3. MATERIALS AND METHODS

3.1. Animals

Rohu (*Labeo rohita*) weighing about 75 ± 6g and mean body length 23 ± 5 cm were collected from a Thiruvankulam fish farm near Ernakulam Kerala, India. The fish were brought to the laboratory and acclimatized for more than 15 days in plastic tank before starting the experiment. The water had a pH of 6.74 ± 0.4 and temperature 32 ± 2°C. The fish tanks were well aerated, and the physical and chemical parameters were kept nearly constant.

3.2. Chemicals

Methylparathion-50% (O,O-dimethyl-O-4-nitrophenyl-Phosphorothioate-Bayer, Germany) a synthetic organophosphorous insecticide was obtained from market in Cochin. All other experimental chemicals were purchased from Sigma (USA), Merck (Germany) and SRL (India).

3.3. Range finding test

The range finding bioassay was conducted following APHA-AWWA-WPCF, (1975) and Reish and Oshida, (1987) with fish exposed to a range of sequential concentrations (0.002, 0.02, 0.2, 2, 20 mg/L) of methyl parathion. Eight fish were released into fish tank containing 50L of water and sequential concentrations of methylparathion. 20% water level was replaced on daily basis during the experimental period. The pesticide loss during this procedure was compensated by adding it in to the water. Mortalities were recorded (24, 48, 72 and 96h) and dead fish were removed immediately.
3.4. Static Bioassays

To determine the lethal concentration (LC$_{50}$) of the pesticide, eight fish of approximately equal size (75 ± 6g) were released into different fish tanks, containing different concentrations (1.8, 3.6, 5.4, 7.2, 9.0, 10.8, 12.6, 14.4, and 16.2 mg/L) of methylparathion. Lethal concentration was determined as per the methods of Reish and Oshida, (1987). Control fishes were maintained separately and mortality was recorded at 24, 48, 72 and 96h. The dead fishes were removed immediately and kept frozen (-20°C) pending analysis.

3.5. Lethal and sub lethal exposures

Six concentrations (1.8, 3.6, 5.4, 7.2, 9.0 and 10.2 mg/L) were selected for lethal (96h) exposures. One group was maintained as control in a tank containing methylparathion free water. Fishes were fed with commercial fish feed, and the tanks were kept well aerated. 20 liters of the medium was replaced every 24 h. At end of exposure, fishes were collected, kept frozen pending analysis. For sublethal studies, 8 numbers of fishes were exposed to the sublethal concentrations. The sublethal concentrations calculated based on the LC$_{50}$ for range finding periods of 15, 30 and 45 days.

3.6. Biochemical assay

At the end of the experiments both lethal and sub lethal, fish were killed by decapitation and organs were analysed for detoxifying and disease diagnostic and/or marker enzymes, proteins and lipids. Liver tissues were dissected, washed in physiological saline (0.9% NaCl), and kept at −20°C until analysis. The tissues were homogenized for 5 min in ice-cold 0.1M Tris-HCl buffer
solution pH 7.2 (1:5 w/v) using Polytron homogenizer (Polytron Model PT3000, Kinematica-Switzerland) and centrifuged (Remi-India) at 8000rpm for 30min. Supernatant were used to determination enzymes.

3.7. Lipid peroxidation and detoxifying enzymes

3.7.1. Lipid peroxides

Lipid peroxide (LPO) content in liver was determined by thiobarbituric acid reaction (Ohkawa et al., 1979). To 0.2 ml of homogenized sample, 1.5 ml of 20% acetic acid, 0.2 ml of 8% SDS and 1.5 ml of 20% TBA were added. The mixture was made up to 5.0ml with distilled water and heated in a boiling water bath for one hour. After cooling, the mixture was centrifuged at 3000 rpm for 10 min. Standards (Tetraethoxy propane-TEP) and blank were treated similarly. The pink colour developed was measured at 532nm in a spectrophotometer (Spectronic 20 Genesys-USA). The lipidperoxide content was expressed as nmol of malonaldehyde formed per mg of protein.

3.7.2. Superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase (SOD) was assayed in liver according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. To 0.1ml of the sample in a cuvette, 1.4ml of Carbonate-bicarbonate buffer, (0.1M-pH 10.2) and 0.5ml of 3mM epinephrine was added, mixed well and immediately read the change in optical density at 480nm for 2min in a UV spectrophotometer (Shimadzu-Japan). One unit of SOD was defined as the amount of protein needed to decrease the absorbance to 50% inhibition of epinephrine auto oxidation.
3.7.3. Assay of catalase (EC 1.11.1.6)

Catalase (CAT) in liver was assayed according to the method of Takahara et al., (1960). To 2.4 ml of 50mM phosphate buffer, 0.1 ml of the enzyme solution was added and the reaction was started by the addition of 1.0 ml of 30mM H₂O₂ solution. The decrease in absorbance was measured at 240 nm at 30sec intervals for 2min in UV spectrophotometer (Shimadzu-Japan). The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as nmol of H₂O₂ decomposed min⁻¹ mg protein⁻¹.

