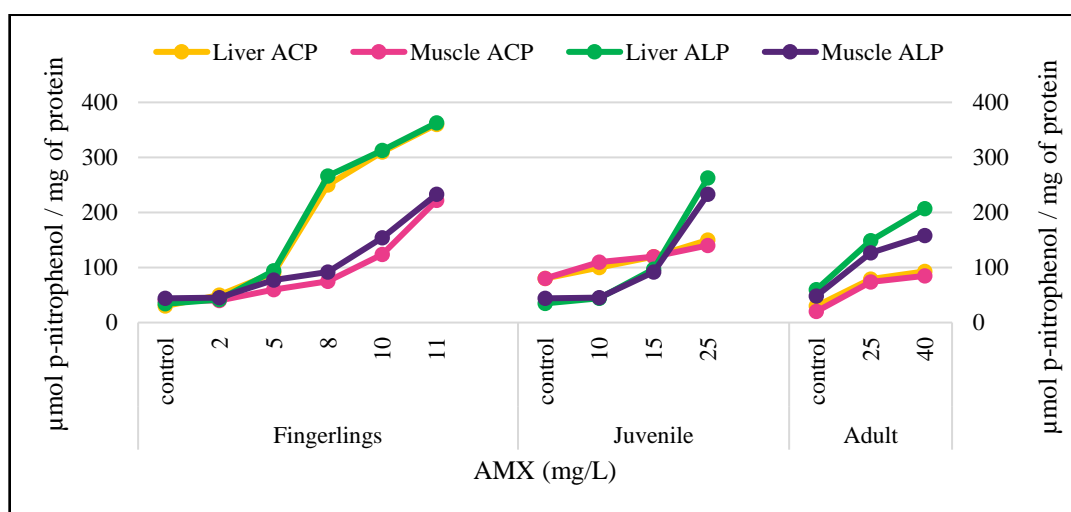
Fig. 4.55: ACP (primary vertical axis) and ALP (secondary vertical axis) levels in Liver and Muscle in Fingerlings, Juvenile and Adult *C. carpio* due to AMX intake significance given at mean ± SD where *P ≤ 0.05*

The ALP activity (Fig. 4.55, 4.56, 4.57) was significantly increased in the liver and muscle of target fish. This explains the involvement of the plasma membrane. AMX (moderately lipophilic), could interact directly with the plasma membrane and cause alteration in its function.
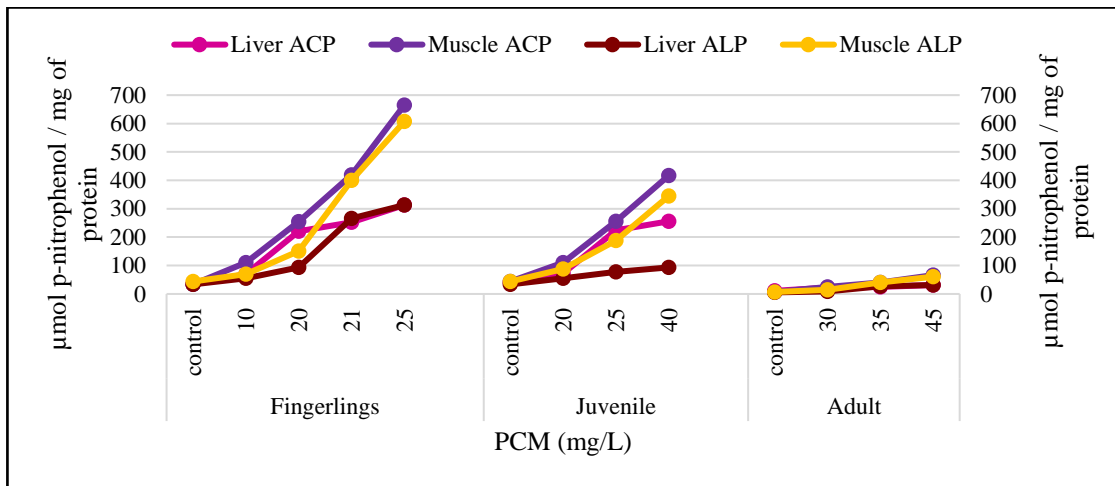
Fig. 4.56: ACP (primary vertical axis) and ALP (secondary vertical axis) levels in Liver and Muscle in Fingerlings, Juvenile and Adult *C. carpio* due to PCM intake significance given at mean ± SD where *P ≤ 0.05*
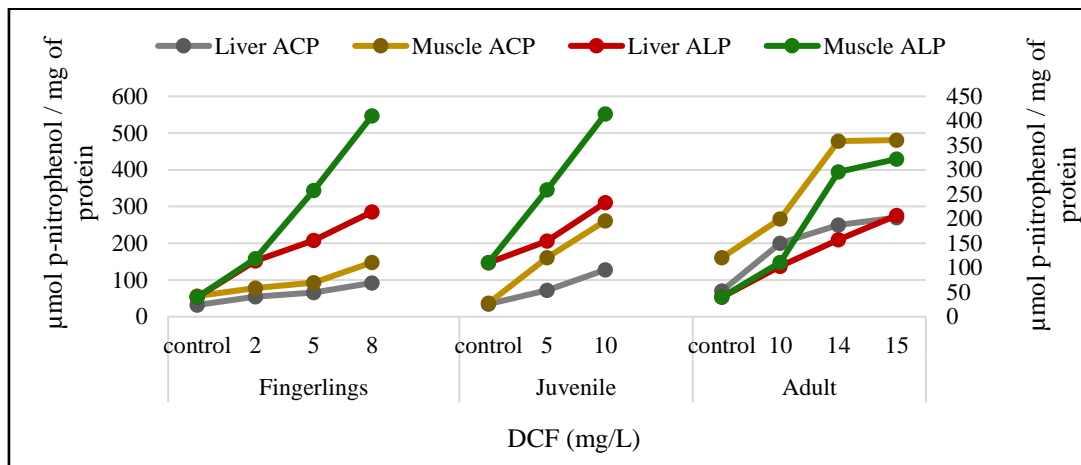
Fig. 4.57: ACP (primary vertical axis) and ALP (secondary vertical axis) levels in Liver and Muscle in Fingerlings, Juvenile and Adult C. carpio due to DCF intake significance given at mean ± SD where *P ≤ 0.05*

ALP is basically a membrane-bound enzyme, and any perturbation in the membrane property caused by interaction with AMX could lead to alteration in the ALP activity. The change in the behavioral pattern of the fish is already described earlier.

Liver AST levels (Fig. 4.58, 4.59, 4.60) were significantly increased in AMX-exposed fish compared to the non-exposed ones. This rise points at the fact that the AMX stimulates the glutamate transaminase activity, which is a mitochondrial enzyme.
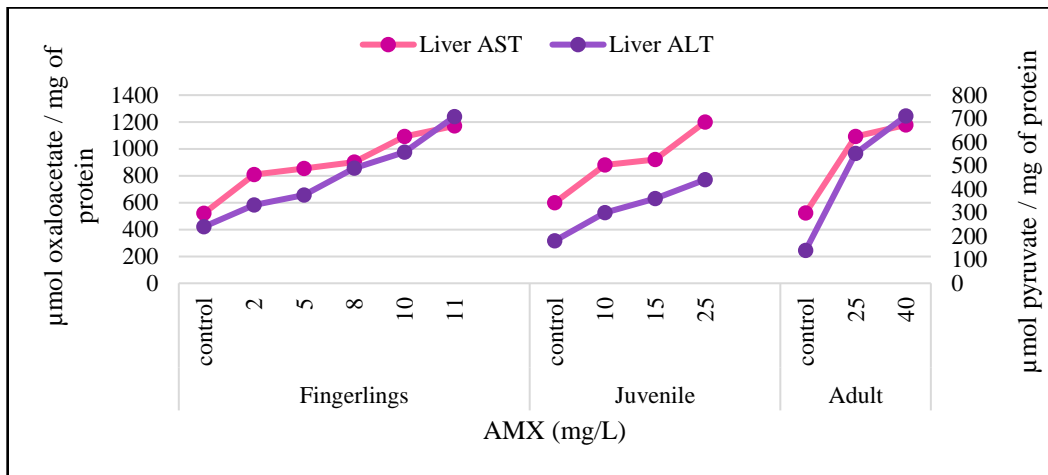
Fig. 4.58: AST (primary vertical axis) and ALT (secondary vertical axis) levels in Liver in Fingerlings, Juvenile and Adult C. carpio due to AMX intake significance given at mean ± SD where $P \leq 0.05$

