Materials and Methods

ANIMALS AND CHEMICALS

ANIMALS

Test fish

Air breathing fish, *C. punctatus* (Bloch) weighing 30-32 g and with mean body length of 12-15 cm were collected from a commercial fish farm. Fish were brought to the laboratory and acclimatized for 2 weeks prior to experimentation. Dechlorinated tap water was used throughout the course of the experiments. The physico-chemical characteristics of the test water were: temperature 26.5±1.0°C; pH 7.5; hardness 79 mg/l (as $CaCO_3$); alkalinity 87 mg/l (as $CaCO_3$) and dissolved oxygen concentration (D. O.) 6.5 mg/l. The LC_{50} value was determined in the laboratory as per the methods of Reish and Oshida (1987) starting with range finding tests to acute toxicity trials. The 96-h LC_{50} was found to be 18 mg/l for dimecron and 25 mg/l for quinalphos.

CHEMICALS

Dimecron

Dimecron, which is a trade name of Phosphamidon (O, O-dimethyl-O- (2-chloro-2-diethyl carbamoyl-1-methyl-vinyl) phosphate). Dimecron, marketed by Hindusthan Ciba-Geigy Limited, India was used throughout the study. For each experiment the required concentrations were prepared from fresh stock solutions.

Quinalphos

Quinalphos, which is a trade name of Basuquin 25 EC (O, O- diethyl O- quinoxalin –2- yl phosphorothiate) marketed by Hindusthan Ciba-Geigy Limited, India was used throughout the study. For each experiment the required concentrations were prepared from fresh stock solutions.

TOXICITY

Sub lethal toxicity

Fishes were fed regularly with dried shrimp powder and a minimum quantity of antibiotics to free from any ailments. The water in the aquaria was changed every 24 hr. to maintain a constant concentration of dimecron (phosphamidon) and quinalphos during the period of exposure. Aeration was provided to each aquarium to prevent hypoxic conditions. Two Sub lethal concentrations of dimecron (1.8 mg/l and 0.36 mg/l) and quinalphos (2.5 mg/l and 0.50

mg/l) were added to the medium (tap water) of aquaria. About 25-30 acclimatized fish were introduced in each aquarium. Simultaneously, an aquarium was set up for the study of control animals. A minimum of five fish was sacrificed each time from treated as well as control aquaria. The analysis was performed at 10, 20 and 30 days of exposure.

STUDY SCHEDULE

OUTDOOR STUDY SCHEDULE

Water quality

Chronic tests were conducted in outdoor earthen vats (circumference 64 cm, mean depth 32 cm.) each holding 60 liters of borehole water and 3.5 kg uncontaminated soil. Fifteen fish (*Channa punctatus* of approximately 12-15 cm. in length and 30-32 gms. in weight) per vat were stocked. Each test had three replicates all accompanied by controls. Fish (*Channa punctatus*) exposed to sub lethal concentrations of dimecron and quinalphos based on acute toxicity data (Reish and Oshida, 1987). The concentrations were used low to high @ 0.36 mg/l to 1.8 mg/l and @ 0.50 mg/l to 2.5 mg/l respectively. Water quality such as pH, alkalinity, dissolved oxygen (DO), free carbon dioxide (CO_2), hardness and water temperature were analysed following APHA (1995) at 10 day intervals. Plankton samples were collected at 10 day intervals by filtering surface water through plankton net (bolting silk no, 25). The collected samples were fixed in 5% formalin on the spot and carefully brought in the laboratory for analysis. Counting was done following standard method (APHA, 1995). Temperature (maximum and minimum), atmospheric pressure, relative humidity and rainfall were also noted during the study.

INDOOR STUDY SCHEDULE

Air breathing fish, *Channa punctatus* (Bloch) were obtained from a commercial fish farm and acclimatized to laboratory conditions for at least two weeks before the experiments commenced. *Channa punctatus* of approximately 12-15 cm. in length and 25-32 gms. in weight were chosen as experimental animals. The fishes were kept in glass aquaria of about 100 litres capacity. Dechlorinated tap water was used throughout the course of the

experiments. The physico-chemical characteristics of the test water temperature 26.9 $\pm 1^0$ C; pH 7.5; hardness 79 mg/l (as $CaCO_3$); alkalinity 87 mg/l (as $CaCO_3$) and dissolved oxygen concentration 6.5 mg/l. The LC_{50} value was determined in the laboratory as per the method of Reish and Oshida (1987) starting with range finding tests to acute toxicity trials. The 96-h LC_{50} was found to be 18 mg/l for dimecron and 25 mg/l for quinalphos. One-tenth and 1/50 th of the 96-h LC_{50}, i.e. 1.8 mg/l and 0.36 mg/l and 2.5 mg/l and 0.50 mg/l were selected for sub lethal test trials.

