CHAPTER-II
MATERIALS AND METHODS

2.1 Selection of Biological Species:

The Indian major carp *Cyprinus carpio* (Linnaeus, 1758) is an important palatable or eatable fish with great profitable worth and this fish, *Cyprinus carpio* is one of the most mutable fresh water fish species in the world and concerning to the shape of body of this fish. The body of the fish will be inherent inborn forms and enhanced strains may differ from elongated to subterranean elliptical in nature. And this Common carp is occurring plentifully in the freshwater reservoirs, ponds, tanks and rivers in and around Tumakuru, Karnataka state, India. And this species easily available throughout the globe and it is largely employed for pond culture which existed national and international hatchery centers throughout the country. It plays a very important leading role in composite fish culture throughout the world. Also its wide availability and commercial importance for import and export levels and this fish is also known for its adaptability to laboratory conditions for GLP-Good Laboratory Practices conditions and it is very suitability to evaluate concentrations in toxicity studies. Therefore, this fish was selected as the experimental biological model for the investigation or problem of research.

![Cyprinus carpio](image)

**Fig. 1** *Cyprinus carpio* (Linnaeus, 1758)
2.2 Classification: Systematic position of *Cyprinus carpio*:

**Taxonomic name:** *Cyprinus carpio* (Linnaeus, 1758)

**Common names:** Carp, Common carp, Leather carp (English)

- Kingdom: Animalia
- Phylum: Chordata
- Sub-Phylum: Vertebrata
- Division: Gnathostomata
- Super Class: Pisces
- Class: Osteichthyes
- Sub class: Actinopterygii
- Super order: Teleostei
- Order: Cypriniformes
- Family: Cyprinidae
- Genus: Cyprinus
- Species: carpio

2.3 Biology of *Cyprinus carpio*:

Today in this present world aquatic species, this fish is commonly called as Common carp which belongs to the class of bony fishes (*Osteichthyes*) the order of *Cypriniformes* and it belong to the family of *Cyprinidae*. In everyday life sport fishers, artisanal and commercial use the short scientific name of Common carp (*Cyprinus carpio*) and also named as leather carp in English in different continent of the world. The outline index is the fraction of the body shows length and height of the fish, the head index is the proportion of body length of the fish and the length of the head, but the width index is the fraction of the height and width of body of the fish. The colour differs from greyish through silver to bronze with yellowish or reddish colour of the abdomen of the fish. This Common carp has one long dorsal fin which consists of 2–3 hard and 17–22 spineless rays. In this type of carp the first largest hard ray is sharp and backward saw-toothed in condition. The morphological characteristic features of that carp are that there are 2–3 and 5–6 anal spines and rays and 36–37 vertebrae are present (Froese and Pauly 2008). The mouth of the fish is
large which opens accordion-like. It is having pairs of barbels of Common carp. And also having one pair is on the upper lips and the other pair is at the corner of the mouth. And there are 5–5 molar-like pharyngeal teeth serving for grinding the consumed food and feed materials. This fish Common carp, *Cyprinus carpio* is more energetic in feeding when the water temperature is over 18–20°C in nature condition. However, this Common carp tolerates high water temperature (around 28–30°C) so this fish was bottom feeder mostly and the optimum temperature of growing is between 20 and 25°C. During those time periods when the water temperature is optimum in condition than about 15–16°C nourishing of Common carp becomes less and less intensive in nature. This type feeding nature practically which stops if the water temperature drops under about 8°C. When the water temperature is under about 5°C Common carp hides in groups in the benthic zone water levels. Almost it depends length of the year when the water temperature is ended 18–20°C it depends on the climatic condition under which the carp is created in this nature. Consequently, the actual total length of table fish production varies between 1 and 3 years of time and finally which shows in the Figure. 1 & 2 the fingerlings Common carp, *Cyprinus carpio*.

The growth potential of common carp is massive in this part of the world. If the water temperature is suitable it’s many forms and strains can attain the individual weight of about 0.2–0.3 kg, 1–1.2 kg and 2.5–3.5 kg within about 2–3, 5–7 and 10–14 months respectively. Still, the economically feasible, individual weights of the different age groups of common carp are about 0.025–0.05 kg/fingerling, 0.25–0.5 kg/grower and 1.2–1.8 kg/table fish.

### 2.4 Carp Procurement and Their Maintenance:

Biological species fish, *Cyprinus carpio* Common carp in which heavy consumption of human being life everyday throughout the world. The biological test system *Cyprinus carpio* weighing about 5 ± 2 g and it measuring an average length of 4-5 cm, and this fish were collected from the State Fisheries Department, Government of Karnataka, Tumakuru, Karnataka, India and these fishes were kept in a large tank/aquarium. In this experiment the fishes were acclimatized to research laboratory conditions for 1 to 15 days or two weeks of time period in tap water and the physico-chemical characters of water were analyzed by the following
international guideline method (APHA, 2005). In this experiment water media was renewed frequently every day by day and it has maintained a 12 hrs day time and 12hrs nite time, photoperiod (light and dark) during acclimatization period. And these fish species were fed with commercial available fish pellets (40% protein content) which we are collected / procured from the local marketplace (area) and keep these fishes in healthy condition throughout the experimental period. The aquarium was cleaned periodically to avoid infection to fish and 1% potassium permanganate solution was sprayed to eradicate any bacterial or fungal infection around aquarium. So, finally in this current study the optimum temperature of water in the aquarium was around about 22 ± 3°C were maintained throughout the course of investigation.

