MATERIALS AND METHODS

CHAPTER II

2.1 Selection of sites:

To study the impact of Ganesh idol immersion, selection of water bodies gained utmost importance. In the present study the sites selected on water bodies are the sites where maximum idol immersions take place every year (Table 1, Source DIG office, Kolhapur) . The water bodies selected are of lentic as well as lotic type i. e.river and lakes from Kolhapur city. Two sites from Panchganga River were selected namely Panchganga Ghat and Rajaram Bandhara where maximum idol immersion takes place. Two sites from Rankala Lake namely Rajghat and Sandhyamath where maximum human interference with washing activities and immersion practices was observed were selected for the study. Irani Khan, a small water body situated near Rankala Lake was selected as idols with height more than 12 ft. are immersed in this area. Kotitirtha and Rajaram lakes were considered looking at the increasing human interference and as they are from the few lakes which can still be protected.

2.2 Survey of idol-makers:

Survey of the idol-makers was carried out to collect the information about the raw materials and their quantity required for manufacturing the Ganesh idols. Twenty five idol-makers surveyed randomly from different areas like Shahupuri, Bapat Camp and Shukrawar Peth from Kolhapur city. The information about raw materials like 'Shadoo', Plaster of Paris, different colours used and their sources were extracted from this survey. This helped to select the colour pigments for the toxicity study.

2.3 Water analysis:

2.3.1 Water sampling:

To study the alterations in the water quality of the selected seven sites sampling and physicochemical analysis was carried out before, during and

after the Ganesh festival for the years 2006 and 2007. Samples we collected before 5 days of the festival, on the 4th day of the festival (1 ½ d immersion), 9th day of the festival (5 days immersion) and after 12 days of t 10th day's immersion i.e. Anant Chaturdashi.

Water sample were collected in clean polythene cans having 2 l capacity. Physico-chemical analysis was carried out within 24 hrs. aft collection of the samples.

2.3.2 Water analysis:

Using standard methods prescribed in A.P.H.A. (1995) carried o analysis of the collected water samples.

pH: pH, the hydrogen ion concentration was measured by digital pH mete using glass electrode.

Turbidity: It was measured by Nephelometric method using standard solutio of 40 NTU.

Dissolved oxygen: Dissolved Oxygen (DO) was measured by modifie Wrinkler's method.

Biochemical oxygen demand: The Biochemical Oxygen Demand (BOD was estimated by calculating initial and final DO by Wrinkler's method.

Chemical oxygen demand: COD was estimated by digesting the sampl with $K_2Cr_2O_7$ and conc. H_2SO_4 and then titrating with Ferrous ammoniu sulphate using Ferroin indicator.

Total dissolved solids: TDS was calculated by weighing the evaporate filtered sample and weighing final residue.

Hardness: Hardness of the water samples was determined by using EDT titrimetric method. The sample was titrated against 0.01 M EDTA with th addition of buffer solution and using Eriochrome Black-T as an indicator.

Nitrates: Colorimetric estimation by Brucine method was used to determin the nitrate levels in the water samples.

Phosphates: Phosphates were estimated by using Stannous chlorid colorimetric method.

Heavy metals: By Atomic Absorption Spectrphotometry method.

2.4 Selection of animal model:

The animal selected for the present study is a freshwater molluscan bivalve, *Lamellidens marginalis* (Lamarck). In selecting this animal the main considerations are:

i. Its geographical distribution all over the Indian subcontinent, availability in rivers as well as in lakes and reservoirs.
ii. Availability of sufficient stock of animals throughout the study period.
iii. Its ecological and economic importance.
iv. Knowledge about their feeding and other requirements.
v. Its freedom from parasites and diseases.
vi. As they are considered as the pollution indicators (Gupta and Sharma, 2005, Jamil *et al.*, 1999).

