MATERIALS AND METHODS
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3.1. Bioassay of Cypermethrin

3.1.1 Test organisms and their acclimatisation

Advanced fry (average length 40 mm and weight 0.323g) and fingerlings (average length 102 mm and weight 11.5g) of *Clarias batrachus* were collected from the ponds of Central Institute of Freshwater Aquaculture, Bhubaneswar, India and acclimatised to laboratory conditions for a period of one week. Fishes were kept in 40 l glass jars with 30 l dechlorinated freshwater at a stocking density of 10 fry or fingerlings/jar. Plankton was given as feed to the fry *ad libitum* and finely ground formulated feed containing crude protein (40%), fat (8.0%), carbohydrate (38%) and vitamin / mineral mixture (0.1%) was given to the fingerlings @ 5% of their body weight on a daily basis. The faecal matter and other waste materials were siphoned off daily to reduce the ammonia content in water. Fifty % of water exchange was carried out every alternate day.

3.1.2 Toxicant

The commercial grade of 'cypermethrin', with 25% EC under the brand name of Ralothrin 25 E of Rallis India Ltd. procured from the local agrochemical outlet and was used as the toxicant in the study. The desired concentrations of toxicants were made in water with pH 7.2, total alkalinity of 112 mg / l CaCO₃, dissolved oxygen 3.8 mg / l, CO₂ 12 mg / l and temperature 25.0 ± 2 °C.
3.1.3 Selection of toxicant concentrations

The 'range finding' bioassays for advanced fry and fingerlings of the test animals were conducted with the toxicant using protocols established as per APHA-AWWA-WPCF(1976). The test specimens were exposed to a range of concentrations in log scale from 0.001 to 1.0 ppm for advanced fry and fingerlings. Ten animals each were released into the glass jars containing 30 l of water with the desired concentration of the toxicant. Based on the quantal response (death of the animal), the final concentrations were selected for each bioassay experiment with advanced fry and fingerlings.

3.1.4 LC$_{50}$ Test

Static bioassay method (Reish and Oshida, 1987; Mohapatra and Rengarajan, 1995) was followed and the test animals were kept in the same experimental medium for the entire experimental period. Animals were not fed during the test period. Each test consisted of a series of 7 test concentrations along with the control with normal water (dilution water), in duplicates. Twenty fish each were exposed to the experimental concentration and control. The cumulative percentage of death after 24, 48, 72 and 96 hr was noted in each experiment. The data obtained from the experiments were processed by 'Probit analysis' (Reish and Oshida, 1987; Mohapatra and Rengarajan, 1995; and Mohapatra and Saha, 2000) for determination of median lethal concentration or LC$_{50}$ with 95% fiducial limits. The 95% limits are the upper and lower 95% confidence values of LC$_{50}$. Fiducial limit is a statement where a 19 of 20 chances of the LC$_{50}$ value falls within the specified limits (i.e., P=0.05). For calculation the following formulae were used:
Slope \((S) = (LC_{84} / LC_{50} + LC_{50} / LC_{16}) / 2\)

Confidence limit \((f_{LC_{50}}) = S^{(2.77/ \sqrt{N})}\)

(Where, \(N\) = the total number of test organisms in all concentrations whose expected results were between 16 % and 84 %; 2.77 = constant)

95% Fiducial limits:  
Upper limit = \(LC_{50} \times f_{LC_{50}}\)
Lower limit = \(LC_{50} / f_{LC_{50}}\)

The 50 % lethal concentrations values were plotted against time for obtaining the toxicity curve for advanced fry and fingerlings.

3.2 Sublethal Effect of Malathion and Cypermethrin on Gonadal Development and Breeding

3.2. Collection of Test Organisms and their aclimatisation

The fish were procured from the grow-out ponds of the Institute for the study (Fig 3). A total of 200 healthy specimens were selected and kept for aclimatisation for a period of one week. Further, the fishes were divided into 5 groups; 4 experimental groups along with the control (in duplicates). Twenty fish (Sixteen female and four male) for each group were separated out and kept in rectangular fiber glass cisterns of 630 l capacity (Fig 2). The cisterns were provided with 5 cm soil base. The water level was maintained at 250 l.
Fig. 2: Experimental cisterns.

