Toxicity evaluation

It is true that the problem of environmental toxicity testing is quite complex because every year thousands of chemicals are being cleared for use. But it is impossible to test the toxicity of each and every product on every organism of the ecosystem. To have a proper understanding of the effect of toxic substance, the laboratory studies need to be followed by the field observations to prevent large-scale deleterious effects.

The quantitative evaluation of the biological changes caused by chemicals/metal aim at the establishment of dose-effect and dose response relationships that are of fundamental importance for risk evaluation. Toxicity is defined as any harmful effect of chemical or a drug on a test organism (NAS/NHS, 1970). The studies on toxicity evaluation triggered by eminent scientist, Henderson, (1960) and attempts of several other scientists to formed a core in this field of research (NTP, 1984).
In nature, the adverse effect of any toxic substance may be seen immediately after the release of the toxicant, if the concentration is more (acute toxicity). But the incidences of the potential deleterious effects are seen on long-term exposure of chronic toxicity.

The deleterious effects produced by chemical agents can be at the cell, molecule, tissue, organ, organism, family and population level through physiological, pathological, hematological and biochemical responses (Green, 1984).

Various methods employed for whole animal toxicity testing in animals are reviewed (Mars Sumura, 1985). The dose, time and mode of administration of chemical to an animal plays an important role in the manifestation of the toxicity.

Biological assay occupies an important position in the toxic evaluation. It is a set of techniques relevant to compare between strengths of alternative but similar biological stimulus (Finney, 1971). It refers to the response produced when doses of toxicants are given to experimental animals. The dose response relationship is an important way of measuring the toxicity. The degree of lethality for any toxic chemical to a particular animal species is represented in the form of dose mortality over a period of time.
Toxicity cannot be defined without referring to the quantity of a substance administered, the method of administration, the duration of the treatment, severity of signs and symptoms of the toxicant to aquatic animals and is usually expressed in terms of lethal concentration 50% (Mean lethal Concentration).

The LC$_{50}$ has been defined as "a statistically derived expression of a single concentration of material that can be expected to kill 50% of the test organisms". The most commonly used methods for calculation of LC$_{50}$ are the graphical method, regression analysis and estimation of confidence limits as proposed by Finney (1971). Several types of toxicity testing procedures have been developed which include acute, sub acute and chronic studies. Major differences between these tests happen to be the dose employed and the length of exposure to the chemical agents. The acute toxicity has been defined as the adverse effects occurring within a short time of administration of single dose or multiple doses given within 24 hrs (Hagan, 1959). The lethal concentration is expressed as mg/l/day. The sub acute toxicity is similar to that of chronic concentration of the chemical where in the concentration is low with a long period of exposure.

Several factors like, chemical, composition, choice of vehicle, impurities and stability of technical concentration, volume of chemical, route, time of administration, sex, age, body weight, maturity, nutritional status, species, strain and environmental factors such as temperature, humidity, housing animals play a major role in influencing the toxicity of a chemical.
In the present investigation evaluation of toxicity of CdCl₂ in fish (Oreochromis mossambicus) was done by a rapid approximate method (1983) for LC₅₀ values. This preliminary work was found necessary to choose the appropriate sub acute dose for long-term response of CdCl₂. Analysis of effects of sub acute exposure of fishes to sub lethal concentration CdCl₂ is interesting and of importance.

Sub lethal concentrations of chemicals offer an excellent opportunity to observe behavioural and physiological changes in animals from close angles over a prolonged period (Edwards, 1973). These studies would also reveal development of lesions, biochemical, behavioural, neural, transient, incipient and permanent. These studies are more relevant and informative providing clues for developing management strategies in Cadmium poisoning.

Experimental Protocol

Fresh water fish Oreochromis mossambicus (10 ± 2g) were starved before they were subjected to CdCl₂ treatment and thereafter food was given ad libitum.

A concentrated 500ppm CdCl₂ stock solution was prepared by using 5 g of CdCl₂ in 100 ml of distilled water and administered into the water in required doses.

Six groups, each of 30 fishes were selected for acute toxicity testing. Each batch of animals when maintained in different troughs each receiving 30 lts of water and batches were divided based on concentration they received.
such as 20, 30, 40, 60, 80 and 100 ppm. All the experimental animals were observed 48 hrs thoroughly for the onset of any noticeable symptoms and mortality was recorded as and when it occurred. The method of calculation is as follows:

**Step 1**
A table (Table 1 A) was prepared with 5 columns, and labeled such as $d_i$, $n_i$, $r_i$, $x_i$ and $y_i$ where $d_i$ indicates increased order of dose (CdCl$_2$), $n_i$ indicates number of individuals, $r_i$ indicates number of animal dead and $x_i$ indicates log concentration of CdCl$_2$ (See Table - I A).