2.5 Distribution, habit and habitat:

The bivalve, *Lamellidens marginalis* is found in freshwater ponds, lakes, rivers and streams and widely distributed in India, Bangladesh, Mayanmar and Sri Lanka (Rao, 1989, Mandal *et al.*, 2007). Patil (1974) reported that these mussels are planktophagous: the foods mainly comprise diatoms, spores of various fungi, microscopic algal and other minute organisms like zooplankton.

2.6 Classification of animal:

Kingdom	–	Animalia
Phylum	–	Mollusca
Class	–	Pelecypoda or Bivalvia
Order	–	Eulamellibranchia
Family	–	Unionidae
Genus	–	*Lamellidens*
Species	–	*marginalis*

2.7 Collection and maintenance of animals:

The animals were collected from Panchganga River at Prayag Chikha near Kolhapur city and carried to the laboratory. The crowded organism were transferred to the glass aquaria. They were acclimatized to th laboratory conditions for fifteen days prior to the test and were maintained i untreated pond water. Ten animals were kept in a glass aquarium with siz 18" X 9" X 12". Water was changed twice daily preferably early in the mornir and late evening to avoid temperature fluctuation and aeration was provide to maintain proper oxygen tension. After acclimatization to laborato conditions bivalves with weight 75-100 gm were selected for the further study

2.8 Test chemical:

The colour pigments Pthalocyanine Green, S. F. Yellow R 135, Toluid Red 405 along with Sumica Pearl were donated by Sudarshan Chemi Industries, Pune, Maharashtra, India for the toxicity study. The laborat analysis of these pigments was carried out for different trace metals such Copper, Chromium, Zinc and Lead as several workers have recorded varic biological effects of these metals on aquatic organisms. These heavy met were detected by Atomic Absorption Spectrophotometry.

The homogenous mixture of red, green and yellow pigments along w mica was prepared in the laboratory. This mixture was having 30% of ea colour pigment and 10% of mica. Test solution was prepared by dissolvi required quantity of the above mixture of colour pigments was added to g desired concentration.

2.9 Measurement of toxicity:

2.9.1 Acute toxicity:

The results of acute toxicity tests are expressed in terms of Leth concentration. Pilot study was conducted by a range of concentrations of t

toxicant randomly from the logarithmic series of concentration given by A.P.H.A. (1995). This helped in determining the range of concentrations for the scale test. To maintain the requisite level of toxicant in the solution, the water was changed every 24 hrs during the 96 hrs experimental periods. Each time the dose of toxicant was added with the change of water in the tanks. Ten animals were used for each test concentration of the toxicant and for the control set. The animals of uniform size weighing between 75 - 100 gms and their shell length ranging from 3.5 – 5.0 cms were selected. Control tests with zero toxicant concentrations were simultaneously performed under the same laboratory conditions. Mortality were recorded after every 24 hrs. for 96 hrs. LC_0 and LC_{50} concentrations was determined after zero and 50% mortality of the total bivalves' population.

2.9.2 Exposure:

The bivalves were exposed to the sublethal concentrations that are $1/10^{th}$ of the predetermined LC_0 and LC_{50} values in acute exposure. The animals were exposed to the sub lethal concentrations for 10 days and 20 days. Large glass aquaria were used for conducting bioassay tests. They were properly cleaned before use by washing with detergent and rinsing with 10% hydrochloric acid followed by a wash with clean tap water. Each test container contained 2 liters of pond water and 10 organisms. The mixture of colour pigments was dissolved in a little quantity of test water and then this paste was introduced in the tank water to form desired concentration. Replicate tests for each test concentration were conducted along with a control group.

2.10 Toxicity studies of POP and Shadoo idols:

2.10.1 Immersion of idols at laboratory conditions:

To study the toxicity of the Plaster of Paris (PoP) and 'Shadoo' cla idols an experiment was carried out at laboratory conditions. Idols of Lor Ganesha made of Plaster of Paris and 'Shadoo' with height 9 inches wer immersed in water in the glass aquaria with 20 liters of tap water. Physicc chemical analysis of the water before and after the immersion of idols wa carried out. The parameters studied include pH, turbidity, TDS, DO, BOL COD, hardness, nitrates and phosphates.