3.7.4. Glutathione peroxidase (EC 1.11.1.9)

Glutathione peroxidase (GPx) activity in liver was determined by the non-enzymatic method (Pagila et al., 1967). Homogenized sample (0.2 ml) was added to a mixture of 0.2 ml 0.4 M phosphate buffer, (pH 7.0), 0.2 ml of 0.4mM EDTA, 0.1 ml of 10mM sodium azide, mixed well and 0.2 ml glutathione (61.4mg Glutathione in 100 ml). The reaction mixture was thoroughly mixed and 0.2 ml of GSH, 0.1ml of 30% hydrogen peroxide and 0.1ml NADPH were added. The content of the test tube incubated in a water bath at 37°C for 10min. At the end of incubation period, 0.5ml of 10% TCA was added and centrifuged at 10,000rpm for 5 min. To 1.0 ml of the supernatant, 2ml Tris buffer (0.4M, pH 8.9) and 0.1 ml DTNB (99mg in 25ml methanol) were added. The absorbance was read at 412nm in a spectrophotometer (Spectronic 20 Genesys-USA). The enzyme activity was expressed as nmole of glutathione oxidized min⁻¹ mg protein⁻¹.
3.7.5. Total reduced glutathione (E.C 1.6.4.2)

The total reduced glutathione (GSH) was determined in liver by the method of Ellman (1959). The method is based on the reaction of reduced glutathione with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to give a compound that has absorbance at 412 nm. To 0.5 ml of homogenized liver sample, 0.1ml of 5 % TCA was added and made the volume up to 1.0 ml using distilled water. The contents were mixed well for complete precipitation of proteins and centrifuged at 4000 rpm for 15min. To 0.5ml of clear supernatant, 2.5ml of 0.2M-phosphate buffer and 50µl of 0.6mM DTNB were added and the absorbance was read at 412nm in spectrophometer (Spectronic 20 Genesys-USA), against a blank containing TCA instead of sample. Series of standards were treated in a similar way to determine the reduced glutathione content. The amount of glutathione was expressed as µmol. g wet tissue⁻¹

3.7.6. Glutathione S-transferase (EC 2.5.1.18)

Glutathione S-transferase (GST) activity was determined in liver by the method of Habig et al., (1974). To a reaction mixture containing 1.0 ml of Phosphate buffer (0.3 M - pH 6.5), 0.1 ml of 30mM CDNB, 0.1 ml of tissue homogenate and 1.3 ml of distilled water were added and pre-incubated at 37°C for 5min. 0.1 ml of 30mM GSH was added and the change in the absorbance was measured at 340nm for 3min at 30sec intervals in UV spectrophotometer (Shimadzu-Japan). The enzyme activity was expressed as µmol of chloro-2,4-dinitrobenzyne conjugate formed min⁻¹. mg of protein⁻¹.
3.8. Acetylcholinesterase activity (EC 3.1.1.7)

Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and propionylcholine (PChE) were assayed by the method of Ellman (1961), taking acetylthiocholine iodide, S-butyrylthiocholine, propionylcholine as substrates. To 3ml of sodium phosphate buffer (0.1mM, pH-8), 50 µl of the enzyme was added and incubated at room temperature for 5min. To this mixture 10µl of 10mM DTNB followed by 20 µl acetylthiocholine iodide (75mM) as substrate was added. The increase in absorbance was recorded at 412 nm on a UV spectrophotometer (Shimadzu-Japan) against blank. Butyrylcholinesterase (150mM) and propionylcholinesterase (150mM) were analysed by the same procedure, but using the appropriate substrate. The enzyme activity was expressed as nmol mg protein⁻¹ min⁻¹.

3.9. Marker and/or disease diagnostic enzymes.

3.9.1. Alanine aminotransferase (EC 2.6.1.2)

The activity of alanine aminotransferase (ALT) was determined by the method of Mohun and Cook (1957). To 1.0 ml of the buffered substrate (0.1M phosphate buffer, pH 7.4, 0.2M DL-alanine, 2.0mM 2-oxoglutarate, 1.5g dipotassium hydrogen phosphate, 0.2g potassium dihydrogen phosphate, 30mg 2-oxoglutaric acid and 1.789 DL-alanine were dissolved in 100 ml distilled water), in a test tube and 0.1 ml sample was added and incubated at 37°C for 30 min. The reaction was arrested by adding 1.0 ml of 200 mM DNPH and left aside for 20 min at room temperature. 10 ml of 0.4N NaOH was added and read at 540nm in a spectrophotometer (Spectronic 20
Genesys-USA) against the reagent blank. The enzyme activity was expressed as μmol pyruate liberated h⁻¹ L⁻¹.

