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3. MATERIALS AND METHODS

3.1. Field Studies

Field study was undertaken in East Kolkata Wetland Area covering the general information such as hydrobiology, catchment areas and land use pattern (Total area, Area comprises of water spreading area, degraded water spreading area, Agricultural area, Garbage dumping area, Urban and rural settlement) floral (mainly phytoplankton and Macrophytes) and faunal (mainly Zooplankton, Mollusk, Prawn and fish) with the composition of total study area. General survey based analysis was undertaken around East Kolkata Wetland (EKW) to locate different industries situated around the East Kolkata Wetland. For this purpose repeated visits were conducted to each and every corner of the East Kolkata Wetlands.

During survey, discharge points of different industrial effluents were spotted out and a number of sewage fed ponds were also studied. Ecological Status of East Kolkata Wetland was analyzed with reference to fish and fish food organisms. Fish production data was collected from a number of fishermen co-operatives by repeated visits.

The entire area of about 12,741 hectares of East Kolkata Wetlands was studied for 180 days. During the survey 4 spots were selected for sampling starting from southern part to the northern part. Sampling spots were selected on the basis of effluent discharge points, Samplings of all the 4 spots were conducted at an interval of 30 days. Each spots was maintained with five replicates and a control spot was selected near to science city, Kolkata as this part of the wetland did not receive any pollutant from any sources. The sampling spots were designated as D1, D2, D3 and D4. The other reasons for destruction of this aquatic ecosystem were also studied through repeated visit at the studied area. The parameters considered for study were planktons, bottom organisms and water qualities.
3.2. Limnological Methods

Initial Survey was undertaken to spot out the discharge points of industrial effluents that were discharged into East Kolkata Wetlands. Four discharged points in main waste water canal were finalized. They were designated as MC1, MC2, MC3 and MC4. Three sampling points in feeder canals were selected and designated as FC1, FC2, FC3. Four sewage fed ponds were also selected for the study of water quality parameters. They were designated as P1, P2, P3, and P4. Physico-chemical nature of the effluents and water quality of sewage fed wetlands were analyzed seasonally. Samplings were done at an interval of 30 days. Following parameters were analysed to find out the composition of different effluents discharged into East Kolkata Wetlands. Effluents were collected in between 6 a.m to 11 a.m. Some parameters were analyzed on spot with multi parameter kit. Effluents were collected in 10 liter plastic jar then preserved accordingly and further analysis was carried out in the laboratory. The parameters analyzed for aquatic ecosystem included water qualities, planktons, and bottom grazing organisms. All these parameters were estimated periodically.

3.2.1. Analysis of Water Quality Parameters at sampling spots

Water quality parameters of sampling spots were studied as follows:

a. Colour: In naked eyes.
b. Odour: By smelling.
c. Temperature: Mercury thermometer.
d. pH: pH of water samples were determined using pH meter with electronic glass electrode (LI 127 of Elico, India).
e. Dissolved oxygen: Dissolved oxygen (DO) was determined by Winkler’s method. Water was sampled carefully in 100 ml reagent bottle and fixed by 48% manganese sulphate (MnSO₄) and alkaline potassium iodide followed by vigorous shaking of the bottle. A thick precipitate appeared which was dissolved by concentrated sulphuric acid (H₂SO₄). Aliqute of the sample was titrated against (0.025N) Na₂S₂O₃ using 1% starch solution as indicator. Disappearance of blue colour determined the end point of the titration.
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f. Total alkalinity: It was also determined by titration with 0.02 (N) H₂SO₄ using phenolphthalein and methyl orange as indicators. Change of colour from yellow to faint orange indicated the end point of titration.

g. BOD: Winkler method (APHA 1998).

h. COD: Titration method (APHA 1998).

i. Total Suspended solid: For TSS, approximately 50-100 ml samples (depending on visual assumption) were collected in 100-ml polyethylene bottle and filtration was done by transferring whole aliquot to filtration funnel fitted with filter paper.


l. Hardness-Hardness of water was calculated by titration against EDTA after adding a few drops of ammonia base. Eriochrome black-T was used as indicator. Change of colour from red to blue indicated the end point of titration.

m. Oil and grease: According to APHA (1998).

Statistical Analysis

The analytical results were processed using various statistical techniques such as mean, median, maximum, minimum, standard deviation, coefficient of variation, correlation coefficients etc. The paired t-test was applied to assess the level of significance of difference among different parameters and among the stations. The p values larger than the critical value of p at the 95% confidence levels were considered significant.

