MATERIALS AND METHODS

CHAPTER II

2.1 Animal Model

The animal selected for present study is a bivalve, *Lamellidens marginalis*. It belongs to phylum mollusca and is commercially important and easily available in fresh water through out the year in large numbers. Animal belongs to family Uniodae, which is found through out the world and includes all fresh water mussels. This family has many genera and about 1000 species. Generally the size of animal is about 5 to 10 cms and its body is soft, unsegmented, bilaterally symetrical and laterally compressed. Body of animal is enclosed in a shell made up of calcium carbonate.

2.2 Selection of Animals

Following points were considered for the time selection of test animal,

i) Animal is easily available in fresh water in large number through out the year.

ii) Animal is used as food therefore having economical and ecological value.

iii) Animal is filter feeders, which may be affected by industrial effluent mixed in water bodies.

iv) Animal can be easily maintained in the laboratory for larger period.

2.3 Distribution, Habit and Habitat

Freshwater bivalves are found in freshwater lakes, rivers and streams, inhabiting the surface layers of the muddy beds of rivers and lakes. In a buried position the posterior tip of its shell remains exposed in water to facilitate entry and exit of the water current, which is helpful both in breathing and feeding. It crawls slowly with the help of its plough-like, wedge-shaped muscular foot that leaves a deep trail all along its journey.

2.4 Classification of Animals

Kingdom	–	Animalia
Sub kingdom	–	Metazoa
Branch	–	Eumetazoa
Class	–	Pelecypoda
Order	–	Eulamellibranchia
Family	–	Unionidae
Genus	–	*Lamellidens*
Species	–	*marginalis*

2.5 Collection of Animal

The animals were collected from river Panchganga at location Prayag Chikhali of Kolhapur city. Animals were carried to the laboratory in plastic container. Many times animals were crowded and stressed, thereby increasing their susceptibility to disease during transport. In the laboratory, shells of animals were cleaned and made free from fouling biomass, adhered mud and kept in clean tap water in glass aquaria.

2.6 Maintenance of Animals

The animals were brought to the laboratory as a single stock for each complete test. They were acclimated to the laboratory conditions for fifteen days prior to the bioassay tests. The animals were maintained in glass aquaria containing 5 liters tap water. During acclimation water in glass aquaria was changed twice daily. To avoid unnecessary stress, animals were not subjected to rapid temperature or water quality changes. It is of utmost importance that animals be kept in excellent condition before the test. Therefore, no abrupt changes in the environmental conditions were made and preferably natural seasonal variations in environmental conditions such as temperature and daylight patterns were followed.

2.7 Food and Feeding

Food of the animal consists of microscopic organisms, both animals and plants, which are fed upon by filter feeding mechanism involving both ciliary as well as mucoid movements. Animal respond to light, touch or some other stimulus by withdrawing its foot and closing the siphons, meant for incoming and outgoing water currents.

2.8 Uses of Animal

The Lamellidens spp. is fat free however curry and soup of foot of *Lamellidens spp.* is used for faster growth and good health. The shell powder of *Lamellidens spp.* mixing with honey is used for the ramedy of giddiness, nervousness and dehydration. This soup is also used to cure cardiac ailments and blood pressures.

2.9 Tannery wastewater

Tannery wastewater is used as test pollutant through the experiment for the toxicity studies. Tannery industry is one of the water polluting industries as wastewater from tannery industry is highly contains organic components. The physicochemical parameters of the tannery effluent viz. colour, odour, pH, turbidity, TSS, TDS, COD, total hardness, chlorides, oil and grease, total calcium, total magnesium, chloride and total chromium were found to be high. For the present investigation tannery wastewater was collected from the tannery industry located at Jawahar Nagar, Kolhapur. This industry is vegetable type of industry.

2.9.1 Collection of tannery waste water

Tannery wastewater was collected from Jawahar Nagar in Kolhapur city. A small-scale tannery is selected for the collection of wastewater. Wastewater was collected in thoroughly washed and dried plastic can and brought to laboratory and stored at cold conditions for further experimental use.

2.9.2 Characterization of tannery wastewater

The physicochemical characteristics of tannery wastewater selected for determination were pH, Turbidity, TS, TDS, TSS, Chlorides, COD, Calcium, Magnesium, Chromium and Oil and Grease, etc. methods used for the determination of these parameters were as per A.P.H.A., (1995).

Methods used for analysis of Parameters in tannery wastewater

a) **Hydrogen Ion Concentration (pH) :**

For the determination of pH digital pH meter was used.

b) **Turbidity :**

Turbidity was recorded on Nephalo- turbidity meter and results are presented in NTU.

c) **Total Solids :**

Total solids were determined as the residue left after evaporation of unfiltered sample. The results are expressed in mg/lit.

d) **Total Dissolved Solids:**

Total dissolved solids were determined as the residue left after evaporation of the filtered sample. The results are expressed in mg/lit.

e) **Total Suspended Solids :**

Total solids were determined as the difference between total solids and total dissolved solids. The results are recorded in mg/lit.

f) **Chlorides :**

Chlorides were estimated by Argentimetric method using silver nitrate as a titrant. The results were expressed in mg/lit.

g) **Chemical Oxygen Demand**

COD was determined by using Reflux method in which organic matter get oxidized by standard $K_2Cr_2O_7$ and titrated with FAS. The results were expressed in mg/lit.

h) Oil and Grease

Oil and Grease were determined by liquid- liquid extraction method in which an acidified water sample is extracted with petroleum ether solvent in a separating funnel. The results were expressed in mg/lit.

j) Metal detection

Chromium metal was determined by atomic absorption spectroscopy

2.9.3 Tannery wastewater for toxicity tests

From tannery wastewater required aliquots were added to the test water to obtain the desired concentrations of the pollutants. Concentration of the pollutants was taken into consideration while calculating the strength of the solution as well as LC_{50} values. The concentrations of tannery wastewater were found in acute toxicity tests. LC_0 and LC_{50} values of tannery wastewater found were 5% and 20% respectively. The LC_0 and LC_{50} values selected for subacute toxicity were 0.5% and 2% of tannery wastewater.

