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## 2. MATERIAL & METHODS

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2.1 Information of test organism

The species has been selected for the present study is of great economic importance and readily available throughout the year. It is a beneficial animal as it feeds on insects which may be harmful to the crops. Amphibians are those first vertebrates who have adapted themselves for life of land to some extent. They pass some of the time of their life cycle in water and on land. Frogs in general are found in freshwater lakes, rivers, ponds, ditches and moist land and is resistant to handling and transportation.

Identification and taxonomical position of the frog is as follows.

- Kingdom : Animalia
- Subkingdom : Metazoa
- Phylum : Chordata
- Subphylum : Vertebrata
- Super class : Anathostomata
- Class : Amphibia
- Order : Anura
- Sub-order : Diplasicoeia
- Genus : Rana
- Species : Cyanophlyctis

2.2. Acclimatization method

The animals were collected around the Amravati (Latitude 20°-56N Longitude 77°-45E) region and brought to the laboratory. Healthy frogs weighing between 80-85 gms were

selected. The difference between largest and smallest specimens used was not more than 1-2 cms and weight 1-10 gms. Immature frog as well as spent frogs were not used for bioassay test. When the frogs were first brought to the laboratory they were transferred to the glass aquarium and were inspected for any possible injury or infection, only the healthy frogs were selected and washed with dilute solution of potassium permanganate ( $Kmno_4$ , 1.0 mg/l) to remove dermal infection if any.

The frogs were acclimatized for 10 days according to APHA/AWWA/WPCA (1975) standard method. The mortality was less than 5 percent within 4 days of preceding test. The campus well water was used as experimental medium for holding different tests. The volume of holding chamber was sufficient, so that each frog had not less than five volumes of water (Compare to its volume). The holding water had dissolved oxygen concentration always greater than 40 percent of saturation (Table 2.1). The test frogs were fed on of boiled egg, small insects, earthworms, and cockroaches. Excess food and faecal matter were removed from the glass aquaria once in a day or twice at least in a week by siphoning. The test frogs were handled carefully so that the stress was minimum.

The renewal technique bioassay method (Committee on

methods for toxicity test with aquatic organisms, 1975) was used. It is an improved static test in the sense that an attempt was made to maintain the water quality. The test solutions were periodically, usually once every (24 h) replaced with fresh test solutions of the same composition. This was achieved by transferring the test organism from one test chamber to another or some time by replacing the test solution in the same chamber periodically.

### Toxicity method

#### Selecting test organisms

The prime consideration in selecting test organisms are, their sensitivity to the factors under consideration, their geographical distribution, abundance, and availability within a practical size-range throughout the year, their recreational, economic and ecological importance, the availability of culture methods for rearing them in the laboratory and knowledge of their requirements and their general physical condition and freedom from parasites and disease. Few studies have been made to determine the important species most sensitive to a potential toxicant. To select a best species consider available information on sensitivity or determine sensitivity with short term tests.

#### Collecting test organisms

Many smaller and medium size (5 to 8 cms) and weight (80.85 mg) animals were collected along the ponds,

river and ditches around the Amravati region by nets and hand. After catching they were quickly transferred to the plastic container. Ensure that organisms are not damaged during collection, transfer and transport.

#### Toxicity methods

For toxicity evaluation the organophosphorous pesticide selected was cyathion. The dilution medium was campus well water. The physiological composition a tested medium is given in the table.

Table : Means and ranges of various physiological characteristic of dilution water.

Characteristics	Mean	Range
Temperature	27°C <sub>±</sub> 2.5	24.5 - 29.5°C
Ph	7.40	6.8 - 7.5
Total solids	12.80	11.50 - 17.25
Suspended solid	9.42	9.23 - 11.20
Dissolved solid	4.36	3.23 - 5.80
Alkalinity as CO <sub>3</sub>	55.00	37.00 - 38.00
Alkanity as OH	5.22	3.50 - 8.22
Dissolved oxygen (D O.)	4.33	3.56 - 6.80
Biochemical oxygen demand	1.70	1.83 - 3.53
Phosphate as C	0.82	0.22 - 1.61

Values except pH and temperature have been expressed in mg/l.



### Exploratory test

Before performing full scale acute toxicity test, ( ) 24 h exploratory tests were made. The test frogs were exposed to widely spaced concentrations of pesticides cythion for 24 hours . Based on this data detailed toxicity tests were made.

