CHAPTER-II

MATERIAL AND METHODS
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Bioassay tests were employed in the present investigations for lethal and sublethal toxicity tests. It is the procedure in which responses of organisms are used to detect or quantify the damage effect of toxic chemicals alone or in combination for toxicity evaluation (APHA, 1998). Hence, this test is considered as one of the fundamental yardsticks for the toxicity studies in the organisms. The potential effects of toxic chemical on aquatic organisms can be assessed with this test. Further, it provides a direct and conclusive result when compared with the physical and chemical quantification method.

Apart from bioassays, O₂ consumption, NH₃-N excretion and behavioral studies were also employed to evaluate the effect of cadmium and cadmium with chelating agents (EDTA and NTA) on fishes.

Experimental fishes:

The fishes under consideration for the toxicity tests are exotic fishes of Poeciliid family. They constitute: 1) Gambusia affinis (Baird & Girard, 1854), commonly called as Mosquito fish and 2) Poecilia reticulata (Peters, 1859), commonly called as Guppy. Both are small larvivorous fishes, employed throughout the world for mosquito control programs (WHO, 1982; Singaravelu, et al., 1997). Both the species can thrive well even in absence of mosquito larvae and pupae as a source of food in nature as well as in laboratories. In India they are found in ponds, well, pools, tanks, swamps and shallow rivulets. They need no special habitats for oviposition since they are ovo-viviprous. Both of them are hardy fishes and the range of resistance to water temperature fluctuation and
the change of quality of medium for the survival are quite wide (WHO, 1982). They are widely distributed through the world (Krumholz, 1948) and considered to be the most widely disseminated natural predator in the history of biological control (Wilson, 1965 and Garcia, 1983). Both the fish species exhibit a strong sexual dimorphism. The physical characters of these fishes are mentioned in Table-2.1.

Collection and maintenance of fish:

The fishes of both the species were collected from same locality with dip netted from a local ponds and acclimated to the laboratory condition for at least two weeks in glass aquarium, filled with dechlorinated tap water and fed with high grade commercial flake fish food ad lebitum. During the first week of acclimation both the species were separated and sexed macroscopically and maintained is separate aquaria. Every care was taken with keen observations to prevent infection and overcrowding of fishes. Only healthy fishes were selected in the second week of acclimation for experiments.

Collection of Fries:

A single large gravid female of specific species (either G. affinis or P. reticulata) was kept in a rectangular plastic net partially submerged in the center of aquarium filled with tap water. The net was supported from the sides of aquarium in such way that the distance between wall of net and peripheral wall of aquarium was 2.5 inches and the distance between floor of net and base of the aquarium was 5 inches. This allows a free movement of gravid female only in the center of aquarium surrounded by the walls of net, whereas the new born fries can move in all the directions by penetrating through the
holes of net. This strategy helps the prevention of cannibalistic attack of mother on its fries. The fries were collected and kept in a separate aquarium after recording their birth time and day. The care was taken not to over feed them. The water medium was changed on alternative days through siphoning with a narrow plastic pipe covered with a fine net on its tip. The fries were retained in the aquarium within a little quantity of water (free from debris of food and fecal matter) while removing the water medium through siphoning. The fresh water was released into the aquarium very gently. This was done to prevent the handling stress and physical disturbance to fries. The healthy fries were later used for lethal toxicity test.

Test medium:-

All the experiments were conducted in dechlorinated stored tap water. The physico-chemical characteristics of water medium were analysed prior to and after the test as per the procedures recommended in APHA, (1998). The details of water quality are given in Table: 2.2.