2.10.2 Animal exposure and toxicity:

Bivalves were exposed to the water dissolved with PoP and shadoo fc 15 days. Water was continuously aerated. After 15 days the animals wer sacrificed and tissues were used for biochemical and enzymological studies Biochemicals studied were proteins, glycogen, cholesterol and lactic acic Enzymes analysis includes ACP, ALP, GOT, GPT, ATPase and LDH. Heav metal accumulation was tested for the whole body of the bivalves. The elements Copper, Zinc, Lead, Chromium and Iron were detected by AAS Light microscopy was carried out for gills, hepatopancreas and gonad tissues.

2.11 Biochemical studies:

The following biochemical parameters were studied:

2.11.1 Protein (Lowry *et al.*, 1951)

Protein content in the muscles, mantle, gills, foot, hepatopancreas and gonads tissues were estimated by Folin-Phenol reagent, as described by Lowry *et al.*, (1951). To 1 ml tissue homogenate (1:10 W/V) 5ml of alkaline copper sulphate ($CuSo_4$) solution was added. After 10 minutes of incubation at room temperature, 0.5 ml of diluted Folin-Phenol reagent was added, and the contents thoroughly mixed. The color developed was

read after 30 minutes using Spectronic 20 spectrophotometer at a wavelength of 750 nm. The bovine serum albumin was used as standard for the estimation. The results were recorded in the table.

2.11.2 Glycogen (Seifter *et al.*, 1950)

Glycogen in muscles, mantle, gills, foot, hepatopancreas and gonads were estimated by Anthrone reagent method, as described by Seifter *et al.*, (1950). Freshly excised tissues of crabs were digested in 30 % KOH. Each KOH digest was diluted to 100 ml with distilled water. To a 5ml aliquot of this solution, 10 ml of freshly prepared Anthrone reagent (0.2 % in concentrated H_2SO_4) was added gradually similarly blank and standard tubes were also prepared using distilled water and glucose solution respectively. The mixture was then boiled for 8 to 10 minutes by keeping the tubes in boiling water bath. After cooling the optical density of the mixture was read at 625 nm using Spectronic 20 spectrophotometer. Standard calibration curve was prepared for calculating glucose values. The glucose values thus obtained were converted into glycogen using Morrison's factor 1.11. The results were recorded in the table.

2.11.3 Cholesterol (Kolmer *et al.*, 1951)

Cholesterol in muscles, mantle, gills, foot, hepatopancreas and gonads was estimated as described by Kolmer *et al.*, (1951). To the tissue homogenate (1:10) prepared in glass-distilled water Ferric chloride ($FeCl_3$) reagent was added and centrifuged after half hour incubation. To 5 ml supernatant 3 ml concentrated Sulphuric acid (H_2SO_4) was added and after 20 minutes optical density was read at 560 nm wavelength using Spectronic 20 spectrophotometer. The results were recorded in the table.

2.11.4 Lactic acid (Hawk *et al.*, 1965)

For the lactic acid estimation, the method of Barker and Summerson, (1974) as described by Hawk *et al.*, (1965) was used. For determining the lactic acid, the excised tissue were accurately weighed

and homogenized in 10% TCA. After centrifugation, the clear supernatant was used for estimating lactic acid. A 2 ml aliquot of the protein free filtrate was treated with 1 ml of 20% copper sulphate solution. The mixture was diluted to 10 ml and 1 gm of calcium hydroxide powder was added to it. It was vigorously shaken till the powder was uniformly dispersed and then allowed to stand for half an hour. The mixture was then centrifuged and 1 ml of clear supernatant from each tube was transferred to wide mouthed test tube to which 0.05 ml of 4% copper sulphate solution was added followed by 6 ml of concentrated sulphuric acid. Then the tubes were placed in a boiling water bath for 5 mins. After cooling, 0.1 ml of p-hydroxydiphenyl reagent was added drop by drop in each tube. The tubes were kept at 30° C for half an hour. Finally, they were placed for 90 seconds in a boiling water bath and after cooling the optical density of the solution was measured at 560 nm.