### Lethal toxicity tests

The Mulrhead Thomson (1971) method was used in which a minimum of four dilution of test solution of test material were chosen in such a way that in the least dilution, so percent of the frog were killed after 24 h; in the second, which was not more than twice as dilute as the Frist. Five percent or less of the frog were killed after 24 hr. In the third 50 percent or more of the frog were killed after 24 h. And in the fourth which was not more than twice as dilute as third. Fifty percent or less of the frog were killed, after 48 h. Ten frogs were exposed to each concentration. The dose loading procedure was followed as that of APHA (1975). Appropriate controls were maintained for each test. The mortality did not exceeds 5 percent during the test period in control. The test duration were 24, 48, 72 and 96. The LC<sub>50</sub> values were determined from direct observation as well as estimated from statistically fitted graphs, plotted from log concentration and percent mortality data ( Litchfield and wilcoxon 1949)

### Statistical analysis and calculations

LC values were calculated by the simplified method of evaluating dose effect experimental mortality (Litchfield and Wilcoxon, 1949).

Slope function (S) was calculated according to the method of Litchfield and Wilcoxon (1949) by using the formula-

$$S = \frac{LC_{84}/LC_{50} + LC_{50}/LC_{16}}{2}$$

Factor of LC<sub>50</sub> was calculated by using the formula-

$$FLC_{50} = \frac{S^{2.77}}{\sqrt{N'}}$$

whereas factor of slope (FS) was calculated by the formula of Litchfield and Wilcoxon (1949).

$$FS = A^{10} (X-1)/K \sqrt{N'}$$

### Biochemical changes

The specimens of Rana cyanophlyctii were collected around Amravati and maintained and acclimated to the laboratory condition for about 10 days. The frogs were exposed to the sublethal concentration of the cythion (organophosphate). The variations in the biochemical levels were studied in different organs such as liver, muscle,



Constituent	Metabolite	Reference
Carbohydrate	a. Glycogen	Carrol <u>et al.</u> , 1958
Proteins	a. Soluble protein	Lawry <u>et al.</u> , 1951
	b. Insoluble protein	-"-
	c. Acidic protein	-"-
	d. Basic protein	- "-
Lipid	a. Total lipid	Folch <u>et al.</u> , 1957
	b. Cholesterol	Zaltikis <u>et al.</u> , 1953

The glycogen was estimated by modified (Carrol et al., 1956) method. The proteins were estimated by Lowry et al. (1951). The total lipid were estimated by Folch et al. (1957) method and Cholesterol by Zaltikis et al. (1953) method.

#### Estimation of glycogen:

Tissue samples were placed in an efficient blender under an appropriate volume of TCA and homogenated for 3 minutes, homogenate were placed into a suitable centrifuge tube. Centrifused and decanted the supernatant liquid upon an acid washed filter paper placed ina funnel draining into a gradual cylinder transferred the residue quantitatively to the blender with an appropriate volume of TCA and homogenized again for one minute. Centrifuged the mixture and pour the supernatant fluid through the same filter. Two more extractions made in the same manner. In test tube 1 ml of TCA taken and 5 ml of 95% ethanol was added kept it for overnight, Centrifused supernatent was decanted, glycogen was dissolved by addition of 2 ml of distilled water.

At this point 10 ml of anthrone reagent was delivered into each tube, than the tube was fightly capped and kept in a cold tap water. After each tubes have reached the temperature of the cold water. They are immersed in a boiling water bath for 15 minute and then removed to cold water bath and cooled to room comperature and read at 620 u on calorimeter with blank and standard glucose values were multiplied by glycogen factor and values were expressed as mg/gm wet. wt tissues.

#### Estimation of proteins

Proteins were estimated by Lowry et al. (1951).

#### Method

One percent homogenates of selected tissues were prepared in 0.25 mM sucrose and centrifused at 1000 rpm for 10 minutes. The supernatent and the residue fractions were separated to estimate the sucrose soluble and sucrose insoluble protein fractions.

#### Sucrose soluble and insoluble protein

Two ml of 10 percent TCA was added to the supernatent and centrifused at 1000 RPM for 10 minutes to the residue (supernatant was discarded) 1 ml of 0.4 N NaOH was added and mixed well to dissolved the residue (TCA PDT) completely and 4 ml biurrete reagent was added, the colour was read at 540 u on colourimeter.

The residue fraction obtained after the first centrifugation was dissolved in a known volume of 0.4 N. NaOH, from this 0.05 ml of the aliquot was taken in a test tube, 4 ml of alkaline copper sulphate was added after 10 minute the 4 m of biuret reagent was added to this. The colour was read at 540 u in a spectro-colourimeter, against a reagent blank. The standard graph was prepared by using Bovine serum albumino.

The protein content was expressed as mg protein/gm wet, wt, tissue.