Preparation of stock solutions:-

1) Cadmium :- 10g of cadmium chloride salt (CdCl$_2$.2 $\frac{1}{2}$ H$_2$O; Mol. Wt.:- 228.35) was dissolved in 100 ml of deionized distilled water. The contribution of cadmium metal in the salt compound is 4.923 mg and 5.077 mg is from Cl$_2$ and 2 $\frac{1}{2}$ H$_2$O per 100 ml of distilled water. Therefore, 1 ml of stock solution = 10mg of total salt solution = 4.923 mg of cadmium metal in stock solution. The toxicity was calculated on the basis of Cd metal alone throughout the experiments.
Table 2.1: Morphology and peculiar characters of experimental fishes.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Sex</th>
<th>Length (cm)</th>
<th>Weight (mg)</th>
<th>Colour</th>
<th>Distinguish characters</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gambusia affinis</em></td>
<td>Male</td>
<td>2.0—2.60</td>
<td>120—200</td>
<td>Bluish grey</td>
<td>Presence of gonopodium (a modified anal fin).</td>
</tr>
</tbody>
</table>
|                    | Female    | 2.4—3.50   | 280—450     | Bluish grey        | a) Presence of anal gravid black spot, which disappears soon after parturition of fries but reappears gradually after 1-2 weeks.  
|                    |           |             |             |                    | b) Highly cabalistic-Immediately try to feed on its own fry at every single release of fry.   |
|                    | Fry       | 0.75—0.90  | 36—45       | Bluish grey        | Presence of prominent large eyes, elongated body with blunt anterior and pointed posterior. |
| *Poecilia reticulata* | Male     | 1.6—2.40   | 90—160      | Multicoloured body and fins | Presence of gonopodium (a modified anal fin)                                           |
|                    | Female    | 2.20—3.50  | 210—450     | Yellowish grey     | a) Presence of anal gravid black spot, which reduces size soon after parturition, but never disappears completely throughout its life.  
|                    |           |             |             |                    | b) Moderate cannibalistic-Sometimes mother spares its fries from consumption even after the parturition of all the fries.   |
|                    | Fry       | 0.75—0.90  | 36—45       | Yellowish grey     | Presence of prominent large eyes, elongated body with blunt anterior and pointed posterior. |

Table 2.2: The details of water quality for the test medium are as follows:-

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>RANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature:-</td>
<td>23 – 28°C</td>
</tr>
<tr>
<td>pH :-</td>
<td>6.5 – 7.6</td>
</tr>
<tr>
<td>Total hardness:-</td>
<td>60 – 102 (as CaCO₃ mg/l)</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg/l):-</td>
<td>6.5 – 7.5</td>
</tr>
<tr>
<td>Dissolved CO₂ (mg/l) :-</td>
<td>0.5 – 1.9</td>
</tr>
<tr>
<td>Chlorine (mg/l) :-</td>
<td>Nil</td>
</tr>
</tbody>
</table>
2) EDTA (Ethylene diaminetraacetic acid) :- The commercial compound of EDTA i.e. Na₂EDTA with Mol. Wt.: 372.24 (From S.D. Fine Chemical Ltd. Analytic grade) was used throughout the experiments. 3.720 mg of Na₂EDTA was dissolved in 100ml of de-ionized distilled water as a stock solution.

1 ml of stock solution = 37 mg of Na₂EDTA

The Molarity conversion of solution in the test medium is as follow:

1 M. of Na₂EDTA = 372.24 g/l
1.0⁻⁴ M. of Na₂EDTA = 37.224 mg/l
1.0⁻⁵ M. of Na₂EDTA = 3.7224 mg/l

3) NTA (Nitrilotriacetic acid):- The commercial compound of NTA with Mol. Wt.: 191.22 (S.D.Fine Chemical Ltd. Analytical grade) was used throughout the experiments.

1.912 g. of NTA was dissolved in 1N.NaOH. The dissolved NTA solution was further diluted to make 100 ml in de-ionized distilled water as a stock solution.

1 ml of stock solution = 19.114 mg of NTA

The Molarity conversion of solution in the test medium is as follow:

1 M. of NTA = 191.14 g/l
1.0⁻⁴ M. of NTA = 19.114 mg/l
1.0⁻⁵ M. of NTA = 1.911 mg/l

LETHAL TOXICITY

Static renewal bioassay tests were adopted as per the method recommended in APHA (1998) to evaluate the acute toxicity of Cadmium and Cadmium with Chelating agents (EDTA and NTA) to Gambusia affinis and Poecilia reticulata. Feeding was withdrawn
one day prior to the commencement of acute toxicity test and they were not fed during experimental period of four days.

Range finding test was conducted before the commencement of actual experiments to find the appropriate concentration of toxicant to be used for the future experiments. The experiments were set within the selected range of concentration (dose). Lethal toxicity tests were conducted duplicate with at least five toxicant concentrations and a control. Sample size was set at 10 fishes per test chamber of glass aquarium containing one litre of test medium. The ratio of the weight of the fish to the volume of the test medium was maintained at 0.8g/l. Response to a toxicant was measured on the basis of mortality due to the metal exposure. The mortality was recorded at fixed hour in morning every day till the completion of experiment. Dead fishes were removed immediately from the test medium to prevent the change in water quality for the remaining fishes in aquarium.

