Chapter - 3

Material and Methods and Scope of Study
Scope of the Present Study

"Heavy metal pollution is one of the burning subjects in the modern biological sciences. An urgent need to enhance the knowledge of metal-metal interactions and to promote research on the responses mounted by all living organisms to environmental, physiological and pathological stresses, is being felt by researchers worldwide."

The present study is based on the above rationale. In this study an attempt has been made to evaluate the environmental stress created by copper and zinc on the commonly available economically important fresh water fish *Catla catla*. The following parameters are to be investigated in liver, muscle and kidney of *Catla catla*:

1) Physico-chemical parameters.
2) Acute toxicity test of copper and zinc.
3) Chronic toxicity test of copper and zinc.
4) Survival and growth under copper and zinc stress.
5) Biochemical analysis.
6) Phytoremediation for heavy metal pollution.
7) Adsorbents to remove metal from Water.
8) Electrophoresis.
Material and Methods

Haryana occupies a total geographical area of 44,000 sq. km and is located in North West part of India. Rohtak is one of the important cities of Haryana. The topography of Rohtak and surrounding areas is bowl or saucer shaped. The underground flow of water from the Shivalik range and Aravali range is towards Rohtak. This has resulted in soil clogging and raise in water table. Leaching of salts has increased soil salinity. Natural draining of water into either rivers or sea is lacking due to the absence of any major river and coastline in the state. Due to these factors heavy metals released through various sources cannot escape and are permanently retained.

Test Animal

For the present study, test animal selected is the Indian Major Carp *Catla catla* (Linnaeus). This fish is a surface feeder and exclusively herbivorous in nature. *Catla catla* has economical, recreational, commercial and ecological importance locally as well as nationally. It is easily available, ideal for toxicity studies in aquatic biology and sensitive to materials or environmental factors under consideration.

Healthy specimens (fry) of the fish *Catla catla* measuring 0.3±0.001mg in weight and 2-3mm in length, hatched from the same lot of eggs were procured.
from State Govt. Center at Sampla. Prior to experimentation, fishes were disinfected with KMnO₄ solution and were maintained for 15 days in laboratory in well-aerated tap water, which was renewed after every 24 hrs. Therefore, fishes were acclimated to experimental containers for a week. During the period of maintenance and acclimation, fish were fed with rice bran, ground nut oil cake and fish meal in the ratio 1:1:1. Food was given at least one hour before the renewal of tank water. Feeding was withheld for 48 hours prior to the commencement of all experiments so as to keep the experimental animal in same metabolic condition and to minimize possible effect on measured parameters during experiment. Fish belonging to either sex were used. The temperature of water in aquaria was maintained between 20±5°C, pH 7.1±0.2; hardness 160±8ppm as CaCO₃; alkalinity 87±9ppm and D.O. 7.5±0.4ppm.

**Location of Study Sites**

For the present study twenty ponds namely Dobh, Bhali, Baniyani, Balam, Khidwali, Basantpur, Sunderpur, Jindrain, Titoli, Brahminwas, Kanheli, Karor, Bhambhewa, Baliana, Kheri, Pehrawer, Sunarian, Shimali, Garhi and Maina (See Map) were selected. These ponds are located with in an area of 25 KM. from the study center. i.e. M.D.U. Campus, Rohtak. Rohtak district has population of 1,15,869 as per 2001 census. The average annual rainfall is 415mm and the
Village Pond ' Dobh' (Rohtak)

Village Pond ' Titoli ' (Rohtak)

Village Pond ' Jindrain' (Rohtak)
average rainy days are 22. Ground water is saline and city is dependent on canal water and shallow tube wells for drinking and irrigation purposes.

**Collection of Water and Soil Samples**

Throughout the study period of two years from May 2002 to May 2004, water samples from middle of the pond in triplicate were collected from twenty different ponds (APHA, 1985) in clean and sterilized polythene bottles except dissolved oxygen. Oxygen was fixed on the spot; temperature as well as pH were also measured on the spot. For various physicochemical parameters, samples were analyzed in the laboratory as per standard methods (APHA, 1985). For estimation of heavy metals, samples were preserved by acidification with nitric acid.