**Step 2**
In this $Y_i$ is calculated and placed in above Table (Table -1 A)

(a) $Y_i$ is calculated as $Y_i = \ln \frac{r_i}{n_i - r_i}$ (if number of animals died is less than number of tested animals)

(b) $Y_i = \ln (2n_i - 1)$ (if number of animals died is equal to number animals tested)

(c) $Y_i = \ln \frac{1}{(2n_i - 1)}$ (If number of animals died is zero)

**Step 3**
Divided the transformed data set into upper and lower halves.

Note (If the number of doses is odd, discard the largest dose to give equal number of doses in both halves)
<table>
<thead>
<tr>
<th>Step 3</th>
<th>Transformed dose (X_i)</th>
<th>Transformed Response (Y_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper half</td>
<td>2 996</td>
<td>-1 386</td>
</tr>
<tr>
<td></td>
<td>3 689</td>
<td>-0 546</td>
</tr>
<tr>
<td>Lower half</td>
<td>4 094</td>
<td>0 134</td>
</tr>
<tr>
<td></td>
<td>4 382</td>
<td>1 012</td>
</tr>
<tr>
<td>Total</td>
<td>15 161</td>
<td>-0 786</td>
</tr>
</tbody>
</table>

Step -4  
In each half, calculated the sum of the transformed doses and the sum of the transformed responses and denoted these values as $Y_u$, $Y_L$, $X_u$, $X_L$, where subscripts $u$ and $L$ refer upper and lower halves of the data set.

Step -4  
$Y_u = -1 386 + (-0 546) = -1 932$

$Y_L = 0 134 + 1 012 = 1 146$

$X_u = 2 996 + 3 689 = 6 685$

$X_L = 4 094 + 4 382 = 8 476$

Step 5  
The mean of all transformed doses and then mean of all transformed responses included in step 3 was calculated and the means were denoted as $\bar{X}$ and $\bar{Y}$ respectively.
Step 5 \[ \bar{X} = \frac{15161}{4} = 3790 \]

\[ \bar{Y} = -\frac{0.786}{4} = -0.197 \]

Step 6 slope was calculated (b)

\[ b = \frac{Y_u - Y_t}{X_u - X_t} = \]

Step 6 \[ \frac{-1932 - 1146}{6685 - 8476} = \frac{-3078}{1791} = 1.718(1.719) \]

Step 7 Calculated the intercept (or) as

\[ a = \bar{Y} - b\bar{X} = -0.197 - (1.718 \times 3.790) = -6.708 \]

\[ \text{LC}_{50} = e^c \]

Where \( C = \frac{(F-a)}{b} \), F is the log of \( \text{LC}_{50} \)

\[ F = \ln \left\{ \frac{50}{100 \sim 50} \right\} = 0 \]

\[ C = \frac{-6.708}{1.71} = 50.30 \]
Step -9 total number of fish tested was calculated

\[ N = 30 + 30 + 30 + 30 = 120 \]

Find \( S_c \)

\[
S_c = \frac{\sqrt{5}}{(b \times \sqrt{N})}
\]

\[
S_c = \frac{\sqrt{5}}{(1.71)^2 \times 120}
\]

\[
= \sqrt{\frac{5}{350.89}}
\]

\[ = 0.119 \]

Step – 11 Standard error (S) is estimated

\[
S (LC_{50}) = LC_{50} \times S_c
\]

\[ = 50.30 \times 0.119 \]

\[ = 5.98 \]

Step – 12 The approximate 95% confidence interval was calculated as

\[
LC_{50} \pm 1.96 \times S (LC_{50})
\]

\[ 50.30 \pm (1.96 \times 5.98) \]

\[ 50.30 \pm 11.72 \]

Upper limit \[ = 50.30 + 11.72 = 62.02 \]

Lower limit \[ = 50.30 - 11.72 = 38.58 \]

\( LC_{50} \) for 48 hr to \( CdCl_2 \) was calculated as

\[ = 50.30 \ (38.58 \ to \ 62.02) \]
Results

In the present study the mortality rates for fishes to different concentrations of cadmium were determined under ambient exposure. The concentrations ranged from 20 ppm to 100 ppm and the concentration at which 50% mortality could be obtained for 48 hrs, exposure was determined by Husen rapid approximate method (1983). The data was tabulated in Table 1A. The mortality started from 20 ppm, 20% mortality at 20 ppm, 36 6% mortality at 40 ppm, followed by 53 3%, 75 3%, 93 3% mortality was observed at 60, 80, 100 ppm respectively.

