Chapter 3

EXPERIMENTAL PROGRAM
Experimental Program:

3.1 Material and methods:

In the present investigations, toxicity of heavy metals cadmium, mercury and nickel to fresh water *Clarius batracus* and mercury or nickel or Cd to has been studied. The procedure for the analysis of water, determination of median lethal conservation and determination of accumulation of heavy metals in fish tissues were followed from APHA1985\(^8\) and EIFAC (1983)\(^50\).

3.2 Material:

3.2.1 Chemicals: the chemical used for the treatment given to the fish is:

a) Cadmium chloride  
b) Mercuric chloride  
c) Nickel sulphate  

The chemicals were of analytical grade purity as packed by E. Merck. The chemicals used for the estimation of metal accumulation in fish were also of analytical grade purity from E. Merck, B.D.G.H., and S. Merck. These chemicals are: -

1. 95% ethyl alcohol  
2. Anhydrous Potassium Sulphate (Na2SO4)  
3. Bromine Water  
4. Chloroform (CHCl3)  
5. Concentrated Acetic Acid (CH3COOH)  
6. Concentrated Ammonium Hydroxide (NH4OH)  
7. Concentrated Hydrochloric Acid (HCl)  
8. Concentrated Nitric Acid (HNO3)  
9. Concentrated Sulphuric Acid (H2SO4)  
10. Cupferron.  
11. Cycloheptanonedioneoixime (Heptoxime)  
12. Disodium Hydrogen Phooshphate (Na2HPO4.12H2O)  
13. Dithizone solution  
14. Hydroxylamine hydrochloride (NH2OH.HCl)
15. Mercuric Chloride (HgCl2)
16. Methyl orange
17. Nickel Sulphahte (NiSO4.6H2O)
18. Potassium Bromide (KBr)
19. Potassium Cyanide (KCN)
20. Potassium Permanganate (KMnO4)
21. Sodium Hydroxide (NaOH)
22. Sodium Permanganate (K2S2O8)
23. Sodium persulphate (K2S2O8)
24. Sodium Potassium tartarate (NaKC4H4O6.4H2O)
25. Sodium tartarate (Na2C4H4O6.2H2O)
26. Tartaric Acid (H2C4H4O6)
27. Thymol Sulfonephthalein Sodium Salts

3.2.2 Equipments:

1. Plastic pool: The plastic pool with 3-meter diameter and 50c.m. Depth was used for the acclimatization of the fish.

2. Auauaria: Glass aquaria measuring 2’ x 1’x 1’ were used for carrying out the experiments.

3. Aerator: Royal mark aerators were used for continuous aeration of the water in the aquarium during treatment given to the fish.

4. Thermostat: Thermostat and heater manufactured by dutta electronics, Jhansi were used for 25°c to 27°c in the aquarium. They were slandered before used.

5. Spectrophotometer: Spectronic 20 spectrophotometer (Baush and Lomb make) was used.

6. Separatory funnels: 125 ml, 250ml and 1000ml with TFE Stopcocks, 125 ml, squibb from with ground glass stoppers.

7. Microtome: Weston Rotary microtome was used for section cutting.

8. Wintrobe ESR Tube:
9. Neubaur Haemocytometer:
10. Sahani’s Haemoglobinometer.

3.3 Method:

3.3.1 Test fish and their collection:
The fish on which the effects of metallic pollutants have been studied are *Labeo rohita* and *clarius batracus*. *Labeo rohita* were collected from Stream of Betwa River near Jhansi. Healthy, living specimens of *C. batracus* in the weight range of weight of 58 ± 2g and length of 18 ± 3 cm were collected from local market. The fish were handled very softly to avoid injury to them. Prior to experimentation the fish were allowed to acclimate to laboratory conditions for 2 wk. The fish were fed twice daily with commercial balanced fish feed in the form of pellets at the rate of 3% of body.