Soil samples were collected from the bottom of all the selected sites at a depth of six inches in duplicates. Mouths of sampling bags were tied with rubber bands and brought to the laboratory for analysis. These samples were air dried, powdered, sieved and homogenized before use.
**GROUPING OF ANIMALS**

<table>
<thead>
<tr>
<th>GROUP NO.</th>
<th>TREATMENT</th>
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<tr>
<td>A</td>
<td>Control</td>
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<tr>
<td>B</td>
<td>1/10&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Cu</td>
</tr>
<tr>
<td>C</td>
<td>1/10&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Zn</td>
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<tr>
<td>D</td>
<td>1/10&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; both Cu</td>
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<tr>
<td></td>
<td>and Zn</td>
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<tr>
<td>E</td>
<td>1/20&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Cu</td>
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<tr>
<td></td>
<td>+ 1/10&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Zn</td>
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<tr>
<td>F</td>
<td>1/10&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Cu</td>
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<tr>
<td></td>
<td>+ 1/20&lt;sup&gt;th&lt;/sup&gt; of LC&lt;sub&gt;50&lt;/sub&gt; of Zn</td>
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</tbody>
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FLOW SHEET- 3
PLAN OF WORK

Water analysis of ponds

Physico-chemical

- pH
- EC
- D.O.
- Cl⁻
- Mg²⁺ ions
- Salinity
- Ca²⁺ ions
- Hardness
- Alkanility
- Ca Hardness

Metal Estimation

- Soil
- H₂O
- Cu
- Soil
- H₂O
- Zn
- H₂O
- Fe
- H₂O
- Pb
- H₂O
- Ni

FLOW SHEET- 3.1
Water analysis

Chemical analysis of water was carried out following the standard methods of APHA (1985).

Hydrogen ion concentration (pH)

The pH was measured by means of pH meter (Model systronics-324).

Conductivity

Conductivity was measured by a direct reading conductivity meter (Model Systronics-303).

Dissolved Oxygen

The D.O. content was determined according to the modified Winkler’s method. Water samples were collected directly from the ponds in 250ml BOD bottles by slowly immersing in water without any access to air bubbles. The bottles were stoppered under water. The oxygen content of water in these bottles was fixed by adding saturated solution of manganous sulphate (2ml) and saturated solution of alkaline potassium iodide, sodium azide (2ml). The bottle was shaken thoroughly and the precipitate formed was allowed to settle down in dark for ten minutes. It was then brought to the laboratory. Concentrated sulphuric acid 2ml was added to dissolve the
precipitate. 50ml of this solution was titrated against 0.025N sodium thiosulphate using starch as indicator until initial blue color became colorless. The D.O. content was calculated by the equation:

\[
\text{DO (mg/l)} = \frac{V_1 \times N \times 8 \times 1000}{V_4 \frac{(V_2 - V_3)}{V_2}}
\]

Where
- \(V_1\) = Volume of titrant (ml)
- \(N\) = Normality of titrant (0.025)
- \(V_2\) = Volume of sample bottle (ml)
- \(V_3\) = Volume of MnSO\(_4\) and alkaline potassium iodide (ml)
- \(V_4\) = Volume of fraction of the content used for titration (ml)

**Total alkalinity**

**Reagents**
- 0.02N \(\text{H}_2\text{SO}_4\)
- Phenolphthalein indicator
- Methyl orange

**Method**

Carbonate and bicarbonate alkalinity was estimated by titrating 50ml of sample water against sulphuric acid using Phenolphthalein and Methyl Orange as indicators.
**Calculation**

\[
\text{Phenolphthalein alkalinity} = \frac{P \times 1000}{S}
\]

(As CaCO₃, mg/l)

\[
\text{Total alkalinity} = \frac{T \times 1000}{S}
\]

(As CaCO₃, mg/l)

Where

- \(P\) = Volume of titrant used against phenolphthalein indicator (ml)
- \(S\) = Volume of sample
- \(T\) = Total volume of titrant used for the two titrations (ml)

**Total Hardness**

**Reagent**

- Ammonia buffer solution
- Eriochrome black-T indicator (4-5 drops)
- EDTA solution (0.01M)

**Method**

To determine the total hardness, 50ml of sample was taken in a conical flask and 1 ml ammonia buffer solution and 4-5 drops of eriochrome black-T indicator were added to it. The contents were
titrated against EDTA (0.01M) solution until the color changed from wine red to blue.