![Fig. 2 Fishes Acclimatization in Aquarium Cyprinus carpio (Linnaeus, 1758)](image-url)
**Methods/Techniques Followed During Study Periods**

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2.4.1 Aspects Influencing Heavy Metal Toxicity:

The following factors which are likely to contribute variations in toxicological investigations were approximately eradicated to a satisfactory level.

2.4.2 Flow of Water Media:

Since, significant difference in the toxicity of heavy metals between static and flowing water in fish was reported by Burke and Ferguson, (1969), the tests of the present investigation were conducted in static-renewal water media, as recommended by Doudoroff et al., (1951).

2.4.3 Temperature:

It was reported that heavy metal toxicity increases with a rise in temperature of the medium (Macek et al., 1969) Therefore, throughout the present investigation the optimum temperature of the water media was maintained regularly at 29 ± 1°C.

2.4.4 Density/Mass of the Fish:

Since increase in fish mass/density was known to augment the toxicity of the heavy metals (Holden, 1970) a constant ratio of fish bio-mass to water volume was maintained by taking one fish per two liter of water media.

2.4.5 Water Media Quality:

Since it is an established fact that chemistry of water influences the toxicity of chemical (Pickering and Henderson, 1966) Care was taken to maintain uniformity of water quality used the quality of water was determined by following APHA, 2005.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>22 ± 3 °C</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.189 mg/l</td>
</tr>
<tr>
<td>pH</td>
<td>8 ± 0.2 at 26 3 °C</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>8.7 to 9.2 mg/l</td>
</tr>
<tr>
<td>Chlorinity</td>
<td>46.3 mg/l</td>
</tr>
<tr>
<td>Sodium</td>
<td>1.22 mM/l</td>
</tr>
<tr>
<td>Potassium</td>
<td>30.5 mM/l</td>
</tr>
</tbody>
</table>
Calcium : 17.04 mg/l
Magnesium : 1 mg/l
Carbon dioxide : 19.66 mg/l
Specific gravity : 1.00374
Hardness of water : 115 mg/l

2.4.6 Toxic Compound Selected:

In the present times, the huge amount of Cadmium chloride is being discharged into the freshwater media and the environment due to the activities of highly industrialized societies. The Studies on the toxicity of this nonessential element stated its harmful nature for the productivity of freshwater fishes, which serve as staple food for human beings. However, most of them are confined to adult fishes but comparative studies on the effect of Cadmium chloride on different physiological, biochemical and histological aspects in selected fish fingerlings and these fishes were exposed to different concentrations levels are very limited. This wide lacuna in the field of Cadmium chloride toxicity on freshwater fishes inflamed to take up this investigation. Since, *Cyprinus carpio* freshwater fish is sufficiently available in the rivers, tanks and ponds in and around Tumakuru district. Therefore the current investigation is carried on this species.

2.4.7 Procurement of Cadmium chloride:

In this globe Cadmium is available in various salts forms like Cadmium chloride, Cadmium Sulphate, Cadmium Nitrate, Cadmium Acetate etc. Commercially available grade transition/heavy metal (CdCl₂) this compound were procured from the local market of Bangalore, Karnataka, India, under the trade name Thermo Fisher Scientific India Pvt. Ltd., this was supplied by Vasa Scientific Co., Bangalore, Karnataka, India. The expiry date of the test compound plaid previous to start of the treatment and so, this was found appropriate to the species exposure. And the necessary required quantity of Cadmium chloride was pinched directly from the stock and then prepared concentrated stock solution by using variable using micropipette. And these heavy metals which are more or less soluble in water media. The degree of solubility of each salt depends on the hardness of water. For instance Cadmium chloride is highly soluble in the laboratory water media (tap water) where
the entire investigation is carried out today. A pure salt of Cadmium chloride with molecular weight 183.32g/mol is used in this investigation.

**CADMIUM CHLORIDE**

Physical or Chemical Properties of Cadmium chloride:

- **Molecular Formula:** Cd-Cl₂
- **Molecular Weight:** 183.32
- **Colour/Form:** Rhombohedra crystals, Small, white crystals, Hexagonal, colourless crystals
- **Odour:** Odourless
- **Boiling Point:** 960 deg C
- **Melting Point:** 568 deg C
- **Density/Specific Gravity:** 4.08 g/cu cm
- **Heat of Vaporization:** 124.3 kJ/mol at 964 deg C
- **Solubilities:** Soluble in acetone; practically insoluble in ether, Slightly soluble in ethanol, In water, 120 g/100 g water at 25 deg C
2.5 METHODS

2.5.1 Evaluation of Cadmium chloride Toxicity:

In this present study the percent mortality of the fish will be shown in different concentrations of Cadmium chloride which was determined at 96 h exposure time period. And the fishes were divided into batches for each replicates ten fishes for twenty liters of water based on the density of the fish and these fishes were acclimatized in this experiment and exposed to different absorptions of Cadmium chloride. These ranges of experiment were found on pilot and error basis mode. And toxicity evaluation was carried out in static renewal of water and in all the concentration of Cadmium chloride the mortality rate was observed and recorded after 96 h. This group of fishes was maintained alongside in freshwater medium without adding Cadmium chloride assisted as control the experiment was repeated thrice for accuracy. Finally, in this study the mean values were derived by following the method of Finney Probit Analysis and Kill Theory (1971). So, the data was subjected to following statistical equations for arriving at 96 h LC50 values were found.