Increased glutamate transaminase activity in the liver could be due to toxic injury brought about by AMX. As a result, fish may exhibit increased respiration as a response to the stimulus. Rao C.V. *et al*. worked with another lipophilic compound (DEP) instead of AMX.
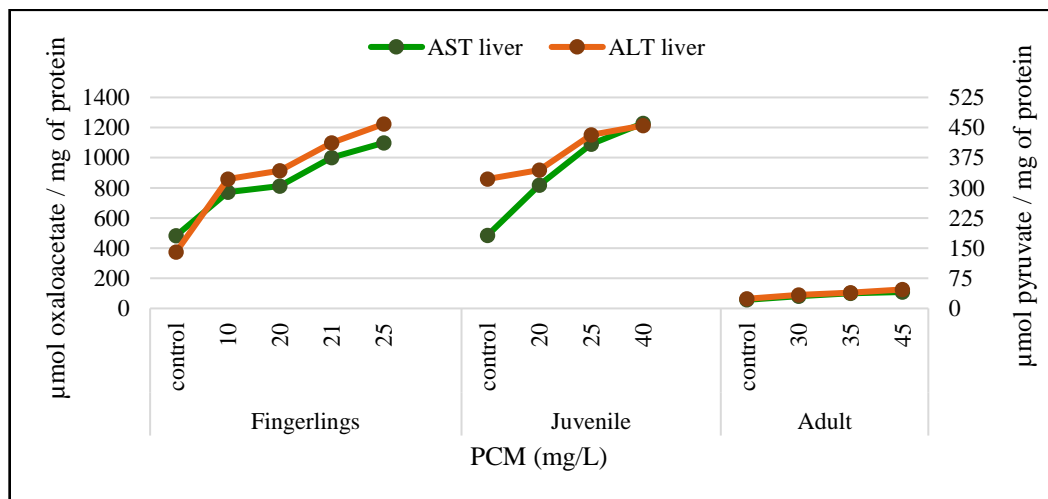
Fig. 4.59: AST (primary vertical axis) and ALT (secondary vertical axis) levels in Liver in Fingerlings, Juvenile and Adult C. carpio due to PCM intake significance given at mean ± SD where $P \leq 0.05$

Increased LDH and glutamate levels seem to indicate that the mitochondria are involved, and its normal functioning is disturbed in the process. It may also stimulate

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tissue repair through protein turnover. It was also concluded that the rise in AST levels confirms the involvement of the mitochondria; also, that it was heavily damaged in the same. Increased levels of liver ALT (Fig. 4.58, 4.59, 4.60) almost 6 times higher than the control was enough proof of the damage incurred to the liver. It highlights the phenomenon that the transaminase activity in the liver is enhanced due to the toxicity. This proves the credibility of the results of AST tests. This leads to the affirmation of the statement that both AST and ALT activity is enhanced due to the AMX toxicity.
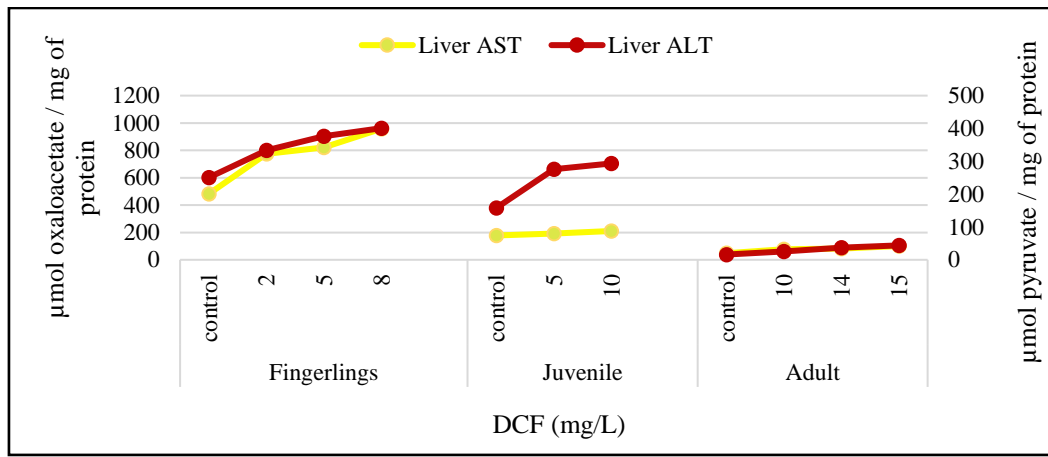
Fig. 4.60: AST (primary vertical axis) and ALT (secondary vertical axis) levels in Liver in Fingerlings, Juvenile and Adult C. carpio due to DCF intake significance given at mean ± SD where *P ≤ 0.05*
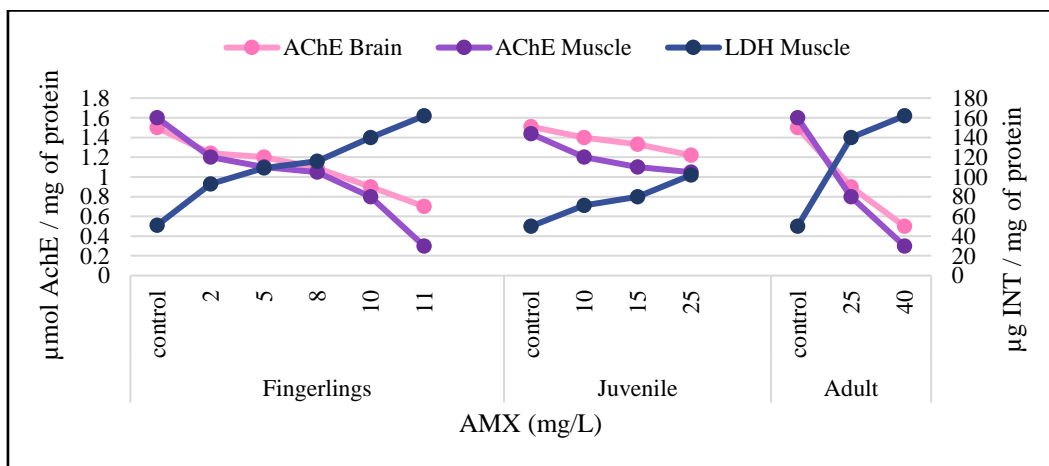
Fig. 4.61: AChE (primary vertical axis) and LDH (secondary vertical axis) levels in Brain and Muscle in Fingerlings, Juvenile and Adult C. carpio due to AMX intake significance given at mean ± SD where *P ≤ 0.05*

Unlike all other test results, the AChE levels (Fig. 4.61, 4.62, 4.63) in both brain and the muscle decrease as the concentration increase. This indicates that AMX< PCM< DCF inhibits AChE activity. This explains the inactive, non-motile behavior of the fish when exposed to the pollutant concentrations of PCM and DCF. AChE activity decreased drastically in brain for DCF exposure. Increased LDH (Fig. 4.61, 4.62, 4.63) and glutamate levels were more glaring by PCM and DCF than AMX. It indicates that the mitochondria are involved, and its normal functioning is disturbed in the process. That stimulates tissue repair through protein turnover.
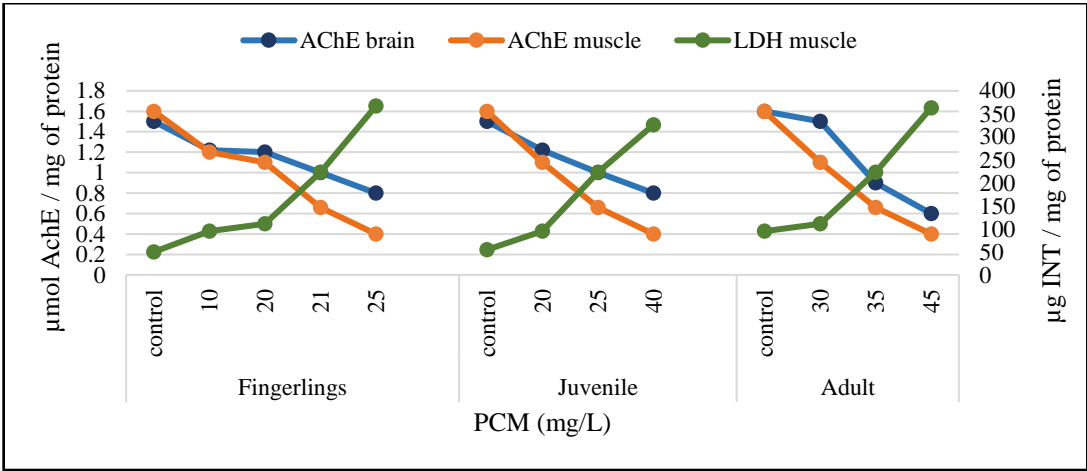
Fig. 4.62: AchE (primary vertical axis) and LDH (secondary vertical axis) levels in Brain and Muscle in Fingerlings, Juvenile and Adult C. carpio due to PCM intake significance given at mean ± SD where P ≤ 0.05
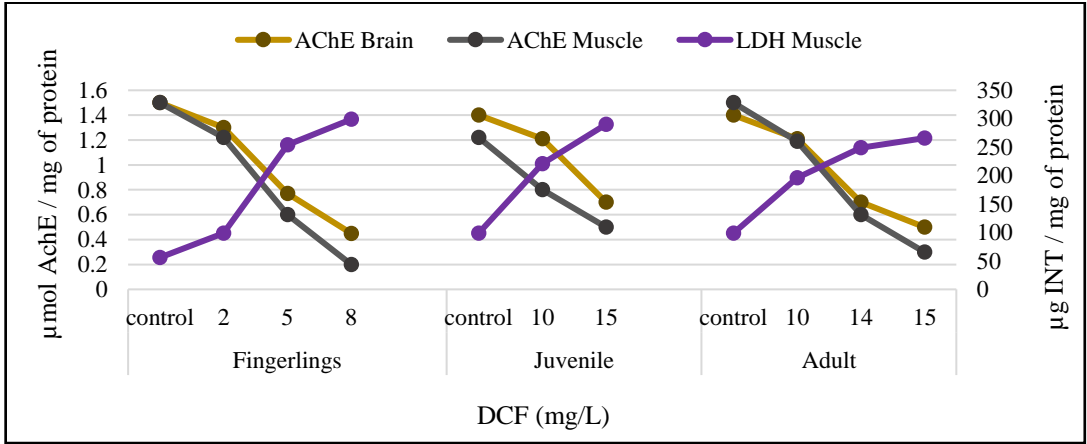
Fig. 4.63: AChE (primary vertical axis) and LDH (secondary vertical axis) levels in Brain and Muscle in Fingerlings, Juvenile and Adult C. carpio due to DCF intake significance given at mean ± SD where P ≤ 0.05

The protein level rise can be linked to the protein turnover stimulated for the tissue repair. Thus, more significant changes in AchE and LDH levels (Fig. 4.61, 4.62, 4.63) are observed by PCM and DCF exposure than AMX. As it was brain that was highly affected compared to liver and muscle, the protein level rose maximum for the brain sample. The ACP, ALP, AST and the ALP assays for AMX, show the liver is extensively damaged. Among the EDCs- AMX showed maximum impact on liver; PCM showed intense harm to the muscle and DCF proved to be hazardous and detrimental to both muscle and brain. The justification follows.