**Calculation**

\[
\text{Total hardness} = \frac{T \times 1000}{S}
\]

(As CaCO₃, mg/l)

Where

\[T = \text{Volume of titrant (ml)}\]
\[V = \text{Volume of sample (ml)}\]

**Calcium**

**Reagents**

- Sodium hydroxide solution (8%)
- Muroxide indicator (a pinch)
- EDTA solution (0.01M)

**Method**

To determine the concentration of calcium, 50ml of sample was taken in a conical flask and 1 ml sodium hydroxide solution and a pinch of murexide indicator were added to it. The contents were titrated against EDTA (0.01M) solution until the pink color changed to purple.
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Calculations

Calcium (mg/l) = \( \frac{T \times 400.5 \times 1.05}{V} \)

Where

\( T \) = Volume of titrant (ml)
\( V \) = Volume of sample (ml)

Calcium Hardness = \( \frac{T \times 1000 \times 1.05}{V} \) (mg/l)

Where

\( T \) = Volume of titrant (ml)
\( V \) = Volume of sample (ml)

Magnesium

Total hardness and calcium hardness of water as mg/l CaCO₃ were determined as mentioned above. From these values magnesium content was calculated as follows:

Magnesium (mg/l) = \( (T-C) \times 0.244 \)

Where

\( T \) = Total hardness (mg/l as CaCO₃)
\( C \) = Calcium hardness (mg/l as CaCO₃)
Chloride

Reagents

AgNO₃ (0.02N)

Potassium chromate indicator 5-6 drops

Method

To determine chloride content, 10ml of the sample was taken in a flask and 5-6 drops of potassium chromate indicator was added to it. The contents were titrated against AgNO₃ solution until yellow color of solution changed to brick red color.

Calculations

\[
\text{Calcium (mg/l)} = \frac{V \times N \times 35.457 \times 1000}{S}
\]

Where

V = Volume of titrant (ml)
S = Volume of sample (ml)
N = Normality of titrant (0.02)

The salinity of water was calculated as follows:

\[
\text{Salinity (ppt)} = 0.03 + 1.805 \times (\text{Cl'} \text{ in mg/l})
\]

Estimation of Metals
Water

100 ml of each acidified sample was digested by triacid (conc. HNO₃ + Conc. H₂SO₄ + 70% perchloric acid) in ratio of 2:1:0.5. The solution was diluted up to 15 ml with triple distilled water and filtered. The solution was used for further AAS analysis.

Copper, iron, lead, and nickel were estimated with hollow cathode lamps at wavelength 324.8, 213.9, 248.3, 283.3 and 232.0 nm respectively, with a slit of 1.3 nm (except iron and nickel at 0.2nm) by atomic absorption spectrophotometer (Hitachi model Z-6100) with air-acetylene mixture as fuel.

Soil

The concentration of copper and zinc were estimated after triacid digestion by AAS (Hitachi model Z-6100) as mentioned before.

Toxicity Tests

Procurement and Preparation of Experimental Fish

Test animal for the present study is the Indian Major Carp *Catla catla*. *Catla catla* is a surface feeder and exclusively herbivorous in nature. From the viewpoint of fish culture, *Catla catla* is most extensively cultivated worldwide and is a native species.

Healthy specimens of the fish 200-300gm in weight and 20±2 cm in length were procured from State Fishery Department, Kishanpura (Rohtak). Prior to experimentation fish were disinfected with KMNO₄.
**PLAN OF WORK**

Acute Toxicity Test

Fingerling 2-5 gm

- Fry 0.3 gm
- Adult 200-300

**LC₅₀ of Cu and Zn for 96 hr**

Probit Mortality Curve

**FLOW SHEET-3.2**
solution and were maintained for 15 days in laboratory condition in well aerated tap water which was renewed after every 24 hrs. Thereafter, fishes were acclimated to experimental containers for a week. During the period of maintenance and acclimation fish were fed with rice bran, ground nut oil cake and fish meal in the ratio 1:1:1. Food was given at least one hour prior to the replacement of tank water. Feeding was discontinued 48 hrs prior to the commencement of all experiments so as to keep the experimental animals in same metabolic condition and to minimize possible effect on measured parameters during experiment. Fish belonging to either sex were used.

Toxicants Used

In the present study two metals, copper and zinc were selected for toxicity tests. Inorganic salt of heavy metals namely cupric sulphate \((\text{CuSO}_4 \cdot 5\text{H}_2\text{O})\) and zinc sulphate \((\text{ZnSO}_4 \cdot 7\text{H}_2\text{O})\) were used and obtained from SISCO research laboratory Pvt. Ltd. Mumbai with following physical properties.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Copper ((\text{CuSO}_4 \cdot 5\text{H}_2\text{O}))</th>
<th>Zinc ((\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula</td>
<td>(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})</td>
<td>(\text{ZnSO}_4 \cdot 7\text{H}_2\text{O})</td>
</tr>
</tbody>
</table>
Molecular weight | 249.68 | 287.55
--- | --- | ---
Color | Blue crystalline | White powder
Purity | 98.5% | 99%

Reason for selection
Both metals are present in agricultural run off, sewage sludge and industrial wastes.
Copper is used as algaecide in fishponds.

