MATERIAL
AND
METHOD
MATERIALS AND METHODS

Live specimens of fish, *Heteropneustes fossilis* were procurred from the Sagar Lake and other fresh water reverine sources of Sagar Division. The stock of about 200 fish was maintained in glass aquariums at laboratory temperatures after disinfecting them with KMnO$_4$ solution. These fishes were acclimatized for two weeks providing artificial food and air. The food consists of live earthworm pieces, dry fish food and wheat flour pallets. The water in the aquariums was changed every alternate day. Before the commencement of the experiments the fishes were starved for about 20-24 hours.

Healthy acclimatized fishes of uniform size and weight, showing normal activity were selected for various experiments. The experiments were set up in glass aquaria of twenty litres capacity. The amount of water used in each aquarium was in the ratio of 1 gm fish wt. : 1 litre water. The stock solutions of the pesticides were prepared. 100 mg of aldrin was dissolved in 0.2ml acetone and distilled water (100 ml.), and 1 ml of fenvalerate was diluted in 100 ml of distilled water to get 10 and 20 ppm solution of aldrin and fenvalerate respectively. For further dilutions ordinary tap water was used.

ACUTE TOXICITY

Static bioassay procedures (APHA 1992) were used to determine the toxicity of the pesticides to the fish, *Heteropneustes fossilis*. A dilution series with tightly spaced concentrations of pesticides were used to find out the concentration range for toxicity tests. Ten fishes of similar size
and weight were exposed to 0.0020-0.0065 ppm solution of aldrin and 0.0045-0.0090 ppm of fenvalerate in the separate aquariums. During bio-testing feeding was disrupted. The physicochemical characteristics such as, temperature, salinity, pH, dissolved oxygen, alkalinity, conductivity and hardness of the experimental water were analysed during the exposure period. Temperature, pH and conductivity were determined by Century Water Analyser Kit (CK 710) and DO, alkalinity and hardness by titration methods as described by Trivedi et al. (1987).

The toxicity tests for the pesticides were carried out with water variables such as pH and hardness and also under normal laboratory conditions for 24, 48, 72 and 96 hours period. The LC 50 values for the same exposure periods were calculated by Probit analysis method applying regression equation (Robert and Boyce 1972).

CHRONIC TOXICITY

For assessing the chronic toxicity of the pesticides, sub-lethal concentrations were selected that were 1/10<sup>th</sup>, 1/15<sup>th</sup> and 1/20<sup>th</sup> ppm of the 96 hours LC 50 values of the pesticides. The values of sub-lethal concentrations of aldrin were, 0.0004, 0.00027, and 0.0002 ppm and for fenvalerate, 0.0007, 0.00046 and 0.00035 ppm. Twenty fishes of 18-20 gm and 15-17 cm in length were exposed to the above mentioned sub-lethal concentrations of each pesticide for sixty days and during this exposure period, survival, feeding and growth of the exposed fishes were studied.

Two sets of controls were maintained simultaneously. One having an equal aliquot of acetone served as control for
the aldrin exposed group whereas the other with only tap water served as control for the fenvalerate exposed group.

Weekly observations were recorded for survival, feeding and growth. For survival data, mortality percent of the test fishes were calculated. The amount of food consumed was determined by taking the difference between the weights of the fish, one prior to feeding and other after feeding. The food consumed was expressed in mg/gm body weight/week. Growth was measured in terms of increase in the wet weight per fish of the group per week. The data on growth were analysed following the methods described by Webb and Brett (1972).

OXYGEN CONSUMPTION

The rate of oxygen consumption in the control as well as exposed groups was determined by Winkler's Iodometry method as described by Strickland and Parsons (1968). Oxygen level in the water was determined under normal condition compared to the experimental ones. The procedure was repeated for 5-6 times and average mean was taken to determine the oxygen consumption in terms of mg/gm of fish/hr/oxygen consumed under normal activity. Data was analysed statistically and the values of 't' were utilized to show the variations in oxygen consumption rate and the level of significance.

HAEMATOLOGICAL STUDY

The fish, *Heteropneustes fossilis* was exposed to sub-lethal concentrations of both the pesticides for about 60 days. After 15, 30, 45 and 60 days fish from control as well as treated groups were sampled for determination of various
haematological parameters. For this purpose blood was collected from the caudal region, in the tubes containing anti-
coagulant mixture.