Fishes were fed regularly with dried shrimp powder and a minimum quantity of antibiotics to free from any ailments. The water in the aquaria was changed every 24 hr. to maintain a constant concentration of dimecron (phosphamidon) and quinalphos during the period of exposure. Aeration was provided to each aquarium to prevent hypoxic conditions. Two Sub lethal concentrations of dimecron and quinalphos were added to the medium (tap water) of aquaria. About 25-30 acclimatized fish were introduced in each aquarium. Simultaneously, an aquarium was set up for the study of control animals. A minimum of five fish was sacrificed each time from treated as well as control aquaria. All the experiments were repeated three times for each biochemical estimation. The analysis was performed at 10, 20 and 30 days of exposure. The liver, kidney and muscle were removed immediately and frozen until required (not more than 1 hr.) for biochemical and enzymological analysis.

DETERMINATION OF BODY WEIGHT AND ORGAN WEIGHT

Body weight of individual fish was determined gravimetrically with the help of sensitive weighing balance. Organ weight was taken after taking the total organ of five fish at a time on a thin slide or watch glass, as the may be, and its wet weight was determined by deducting the tare weight.

BEHAVIORAL STUDY

The behavioural changes of exposed and control fish were observed regularly at eight hours intervals throughout the experiment.

STUDY OF PROTEINS

QUANTITATIVE STUDY (LIVER, KIDNEY, AND MUSCLE)

Total quantity of protein was estimated following the method of Lowry *et al.*, (1951). Weighed individual tissue of fish was homogenised in 0.1 N sodium hydroxide and centrifuged at 3000 g for 15 minutes. The supernatant was collected and quantitatively transferred in a neutral glass test tube. This served as the aliquot.

A definite volume of the stock solution was taken in a glass test tube and diluted with 0.1 N sodium hydroxide to make a final volume of 1 ml. Then 5 ml of reagent C (prepared by mixing reagent A and B at ratio of 1: 50) was added. After 5 minutes, 0.5 ml of reagent D was added to the sample and mixed thoroughly. An analysis blank with 1 ml of 0.1 N sodium hydroxide, 5 ml of reagent C and 0.5 ml of reagent D was run simultaneously. This served as control. All the preparations were kept in the dark for thirty minutes at room temperature. After the stipulated period, the color intensity of the solutions was recorded in a spectrophotometer (Milton Roy Co., USA) at 750 nm. The concentration of the protein in the sample was estimated from the standard curve prepared in an identical way with a known concentration of analytical grade bovine serum albumin (Sigma, USA).

Reagent A: A solution of 0.5 % copper sulphate and 1.0% potassium sodium tartrate prepared freshly and stored in colour bottle.

Reagent B: This consists of 2.0 % sodium carbonate in 0.1 N sodium hydroxide (w/v) and was always prepared fresh when used.

Reagent C: This was prepared by mixing 50 ml of fresh reagent B with 1 ml of reagent A. This mixture was vigorously shaken.

Reagent D: It is Folin phenol reagent. This consists of 100 g of sodium tungstate, 25 g of sodium molybdate, 700 ml glass distilled water, 50 ml 85% phosphoric acid and 100 ml concentrated hydrochloric acid, boiled under reflux for 10 hours followed by the addition of 150 g lithium sulphate, 50 ml of water and few drops of bromine. The mixture was boiled for nearly 15 minutes to remove the excess of bromine, cooled, diluted to 1 litre and stored in a

stoppered bottle after filtration. In fresh condition, it is yellowish in colour. It was diluted with double the amount of glass-distilled water, when it was used.

The quantity of protein was estimated on wet weight basis and the value of concentration of protein was expressed as mg/gm of tissue.

QUALITATIVE STUDY (LIVER, KIDNEY AND MUSCLE)

The qualitative analysis of protein from liver, kidney and muscle were done through the separation of the component bands with their molecular weight in the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

PREPARATION OF TISSUE MATERIAL

A definite amount of liver, kidney and muscle tissue were homogenised in 0.9% sodium chloride solution. The homogenate was centrifuged at 3000 g at 4^0C for 15 minutes. The supernatant was collected and kept at 4^0C for subsequent study.