The Gluconeogenesis is a process of maintaining, augmenting and replenishing the glucose reserves in the body. Similarly, lipogenesis is conversion of nonlipid dietary components to lipid- for the sake of growth and maintenance. In fishes, both involve active participation of the mitochondria in liver, kidney and muscle tissues. As we can see, the rise in AST levels indicate anomaly in liver. This affects the mitochondria in there and thus directly/ indirectly affecting the various enzyme pathways that are interlinked with gluconeogenesis and lipogenesis. Due to the disturbance in the various enzyme pathways responsible for growth and metabolism, the fishes begin to show the symptoms such as reduced appetite, lethargy etc.

On the other hand, we are dealing with the muscle tissues and the nervous system. Nervous system is vital. This network is responsible for the fluid, quick, movements of the fish along with responses to stimuli, threat recognition-evasion impulses. Muscles are one of the most important and active organs in the fish. The skeletal muscles of the fishes are a significant proportion of the fish's biomass. These have a variety of key roles from powering locomotion to regulating whole-body metabolic homeostasis. These two systems together act as the physical defenses of the fish. They are strong when neither is negatively affected. When one is negatively affected, the other bears the brunt of defense-responsibility overload. The system eventually breakdowns under extra pressure and the fish health is destroyed. Researchers say that, the fingerlings have maximum potency for muscle plasticity and myogenesis. The potency reduces with progress in age while immunity increases. Myogenesis is the creation of new muscle fibers by multiple distinct processes. Muscle plasticity is of two types: phenotypic and the genotypic. The plasticity of the muscles provides them the opportunity to survive in unfavorable conditions. The dormant genes responsible for plasticity are activated by

drastic changes in their habitat. The pathway that regulates muscle growth favors the protein formation over the protein degradation.

The fishes do not develop enough scales in the fingerlings stage. The scales begin to form very early in life, but they are not hard and fully functional in fingerlings. Hence when a pollutant is added to the water bearing the subject, the intake of pollutant in fingerlings is via mouth, gills and body muscles. The scales are permeable and allow not just the pollutant, but air for breathing too (in case of emergency only). They allow only oxygen molecules to penetrate within relieving the fish from lack of oxygen. The fishes will grow considerably hard scales in juvenile stage. It basically depends on the personal growth rate of the fishes. The fish immunity and resistance are quite strong by then. This is a phase where the growth curve of the fish takes a turn towards stability. It's a highly unpredictable stage as most of the factors are based on individual growth rate and resistance ability of the fish. The adult stage is very predictable, stable and at its strongest from an immunity point of view. The scales are fully formed and are impermeable to almost all compounds, except for a few lipophilic compounds. The oxygen intake is strictly through gills. The pollutant intake is also mainly from gills and mouth (and muscles- only if it is a lipophilic substance).

The PCM and DCF are enough lipophilic to enter the muscle tissues. Once inside, PCM binds onto certain receptors which are meant for the relaxation-contraction cycle of the muscles. This binding comes in the way of normal functioning of the muscles; thereby rendering them non-flexible and stiff. The DCF works in a similar way, except that it penetrates deeper and affects the brain-nervous system of the fish. The fingerlings have more pollutant intake – least immunity – highest plasticity. The juveniles have medium pollutant intake – average immunity – medium plasticity whereas the adults face less pollutant intake – highest immunity – complex plasticity. The degree of pollutant intake is by considering a ratio of fish biomass to the pollutant consumed.

4.6.2. *Symptoms observed during the Enzyme Assays and their Causes:*

Generally, when a fish dies, it first has breathing problems, then normal movements are affected along with less appetite. In some hours' time (approximately 4hrs.) the fish is found dead and floating on the water. It is either found floating in upside-down position (stomach-up position) or in horizontal position (floating on its sides). The position gives

an estimate of the how long has the fish been dead. The following table (Table 4.4) represents the same. The following table gives a general overview of the situation after death, given- a) the fishes mentioned are the freshwater fish *C. carpio*; b) the fishes are bred in natural water maintained at natural conditions (with no added pollutants). The adult fishes behave differently than the juvenile and the fingerlings. They tend to eat up the smaller dead ones, thereby interrupting in the natural decay process. That is, adult eat up both the juvenile and the fingerlings while the juvenile only eat fingerlings. In such cases, the below mentioned situations will be hardly witnessed in the same order and time frame.

Table 4.4: Post-mortality changes of freshwater fish

Appearance	Time passed after death
Floating upside down	Immediately – 4hrs.
Floating sideways	About 8hrs.
Floating sideways + change in muscle color (natural to paler hues) + mild stink	About 16hrs.
Floating sideways + discoloration of muscle and fins (natural to pale yellow) + strong stink	About 24hrs.
Floating sideways + disintegration of muscle and eyes + strong stink	More than 48hrs.
Complete disintegration of fish into strands and lumps	About 4 days
Remains of fish settle at the bottom	After 5 days

The damage inflicted on muscle reduces the fish motion (distance travelled) as well as the potency (speed). The same is depicted by the following graphs plotted after careful repetitive manual observations of the fish behavioral and swimming patterns during each run. The behavior was video-recorded for reference purposes and the most probable plot of its motion was plotted after going through the videos 20 times each for most-possible accuracy. The experimental tanks were scaled and plotted on the standard graph papers, valued obtained; then electronically plotted as Fig. 4.64 to 4.72. The Figures represent the behavioral responses in terms of motion: swimming potency (distance and speed). The downward arrow of a trendline indicates the drop in the "distance covered" and "speed" of the target fish.

- *Abnormal breathing patterns*

The gas exchange and blood gas transport take place in a pair of specialized air sacs extending from the pharyngeal cavity. Reduced rates of blood flow during normal breathing and the fall in blood pressures forced the fish to survive with air breathing. Generally, *C. carpio* does breathe in the aquatic environment. But during health disorders, when the dissolved oxygen does not *seem* to be enough, it tends to move to the surfaces for direct oxygen consumption. This response-to-inconvenience is very similar to human reaction.

Fig 4.64 (a, b): Behavioral responses in Fingerlings in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr AMX concentrations.

Whenever a person feels suffocation or dizziness, he prefers more oxygen intake. He immediately moves towards a more spacious location where he can feel fresh and breathe in more air. This condition occurs due to the lack of oxygen supply to the brain.

During this, the normal functioning of the brain is not possible. The loss of control over the voluntary muscles of the body, such as locomotory organs, is also possible.

- ***Reduced food intake***

A vital area in lipid nutrition in fish is the provision of sufficient amounts of the correct EFA to satisfy the requirements for normal growth and development, requirements that can vary quantitatively during the life of the fish. Biochemical studies have been advanced by the use of cell cultures which have elucidated key parts of the pathway. Understanding this pathway is of increased importance due to the varied responses at different pollutant concentration. The fishes lose their appetite due to consumption of some pollutants. Initially when the dosage is low, they degrade the pollutants within their body. This is then either excreted or deposited in the fish muscle tissues. In some cases, the traces are found in the fish blood too (Sole, *et al.,* 2010; Stalter, *et al.,* 2010).

Fig 4.65 (a, b): Behavioral responses in Juveniles in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr AMX concentrations.

- *Decreased movement; sluggish behavior*

The muscle is very important to the fish. The scaly exterior not only protects it soft interior systems but also helps in movement in water. The muscle tissues have lot of lipid content stored in them that help the fish to retain body temperature when the temperature in the aquatic habitat drops during seasonal variations and due to pollution impacts (Tocher, D. R., 2010).

As the fingerlings have very less scales, the pollutant affects both the outer and inner surface of the fish far more easily than in an adult. This leads to the reduction in the inflexibility of the muscle tissue making the fish move lesser and slower. Extreme damage stiffens the entire fish body making it movement impaired and eventually dead.

Fig. 4.66 (a, b): Behavioral responses in Adults in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr AMX concentrations.

- *Non-functional gills; Mouth, gills, muscle reddened*

Since the pollutant is added into the water, the gills become the means of uptake of the pollutant. Hence the gills are very much affected. The reddening is a physical proof of the irritation caused as well as the abnormality caused therewith. Due to continuous exposure to pollutant, the gills, mouth and the muscle were reddened. When there is any unwanted chemical in the body, the body reacts by sending out signals to the defense mechanism in the body. The first ones are the inflammation, reddening, irritation, swelling and finally, organ malfunction.