3.2.2. Plankton and Bottom organisms

For collection of plankton, water samples were collected from different wetland at subsurface level through plankton net of 40 micron size. Samples were preserved in 1% formalin for laboratory analysis and identified accordingly. Mollusks are collected from the lake’s bed and side by collecting buskets and then preserved dry for future analysis.
3.3. Fish production trend in East Kolkata Wetlands

Fishery practice is one of the main activities in the East Kolkata Wetland. A number of fishermen co-operatives were formed for the upliftment of the fishermen community. Previous and recent data of fish production were collected from fishermen co-operatives time to time. Collected data were compared with each other.

In Study I, the various fishponds were surveyed and fish species were identified. A detailed data of fish species of the East Kolkata Wetlands was recorded with abundance of each fish species for a period of two years. Besides, the local fish markets at Anandapur, Gariahat, Kasba and Chingrihata (places around the wetlands at Kolkata) were also surveyed along with the major fish landing stations at Bantala and Choubhaga. Fishes were caught by professional fishermen usually by drag nets. After being caught, fishes were identified, measured, weighed, and stored with formalin (10%). Identification was done with the aid of taxonomic keys following the taxonomic descriptions and biological features by Menon (1991), Talwar and Jhingran (1992), Jayaram, (1999) and Daneils. (2002). Conservation Status of fish was followed from the CAMP report (2010) and Menon (1991) in accordance to the IUCN Red List category.

Study II was designed to find out the relative abundance of some of the frequently sampled and commonly occurring fish species.

Relative Abundance \( = \frac{P}{\Sigma P} \) where \( \Sigma P \) is the total number of species.

3.4. Social Impact Assessment

Social Impact Assessment of environmental changes was carried out around the East Kolkata Wetland area. Pollution of aquatic Environment directly and indirectly altered the social life of the surrounding people. They faced a lot of problems in their daily life for the environmental changes. Interview programs were carried out among the local people and local fisher folks of three selected spots of East Kolkata wetland area. The types of social Impact Assessment that occurred as a result of any developmental project related changes were grouped into six overlapping categories as per Vanclay (1999), EIA training Resource Manual (2002) and International Association of Impact Assessment Guidelines (Vanclay,
2003). Then based on these categories the following variables were finalized for the Social Impact Assessment.

3.4.1. Demographic Assessment
   i. Sex Ratio
   ii. Age structure
   iii. In and out migration rate

3.4.2. Cultural Impact
   i. Change in share customs and traditions.
   ii. Change in value system.
   iii. Change in language.
   iv. Changes in traditional and recreational activities.

3.4.3. Community Impact
   i. Changes in social structure.
   ii. Changes in social organization and relationship.
   iii. Effect on cohesion and stability.
   iv. Effect on sanitary and health condition.

3.4.4. Socio-psychological impact
   i. Change in individual quality life.
   ii. Sense of security on belonging.
   iii. Perception on hazards.
   iv. Effect on mental stress

3.4.5. Economical Impact
   i. Changes in income
   ii. Pressure on service, transportation and infrastructure
   iii. Effect of the Economical stress.
   iv. Impact on traditional economic activities.

3.4.6. Other Impacts
   i. Changes in Environment.
   ii. Alteration to land use pattern.
   iii. Alteration to natural habitat.
iv. Change in Institution.
v. Changes in local leadership.
vi. Impact on historical structure.

3.4.7. Sampling process for generating primary data

On the basis of the livelihood strategies adopted by the households in the EKW area, they are categorised into three groups. The first category of households depends on the wetland based livelihood activities, which included farming using waste water from the city, fishing in bheries whether in own bheries or as wage labour in bheries, collection of food wastes from the hotels of Kolkata and supply of these wastes as foodstuff for fish in the bheries, several works related to fisheries other than fishing or drawing nets etc. The second category of households depends on both wetland based livelihood as well as non wetland based livelihood activities. The third category of households depends on non wetland based livelihood activities for their earning. The non wetland based livelihoods comprises wage labour in formal and informal sectors in the nearby urban areas, domestic labour, construction labour, one village from each category had been chosen as representative of the three communities for the purpose of the survey. The reasons behind the choice of these three villages were purposive and strategic. The sample size for the purpose of the survey had chosen practically considering time, space and budget for the field work. The sample represented 4.26% of the total population of the sample villages.

