Materials
And
Methods
The Bhatye estuary is located in between 17° 20’ N and 73° 20’ W in the Ratnagiri district in the Konkan region and this is the major estuary on the west coast of Maharashtra state. The experimental clams, *Katelysia opima* used for the present study were collected from Bhatye estuarine region, Ratnagiri coast, Maharashtra state (Plate 1 and 2). During low tide, the collection site was fully exposed, while during the high tide this area it gets submerged to approximately one meter. The clams of medium size (4.0 – 4.4 cms) (Plate 3) were selected, brought to the laboratory and stocked in the plastic containers containing filtered, aerated estuarine water, for 48 hours. Clams well acclimatized to the laboratory condition were grouped in tens and kept in plastic containers containing 5 liters filtered estuarine water. Static bioassay tests were conducted for 96 hours by using Cypermethrin (25% EC). For every experiment, a control group of clam was also run simultaneously. For the formulation of test concentration, pilot experiment was conducted and range of concentration selected was such that it resulted in zero to total mortality. The formula used to prepare the pesticide solution is as follows:

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\text{No. of ml for required volume} = \frac{\text{required ppm X required volume}}{\text{Stock solution}}
\]

The volume of the container was maintained at 5 L for each. Observations were made at 12, 24, 36, 48, 60, 72, 84 and 96 hours behavioural changes of the clams after introduction into different pesticide concentration were determined.

For the selection of test concentrations, some pilot tests were performed to determine the range of toxicity of the pesticide. The range of concentrations selected was such that it resulted in zero to hundred percent
mortality for short term exposures. The LC$_{50}$ value for each time period was estimated by a regression analysis determined for the log of concentrations and percentage survival of the clam. The percentage mortality in various concentrations at particular period were converted into probit values and plotted against the log of concentrations (Gosh, 1962).

The toxicity tests were repeated three times and LC$_{0}$ and LC$_{50}$ values were determined. The regression equation between the log of concentration (X) and probit mortality (Y) were determined statistically for acute toxicity using the formula Y = $\alpha + \beta \log (x)$ and 95 % fiducial limits were established according to Finney (1971).

All the experiments were carried out on freshly collected clams in three different seasons i.e. summer (March & April, 2008), monsoon (June & July, 2008) and winter (December & January, 2009). Based on the LC$_{50}$ values, $1/10^{th}$ concentration of the LC$_{50}$ of Cypermethrin was selected for sub-lethal toxicity (30 days) studies.

Sub-lethal exposure was done in static system where water and pesticide medium were renewed every 12 hours to obtain the desired pesticide concentration. A control, free of pesticide, was also maintained in each experiment. All the experiments were done in triplicates. The water parameters like temperature, salinity, pH and dissolved oxygen were measured using standard procedures. The temperature recorded with a standard mercury thermometer; salinity recorded with hand salinometer (Hanna Instruments, Italy), pH was recorded by hand pH meter (Hanna Instruments, Italy) and universal indicator method, while dissolved oxygen was measured by Winkler's titrimetric method (Welsh and Smith, 1961).
While running the bioassay, the animals were closely observed for their general behaviour, health and number by considering clams from the control group as a normal for the comparison with the behaviour of the experimental clams. The observations were noted under the following guidelines.

**Condition:**

A- The clams remained with the shell valves tightly closed with lapse of time. They slightly opened the shell valves to protrude pallial edges.

B- The clams opened the shell valves and protruded the foot.

C- The gentle mechanical stimuli made the clams to react and to take in these organs inside the shell valves, excreta and mucus secretion occurred.

D- The clams opened the shell valves slightly and extended the foot outside shell valves. The adherence of two pallial edges to the inner part of each shell valve did not remain firm. No excretion occurred but mucus secretion seen.

E- The clams died, the shell valves got widely open, shrinking of foot and siphons occurred, these organs did not respond to mechanical stimulus. Mucus secretion was considerable.

Oxygen consumption experiments were performed in specially designed glass jars of one liter capacity fitted with rubber lid containing inlet and outlet rubber tubes. The marked clams were kept, one in each jar and immediately filled with filtered estuarine water through a siphon and then clamped at both the ends and were kept aside for one hour. Dissolved oxygen
was determined by Winkler’s method from the estuarine water (Welsh and Smith, 1961). The rate of oxygen consumption of the LC_0 and LC_{50} groups of the clams along with control after every 12 hours time interval was determined. All the values were subjected to statistical analysis. Rate of oxygen consumption was calculated in terms of ml/hr/gm wet weight. Comparing the results with control, the changes in the rate of oxygen consumption from LC_0 and LC_{50} concentration were statistically calculated (Dowdeswell, 1959). The experiment was repeated three times for confirmation of the results. Such experiments were performed during summer, monsoon and winter seasons of the year 2008-2009.

After studying 96 hours (acute) and 30 days (chronic) toxicity of Cypermethrin to *Katelysia opima* in different seasons, various tissues (*i.e.* gills, mantle, hepatopancreas, foot and male and female gonad) of control, LC_0 and LC_{50} groups from acute exposure and 1/10^{th} concentration of LC_{50} groups from chronic exposure were removed from each group and were blotted with filter paper to remove excess moisture. The tissues were then dried in an oven at 60°C until a constant weight obtained. Tissues were powdered and oven dried tissue powder was used for biochemical analysis. 100 mg of dry powder of each tissue was taken for biochemical analysis. The total glycogen content was estimated according to the method proposed by De-Zwaan and Zandee (1972), using glucose as standard. Protein was determined by the method proposed by Lowry *et. al.* (1951) using Bovine Serum Albumin (BSA) as a standard. The method used for determination of lipid was Sulpho-phospho-vanilline method proposed by Barnes and Blackstock (1973). The results are expressed as milligram content per 100mg
of dry tissue. Triplicate values of each biochemical constituents were subjected to statistical confirmation using students’ test (Dowdeswell, 1957). Standard deviations were calculated during each season.

**Microtomy:**

After studying 96 hour (acute) and 30 days (chronic) toxicity of Cypermethrin to *Katelysia opima* in different seasons, various tissues (gills, mantle, hepatopancreas, foot, male and female gonad) of control, LC₀ and LC₅₀ groups from acute exposure and 1/10th concentration of LC₅₀ groups from chronic exposure were removed and fixed in a neutral buffer formalin for 48 hour for proper fixation. Tissues were washed in distilled water, then dehydrated in Ethyl alcohol, cleared in Xylol and embedded in tissue mat (at 58-60 °C melting point) and then they were sectioned at 5 to 6 µm thickness on a rotary microtome (Erma, Japan). Theses Sections were stained with Ehrlich’s Hematoxyline and alcoholic Eosin stain and mounted in DPX. All the observations for microphotography were done under Trinocular research microscope attached to the camera (Carl Zeiss, model: Axiostar).
PLATE - 1

BHATYE ESTUARY, RATNAGIRI, (MAHARASHTRA).
PLATE – 2

STUDY AREA
BHATYE ESTUARY,
RATNAGIRI, (MAHARASHTRA).
Katelysia opima (Gmelin, 1791)