2.12 Enzyme studies:

The following enzymatic parameters were studied

To estimate ACP, ALP, GOT, GPT, LDH and ATPase in muscles, mantle, gills, foot, hepatopancreas and gonads were dissected out at the end of the experimental period by sacrificing the bivalves. The tissue homogenates were prepared in saline using glass-distilled water under chilled conditions, centrifuged at 3000 rpm and the clear supernatant obtained were used for enzyme estimation.

2.12.1 Acid Phosphatase (Butterworth and Probert, 1970)

The ACP activity was assessed by the method of Butterworth and Probert (1970). The assay medium consisted of 0.5 ml buffer (veronal-acetate; pH 5.5) 0.5 ml substrate (3.2×10^{-2} M 4-nitrophenyl phosphate disodium salt) and 0.1 ml tissue homogenates. The reaction mixture was incubated at 30^0 C for 1 hour and reaction was arrested by adding 5.9 ml of freshly prepared NaOH solution. A control with 5.9 ml of 0.5 N NaOH,

0.5 ml substrate and 0.5 ml buffer was maintained. A blank solution with ice-cold distilled water in place of substrate was used and 6 ml 0.05 N NaOH was added to it and prepared. The p-nitrophenol (PNP) liberated due to hydrolytic activity of the enzyme was measured at 410 nm in a Spectronic 20 Spectrophotometer. The protein contents of the sample were estimated by the method of Lowry *et al.,* (1951) and the enzyme activity expressed as µ mole PNP liberated/mg protein/hour.

2.12.2 Alkaline Phosphatase (Butterworth and Probert, 1970)

The ALP activity was assessed by the method of Butterworth and Probert (1970). The assay medium consisted of 0.5 ml buffer (veronol-acetate; pH 9.5), 0.5 ml substrate (3.2 X 10^{-2} M 4-nitrophenyl phosphate disodium salt) and 0.1 ml tissue homogenate. The reaction mixture was incubated at 37 ^{0}C for 1 hour and reaction was arrested by adding 5.9 ml of freshly prepared NaOH solution. A control with 5.9 ml of 0.5 N NaOH, 0.5 ml substrate, and 0.5 ml buffer was maintained. A blank solution with ice-cold distilled water in place of substrate was used and 6 ml 0.05 N NaOH was added to it and prepared. The p-nitrophenol (PNP) liberated due to hydrolytic activity of the enzyme was measured at 410 nm in a Spectronic 20 Spectrophotometer. The protein content of the sample was estimated by the method of Lowry *et al.,* (1951) and the enzyme activity expressed as µ mole PNP liberated/mg protein/hour.

2.12.3 Glutamic Oxaloacetic Transaminase (Reitman and Frankel, 1957)

The GOT activity was assessed by employing the method of Reitman and Frankel, (1957). The reaction mixture consists of 1.0 ml of the substrate buffer solution (0.1 N Phosphate buffer pH 7.4, 0.2 M L-aspartate, 2 X 10^{-3} M Oxoglutarate) and 0.2 ml. of tissue homogenate. This mixture was incubated for one hour at 30°C and the 1.0 ml. of ketone reagent (10 N 2-4 Dinitrophenyl hydrazine) was added to it. After 20

minutes at room temperature 10 ml. of 0.4 N NaOH was added and the O.D. was read after 5 minutes at 540 nm in a Spectronic 20 Spectrophotometer. The blank was prepared in a similar manner, except for the tissue homogenate, distilled water was used and without incubation ketone reagent was added.

The specific activity of GOT is expressed as units per gram protein. One unit of enzyme is defined as that the amount which catalyzes the turnover of one mole of substrate per minute under the condition of the assay.