Field survey was conducted with the help of structured questionnaire as well as open ended interviews to realize the perception of the people living in the wetland area regarding the conversions of the wetlands.

The Questionnaires

The questionnaires used for the household level survey had comprised of following section of information:

Section 1: general information of the sample household.
Section 2: demographic information of the sample household.
Section 3: socioeconomic condition of the household, household assets and income.
Section 4: livelihood information of the sample household.
Section 5: development indicators, indebtedness and saving status of the sample household.
The survey was conducted over a period of three months; June, July and August 2011. The local people were arbitrarily chosen from the three villages for the purpose of the interview. The household selected to be surveyed were chosen randomly on the basis of latest voter list available of these three villages at the Panchayet level. The person chosen from the voter list was respondent of that particular household. Only in few cases the unavailability of the particular respondent in the household at the time of survey, made to choose either the head of the household or any other respondent from the particular household for the survey.

3.4.8. Secondary data

The secondary data was collected from Government Offices and NGOs working in the wetland area. The sources of the secondary data were Department of Environment, Government of West Bengal, India, and the NGO SEED working in the wetland area. Website of Census, India and various other websites were also used to obtain the secondary data in this investigation.

3.5. Experimental Design for Toxicity test

All laboratory and outdoor tests were carried out on the basis of randomized block design with several treatments. Laboratory studies were conducted on acute toxicity for 96 hours; outdoor studies were done on chronic effects on fish and aquatic ecosystem for 90 days. For both laboratory and outdoor test, five replicates per concentration with appropriate controls were maintained. All data were then statistically analyzed by using SPSS software and the significant of any change was tested at 5% level of probability.

3.5.1. Acute Toxicity Tests of tannery effluents on fresh water fish

Test Organisms

*Labeo rohita* commonly known as ‘rohu’ is one of the economically most important species among freshwater fish cultured in India. Rohu is considered as a bottom-column feeder and prefers plant matters including decayed vegetation (Khan and Jhingran, 1975). The fish is widely distributed all over India and many other Asian countries viz.,
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Bangladesh, Pakistan and Myanmar (Menon, 1974). It is found abundantly in ponds, tanks and flood-plain wetlands.

The systematic position of *Labeo rohita*, according to Berg (1940) is:

- **Phylum**: Chordata
- **Sub-phylum**: Vertebrata
- **Super class**: Gnathostomata
- **Series**: Pisces
- **Class**: Teleostomi
- **Sub-class**: Actinopterygii
- **Order**: Cypriniformes
- **Sub-order**: Cyprinoidei
- **Family**: Cyprinidae
- **Genus**: Labeo
- **Species**: *Labeo rohita* (Hamilton, 1822)

Freshwater fish, *Labeo rohita* of about 6-8 cm in length and 32–58 gms in weight were collected from fish seed farm, Naihai. They were treated with 0.1% of KMnO4 solution for 30 minutes to remove any external infections and Fishes were fed with pelted feed and feeding was stopped 24h prior to acute toxicity test. Each experiment was performed in triplicate.

Acute toxicity tests on test fish were conducted in 15 liter glass Aquaria each holding 10 liters of unchlorinated tap water (pH 7.3± 0.2; DO 10 mg /liter; DO 10 mg/liter; alkalinity 240mg/liter as CaCO3 ; hardness 260 mg/ liter as CaCO3; temperature 28±2°C ). These fishes were exposed only to tannery effluent. Five fishes per aquarium were tested at a time. If any organisms were found dead it was immediately removed from the test container to avoid contamination due to rotting. Death was assumed when moment ceased and there was no response to mechanical stimulation.

However, this could not be definitely stated in case of gastropods. The death of gastropods was evident from the following facts. The gastropods lost their ability to retract their protruded mantel and could not adhere to substratum. For test of gastropods, a heavily perforated alluminium foil was placed in ach beaker half inch above the surface of test solution to prevent crawling out of the gastropods from the beaker. Oxygenation of the
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Water was not hampered due to perforation of the aluminium foils. The length and weight of fish used in toxicity tests were given in respective chapters. Lethal concentration at which 5% (LC$_5$), 50% (LC$_{50}$) and 95% (LC$_{95}$) died in 96 hours were estimated. The 96h LC$_{50}$ value of tannery industry effluent was calculated by following the method given by Finney (1971). For this, 10 fishes each were exposed to 50L of normal water (control) and five concentrations (2%, 4%, 6%, 8% and 10%) of tannery industry effluent. Mortality of fishes was recorded in each concentration for 96h. The values of mortality were converted into Probits and concentrations into log values and graph was plotted to determine 96h LC$_{50}$ value. Control group was also monitored. Behavioural responses and morphological changes in three sub lethal Concentrations (3.53%, 1.76% and 0.88%) of LC$_{50}$ value of effluent were recorded after 24h, 48h, 72h and 96h durations of exposure. Control group fishes were also monitored to assess the normal behaviour.