Fig. 4.67 (a, b): Behavioral responses in Fingerlings in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr PCM concentrations.

- *Abnormal swimming patterns; Stiffness of entire body*

The pharmaceutical pollutants had visible impacts on the species. These behavioral changes were observed to have occurred since 10mg/L AMX, 21mg/L PCM and 5mg/L

DCF. All three concentrations did cause mortality. Hence, it can be said that this symptom is one of the unmistakable signal of mortality in freshwater fishes. As the target species is known to be quite tolerant, it can be understood that the other more vulnerable kinds would show these symptoms at a lower concentration.

Fig. 4.68 (a, b): Behavioral responses in Juveniles in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr PCM concentrations.

The random moving-with-the-water-currents swim patterns can also be caused due to the rupture of the swim bladder. The swim bladder keeps the fishes afloat. It also helps maintain body temperature in adverse conditions. The rupture of the swim bladder guarantees the loss of buoyancy of the fish; hence drowning the fish. Simultaneously, due to the PCM and DCF intake, the muscles and fins go rigid and stop functioning-leading to a total impairment of movement of the fishes. Additionally, losing out on the

body temperature regulation mechanism, the fish is more prone to adversity (Tudorache, *et al.,* 2008; Tu, *et al.,* 2009; Stepanova, *et al.,* 2013).
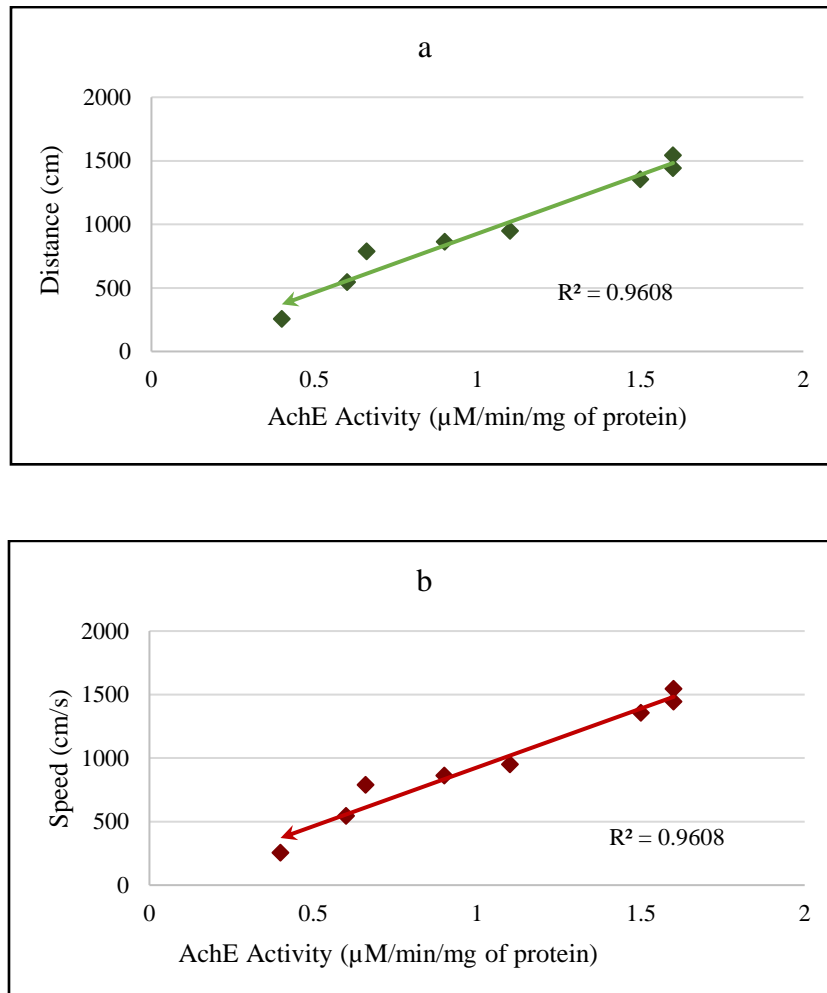
Fig 4.69 (a, b): Behavioral responses in Adults in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr PCM concentrations.

The fishes were observed moving downwards in the water- either head-first or tail-first position. While the nose-dive position of the fish may seem normal to a person, close observation reveals that it can be abnormal too. The nosedive of the fish is normal if all the fins are working fine. Due to DCF pollution, it was observed that the fins were stiff and got impaired. The tail fin was non-functional. The upper part of the fish (the head to stomach) lost coordination with the lower part (stomach to tail fin). This disconnection may be between (i) the bone structures running right through the fish-head to tail; (ii) non-transmission of the signals from the brain to the fin.
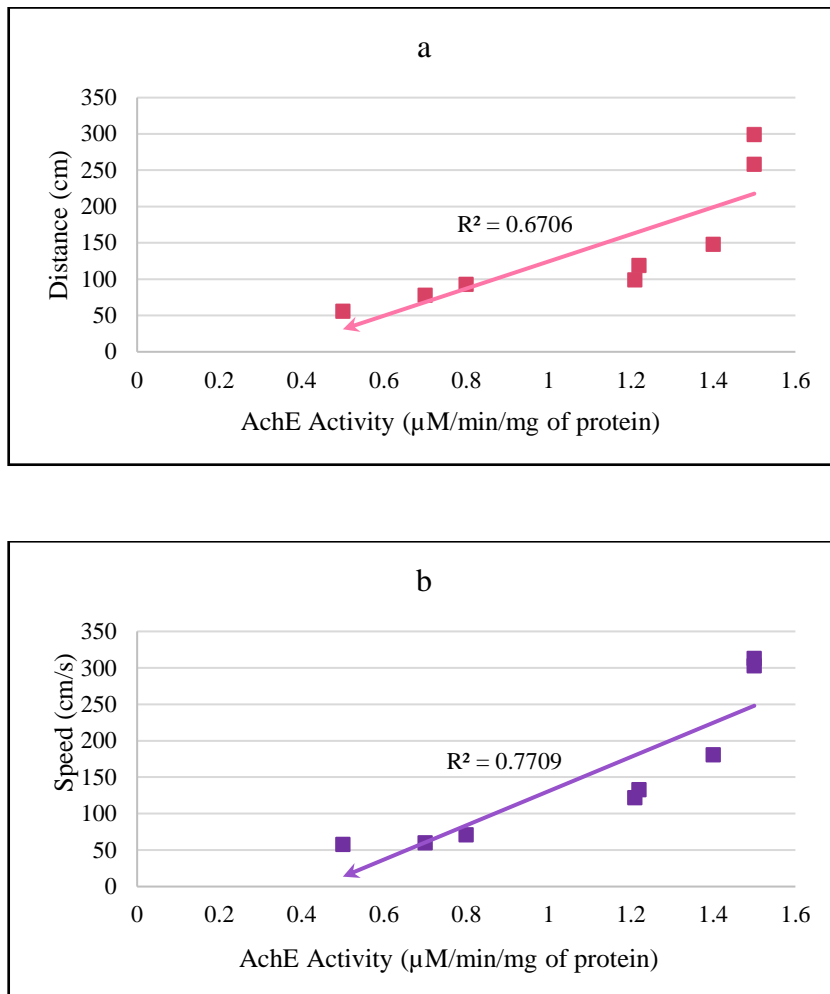
Fig. 4.70 (a, b): Behavioral responses in Fingerlings in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr DCF concentrations.

Observations during PCM impact analysis: When the tail fin goes non-functional, the fish loses it balance. So, it can no longer move in a horizontal direction. That makes it move haphazardly in the water- an absolute non-voluntary movement. The head, moves downward after this loss of balance. At a later stage, fish is observed to be drowning in the tail-first way. This too is the result of imbalance. Once the fins and tail fin are damaged, they get stiff eventually. That part of the fish can be considered dead. As the toxic effects spreads to the other muscles, they too stiffen up. The muscle loses its texture, flexibility, semi-permeability and color.

This "stiffness of muscles" is one of the prominent symptoms of Seizure. The non-motility, fin degradation are other indicators of Seizure. The PCM induced partial paralysis to muscles and in compensation, attacked the liver more than DCF could. On

