CHAPTER-3

EXPERIMENTAL

PROGRAMME
3.1. **Material and method** :-

3.1.1. **Collection of the specimens** :-

    Healthy specimen of *Cyprinus carpio* and *Heteropneustes fossilis* were collected locally from river Yamuna at Kalpi, Distt. Jalaun, Uttar Pradesh and acclimatized in the laboratory condition for 30 days in large plastic pool containing tap water. During acclimatization they were fed with fish food (tokyu) every day for 3 times a day. Water was renewed after every 240 hours with routine cleaning of aquaria, removing faecal matter, dead fish (if any) or unconsumed food.

3.1.1.1. **Morphology of Cyprinus carpio (Linn.)** :-

    The common carp or European carp (*Cyprinus carpio*) is widespread fresh water fish (P-1). Common carp are native to Asia and Europe. It has been introduced into the environments worldwide. It can grow to a maximum length of 5 feet, a maximum weight of over 37.3 Kg. Although they are very tolerant to most of conditions, the common carp prefer large bodies of standing water and soft vegetative sediments. As a schooling fish, they prefer to be in group of 5 or more. They are native in a temperate climate in fresh or brackish water and soft vegetative sediment. The common carp eat a vegetarian diet
of water plants but eventually also take insect, crustaceans or even dead fish.

3.1.1.2 Morphology of *Heteropneustes fossilis* (Bloch):

*Heteropneustes fossilis* (Bloch) is commonly called singhi in Hindi, inhabits the fresh water and swampy pools (P-2). Body is elongated and laterally compressed with a great depressed head, measuring about 30 cm in length and its skin without scale. Barbles are long and four pairs. Pectoral fins are stronge with poison spine and associated venom gland. Assesory breathing organs are present. They have long air sacs that extends from the gill chamber. It is capable of living outside the water for a long time due to having the air breathing organs. They are found in fresh water throughout India. They are predatory in nature but not piscivorous. Adult and juveniles mostly prefer insects, shrimps and organic debris, but larva and young fries are feeding mainly on microcrustaceans. They subsist largely on a diet of mud from the swamp bottom.

3.1.2. **Equipments** :-

1. Fish aquaria 45 x 30 x 25cm.
2. 20 litre pails for transfer and holding fish aquaria.
3. Fish measuring board (Metric).
4. Monopan Balance (Dhona).
5. Stainless steel forceps.
7. Stainless steel scalpal blades.
8. Tubes for histological samples.
9. Tube racks.
12. Thermostat.
13. Microscope (Olympus).
15. Microtome.
16. Aerators (Royal Mark).
17. Atomic absorption spectrophotometer (Spectr AA-20).
18. GTA-96 Graphite Tube Atomizer.
19. pH Meter.

3.1.3. **Chemicals** :-

1. Cadmium chloride (E.Merk).
2. Mercuric chloride (E. Merk).
3. Copper sulphate (E.Merk).
4. Potassium permanganate.
5. Metal free water.
6. Distilled water.
7. Concentrated nitric acid.
8. Concentrated sulfuric acid.
9. Perchloric acid.
11. Potassium persulphate solution.
12. Sodium chloride.
13. Hydroxilamine sulphate solution.
15. Hydrochloric acid.
16. Calcium solution.
17. Lanthanum solution (Lanthanum oxide and con. HCl).

3.2. **Method** :-

In present study *C. carpio* (Linn.) were treated with Mercuric chloride and Cadmium chloride and *H. fossilis* (Bloch) were exposed to Copper sulphate solution. The methods for suggesting these experiments followed APHA (1992) and EIFAC (1983).

3.2.1. **Acclimatization of fish for toxicity test** :-

Live and healthy fish were collected from Yamuna river at Kalpi. Fish were checked for injury and disease and
then washed in 1% KMnO₄ solution for 5 minute. Fish were acclimatized for 15 days in laboratory condition. Collected fish were kept for quarantine for at least 7 days for parasites and disease. If more than 10% of collected fish died after second day or if they were parasitized or diseased beyond control, they were discarded. Clean and sterilized containers and equipment were used. After quarantine period the disease free fish were transported to stock tanks.

3.2.2. Experiment design :-

The test fish were exposed in at least duplicate aquarium. Each test consisted of minimum of five concentration and a control. Before treatment fish were divided in to six group, comprising 10 fishes each, placed in individual glass aquarium of 20 litre capacity and used for treatment. An untreated group of 10 fish, were maintained in separate tank, as control group.

