CHAPTER III

GENERAL MATERIALS AND METHODS
Experimental fish:

Live specimens of the fresh water fish, *Labeo rohita* were collected from the Government fish farm, Mahan (Distt. Akola), and were brought to the laboratory in well oxygenated bags without any injury. They were washed with 1% KMnO₄ and acclimated to laboratory conditions for a fortnight. Chlorine free aged tap water was used in the aquaria. The water had pH 8.2 ± 0.2; hardness 280 mg/l; D.O. 6.2 mg/l; total alkalinity 310 mg/l and temperature 25 ± 2°C. The fish were fed with rice bran daily at 10.30 am. The water in the aquaria was changed daily after the consumption of food supplied.
After 15 days of acclimation healthy and active fishes of uniform size and weight (length $20 \pm 1$ cm; weight $125 \pm 2$ g) were sorted out and kept in separate aquaria.

**The heavy metals and their compounds:**

Three heavy metal compounds were selected for the present investigation and they were,

1. Chromium chloride ($\text{CrCl}_2$)
2. Nickel chloride ($\text{NiCl}_2$)
3. Zinc chloride ($\text{ZnCl}_2$).

(A) Chromium:

Chromium belongs to the group VI B and 4$^{th}$ period of periodic table having atomic number 24 and atomic weight 51.996. Electronic configuration of chromium is $1s^2 \ 2s^2 \ 2p^6 \ 3s^2 \ 3p^6 \ 3d^3 \ 4s^1$. For chromium, the highest oxidation state is that corresponding to the total number of 3d and 4s electrons. The most stable and generally important states are Cr (II) and Cr (III).

Occurrence:

The chief ore is chromite ($\text{FeCr}_2\text{O}_4$), which is a spinel with Cr (III). Chromium is found in nature at levels of 100 ppm in the earth’s crust. Chromium is a white, hard, lustrous and brittle metal with melting
point 1903± 10°C. It is extremely resistant to ordinary corrosive agents, which accounts for its extensive use in electroplating.

Properties of chromium chloride:

- **Appearance:** White
- **Molecular weight:** 122.90
- **Melting point:** 824 °C
- **Boiling point:** 1300 °C (Sublimes)
- **Solubility:** In water

(B) Nickel:

Nickel belongs to the VIII (10) group and 4th period of the periodic table having atomic number 28 and atomic weight 58.69. Electronic configuration of nickel is 1s² 2s² 2p⁶ 3s² 3p⁶ 3d⁸ 4s². Its common oxidation state is Ni (II).

Occurrence:

Nickel occurs in nature mainly in combination with arsenic, antimony and sulphur, for example as millerite (NiS), as red nickel ore that is mainly NiAs, and in deposits consisting chiefly of NiSb, NiAs₂, NiAsS or NiSbS. The most important deposits commercially are garnierite a magnesium-nickel silicate of variable composition, and certain varieties of the iron mineral pyrrhotite, which contain 3 to 5% Nickel. It occurs in the earth's crust at a level of 80 ppm. Elemental nickel is also found alloyed
with iron in many meteors, and the central regions of the earth are believed to contain considerable quantities.

Nickel is silver-white with high electrical and thermal conductivities and melting point 1452 °C, and it can be drawn, rolled, forged and polished. It is quite resistant to attack by air or water at ordinary temperatures when compact and is therefore often electroplated as a protective coating.

**Properties of nickel chloride (NiCl₂):**

- **Appearance:** Yellowish
- **Molecular weight:** 129.62
- **Melting point:** 1001°C
- **Boiling point:** 973 °C (sublimes)
- **Solubility:** In water

**Zinc:**

Zinc belongs to the group II B (12) and 4th period of the periodic table having atomic number 30 and atomic weight 65.4. Electronic configuration of zinc is 1s² 2s² 2p⁶ 3s² 3p⁶ 3d¹⁰ 4s². Melting point is 419 °C and boiling point is 907 °C.
Occurrence:

Zinc have relatively low abundance in Nature (of the order of $10^6$ of the earth’s crust, but have long been known because they are easily obtained from their ores. Zinc occurs widely in a number of minerals, but the main source is Sphalerite \((\text{ZnFe})_2\text{S}\), which commonly occurs with galena \((\text{PbS})\).

Properties of zinc chloride:

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>White</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>136.28</td>
</tr>
<tr>
<td>Melting point</td>
<td>283 °C</td>
</tr>
<tr>
<td>Boiling point</td>
<td>732 °C</td>
</tr>
<tr>
<td>Solubility</td>
<td>In water</td>
</tr>
</tbody>
</table>

Bioassay studies:

96 h LC$_{50}$ and sublethal concentrations of all the three heavy metal salts for the fish, *Labeo rohita* were investigated to carry out further studies. Static bioassay was carried out as per standard methods (APHA, 1998) to determine 96 h LC$_{50}$. The physico-chemical characters of water used were also analysed by using standard methods (APHA, 1998).

As the toxicants were of unknown toxicity, first literature survey was made and from that probable concentrations were selected.
Aqueous solutions of selected heavy metal salts (chromium chloride, nickel chloride, and zinc chloride) ranging from 10 to 100 ppm were added to the glass aquaria, containing 25 liters of water. The toxicant solution was added drop by drop with constant stirring and then acclimatized 25 fish were transferred to the glass aquaria (45 X 25 X 25 cm) containing 25 liters of toxicant treated water. The fishes were fed (25 mg rice bran / gm fish / day) once in a day specially at 10.30 am. Observations are made for 24 hours, from which the different concentrations were selected for the full-scale experiments; behavioural changes in the fish were observed and recorded. The time, at which the fish looses its sense of balance and float on its side or upside down, was noted. Keeping these observations in mind different 5 concentrations of each toxicant were selected for the final experiment.