SDS-GEL ELECTROPHORESIS

For the qualitative study of tissue proteins, sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was done by following the method of Laemmli (1970). Acrylamide, N, N, N', N'-tetramethyl ethylene Diamine, Tris- buffer, Sodium dodecyl sulphate, Coomassie Brilliant Blue R-250 etc. were purchased from Sigma Chemical Co., USA. 10.0 % separating gel and 5.0 % stacking gels were made. Before loading onto the gel slots, proteins were extracted from the tissues by homogenizing with cold normal saline and the supernatants were isolated by centrifuging at 3000 g for 15 minutes. The supernatant was then mixed with equal volume of sample buffer (1:1) and the content was then boiled for 3 min in a boiling water bath. Before loading proteins, the slots were washed with electrode buffer and then blotted with blotting paper. About 0.02 ml to 0.03 ml of protein sample buffer mixture containing about 100-125 μg of protein was loaded into each slot. The gel apparatus was then connected with the power pack. Electrophoresis was performed at 4^0C. 100 V was applied first and when the dye marker crossed the stacking gel area voltage was increased to

150 V and constant current was applied. After 3-4 hours, when the tracking dye reached near the end of the gel, apparatus was disconnected from the power pack, gel was removed from the glass plates, fixed in 20 % TCA solution with constant rocking movement for 20 minutes and then kept in Coomassie Brilliant Blue R-250 staining solution overnight. On the next day, gel was removed from the staining solution and placed in destaining solution until the bands became clearly visible. The gels were then stored in 6% acetic acid for future studies.

REAGENTS PREPARED

i) 4 X Separating gel buffer

16.95gm Tris (1.5 M)

90 ml distilled Water

pH adjusted to 8.8 with HCl

4 ml 10 % SDS

Volume brought upto 100 ml. with distilled water.

ii) 10 X Electrode buffer

30.25gm Tris (0.25 M)

144 gm glycine

850 ml distilled Water

pH adjusted to 8.3 with HCl

100 ml 10 % SDS

Volume brought upto 1000 ml. with distilled water.

iii) 2X Stacking gel buffer

3 gm Tris (0.25 M)

90 ml distilled Water

pH adjusted to 6.8 with HCl

2 ml 10 % SDS

Volume brought upto 100 ml. with distilled water.

iv) 2X Sample buffer

0.15 gm Tris (0.125 M)

4 ml distilled Water

pH adjusted to 6.8 with HCl

1 ml glycerol

20 mg Bromophenol blue

0.4 ml β- mercaptoethanol

Volume brought upto 10 ml. with distilled water.

v) Fixing Solution

20 gm Trichloroacetic acid

50 ml distilled water

Volume brought upto 100 ml with distilled water.

vi) Staining Solution

200 mg Coomassie Brilliant Blue R-250

50 ml methanol

10 ml glacial acetic acid

40 ml distilled Water.

vii) Destaining Solution

50 ml methanol

10 ml glacial acetic acid

40 ml distilled Water.

viii) Fixing Solution

6% acetic acid

PREPARATION OF GEL

30 % Acrylamide: Bisacrylamide (28:2) solution was prepared first in distilled water. For 10 % gel 20 ml of Acrylamide: Bisacrylamide stock 15 ml 4 X separating gel buffer and 50 ml distilled water were added. After that 0.1-ml ammonium persulphate solution and 0.03 ml (30 µl) of TEMED was added. This mixture was gently poured in the already set glass plates and a thin layer of water was made over the gel solution to make sharp, flat gel surface. After 30-40 minutes the upper unpolymerized liquid was poured out and stacking gel solution (5%) was prepared by adding 1 ml 30 % Acrylamide: Bisacrylamide stock, 4 ml water, 5 ml 2 X stacking gel buffer, 0.01 ml ammonium persulphate and 5 µl TEMED. The mixture was then quickly poured on the polymerized separating gel. The slot former was also quickly placed on

the stacking gel and the mixture was then allowed to polymerize. It took 3-5 minutes to polymerize. The gel plates used were 10x10 cm. with 0.5-mm spacers.

The Standard molecular weight proteins were purchased from Sigma Chemical Co., USA.