The concentration–mortality data obtained from the experiments were plotted on Log-probit graph and further crosschecked on Semi-Log graph. The \( LC_{50} \), factor for \( LC_{50} \) \( (fLC_{50}) \), Slope \( (S) \), factor for Slope \( (fS) \), Slope ratio \( (SR) \), factor for Slope ratio \( (fSR) \), Potency ratio \( (PR) \), factor for Potency ratio \( (fPR) \) and 95% confidence limits were evaluated statistically with the methods proposed by Litchfield and Wilcoxon (1949), and simplified method by Bengeri and Patil (1997).

The following symbols are used in the statistical evaluation of the data collected from the acute toxicity tests. -

\[
\begin{align*}
K & = \text{The number of doses plotted.} \\
LC_{50} & = \text{Median Lethal concentration.} \\
S & = \text{Slope function.}
\end{align*}
\]
\[ \text{fLC}_{50} = \text{Factor for LC}_{50}. \]
\[ \text{fS} = \text{Factor for Slope} \]
\[ \sqrt{N} = \text{Total number of animals used between 16 and 84 percent expected effects.} \]
\[ R = \text{The ratio of largest to smallest dose plotted.} \]
\[ A = \text{A value derived from S and R.} \]
\[ \text{SR} \& \text{PR} = \text{Slope Ratio and Potency Ratio respectively.} \]
\[ \text{fSR} \& \text{fPR} = \text{Factor for Slope Ratio and Potency Ratio.} \]

a) Slope function (S) was calculated by measuring the LC_{16}, LC_{50} and LC_{84} values from the Log-probit graph.
\[
S = \frac{\{LC_{84} \div LC_{50} + (LC_{50} \div LC_{16})\}}{2}
\]

b) The factor for LC_{50} (fLC_{50}) was calculated by the formula:
\[
fLC_{50} = (S)^{2.77} \div \sqrt{N}
\]
Where \( N \) is the number of animals used between 16 and 84 percent expected effects.

c) Confidence limits of LC_{50} were calculated by multiplying and dividing the LC_{50} by its factor for LC_{50} to get the upper and lower limit of 95 percent probability. Thus:
\[
\text{Upper Limit} = \text{LC}_{50} \times \text{fLC}_{50} \quad 95\% \text{ Probability.}
\]
\[
\text{Lower Limit} = \text{LC}_{50} \div \text{fLC}_{50}
\]
d) The dosage range was estimated as follow:
\[
R = \text{Largest} \div \text{Smallest dose plotted.}
\]
e) Using the value of R and S, the value designated as A was read from the Nomograph No.3 by laying the straight edge across the correct scale values.
f) Factor for Slope was estimated by the following formula:

\[ fS = A^{10(K-1)/\sqrt{N'}} = A^{\text{exponent}} \]

Where \( K \) = the number of doses plotted.
\( N' \) = Total number of animals used between 16 and 84 percent expected effects.

With this exponent and the value of \( A \), the factor for slope can be read from the Nomograph No.2.

g) Confidence limits of slope were calculated using the formula:

\[
\begin{align*}
\text{Upper Limit} &= S \times fS \\
\text{Lower Limit} &= S \div fS
\end{align*}
\]

95% Probability.

h) The significance of difference between any two estimated LC\(_{50}\) value was calculated as follows:

\[
\text{Potency Ratio (PR)} = \frac{\text{LC}_{50,1}}{\text{LC}_{50,2}}
\]

Where LC\(_{50,1}\) is the larger value.

i) Factor for Potency Ratio was read from the center scale of Nomograph No. 4 using \( f\text{LC}_{50,1} \) and \( f\text{LC}_{50,2} \).

The difference was considered significant if the PR value exceeded the \( f\text{PR} \).

j) Confidence limits of Potence Ratio was calculated as follows:

\[
\begin{align*}
\text{Upper Limit} &= \text{PR} \times f\text{PR} \\
\text{Lower Limit} &= \text{PR} \div f\text{PR}
\end{align*}
\]

95% Probability.

k) Slope Ratio was estimated as follows:

\[ \text{SR} = S_1 - S_2 \]

Where \( S_1 \) is the larger value.
j) Factor for Slope Ratio was read from the center scale of Nomograph No.4 using $f_{S_L}$ and $f_{S_2}$.