I. A graph was drawn between percent mortality and log concentrations of the heavy metal and the sigmoid curve was obtained.

II. A graph was drawn between probit mortality and log concentration of the heavy metal and a linear curve was obtained.

III. To know the validity of data it was subjected to Dragstedt and Behren’s equation (Carpenter, 1975) and is given below,

\[
\log_{10} \text{LC}_{50} = \log A + \frac{50 - a}{b - a} \times \log \frac{B}{A}
\]

Where,

\( A = \) concentration of heavy metal, whose concentration is just below 50% mortality.

\( B = \) concentration of heavy metal, whose concentration is just above 50% mortality.

\( a = \) percent mortality observed immediately below 50% mortality.

\( b = \) percent mortality observed immediately above 50% mortality.

The mean LC50 value was calculated from the values obtained from the above three methods namely percent, probit mortality and Dragstedt and Behren’s method.
2.5.2 Fixation of Lethal and Sub-lethal Concentrations:

In this experiment effect of a metal on fish becomes steady within 96 hr of exposure (Eisler, et al., 1977). The LC₅₀/96 hr of Cadmium chloride was taken as lethal concentration (14 mg/L), to evaluate the behavioral, physiological, morphological, biochemical, hematological and histological responses of fish Common carp. However knowledge on the concentration of the toxicant that kills 50% of the test animals in a fixed period of time could become insufficient to assess various responses of the animal to the toxicant (Hoppenheit, 1977). Further, studies on acute toxicity have significant limitation such as the incidence of adaptation of test animals to the enforced toxicity studies (Stockner and Anita, 1976). Hence, Perkin 1979 also reviewed the need for sub-lethal studies become distant changes involving an arrangement of trials in the responses of the test species could occur in sub-lethal concentration. So about one fifth of the 96 hr LC₅₀ i.e., 2.8 mg/L was taken as sub-lethal concentrations for further studies.

2.5.3 Fixation of Exposure Periods:

In this present study since the duration of exposure is having a great influence on toxicity of a metal in organism (Radhakrishnaiah and Busappa, 1986). A lethal and sub-lethal concentration of the Cadmium chloride was shows the effects on fishes this was studied at different exposure time periods and in order to comprehend the inspiration of period over an evaluating toxicity. In this experiment the lethal absorption exposure period will be day 1, day 2, day 3 and day 4 and in the sub-lethal concentration exposure period will be day 1, day 5, day 10 and day 15 were selected to detect the short-term and long-term acute and chronic effects of Cadmium chloride on the fish, Cyprinus carpio, respectively.

2.5.4 Experimental Design:

In this present investigation Six groups of ten fishes were arranged and each fish were exposed to lethal concentrations of Cadmium chloride at a concentration level will be (9 mg/L, 11 mg/L, 14 mg/L, 18 mg/L & 22 mg/L) was prepared and exposed to ten fish per concentration along with 20 Liter of tap water for each concentration with control replicates and (LC₅₀) of Cadmium chloride was found to be 14 mg/L. Another two group of ten fishes each fish were exposed to sub-lethal
concentration of Cadmium chloride including ten fish was taken as control group of (1/5th of the LC\textsubscript{50} i.e., 2.8 mg/L).

In this present study selection of the gill, kidney, and liver in fish \textit{Cyprinus carpio} was to understand the difference in the effects of Cadmium chloride in different tissues. Prior to each experiment, fishes were exposed to their specific lethal and sub-lethal concentration of Cadmium chloride and were maintained in these concentrations up to the specified period of exposure. At the end of exposure time period the fishes were staggered to death and the target organs were dismembered out from each animal using sterilized instrument. The organs were weighed on an electrical semi-microbalance and transferred into ice-jacketed micro beakers containing fish ringer solution. The fish ringer existed prepared as per the arrangement given by Ekenberg, (1958). An equilibration time of 15 min was allotted to the organs to regain normalcy from a state of shock, if any, due to the handling and dissecting procedures.

2.5.5 Behavioural Studies:

The Behavioural changes on exposure to Cadmium chloride was observed both in control and treated fish (Lethal and sub-lethal) as described by Murty, 1987 as modified by David, 1995. In the laboratory fish \textit{Cyprinus carpio} shows the behavior activity of the fish it can be a delicate/sensitive biomarker of toxic compound which induced stress factors in this type of conditions of the aquatic living organisms. Therefore, an endeavor of the experiment which has been made to study the behavioral changes of the fish, physical, morphological and histological aspects in Cadmium chloride exposed fish species.