3.9.2. Aspartate aminotransferase (EC 2.6.1.1)

The activity of aspartate aminotransferase (AST) was assayed by the method of Mohun and Cook (1957). To 1.0 ml of the buffered substrate (300mg of L-aspartic acid and 50mg of α-ketoglutaric acid dissolved in 20-30 ml of the phosphate buffer and 10% sodium hydroxide added to bring the pH to 7.5 and was made up to 100 ml with phosphate buffer) in a test tube, 0.1 ml of the sample was added and incubated for one hour at 37°C. Then 1.0 ml of 200 mM DNPH was added and left for 20 min. 10 ml of 0.4N NaOH was added and the absorbance was read at 540nm in a spectrophotometer (Spectronic 20 Genesys-USA) after 10 min. The blank and standards were also treated similarly. The enzyme activity was expressed as μmol pyruate liberated h⁻¹ L⁻¹.

3.9.3. Lactate dehydrogenase (EC 1.1.1.27)

The lactate dehydrogenase (LDH) activity was assayed according to the method of King (1965a). The amount of pyruvate formed in the forward reaction was measured colorimetrically. To 1.0 ml of the buffered substrate (2.76g of lithium lactate was dissolved in 125 ml of glycine buffer containing 75 ml of 0.1 N sodium hydroxide to adjust the pH 10), 0.1 ml of the sample was added and the tubes were incubated at 37°C for 15 min. After adding 0.2 ml of 5.0mg NAD solution, the incubation was continued for 30 min and then 1.0 ml of 200mM DNPH reagent was added, and the tubes were incubated further 15 min. Then 7.0 ml of 0.4N NaOH was added and the colour
developed was read at 540nm in a spectrophotometer (Spectronic 20 Genesys-USA) against the reagent blank. Suitable aliquots of the standards were also treated in the same procedure. The enzyme activity was expressed as μmol pyruvate liberated h⁻¹ L⁻¹

3.9.4. Alkaline phosphatase (EC 3.1.3.1)

Alkaline phosphatase (ALP) was assayed by the method of King (1965b) using disodium phenyl phosphate as the substrate. The incubation mixture contained the following components in a final volume of 2.8-ml. 1.5 ml of carbonate- bicarbonate buffer (pH 10.0), 1.0 ml of substrate (0.01 M disodium phenyl phosphate) and 0.1 ml of 0.1M magnesium chloride and 0.2ml of enzyme. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of 1:2 Folin's phenol reagent and tubes were centrifuged. Controls without enzyme were also incubated and the enzyme source was added after the addition of Folin's phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37°C and read at 640 nm in a spectrophotometer (Spectronic 20 Genesys-USA) against a blank. The standards were also treated as for samples. The activity of the enzyme is expressed as μmol phenol liberated h⁻¹ L⁻¹

3.9.5. Assay of acid phosphatase (EC 3.1.3.2)

Acid phosphatase (ACP) was assayed by the method of King (1965b) using disodium phenyl phosphate as the substrate. The incubation mixture contained the following components in a final volume of 3.0 ml - 1.5 ml of 0.1 M citrate buffer (pH 4.9), 1.0 ml of substrate (0.01 M Disodium phenyl
phosphate), 0.3 ml of distilled water 0.2 ml of enzyme solution. The reaction mixture was incubated at 37° C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of Folin's phenol reagent. If turbidity appeared, the tubes were centrifuged. Controls without enzyme sources were also incubated and the enzyme source was added after the addition of Folin's phenol reagent. 1.0 ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37° C. The blue colour developed was read at 640 nm in a spectrophotometer (Spectronic 20 Genesys-USA) against a blank. Blank and standards were done in the same way. The activity of the enzyme is expressed as µmol phenol liberated h⁻¹ L⁻¹.