Discussion

From the results, it is evident that the lethal concentration (LC50/48 hr) of cadmium chloride to fish O. mossambicus was 50 33 ppm/liter. Results indicate that the percent mortality increases with increased concentrations of CdCl2. Previously the LC50 for CdCl2/48 hrs to the fish O. mossambicus was reported from this lab (Usha Ranj, 1986) by adopting Probit method of Finney (1974) and the LC50/48 h, was 50 ppm of CdCl2.

To standardize the earlier method of LC50/48h for CdCl2, in the present study LC50 for CdCl2 for 48 hrs was carried out by the above mentioned method and was found in confirmation as 50 33 (See table 1 A), which, coincides with the earlier findings. Hence from this, a sub lethal concentration (1/10 LC50) was presented as 5 ppm/ L to carry out the further studies.
b. Analysis of Metallothionein bound cadmium/ Analysis of Cytosolic cadmium concentration

The technological advancement has its ultimate impact on aquatic fauna in the form of industrial effluents, which are continuously polluting water bodies with toxic heavy metals and chemicals (Vincent et al., 2002). These metals after entering the water, may precipitate or adsorb on solid surfaces, remain soluble or suspended in water or may be taken up by fauna and flora (Madhyastha, 1996). Fish constitute the group most vulnerable to heavy metal toxicity under the imminent danger of unchecked aquatic pollution. A very significant biological property of metals is their tendency to bioaccumulate (Waldichuck, 1974). Continuous discharge of heavy metals into the aquatic environment has prompted the bioaccumulation studies. A number of biotic and abiotic factors are found to influence the bioaccumulation (Balaji & Sathyanarayana Rao, 2000). This uptake of heavy metals is mostly tissue specific and may even biomagnify in animals of higher trophic levels, including humans. It is this state of the art of the metal that is posing a threat to non-target organisms of the trophic level (Usha Rani, 2000).

Organisms can accumulate metals directly from water or indirectly from food. Both laboratory, and field studies reported on bioaccumulation of cadmium in different animals, such as Oreochromis mossambicus (Usha Rani, 2000, Mason et al., 2000, Romeo et al., 2000). Metal content in these studies were determined by using acid digestion method (3 1 perchloric acid and conc nitric acid) followed by Atomic Absorption Spectro photometer (AAS) above these studies gives whole organ or whole animal bioaccumulation.
The study focuses on metal concentration in cytosolic fractions or in metallothionein protein that was bound or recovery by MT protein which in turn reflects bioaccumulation.

**Results:**

In the present study, cadmium containing peak cytosolic fractions obtained from Sephadex – G75 column and DEAE – 32 cellulose ion exchange column, were pooled separately for each tissue, (See fig 4 IA, 4 IIA, 4 IB, 4 IIIB, 4 IC, 4 IIC, 4 ID, 4 IID, 4 IE, 4 IIE) and monitored in Atomic Absorption Spectrophotometer, for cadmium.

The metal content, so determined, revealed, the metal concentration at MT protein level induced under 30 days of cadmium exposure. Comparatively, Sephadex profile, contain more cadmium level than DEAE- 32 ion exchange fractions. Among gel filtration eluted profiles of cadmium, the concentrations were more in kidney (12 10 ± 0 28ppm) followed by liver (7 142 ± 0 05ppm), gill (2 72 ±0 016ppm), brain (1 63 ± 0 02ppm) and muscle (0 752 ± 0 0009ppm). Similarly ion exchange profile also showed concentrations of Cd in tissues like kidney (11 02 ± 0 066ppm) liver (6 075 ± 0 15ppm) gill (2 55 ± 0 043ppm) Brain (2 175 ± 0 023ppm) and muscle (0 55 ± 0 033ppm) (See Table 1 B)

**Discussion:**

A comparison of the tissue distribution of Cd in fish after chronic exposure to CdCl₂ showed significant differences see Table (1 B). The recovery of Cd concentration by induced MT in kidney is more. This is because of
subsequent depletion of hepatic MT in the CdCl₂ exposed *Oreochromis* fish may be transported to kidney as Cd-thionein (Dalal and Bhattacharya, 1991). This also supports the further step in the present investigation of MT quantification, where we could observe more MT levels in the kidney (See Table 4 A). Likewise more cadmium concentration in kidney was also observed in mice after CdCl₂ administration (Cherian, 1983, Tandon et al., 2001).

The results of the present study are in clear agreement with the earlier reports of Kito et al., (1986) in carp exposed to cadmium, wherein Cd concentration is more in kidney followed by liver, gill and muscle, when observed in sephadex, and DEAE ion exchange profiles. As liver is the site for all metabolic activities and also is a good storage organ, there might be active accumulation sites for Cd, but during chronic exposure, liver transports Cd as Cd-thionein to kidney. It is attributed that this may be the reason, that for less MT levels in liver and more in the kidney (Kito et al., 1986, Tandon et al., 2001) in the present investigation also liver showed less Cd in fish. Since the gills are the first organs to be exposed to water borne contaminants, this tissue is of importance in the detoxification and elimination of contaminants.