After acute toxicity (96 hr.) experiments for tannery effluents, alive fishes (collected from fish farm, Naihati) were immediately sacrificed (5 from each group) from control, LC$_0$ and LC$_{50}$ group separately to obtain gill, liver, muscle and kidney. The pooled samples of these organs were properly blotted; weighed and used for biochemical estimations i.e. total glycogen (De Zwaan and Zandee, 1972), total protein (Gornall et al., 1949) and total lipid (Barnes and Blackstock, 1973).

3.5.2. Chronic toxicity tests of tannery effluent’s component, Chromium on fresh water fish

Test chemicals:

The toxicity of tannery effluent’s components heavy metal, Chromium was tested in the present study. The bioassays were performed with their inorganic salts. Their source of procurement, chemical composition and physico-chemical properties are described in Table 3A.
Table 3A: Details of the Tannery effluent’s component heavy metal used in the present study

<table>
<thead>
<tr>
<th>Chemical used</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of heavy metals</td>
<td>Chromium</td>
</tr>
<tr>
<td>Name of compounds used</td>
<td>Chromium chloride</td>
</tr>
<tr>
<td>Chemical formula</td>
<td>CrCl₃</td>
</tr>
<tr>
<td>IUPAC Name</td>
<td>Chromium (III) Chloride</td>
</tr>
<tr>
<td>Source of procurement</td>
<td>E. Merck (India) Ltd. Mumbai</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physico-chemical properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity (%)</td>
<td>98</td>
</tr>
<tr>
<td>Molar mass (gm/mol)</td>
<td>158.35 (anhydrous)</td>
</tr>
<tr>
<td>Density (gm/cm³)</td>
<td>2.76</td>
</tr>
<tr>
<td>Physical grade properties</td>
<td>Violet coloured solid</td>
</tr>
<tr>
<td>Melting Point</td>
<td>1150°C (an-hydrous)</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>1300°C decomposes</td>
</tr>
<tr>
<td>Solubility</td>
<td>Less soluble in water</td>
</tr>
</tbody>
</table>

These tests were conducted in cemented vats (diameter 62 cm and mean depth 30 cm), each holding 60 liters of bore hole water used in acute toxicity test. In each vat, five kg of uncontaminated soil were added. Then fifteen numbers of fish were introduced in each vat. They were fed 1:1 mixture of rice bran and mustard oil cake every day, at the rate of 5% of their body weight. Fish were exposed as many as 13 times at 7 days intervals with different sub lethal concentration of tannery effluent and mixture of tannery effluents and other effluents such as electroplating industrial effluents and dye effluents. This test was carried out for 90 days to see the impact of pollutants on behavioral, biochemical and physiological changes of fish. Various schedule such as treatments, feeding and measurement of various parameters in this studies were given in Table 3B. The length and weight of fish used in chronic tests are given in Table 3B.
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Table 3B: Time schedule for Outdoor Experiment

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Observations made on days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0,7,14,21,28,35,42,49,56,63,70,77,84,90</td>
</tr>
<tr>
<td>Fish behavior and survival</td>
<td>Daily</td>
</tr>
<tr>
<td>Growth measurement</td>
<td>Daily</td>
</tr>
<tr>
<td>Gastro Somatic Index (GIS) and condition factor (K)</td>
<td>90</td>
</tr>
<tr>
<td>Maturity Index</td>
<td>90</td>
</tr>
<tr>
<td>Water analysis</td>
<td></td>
</tr>
<tr>
<td>DO, Alkalinity, hardness, free CO₂, pH</td>
<td>0,7,14,21,28,35,42,49,56,63,70,77,84,90</td>
</tr>
<tr>
<td>Temperature, colour, odour</td>
<td>Daily</td>
</tr>
</tbody>
</table>

3.5.2.1. Behavior, Survival, Growth parameters

Behavior and survival of fish were studied only on daily observation basis. After completion of 90 days period of outdoor studies, all fish sampled from the vats for analyzing the growth parameter (yield, condition factor and gastro somatic index) following Lagler (1959).