The molecular weight calculated from the Rf values of the Standard run under the same conditions. The gel percentage used was 10 %.

NUCLEIC ACIDS

EXTRACTION OF NUCLEIC ACIDS

The tissues were dissected out, kept in an ice bath. For determination of nucleic acids, the method of Cherry (1962) was followed. Definite amount of tissue was taken for homogenisation with cold methanol. The tissue was centrifuged at 3000 g for 20 minutes in cold in a centrifuge (SCR 20BA, Hitachi Himac centrifuge, Japan). The supernatant was discarded and to the precipitate 0.02M perchloric acid was added. After waiting for 5 minutes the content was centrifuged at 3000 g for 20 minutes. The supernatant was discarded and to the precipitate ethanol: ether (1:1) was added and incubated at 50^0 C in a water bath for 30 minutes. Again it was centrifuged and the supernatant was discarded. To the precipitate known amount of 5% perchloric acid was added and incubated overnight in cold. On the next day the tissue was centrifuged and here the supernatant was collected. From this supernatant the DNA and RNA concentration were estimated by the diphenylamine and orcinol reactions respectively as described by Plummer (1988).

ESTIMATION OF DEOXYRIBONUCLEIC ACID (DNA)

The DNA was estimated through diphenylamine reaction. Diphenylamine reagent was prepared by mixing 1 g of DPA, 100 ml of glacial acetic acid and 2.5 ml of concentrated sulphuric acid (36N). From the stock solution prepared previously a measured amount was taken in a test tube and the volume was made 1 ml by the addition of 5% PCA 5 ml of DPA reagent was further added to the tube. An analysis blank with 1 ml 5% PCA and 5 ml DPA was prepared. All the tubes were kept in a boiling water bath for 10 minutes. It was cooled,

and the intensity of blue colour was determined at 595 nm in a spectrophotometer (Milton Roy Co., USA). The concentration of DNA was determined from the standard curve and the value expressed as mg/g.

Composition of diphenylamine Reagent (DPA): Diphenylamine reagent was prepared by mixing 1 g DPA, 100 ml glacial acetic acid and 2.5 ml concentrated sulphuric acid.

ESTIMATION OF RIBONUCLEIC ACID (RNA)

RNA was estimated through orcinol reagent. It was prepared by mixing 3.5 ml of solution A with 100 ml of solution B. A measured amount of the same stock solution (as prepared) was taken in a test tube and final volume of 2 ml was made with 5% PCA. 3 ml of orcinol reagent (freshly prepared) was further added to it. An analysis blank with 2 ml of 5% PCA and 3 ml of orcinol reagent was also prepared. Both the control and experimental tubes were heated for 20 minutes in a boiling water bath and brought to room temperature and the intensity of green color was estimated in a spectrophotometer (Milton Roy Co., USA) at 665 nm against a blank. The concentration of RNA was determined by the standard curve and the value was expressed as mg/g.

Composition of orcinol reagent: It was prepared by mixing 3.5 ml of solution A with 100 ml of solution B.

Solution A: This consisted of 6% orcinol.

Solution B: It was prepared by dissolving 100 mg ferric chloride in 100 ml concentrated HCl.

ENZYMES

EXTRACTION OF PHOSPHATASES

A definite amount of liver, kidney and muscle of fish was taken and homogenised in 0.9% sodium chloride. The homogenate was centrifuged at 4000 g for 10 minutes at 4^0C. The supernatant was quantitatively collected and used for further analysis.

ESTIMATION OF ACID PHOSPHATASE

Acid phosphatase enzyme activity was determined by the method of Walter and Schutt (1974). For the estimation of acid phosphatase, 1 ml acid buffer (pH 4.8) was taken in two separate test tubes. In one of the tubes, marked as control, 2ml 0.1 N sodium hydroxide was added prior to the addition of 0.2-ml enzyme extract. Both the tubes were prewarmed at 25^0C & then 0.2-ml enzyme extract was added in each tube and incubated for 30 minutes in a 25^0C water bath. At the end of stipulated period, the reaction in the experimental tubes was stopped by the addition of 2 ml of 0.1 N sodium hydroxide. The amount of 4-nitrophenol in the medium was estimated by measuring the yellow colour at 405 nm in a spectrophotometer (Milton Roy Co., USA) against a blank. Activity of the enzyme was determined in terms of the protein present per mg of the tissue sample.