2.5.6 Oxygen Consumption by the Whole Animal:

In this present investigation the rate of oxygen consumption by the whole animal was projected to adopting Winkler’s iodometric method which as designated by Welsh and Smith in (1953) and the devices/instruments setup was made and the same as described by Saroja in (1959). The differences shown in the oxygen content of the initial and final samples were taken as the amount of oxygen consumed by the fish during the exposure period of experimentation. The oxygen consumed by the whole animal of the fish was normal and Cadmium chloride exposed fish was
determined. After the experimentation of exposure period the fishes were individually weighed and their unit metabolism/absorption was calculated and expressed as ml of oxygen consumed/g wet wt./h.

2.5.7 Ionic Composition and Associated ATPase’s:

In this present investigation the activities of Na\(^+-\)K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) ATPases were estimated in the fish organs like: gill, liver and kidney of fishes under this study and levels of sodium ions, potassium ions and calcium ions were studied on fish species.

2.5.8 Estimation of Sodium ions, Potassium ions and Calcium ions:

In this current research work organs of the exposed fish were exploited and the balanced tissues/organs were kept in wet ashed in 50:50 (V/V) concentrated perchloric and nitric acids (Dall, 1967). After some time keeping for half an hour, in the wet ash solutions the organs were totally dissolved until to get result, in the container and they were vaporized at 100 °C to 200 °C temperature. The extracted residues were dissolved in glass vessel with distilled water and made up to 10 ml. and this residue was filtered through whatman No.1 filter paper. Further, the suitable dilutions were made prior to approximations and, the sodium ions, potassium ions and calcium ions were projected with the help of flame photometer (Elico Pvt. Ltd., Model CL-22A). The Standard solutions of sodium ions, potassium ions and calcium ions were prepared by using analar grade chemicals. The values are expressed as \(\mu\)M/g wet wt. of the organ.

2.5.9 Na\(^+\)-K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) ATPase activities (ATPase Phosphorylase e.c. 3.6.1.3.):

Na\(^+\) - K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) ATPase activities were projected individually in the organs by using the method described by Watson and Beamish, (1981) and with slight modification in the entire study. Took 1% tissue homogenate (W/V) were prepared in ice-cold 0.25 M sucrose solution it containing 5 mM EDTA (Prepared in 40 mM tris-buffer at pH 7.5) and 0.1 M imidazole solution were taken. Prepare the homogenates were centrifuged at 2500 rpm for 10 min of time period and the
bottomed settled supernatants were taken as crude enzyme extract for the assay of the ATPase enzyme activities.

In this subsequently owing some standardization of enzyme kinetic parameters, there was a three sets of incubation blends were prepared in laboratory condition. In a total volume of 2 ml, the first set consisted of 100 mM disodium adenosine triphosphate (prepared in 20 mM tris-HCl buffer at pH 7.5), 100 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$ and 0.3 ml of enzyme extract. The second set consisted of 100 mM disodium ATP (prepared 2 mM tris-HCl buffer at pH 7.5), 100 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$, 1 mM ouabain (potent inhibitor of Na$^+\text{-K}^+$ ATPase) and 0.3 ml of enzyme extract and the third set consisted of 100 mM disodium ATP (prepared in 20 mM tris-HCl buffer at pH 7.8), 5 mM Cad and 0.3 ml of enzyme extract. All the three incubation sets were incubated at 37 ºC for completion of experiment upto exactly 15 mins and then the reaction was detained by adding 2 ml of cold 10% TCA buffer. In this method the inorganic phosphates liberated were estimated by Fiske and Subba Rao, (1925). Finally the absorbance reading was measured at 660 nm. And the standard blanks were prepared to find out the endogenous inorganic phosphates activities. Another blank was prepared without using the co-factor to deduct the sodium salt stimulated activity as the co-factor used was a disodium salt of ATP.

In this the first set gave the total ATPase activities of Na$^+\text{-K}^+$ and Mg$^{2+}$, whereas the second set gave only the Mg$^{2+}$ ATPase activity as ouabain prevents Na$^+\text{-K}^+$ stimulated to ATPase activities. Therefore, the Na$^+\text{-K}^+$ activity was derived by subtracting the Mg$^{2+}$ ATPase from total and of Na$^+\text{-K}^+$ and Mg$^{2+}$ ATPase activities. The third set directly gave the Ca$^{2+}$ ATPase activity. All these three ATPase activities are expressed as µM Pi liberated/mg protein/h.