3.2.3. Selection of the test concentrations :-

At least five metal concentration were tested. The concentration was spaced at approximately logarithmic interval. The concentration were arranged so that complete mortality occur with in a day at highest concentration used and no mortality occur in lowest concentration in the period of test.
A partial mortality in at least two intermediate concentration were obtained with in the test period. The test continued for 48 hours and 96 hours. The fish were not fed during this period.

3.2.4. **Biological data and observations** :-

In short term tests the number of dead fish in each container were counted at least daily throughout the test. The dead organism were removed as soon as they were observed. It was more important to obtain data that defined the shape of the toxicity curve than to obtain data at pre specified time. Death is the adverse effect most often used to reflect acute toxicity.

Acute toxicity test was used to determine the harmful properties of a heavy metal. Acute toxicity test demonstrated the results with in short period (4d) of exposure. A Chronic toxicity test usually continues for a relatively longer period of time often for a month or so. A chronic toxicity effect can be measured in terms of reduced growth, histological alteration and bioaccumulation of metal in gill, liver and kidney. Acute toxicity test result generally are characterized by median lethal concentration LC 50.
3.2.5. **Data analysis and results** :-

Statistical methods are used for analyzing data of acute and chronic toxicity tests. Acute toxicity test results are generally characterized by median lethal concentration. Lethal concentration (LC 50) is calculated by parametric procedure such as probit analysis (Finney 1981). Probit method probably is the most widely used LC 50 calculation procedure and uses the probit transformation of mortality, data in combination with a standard curve fitting technique.

This method involves manually plotting dose response data and then drawing a best fit regression line. To construct the graph, percent mortality is plotted as the ordinate against concentrations and is observed on probit paper.

3.2.6. **Toxicity curve** :-

It is a curve produced when, the median periods of survival of test batches of fish exposed to different concentrations are expressed in graphical form. (EIFAC 1983).

3.2.6.1. **Time-response curve** :-

The curve obtained by plotting the cumulative
percentage response of a test batch of fish to a single concentration of toxicant against time and a regression line is drawn to analyse the result.

3.2.6.2. Concentration response curve :-

After a given period of exposure, when the different percentage responses of batches of fish exposed to different concentrations of poison are plotted against those concentrations. A regression line is drawn with the help of analysed data.

3.2.7. Physico chemical analysis :-

The physico chemical properties of water used for the experiment are given in table 2. Water temperature varied according to the ambient laboratory conditions but average temp. (24-26°C) and a photoperiod of 12L, 12d was maintained with the help of fluorescent tube. The dissolved oxygen, pH, and hardness were measured regularly in the laboratory.

3.3. Toxicity test :-

3.3.1. Treatment of Cadmium chloride to C.carpio :-

C.carpio (Linn.) has been exposed to different concentration of Cadmium chloride for toxicity of metal
Cadmium on fish. There was no significant difference (P > .05) between mean weight of the fish used in experiments. Because metabolic activity change with size and effects the parameters to be measured individual of similar size and length were used in experiment (Canli and Furness 1993). Six aquaria, one of which was designated as control, were used to conduct the experiments. Cadmium chloride was utilized for preparation of stock solution. Five aquaria were filled with 20 L of tap water and Cadmium stock solution was added to each aquaria to make the final concentrations 3.0, 3.5, 4.0, 4.5, and 5.0 mg/L cadmium, the sixth aquaria was used as control. Ten fish were added to each aquaria and the acute effect of Cadmium concentration on mortality in 24 hours intervals was investigated. Fish were not fed during acute toxicity test. 96 hours lethal concentration of Cadmium chloride to *C. carpio* was estimated following the Trimmed Sperman Karber method. For chronic toxicity test three groups of 10 fish each were exposed separately in 3 separate aquaria (marked 240h, 480h and 720h) 20L water in each of aquaria with 0.32 mg/L (10% 96h LC50) Cadmium chloride solution prepared in tap water were set. Ten fish were added in each aquaria, the effect of Cadmium concentration in Gill, Liver, and Kidney were observed in 240h, 480h and 720h.
Five fish were sacrificed in different exposure duration for
determination of Cadmium accumulation and histopathological
abnormalities.