3.5.2.2. Growth and reproduction study

At every 30d interval with a total exposure period of 90d all fish were sampled from the vats and preserved in 4% formalin. Within short period body length and weight, liver and gonad weight of the fish were recorded. Total number of ripening eggs per female fish as their fecundity was also measured.

3.5.2.3. Hematological test

Blood parameters are considered as an essential index of the whole body and therefore are important in diagnosing the structural and functional changes establishing the health status of fish exposed to toxicants (Al-Attar, 2005). Haematological changes in fish were analyzed at every 30d interval with a total exposure period of 90d during outdoor bioassay in a separate earthen vat with 4 adult, healthy fishes exposed to two sublethal concentrations of toxicants mentioned in the respective chapter. Every treatment of the test chemicals consisted four replicates and one control. Actually aquatic pollutants in the
medium induce marked haematological alteration and thus blood parameters can be applied as indicators for adverse environment (Kumar et al., 2009).

The blood samples were analyzed separately for each fish. Blood was drawn from the control and treated fishes by severance of caudal peduncle (Dacie and Lewis, 1991). Fish were randomly collected from both control and treatments, and immersed in 0.5 mg/l MS 222 (3-aminobenzoic acid ethyl ester; Sigma) for 1-2 min to anaesthetize the fish and then blood was collected in non-heparinized Eppendorf tubes with the help of micropipette. The initial drops were discarded and about 4ml of fresh blood was collected from each fish. Any blood adhering to the outside of the pipette was wiped before transferring the blood into the tube (Mgbenka et al., 2003; Shaheen and Akhtar, 2012). Blood in tubes containing 0.02 ml of 10% EDTA (dipotassium salt of ethylene diamine tetra acetic acid; Sigma) as anticoagulant was used for determination of haemoglobin content (Hb) as well-known stress indicators (Kumar et al., 2009).

**Estimation of blood haemoglobin (Hb)**

Estimation of blood haemoglobin is used to assess the functional status of the oxygen carrying capacity of the blood stream and has been used as indicator of heavy metal pollution in the aquatic ecosystem (Ergönül et al., 2012). Haemoglobin content in fish blood was determined by Sahli's acid haematin method (Mukherjee, 1988) with the help of a haemometer (Marienfield, Germany). The graduated tube was filled by (N/10) HCl upto the mark. Now 20 cm$^3$ of blood sample was added to it with the help of a micropipette. The contents of the tube were stirred and allowed to stand for 3 minutes. The acid haematin formed was gradually diluted by stirring and adding distilled water. The reading was noted when the colour of the graduated tube matched with that of the sealed tube. The reading was noted directly in gm/dl.

**3.5.2.4. Patho-Physiological indices**

Fishes have a larger surface area which facilitates the uptake of materials from the water to the body inside. Under stress, caused by toxicants different body tissues like liver, ovary etc. are affected in several ways, which alters the physiological state of those organs such type of alteration may be expressed by the target organ and body weight ratio as an index. The result may also be a good bioindicator for pollution and be useful in
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determining the extent of damage from pollutant exposure (Hauser-Davis et. al., 2012; Sadekarpawar and Parikh, 2013). However, the target organs tested in the experiment were as follows:

(a) Liver as one of the very important organ considering the action of toxic chemicals on the fish.

(b) Reproductive organs i.e. ovary and testes as organs for propagation.

Hepato somatic index (HSI) and gonado somatic index (GSI) were calculated at every 30 days interval for fishes treated with toxicants to assess the changes in their physiological condition during the experiment using the formulae (Delahunty and de Vlaming, 1980; de Vlaming et. al., 1982; Heath, 1995) as follows:

**Hepatosomatic Index (HSI)**

$$\text{HSI} = \frac{L}{W} \times 100$$

Where, $L = \text{liver weight of fish (gm)}$

$W = \text{body weight of fish (gm)}$

**Gonadosomatic Index for male and female fish (GSI)**

$$\text{GSI} = \frac{G}{W} \times 100$$

Where, $G = \text{gonad weight of male and female fish (gm)}$

$W = \text{body weight of male and female fish (gm)}$

3.5.2.5. Statistical analysis:

Mortality rate of *Labeo rohita* at different concentrations of each toxicant and at different times of exposure (24, 48, 72, 96h) was analyzed using the computer software R version 2.14.0 (US EPA, 1999) and probit analysis by Finney (1971) for determining 96h lethal concentrations (LC$_{10,50,90}$) with 95% confidence limits of different heavy metals to the test organism. The relation between mortality rate, feeding rate and opercula movement with exposure time and doses was determined by analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) (Gomez and Gomez, 1984). The relation between the mean values of the various haematological, pathophysiological indices and limnological parameters for the control and treatments for each toxicant during chronic experiment
with exposure time and concentrations of each toxicant were also analyzed by one-way ANOVA followed by DMRT.

3.6. Acute and chronic toxicity of tannery effluent’s component, Chromium on freshwater snail

Test organism:

Freshwater snail, *Bellamya bengalensis* Lamarck, 1882 was used in the present investigation as test organisms.

Systematic position (after Ramakrishna and Dey, 2007):

- **Phylum**: Mollusca
- **Class**: Gastropoda
- **Sub-class**: Prosobranchia
- **Order**: Mesogastropoda
- **Super family**: Viviparoidea
- **Family**: Viviparidae
- **Sub-family**: Bellamyinae
- **Genus**: *Bellamya*
- **Species**: *bengalensis*

Freshwater snail *Bellamya bengalensis* is a common mollusc in India and usually found in stagnant water of ponds, ditches, lakes, paddy fields etc. Sometimes they also occur in the slow flowing streams, rivulet etc. (Ramakrishna and Dey, 2007). With few greenish-black bands on the smooth, thin and moderately large shell, these snails are popularly known as Googli in West Bengal.

Mature stock of *Bellamya* was collected from the natural, unpolluted ponds by leaf trapping. Among them, healthy and active snails were selected and used in the study. After collection from the littoral zone of the pond they were cleaned in freshwater and then undertaken bath treatment of 0.1% KmnO₄ solution for disinfection, if any. They were kept in outdoor 1.0 X 1.0 m cemented tank and with aeration facilities. During acclimatization they were also provided with their natural food like *Hydrilla, Pistia, Lemna* etc. In case of mortality of the test organisms exceeding 5%, entire lot of the animals was discarded. Finally before starting the acute toxicity test in the laboratory the snails were again...
acclimatized for 24h prior to experiment in well aerated glass aquaria filled with unchlorinated tap water at room temperature. During chronic toxicity test in the outdoor earthen vats the snails were tested directly taking from the culture stock.

**Test chemicals:**

The toxicity of six heavy metals which are frequently found in the fly ash as ingredients was tested in the present study. The bioassays were performed with their inorganic salts. Their source of procurement, chemical composition and physico-chemical properties have been described in Table 3A.

**Stock solution:**

A 1000 mg/l stock solution of each test compound was prepared by mixing the analytical grade compound of respective heavy metal into the distilled water as solvent. The mixture was then stirred with a magnetic stirrer for uniform mixing of the test chemical. Stock solutions were used within 2h after preparation and were prepared fresh when required further.

Bioassays test conducted in the laboratory for 96h acute toxicity tests and in the outdoor enclosures for 28d chronic toxicity test using *Bellamya bengalensis*. Water chemical analysis and the bioassays were done following the methods outlined in American Public Health Association (APHA, 2005). Deep tube well water (temperature 27 ± 2.05 °C, pH 7.4 ± 0.21, free CO$_2$ 8.0 ± 0.21 mg/l, DO 5.54 ± 0.42 mg/l, alkalinity 176 ± 7.01 mg/l as CaCO$_3$, hardness 120 ± 7.0 mg/l as CaCO$_3$) stored in an overhead tank was used as a diluent medium. Only healthy, active and mature snails were selected at random from a single stock before the experiment. Test organisms used in the bioassay comprised of the snail with mean shell height 2.28 ± 0.49 cm and mean weight 2.83 ± 0.71 gm. Detail account of each bioassay is given below:

**3.6.1. Acute toxicity test:**

The acute toxicity tests for molluscs were conducted in 15 litre glass aquaria containing 10 litre of water. A set of four aquaria were exposed to one concentration of toxicants to make four replicates per concentration. Each set of tests was accompanied by four replicates and control. Test concentrations were prepared by diluting appropriate aliquots of stock solutions into the test medium. Ten test organisms were used in each
replicate. Furthermore, test animals were not fed 24h before and during the 96h bioassays to avoid interference of excretory substances on the toxicity of test solution (Verma et al., 1980) and also to avoid dissolved toxicant losses due to particulate adsorption (Kasherwani et al., 2009).