The deviation between parallelism of two lines was considered as significant if the value of SR exceeded that of fSR. If SR is less than fSR, then the curves may be considered parallel within the experimental error.

SUBLETHAL TOXICITY

Sublethal concentration of cadmium was fixed at 1/10 of their 96hr LC$_{50}$ values. The chelating agents were also fixed at 1/10 of concentration treated on lethal toxicity. The duration of exposure was fixed at different interval of days up to 30days (as the longest exposure day). Change in the oxygen consumption rate, NH$_3$-Nitrogen excretion, bioaccumulation of cadmium, preference and avoidance behavior of fishes was studied at different time intervals.

OXYGEN CONSUMPTION

Modified Winkler's Azide method (APHA, 1980) was employed in the present investigation to measure the dissolved oxygen in test medium. In this method one molecule O$_2$ $\equiv$ 2 molecules of iodine produced at the end of the reaction in the bottle. Therefore, 1 ml of standard Sodium thiosulphate (0.025N)= 0.2 mg of dissolved oxygen in 200 ml of water.

Therefore dissolved oxygen mg/l = 0.2 x 5 $\equiv$ 1mg/l

Hence, the burette reading directly gives the amount in weight of oxygen dissolved.

Closed chamber method (Patil and Kaliwal, 1983) was adopted for the measurement of oxygen consumption. The fishes were confined in a fixed volume of water in the
respiratory bottles for a particular length of time. The volume of water was in proportion to size and weight of fish. For each set of experiment a parallel control with fish (without any addition of the toxicant) and a blank was set. Blank was used to deduct the oxygen consumption of microorganisms and other oxidizing materials.

Fishes were transferred to respiratory bottles containing the same concentration of toxicant to which they were exposed. After a known interval of time the dissolved oxygen of the water in the bottles was estimated. Similarly the dissolved oxygen content of the blank, dissolved oxygen of the water in which the experimental fishes were kept, from these the amount of oxygen consumed by the test fishes was calculated. The weight of the control and the exposed fishes was noted and $O_2$ consumption was expressed as mg of $O_2$/h/g body weight. The consumption rate of control was taken as 100% (normal rate). The consumption rate of toxicant exposed fishes was represented as percent (increase/decrease) of control. At least six fishes were studied at each experimental condition in one toxicant group.

**AMMONICAL NITROGEN EXCRETION**

Nesslerization method (APHA, 1980) was employed in the experiments to determine the effect of the cadmium on the rate of total ammonical Nitrogen (NH$_3$-N) excretion. The graduated yellow to brown colour produced by the Nessler-ammonia reaction absorb strongly over a wide wave length range. The reddish brown colour characteristic of ammonia nitrogen concentration can be measured with acceptable sensitivity on the wavelength range from 450-500nm when 5 cm light path is available. The absorbance or transmittance was measured with Klett-Summerson colorimeter. Calibration curve was
prepared from the standard solution of ammonium chloride with 1.0 ml = 10 mg, N = 12.2 mg of NH$_3$-N concentrations.

Estimation of ambient total Ammonia - Nitrogen as a factor of nitrogen excretion was adopted from Brett and Zala (1975). The fishes were confined to a fixed volume of water in experimental jars for a particular length of time. The volume of water was in proportion to the size and weight of the fish. For each set of the experiments a parallel control with fish and a blank were set. Blank was used to deduct the nitrogen excretion of micro-organism. After a known interval of time the Ammonical-Nitrogen was measured at 435 to 480 nm using blue filter in colorimeter. Standard ammonium chloride was used as reference standard. The difference in concentrations of Ammonical – Nitrogen excretion between the test group and the mean values of control group of each set solution at the end and the beginning of the experiment and its intervals was converted to Ammonia – Nitrogen excretion (µ mol/g/h) by multiplying the values of the water volume and then dividing the result by w/v/g and time lapsed hr. The excretion rate of control was taken as 100% (normal rate). The excretion rate of toxicant exposed fishes was represented as percent (increase/ decrease) of control.

**BIOACCUMULATION**

Van Hoof and Van San (1981) and APHA (1980 and 1998) were employed with slight modification for the estimation of cadmium content in the whole body of the fishes in both acute and chronic exposed cadmium and cadmium with chelating agents.