**Determination of LC$_{50}$**

Preliminary toxicity tests were conducted under laboratory conditions to determine the LC$_{50}$ value for 96hrs of copper and zinc according to APHA (1985). Stock solutions of copper and zinc were prepared. Working solutions of the two metals were prepared by simple dilution technique to obtain the desired degree of concentration based on the progressive bisection of interval on the logarithmic scale, as given by Duodoroff et al., (sited in Standard Methods, 1951).
The test fish were examined carefully for pathological symptoms and were transferred from the acclimation tanks to experimental containers. Requisite quantity of water was added to each container ensuring the availability of at least one-liter water for each gram biomass of the fish. From the stock solution of both copper and zinc, a measured quantity was added separately in each test container. Thus there were two sets, one for copper and other for zinc testing. All the precautions prescribed in the ‘Standard Methods’ were followed. Hundred healthy fishes (fry 2±0.6mg, fingerling 50±9mg and adult 180±10 in weight) were divided into 10 groups of 10 each for acute exposure. Fish in batches of 10 each were transferred to glass aquaria 4”x3”x2” size and 400 cubic meter volumes for 96 hr to different toxicant concentrations, since 96 hr is often a convenient and useful exposure duration. No food was provided during the exposure period. Water was renewed after every 24 hr. After 96 hr exposure, the mortality of the fish at each concentration was recorded.

Whenever mortality occurred in the control experiments due to some other reason, a correction was made in the experimental values by following Abbot’s formula:

\[
CM = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100
\]
Where CM = Corrected mortality

The mortality of fish was recorded at the end of 24, 48, 72 and 96 hrs of exposure to each concentration of copper and zinc separately. The fish were considered dead when respiration ceased and they did not respond on being prodded with a glass rod. Any alteration in behavior of the fish due to exposure to toxicants was also recorded. The LC50 values for 96 hr were obtained by Probit analysis method of Finney, (1971).

The percent mortality when plotted against log concentration separately for copper and zinc yielded sigmoid curve. To obtain a straight-line plot, the percent mortality was transformed into probit mortality and graphs were plotted against the respective concentrations.

Chronic exposure

Determination of Sub lethal Concentration

To observe the chronic effect of copper and zinc, six different sets of experiments were designed. 1/10th and 1/20th concentrations of 96 hours LC50 values were selected as the sublethal concentrations. Sets were designed as follows:
Chapter-3: Materials and Methods

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<tr>
<td>A</td>
<td>Control</td>
</tr>
<tr>
<td>B</td>
<td>1/10th of LC50 of Cu</td>
</tr>
<tr>
<td>C</td>
<td>1/10th of LC50 of Zn</td>
</tr>
<tr>
<td>D</td>
<td>1/10th of LC50 both Cu and Zn</td>
</tr>
<tr>
<td>E</td>
<td>1/20th of LC50 of Cu + 1/10th of LC50 of Zn</td>
</tr>
<tr>
<td>F</td>
<td>1/10th of LC50 of Cu + 1/20th of LC50 of Zn</td>
</tr>
</tbody>
</table>

Fishes were regularly checked for infection, disease and other unhealthy conditions during entire tenure of experimentation. Fishes were exposed for a period of 60 days. After every 24 hrs water was renewed and fresh toxicants added. At the expiry of each experimental period, control and exposed fishes were processed simultaneously for
metal uptake, biochemical and enzymological studies in different tissues viz. muscle, liver and kidney.

Estimation of Copper and Zinc in Fish Tissues

For determination of copper and zinc content of fish tissues, muscle, liver and kidney were excised from control and exposed groups of 6 different sets. Each tissue was digested with a mixture of Conc. HNO₃, Conc. H₂SO₄ + 70% perchloric acid in 2:1:0.5 ratios on a hot plate until dissolved. The digest was then diluted with double distilled deionized water and the level of copper and zinc in different tissues of the fish was determined by AAS (Hitachi model Z-6100) as described for water analysis.