![Fish images]

During acclimation they were fed with minced goat liver every day (d), for 3 hours (hrs). Water was renewed after every 24 h with routine cleaning of the aquaria, leaving no faecal matter, dead fish (if any) or unconsumed food. Prior to the commencement of the experiment, 96 hrs median lethal concentration (96 hrs LC50) of cadmium chloride E. Merck, India to *C. batracus* was estimated following the Trimmed Spearman Karber method.
HAMILTON et al., (1977)\textsuperscript{70} and 24 hrs renewal bioassay system, and was 1gm found to be 70 ppm (95\% confidence limit). For the analysis of sublethal toxicity, 24 hrs renewal bioassay systems were followed. Six groups of 10 fish each were exposed separately in six separate aquaria (marked as 10d, 20d, 40d, 60d, and two extras as ext-1 and ext-2) containing 100. liters (L) each of 7-ppm (10\% of 96 h LC50) cadmium chloride solution prepared in well water having dissolved oxygen 5. 8 ppm, pH 7.4, water hardness 30.0 mg/L ANONYM. 1992 and a water temperature of 27 ± 2 0C. Parallel groups of 10 fish each were kept in separate aquaria containing 100 L of well water (without the addition of cadmium chloride) as controls. Feeding was allowed in the experimental as well as control groups every day for a period of 3 hrs before renewal of the medium throughout the period of the experiment. Random checking of goat liver for the presence of cadmium on 10 different days during the experiment did not reveal any detectable amount. After the expiry of 10th, 20th, 40th and 60th days of exposure, 3 fish each from the respectively marked experimental, as well as control aquaria, were sacrificed. For estimating the cadmium content, first a pair of gills, kidneys and liver was excised from the experimental fish as well as control fish separately and the tissues were placed in separate Petri dishes to dry at 80 °C until reaching a constant weight. Five hundred mg each of the dried tissues were placed in separate digestion flasks and nitric-perchloric acid (4:1) mixture was added. The digestion flasks were gradually brought to and kept at 130 °C on a hotplate until all materials were dissolved and the digests were diluted with deionized water. All the dissection instruments and glassware were acid washed and rinsed with deionized water. Metal concentrations in samples were measured using a Perkin Elmer A Analyst 800 atomic absorption spectrophotometer and is given in ppm. Obtained data were subjected to standard statistical processing based on random sampling of three different samples of experimental, as well as control groups, of each tissue at each sampling period. One-way analysis of
variance followed by Duncan's multiple range test was performed
BRUNING and KINTZ, (1968)26 to determine whether the bioaccumulation
of cadmium in the various tissues studied was influenced significantly by
the exposure periods. Since there were no significant variations in the
values of the respective control tissues collected at the various exposure
periods, the average value of each of the control tissues was taken into
account.

3.3.2 Acclimatization:
The collected fish were first treated with 0.1 kmno₄ solution for removal of
any possible fungal infection. The fish were acclimatized in the ordinary
tap water in the plastic pool for 10 days. Under laboratory condition. The
similar tap water was used in the aquarium during fish treatment. The fish
were fed once a day on standard fish food. These were starved for 24
hours before being used for bioassay test.

3.3.3 Preparation of stock solution:
100μg of mercuric chloride (HgCl₂) was dissolved in 1 liter of distilled
water (1N = 100mg HgCl₂). For cadmium, 1gm. of cadmium sulphate was
dissolved in distilled water (1ml = 1mg CdCl₂). For nickel, 1g of nickel
sulphat (NiSo₄) was dissolved in 1liter of distilled water (1ml = 1mg NiSo₄)

3.4 Toxicity Tests:
To determine the toxicity of cadmium chloride, mercuric chloride and
nickel sulphate, Bioassay tests were conducted screening tests were
arranged prior to bioassay test, to ascertain the concentration of these
metallic pollutants to be used for final bioassay test

3.4.1 Bioassay Test:
For chronic toxicity and histopathological studies long-term bioassay test
were conducted while for acute toxicity of the metal was tested by short-
term bioassay test. Dead fish were removed immediatately and the time and number of their mortality was recorded.