the other hand, DCF has the access not only to the muscle tissue but also to the nervous system of fishes. Hence the DCF launched full-fledged Paralytic Stroke lending a slow and steady death to the target fishes. The DCF took a while to spread to the brain. Meanwhile it kept penetrating the muscles deeper and deeper; incapacitating the muscles and nerves all along. Once it got a good hold over the brain cells, the fish went brain dead. And was thus relieved of the prolonged extensive torture. Even though certain scientists claim the fish cannot "feel" and "express" pain, the rise and fall in certain enzymes responsible for feeling and expressing pain eradicate all doubts about the level of damage caused and the "stress" experienced by the fish.
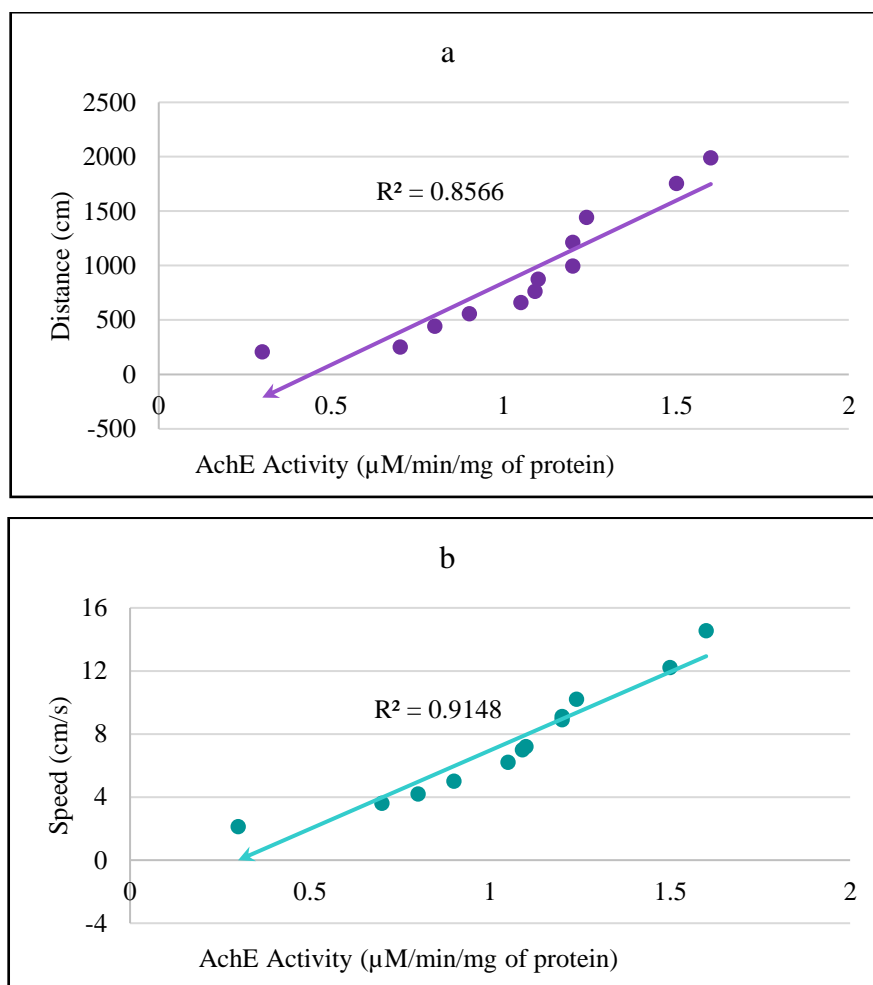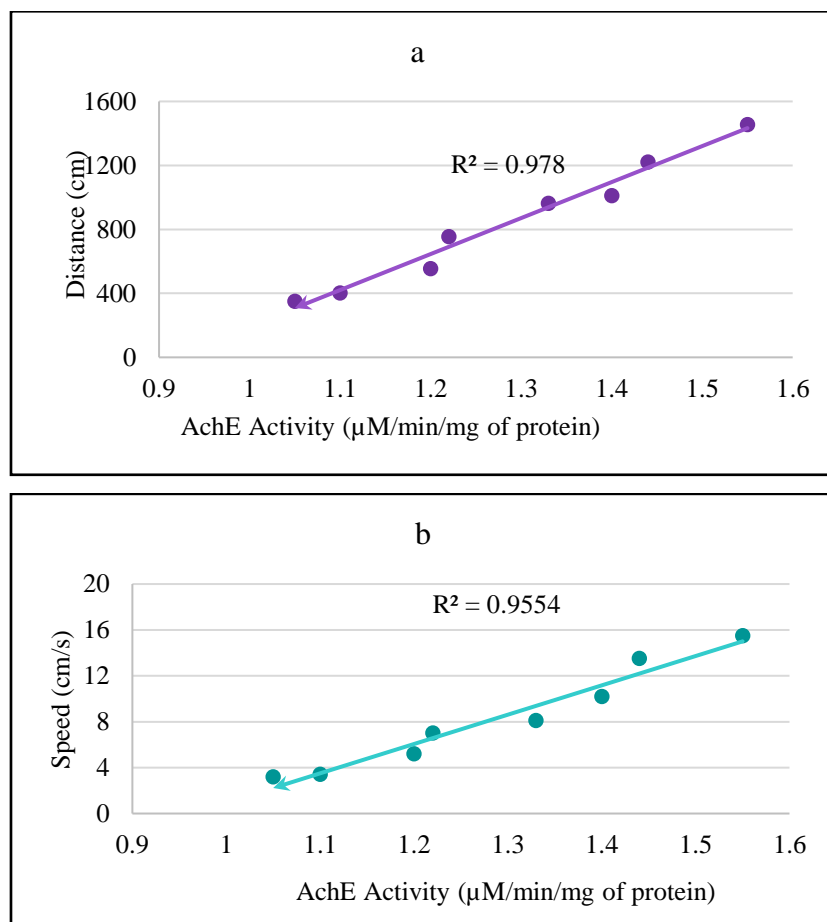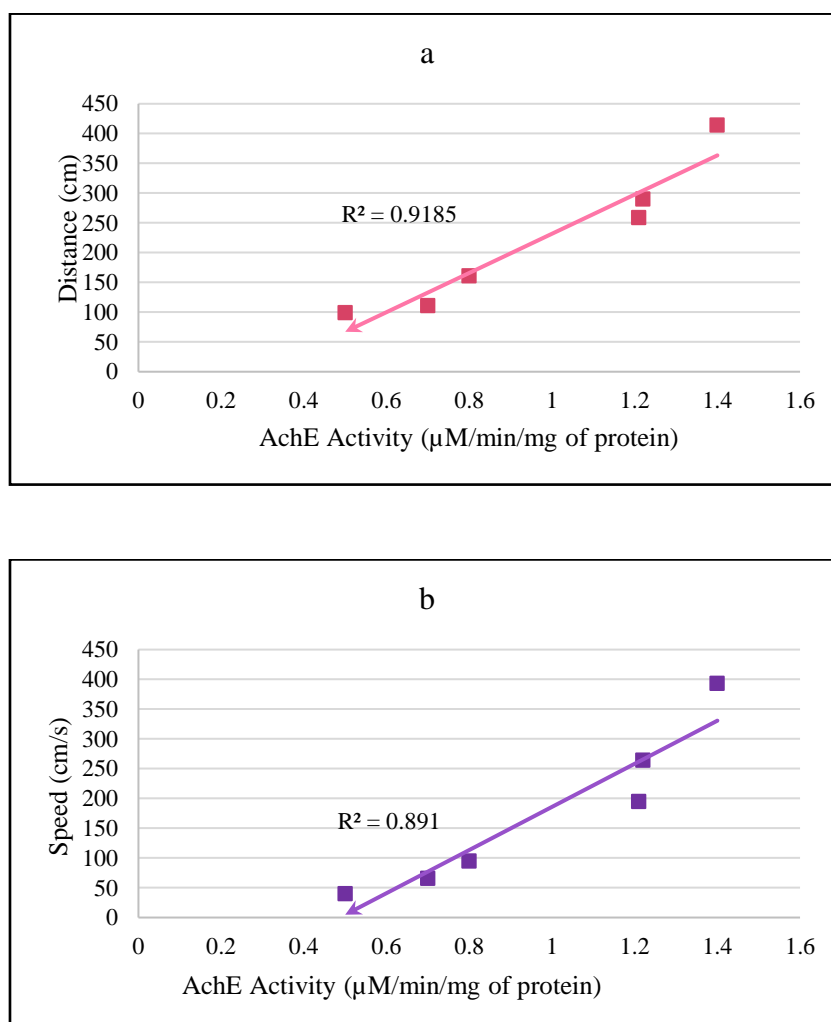

Fig. 4.71 (a, b): Behavioral responses in Juveniles in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr DCF concentrations.