Biochemical and Enzymological Methods

Total Protein

The method of Lowry et al (1972) was adopted for the estimation of total protein using bovine serum albumin as standard. 10% w/v homogenate was prepared in phosphate buffer saline. The homogenate was centrifuged at 1000x RPM for fifteen minutes. The supernatant fluid was collected and utilized for soluble protein and residue was used for insoluble proteins. To 100μl of supernatant, 2.9
**PLAN OF WORK**

Chronic Toxicity Tests

*Catla catla 200-300 gm*

- Gp. A: Control
- Gp. B: 1/10<sup>th</sup> LC<sub>50</sub> Cu
- Gp. C: 1/10<sup>th</sup> LC<sub>50</sub> Zn
- Gp. D: 1/10<sup>th</sup> Cu & Zn
- Gp. E: 1/20<sup>th</sup> Cu + 1/10<sup>th</sup> Zn
- Gp. F: 1/20<sup>th</sup> Cu + 1/20<sup>th</sup> Zn

Biochemical Estimation for 15, 30, 60 days

- Protein
- Carbohydrate
- Lipid

Liver

Muscle

Kidney

*FLOW SHEET- 3.4*
ml distilled water and 1ml 24% TCA was added and centrifuged at 12000 RPM for 15 minutes. The supernatant was discarded.

To both the residues obtained 1.5ml of reagent C (Reagent A- 2% Na\textsubscript{2}CO\textsubscript{3} in 1N NaOH; Reagent B- 5% CuSO\textsubscript{4} + 1% C\textsubscript{4}H\textsubscript{4}KNaO\textsubscript{6}.4H\textsubscript{2}O in 1:1; Reagent C-; 50:1 A and B respectively) were added and 150\textmu{l} of Folin's reagent (1:1 dilution with DW). After mixing well, the mixture was allowed to stand for 30 minutes. The OD was measured at 570nm.

**Total Carbohydrates**

Total carbohydrates were estimated by the method of Folin and Wu (1920). The tissue was hydrolyzed by keeping in a boiling water bath for 3 hr. with 5ml of 2.5 N HCl and cooled to room temperature. The mixture was neutralized with solid sodium carbonate until effervescence ceased. It was made up to 100ml and centrifuged at 1000 RPM for 10 minutes. The supernatant was collected and used for carbohydrate estimation. To 0.5 ml of the supernatant, 2 ml alkaline copper reagent was added and heated for 20 minutes in a boiling water bath, cooled rapidly and then 1ml of phosphomolybdate reagent was added to the solution and blue color was read at 430 nm.
**PLAN OF WORK**

Chronic Toxicity Tests

Catla catla 200-300 gm

Gp. A  
Control

Gp. B  
1/10th LC₆Cu

Gp. C  
1/10th LC₆Cu & Zn

Gp. D  
1/10th LC₆Z

Gp. E  
1/20th Cu + 1/10th Zn

Gp. F  
1/20th Cu + 1/10th Zn

Enzymological Estimation  
15, 30, 60 days

Glucose-6-phosphatase

LDH  SDH  GOT  GPT

Fructose-1,6-diphosphatase

Liver  Muscle  Kidney

**FLOW SHEET: 3.3**
Total Lipids

The lipid content was determined by adopting the method of Folch et al. (1957). Known quantity of the tissue was placed in an oven at 60°C for dehydration until the weight became constant. The dehydrated tissue was then transferred to a filter paper and was introduced into the fractionating column of Soxhlet extractor of 100 ml capacity. For the extraction of lipids, chloroform was used as the solvent. The flask containing chloroform was heated in a water bath for 6 hrs until the chloroform became colorless. The lipid free tissue was placed in an oven at 47°C to evaporate chloroform until the weight became constant. The difference between initial and final weight of the sample gave the lipid content of the tissue.

Enzyme Assays

For the estimation of the activity of different enzymes, muscle, liver and kidney were immediately removed from the control and exposed groups of fishes. The tissues were separated from the adjoining tissue and blotted free of blood with filter paper. The tissues were weighed to the nearest milligram and homogenized in cold. The homogenates were adjusted to 10% (w/v) strength with 0.25M chilled sucrose solution. The homogenates were centrifuged under refrigeration at 1000×g for 20 minutes and the clear supernatant fluids were used as the source of enzyme.
Glucose-6-phosphatase

The enzyme activity was determined by adopting the method of Swanson (1955). The substrate used was sodium salt of glucose-6-phosphate. The incubation mixture consisting of 0.1ml of 0.1M substrate solution and 0.3ml of citrate buffer (pH 6.6) was maintained in a water bath and 0.1 ml of homogenate was added. The incubation time was 30 minutes at 23°C. The activity was stopped by the addition of 1.0 ml of 10% TCA. The mixture was chilled in ice and the inorganic phosphate was estimated by the method of Fisk and Subba Rao (1925).

