MATERIALS AND METHODS

Material and Methods

2.1 Water analysis:

Water samples were collected from the sites from where experimental animals were obtained. The essential physico-chemical analysis of water was done within 24 hours. During sample collection, sample containers were rinsed 2-3 times with the sample to be analyzed before filling. Tightly capped container was bought to laboratory for further analysis. Using standard methods prescribed in A.P.H.A. (2001) analysis of the collected water samples was carried out.

2.2 Physico chemical characteristics of water:

Various changes in parameters are observed in water present in different environment e.g. between river water and sea water or river water and ground water. Thus, while using this water for investigation or experimentation it is necessary to have knowledge about various constituents of water. Thus, physicochemical parameters play important role governing ecological distribution, productivity and physiology of flora and fauna.

Various physico-chemical characteristics of water included were as follows:

A) Physical parameter:

1. Temperature

B) Chemical Parameters:

1. pH
2. Dissolved Oxygen (DO)
3. Biochemical Oxygen Demand (BOD)
4. Chemical Oxygen Demand (COD)
5. Nitrogen Nitrate (NO_3-N)
6. Calcium.

C) Heavy metals:

1. Lead

All these parameters were measured by standard methods described in the handbook of APHA 20th edition, (2001), Handbcok of methods in Environmental Studies by S.K Maiti, Vol 1(2001).

2.2.1 Physical parameter:

1. Temperature:

Temperature was recorded from two sites i.e. animal collection site and tap water, which was used as experiment water. The temperature was recorded in °C by using standard mercury thermometer (100 °C thermometer with 0.5 °C graduation).

2.2.2 Chemical parameters:

1. pH:

pH being important parameter helps in knowing whether the nature of water is acidic or alkaline. The Hanna instrument, Mauritius (Digital PH meter) was used for pH measurement of experimental water. At animal collection site pH strips were used for detection.

2. Dissolved Oxygen (DO)

Dissolved oxygen was determined by standard Winkler's method.

Reagents:

1. Monohydrate manganous sulfate
2. Pottasium iodide
3. Conc Sulphuric acid (sp.gr. .84)
4. Sodium thiosulphate (0.025 N)
5. Pottasium dichromate
6. Sodium hydroxide
7. Sodium azide and Starch.

Protocol:

1.Sample was collected in well rinsed BOD bottle (300 – ml capacity). Care was taken to avoid bubbling

2.Dissolved oxygen was fixed at site by adding 1 ml manganous sulphate and alkaline potassium iodide solution and 3 to 4 samples were taken from different location to check the variation.

2. Carefully removed stopper and immediately added 1 ml conc. H_2SO mixed with gentle inversion until the precipitate completely dissolve.
3. Titrated 100 ml sample contents of the bottle with fresh Sodium thiosulphate (0.025 N) solution using starch as an indicator.
5. End point was dark blue to colorless.

DO was calculated by using formula

$$\text{Dissolved oxygen} = \frac{\text{ml of titrant x Normality x 8 x 1000}}{V2}$$

Where,

V2 = Volume of the contents titrated in ml.

Results were expressed as mg/l of DO

3. Biochemical Oxygen Demand (BOD):

Amount of oxygen required by the microorganisms in stabilizing the biologically degradable organic matter in a sample under aerobic condition at 20 °C over period of 5 days. The test for biochemical oxygen demand (BOD) is a bioassay procedure that measures the oxygen consumed by bacteria for the decomposition of organic matter

BOD was measured by 5 days BOD test.

Reagents:

1. Phosphate buffer
2 .Magnesium sulphite
3 .Calcium chloride
4. Ferric chloride
5. Sodium sulphite solution (0.025 N)

Protocol:

1. Sample was collected in well rinsed BOD bottles (300 ml capacity) care was taken to avoid bubbling.
2. Sample was aerated by providing sufficient air by air bubbler.
3. Dissolved oxygen of one set was carried out for initial DO measurement.
4. The second set of the bottles were kept in BOD incubator at 20°C for 5 days.

5. DO was determined in the sample bottle immediately after the completion of 5 days incubation.
6. Biochemical oxygen demand was calculated by difference in initial and final dissolved oxygen.

   BOD was calculated by using formula

   BOD = (D1 - D5) x Dilution factor

   D1 = Initial DO in the sample

   D5 = DO after 5 days.