Composition of Acid buffer: This was prepared by mixing 0.41 g citric acid, 1.125 g sodium citrate, 0.203 g 4-nitrophenyl phosphate and 100 ml glass distilled water.

ESTIMATION OF ALKALINE PHOSPHATASE

Alkaline phosphatase enzyme activity was determined by the method of Walter and Schutt (1974). For the estimation of alkaline phosphatase 2 ml alkaline buffer (pH 9.8) was taken in two separate test tubes. In one of the tubes, marked as control, 10 ml 0.05 N sodium hydroxide was added. Both the tubes were prewarmed at 25^0C prior to the addition of enzyme extract. 0.05 ml cold enzyme extract was added in both the tubes and incubated for 30 minutes in a 25^0C water bath. At the end of stipulated period, the reaction in the experimental tubes was stopped by the addition of 10 ml of 0.05 N sodium hydroxide. The amount of 4-nitrophenol in the medium was estimated by measuring the yellow colour at 405 nm in a

spectrophotometer (Milton Roy Co., USA) against appropriate blank. Activity of the enzyme was determined in terms of the protein present per mg of the tissue sample.

Composition of Alkaline buffer: This was prepared by mixing 1.052 gms diethanolamine, 8.0 ml of 0.1 N HCl, 46.4 mg 4-nitrophenyl phosphate in 85 ml double distilled water, pH adjusted to 9.8 by addition of 0.1 N HCl. Volume made upto 100 ml with distilled water.

TISSUE PREPARATION FOR LPO, GSH AND CYTOCHROME P-450

The liver, kidney and muscle were quickly isolated after fish were sacrificed. A definite amount of tissue was homogenised with ice-cold 1.15% KCl (10 % w/v) followed by centrifugation at 7000 g for 15 min. The supernatant was then centrifuged at 100000 g for 60 min in a Sorvall ultracentrifuge at 4^0 C. The firmly packed microsomal pellet was washed twice and resuspended in 1.15 % KCl to make 10-20 % suspension calculated from the original weight of tissue.

ESTIMATION OF LIPID PEROXIDATION

Lipid peroxidation was determined from the homogenate by the method of Ohkawa *et al.*, (1979). To 0.2 ml homogenate 0.2 ml 8.1% (w/v) Sodium dodecyl sulphate, 1.5 ml 20 % acetic acid, adjusted to pH 3.5 with 20 % (w/v) sodium acetate and 1.5 ml 0.8 % (w/v) thiobarbituric acid was added. The mixture was diluted to 4 ml with water and then heated for 60 min in a boiling water bath. Then it was cooled to room temperature and 1 ml water followed by 5.0 ml of a mixture of n- butanol and pyridine (15: 1 v/v) was added. The mixture was shaken vigorously and centrifuged at 1500 g for 15 min. The absorbance of the organic layer was measured at 532 nm. The results were expressed as nmol of malondialdehyde (MDA) formed/g wet tissue.

ESTIMATION OF REDUCED GLUTATHIONE

The reduced glutathione (GSH) in liver, kidney and muscle was determined by the method of Ellman (1959). 1-ml cytosol was mixed with 1 ml 4 % (w/v) sulphosalicylic acid and the mixture was centrifuged at 1500 g for 15 min. The supernatant was allowed to react with 0.1

mM 5, 5'- dithio-bis-2-nitrobenzoic acid. The solution was then kept at room temperature for 10 min and read at 412 nm. The result was expressed as m mole GSH/g wet tissue.

ESTIMATION OF CYTOCHROME-P-450

The microsomal pellet obtained after ultra centrifugation was resuspended in isotonic KCl solution and centrifuged as above. The twice washed microsome was finally suspended in isotonic KCl to make 10 –20 % solution and Cyt-p-450 content was assayed according to the method of Omura and Sato (1964) on an Aminco DW-2a UV/Vis spectrophotometer at 520 nm. The result was expressed as n mole/mg microsomal protein.

Protein was estimated from the cytosolic and microsomal fractions by the method of Lowry *et al.*, (1951) using bovine serum as a standard.

STATISTICAL ANALYSIS

The student's t-test and "ANOVA" (One Way) were conducted for testing significance at 5% and 1% level respectively between data of control and treated series at different fixation intervals. Data were analysed statistically using analysis of variance (ANOVA) in MS-EXCEL programme.