The fishes at the end of 96h period (for acute toxicity), 5, 11, 21, and 31 days (for chronic toxicity) exposure were sacrificed, rinsed in distilled water and swapped with blotting paper to remove the adhering water on body surface. The fishes were weighted
on digital single pan balance for wet body weights observation and dried them in oven for 48hr. The oven-dried fish was weighted again and record the dry weight. This revealed the loss of water through oven drying. The known weight dry fish was transferred into 100 ml clean (acid washed) beaker and to this 2.5 ml of concentrated HNO₃ (14N Analytical grade) was added and gently shaken till the entire fish was dissolved in acid. On that solution 7 ml of 30% Hydrogen peroxide was added. The sample solution was gently shaken briefly at an interval of 2 to 3 hrs and kept for over night to prevent the over flow of solution through frothing and foaming process on reaction. The over night kept sample was gently boiled on a hot plate (using sand bath). The digestion was continued until a white residue remained in the beaker. The digested residue was diluted with 2% Nitric acid to 50 ml and filtered through Watman filter paper No.2 into a clean (acid washed) sample bottle for further process for metal analysis.

Atomic Absorption Spectrophotometer (Spectra AA 20 model) values were recorded as µg/g on dry weight basis.

**BEHAVIOURAL STUDY**

The toxicant actions are manifested with symptoms of poisoning at certain concentration or dose in organisms. In fishes them self through its sensory perceptions can detect the toxicant of certain concentrations. The symptoms of toxicant action vary with fishes. But the following successive stages are commonly observed (Metelev et al.,(1971)):-

1) Beginning of restlessness.
2) First symptoms of loss of sensitivity.
3) Stage of enhanced or deduced excitability.
4) Loss of equilibrium
5) Total loss of equilibrium, total ataxia
6) Rigor mortis.

**Preference and Avoidance to Toxicants**

Fishes are behaviorally responsive to a large variety of chemicals. Preference/avoidance studies with fish may be a sensitive method for evaluating the response of an organism to a pollutant. The behavioral response of an organism to pollutant is the initial reaction that may determine whether the organism will remain in contact with potentially toxic materials or retreat to a more diluted or less contaminated/uncontaminated area.

**Toxicant delivery system**

The continuous-flow delivery system was constructed along the guidelines of Garton (1980). Flow of the toxicant to the avoidance chamber depends upon size of delivery tube and hydraulic head of the tubes. Constant head toxicant boxes were used to maintain a constant head which supplies toxicants through restricted delivery tubes directly to the avoidance chamber. Flow was determined by restriction of the tube and concentration was adjusted in the stock solution. Constant head toxicant boxes were kept high enough to provide gravity flow from them to the avoidance chamber.

**Avoidance chamber**

Steep gradient avoidance principle (Jones, 1947) was undertaken with the modification made by Sprague (1964a & b) and Steel et al., (1990). The avoidance chamber was designed and fabricated with plexiglass. The chamber consisted of rectangular trough with a central drain at the bottom. The rectangular container was compartmentalized into six regions with opaque glasses. However, the design facilitated the fish to move into
any compartment of preference.

Toxicant solutions and fresh water entered the two ends and passed along the compartments and drained in the mixing zone. Fig. 2.1 shows the specifications of avoidance chamber and Fig. 2.2 the flow chart of the present investigation.

**Avoidance study**

Fishes were exposed to lethal and sublethal concentrations of cadmium or cadmium with chelating agents from one end and fresh water from other end. The fishes were not fed for 24 hour prior to the experiment and also during the experiment. This was meant to elicit an unambiguous response from the experimental fishes and also to observe a useful behavioral and toxicity bioassay for behavioral altering chemical.

The toxicant delivery system was set to discharge toxicant at the flow rate of 140 ml/min. 10 fishes of same sex and relatively same size were used in each study on the preference and avoidance test. Position and number of fishes were noted visually at every 30 seconds interval for the period of 30 minutes. The experiment was repeated with change in position of toxicant and fresh water delivery tubes on either sides.

**STATISTICAL ANALYSIS**

The statistical analysis of the experimental results was performed with ANOVA tests as per the procedure described else were (Zar, 1974), and application of Biostatistical Computer Programme.
A, B and C are different zones of fish mobility. B is the mixing zone.
Fig: 2:2 Flow Chart for Monitoring Avoidance of Aquatic Metal Pollution

A, B and C are different zones of fish mobility. B is the mixing zone.