   Results were expressed in mg/l of BOD

4. Chemical Oxygen Demand (COD):

COD is the amount of oxygen required by the organic and inorganic substances in water to oxidize them by strong oxidant.

COD was analyzed by Reflux method.

Reagents:

1. Standard Potassium dichromate
2. Conc. sulphuric acid
3. Sulphamic acid
4. Silver sulphate
5. Ferrous ammonium sulphite
6. Mercuric sulphate.

Protocol:

1. 20 ml well diluted sample was taken in a 250 ml COD flask (Round bottom flask).
2. Add pinch of $HgSO_4$ and shake thoroughly to maintain the ratio 10:1 for high chloride sample.
3. Add few glass beads followed by 10 ml 0.25 N Potassium dichromate solution.
4. Add slowly 30 ml of conc. H_2SO_4 and pinch of Ag_2SO_4 reagent and mixed thoroughly.
5. After connection of flask to reflux condenser, sample was refluxed for 2 hrs at 150 ^{0}C.

6. The sample was titrated against 0.1 N Ferrous ammonium sulphite using starch as indicator. Sharp colour changed blue green to wine red was indicated as end point.
7. Similar procedure was carried out for blank sample.

$$\text{Chemical Oxygen Demand} = \frac{(\text{Blank} - \text{Sample}) \times M \times 8 \times 1000}{\text{ml of Sample}}$$

Where,

8 = Miliequivalent weight of oxygen.

Results were expressed as mg/l of COD.

5.Hardness:

The hardness of the water was estimated by EDTA method

Reagents:

1. Ethylene diamine tetraacetic acid (0.01M)
2. Buffer solution
3. Erichrome Black-T
4. Sodium sulphate solution.

Protocol:

1. Take 50ml sample in a conical flask and add 1 ml buffer solution to it.
2. Add about 1 - 2 ml of sodium sulphate solution to the flask.
3. Add a pinch of Erichrome Black-T indicator to the solution which results in wine red color solution.
4. Titrate the content against standard EDTA solution till the wine red color changes to blue.
3. Note the volume of EDTA required.

Following formula was used for calculation:

$$\text{Total hardness as } CaCO_3 = \frac{\text{Burette reading} \times 1000}{\text{ml of Sample.}}$$

Results were expressed as mg/l.

6.Nitrogen (NO_3 - N):

Brucine Sulphanilic acid method was used to determine the nitrate.

Reagents:

1. Brucine Sulhpanalic acid
2. Sulphuric acid
3. Sodium chloride
4. Sodium arsenite solution
5. Standard nitrate solution.

Protocol:

1. 50 ml cleared sample was taken in large tube and put on wire rack.
2. Rack was placed in a cool water bath with the addition of 10 ml H_2SO_4 in the sample, the contents were mixed thoroughly swirling by hand.
3. 0.5 ml Brucine reagent was added and placed on a hct water bath for 20 minutes.
4. Standard curve of nitrate (1 to 10 mg/l) was prepared in different concentrations.
5. Similar procedure was carried out for the blank.
4. Absorbance of the sample and blank was recorded at 220 nm.
   Nitrate was calculated by standard curve with absorbance.
   Results were expressed as mg/l.

2.2.3 Atomic Absorption Spectrophotometry :

1. Lead:

Since lead acetate had to be induced in animals it was essential to know the actual amount of lead in both the water i.e. water at animal collection site and water used for experiment. Perkin Elmer Atomic Absorption Spectrophotometer (AAS) was used to detect the heavy metal in water. AAS is available in the Common Facility Center (CFC), Shivaji University, Kolhapur.

Protocol:

1. Sample was collected from selected site and kept in refrigerator for heavy metal analysis. 100 ml sample was taken for each element analysis.
2. Carefully filtered by using Whatman filter paper No 42. Standard solution

was prepared according to element detection method.

3. Heavy metals were detected by Perkin Elmer Atomic Absorption after Lead (Pb) (1 ml = 100 µg Pb): Dissolve 0.1598 g lead nitrate in HNO_3 solution and add 10 ml conc. HNO_3 soln. Dilute it to 1 Liter.

2. Calcium:

Calcium being essential constituent for metabolism and growth of aquatic organisms specially shell formation, since its lack or excess presence can result in various alterations in physiological condition of animals. Calcium was detected by Atomic Absorption Spectrophotometer (AAS).