Initially, rough range finding tests were conducted for all toxicants to determine the dose range at which mortality occurs. The selected test concentrations of toxicants were finally used for the determination of 96h LC\textsubscript{10,50,90} to the test organism.

**Table 3C: Concentrations of Tannery effluent’s component, Chromium used during their 96 hrs acute toxicity tests to Bellamya bengalensis.**

<table>
<thead>
<tr>
<th>Name of toxicant</th>
<th>Concentrations used during acute toxicity test (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>0.0  60.0  62.5  65.0  67.5  70.0  72.5  75.0  77.5 80.0  82.5  85.0  87.5  90.0  92.5  95.0  97.5 100.0 102.5 105.0 107.5 110.0 - - - - - -</td>
</tr>
</tbody>
</table>

The vitality of the snails was frequently checked using soft tweezers, and they were considered dead when there was no response to physical stimulation. The number of dead snails was counted every 24h and removed immediately from the test medium to avoid any organic decomposition and oxygen depletion (Bhunia, 2000; Dhara et al., 2013). The test medium was replaced every 24h by freshwater and the desired quantity of respective heavy metal was immediately added to the water to assure a constant concentration of the toxicant in the solution and also to avoid other abiotic factors interfering with the animals’ performance (Al-Attar, 2005; Dhara et al., 2013). Cumulative mortality of the test organisms after 96h was used to estimate LC\textsubscript{10,50,90} values with 95% confidence limits by a computer program (US EPA 1999).

3.6.2. **Behavioural study:**

During the 96h bioassay the behavioural changes of different test organisms exposed to the toxicant were recorded systematically. The ethological changes like crawling activity, clumping tendency and touch reflex of the snail were recorded systematically by
naked eye observation during the entire experiment period following the method of Rand (1985).

3.6.3. Chronic toxicity test:

Chronic toxicity tests were conducted in outdoor earthen vats of 60 l capacity for 28 days. Vats were arranged in 3 blocks each with 4 vats as per Randomized Block Design thereby giving four replicates for each treatment (Gomez and Gomez, 1984). For toxicant three treatments (two sublethal concentrations and a control) were used during chronic toxicity tests (Table 3D). The sublethal concentrations of a toxicant were determined on the basis of its 10% and 20% of 96h LC₅₀ value to *B. bengalensis*. Each vat was filled with 5 kg uncontaminated soil. Then it was filled with water and manured by cow dung at the rate of 5000 kg/ha. It was kept in this condition for one month in order to grow sufficient plankton which serves as natural food for the test organisms (Bhunia, 2000). Such conditioned vats also show minimum percolation of water and have been found suitable for evaluating chronic toxicity of toxicants (Kaviraj et al. 2004). Each vat was stocked with fifteen healthy, active and acclimatized adult *B. bengalensis* for haematological and biochemical studies under chronic exposure. 10% of test medium was replaced weekly during experiment. The stocked snails were fed with aquatic macrophytes, *Lemna perpusilla* in addition to the algae developed naturally on the inner wall of the vat.

**Table 3D: Sublethal concentrations of tannery effluent’s component, Chromium used in the chronic toxicity test for *Bellamya bengalensis***

<table>
<thead>
<tr>
<th>Name of the toxicant</th>
<th>Concentrations used during chronic toxicity test (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Haematological study**

The blood samples of both the treated and control *B. bengalensis* were analysed for haemocyte count at every 7 days interval with a total exposure period of 28 days following the method as described by Kambale and Potdar (2010). Before collection of haemolymph, snails were washed with cold water in order to remove the faeces and excess mucus. Then
water adhering to shell of the snails was soaked. A 26 G½ (0.45mm × 13mm) sized needle attached to a 5 ml sterile syringe was inserted in foot of the snail through their operculum. The pressure was applied by withdrawing the plunger of the syringe and needle was slowly moved deeper until drops of almost colourless haemolymph were aspirated. About 0.5 ml of oozing haemolymph was collected aseptically from each snail and it was immediately stored at 4°C to avoid haemocyte clumping. The number of haemocytes/mm³ of haemolymph was counted in a Burker-Turk haemocytometer (Van der Knaap et al, 1981). During counting of haemocytes the first few drops of haemolymph were expelled and the next drop was discharged into a haemocytometer. Total haemocytes present in the haemolymph were counted after two minutes.