While some scientists may argue that the fish tend to display many "kinds of stress"; One of them being the "social" stress. In plain words, it means "accepting defeat / surrender or accepting the superiority of the best fish in the particular school of fishes". It can also mean having the common sense to avoid confrontation with the best one in the stock. The assays conducted on such "subdued" fishes show a lot of stress related enzyme level hike and death (at the last stage). But here, this is not the case. Because, i) the observations did not reveal any heightened animosity among fishes in the same tank; ii) all the fishes- irrespective of size and shape, displayed these symptoms before tormented death. Evidently, the stress experienced by the fishes in this study is absolutely pollutant-induced.
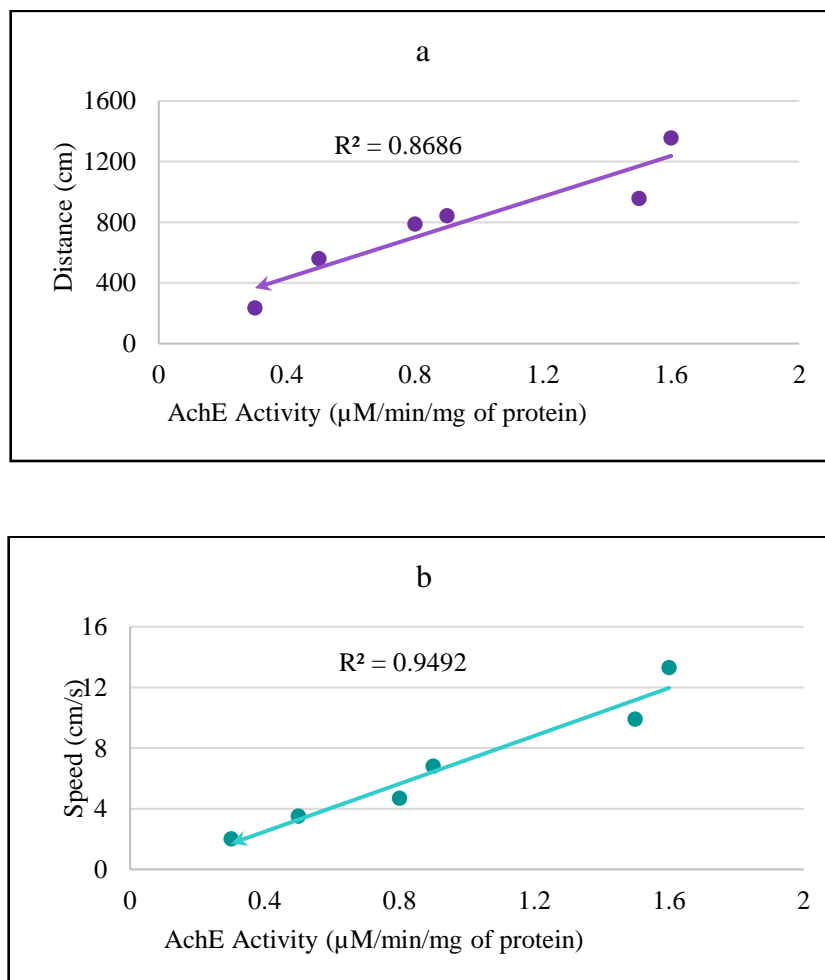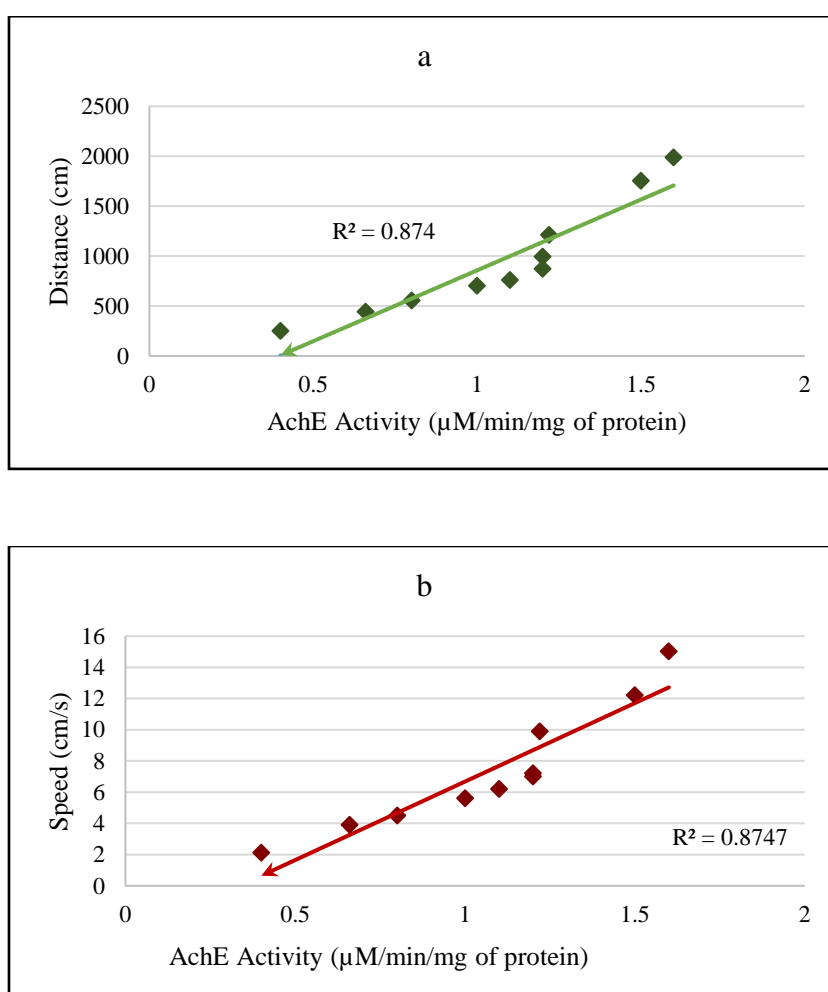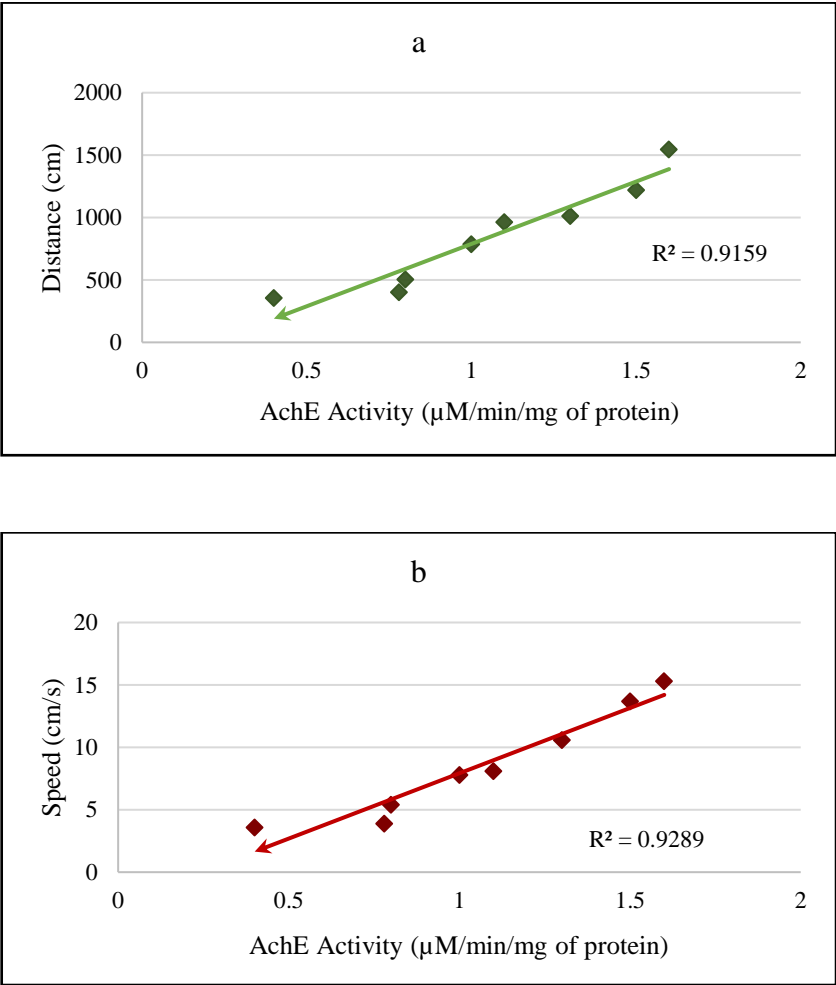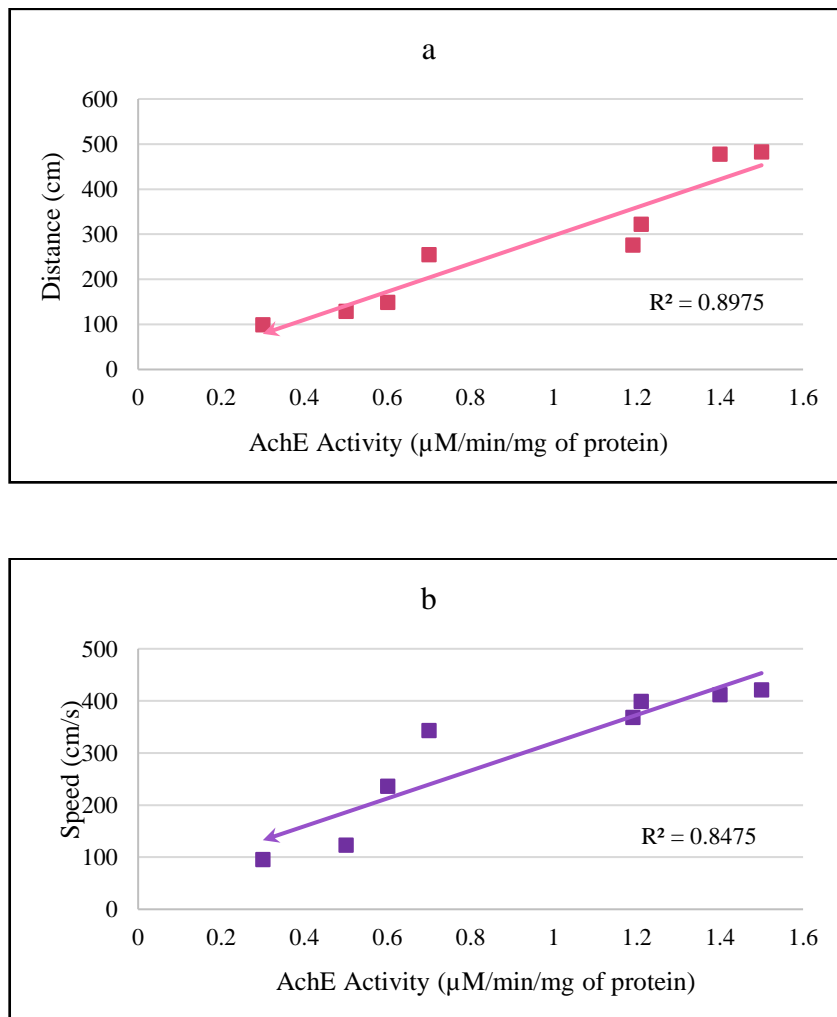

Fig 4.72 (a, b): Behavioral responses in Adults in accordance with the changes in the AchE levels of brain and muscle, when exposed to a 96-hr DCF concentrations.