Fructose-1, 6-diphosphatase

The enzyme activity was determined by the method of Sadao and Shimeno (1982). The substrate used was sodium salt of Fructose-1, 6-diphosphate. 1.8ml of substrate mixture containing 2 μM Fructose-1, 6-diphosphate, 10 μM of Mg Cl₂ and 45 ml of veronal buffer (pH 7.0) were preincubated at 23 °C. The mixture was stirred occasionally. The activity was stopped with 1.0ml of 10% TCA. For control, TCA solution was added to the incubation mixture prior to the addition of enzyme extracts.

The mixture was chilled in ice and inorganic phosphate was estimated by the method of Fiske and Subba Rao (1925).
Dehydrogenases

The activity of LDH and SDH enzymes was estimated by triphenyl tetrazolium chloride method of Srikantan and Krishnamoorthy (1955). 0.5M solution of sodium lactate and sodium succinate were used as substrates for LDH and SDH respectively. The incubation time was 1 hr at 23°C. The reaction was stopped by the addition of 4.0 ml of glacial acetic acid. Formazan formed was extracted with xylene at 0°C by keeping overnight in a refrigerator. The optical density of the colored xylene layer was read at 495nm. The enzyme activity was expressed in μg formazan formed per mg of protein per hour.

Transaminases

The activity of GOT and GPT were estimated by the method of Bergmeyer (1974). To 1.0ml of buffer substrate solution (0.1M phosphate buffer, pH 7.4, 0.1M aspartate for GOT and Dl- alanine for GPT, 2mM of 2 oxoglutarate) 0.2ml of homogenate was added and mixed thoroughly. The incubation time was 30 minutes for GOT and 60 minutes for GPT respectively at 23°C. To this 1.0ml of 1mM 2-4 dinitrophenylhydrazine (DNPH) was added and after 20 minutes at room temperature, 10.0ml of NaOH solution was added. The color was recorded at 550 nm after an interval of 5 minutes.
Phytoremediation

Fresh *Lemna minor* and *Azolla pinnata* plants were collected from wastewater drains and small ditches in front of sports complex of M.D.University, Rohtak respectively. The *Lemna minor* and *Azolla pinnata* were acclimated for 7-8 hr to the laboratory conditions and after acclimation these were maintained in 3% Hoagland solution at 25±0.5°C and 16 hrs light and 8hr dark photoperiods were provided.

The experiments were carried out in PVC coated (4"X2"X3") cement tanks located outside the animal house of M.D.University, Rohtak. Initially the tanks were filled with fresh water supplied by the university water works and allowed to stabilize for three days before the commencement of the experiments. A stock solution of copper sulphate and zinc sulphate containing 100mg/l of copper and zinc were prepared and diluted to the desired concentration (0.23 ppm for copper and 0.90 ppm for zinc) by adding the calculated volume of the stock solution to 250 liter of water in tanks. Experiments were carried out to test for the removal efficacy of *Lemna minor* and *Azolla pinnata* (wet and dry) by adding different quantities ranging from 0.5g/l to 5.0g/l to 250 l. of water in the tanks. The experiments were carried out for 96hr. After every 8hr, 100ml of the samples from the tanks were withdrawn and concentration of copper was determined by Hitachi Z-6000 atomic absorption spectrophotometer at 324.8nm
wavelength using air- acetylene gas mixture as fuel. Standard metal solution was used for calibration purpose.

**Exposure of Test Fish**

Healthy living specimens of the fresh water fish *Catla catla* were collected from the local ponds of Rohtak. The fishes were maintained in glass aquaria. Artificial aeration was also provided, whenever necessary. Preliminary toxicity tests conducted earlier have shown that 0.32ppm/l of copper and 0.65ppm/l of zinc were the 96hr LC$_{50}$ to the test fish. Fishes in batches of 10 each were transferred to six glass aquaria and exposed with *Lemna minor* and *Azolla pinnata* (1.0g/l) separately for 96hr. Six different concentrations of copper between 0.30 and 0.55 ppm were tested. After 96hrs exposure, the mortality of fish at each concentration was recorded. The obtained data, like number of fish dead, concentration of chemical, percent mortality and probit mortality were statistically analyzed by probit method of Finney (1971) to compute the LC$_{50}$ value.