3.4.2 Screening test:

Seven widely spaced concentrations of each salt were tested to find out the toxicity range of the metal concentrations. Three fish used for this preliminary-screening test. Results of screening test were used to decide the range of the concentration of the metal salts to be used for toxicity test.

3.4.3 Cadmium Treatment:

Starved fish for 24 hours were placed to aquarium containing different concentration of cadmium chloride, ranging from 0 mg/lit to 5.0 mg/lit. Each aquaria containing 20 liter of water, ten fish in each aquarium. Those fish, which are found in cadmium chloride, free aquaria (0 mg/l) for control set. The size of the fish ranged from the weight of the fish "Clarias batrachus" is ranged 58 ± 2g gm. The fish were observed after intervals of 24 hrs, 72hrs, 96hrs, 10days, 20days and 30days for the following.

a) Percent mortality was recorded with time by using short-term toxicity tests.

b) The change in the behavior of the fish was also observed.

c) The growth of the fish in terms of length and weight was recorded and compared with the controlled fish.

d) The fish were dissected and gills, liver and kidney were preserved in 20% neutral fromalone for histopathological studies.

e) The organs gills, liver and kidney were taken out and preserved in concentration HNO3 for the estimation of metal accumulation in these organs APHA (1985)\textsuperscript{12}.
3.4.4 Mercury treatment:

The toxicity of mercury has been studied by repeating the above experiment using mercury as mercuric chloride. It has been used in different concentration ranging from 0 μg/l to 1000 μg/l. 20 liters of water used in each of the aquaria mercuric chloride for toxicity test. The size of the fish *Labeo rohita* ranged 65 mm in length and the weight ranged 6.284 gm the fish were observed after the intervals of 24 hrs. 48 hrs, 72 hrs, 96hrs, 10days, 20days and 30days. In mercury treatment the observation have been reported for 23rd and 25th days, as mortality was observed on these days. All the observation from a to e were made as in cadmium treatment.

3.4.5 Nickel treatment:

Nickel is found in plating, metal pickling and metal cleaning wastewater. The nickel from the plating wastewater can be chelated from as nickel sulfamate from sulfamate nickel plating, or nickel lactate from electroless nickel plating. Nickel cleaning and stripping solutions are often having nickel cyanide and nickel EDTA complex. Nickel that isn’t complexed or chelated can be precipitated as nickel hydroxide by adjusting the pH to 10.5 or higher. Complexed nickel must be treated with sulfide or other strong reducing agents that can maintain a negative ORP of −700 mv or less. Even then the treatment may be difficult to reach acceptable limits. Precipitating the nickel as a carbonate is not effective due to the high solubility of nickel carbonate. The precipitation of nickel phosphate is effective for lightly complexed nickel using a two-step process described in the copper section. To precipitate the nickel as the metal using ORP, a strong reducing agent, such as ferrous ion or sulfide ion, must be used at a pH greater than 10.5. Raising the pH to 11.5 and allowing the nickel to plate out using the residual reducing agent, sodium hypophosphite, in the solution before continuing the treatment should pretreat Electroless nickel solutions. The plating out or dropping out of
electroless nickel solutions releases significant quantities of hydrogen so the treatment vessel must be exhausted to keep the hydrogen below the explosion limits.

Nickel can be removed with cation resins and chelated anion resins. Using a reducing agent and sand filter or other media column to precipitate the nickel metal on the surface of the media can attain low concentrations. If nickel concentrations below 1 mg/l are required, a three-step process of precipitation, oxidation and then final precipitation or adsorption will be required. The treatment equipment for removing nickel is similar to that used for copper adding forced exhaust to prevent hydrogen build up during the treatment of electroless nickel baths. The toxicity of nickel sulphate. Defferent concentration ranging from 0 mg/l to 12 mg/l were maintained in glass aquaria containing 20 liters of water and ten fish in each of them. The fresh water *Clarias batrachus* ranging in size is 18 cm. and in weight is 58 ± 2g gm were to different concentration of nickel sulphate. The fish were observed at the intervals of 24 hrs, 48 hrs, 72 hrs, 96 hrs, 10 days, 20 days and 30 days. All the observation from a to e were made as in cadmium and mercury treatment.