### 2.5.10 Bioaccumulation:

The present investigation shows that the Cadmium chloride accumulated in the organs of fish, *Cyprinus carpio* was estimated separately with (AAS) atomic absorption spectrophotometry (Varian Techtron model 1000). Prior to estimation, the organs were digested by wet digestion method as described by Humpries (1956). To begin with, the organs of the fish were reserved in a hot air oven at 85ºC for 24 hours to remove water. Then each organ was transferred into 300 ml Kjeldhal flask,
and 4.0 ml of perchloric acid, sufficient nitric acid (about 7.0 ml per every gram material) and 5.0 ml of concentrated sulphuric acid were added to ensure complete oxidation of organic matter. After the addition of three acids the contents were mixed gently at low heat for 3-5 minutes until the first appearance of thick brown fumes. Then the flask was removed from the heater for 5 minutes to subside the reaction. The flask was replaced on the heater and digestions continued, slowly allow to heat until the arrival of thick white fumes of sulphuric acid. The digestion was continued for 5 to 10 minutes, 1 to 2 minutes at increased temperature, after the arrival of thick white fumes. Then the digestate was cooled and the colorless liquid was neutralized by the addition of 20% sodium hydroxide. In this the entire digestate was dilute to 50 ml with double distilled water. The diluted digestate was fed directly into the atomic absorption spectrophotometer and Cadmium chloride content was measured at a wavelength of 198.15 nm by using Cadmium cathode lamp. Cadmium standards were also prepared and fed similarly. The amount of Cadmium chloride present in the organs of the fish is expressed as µg/g wet wt. of the tissue.

2.5.11 Some Aspects of Carbohydrate Metabolism:

2.5.11.1 Estimation of Glucose:

In this present experiment the glucose in the samples was determined by colorimetric technique as which as defined by Nelson and Somogyi (1952). 0.1 ml of blood was collected, to it 3.9 ml of deproteinizing solution prepared and (5% zinc sulphate and 0.3 N sodium hydroxide in 1:1 ratio form) and this were added to this and the mixture was centrifuged at round 3000 rpm for 10 min and to extract 1 ml of the supernatant solution from each of these mixtures and add 1 ml of alkaline copper reagent to this and this was shaken vigorously and heated in a boiling water bath exactly for up to 20 min. Then it was chilled and 1 ml of arsenomolybdate colour reagent was added to this solution. Finally entire solution was made up to 10 ml with distilled water and the absorbance was measured in a spectrophotometer at a wavelength of 540 nm. A blank and glucose standards were also run simultaneously. The glucose level content was uttered as mg of glucose/100ml of blood.
2.5.11.2  Estimation of Glycogen:

The present study shows that the glycogen content in the tissues of the fish was projected by using the anthrone reagent technique as described by Caroll et al., (1956). Since glycogen concentration in tissues is known to vary in different regions of body (Amano et al., 1953; Fraser et al., 1966). Hence, care was taken in dissecting out this sample from the same region of body of fishes i.e. the anterodorsolateral region of the trunk. The organs were digested with 3 ml of hot 30% potassium hydroxide (Hassid and Abraham, 1957). The digest was cooled and 3.75 ml of unconditional/absolute ethanol was added to it. The entire mixture were kept overnight in a refrigerator. Then the mixture was centrifuged for 15 min of time period at around 2500 rpm. Supernatant solution was transferred to it and 10 ml of warm purified/distilled water was added to the residue to dissolve the precipitated glycogen. And it has been added 0.2 ml of this 1.8 ml of distilled water and 0.5 ml of 2% anthrone reagent dissolved in 72% concentrated sulphuric acid were added to this and heated in a boiling water bath exactly for 10 min of time. The mixture was cooled and colour developed was measured and the optical density of the spectrophotometer at a wavelength of 620 nm. The standard blank was run and glucose standards were also run similarly. Finally the glycogen content is expressed as mg of glycogen/g wet wt. of the organ.

2.5.11.3  Estimation of Glycogen Phosphorylase, (1-4-Glucanorthophosphate Glucosyl Transferase, e.c. 2.4.1.1) activity in the fish:

Phosphorylase activity in the tissues of the fish was projected using; the method described by Sutherland (1955). Homogenates (5%) was prepared in 0.1 M sodium fluoride solution (pH 6.5) and the homogenate was centrifuged at around 1500 rpm for 15 min time period, thus extracting the enzyme into the supernatant. The supernatant was diluted four times with the cold sodium fluoride solution. The incubation mixture consisted of 0.2 ml of 2% glycogen and 4 ml of diluted enzyme added and this mixture was incubated at 37 °C and the response was started by adding 0.2 ml of 0.016 M glucose-6-phosphates and 0.004 M adenosine-5-monophosphate (1:1 ratio). The response was detained after 15 min by adding 5 ml of 10% trichloroacetic acid. A blank was also run similarly. Finally, the inorganic phosphates liberated were projected by the technique which shown off by Fiske and
Subba Rao (1925). For this, to an aliquot of the above reaction mixture 4.5 ml of 0.44% ammonium molybdate and 0.2 ml of 1-amino-2-naphthol-4-sulphonic acid (ANSA) were added. The contents were mixed well and heated in boiling water for 10 min. After cooling the volume was made to 10 ml with distilled water and the colour developed was measured in a spectrophotometer at a wavelength of 660 nm and the activity expressed as µM Pi liberated/mg protein/h, using phosphate standards.