Physico-chemical analysis results were analysed statistically. Standard deviation (± S.D.) was calculated with the mean of 3 samples. The student t - test was used for determining the significance of the mean. The level of significance was set at $p < 0.05$ considering 5% error.

2.3 Animal model:

The animal selected for the present study is a freshwater bivalve, *Lamellidens marginalis* (Lamarck). It is common fresh water bivalve, commercially important and easily available on large scale between the months of February to May.

2.3.1 Selection of Animal:

The selection of animal was done considering following points as they are considered as the pollution indicators (Gupta and Sharma, 2005; Jamil *et al.*, 1999.

- Its geographical distribution all over the Indian subcontinent, availability in rivers as well as in lakes and reservoirs.
- Availability of sufficient stock of animals throughout the study period.
- Its ecological and economic importance.
- Knowledge about their feeding and other requirements.
- Its freedom from parasites and diseases.

Before selection of any species for such exposure, it's important to have possible information and tolerance of that species. Bivalves may be unisexual or

hermaphrodite indicated phenomenon of sex reversal (Coe, 1932). In this bivalve the respiratary organs are gill and mantle. The gills are also helpful for feeding. The cilia present over the surface of the gill create water current which enters into mantle cavity. That means the dissolved materials or any types of toxicant firstly interact with gill and mantle. Also epithelium cells of these tissue performs major defence activity of foreign bodies (Motokawa *et al.*, 1975). Since our interest lies in study of effect of heavy metal lead (Pb) on gill and mantle and *Lamellidens marginalis* is recognized for its capacity to concentrate metals from environment (Phillips, 1980). Their tolerance of the resulting metal has been attributed to the existence of an effective detoxification mechanism involving the trapping of the incoming metals by the specific ligands in the cytosol (Viarengo, 1989). Since major part of investigation includes remedial detoxification of induced lead by mechanism using calcium.

2.4 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 and the food mainly comprises of diatoms, spores of various fungi, and microscopic algal and other minute organisms like zooplankton.

2.5 Classification of Animal:

The systematic study shows the characteristic features of shell, gills and foot are considered by the earlier contributors (Goldfuss, 1820; De Blainvilli, 1825; Simpson, 1900; Ortmann, 1911). Accordingly they have been designated as bivalves, Lamellibranchiata or Pelecypoda under phylum Mollusca. Simpson (1900) included the freshwater mussels in the family Unionidae which included genera like, Unio, Anodonta, Lamellidens, Pseudodon and Passeysia having economic importance. Preston (1915), Patil (1965, 1918b), Satyamurti (1960), Patil (1965, 1968) having taken a systematic survey of fresh water mussels in India.

The systematic classification of *Lamellidens marginalis* can be stated as follows:

Kingdom : Animalia
Phylum : Mollusca.
Class : Pelecypoda.
Subclass : Lamellibranchia
Order : Eulamellibranchia
Genus : *Lamellidens*
Species : *marginalis* (Lamark)

2.6 Collection of animals:

The animals were collected from Panchganga River at Prayag Chikhali near Kolhapur City and carried to the laboratory. The crowded organisms were transferred to the plastic containers.

2.7 Maintenance of animals:

The animals were brought to the laboratory as single stock for complete test. The animals were acclimatized to the laboratory conditions for fifteen days prior to the test and were maintained in untreated pond water. Ten animals were kept in plastic containers. Water was changed twice daily preferably early in the morning and late evening to avoid temperature fluctuation and aeration was provided to maintain proper oxygen tension. After acclimatization to laboratory conditions bivalves with weight 75 - 100 gm were selected for the further study. To avoid unnecessary stress animals were not subjected to rapid temperature or water quality change.

2.8 Heavy Metal:

The test pollutant selected is lead (Pb) in the form of lead acetate.

Test Pollutant:

Lead is among the oldest and most versatile of the common metals.

Symbol : Pb.
Period : 6.

Group	:	IV.
Atomic number	:	82.
Boiling point	:	1740°c.
Melting point	:	327.5°c.
Density (gcm^{-3})	:	11.34.
Electronegative	:	1.6.
Electronic configuration	:	[Xe] 4F14 5d10 6s2 6p2.
Atomic weight	:	207.2.
Oxidation status	:	2.4.