(I) Differential Leucocyte Count (DLC):

For differential cell count blood smears were prepared of exposed and control groups. The slides were stained with Haematoxylin-Eosin and Leshman’s stain. Differential leucocyte counting was done from these stained slides at a magnification of 1000 X.

(II) Total Erythrocyte Count (TEC):

Red blood cells were counted by, Neubauer’s Improved Hemocytometer using Hayems fluid as the diluting fluid. The counting was done in five squares including four corners and one central each with 16 smaller squares. Total number of cells in five squares multiplied by $10^4$ gave the number of erythrocytes/mm$^3$.

Formula:

$$\text{TEC} = \frac{\text{Total number of cells counted} \times \text{dilution} \times 4000}{\text{Number of small squares in which counting has been done}}$$

$$\text{TEC} = \frac{Xn \times 200 \times 4000}{80} = Xn \times 10^4$$
(III) Total Leucocyte Count (TLC):

WBC's were also counted by the same Hemocytometer slide, but the diluting fluid and the pippete used were different. The diluting fluid was Turk's fluid. Counting was done in two of the chambers. Total number of the cells counted multiplied by 50 gave the total leucocyte count.

Formula:

\[
\text{TLC} = \frac{\text{Number of WBC} \times \text{dilution} \times 10}{\text{Number of small squares in which counting has been done}}
\]

\[
\text{TLC} = \frac{Xn \times 20 \times 10}{4}
\]

\[
= Xn \times 50
\]

(III) Haemoglobin Estimation:

Haemoglobin percentage of the blood was determined by, Sahli's Hemoglobinometer with N/10 HCl solution. Dilution of the blood is read on the haemoglobin tube in terms of gram percentage. If the reading is 50 it means that the blood contains 50% of the normal hemoglobin.

(iv) Packed Cell Volume (PCV):

Hematocrit (PCV) of the blood was estimated by Wintrobe's method. The volume of the packed cell is read directly as percentage. The PCV is expressed as the % of the original column of blood. If X mm be the upper level of the packed cells in the tube, the PCV will be 'X' percent (%).

(v) Erythrocyte Sedimentation Rate (ESR):
ESR was also measured with Wintrobe's tube and sodium citrate as the anticoagulant mixture. Readings in the tube were noted after I and II hour. The mean of the two readings then taken as, ESR in mm/hour.

**ABSOLUTE INDICES**

The absolute indices ie; MCV, MCH, and MCHC were calculated by the readings of PCV, Hb and TEC.

**Mean Cell Volume (MCV):**

MCV of red cells was calculated, according to the following formula,

\[
MCV = \frac{PCV/1000 \text{ ml of blood}}{TEC \text{ in millions/ cu mm}}
\]

Or

\[
MCV = \frac{PCV \times 10}{RBC}
\]

It is expressed as \(\mu m^3\).
Mean Cell Haemoglobin (MCH):

MCH was calculated from haemoglobin per 1000 ml of the blood and TEC in millions / cu mm of blood according to following formula:

\[
\text{MCH} = \frac{\text{Hb in grams}}{1000 \text{ ml of blood}} \times \frac{\text{TEC in millions}}{
\]

\[
\text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC}}
\]

It is expressed in picogram (1 pg \(10^{-12}\)gm).

Mean Cell Haemoglobin Concentration (MCHC):

MCHC was calculated from the following formula;

\[
\text{MCHC} = \frac{\text{Hb/ 100 ml of blood} \times 100}{\text{PCV in } \%}
\]

It is expressed as %.