2.5.11.4 Estimation of Glucose-6-Phosphatase (e.c. 3.1.3.9) activity:

Glucose-6-phosphatase activity in the tissues of the fish which was projected by using the technique of Young et al., (1970). A 5% homogenate (w/v) was prepared in and added 0.25 M ice-cold sucrose solution. 0.5 ml of 0.2 M tris-malate buffer, 0.2 ml of 0.05 M glucose-6-phosphate and 0.2 ml of distilled water were incubated at 37 °C for 5 min and to this 0.1 ml of homogenate was added and incubated exactly for 10 min of time. This reaction was stopped by adding the 1.0 ml of 10% trichloroacetic acid. A blank was also run similarly. Finally the inorganic phosphates liberated were projected the technique of Fiske and Subba Rao (1925) at a wavelength of 660 nm and the activity is expressed as µM Pi liberated/mg protein/h.

2.5.11.5 Estimation of Pyruvate:

Pyruvate in the organs of fish was projected using the technique of Friedman and Haugen (1942). A 5% homogenates (w/v) were prepared in 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 min of time. The supernatant was used for the approximation/estimating of pyruvate. And 1 ml of supernatant was taken and to it 1.0 ml 0.001 M 2, 4-dinitrophenyl hydrazine and 3 ml of 0.4 N sodium hydroxide were added. After 10 min the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 540 nm against the reagent blanks. Pyruvate standard values were prepared alongside for evaluating the results. The pyruvate content in the organs is expressed as mg pyruvate/g wet wt of the organ.
2.5.11.6 **Estimation of Lactate:**

Lactate in the organs was projected using the technique of Barker and Summerson, (1941) as modified by Huckabee, (1961). A 5% homogenate (w/v) was prepared in cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 min of time period. The supernatant was used for the estimation of lactate. To 1.0 ml of supernatant and 1.0 ml of 20% copper sulphate was added and the mixture was made to 10 ml with distilled water continuously. Then 1 g of powdered calcium hydroxide compound was added to this and shaken vigorously and kept it for 1 hour at room temperature with intermittent shaking. And later the contents were centrifuged at 3000 rpm for 10 min and to add 1.0 ml of the supernatant, 0.5 ml of 4% copper sulphate was added and followed by 6.0 ml of concentrated sulphuric acid. The substances were mixed by lateral shaking, kept it in a boiling water bath for exactly 6.5 min and cooled. When the contents were adequately cooled and to add 0.1 ml of 1.5% p-hydrophenyl (prepared in 5% of sodium hydroxide) which was added and the precipitate in nature was formed which was kept at laboratory temperature for 30 min of time and then the contents were placed in boiling water bath for 1.5 min, cooled and take the measurement of the optical density of the colour developed was in a spectrophotometer at a wavelength of 560 nm against reagent blank. Lactate standards were prepared alongside for comparison. The lactate content is expressed as mg lactate/g wet wt. of the organ.

2.5.11.7 **Estimation of Succinate Dehydrogenase (Succinate acceptor oxido reductase, e.c. 1.3.99.1) activity (SDH):**

The succinate dehydrogenase activity in the organs was projected using the colorimetric technique of Nachalas et al., (1960). A 5% homogenate (w/v) was prepared in 0.25 M ice cold sucrose solution, centrifuged at 3000 rpm for 10 min and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 0.2 ml of 0.4 M phosphate buffer (pH, 7.7), 0.2 ml of 0.2 M sodium succinate, 0.1 ml of 0.004 M 2-(p-indophenol)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT), 0.1 ml of 0.005 M phenazine methosulphate and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37 °C for 30 min of time and the reaction was immobile by adding 6.0 ml of glacial acetic acid. The formazon formed was extracted into 6.0 ml of toluene overnight at 50 °C and the optical density of the colour developed which was measured in a spectrophotometer at a wavelength of
495 nm. A blank was taking and added 0.5 ml of distilled water and control taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as µM formazon/mg protein/h.

2.5.11.8 **Estimation of Lactate Dehydrogenase (1-lactate nad oxido reductase e.c. 1.1.27) activity (LDH):**

Lactate Dehydrogenase activity in the organs which was measured using the technique of by srikanthan and krishnamoorthi (1955) as modified by govindappa and Swami (1965). A 5% homogenate (w/v) was prepared in 0.25 M ice cold sucrose solution added and centrifuged at 2500 rpm for 15 min of time and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 1.0 ml of 0.4 M phosphate buffer (pH 7.4), 0.5 ml of M lithium lactate, 0.1 ml of 0.0001 M. nicotinamide adenine dinucleotide (NAD), and 0.1 ml of 0.004 M 2-(p-indophenol)-3-p-nilrophenyl-5-phenyltetrazotium chloride (INT) and 0.5 ml of 5% enzymes preparation was prepared and the mixture was incubated at 37 °C for 30 min and then the reaction was stopped by adding 6.0 ml of glacial acetic acid to the experiment. The formazon formed was extracted into 6 ml of toluene overnight at 50 °C. The optical density colour was developed and measured in a spectrophotometer at a wavelength of 495 nm, a blank taking 0.5 ml of distilled water added and control by taking 0.5 ml of boiled enzyme were also run similarly at a same time. INT standards were prepared alongside for comparison. The enzyme activity is expressed as µM formazon/mg protein/h.
2.6 Haematology

2.6.1 Collection of Blood:

Blood was collected by the method of Steuke and Schoettgr, (1970) by severing the caudal peduncle. Before considering the blood for hematological observation, a drop was placed on a clean slide and examined for the presence of parasites if any.