3.3.2. Treatment of Mercury chloride to C.carpio :-

Similar above experiment were conducted for
determination of toxicity of Mercury on C.carpio (Linn.). Five
aquaria were filled with 20 liter of tap water and Mercury stock
solution were added to each aquaria to make the final
concentration of .30, .35, .40, .50 mg/L Mercury. Ten fishes
were added to each aquaria and effect of Mercury concentration
on mortality were investigated in 24 hours intervals. Fish were
not fed during acute toxicity test. For chronic toxicity tests three
group of 10 fish were exposed in 3 separate aquaria (marked
240h, 480h and 720h) containing 20 liter tap water each with
.03 mg/L (10% 96h LC 50) Mercuric chloride solution prepared
in tap water. 10 fish were added in each aquarium and the
effect of Mercury concentration on Gill, Liver, and Kidney was
observed. Fish were sacrificed in different exposure duration
for the determination of Mercury accumulation and
histopathological abnormalities in Gill, Liver, and Kidney.
3.3.3. **Treatment of Copper sulfate to *H. fossilis*** :-

Similar above experiment were conducted for determination of toxic effect of Copper sulphate on *H. fossilis*. Five aquaria were filled with 20 liter of tap water and Copper stock solution was added to each aquarium to make final concentration to 10, 20, 30, 40, 50 mg/L Copper. Sixth aquarium was used as control. Ten fish were added to each aquarium and effect of Copper concentration on mortality were investigated in 24 hours interval. For chronic toxicity tests three group of ten fish were exposed in 3 separate aquaria marked as (240h, 480h and 720h) containing 20 L tap water each with 2.24 mg/L (10% of 96h LC 50) Copper sulphate solution. Ten fish were added to each aquaria and effect of Copper concentration on Gill, Liver and Kidney were observed. Fish were sacrificed in different exposure duration for determining Copper accumulation and histopathological abnormalities in Gill, Liver and Kidney.

3.3.4. **Determination of accumulation of metal in vital organs of metal exposed fish** :-

The accumulation of metal in the organs of fish has been determined by atomic absorption spectrometer. Sample containing organic matter generally require pretreatment before
analysis. Making Colorless, transparent sample, containing a turbidity < 1 NTU, odorless and only in liquid phase may be analyzed directly by Atomic Absorption Spectrometer.

Fish is dissected with clean instruments. Gill, Liver and Kidney tissue are put in digestion flask after excising from the body of fish. Preliminary digestion of metal is done by nitric acid– perchloric acid method (Approved by standard method committee 1991). Gill, Liver, and Kidney are digested separately for determination of accumulation of Copper, Mercury, and Cadmium by Atomic Absorption Spectrometer. Gill, Liver, and Kidney are separately transferred to suitable conical flasks. Sample is acidified with conc. HNO₃ and tested with methyl orange end point. 10 ml each of conc. HNO₃ and HClO₄ is added in cool flask or beaker between addition. Evaporation is done gently on a hot plate until dense white fumes of HClO₄ just appear. If solution is not clear, cover container with watch glass and keep solution just boiling until it appears clear. 10ml conc. HNO₃ is added to complete digestion solution and is then cooled and diluted to about 50 ml with water and boiled to expel any chlorine or oxides of nitrogen.
3.3.4.1. **Determination of Mercury** :-

Cold vapor atomic absorption method is used for determination of Mercury (APHA 1992).

3.3.4.1.1. **Principle** :-

Atomic absorption spectrometer resembles emission flame photometry in that a sample is aspirated in to a flame and atomized. The major difference is that in flame photometry the amount of light emitted is measured, where as in atomic absorption spectrometry a light beam is directed through the flame into a monochromator, and on to a detector that measures the amount of light absorbed by the atomized element in the flame. For some, atomic absorption spectrometer exhibits superior sensitivity over flame emission. Because each metal has its own characteristic absorption wavelength, a source lamp composed of that element is used. This makes the method relatively free from spectral or radiation interferences.

3.3.4.1.2. **Apparatus** :-

Glassware for Mercury analysis, Atomic Absorption Spectrometer, absorption cell, cell support, air pump, reaction flask, drying tube etc.
3.3.4.1.3. **Reagents** :-

A:- **Metal free water** :-

Metal free water is used for preparation of all the reagents and calibration standards and dilution water. Metal-free water is prepared by deionizing tap water. (NOTE: If the source water contains Hg or other volatile metals water may not be suitable for trace analysis.)

B:- **Stock Mercury solution** :-

.135 g Mercury chloride HgCl₂ is dissolved in about 70ml water, 1ml conc. HNO₃ is added and diluted to 100 ml with water (1.00ml=1.00 mg Mercury).

C:- **Standard Mercury Solution** :-

A series of standard Mercury solution is prepared containing 0 to 5 ug/L. Appropriate dilution of Stock Mercury solution is done with water containing 10 ml conc.HNO₃/L. Standards solution is prepared daily.

D:- **Nitric acid** :-

HNO₃ Conc.
E:- Potassium permanganate solution :-

50 g KMnO₄ is dissolved in 1L of water.