**Adsorbents**

The experiments were carried out in large PVC coated (4"× 2" × 3") cement tanks located outside the animal house of M.D. University, Rohtak. Samples of the adsorbents i.e. sugarcane bagasse, sugarcane leaves, rice husk, wheat husk, groundnut husk, etc. were collected from the adjacent local agricultural fields in Rohtak district.
adsorbents were washed with de-ionized water and dried at room temperature. Dry adsorbents were crushed in a mixer to convert them into small uniform sized particles so as to increase their surface area.

Initially the tanks were filled with tap water (Copper 0.08±0.003ppm and zinc 4.15±0.33ppm) and allowed to stabilize for 3 days before the starting of the experiment. Water temperature was 25±3°C, pH 7±0.3, hardness 170±10mg/l, alkalinity 90±8mg/l, and DO 7.6±1mg/l. The concentration of copper and zinc were not in detectable limits in the waste. A stock solution of copper sulphate and zinc sulphate containing 100mg/l of copper and zinc were prepared and diluted to the desired concentration (0.23, 0.56mg/l) by adding the calculated volume of the stock solution to 300-liter water in the tanks. Experiments were carried out to test for the adsorbing efficacy of the biological wastes by adding different quantities ranging from 0.5g/l to 5g/l to 300/l water in the tanks. The experiments were carried out for 96 hrs. After every 8-hrs 100ml of the water from the tank was withdrawn from surface and concentration of copper and zinc were determined by Hitachi Z-6000 Atomic Absorption Spectrophotometer at 324.4nm wavelength. Standard metal solutions were used for calibration purpose.

Adsorption on Activated Carbon
The experiments were conducted under the same conditions as in the adsorption by natural wastes. These experiments were done to test the adsorbing efficacy of the activated carbon by adding different quantities ranging from 0.1 g/l to 0.7 g/l by mixing with different quantities of natural wastes to 300-liter of water in the tanks for 96 hrs. After every 8 hrs, 100 ml of the sample from the tanks were withdrawn and the concentration of copper and zinc were determined by Hitachi Z-6000 Atomic Absorption Spectrophotometer.

Exposure of Test Fish
Healthy living specimens of the fresh water fish *Catla catla* were collected from the local ponds of Rohtak. The fishes were maintained in glass aquaria (4"x 3"x 2" size and 400 cubic meter volumes) with 10 fishes in each batch. LC50 of copper and zinc with different agricultural wastes separately for 96 hrs to six different concentrations were determined, since 96 hrs is convenient and useful exposure duration. Water is renewed after every 24 hr no feeding is done during the exposure period. After 96 hr exposure, the mortality of fish at each concentration was recorded. The obtained data, like number of fish dead, concentration of chemicals, percent mortality and probit mortality were statistically analyzed by probit method of Finney (1971) to compute the LC50 value.
Protein Gel Electrophoresis

Preparation of Samples

The protein content of the muscle and liver was quantified by a standard method (Lowry et al, 1971). The sample was then subjected to SDS-PAGE under 6% stacking gel and a 10% separating gel (Laemmli, 1970). Electrophoretic study of protein pattern in muscle and liver of *Catla catla* was carried out as mentioned below.

Preparation and Casting of Gel

The gel plates were assembled according to the manufacturers instructions and the volume of the gel mould was determined in a conical flask. The acrylamide mixture for 10% resolving gel was prepared by mixing 10 ml acrylamide + 7.5 ml resolving buffer, pH 8.8 +12.3 ml distilled water +150 μl ammonium per sulphate (freshly prepared10% stock) and 50μ1 TEMED (N, N, N/ Tetra Methylene Diamine).

The components were mixed and without delay the acrylamide mixture was poured into the glass mould till the lower mark. A layer of distilled water or isopropyl alcohol was overlayed to facilitate proper polymerization. After polymerization (30 minutes) the layer was removed and the gel top washed with distilled water.
Stacking Gel

6% stacking gel was prepared using 2 ml acrylamide (30% stock) + 3 ml stacking gel buffer 1 (pH 6.8) distilled water + 75 μl ammonium per sulphate (10% stock) + 25 μl TEMED. The components were mixed properly and poured over the resolving gel. The comb was immediately inserted and the gel was allowed to polymerize.

Loading of Sample

Samples were prepared using required volume of sample (100μg protein/lane) plus equal volume of sample buffer (7.25 ml distilled water + 1.25 ml stacking gel buffer + 1ml glycerol + 0.5 ml β-mercaptoethanol + 150 mg SDS and a pinch of bromophenol blue).