2.6.2 Sampling of Blood:

Fish were euthanized by an overdose of MS-222 (Tricaine Methanesulfonate) and then weighed and measured. Blood was sampled by caudal severance from the disease free test species during early hours of the day and stabilized with 50 IU sodium heparin (anticoagulant) per 1 ml blood. Blood free from any infection was used to study the hematological parameters to avoid variation; the samples were taken at a particular time during early hours of the day. Ethylene diamine tetra acetic acid (EDTA) was used as an anticoagulant in this study.

2.6.3 Haematological examination:

In this present investigation the haematological blood parameters was adjustable analyzed were red blood cells count (RBCC), haemoglobin (Hb), white blood cells count (WBCC), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were studied in this current experiment.

2.6.4 Determination of Red Blood Corpuscles count (RBCC):

Blood is an important source of every living organisms and RBC count was determined with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969). This blood was collected in a vial rinsed with 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant which was used. The blood was sucked up to 0.5 marks on the RBC pipette and immediately RBC diluting fluid (Hayem’s fluid) was drawn up to 101 mark and the pipette was rotated between the thumb and the forefinger to facilitate adequate mixing of the solution (dilution 1:200).
The blood counting chamber and the cover cut-glass were cleaned thoroughly and the cover glass was placed in position over the ruled area based on the size. The fluid from the stem of the pipette was expelled as it contains only the diluting fluid. The pipettes out the fluid exact an angle of 45 degrees with the tip of the pipette and at the junction of the edge of the cover glass and the counting chamber. A drop of blood was placed from the tip of the pipette on the central platform and near the edge of the cover slip of the chamber, so that the drop was sucked up between the central platform and the cover slip by the capillary force. The cells were allowed to settle for two or three min.

The main area of the blood counting chamber was focused under the microscope and the numbers of RBCC were counted in 80 small squares (4 squares of 16 at the four corners and one of 16 at the center). The cells touching the upper and left hand lines were counted. The cells touching the lower and the right hand lines were absent.

2.6.5.1 The numbers of RBCC per sq mm were calculated as follows:

The area of a small square: 1/400sq mm

The depth of the counting chamber: 1/10 mm

Therefore the volume of a small square is: 1/400x1/10 = 1/4000 Cumm

The dilution of the blood is: 1/200

Total RBC = n/80 x 4000/1 x 200/1

N = No. of cells counted in 80 small squares

2.6.6 Determination of White Blood Corpuscles count (WBCC):

White Blood Corpuscles count was complete as per the procedure were defined and followed by Donald Hunter and Henrry, (1969). The blood was collected in a sterilized vial containing 2% EDTA as an anticoagulant in this study and the blood was slowly drawn up from the fish and it has to exact 0.5 mark of WBC pipette out and immediately transform in to the diluting fluid was drawn up to the 101 mark above the bulb (the dilution fluid consists of 1.5 ml of glacial acetic acid and 1 ml of aqueous gentian violet solution and this was made up to 100 ml with distilled water); The solution was mixed thoroughly by shaking gently. It was
allowed to stand for 2 to 3 min. The Neubauer counting chamber and cover glass were cleaned and the cover glass was placed over the ruled area. Excess solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 40 degrees. It was allowed to stand for 2 to 3 min and the WBCC were counted in the four corner square millimeters. The numbers of WBCC per cubic millimeter were calculated.

2.6.7 Estimation of Haemoglobin (Hb):

Haemoglobin content in the blood was projected by cyanomethaeglobin technique which as described by Dacie and Lewis, (1961). According to this method, haemoglobin is altered into cyanmethaeglobin by the addition of potassium ferricyanide. The colour of cyanmethaeglobin is recited in a photoelectric colorimeter at 540 nm against a standard solution, since the cyanide toxin has the maximum affinity for haemoglobin. 20 ml of blood was removed with the help of a haemoglobin pipette into a test tube containing 5 ml of Drabkin’s solution. The test tubes were vigorously shaked and readings were taken in a photoelectric colorimeter at 540 nm. The reagent blank is adjusted to zero.

2.6.8 Determination of Packed Cell Volume (PCV) or Haematocrit Value:

Packed cell volume was determined by micro haematocrit technique of Schalm et al., 1975. The heparinised fluid was filled up to the mark 100 of the haematocrit tube with the help of Pasteur pipette and centrifuged at around 3000 rpm for 30 min of time and the relative volume of height of the RBC’s packed at the bottom of the haematocrit tube was recorded as packed cell volume in terms of percentage of total blood column taken in the haematocrit tube.