After studying the literature on the biochemical effects of the pharmaceutical wastes-their impacts on cells, tissues, muscle, liver, brain, gills; enzyme levels in various organs; enzyme pathways affected; respiratory systems in fish; It can be concluded that the lower body of fish had- i) non-motile fins, ii) dead cell mass, iii) dead protein pile-up from muscle tissues, iv) the lipid content, v) new enzymes released due to toxicity in tissues, vi) increased levels of key enzymes that participate in different pathways, vii) non-excreted urea deposition. Also, when the muscle is more affected than the brain, the fish dies after a prolonged time of suffering the symptoms.

Comparing all the results of all enzyme assays on all the age group of fishes (fingerlings, juvenile and adult) for all pollutants (AMX, PCM, DCF), we can establish that AMX damages the liver more than muscle and brain; PCM causes the muscle tissue damage; DCF causes brain as well as muscle damage. These "damages" are intense enough to lead unto death. The AMX is observed to cause intense liver malfunctioning and death follows soon. The PCM is seen to cause partial muscle seizure followed by de-capacitation and death. The DCF affects the muscle first, then spreads further into the body affecting nervous network finally reaching for the brain. It causes complete seizure or paralysis in the fish. This slows down the death i.e. instead of dying in few hours after the symptoms of death shows up, it dies after many days.

The ratios of the damage-impact are calculated for liver damage for AMX, muscle and brain damage for PCM and DCF. Considering the concentration set of fingerlings as 1, the following ratios x: y: z is derived, where the x = fingerlings, y = juvenile and z = adults. The AMX goes by 1: 3.85: 7.85. The PCM shows 1: 1.31: 0.78. The DCF has 1: 1.88: 0.88. Adding the standard error factor percentage of 5% to the realistic difference in the health status of the target fish (factual age, health, immunity and gender), round it off as- AMX 1: 4: 8; PCM 1: 0.9: 1.5; DCF 1: 0.25: 1.25.

This clearly tells the liver damage was highest in fingerlings due to AMX. The muscle due to its plasticity feature coped better against the pollutants. The fingerlings have plasticity attribute, but the juvenile plasticity is very active and more easily adaptable. The adults have least plasticity since the muscle tissues are already fully developed. So, the PCM and DCF could not cause as much damage to muscle tissues of juvenile as much to the fingerlings and adult.

4.6.3. Interference Analysis:

The Interference Tests produced interesting results. The reduction in concentration was observed for all cases. But the rate of concentration reduction was not consistent for all the cases. The results point out that the plants and the aeration compete for degradation of pollutant. The effects of either ones individually were a confirmation of the same. Also, the reduction in concentration in the last case, where neither aeration nor plants were supplied in the test environment, was another affirmation for the same conclusion. All-in-all, the reduction was observed over time for all the cases. The plants tend to have an upper hand in the reduction of the pollutants than the aeration factors. But, all this was the analysis and conclusions for the lab-scale set-up. The actual scenario can possibly be entirely different. It would depend on the biodiversity of the freshwater source in question and the nature of the pollutants flushed into the freshwater.

4.6.4. Predictive Toxicological Analysis:

The common carp is one of the most abundant and most used species for both *in vivo* and *in vitro* studies. This species is almost tolerant to a wide range of water characteristics. If a species a too sensitive, analysis may seem false-positive or false-negative at times. The genome of common carp is the one with highest number of chromosomes (50) and also the gene pool has evolved a lot compared to the other carp species and other freshwater species that are used for bioassays or bioinformatic procedures.

The BLAST results for *Cyprinus carpio* (common carp) with *Cirrhinus mrigala*, *Salmo gairdneri* (rainbow trout), *Carassius cuvieri* (Japanese crucian carp), *Carassius auratus* (goldfish) and a goldfish- common carp hybrid is a clear indication that the closer a species is to the other one, taxonomically, the higher are the chances of a match/similarity.

Out of all the species considered, common carp is closest to the Japanese crucian carp taxonomically. Next comes the mrigal carp, goldfish and the rainbow trout. One hybrid was considered with the intention of highlighting the importance of usage of mitochondrial genome set. The goldfish-common carp hybrid considered would have had the common carp as the maternal genome donator since it has the highest of 99% match (a difference of 377 nucleotides) with it.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1. SUMMARY

The endocrine system basically works on a simple principle: receive hormone signals – process the signals appropriately - produce the hormones – express them. When an EDC affects an organism, it generally interferes and disrupts that process. Instead either signals are not sent in properly or not processed; or not let to produce hormones/proteins or express them adequately. This results in malfunctions in the various enzyme pathways simultaneously. The fish immune system finally collapses, and the target fishes are either left with serious damage to some of the vital tissues or death. Even death can be either a quick death due to abrupt vital organ failure as in case of AMX or death by inability for normal breathing accompanied by extensive loss of locomotion and food intake as in case of PCM or a slow tortured painful death creeping in by the hour, that first puts the target body in a total Seizure and then slowly and steadily leads it to death- as in case of DCF.

The researchers believe that the EDCs have a potential for bioaccumulation, bio magnification and potency for multigenerational effects through changes in the epigenome. This means that the EDCs can alter the basic DNA of the target body- that are inheritable by the offspring. Not all genes are inheritable, thus making it a too complex a problem. That way, it can affect not only current generation negatively, but also cause permanent damage to all the future generations. EDCs can also cause altered sexual development, that is, change of gender of the fishes or render them neutral and impotent/infertile. Literature data says these subtle, yet powerful changes can be brought about by the ng/L concentrations alone. The mg/L concentrations as studied in this study focus on identifying the mortality rates and other major organ failures associated with mortality of the subject.

The importance of Predictive Toxicological Analysis is that the results obtained for a single species can be used not only to interpret and understand the toxic and lethal impacts on the target species but can also be useful to predict the impact on the relative

species also. The work carried out was with the combination of the lab results, genome data, gene sequences, structure and function of both pollutants and the enzymes affected. The Ensembl, NCBI database and BLAST tools help in predicting the toxicity (at a basic level) *without inflicting* experiments on all concerned species.

In this study, we have focused on the mortality of the freshwater fishes. Fish death leads to rotting and disintegration of the bodies within the next 48-60 hours. This eventually leads to the contamination of water resources to the point of harm to other aquatic beings. Loss of a freshwater source is a major setback for the human population. All life forms would face either drought, famines or water-borne infections and epidemics. So, it is always better to know where and how it all begins. Hence this study, where we have understood the harms caused by crossing certain limits.

5.2. CONCLUSIONS

From the current study, it was concluded that

1. While Amoxicillin, Paracetamol, Diclofenac all lead to mortality eventually, AMX is responsible for the liver failure; PCM for muscles and DCF for both muscle and brain. Both PCM and DCF cause death by partial and complete seizure respectively.

2. The liver damage was highest in fingerlings. The PCM and DCF could not cause as much damage to muscle tissues of juvenile as much to the fingerlings and adult.

3. The ratios of the damage-impact state that AMX goes by 1: 4: 8. PCM shows 1: 0.9: 1.5. The DCF has 1: 0.25: 1.25

4. Water-plants (hydrilla) and oxygen recharges do influence the reduction of pollutants in waters significantly.

5. The physical, behavioral and biological responses to toxicity due to AMX, PCM, DCF would be similar in other Freshwater Fish Species chosen in this study for Predictive Toxicological Analysis, but for lower amounts and over a longer time.

6. The longevity of an organism is not just dependent on the species but also on the life-stage when it is exposed to the pollutant.

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APPENDICES

APPENDIX A: Enzyme monitored for Enzyme Assays

- ***Acid phosphatase (ACP) and Alkaline phosphatase (ALP)***

Acid phosphatase (ACP) and Alkaline phosphatase (ALP) are enzymes capable of catalysing the hydrolysis of various phosphate esters at acid and alkaline pH respectively.

The activities of these two enzymes were determined in the liver homogenates prepared in saline by using the procedure of Andersch and Szcypinski with slight modifications. Here, p-nitrophenol phosphate was used as a substrate and was hydrolysed to p-nitrophenol and inorganic phosphate, in the presence of enzymes. The p-nitrophenol, a strong yellow compound was then measured at 420nm. The phosphate activity is directly proportional to the amount of p-nitrophenol liberated per unit time.