F:- Sodium chloride-hydroxylamine sulphate solution :-

Dissolved 120g \((\text{NH}_2\text{OH})_2\cdot\text{H}_2\text{SO}_4\) in water and diluted to 1L. A 10% hydroxylamine hydrochloride may be substituted for hydroxylamine sulphate.

G:- Potassium perforate solution :-

50g K₂S₂O₈ is dissolved in water and diluted to 1L.

H:- Stannous Ion \(\text{Sn}^{2+}\) solution :-

10 g SnCl₂ is dissolved in water containing 20ml conc. HCl and diluted 100 ml.

I:- Sulfuric acid :-

\(\text{H}_2\text{SO}_4\) conc.

3.3.4.1.4. Procedure :-

A:- Instrument operation :-

Set wave length to 253.7 nm. Install absorption cell and align in light path to give maximum transmission. Connect associated equipment to absorption cell with glass tubing. Turn on air and adjust flow rate to 2 L/min. Allow air flow continuously.
**B: Standardization :-**

100 ml of each 1.0, 2.0, 5.0 μg/L mercury solution and blank of 100ml water is transferred to 250ml reaction flasks. Add 5 ml conc. H₂SO₄ and 2.5 ml conc. HNO₃ to each flasks. Add 15 ml KMnO₄ solution to each flask and let that stand for at least 15 min. Add 8 ml K₂S₂O₈ solution to each flask and heat for 2 hours in a water bath at 95°C, cool to room temperature.

Each flask is treated individually with enough NaCl. Hydroxyl amine sulphate solution is added to reduce excess KMnO₄ and then 5ml SnCl₂ solution is added and immediately flask is attached to aeration apparatus. Mercury was volatized and carried to the absorption cell. Absorbance is increased maximum for few seconds. As soon as recorder returns approximately to base line stopper is removed holding the frit from reaction flask, and replace with a flask containing water. Flush system for few seconds and run standard curve by plotting peak highest versus micrograms Mercury.
3.3.4.1.5 Calculation :-

Determine peak height of sample from recorder chart and read Mercury value from standard curve preparation.

3.3.4.2. Determination of Cadmium and Copper :-

Direct air-acetylene flame method is used for determination of Copper and Cadmium (APHA 1992).

3.3.4.2.1. Principle :-

Principle is same as that of determination of Mercury.

3.3.4.2.2. Apparatus :-

Atomic absorption spectrometer and associated equipment.

3.3.4.2.3. Reagents :-

A: Air :-

Clean and dried through a suitable filter to remove, oil, water, and other foreign substances.

B: Acetylene :-

Standered commercial grade.
C:- Metal free water :-

Use metal free water for preparing all reagents and for dilution in water. Prepare metal free water by deionizing tap water. (NOTE: If the source water contains Hg or volatile metals, single or redistilled water may or may not be suitable for trace analysis because these metals distil over).

D:- Calcium solution :-

Dissolved 630 mg calcium carbonate (CaCO₃) in 50ml of 1+5 HCl. If necessary, boil gently to obtain complete solution. Cool and dilute to 1000ml with water.

E:- Hydrochloric acid :-

HCl 1%, 10%, 20%, 1+5, and Conc.

F:- Lanthanum solution :-

Dissolved 58.65g lanthanum oxide La₂O₃, in 250 ml conc. HCl. Add acid slowly until the material is dissolved and dilute to 1000ml with water.

G:- Hydrogen peroxide :-

30%
H: - **Nitric acid**:

HNO$_3$ 2%, 1+1 and conc.

I: - **Aqua regia**:

Add 3 volumes conc. HCl to 1 volume conc. HNO$_3$.

J: - **Standard metal solution**:

Prepare a series of standard metal solution in the optimum concentration range by appropriate dilution of following stock metal solution with water containing 1.5ml conc HNO$_3$/L.

[ I ]: - **Cadmium**:

Dissolve .100gm Cadmium metal in 4ml conc. HNO$_3$. Add 8ml conc. HNO$_3$ and dilute to 1000ml with water; 1.00ml=100$\mu$g Cd.

[ II ]: - **Copper**:

Dissolve .100g Copper in 2ml conc.HNO$_3$ and dilute to 1000ml with water; 1.00ml=100$\mu$g Cu.
3.3.4.2.4. **Procedure :-**

A. **Instrument operation :-**

Install a hollow–cathode lamp for the desired metal in instrument and approximate wave length is set. Set the width according to manufacture’s suggested setting for element being measured. Turn on instrument, apply hollow–cathode lamp to the current suggested by the manufacture, and let instrument warm up until energy source, stabilizes, generally about 10 to 20min.. Read the current just necessary after warm up. Optimum wave-length is maintained by adjusting wavelength dial until optimum energy gain obtained.