Anders et al., (2000), observed more cadmium in gills but less than liver and kidney concentrations during chronic exposure periods, and this trend was also observed by Tayal et al., (2000) using Electron microscopy and its specific quantification by electron probe X-ray micro analysis (EPMA) in teleost fish *Colisa fasciatus*. The reports of the present investigation also coincides with these reports. The cadmium concentration in muscle is not at significant level.
when compared to other tissues, in the present study. The same trend was also observed by De Conto 
ciner et al., (1997) and De Conto ciner et al., (1999) in carp using inductive coupled plasma mass 
Spect during long term high exposure, and noted significant increase in cadmium concentration only after 3 
months, Their findings, suggest that when the storage capacity limits of the liver 
and kidney are reached, cadmium accumulation in muscle is stimulated.

In the present study, i.e., recovery of Cd by MT protein by kidney was observed, 12 folds than muscle, 10 folds than brain, 9 folds than gill and 15 folds than liver (See Table 1 B) in turn reflects the bioaccumulation of cadmium 
in different tissues. The change between sephadex G-75, and DEAE - 32 cellulose ion exchange profiles is due to fine purification of Cd-protein which gives only MT bound cadmium concentrations. Hence, in Ion exchange column, 
the Cd concentrations were less than gel filtration profile.

Cross and Sunda (1978) used thermodynamic considerations to explain 
the dependence of the bioaccumulation of these metals on their free ion activity. 
The free metal ion activity is a measure of the free energy of the metal and, as such, reflects the potential per interactions between the metal and the available 
ligands. Many metals including Cd (Gutknecht, 1983) require a protein to 
mediate transport across cell membranes. Metal uptake then will depend upon 
the interactions between the metal and the transport proteins and the free metal 
ion activity reflects the potential for these interactions.
Metal concentration at MT protein level reveals sub cellular distribution. Jenkins and Sanders, (1986) reported that within the cell much of the cadmium was found in the cytosol where it ranged from 30-66% of the total tissue burden of Cd. Hence, present study reveals the Cd recovery by MT protein induced during cadmium stress, which in turn reflects bio-concentration/ bioaccumulation of cadmium for detoxification.
TABLE 1.A  Determination of LC\(_{50}\) for 48 hrs to cadmium chloride by Husen rapid approximate method for *Oreochromis mossambicus*

<table>
<thead>
<tr>
<th>Concentration ppm</th>
<th>Number of animals</th>
<th>Number of animals dead</th>
<th>Log concentration</th>
<th>% mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>30</td>
<td>6</td>
<td>2.9963</td>
<td>-1.386</td>
</tr>
<tr>
<td>40</td>
<td>30</td>
<td>11</td>
<td>3.689</td>
<td>-0.546</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>16</td>
<td>4.094</td>
<td>0.134</td>
</tr>
<tr>
<td>80</td>
<td>30</td>
<td>22</td>
<td>4.382</td>
<td>1.012</td>
</tr>
<tr>
<td>100</td>
<td>30</td>
<td>28</td>
<td>4.605</td>
<td>2.639</td>
</tr>
</tbody>
</table>
Cadmium recovery by MT protein in different tissues of *O. mossambicus* during chronic exposure to sub lethal concentration of cadmium (Each value is mean ±SD of 4 individual observations)

<table>
<thead>
<tr>
<th>S NO</th>
<th>NAME OF THE TISSUE</th>
<th>CONTROL Cd concentration (ppm)</th>
<th>30 DAY EXPOSURE Cd concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SEPHADEX POOLED FRACTIONS</td>
<td>DEAE-32 CELLULOSE POOLED FRACTIONS</td>
</tr>
<tr>
<td>1</td>
<td>LIVER</td>
<td>0.19 *</td>
<td>0.14*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.0002</td>
<td>±0.001</td>
</tr>
<tr>
<td>2</td>
<td>KIDNEY</td>
<td>0.33 *</td>
<td>0.26*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.001</td>
<td>±0.001</td>
</tr>
<tr>
<td>3</td>
<td>BRAIN</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MUSCLE</td>
<td>0.02 *</td>
<td>0.017*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.0001</td>
<td>±0.0001</td>
</tr>
<tr>
<td>5</td>
<td>GILL</td>
<td>0.02 *</td>
<td>0.016*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.0001</td>
<td>±0.001</td>
</tr>
</tbody>
</table>

* Indicates may be negligible concentrations

**indicates P<0.001