BIOCHEMICAL STUDIES

The fish, Heteropneustes fossilis were exposed to three sub-lethal concentrations of each pesticide in different groups for sixty days. Biochemical estimations were done after fortnight ie; on 15\(^{th}\), 30\(^{th}\), 45\(^{th}\) and 60\(^{th}\) day of the experiment. The fish from the control and exposed groups were sacrificed and dissected in an ice tray. Liver, kidney and body muscles were quickly removed, wet weighed and homogenized in the glass homogenizer as per requirements. The homogenates, then processed for the estimation of Protein, Lipid, Glycogen and Ascorbic acid content.
PROTEINS

Total soluble protein content of the tissues was estimated by the method of Lowry et al. (1951). The tissues were homogenized in 5% trichloroacetic acid in a glass homogenizer to precipitate all the proteins. Folin phenol reagent was used for colour development. The blue colour formed, measured at 650 nm. (Spectrophotometer- Shimadzu, UV-190). A proper blank with distilled water was used. The standard graph was drawn from the absorption values of the standard protein solution (Bovine serum albumin) processed in the same way.

GLYCOGEN

Glycogen content of the tissues was estimated by the method of Carrol et al. (1956). Tissue was homogenized in 5% TCA solution, centrifuged and supernatent fluid decanted. Glycogen, was then precipitated by adding twice the volume of 45% ethanol or 2 ml of 1N KOH solution. Anthrone reagent was added to the dissolved precipitate for the colour formation. The optical density of it was recorded immediately after cooling, at 540 nm against distilled water blank. Glucose solution was used as standard and the graph was drawn.

LIPIDS

Lipids were extracted from the tissues following the method of Folch et al. (1951) and then were estimated by the use of vaniline reagent. The colour formed was read at 530 nm. Standard solutions were prepared from the total lipid solution supplied in the kit.
ASCORBIC ACID

The method described by Roe (1967) was followed for the estimation of ascorbic acid, using 2,4-dinitrophenyl hydrazine reagent. The tissues were homogenized in 10% trichloroacetic acid, centrifuged at 3000 rpm for 10 minutes and Norit charcoal was added to convert the ascorbic acid into dehydroascorbic acid and filtered. The colour formed was read at 540 nm after 30 minutes on the spectrophotometer. Standard solutions were prepared with the vitamin 'C' tablets and the graph was drawn.

A standard curve was prepared for protein, glycogen, lipid and ascorbic acid. The absorption values were then converted into mg by applying appropriate conversion factors for each of the biochemical parameters. The values thus obtained were expressed in mg/100mg wet weight of the tissue concerned.

STATISTICAL TOOLS:

APHA (1992) opined that the data has to be gathered and inferred according to statistical methods. In the toxicological studies also, the interpretation of the gathered data by the use of statistical methods is an inevitable and integral part of the investigation. The data collected may be too meagre to draw any apparent conclusion. Therefore statistical methods are applied to organize and summarize the data to get the important hidden information.

In the present study following statistical tools were used:
I. Data obtained are presented in the form of frequency tables, on the basis of exposure periods of the pesticides to the fish.

II. Graphs, photographs and other diagrams are prepared in order to compare the mortality, feeding, growth, rate of oxygen uptake and changes in the various biochemical and haematological parameters of the exposed fish with those of the controls at different time intervals.

III. Analyses of variance, Students ‘t’ test, co-efficient of correlation and regression analyses are used in order to decide the significance level and nature of correlation of different parameters analysed.

Mortality of the test fish has been found to be the function of the pesticide concentration and period of exposure. Regression analyses and correlation techniques were used to explain the effects of pesticide concentration on the mortality of fish and find out the type of relationship existing between the two parameters.

**Regression Analyses:**

\[
y = a + bx \\
a = \bar{y} - bx \\
b = \frac{\Sigma xy - \bar{x} \Sigma y}{\Sigma x^2 - \bar{x} \Sigma x}
\]

Where,

a and b are constants  
x and y are variables  
Degree of freedom= n-2
Probability level = 5%, 1%, 0.1%.

**Coefficient of Correlation:**

\[
r = \frac{\sum xy - \bar{x} \sum y}{\sqrt{(\sum x^2 - \bar{x} \sum x)(\sum y^2 - \bar{y} \sum y)}}
\]

Where,

\(X = \) concentration (ppm)

\(y = \) mortality (%).

Degree of freedom = n-2

Probability Level = 5%, 1%, 0.1%.