2.8.2 Uses:

With the early use of metals, there was little concern about environmental contamination. The salts of metals were utilized into commercial and industrial applications. Lead is used in plastic water pipes as a stabilizer which leads to water contamination. Lead is also in storage batteries, insecticides, food, beverages, ointments and medicinal products for flavoring and sweetening (Sharma, 2000).

2.8.3 Effects:

It was suggested that Roman civilization was deteriorated lead poisoning especially among ruling class. Mental retardation, infant mortality, sterility in wealthy families was found in wealthy Romans (Blood, 1969). Lead has two quite distinct toxic effects on human beings, physiological and neurological. The immediate effect of acute lead poisoning is ill defined symptoms, which include nausea, anemia, irritability; mood disturbance and loss of co-ordination. In more severe situations neurological effects such as restless, hyperactivity, confusion and impairment of memory can result as well as coma and death (Grand jean *et al.*, 1979). Lead exposed animals adversely affect reproductive function in both sexes. Lead poisoning also causes cardiovascular effects, mutagenic effect and cancer (WHO, 1979).

2.8.4 Test Solution:

The stock solution of toxicant metal salt was prepared in glass diluted water in such a way that all solution contained the same quantity of the actual metal. From this solution required aliquots were added to the test water to obtain the desired concentration of toxicant. Concentration of metal alone was taken into consideration while calculating the strength the solution as well as LC_{50} values. The test solution was prepared in 10 lit water with desired concentration.

2.8.5 Selection of Test Concentration:

Pilot study was conducted by the range of concentration of the toxicants randomly from the logarithmic series of concentration given by A.P.H.A (2001). This helped in determining the range of the concentration for the scale test. Another set of experiment was conducted and tanks were set in duplicate to increase precision of result. To maintain the required level of toxicant in the solution the water was changed after every 24 hours during the 96 hours experimental period. Each time the dose of toxicants were added in the tanks. Control test with zero toxicants concentration were simultaneously performed under the same laboratory conditions.

2.9 Stock Animals and Acclimatization:

The animals were brought to the laboratory as a single stock for each complete bioassay test. They were acclimatized to the laboratory conditions for couple of weeks prior to bioassay test during which they were maintained in big plastic container. During acclimation water in the aquaria was changed daily. Only healthy animals were selected for bioassay.

2.10 Nature and Size of the Test Animals:

Among the healthy animals well acclimated to the laboratory condition, 10 animals were kept in each dose container. These animals were given the LC_{50} dose of the selected test solution. The sexually matured animals were preferred for experiment and they were chosen according to the size i.e.about 5 cm to 7 cm of length.

2.11 Test Containers:

Large plastic containers were preferred for conducting bioassay test. They were properly cleaned before use by washing with detergents and rinsing with tap water. Each test container contains 10 liter water and 10 bivalves. Required dose of toxicants was added to each tank. Repeated tests were performed along with control group.

2.12 Test Water:

Tap water was used for experiment. Physico-chemical properties of the test water and collection site water were performed. Temperature, pH, Nitrates, Phosphate, BOD and COD, dissolved oxygen, Calcium and Lead were determined as per procedures recommended in A.P.H.A (2001).

2.13 Measurement of toxicity and calculation:

After these physicochemical parameter studies it is essential to evaluate the LC_0 and LC_{50}. The acute toxicity and chronic study provides information about the relative toxicity of the test material or toxin. The test designed to determine the highest concentration of a toxicant that is sufficient to affect 0% of the test organisms is called the LC_0 value, and the concentration of toxicants that is sufficient to affect 50% of test organisms is LC_{50}. This concentration is estimated by exposing test organisms to a graded logarithmic series of concentrations of toxicants and observing their responses.

For determining the value of LC_0 the organisms were exposed to different concentrations of test material and the highest value where no death of organism occur is determined. For this about 10 different concentrations were taken and animals were exposed. Similarly the LC_{50} value was determined at which the highest concentration where 50% of animals were dead.

2.13 Experimental Exposures:

For the present study we choose only chronic exposure. The animals were exposed to concentrations of lead acetate which was 1/10 of its LC_{50}. A

concentration of 28 ppm were prepared and introduced into the containers and bivalves were exposed to these solutions for two exposure periods, 8 days and 16 days.

As this experiment also include remedial study calcium was used as the chelating agent for removal of lead acetate from the tissues and body of organisms, the calcium was used in different concentrations to determine the curative effects in different organs of *Lamellidens marginalis*. Thus the bivalves were divided into 8 different groups according to the dose given.