Fig. 3: *Clarias batrachus* haul.
3.2.2 Test Chemicals

Malathion with the brand name of Suthion®% with 50 % EC and cypermethrin with the brand name of Ralothrin 25E with 25 % EC was procured from the local market and used in these experiments.

3.2.3 Physicochemical Parameters

The physico-chemical parameters of the cisterns were monitored at the beginning and the end of the experiment (Table 3).

3.2.4 Test experiments:

Four different experiments in duplicates were set up for observing the sublethal effects of the desired pesticides (in duplicate):

*Experiment 1*: Calculated amount of malathion at the 1/10th concentration of 96 h LC$_{50}$ (*i.e.*, 4ppm) (Sadhu and Mukhopadhyay, 1984) was added to the cisterns.

*Experiment 2*: Calculated amount of malathion at the 1/50th concentration of 96 h LC$_{50}$ (*i.e.*, 4ppm) (Sadhu and Mukhopadhyay, 1984) was added to the cistern.

*Experiment 3*: Calculated amount of cypermethrin at the 1/10th of 96 h LC$_{50}$ (*i.e.*, 0.220ppm) (Mitra *et al.*, accepted in 2002) was added to the cisterns.

*Experiment 4*: Calculated amount of cypermethrin at the 1/50th of 96 h LC$_{50}$ (*i.e.*, 0.220ppm) (Mitra *et al.*, accepted in 2002) was added to the cisterns.
3.2.5 Duration of Experiment:
The experiment was conducted for a period of 45 days and extended till the completion of 60 days. During this period, 50% of the experimental solution was replenished once a week.

3.2.6 Feeding:
Fish were fed @ 5% of body weight with a balanced pelleted diet consisting of fish meal (40 %), rice bran (23.7 %), groundnut oil cake (22.6%), soyabean flour (13.6 %), wheat flour (10 %) and supplemented with required amount of vitamin and mineral mixture (0.1%).

3.2.7 Gonado Somatic Index (GSI)
After 15 days of experiment, four male female fish each were sacrificed and their GSI were calculated as follows -

\[ GSI = \frac{\text{Weight of Gonads}}{\text{Weight of the fish}} \times 100 \]

3.2.8 Histology of gonads
The gonads of the above fish were fixed for histological studies in Bouin’s solution. The tissues were then processed and the paraffin blocks were prepared using wax with melting point of 58-60°C procured from M/s E-Merk. Subsequently, sections of the tissues were cut at 5μ using a microtome. The sections thus obtained were further processed with a series of gradual hydration and dehydration and stained using Hemotoxilin-Eosin stain (Delafield’s hemotoxilin and alcohol soluble eosin procured from S.D.fines chemicals). Finally, the sections were observed under binocular microscope.
3.2.9 Position of Germinal Vesicle

On the onset of the breeding season (after 40 days of exposure), the female fish were examined to find out the stage of maturation. The sample of eggs were taken out with the help of a catheter and treated with clearing solution (30 parts absolute alcohol and 70 parts glacial acetic acid) for 5 minutes. The position of germinal vesicle (GV) was observed under the microscope ("Endomatrial biopsy"-Laboratories CCD60, Paris). The GV position was graded as A, B and C.

A = The Germinal vesicle in the centre
B = The Germinal vesicle in between centre and periphery
C = The Germinal vesicle very close to periphery

3.2.10 Breeding

After the completion of 45 days half of the experimental and control fish i.e., six each were taken for spawning trial. The fish from all the groups were administered with ovaprim @ 0.8 ml/kg body weight. The fishes were stripped after a latency of 14 hours. The eggs were artificially fertilized with the sperm suspension already prepared in 0.89 % sodium chloride. The males in this study used were procured from untreated sources to restrict the breeding results purely on egg properties. Subsequent spawning trial was repeated with the rest of the fish at the end of 60 days in similar manner.