#### Acidic protein and Basic proteins

For detection of acidic and basic proteins the sucrose soluble fraction was used. In the 2 ml supernatant 4 m of acidic buffer was added (0.2 M acetate buffer pH 4.0) in one test tube for acidic proteins estimation and in the another test tube 2 ml supernatant was taken in that 4 ml basic buffer (0.2 in carbonate bicarbonate buffer pH 10.5) was added. This two tubes kept overnight after 24 hour, 2 ml TCA was added (10 %) and 1 ml of 0.4 N NaOH was added, and after 10 minute, 4 ml biuret reagent was added in both the test tube and the, colour was read against blank at 540 u of colourimeter.

#### Estimation of lipid

For the rapid and complete removed of lipids fraction from the tissues, various combinations of lipid solvents were suggested in methods in Enzymology (Vol.3)

Among them, the procedure given for extraction of lipids from the tissue was used in the present study and the extracted aliquots were employed for estimating various fractions of lipids in the present investigation.

#### Estimation of total lipids

The total lipid content in the tissues was estimated by the method of Folch et al (1957) as described by Overturf and Dryes (1969). In the method 50 percent homogenate of the tissue was prepared in chloroform, methanol mixture (2:1). The tissue was first homogenized in the chloroform, methanol mixture and the suspension was kept in the refrigerator for 30 min. The supernatant was then filtered through filter paper moistened with chloroform; methanol. The precipitate was rehomogenized in chloroform: methanol mixture and filtered into the supernatant. The filtrate constitutes the crude lipid extract. Immediately 0.2 ml of 0.05 M sodium chloride solution was added to the filtrate, mixed thoroughly and was kept overnight in the refrigerator. The mixture which became biphasic contains purified lipids in lower phase. The upper phase which contains essentially the non-lipid contaminants was removed with a fine-tipped pipette. The purified lipid fraction was evaporated in a vacuum evaporator to a known volume. Three

aliquots of 0.5 ml each were removed and put into tared aluminium boats whose weights were already determined. The aluminium boats were heated on a hot plate at 80°C for 5 min. They were transferred with forceps to the vacuum desiccator containing potassium hydroxide pellets and allowed to cool for 5 min. and weighed. By subtracting the empty tared boat's weight from the dried gross weight, the net dry weight of the sample was obtained. The average dry weight of the three samples was taken and the total lipid content was expressed as mg total lipid per gm dry weight of tissue.

#### Estimation of cholesterol

The total cholesterol was estimated using Liberman-Burchard reaction as described by Natelson (1971a). The tissues were homogenized in 4 ml of 1 N sulphuric acid. So this 4 ml of chloroform was added. The contents were transferred into a tube having a tight stopper and shaken vigorously for 20 min. The contents were centrifuged.

The supernatant acid and the protein at the interface were then aspirated off with the help of water. From the remaining chloroform 2 ml of aliquot was taken and to it 1 ml of acetic anhydride mixture (acetic anhydride: conc. sulphuric acid 20:1.5) was added. The colour was developed for 9 min. at 25°C in dark and then read in spectrophotometer at 625 m $\mu$ . The standard consisted of 2 ml of working standard (0.05  $\mu$ g/ml) and 1 ml of acetic anhydride mixture.

The colour of the standard was developed and read in the same manner. The blank consisted of 2 ml of chloroform and 1 ml of acetic anhydride mixture. The cholesterol content was expressed as mg cholesterol per gm dry weight of tissue.

### Enzyme activity

Rana cyanophlyctis specimens were collected around Amravati region, collection and maintenance of animals and group differential were same as that of biochemical change study.

Enzymes were estimated from the tissues like liver, kidney, muscle, brain, gonad, stomach and intestine etc. exposed to lethal concentration of cythion i.e. LC<sub>50</sub>/96 h at 24, 48, 72 and 96 h.

Methods used for enzyme analysis of cythion lethal concentration are as follows.

Enzymes	Reference
Protease	Davis and Smith (1955)
AAT	Raitman and Frankel (1957)
ALAT	Reitman and Frankel (1957)
Acid phosphatase	Fiske and Subbarao (1925)
Alkaline phosphatone	Fiske and Subbarao (1925)

### Protease

Protease activity was assayed by the method described by Davis and Smith (1955) with some modifications as follows. Five percent tissue homogenate were prepared in cold distilled water and centrifused at 1000 xg for 15 min. The supernatants obtained were used for the assay of protease. Citrate buffer (PH7) was used. The reaction was carried out at 37°C for 30 min. than stopped by addition of 2 ml of 20 percent TCA. The content were filtered and was used for estimation of amino acids. The activity is measured as moles of tyrosin/mg/protein/h in the tissue.