Animals were divided in to 8 groups treated with following doses of Lead acetate and Calcium acetate for 8 days and 16 days.

Group A: Control animal kept in untreated water.

Group B: Animals treated with 28 ppm of Lead acetate.

Group C: Animals treated with 28 ppm of Calcium acetate.

Group D: Animals treated with 56 ppm of Calcium acetate

Group E: Animals treated with 84 ppm of Calcium acetate

Group F : Animals with 28 ppm of Lead acetate + 28 ppm of Calcium acetate.

Group G: Animals with 28 ppm of Lead acetate + 56 ppm of Calcium acetate.

Group H: Animals with 28 ppm of Lead acetate + 84 ppm of Calcium acetate.

On completion of exposure periods the bivalves were dissected and organs such as hepatopancreas and gonads were separated and used for biochemical as well as histochemical assays. Few animals from each group were also preceded for Atomic Absorption Spectroscopy.

2.15 Biochemical study:

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

Total proteins were estimated using Folin-Ciocalteu-Phenol reagent (Lowry *et al*, 1951).

Reagents:-

1. Lowry's A – 25 gm sodium carbonate in 0.1N NaOH.

2. Lowry's B – 0.5% Copper sulfate in 1% Sodium tartarate

3.Lowry's C – 50 ml Lowry's A+ 1 ml Lowry's B. Prepare fresh just prior to use.

Folin-Ciocalteu-Phenol reagent :

Sodium tungstate ($Na_2MO_4.2H_2O$)	100 gms
Sodium molybdate ($Na_2MO_4.2H_2O$	25 gms
Distilled water	700 ml
Conc. HCL	100 ml
Phosphoric acid (85%)	50 ml

Reflux the above mixture for 10 hours in glass apparatus. Then add to this mixture.

Lithium sulphate	150 gms
Distilled water	50 ml

Add 5 drops of bromine water and boil this to remove excess bromine water. Dilute to 1N and use.

Assay of Protein:-

	Blank	Sample
Distilled water	1.0ml	0.9ml
Homogenate	0.0ml	0.1ml
Lowry's C	3.0ml	3.0ml
	Wait for 15 minutes	
Folin-Ciocalteu-Phenol reagent		
	0.5ml	0.5ml

Wait for 60 minutes. Then take reading at 660 nm against blank.

Calculation: Optical density was converted into protein value from the standard graph plotted as optical density versus concentration of protein. Calculate the amount of protein per gm tissue.

2.16 Enzyme Study:

Enzymes are biochemical catalysts for chemical reaction that occur in the biological system which are necessary for the metabolic process within the tissue. Studies on the enzyme activities help a great deal in giving deeper insight into the vital phase of metabolic pathways. Persusal of literature shows that enzymes in various organisms are studied by Eisler and Edmunds

(1966), Thomas *et al.*, 1995, Sastry *et al.*, 1997, Ravikrishnan *et al.*, 1997 and Shukla and Sastry, 1998.

2.16.1 Acid Phosphatase (Leinhardt and Walter ,1965)

Acid phosphatase activity was determined according to Leinhardt and Walter (1965) by using citrate buffer (0.1M, PH 4.8)

Reagent:

1. Citrate buffer (0.1M, PH 4.8): Dissolve 410mg citric acid and 1.125 gm sodium citrate in distilled water and make the volume of 100 ml.
2. Substrate buffer solution: Dissolve 165mg P-nitro phenyl phosphate in 100ml above citrate buffer.
4. NaoH (0.1N): Dissolve 4.0gm NaoH in final volume of distilled water of 1000 ml

Procedure:

1. Bioassay of acid phosphatase activity was carried out for the different groups of animals stated above.
2. 1.0 ml of substrate buffer (citrate) was added to each of the tube and the tubes were equilibrated at 37°C for 10 min.
3. To the sample tube 0.2 ml of the respective homogenate was added.
4. All the tubes were incubated at 37.5°C for 30 min.
5. The activity was arrested by addition of 4.0 ml 0.1N NaoH in all the tubes.
5. After arresting the enzyme activity, 0.2 ml of homogenate was added to blank tube, if necessary the tubes are centrifuged.
7. P- nitrophenly liberated in the assay was measured as optical density at 405 nm with spectronic- 20 adjusting blank at zero.