The test concentrations for the fish, Labeo rohita selected were 100 to 400 mg/l, 20 to 60 mg/l and 10 to 40 mg/l for CrCl₂, NiCl₂ and ZnCl₂ respectively. In all these cases treatment (static bioassay test) was carried out for a period of 96 hours and after every 24 hours the water in aquarium with toxicant was replaced by freshly prepared solution of corresponding concentrations. Suitable master controls were also run along with the experimental sets. Mortality was recorded and tabulated after every 24 hours. Three replicates were carried out for each toxicant. The data was then subjected to statistical analysis (Probit analysis) to find out the 96 h LC₅₀ (Finney, 1971). The statistically calculated 96 h LC₅₀ values of the three heavy metal salts were found as below
Chromium chloride: 58.62 mg/l
Nickel chloride: 40.15 mg/l
Zinc chloride: 19.22 mg/l

From the calculated 96 h LC₅₀ values, approximately ten times less concentrations were selected as chronic sublethal dose and the fishes were exposed to these concentrations separately for 30 days to study the alterations in histological structures of various tissues, biochemical constituents, and melanophores.

For the above studies the acclimated fish were divided into four groups as below-

Group I: Containing fishes in aged tap water which served as control,

Group II: Consisted fish kept in toxicant water containing 6 mg/l chromium chloride for 30 days.

Group III: Consisted fish kept in toxicant water containing 4 mg/l nickel chloride for 30 days.

Group IV: Consisted fish kept in toxicant water containing 2 mg/l zinc chloride for 30 days.

The toxicant solutions and aged tap water (control) were renewed everyday to maintain uniform test concentrations throughout the experimental period. Feeding was continued till the end of exposure period (30 days).
**Behavioural study:**

During biotesting no mortality was recorded in control fish and they showed normal swimming movements, however the experimental fish exhibited abnormal swimming movements. Similarly behavioural observations were also noted in chronically treated fish, which were exposed to sublethal doses of chromium chloride, nickel chloride and zinc chloride separately.

**Histopathological studies:**

10 fish in each group were tested for histopathological studies of the fish organs like liver, gill, kidney, intestine and gonads (testis and ovary). For this, fish from control and experimental groups were sacrificed after 10, 20 and 30 days of treatment.

**Biochemical studies:**

Fifteen fish in each group were used for studies of various biochemical parameters in blood and tissues like liver and muscles. The biochemical studies were carried out as per the methods given in the following table. The blood from the control as well as experimental fishes was removed from caudal peduncle. The tissues, liver, and muscle were homogenized in sucrose solution for the estimations.
Table 3.1: Methods used to carry biochemical estimations in the fresh water fish, *Labeo rohita* exposed to sublethal concentrations of heavy metal salts, chromium chloride, nickel chloride and zinc chloride.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Parameters</th>
<th>Methods used</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood glucose</td>
<td>GOD/POD</td>
<td>Tietz (1976)</td>
</tr>
<tr>
<td>2</td>
<td>Serum protein</td>
<td>Biuret</td>
<td>Rosenthal <em>et al.</em>, (1956)</td>
</tr>
<tr>
<td>3</td>
<td>Serum cholesterol</td>
<td>Ferric chloride-acetic acid</td>
<td>Zlatkis <em>et al.</em>, (1953)</td>
</tr>
<tr>
<td>4</td>
<td>SGOT</td>
<td>2-4-DNPH</td>
<td>Reitmann and Frankie (1957)</td>
</tr>
<tr>
<td>5</td>
<td>SGPT</td>
<td>2-4-DNPH</td>
<td>Reitmann and Frankie (1957)</td>
</tr>
<tr>
<td>6</td>
<td>Tissue protein</td>
<td>Folin-Phenol</td>
<td>Lowry <em>et al.</em>, (1951)</td>
</tr>
<tr>
<td>7</td>
<td>Tissue glycogen</td>
<td>Montgomery</td>
<td>Montgomery, R. (1957)</td>
</tr>
<tr>
<td>8</td>
<td>Tissue cholesterol</td>
<td>Liberman, Burchard</td>
<td>King and Wolte (1959)</td>
</tr>
</tbody>
</table>

**Haematological studies:**

Fifteen fish in each group were used for haematological studies. The blood parameters like RBC, WBC, DLC, Hb%, PCV, MCHC, MCV and MCH were studied in control as well as experimental fish after 10, 20 and 30 days of exposure by using the standard haematological methods (Talib, 1988).
**Melanophore studies:**

The scales from the control as well as experimental fish were removed from the 4\textsuperscript{th} row just below the lateral line above the ventral fin with the help of tweezers. They were cleaned in fish saline and then dehydrated, cleared and mounted in DPX. The slides were observed for the shapes of the melanophores and the photomicrographs were taken at x400.

**Statistical analysis:**

The data of bioassay studies was subjected to probit analysis to find out the 96 h LC\textsubscript{50} (Finney, 1971). Statistical analysis of results for the level of significance was done by student's t-test (Fisher, 1950).