3.2.11 Fertilization and Hatching

The rate of fertilization and hatching were recorded. The fertilized eggs were kept in a flow-through system for hatching i.e. 24^-26 hrs.
3.2.12 Growth of offspring

Further, the hatchlings were reared in indoor in the outdoor larval rearing tanks (LRT) of 500 l capacity and outdoor LRTs of 640 l capacity to observe their growth performance. During the indoor rearing the fry were fed with plankton *ad libitum* and sieved egg custard particles. Here, they were reared for fifteen days and provided with aerators for artificial aeration. Thereafter, they were transferred to the outdoor LRTs. A balanced formulated diet consisting of fish meal (40 %), rice bran (23.7 %), groundnut oil cake (22.6%), soyabean flour (13.6 %) and supplemented with required amount of vitamin and mineral mixture (0.1%) were fed @ 2% of their body weight and plankton were fed twice daily during the outdoor rearing.

Growth was recorded at 7, 14, 21 and 28 days for the offspring by taking atleast 20 numbers at a time from both treated and control groups and then recording their length and weight.

3.3 Effect of pesticides on Biochemical, haematological and immunological parameters

3.3.1 Collection of fish

Live and healthy specimens (average length 20.03±1.12 cm and average weight 128±2.8 g ) of *Clarias batrachus* were collected from the grow-out ponds of the institute at Kausalyaganga, Bhubaneswar and kept in cemented tanks of 630 l capacity after giving them a bath in 500 ppm solution of KmnO₄, to avoid any bacterial or fungal infections. Water level was maintained in the cisterns at 250 l. Fish were acclimatised for a period of one week in the hatchery before the experimentation. During this period, fish were fed with artificial
formulated feed (Rice Bran 20%, Groundnut Oil Cake 20%, Fish Meal 35%, wheat 10% and trace minerals & vitamin mix 0.1%), pelletised in a California pellet meal. The pellets (2 mm) were provided to the fish once a day in the morning hours.

The physico-chemical parameters of the cisterns were determined before the start of the experiment. The pH, total alkalinity (as CaCO₃), dissolved oxygen, CO₂ and temperature was found to be 7.2-7.8, 110-118 mg / l, 3.5-3.8 mg / l, 10-14 mg / l, 25.0 ± 2 °C respectively.

3.3.2 Collection of blood and analysis

Blood samples from experimental fish were collected from the caudal vein near the genital pore. The 2.0 ml injection syringe was inserted in the caudal vein and blood was drawn keeping the fish was vertically held with the head upwards. The blood was collected in 2.5ml eppendorff tubes and was immediately used for various haematological tests. No anti coagulant was used during the course of study. Biochemical and immunological studies were carried out with the serum collected after centrifugation of blood at 5000 rpm for 15 minutes and under refrigeration (-20°C).

3.3.3 Biochemical parameters

3.3.3.1 Glucose

The serum glucose level was measured spectrophotometrically at 505 nm by GOD/POD Method using glucose kit procured from Qualigens diagnostics.
3.3.3.2 Total Protein and albumin

The total protein and albumin content of the serum was measured at spectrophotometrically 565 and 630 nm, respectively by Biuret and BCG Dye Binding Method with the help of total protein and albumin kit procured by Qualigens diagnostics. Then the serum proteins were also analysed by SDS-PAGE.

**Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was done to determine the molecular weight of different proteins. Initial sealing of two glass plates (7 x 6 cm²) separated by spacers of 1.5 mm was done with agarose. For gel casting, two types of gels such as separating gel and stacking gels were prepared. 5 ml of separating gel (10%) was poured between the slide cassette upto a level below 2 cms from the glass top and allowed to polymerize. The stacking gel (4%) was then prepared and poured between the slide cassettes. Wells were made in stacking gel using a teflon comb. The gel was allowed to polymerize for half an hour. Samples were prepared by boiling bacterial WCL antigens with sample buffer for 5 to 7 min at a ratio 1 : 2 inside a water bath. The comb was removed gently and the samples, 20μl in volume were loaded into the wells. Molecular weight marker was mixed with sample buffer (1:2 ratio) and
loaded to a separate well. The gel was run initially at 50 V for 45 min to 1 hr followed by 75 V for 2 hrs. The gel was run till the dye front reached the bottom of the gel. The gel was fixed and stained in Coomassic brilliant blue (CBB) for 2 hrs and destained with destaining solution for 7-8 hrs. Molecular weight was determined as described by Shapiro et al. (1967) comparing the bands with those of the standard marker. The molecular weight of the proteins represented as bands were determined by regression analysis with a logarithmic transformation of the molecular weight using molecular weight standards.