3.10. Protein

3.10.1. Extraction of muscle protein (Sarcoplasmic and myofibrilar) fractions

Sarcoplasmic (SP) and myofibrilar (MP) proteins were analysed by the methods of Sankar et al., (2001) and King and Poulter, (1985) respectively. 2g of mince muscle was homogenized with 0.02M sodium bicarbonate buffer, (pH 7.25 buffer), maintaining a meat buffer ratio of 1:5 in a homegeniser (Polytron Model PT3000, Kinematica, Switzerland) at 10000 rpm for 1 minute. The homogenate was centrifuged using a refrigerated centrifuge (REMI R24, India) at 10000 rpm for 15 min maintaining a temperature of 0°C. Supernatant was transferred into test tube and residue was re-extracted and centrifuged as above and the pooled supernatant was taken as sarcoplasmic protein. The residue as extracted with cold 5% NaCl containing 0.02M NaHCO₃ (as above) twice and the supernatant was taken as myofibrilar protein.
3. 10.2. Estimation of protein

The protein content in the sample was estimated by the method of Lowry et al., (1951). Sample 0.1ml was made up to 1.0 ml with distilled water. Exactly 4.5 ml of alkaline copper reagent was added to all the tubes and left at room temperature for 10 minutes after which 0.5 ml of Folin’s phenol reagent (1:3) was added. The blue colour developed was read after 20 minutes at 640 nm in a spectrophotometer (Spectronic 20 Genesys-USA) against the reagent blank. Bovine serum albumin (BSA) was used as standard 20-100µg, protein concentration calculated by regression and the protein concentrations were expressed as mg g⁻¹.

3.10.3. Electrophoresis separation of proteins - Sodium Dodecyl sulphate polyacrylamide gel electrophoresis-SDS PAGE

Sarcoplasmic and myofibrilar proteins were separated by SDS-PAGE as described by Laemmli (1970) using Mini-PROTEAN II electrophoresis system (Biorad, USA). The proteins were separated using 7.5% gel concentration with staking gel (4%). In the presence of 10% SDS and 2-mercaptoethanol, proteins dissociate into sub units and bind large quantities of the detergent which mask the charge of the proteins giving a constant charge to mass ratio, so that the proteins move according to their molecular weight in an electric field.

After the run (200V, current) in 0.38M Tris-Glycine-SDS buffers, the gel were stained with 0.1% coomassie brilliant blue R250 in methanol, distilled water and acetic acid in ratio 4:5:1 respectively for 30 min. They were distained with 7% acetic acid. The developed gels were documented using Gel
documentation system (Biorad-USA) and the densitometry analysis was made.

3.11. Lipids

3.11.1. Extraction of total lipids

The lipid content was extracted by Folch et al., (1957). The weighed organs (meat, liver and brain) were subjected to lipid extraction (1:15 w/v) using chloroform-methanol mixture (2:1). The extraction was repeated twice with fresh solvent. The lipid extracts were transferred to a separating funnel and added 20% water. The separated lower layer was filtered, through sodium sulphate and concentrated by flash evaporator and stored at -23°C until analysis.

3.11.2. Lipid class separation

Cholesterol, cholesterol ester, triglycerides and phospholipids were determined by the method of Ogasawara et al., (2002), by TLC-FID method (latroscan MK-6s, Japan). The extracted lipid sample was made up with 1ml of the chloroform and 1μl of made up solution was spotted on the chromarods with Drummond Micro dispenser. The spotted chromarods were developed in chloroform- methanol-water-25% ammonia (47:20:2.5:0.28) at 20°C up to 7cm. Then after drying at 110°C for 3min, the chromarods were developed in hexane-diethylether (60:10) up to 10cm in the second stage of development and dried at 110°C for 3min and finally the rods were scanned by latroscan-MK-6s (scan speed 40s/rod, 160ml/min hydrogen flow-rate and 1500ml/min airflow rate).
3.12. Histopathological studies

The slices of the gills, brain and liver were fixed in 10% neutral buffered formaldehyde. Fixing prevent autolysis and putrification of tissues. Then they were dipped in different concentration of alcohol in ascending order and finally in absolute alcohol (10 min each) for removing water. They were then kept in methyl benzoate until it sank and dipped in benzene for removing alcohol. The tissues were then infiltrated with molten paraffin (60-70 °C) for 1 h and 15 min. A boat was made filled with molten paraffin and the tissues were placed in it. The paraffin was then cooled until it hardened, enclosing the tissue.

Using a rotary microtome, section of 4 to 5μ paraffin infiltrated tissues were made. The tissues were de-paraffinised with xylene and treated with 100%, 90% and 70% alcohol (10 min each) for removing undesirable pigment and other materials. The sections were then stained with haematoxylin and counter stained with eosin and dehydrated with 70%, 90% and 100% alcohol for 10 min each. The sections were mounted using dibutylphthalate in xylene and examined under microscope.

3.13. Statistical analysis

Lethal concentration (LC50) was calculated on the basis of initial treatment mortality data. The percentage mortality data were converted to probit values and toxic concentrations were log transformed (Reish and Oshida, 1987). Weighed linear regression was performed on the transformed data to calculate LC50 values. Mean cumulative mortalities were calculated across the treatment duration for each of the three trials and one-way ANOVA was employed followed by Duncan’s new multi-range test to calculate the significant difference between control and experimental mean (Daniel, 1987).