- ***Aspartate aminotransferase (AST/ GOT) and Alanine aminotransferase (ALT/ GPT)***

AST was formerly called Serum Glutamic Oxaloacetic Transaminase (SGOT) and ALT is Serum Glutamic Pyruvic Transaminase (SGPT). The AST test may be done at the same time as a test for alanine aminotransferase, or ALT. The ratio of AST to ALT sometimes can help determine whether the liver or another organ has been damaged. Both ALT and AST levels can test for liver damage.

$$\text{L-Aspartate} + \alpha\text{-Oxoglutarate} \underset{}{\overset{\text{AST (GOT)}}{\rightleftharpoons}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{L-Alanine} + \alpha\text{-Oxoglutarate} \underset{}{\overset{\text{ALT (GPT)}}{\rightleftharpoons}} \text{Pyruvate} + \text{L-glutamate}$$
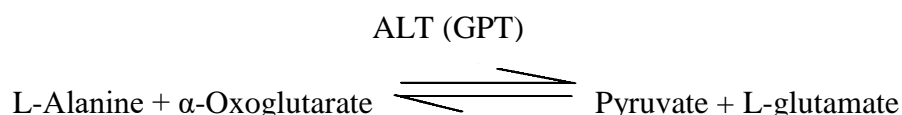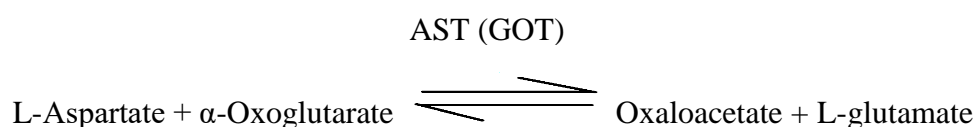
These enzymes catalyse transamination reaction in which the α-amino group of an acid is transferred to the α carbon atom of an α-keto acid, leaving behind the corresponding

α-keto acid analog of the amino acid and causing the amination of the α-keto acid (mostly α-oxoglutarate) to L-glutamate.

The activities of both the aminotransferase were determined by the method of Reitman and Frankel. This procedure involves the colorimetric estimation of oxaloacetate and pyruvate formed as a result of the transfer of the amino group from aspartic acid and alanine respectively to α-oxoglutarate using 2,4-dinitrophenyl hydrazine (DNPH) as a ketone reagent.

- *Lactate dehydrogenase (LDH)*

The enzyme is involved in the glycolytic pathway, responsible for the reversible conversion of pyruvate to lactate. It catalyses removal of two hydrogen atoms from the CH(OH) group of the lactate, thereby, oxidising it to pyruvate.
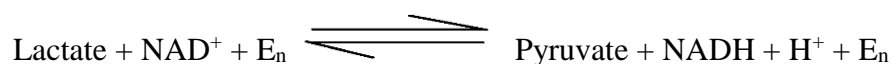
$$\text{Lactate} + \text{NAD}^+ + E_n \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{H}^+ + E_n$$

The reducible enzyme of LDH which is NAD^+ functions as a hydrogen acceptor in this case. Liver homogenates prepared in saline were used for the determination of LDH activity using the method of Bergmeyer and Bernt.

The method involves enzymatic reduction of INT (Iodonitrophenyl tetrazolium salt) in the presence of PMS (Phenazine methosulphate) which acts as an intermediate electron carrier. Reduction of INT forms intensely colored formazan granules which is a measure of LDH activity.

- *Acetylcholine esterase (AchE)*

This is an important enzyme of the nervous system, catalysing the hydrolysis of acetylcholine which is responsible for neurotransmission and it operates from nerve endings to effector cells or from a nerve ending to a second nerve cell. AchE splits the susceptible ester linkage between choline and the acetyl group to form acetic acid and choline, thereby preventing acetylcholine accumulation in tissues.

$$\text{Acetylcholine} + \text{H}_2\text{O} \xrightarrow{E_n} \text{Acetic acid} + \text{Choline}$$
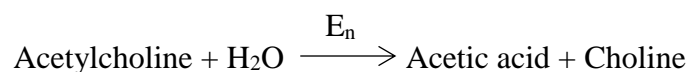
The activity of AchE was determined from the brain homogenates according to the method of Hestrin. This method is based on the reaction of unreacted acetylcholine with

alkaline hydroxylamine. The acetylhydroxamic acid thus formed gives a purple color with ferric ions in acid solution which can be read at 540nm.

APPENDIX B: Top level EC numbers and EC numbers of the enzymes monitored in the current study

Group	Enzyme examples	Enzymes levels monitored in this study	EC number
EC 1 Oxidoreductases	Dehydrogenase, Oxidase	Lactate dehydrogenase (LDH)	EC 1.1.1.27
EC 2 Transferases	Transaminase, Kinase	Aspartate aminotransferase (AST/ GOT)	EC 2.6.1.1
		Alanine aminotransferase (ALT/ GPT)	EC 2.6.1.2
EC 3 Hydrolases	Lipase, Amylase, Peptidase	Acetylcholine esterase (AchE)	EC 3.1.1.7
		Alkaline phosphatase (ALP)	EC 3.1.3.1
		Acid phosphatase (ACP)	EC 3.1.3.2
EC 4 Lyases	Decarboxylase	-	-
EC 5 Isomerases	Isomerase, Mutase	-	-
EC 6 Ligases	Synthetase	-	-

APPENDIX C: Activity and mortality of target fish for different concentrations of AMX

Concentration (mg/L)	Activity of target fish	Exposure (hrs)	Mortality (x/6)
FINGERLINGS			
2	-	96	-
5	-	96	-
8	-	96	1
10	Decreased movement, sluggish behavior	96	2
11	Abnormal movement patterns, breathing problems	72	3
13	Frantic schooling and swimming only at the bottom of the tank	48	5
15	Uncoordinated and irregular movements	36	6
JUVENILE			
10	-	96	1
15	Decreased movement, sluggish behavior	96	2
25	Abnormal movement patterns, breathing problems	84	3
35	Frantic schooling and swimming only at the bottom of the tank	72	5
37	Uncoordinated and irregular movements	48	6
ADULT			
25	Decreased movement, sluggish behavior	96	1
40	Abnormal movement patterns, breathing problems	72	3
70	Frantic schooling and swimming only at the bottom of the tank	48	5
80	Uncoordinated and irregular movements	48	6

APPENDIX D: Activity and mortality of target fish for different concentrations of PCM

Concentration (mg/L)	Activity of target fish	Exposure (hrs)	Mortality (x/6)
	FINGERLINGS		
10	**Mouth, gills (on the inside), stomach (outer side) reddened**	96	-
20	Decreased movement, restricted to the bottom of the tank	96	-
21	Decreased movement, sluggish behavior	96	1
25	Reduced food intake, sluggish behavior	72	3
30	**Non-functional gills, abnormal breathing patterns**	72	4
40	Stiffness of entire body	24	6
	JUVENILE		
20	**Mouth, gills (on the inside), stomach (outer side) reddened**	96	-
25	Decreased movement, sluggish behavior	96	1
40	Reduced food intake, sluggish behavior	84	3
48	**Non-functional gills, abnormal breathing patterns**	24	5
50	Stiffness of entire body	24	6
	ADULT		
30	**Mouth, gills (on the inside), stomach (outer side) reddened**	96	-
35	Decreased movement, reduced food intake	48	1
45	**Non-functional gills, abnormal breathing patterns**	24	3
50	Stiffness of entire body	24	6

APPENDIX E: Activity and mortality of target fish for different concentrations of DCF

Concentration (mg/L)	Activity of target fish	Exposure (days)	Mortality (x/6)
FINGERLINGS			
2	-	4	-
5	Decreased mobility, **Change in muscle color, Slow & steady progress to death**	2	1
8	Abnormal breathing patterns, **Slow & steady progress to death**	10	3
10	Irregular movement patterns, **Slow & steady progress to death**	14	4
14	Stiffness of entire body, Slow & steady progress to death	16	6
JUVENILE			
5	Decreased mobility, **Change in muscle color, Slow & steady progress to death**	4	1
10	Abnormal breathing patterns, **Slow & steady progress to death**	8	3
15	Irregular movement patterns, **Slow & steady progress to death**	10	5
17	**Stiffness of entire body, Slow & steady progress to death**	14	6
ADULT			
10	Decreased mobility, **Change in muscle color, Slow & steady progress to death**	6	1
14	Abnormal breathing patterns, **Slow & steady progress to death**	9	2
15	Irregular movement patterns, **Slow & steady progress to death**	11	3
18	**Stiffness of entire body, Slow & steady progress to death**	13	6

LIST OF PUBLICATIONS

International Journals:

- **Rekha Rao**, Basavaraju Manu and Arun Kumar Thalla, (2017). "Behavioral, Physical and Biochemical Responses of Cyprinus Carpio for Paracetamol Exposure". International Journal of Emerging Research in Management &Technology, 6(2), 215-219, ISSN: 2278-9359.

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- **Rekha Rao**, Basavaraju Manu and Arun Kumar Thalla, (2017). "Behavioral, Physical and Biochemical Responses of Cyprinus Carpio for Diclofenac Exposure" has been published in the conference proceedings with ISBN 13:978-81-930222-3-8 and recommended for International Journal of Renewable Energy and Environmental Engineering.

- **Rekha Rao**, Basavaraju Manu and Arun Kumar Thalla, (2017). "Behavioral, Physical and Biochemical Responses Induced by Paracetamol and Amoxicillin Exposure from Cyprinus carpio" has been accepted for publishing in International Journal on Advanced Science, Engineering and Information Technology (IJASEIT), ISSN: 2088-5334, e-ISSN: 2460-6952.

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- **R. Rao,** B. Manu, C.V. Rao, (2013). "Toxicity Study of Amoxicillin on *Cyprinus carpio*". Environmental Health 2013, 3rd-6th March 2013 at Boston, USA.

- **Rekha Rao**, Basavaraju Manu and Arun Kumar Thalla, (2017). "Behavioral, Physical and Biochemical Responses of *Cyprinus carpio* for Paracetamol Exposure". International Conference on Emerging Trends in Engineering (ICETE) – 2017, 29th January 2017 at Bengaluru.

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Publications:

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Educational Qualification

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M.Tech (Environmental Engineering)	NITK, SURATHKAL	2012	84.40

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