### Estimation of aspartate amino transferase (AAT) (L-aspartate - 2-oxoglutarate amino transferase

AAT activity was assayed by method of Reitman and Frankel (1957). The incubation mixture in a final volume of 1 ml contained 100 u mol of potassium phosphate buffer (pH 7.4), 100 u mol of L-aspartic acid (pH 7.4), 2 u mol of -ketoglutaric acid (pH 7.4) and 0.1 of 10 percent (w/v) freshly prepared homogenate in ice cold 0.25 M sucrose solution. After incubation both for 1 h at 37°C the reaction, was stopped by the addition of 1 ml of 2, 4-dinitrophenyl-hydrozine solution (0.001 M in 1 NHCl) and allowed to stand at room temperature for 30 minute. Zero time controls were maintained for all the samples by the addition of 2, 4-dinitrophenyl hydrazim solution prior to the addition

of the homogenate. Colour was developed by the addition of 10 ml of sodium hydroxide (0.4 N) in all the tubes, which were read at 545 m u in spectrophotometer. The enzyme activity was expressed as u mol sodium pyruvate formed per mg protein per hour.

Estimation of Amino transferase (ALAT) DL-alanine  
2-oxoglutarate aminotransferase

ALAT activity was determined by the method of Reitman and Frankel (1957). The incubation mixture in a final volume of 1 ml contained 100 u mol of DL-alanine (pH 7.4), 2 u mol of  $\alpha$ -ketoglutarate (pH 7.4) and 0.1 ml of 10 percent (v/v) homogenate prepared in ice cold 0.25 M sucrose solution.

After incubation for 30 min. the enzyme reaction was stopped by the addition of 1 ml of 2, 4-dinitrophenyl hydrazine solution (0.001 M in 1 N HCl) and allowed to stand for 30 min. at room temperature. Zero time controls were prepared for all the samples by the addition of 2, 4-dinitrophenyl hydrazine solution prior to the addition of homogenate. Then the colour was developed by the addition of 10 ml of sodium hydroxide (0.4 N) and the developed colour was read at 545 m u in spectrophotometer. The enzyme activity was expressed as u mol sodium pyruvate per mg protein per hour.

Acid phosphatase

Acid phosphatase activity was determined by Fiske and Subbarao (1925). After sacrificed the animal, tissues were

were kept in ice cold petridishes, tissues were blotted free of blood and five percent homogenate was prepared in 0.25 M sucrose solution with homogenizer, homogenate were centrifused under cold conditions at 2500 rpm and supernatant was used for enzyme assay. The reaction mixture in a final volume of 10 ml contained 0.4 ml of the supernatant, 9 ml of acid phosphate substrate (acid phosphate substrate was prepared by adding 3 ml of petroleum ether, 80 ml of distilled water, 0.5 gm of sodium B-glycerophosphate, 0.424 gm of sodium diethylbarbitesrate and distilled water to make up its volume to 100 ml and pH was adjusted to 5.0 by using glacial acetic acid) and 0.6 ml of the distilled water. the contents were incubated at 37°C for 1 hr in a thermostatic water bath. The reaction was arrested by adding 2.0 ml of 30 percent trichloroacetic acid solution and centrifused. Control tubes received 0.6 ml of distilled water, 9 ml of substrates, 2 ml of 40 percent trichloroacetic acid and 0.4 ml of the supernatant. The content were mixed well and centrifused.

Inorganic phosphate of the supernatant was estimated by the method of Fiske and Subbarao (1925). The enzyme activity was expressed as  $\mu$ g pi liberated per mg protein per hour.

#### Alkaline phosphatase (ALP)

Orthophosphoric monoester: Phosphohydrolase,  
Alkaline phosphatase activity was determined by Fiske and

Subbarao (1925). The substrate for acid phosphatase activity was adjusted to pH 5.0. rest of the procedure was similar to that followed for acid phosphatase. Alkaline phosphatase activity was also expressed as u g pi liberated per mg protein per hour.

### Histopathological (Endocrinological) study

#### Endocrine histopathological studies

Collection of Rana cyanophlyctis, its maintenance and test procedure was similar as determined in biochemical estimations method. Frogs were exposed to lethal concentration of cythion (organophosphate) pesticide i.e. LC<sub>50</sub> 96/h.

Frogs were divided mainly into two groups.

Group I : Served as control for all exposure intervals.

Group II : Frogs were exposed to lethal concentration of cythion i.e. LC<sub>50</sub> 96 h (Cythion 0.925 mg/lit).

Each major group is further subdivided into four groups. These subdivided groups were exposed to cythion lethal concentration and were sacrificed at regular time intervals like 24, 48, 92 and 96 hrs. Frogs were dissected and endocrine glands like pancreas, thyroid, gonad, adrenal, were fixed in Bouins fixative for 18 hrs. Then dehydrated using increasing concentration of alcohol.

Cleared in xylol and embaded in paraffin wax. The blocks thus prepared were cut and serial sections at different u (thickness) were taken. The sections were stained with Delafield haematoxylin and eosin counter staining method.