**Analyses of Variance:**

One/two way analyses of variance was used to understand the difference in the biochemical and haematological values and rate of oxygen consumption among the four exposure periods and also in the data using three concentrations of the pesticides.

\[
\text{Variance (S^2)} = \sqrt{\frac{\sum (x-x)^2}{n-1}}
\]

Degree of Freedom = n - 1
Probability level 5%, 1%, 0.1%

**Student's t test:**

To find out whether the differences in biochemical contents, haematological value and oxygen consumption capacity of the control and the treated fish were significant or not, t test was utilized.

\[
t = \frac{x_1 - x_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}
\]

Degree of Freedom = n - 1
Probability level 5%, 1%, 0.1%
### Some physical and chemical properties of Aldrin

| Physical State | Crystalline Solid  
| (95% HHDN is a Waxy Solid) |
| Colour | Colourless  
| (95% HHDN is a tan to dark brown) |
| Odour | Mild "Chemical" odour |
| Relative Molecular Mass | 364.93 |
| Melting point | 104-104.50°C  
| (95% HHDN -49-60°C) |
| Water solubility (27°C) | 27µg / litre  
| Solubility in organic solvents | soluble

* Acetone, Benzene, Xylene > 600 g/litre

| Relative density (20°C) | 1.54 g/ml  
| Vapour pressure (20°C) | 8.6 mPa  
| (6.5x10⁻⁵ mmHg at 25°C) |
Table: 2

Some physical and chemical properties of Fenvalerate

<table>
<thead>
<tr>
<th>Physical State</th>
<th>Viscous Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Yellow or brown</td>
</tr>
<tr>
<td>Odour</td>
<td>Mild &quot;Chemical&quot; odour</td>
</tr>
<tr>
<td>Relative Molecular Mass</td>
<td>419.9</td>
</tr>
<tr>
<td>Boiling point</td>
<td>300° C at 4.93 kPa (37 mmHg)</td>
</tr>
<tr>
<td>Water solubility</td>
<td>2μg / litre</td>
</tr>
<tr>
<td>Solubility in organic solvents</td>
<td>soluble(^a)</td>
</tr>
<tr>
<td>Relative density (25°C)</td>
<td>1.175</td>
</tr>
<tr>
<td>Vapour pressure (25°C)</td>
<td>0.037 mPa</td>
</tr>
<tr>
<td>Log octanol water partition coefficient</td>
<td>6.2</td>
</tr>
<tr>
<td>(log (P_{ow}))</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Acetone (> kg/kg), Hexane (155 g/kg), Xylene (> 1 kg/kg). Ethanol, Cyclohexanone, Ether, Kerosene, Chlorotrom.
### Chemical identity of Fenvalerate and its various stereoisomers

<table>
<thead>
<tr>
<th>COMMON name/ CAS Registry no./ NIOSH Accession no. a</th>
<th>CAS Index name (9CI)</th>
<th>Stereoisomeric composition c</th>
<th>Synonyms and trade names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenvalerate</td>
<td>Benzeneacetic acid 4-chloro-alpha-(1-methylethyl)-cyano3-phenoxyphenyl methyl ester (RS)-alpha-cyano-3-phenoxybenzyl (RS) -2 (4- chlorophenyl )-3 methylbutyrate</td>
<td>(1):(2):(3):(4) =1:1:1:1</td>
<td>Sumicidin, Belmark Pydrin S-5602 SD43775, WL43775</td>
</tr>
<tr>
<td>CY1576350</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alpha-Fenvalerate</td>
<td>Same as Fenvalerate Benzeneacetic acid, 4- chloro-alpha-(1-methylethyl ) -, cyano-3-phenoxybenzyl ester , [S-(R*,R*)] -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66230-04-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta-Fenvalerate</td>
<td>Same as Fenvalerate Benzeneacetic acid, 4- chloro-alpha-(1-methylethyl ) -, cyano-3-phenoxybenzyl ester , [(R,R*,S*)] -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66267-77-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S,S) Fenvalerate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CY1576350</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Registry of Toxic Effects of Chemical Substance (1981-1982 edition)
b (25),d, (+) or (2R), (1) ,(-) in the acid part of fenvalerate signify the same stereospecific conformation, respectively
c Numbers in parantheses indentify the structures shown in fig
REFERENCES


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Lal, B., Amita Singh, Anita Kumari and Neelima Sinha (1986): Biochemical and haematological changes following malathion treatment in the freshwater catfish,