2.6.9 Determination of Mean Corpuscular Volume (MCV):

Mean corpuscular volume indicates that the normal size of the red blood cells in a given sample of blood. MCV was calculated by the following formula and represented in cubic microns.

\[
MCV = \frac{PCV \times 10}{RBC \text{ count (In millions per Cummm)}}
\]
2.6.10 Determination of Mean Corpuscular Haemoglobin (MCH):

Mean corpuscular haemoglobin (MCH) which represents the average weight of the haemoglobin contained in each red blood cell in a given volume of the blood. Mean corpuscular haemoglobin was influenced by the size of the cells and it has the concentration of the haemoglobin and finally the Mean corpuscular haemoglobin was calculated by the following formula and expressed in pictograms.

\[
\text{MCH} = \frac{\text{Haemoglobin (grams/deciliter)}}{\text{RBC count}}
\]

2.6.11 Mean Corpuscular Haemoglobin Concentration (MCHC):

The Mean corpuscular haemoglobin concentration which reflects the average concentrations of the haemoglobin in the red blood cells in a given volume of the blood (unlike MCH, MCHC is not influenced by the size of the cell) and the Mean corpuscular haemoglobin concentration was obtained by the following formula and expressed in terms of percentage.

\[
\text{MCHC} = \frac{\text{Haemoglobin (grams/deciliter)}}{\text{Packed cell volume}}
\]
2.7 Histopathology:

In this present investigation the histological sections of the kidney and liver of fingerling which were occupied by accepting the procedure as described by Humason (1967) and (1972). The above said that the tissues of fish Common carp were isolated from control and Cadmium chloride treated fishes were gently rinsed and washed with physiological saline solution (0.9% NaCl) which were prepared in the laboratory conditions and this was to remove mucus and other debris adhering to them. After removing the vital organs of the fish they were fixed in Bouin’s fluid exactly (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and 5 ml glacial acetic acid) for 24 hours of time period and this fixative was removed by washing through running tap water for overnight. Then these tissues were processed for dehydration and the ethyl alcohol was used as the dehydrating agent in this experiment, as it is the most suitable and economical further and it shows the hardening effect in tissues. The alcoholic transfer procedures were so arranged as to utilize both dehydration and hardening effect and the tissue processing were passed through successive series containing grades of alcohol 30%, 50%, 70%, 80%, 90% and 95% and absolute alcohols and then processing the tissues were cleared in methylbenzoate solution and the process of embedding in paraffin wax tissues were fixed as per section wise exactly of 5 μm section removed by using a rotatory microtome (Leica RM 2235 Germany) then this sections were stained with hematoxylin stain were used and counter stained with eosin as used then dissolved in 95% alcohol. After dehydration and cleaning process completes the each sections were mounted in Canada balsam solution and finally the prepared sections were examined and photographically enlarged using light microscopy (Hamilton compound photomicroscope) Photomicrography equipment were used in this experiment.
HISTOPATHOLOGY - HISTOTECHNIC FLOW

(I) PREPARATION OF BLOCKS

BOUIN’S FIXATIVE
WASH IN RUNNING WATER (10 Mins to 1 hrs)
TRANSFER 50% ALCOHOL (15 Mins)
70% ALCOHOL (Any length of time up to 3 months)
80% ALCOHOL (20-30 Mins)
90% ALCOHOL (20-30 Mins)
95% ALCOHOL (20-30 Mins)
100% ALCOHOL (3 changes of 15 mins each)

At 3rd stage
(Use glass viles) Abs-ALCOHOL-100%+CHLOROFORM (15 Mins)
CHLOROFORM or BENZENE (30 Mins to 1hrs)—[observe brittleness & colour change from yellow to brownish transparent]
INCUBATE for 30 Sec / 1 mins
Pour half chloroform outside & mix the wax and keep in incubator. Observe homogenous mixture (15-20 Mins)
After mixing keep it for (15-20 Mins) take out and pour the wax outside and place fresh wax & keep in incubator for (15-20 Mins)
It can be stopped at this stage to continue with infiltration, allow it for 3 to 4 hrs (maximum 4 hrs)
Prepare blocks. [Glycerin, brush L-blocks, spirit lamp, forceps & scalpel]

(II) STAINING PROCEDURE

XYLENE-I (15 Mins)
XYLENE-II (15 Mins)
Abs-ALCOHOL-100% (10 Mins)
90% ALCOHOL (10 Mins)
80% ALCOHOL (10 Mins)
70% ALCOHOL (10 Mins)
50% ALCOHOL (10 Mins)
DDW (15 Mins)

HAEMATOXYLIN (5-10 Mins, minimum)

RUNNING TAP WATER (5-10 Mins)
70% ALCOHOL (10 Mins)
80% ALCOHOL (10 Mins)
90% ALCOHOL (10 Mins)
EOSIN-3-dips (1-2 Mins)
90% ALCOHOL (10 Mins)

Abs-ALCOHOL-100%-I (10 Mins) [2 Mins not suitable for fish ovary]
Abs-ALCOHOL-100%–II (10 Mins) [2 Mins not suitable for fish ovary]

XYLENE-I (10 Mins)
XYLENE-II (10 Mins)
MOUNT IN DPX
2.8  **STASTISTICAL DATA ANALYSIS:**

In this present investigation each exposure periods and replicates of the work were frequent 6 stretches and the dictated each value were calculated based on the available results and then the mean value was calculated in every time of the current experiment carried out. This shows that the data were obtained are scrutinized statistically by using or following the original software Graph-pad prism, (DMRT) Duncan’s multiple range test (Duncan, 1955).