The samples were heated in boiling water bath for 2 minutes to denature the protein and kept on ice to retain the denatured stage. The comb was then removed from the mould and the wells were washed with distilled water. The gel was mounted on electrophoretic apparatus. Electrophoretic buffer (Tris 3 gm, glycine 14.4 gm and SDS 1 gm in 1000 ml distilled water, pH 8.3) was added to the top and bottom reservoir of the electrophoretic apparatus. The samples were loaded along with marker into the lanes/wells.
Electrophoresis

The apparatus was connected to power supply unit. A current of 8v/cm for gel (70v) and 15v/cm for resolving gel (150-200v) was applied. The electrical contact between the two buffer tanks was through the slab gel. Care was taken to avoid air bubbles, while adding electrode buffer in the tanks as the air bubbles inhibit electrophoretic mobility.

The gel was run until the bromophenol blue dye reached the bottom of the resolving gel. The power supply was turned off and the gel was removed from the sand witched plates from the apparatus and placed on a paper towel.

The plates were removed using a spatula and the orientation was marked. The temperature for electrophoresis was kept constant in an air-conditioned room at 25°C.

Staining and Distaining

This gel was immersed in 5 volumes of staining solution (200mg Coomassie Brilliant Blue R, 250+ 50 MeOH + 7ml acetic acid solution +63 ml distilled water) every half an hour. This was followed by two to three washes. The gel was then stored in 7% acetic acid solution.
When visualized under the illuminator, the protein zones were visible as blue bands after 24-48 hr of destaining. The results were recorded by observing the relative electrophoretic mobility of protein zones for each sample and the run repeated for the samples that did not show clear-cut zones. The protein profiles of the gel obtained are manually observed and compared with the various protein bands in the standard, control and the experimental samples subsequently treated with staining and de-staining solution and identified the additional bands with varied KDa protein.

**Densitometric analysis**

In view of determining the quantity of the proteins present in the SDS gel, the gel was placed under the densitometer and the number of protein bands and their densities were measured with LKB Ultrascan Model 2202 Scanning Laser Densitometer.

**Calculation of Relative Mobility (Rm) Values**

Calculations of Relative Mobility (Rm) values were made from the stained gels. The distance migrated by each protein band and the indicator dye was measured and Rm value of each band was calculated using the following formula;
Distance migrated by protein band

\[ R_m \text{ value} = \frac{\text{Distance migrated by protein band}}{\text{Distance migrated by Bromophenol blue}} \]

The observed protein fractions were tentatively designed as band 1, 2, 3... in increasing order of their mobility. Electrophorogram and the densitometry curves depicting the number of protein bands were prepared for tissues.

Statistical Methods

Standard deviation

\[ S = \sqrt{\frac{\sum Y^2 - (\sum Y)^2}{N}} \]

\[ \text{Standard error} \]

\[ \text{SEM} = \frac{S}{\sqrt{N}} \]

The standard form of the mean is a measure of the reliability of the mean calculated from a set of observations. \( S \) is the standard deviation, \( N \) is the number of observations.
Test of significance

Student's t - test was employed to calculate the significance of the difference between control and experimental mean and 'P' values of 0.05 or less were consider significant. Gossett, W.S (1985) gave a formula.

\[
T = \frac{(\bar{Y}_2 - \bar{Y}_1) \sqrt{N}}{\sqrt{(S_1)^2 + (S_2)^2}}
\]

In case where the numbers of observations in two groups were different, the formula followed was:

\[
T = \frac{(\bar{Y}_2 - \bar{Y}_1)}{S \sqrt{1/N_1 + 1/N_2}}
\]

Analysis of variance was applied to compare "between groups variation" and "with in variation". Variance is defined as the sum of squares divided by degrees of freedom.

\[
\text{Variance (S2)} = \frac{Y^2 - (Y^2)}{N - 1}
\]

The ratio between group variance divided by the within group variance gives the value of 'F' at variance ratio. The calculated value of 'F' is then compared with the tabulated of 'F' at P =5% level or P =1% level.
Coefficient of variation  [C V]

It is used to compare the variability of one character in two different groups having different magnitude of valued or two characters in the same groups by expressing in percentage. If coefficient of variation is greater for a sample (group) then that groups said to more variable or conversely less consistent, less uniform, less stable and less homogenous. On the other hand, the series for which coefficient of variation is less is said to be less variable or more consistent, more stable and more homogenous.

\[
CV(\%) = \frac{\sigma \times 100}{X}
\]

Where \( \sigma \) = Standard deviation

\( X \) = Mean