Unit of enzyme activity was defined as the amount of p-nitro phenyl phosphate required to release 1μ M of p-nitro phenol per minute under standard assay conditions.

2.16.2 Alkaline Phosphate (Leinhardt and Walter ,1965) :

Alkaline phosphatase the enzyme activity was assayed according to method described by Bergmer, 1965.

Reagent

1. Substrate buffer -

Glycine	375 mg
NaOH	166 mg
$MgCl_2$	10 mg
P-nitro phenyl phosphate	165 mg

Dissolve above chemicals in distilled water and make final volume 100 ml. The pH of the solution will be 9.2

2. NaOH (0.02 N) - Dissolve 80 mg NaOH in 100 ml distilled water.

Procedure:

1.Take 1.0 ml substrate buffer and 0.1 ml suitably diluted serum and incubate at 37^0 C for 30 minutes.

2. Stop the reaction by the addition of 10 ml 0.02 N NaOH. Do not add the serum to control tube during incubation.

3. Add serum to control tube stopping the enzyme activity by NaOH. Take reading at 405 nm.

Calculate the activity by converting OD to the amount of p-nitro phenol released.

Unit of enzyme activity was defined as the amount of p-nitro phenyl phosphate required to release 1μ M of p-nitro phenol per minute under standard assay conditions.

2.17 Light Microscopy:

The normal histology of an organ is the refraction of the normal physiology and absence or presence of pathological alterations in histology are used to decide the conditions of protection or efficacy of the induced chemical. Histology also revealed the transitory alteration through which gill and mantle pass during dose and duration dependent cure or protection. Therefore during present work the histopathological alterations in the like gills and mantle were studied.

2.17.1 Preparation of tissue for histology:

Gill and mantle were removed and proceeded for histological preparations. Always new razor blade was used to cut tissue into pieces. Gills and mantle were dissected out and cut with the precautions, into pieces of 0.5 x 0.5 x 0.5 cm, and fixed in Bouin's fixative. The fixative as prepared as follows:

Bouin's fixative: It was prepared as described by (Thompson, 1966).

1. Saturated picric acid (tinitrophenol)	20 g
2. Distilled water	1000 ml

Dissolve picric acid in distilled water with the aid of heat; allow cooling and decanting the supernatant.

Bouin's stock solution

Solution 1	750 ml
Para formaldehyde (4%]	250 ml

Bouin's fixative

Solution 2	95 ml
Picric acid	5 ml

Add glacial acetic acid to stock Bouin's fluid just before the fixative is to be used. The pH of this fixative should be approximately 1.5 to 1.7.

The tissues were processed for wax sectioning as describe by Pears (1968) and Thompson (1966). The sections were cut of 5 µ and stained with hematoxylin and eosin and PAS for neutral mucosubstances (Spicer *et al*

1965; McManus, 1946; Hotchkiss, 1943). In present work we decided to study hitology of Collagen fibers was carried out by Van Gieson's Picric acid Acid fuchsin stain.

Reagents :

1. Harris' hematoxylin

Hematoxylin (national aniline No. 564)	5 g
Ethyl alcohol (absolute)	50 ml
Aluminum ammonium sulfate	100 g
Mercuric oxide	2.5 g
Distilled water	1000 ml

Dissolve aluminum ammonium sulfate in 900 ml boiling water. Similarly dissolve 5 g hematoxylin in small quantity of absolute alcohol. Mix above two solutions and boil rapidly. Remove from the heat and add mercuric oxide very slowly. Shake well and plunge in cold water. This is ready for use as soon as it cools and this stain can be stored indefinitely.

2. Eosin

Eosin Y (National aniline No. 516)	2 gm
Absolute ethyl alcohol (AA)	200 ml

Dissolve eosin in absolute alcohol and filter the stains.

A critical review of biochemical and histochemical cata is obtained by various work in the field discern many physiological and biochemical gaps in our information. The histochemical studies give reliable information about different chemical constituent and the cytological architecture and consequently in the organ to explain the probable roles. The outcome of such research provides substantial background for wider research in comparative morphology, anatomy and physiology.

1. Appropriate size of tissue i.e. gills and mantle were fixed in Bouwins fixative for 24 hours.
2. The tissue is preceded further by washing in chilled distilled water or running tap water.