3.5 Physico Chemical Analysis of Water:
Physico-chemical analysis of water was done every 24 hrs to maintain the standards of the test. The mean values of physico chemical properties of water are given in the Table No.1

3.6 Mortality:
Those fishes, which show no activity and not respond to mechanical stimuli, were counted for mortality. The number of dead fish was recorded in each concentration with time and were removed immediately form the test aquarium.
3.7 Analysis of results of toxicity tests:

Data, which are collected from different sets of toxicity, were processed to determine the lethal threshold concentration and median lethal concentrations for 24 hours, 48 hours, 72 hours and 96 hours exposure durations.

3.7.1 Lethal threshold concentration:
That minimum concentration of the metallic pollutant used, that caused the firsts death of the test fish, was recorded as lethal threshold concentration.

3.7.2 Median Lethal concentration:
It is term as LC50 value and the concentration of the pollutant, lethal to one half of the test population of fish EIFAC 1983)\(^{50}\) it is determined the for the test durations, such as 24 hrs LC 50, 48 hrs LC 50 etc. The percentage mortality was plotted on ordinate on probit scale and concentration of the metallic pollutant on the abscissa on logarithmic scale. Finney (1971)\(^{57}\) and Sprague (1973)\(^{185}\) suggested of LC 50, Probit analysis.

3.7.3 Toxicity curve:
Using LC50 values of different metal salts for different exposure durations does it. Plotting exposure time on ordinate and corresponding LC50 Values on abscissa plots curves on logarithmic graph paper.

3.8 Histopathology:
Histopathological studies were undertaken to find out pathological changes in cytoarchitecture of different organs. The fish for this study were kept in varying concentrations and were sacrificed after different exposure times. The organs liver, kidney and gills were studied for histopathological changes. These organs were fixed in 20% neutral formaline and paraffin
section of 6-8μ thickness were cut. For histopathological studies the sections were stained with Delafied's Hematoxylin and Eosin.

Organ tissues, liver, kidney, gill and collected in 10% neutral buffered formalin were processed for paraffin blocks (56-58 °C) and sectioning at 3-5 μm. Stained sections were examined under a Zeiss compound binocular microscope (Axiophot, Germany) fitted with a photomicrographic attachment. Kumar Ravindar (2000) studied on the effect of chronic exposure to a test concentration (70 ppm) of ammonia has been observed in kidney of teleost fish Channa punctatus (Bloch). In kidney, proximal tubules were highly affected. The nucleus in all the tubular cells was pycniontive and cytoplasm was granular. It was observed that changes at 28 days were more gradual in comparison to 14 days of ammonia intoxication.

3.8.1 Stains:
Delafiel's hematoxylin and Eosin were used for routine double staining process for histological studies. They were prepared by the following method adopted from Johansent (1940)81

(a) Delafiel's Hematoxylin: It was prepared by adding drop by drop a solution of 4g. hematoxylin in 25 ml of 95 % ethyl alcohol to 400 ml of saturated aqueous solution of ammonium aluminium sulphate. It was kept exposed to light and air for 10 days for ripening. 10 ml of c. p. Glycerine and 100 ml of methyl alcohol was added to it. It was allowed to stand for a period of 1 month exposed to air. The colour became sufficiently dark. It was mixed with equal amount of distilled water.