2.9.4 Concentrations of tannery wastewater

Pilot study was conducted by a range of concentrations of the tannery wastewater 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. Another set of experiment was conducted and tanks were set in duplicate to increase precision of results. To maintain the requisite level of pollutants 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. Control tests with zero toxicant concentrations were simultaneously performed under the same laboratory conditions.

2.10 Stock Animals and Acclimation

The animals were brought to the laboratory as a single stock for each complete bioassay test. They were acclimated to the laboratory conditions for a

couple of weeks prior to bioassay tests during which they were maintained in large glass aquaria containing tap water. During acclimation water in the aquaria was changed daily. Only those stock animals showing less than 2 % mortality were subjected to bioassays. At the end of the successful acclimation, the healthy looking bivalves of approximately of uniform size and weight were selected for the bioassay tests.

2.11 Number and Size of the Test Animals

Ten animals were used for each test concentration of the toxicant and for the control set. The animals of uniform size (7 to 9cms)weighing between 65-100 gms were selected. They were acclimatized for 15 days in the laboratory conditions.

2.12 Test Containers

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. The size of aquaria was 18"x 12"x 9" (length, height and breadth) and each test aquria contained 5 liters of tap water and 10 bivalves. The required dose of toxicant was added to each tank. Replicate tests for each test concentration were conducted along with a control group.

2.13 Measurement of Toxicity and Calculations

The results of toxicity tests are expressed in terms of LC_0 or LD_0 (Lethal concentration or lethal dose) values for 24, 48, 72 and 96 hrs. In the present study the values are expressed as the LC_0 and LC_{50} values found during the tests. The concentration of tannery wastewater at which all bivalves survived at 5% i.e. LC_0 value and the concentration of tannery wastewater at which 50% bivalves showed mortality was 20% i.e. LC_{50} value. For the subacute toxicity studies $1/10^{th}$ dose of LC_0 and LC_{50} were taken 0.5% and 2% respectively.

2.14 Exposure to tannery wastewater

The animals were exposed to a concentration of tannery wastewater that was directly introduced in the tank water. The required amount of tannery wastewater was added to the tap water to obtain the desired concentrations of the toxicant. A concentration of 0.5% and 2% were prepared which were $1/10^{th}$ value of LC_0 and LC_{50} respectively and introduced in the water tank. The bivalves were exposed to LC_0 concentration of 0.5% and LC_{50} concentration of 2% for two different treatment periods that is 10 and 20 days for each. The bivalves exposed to LC_0 concentration of 0.5% of tannery wastewater showed 100 % survival. In case of the bivalves exposed to LC_{50} concentration of 2%, the animals were observed to be normal and sensitive till the second interval period of 15 days.

2.15 Biochemical Studies

The following biochemical parameters were studied:

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

Protein content in the gills, hepatopancreas, gonads, muscles, mantle and foot 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.

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

Glycogen in gills, hepatopancreas, gonads, muscles, mantle and foot were estimated by Anthrone reagent method, as described by Seifter *et al.*, (1950). Freshly excised tissues of bivalves were digested in 30 % KOH. Each KOH digest was diluted to 100 ml with distilled water. To a 5 ml 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.

2.15.3 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 sulfate 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 sulfate solution was added followed by 6 ml of concentrated sulfuric 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 were measured at 560 nm.

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

Cholesterol in gills, hepatopancreas, gonads, muscles, mantle and foot 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 Sulfuric 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.

2.16 Enzymes Studies

The following enzymatic parameters were studied

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

2.16.1 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.

2.16.2 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.

2.16.3 Glutamic Oxaloacetic Transaminase (Reitman and Frankel, 1957)

The GOT activity was assessed by 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.

2.16.4 Glutamic Pyruvate Transaminase (Reitman and Frankel, 1957)

The GPT activity was assessed by employing the method of Reitman 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 NaOH 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.

2.16.5 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 30^0 C.

2.16.6 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 (8x10-3M) 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.

2.17 Light Microscopy

The pieces of gills and hepatopancreas were fixed in aqueous 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 4 to 5 μ and transferred on glass slide. The sections were stained with Delafield's hematoxylin and eosin as counter stain. The stained slides were observed and photographed.

2.18 Atomic Absorption Spectrophotometry

For the accumulation studies the bivalve, *Lamellidens marginallis* were exposed to tannery wastewater for 10 and 20 days. The tissues like gills, gonads, hepatopancreas, muscle, mantle and foot were removed from the bivalves and dried for 2 days in the oven al 65^0C. 500 mg of each tissue was digested gently at room temperature with 4:1 nitric aid and perchloric acid. This was heated without boiling till all fumes disappeared. The residue was dissolved in 0.1 N HCL up to 25-ml volume with double distilled water. The samples were stored in refrigerator and metal accumulation was measured by Atomic Absorption Spectrophotometer. The data is expressed as μg/gm for bivalve tissues.

2.19 Statistical Analysis

Data presented in this study are mean of 4 samples with standard deviation (X ± S.D) calculated by standard statistical methods. The student 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 $p < 0.05$.