3. Dehydration is done by different grades of alcohol i.e. 30, 50, 70, 90 and absolute.
4. Clearing is done by xylene and impregnation by paraffin wax.
5. Embedded in paraffin wax and sections are taken at 5 to 6 µm.

2.18. Heamatoxylene eosine staining:

1. Dewax the sections for 30 minutes in xylene.
2. Hydrate through descending alcohol grades.
3. Wash with distilled water.
4. Treat the sections with Harris' hematoxylin for 8 minutes at room temperature.
5. Wash with distilled water repeatedly.
6. Dehydrate through ascending grades of alcohol.
7. Bring slides in absolute alcohol.
8. Stain with eosin for 1minute.
9. Wash with absolute alcohol.
10. Clear in xylene.
11. Mount in DPX.

2.19 Histological demonstration of Collagen fibers :

For the demonstration of collagen fibers in tissue section we used Van Gieson's Picric acid Acid fuchsin stain (Thompson, 1966) was used.

Chemicals:

1) Weigert's Iron Hematoxylin:

i) Alcoholic Hematoxylin, (1%)

Hemotoxylin (National aniline no. 564) – 1g

Ethyl alcohol, 95 % - 100 ml

ii) Aqueous Ferric chloride, (29%)

Ferric chloride ($FeCl_3.6H2O$) – 29 gm

Distilled water makes volume – 100 ml

iii) Acidified Ferric chloride

Solution ii – 4 ml

Distilled water – 95 ml

Hydrochloric acid (concentrated) – 1 ml

iv) Weigert's Iron Hematoxylin

Solution i – 20 ml

Solution iii – 20 ml

Prepared at the time that it is to be used.

2. Aqueous Acid Fuchsin (1%):

i) Acid fuschin (National aniline no. 402) - 1gm

ii) Distilled water makes volume - 100 ml

3.Saturated Aqueous Picric Acid:

i) Picric acid (Fisher certified reagent No. A 253) – 6 gm

ii) Distilled water makes volume – 200 ml

Dissolved the picric acid with the aid of heat. Cool to room temperature and decant the supernatant fluid

4.Van Gieson's stain:

I) Solution 2 – 5 ml

ii) Solution 3 – 95 ml

5.Saturated Alcoholic picric acid:

i) Picric acid – 7 ml

ii) Ethyl alcohol (95%) – 100 ml

6. Picric Acid-xylene

i) Solution 5 – 6 drops

ii) Xylene – 100 ml

7. Acidified xylene

i) Glacial acetic acid (CH3COOH) - 0.5m

(99.7% acetic acid)

ii) Xylene – 100 ml

Staining procedure:

1. Dewax the sections for 30 minutes in xylene.
2. Hydrate the section.
4. Wash in distilled water.
5. Treat with Weigert's Iron Hematoxylin sol.1 – 10 min.
5. Again rinse in distilled water.
6. Give the treatment of Van Gieson's stain – sol.4 – 1 - 3 min.
7. Dehydrate through Ethyl alcohol, 95%.

8. Rinse in Ethyl alcohol, absolute.
9. Again rinse in Ethyl alcohol, absolute.
9. Treat with Picric Acid-xylene - sol. 6.
11. Again give teatment of Picric Acid-xylene - sol. 6.
12. Wash the exess stain with acidified xylene - sol. 7.
14. Clear the sections in Xylene.
15. Mount in DPX to make a permanent preparation.

2.19. Atomic absorption spectroscopy:

For Atomic Absorption Spectroscopy tissues were to be separated from animal after exposure and kept in oven at 60°C for 72 hours. The dried sample i.e. 100 mg sample is digested with 10 ml nitric acid and perchloric acid at the ratio 1:1 till clear solution obtained. Then the sample is cooled, filtered by Whattmans filter paper and diluted with concentrated hydrochloric acid (5 ml) and again diluted with glass distilled water (35 ml). Then, these samples were analyzed for Atomic Absorption Spectroscopy (Perkin Elmer Model No. 3030 USA) (Lithnor, 1975).

2.20. Statistical Analysis:

The results of various experiments were analyzed statistically. The statistical calculations were carried out according to the methods given in the text book of statistics (Agarwal. 1990). Data presented in the study are the mean of 4 samples with standard deviation ($\bar{X} \pm$ S.D) calculated by standard statistical methods. The ANOVA TWO FACTOR test is used for determining the significance of difference between the mean values of the control and experimental groups. The level of significance was set at $P< 0.05$.