3.3.3.3 Cholesterol

The serum cholesterol was measured spectrophotometrically at 505 nm, by CHOD / PAP Method with the help of a cholesterol kit procured from Crest Biosystems.

3.3.3.4 Ca^{++} ion

The amount of Ca^{++} ions present in the serum was measured with the help of Flame Photometer (Systronics) with a calcium filter.

3.3.3.5 Acid Phosphatase

Acid phosphatase of the serum was measured spectrophotometrically at 510 nm, by KING’S Method with the help of an acid phosphatase (ACP) kit procured from Qualigens Diagnostics.
3.3.3.6 Alkaline Phosphatase

The alkaline phosphatase of the serum was measured spectrophotometrically at 510 nm, by KIND & KING’S Method with the help of an alkaline phosphatase (ALP) kit procured by Qualigens Diagnostics.

3.3.4 Haematological parameters

The investigations on the effects of malathion & cypermethrin on the haematological parameters of Asian catfish, *Clarias batrachus* were studied.

3.3.4.1 Total Erythrocyte counts (TEC)

Red blood corpuscule counts were made in a Neubaur’s improved haemocytometer by diluting 1:200 with Henedrick’s solution (Hesser, 1960).

3.3.4.2 Total Leucocyte counts (TLC)

For counting the total leucocytes, blood was diluted 1:20 by 3 % acetic acid solution containing two drops of methylene blue per 100 ml solution using a standard WBC diluting pipette. The WBC counting carried out, using corner blocks of the haemocytometer.

3.3.4.3 Differential counts (DC)

Thin smears of blood were prepared on alcohol-cleaned slides and fixed in methanol after air-drying. Leishman’s stain was used for staining, which was commercially available. The methanol fixed slides
were immersed in Leishman's stain for 1 to 2 minutes and then 2 to 3 drops of distilled water were added to it and left for 10 minutes. Further, the slides were rinsed with distilled water and air-dried. After selecting about 100 leucocytes from each smear under oil immersion did differentials counting of leucocytes. Percentages lymphocytes, monocytes, neutrophils and eosinophils were calculated by counting at least 100 cells.

3.3.4.4 Thrombocyte counts

The thrombocytes were counted from the blood smears prepared.

3.3.5 Immunological parameters

3.3.5.1 Preparation of magur globulin:

Serum was collected from healthy magur. An equal volume of saturated ammonium sulphate (AS) solution was added to it drop by drop to the serum and was placed on a magnetic stirrer for overnight at 4°C. The sample mixture was centrifuged at 1000 x g for 10 min at 4°C and the volume was made to 5 ml with carbonate-bicarbonate buffer (pH 9.6). Precipitation of globulin was repeated twice. After precipitation, the sample was centrifuged at 1000 x g for 10 min at 4°C. The pellet was collected and the volume was made to 2 ml with carbonate-bicarbonate buffer (pH 9.6). The globulin solution was dispensed into a dialysis membrane (Snakeskin) and dialysed against PBS (pH
7.2) for 48 hrs at 4°C. After 48 hrs, the globulin was collected in eppendorffs and stored at -20°C.

3.3.5.2 Raising of Hyperimmune sera in rabbits

Two healthy purebred New Zealand white male rabbits, of about 6 months old were used for immunization purpose. They were injected intramuscularly with 0.5 ml of globulin emulsified with equal volume of Freund's incomplete adjuvant (FIA). Three booster doses were given for the specific antigen at fortnight intervals. Rabbits were bled from the marginal vein of ear after 10 days of last injection. About 5 to 10 ml of blood was collected in a single bleeding. The blood was allowed to clot and the serum was collected, heat inactivated at 56°C for 30 min and stored at -20°C.