2.12.4 Glutamic Pyruvate Transaminase (Reitman and Frankel, 1957)

The GPT activity was assessed by employing the method of Re tman and Frankel, (1957). In this method 1.0 ml. of substrate buffer were taken (pH 7.4; 0.2 M DL alanine and 2 X 10^{-3} M Oxoglutarate) and 0.2 ml of tissue homogenate was added and incubated for 30 minutes at 37°C. The 1.0 ml of ketone reagent (10 N 2-4 Dinitrophenyl hydrazine) was added to it. Kept it for 20 minutes at room temperature and 10 ml of 0.4 N Na OH was added to it. The O.D. was read after 5 minutes at 540 nm in a Spectronic 20 Spectrophotometer.

The specific activity of GPT is expressed as units per gram protein. One unit of enzyme is defined as that the amount which catalyzes the turnover of 1 mole of substrate per minute under the condition of the assay.

2.12.5 Adenosine Triphosphatase (DuBoise and Potter, 1943)

In this reaction ATPase hydrolyzed to ADP and the inorganic phosphate thus liberated was measured by the method of DuBoise and Potter (1943) and Fiske and Subba Rao, (1925). A mixture of 0.25 ml Veronol buffer (0.05M, pH 7.4), 0.1M of $CaCl_2$ solution (0.04M), 0.25 ml of ATP solution (0.02M, pH7.4) and 0.4 ml of distilled water was incubated for 10 minutes at 37° C. After addition of 0.2 ml homogenate it was further

incubated for 15 minutes and then the reaction was stopped by adding 1 ml of 5 % TCA solution. A control was prepared by adding 5 % TCA solution to the mixture before incubation. The mixture was centrifuged and the phosphorus content of the clear supernatant was determined as per the procedure described for ATP.

Specific activity of the ATPase was calculated in terms of mgs of inorganic phosphate released / hour / mg protein at 30° C.

2.12.6 Lactate Dehydrogenase (Bergmeyer and Bernt, 1965a)

All the reagents were brought to the room temperature (26° - 30° C) and 2.85 ml of phosphate-pyruvate solution (0.05 M phosphate, pH 7.5, $3.1x10^{-4}$ M pyruvate) and 0.05ml of NADH solution ($8x10^{-3}$M) were successively pipetted into a cuvette. The reaction as started by adding 0.1 ml of the suitably diluted homogenate. The contents of the cuvette were quickly mixed and the decrease in O.D. was measured on Spectronic 20 at 340 nm at one minute interval for 5 minutes.

Specific activity for LDH was calculated as units per mg protein. The unit is defined as the amount of LDH which changes the O.D. of NADH by 0.001 per minute at 340 nm under the assay conditions.

2.13 Atomic Absorption Spectrophotometry:

For the accumulation study the bivalves, *Lamellidens marginalis* were exposed to colour pigments for 10 days and 20 days. Whole soft body was used for the accumulation study by drying for 2 days in the oven. 100 mg of each tissue was digested gently at room temperature with 4:1 nitric acid and perchloric acid. This was heated without boiling till all fumes disappeared. The residue was dissolved in 0.1 N HCl upto 25ml volume with distilled water. The samples were stored in refrigerator and heavy metals accumulation was measured by Atomic Absorption Spectrophotometry.

2.14 Light microscopy:

The pieces of gills and hepatopancreas tissues were fixed in Bouin's fixative for 24 hours. The tissues were washed in water to remove the excess fixative and dehydrated through graded series of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections were cut at 5 μ and fixed on glass slide. The sections were stained with Delafield's hematoxylin and eosin as counter stain. The stained slides were observed and photographed.

2.15 Statistical analysis:

2.15.1 Water analysis:

Results presented in the water analysis before, during and after the festival are the mean of 4 samples with standard deviation (X ± S.D.). The students t -test was used for determining the significance of the mean values of different parameters after the idol immersion. The level of significance was set at $p < 0.05$ considering 5% error.

2.15.2 Toxicity study:

Data presented in toxicity study are mean of 4 samples with standard deviation (X ± S.D) calculated by standard statistical methods. The students t-test was used for determining the significance of difference between the mean value of control and experimental groups. The level of significance was set at $p < 0.05$.

Water Collection Site from Kolhapur City

Scenario after Ganesh Immersion