Install suitable burner head and adjust burner head position. Turn on air and adjust flow rate to that specified by manufacturer to give maximum sensitivity for metal being measured. Turn on acetylene, adjust flow rate to value specified and ignite flame. Let flame stabilize for a few minutes. Aspirate a blank consisting of either deionized water or an acid solution containing the same concentration of acid in standards and sample. Zero the instrument. Aspirate a standard solution and adjust aspiration rate of the nebulizer to obtain maximum sensitivity. Adjust burner both vertically and horizontally to obtain maximum response. Aspirate blank again and re-zero
the instrument. Aspirate a standard near the middle of linear range. Record absorbance of this standard when freshly prepared with a new hollow cathode lamp. Refer to these data on subsequent determination of same element to check consistency of instrument setup and aging of hollow cathode lamp and standard.

The instrument now is ready to operate. When analyses are finished, extinguish flame by turning off first acetylene and then air.

**B. Standardization :-**

Select at least three concentration of each standard metal solution. Aspirate blank and zero the instrument. Then aspirate each standard in turn to flame and record absorbance.

Prepare a calibration curve by plotting on linear graph paper the absorbance of standards versus their concentration.

**C. Analysis of sample :-**

Rinse nebulizer by aspirating water containing 1.5 ml conc. HNO₃/L. Atomize blank and zero instrument. Atomize sample and determine its absorbance.
3.3.4.2.5. **Calculation** :-

Calculate concentration of each metal ion, in microgram per liter for trace elements, by referring to appropriate calibration curve prepared according to standardization. Alternatively, read concentration directly from the instrument. Read out if the instrument is so equipped.

3.3.5. **Histopathology** :-

Histopathological studies were used to determine pathological alteration in the structure of tissue. In this investigation fish *C. carpio* has been treated with Mercury and Cadmium and *H. fossilis* treated with the Copper for different periods. Due to metallic treatment, some abnormality appeared in selected organs Gill, Liver and Kidney of fish.

3.3.5.1. **Sample collection for Gill histology** :-

The Gill tissue begin to degrade almost immediately after death and must be removed as quickly as possible. After opening the opercula second Gill arch on both sides of fish is selected. The second gill arches are cut with a pair of scissors taking care to cut it from the base to dorsal and ventral insertion. The gill arches are placed in a labeled sample tube and filled with 10% neutral buffered formalin.
3.3.5.2 Sample collection for liver histology :-

Make longitudinal section through the middle of the liver and remove a strip of tissue. Place the sample in to sample tube and preserved in 10% neutral buffered formalin. For histology sample, the liver tissue should originate from a portion of liver away from bilir duct.

3.3.5.3. Sample collection for kidney histology :-

Preserve the kidney in sample tube in 10% neutral buffered formalin.

3.3.5.4. Staining Technique :-

Haematoxylin(nuclear stain) and eosin (cytoplasmic stain) were used for double staining process for histological studies.

A. Ehrlich’s acid Haematoxylin :-

A:- Haematoxylin 2gm
B:- Absolute alcohol 100ml
C:- Glycerine 100ml
D:- Glacial acetic acid 10ml
E:- Distilled water 100ml
F:- Alum in excess
Haematoxylin is dissolved in absolute alcohol, acetic acid is added and then the glycerine and water is mixed. The mixture is allowed to ripen in the light until it acquires a dark red colour.

**B. Eosin :-**

- A: Eosin powder 1.0gm
- B: 90% alcohol 100ml

Dissolved 1 g of eosin in 100 ml of ethyl alcohol.

The slide is dipped in xylol. In this step individual slide require two changes in xylol (15 minutes in the first and 5 minutes in the second). The wax is completely dissolved and removed and only section material remains on the slide. Now pass the slide in degrading series of alcohol. Absolute, 90%, 70%, 50%, 30% distilled water. Fine result are obtained by keeping the slide in these series for 8-10 minutes. Two change in distilled water are required each of 5 minutes. Then stain the section in aqueous Haematoxylin for 2-5 minute. After staining dip the slide in distilled water. Then dehydrate the section on the slide through ascending series of alcohol 30%, 50%, 70% and 90%. Then stain the section in alcoholic eosin. Then the slide is kept in a staining trough containing absolute alcohol. Add xylol for 5 minute and then finally the slide is transferred in the pure xylol for 15 minute. Then the slide is mounted with Canada balsum.