3.3.5.3 Purification of rabbit anti-magur-globulin

The globulin from immunized rabbit sera was purified in the same way as magur globulin.

3.3.5.4 Serological Tests

3.3.5.4.1 Agarose gel precipitation test (AGPT)

4 ml. of 1% agarose in normal saline solution (NSS) was poured on a clear glass slide and allowed to solidify. Wells of 5 mm in diameter, 4 mm apart were prepared by punching in the agarose gel and the bottoms of the wells were sealed with 10μl of agarose.
Freshly thawed soluble antigen @ 20µl / well was added to the duplicate outer peripheral wells and undiluted antiserum was added to the central wells. Gels were incubated for 24 hrs at 30°C in a humidified chamber. After incubation the gel was transferred to a petridish and washed thoroughly in PBS (pH 7.2) several times and then stained in Coomassie brilliant blue solution for 3 hrs followed by destaining for 7-8 hrs to visualize the precipitation bands.

3.3.5.4.2 Counter immunoelectrophoresis (CIE)

Wells were prepared in 1% agarose gel and antigen and antiserum were loaded into the wells in a similar fashion as done in AGPT. The antigen well was placed towards the anode side. Wicks of filter paper dipped in tank buffer were placed in either side of the glass slide touching the gel at one end and the buffer at the other. A voltage of 50V was supplied for 3 hrs. After 3 hrs, the gel was transferred to a Petridish and washed thoroughly in PBS (pH 7.2) several times and then fixed in destaining solution.

3.3.5.4.3 Agglutination of bacteria

For the agglutination test, 25µl of sterile NSS was added to each ‘U’ shaped well of a microtitre plate. About 25µl of serum was dispensed to the first well followed by serial two-fold dilution onwards. To each well, 25µl of bacterial cell suspension was added. The bacteria used in this study were Flavobacterium...
branchiophilum, which is a Gram –ve filamentous rods. The bacteria were grown in nutrient broth (Himedia, Mumbai, India) for 24 hrs. They were enumerated by colony counting methods using nutrient agar plates (Himedia, Mumbai, India) and then they were inactivated and using 1 % formalin and the bacterial suspension was adjusted to 10⁹ CFU / ml. The plate was kept undisturbed overnight at 37°C and the titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of the bacterial cell.

3.3.5.4.4 Haemolytic activity of serum

For this test 50µl of sterile NSS was added to each to each ‘U’ shaped well of a microtitre plate. About 50µl of serum was dispensed to the first well followed by serial two-fold dilution onwards. Subsequently, to each well 10µl of 1% sheep RBC was added. The plate was gently shaked for thorough distribution of RBC in the suspension and was incubated overnight at 37°C. The titre was calculated as reciprocal of the highest dilution of serum showing complete haemolysis of the RBCs.
Immunological estimation of total serum globulin using Single radial immuno diffusion test (SRID)

One gram of agarose was dissolved in 100ml of 1 x Assay buffer by heating and allowed to cool to 55°C. Hundred μl of antiserum was mixed with 5 ml of the above solution. The agarose solution containing the antiserum was poured on a grease free glass plate that had previously been set on a horizontal level surface. The gel was allowed to form after cooling. Further, using a gel punch, wells of 4mm diameter were cut into the gel into which 20μl of the given standard antigens and samples were added. The gel plate was then left in a box containing wet cotton and incubated overnight at room temperature.

The diameter of the disk can be measured by marking the edges of the circle. A standard graph (Fig.1) was then constructed by plotting the diameter of the disk against the concentration of antigen in a semilog graph sheet. This test was performed with the help of SRID kit provided by Bangalore Genei pvt.Ltd.
Fig.1: Standard graph for estimating magur serum globulin

3.4 Statistical Analysis

Data were analysed using the Duncan’s Multiple Range Test (DMRT) to determine levels of significance at $P<0.05$. Statistical analysis system (SAS) version-6.12 was used to analyse the data.