(b) It is a fluorance derivative with 2.18 % solubility in alcohol. It is a cytoplasmic stain for animal tissues. It was prepared by dissolving 1gm.of Eosin in 100 ml of 95 % ethyl alcohol. The deparaffinised section of tissues were passed through down grade series of alcohol
and finally brought to distilled water. They were treated further in the following manner.

a. Haematoxylin for 2 min.
b. Tap water for 2 min.
c. A dip in Acid water
d. Distilled water for 2 min.
e. 30 % alcohol for 5 min.
f. 50 % alcohol for 5 min.
g. 70 % alcohol for 5 min.
h. 90 % alcohol for 5 min.
i. Eosin solution for 1 min.
j. A dip in Acid alcohol
k. A dip in n90 % aclohol
l. 100 % alcohol for 5 min. 1st change
m. 100 % alcohol for 5 min. 11nd change
n. Xylool for 3 min.
O Mounted in DPX.

3.9 Haematology:
The fish *Labeo rohita* were netted from the Betwa River passing from Jhansi district in the first week of each month, from Sept. 2005 to Oct. 2005, between 8-9 a.m. Blood was collected immediately after capture by severing the caudal peduncle. Heparin (0.1 mg 1 ml-1 of blood) was used as anticoagulant. The erythrocyte count was determined by an improved Neubauer haemocytometer with Yokoyama's solution as the diluting medium. Blood haemoglobin was estimated colorimetrically following Wong's method. Haematocrit was measured by Wintrobe Haematocrit. Leucocyte counts were made using a Neubauer haemocytometer, diluted in Yokoyama's solution. Clotting time was determined by taking blood direct from the fish in a capillary tube of 0.5 mm diameter.
The fish *Clarias batrachus* were collected and the similar procedure was adapted for haematological studies were made. Disease-free fish, *Clarias batrachus* were bathed in 1% KMnO4 solution and acclimatised in big glass aquarium of Glass aquaria measuring 2’ x 1’x 1’ capacity for a period of 72 hrs. The healthy fish of both the sexes and uniform length and weight (18c.m. and 58.500 gm ±) were selected from the lot for the experimental purpose. Initially 24 hrs 96 hrs LC50 doses were determined for nickel heavy metal compounds by the method as described in standard methods by APHA, (1998)13. The fishes were divided into three groups.

Group- I: Consisting of 15 fish in aquarium. Water was changed, every day in the morning after removing the unused food. The controls as well as experimental fish were sacrificed on the day 10, 20 and 30. The blood was collected into vials containing heparin as anticoagulant by severing the caudal peduncle, after 10, 20 and 30 days of exposure. Haemoglobin (Hb%) was measured by Sahali’s haemoglobinometer, RBC (TEC) and WBC (TLC) were counted by using Neubaur’s haemocytometer.

Group- II: Consisting of 15 experimental fish exposed to sublethal dose of nickel sulphate (4mg/l) for 30 days.

Group-III: Consisting of 15 experimental fish exposed to sublethal dose. The fish were exposed to 4 mg/l of nickel sulphate, which are the 1/10th of their 96h LC50 concentrations. To avoid the effects of starvation, the fish were fed on the rice bran at the average feeding rate of 25 mg food / gm fish / day. WBC were counted by Neubaur’s haemocytometer and Hayem’s and Tuerk’s solutions as diluting fluids, respectively and Packed cell volume (PCV) by Wintrobe’s method (300 rpm for 1 hour). Differential leucocyte count (DLC) was carried out by preparing a thin blood smear and staining it with Leishman’s stain. Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean cell volume (MCV) were calculated using standard formulae (Dacie and Lewis, 1982)39.
3.10 Growth rate:

The fish growing at normal rate is presumed to be healthy while the condition of food and other things like temperature, quality of water are kept natural. The growth of the fish, *C. batrachus* and *L. rohita* maintained in aquarium with water without metal is measured in length and weight in 30 and 20 days and is registered as normal growth. These fish sp. were subjected to sublethal concentration of cadmium sulphate, nickel sulphate for 30 days and mercuric chloride treatment was given for 20 days. The growth of these fish in terms of weight and length was compared with that of control fish.