**Biochemical study**

For determination of protein contents of the hepatopancreas and gonads, the organs of the treated and control *B. bengalensis* were collected at every 7d interval with a total exposure period of 28d. The organs were dried in oven at 75°C to 80°C and blended into dry powder. Protein contents of these dried powders of different tissues of control and treated snails were estimated following the method of Lowry *et al.* (1951). A sample of 10 mg dry weight of each tissue was transferred into a test tube. Then 5 ml of l (N) NaOH was added to it and warmed for 10 minutes without overheating. The tubes were then cooled and their volumes were adjusted to 10 ml with distilled water. Finally they were centrifuged for 5 minutes at 3500 rpm. 1 ml of the supernatant was then taken and mixed with 5 ml of alkaline copper reagent properly. The mixture was then incubated for 10 minutes and mixed carefully with 0 .5 ml of Folin’s phenol reagent. The optical density of the sample was determined spectrophotometrically at 500 nm after 30 minutes. The concentration of total protein is expressed as mg/100gm dry weight of the tissues.

**Statistical analysis**

Mortality rate of *B. bengalensis* at different concentrations of each toxicant and at different times of exposure (24, 48, 72, 96h) was analyzed using the computer software R version 2.14.0 (US EPA, 1999) and probit analysis by Finney (1971) for determining 96h lethal concentrations (LC₁₀,₅₀,₉₀) with 95% confidence limits of different heavy metals to the test organism. The relation between mortality rate with exposure time and doses was determined by analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test.
Materials and Methods

(DMRT) (Gomez and Gomez, 1984). The relation between the mean values of the various haematological and biochemical parameters with exposure time and concentrations of each toxicant were also analyzed by one-way ANOVA followed by DMRT.

3.7. Acute and chronic toxicity of tannery effluent’s component, Chromium on behavioral changes of freshwater worm

Test organism

The freshwater macrobenthic oligochaete worm, Branchiura sowerbyi was selected as test organism

Systematic position:

Phylum : Annelida
Class : Oligochaeta
Order : Archi oligochaeta
Family : Tubificidae
Sub-family : Branchiurinae
Genus : Branchiura
Species : B. sowerbyi

Scientific name : Branchiura sowerbyi Beddard, 1892

This oligochaete worm, Branchiura sowerbyi is small, slender, usually reddish gray in colour. They measure about 2-4 cm in length. The worms live with their heads buried in the mud whereas the tails wave actively about in water. Branchiura sowerbyi is broadly distributed in the sediments of freshwater bodies like lakes and rivers and occasionally in sewer lines preferably in a gentle waved or wave less water forming a colony (Tyler, 2009).

Mature populations of worm were collected from the local unpolluted sources. They were washed in a freshwater and kept in outdoor 1.0 x 1.0 m cemented tank. A gentle flow of uncontaminated as well as unchlorinated tap water was maintained in the tank from an overhead reservoir. In case of mortality of the test organisms exceeding 5% entire lot of the test organisms was discarded. Finally before starting the experiment the worms were acclimatized for 48h prior to experiment in a well aerated glass beaker filled with unchlorinated tap water at room temperature under laboratory condition.
Test chemicals:

The toxicity of chromium as components of tannery effluent’s was tested in the present study. The bioassays were performed with their inorganic salts. Their source of procurement, chemical composition and physico-chemical properties are described in Table 3A.

Stock solution:

A 1000 mg/l stock solution of each test compound was prepared by mixing the analytical grade compound of respective heavy metal into the distilled water as solvent. The mixture was then stirred with a magnetic stirrer for uniform mixing of the test chemical. Stock solutions were used within 2h after preparation and were prepared fresh when required further.

Bioassay methods:

Static replacement bioassays were conducted in the laboratory during 96h acute toxicity tests of different heavy metals to determine the relative sensitivity and ethological changes of *B. sowerbyi*. Water chemical analysis and the bioassays were done following the methods outlined in American Public Health Association (APHA, 2005). Tap water stored in the glass aquaria (temperature 27 ± 0.45 °C, pH 7.4 ± 0.21, free CO₂ 8.0 ± 0.21 mg/l, DO 5.54 ± 0.42 mg/l, alkalinity 176 ± 7.01 mg/l as CaCO₃, hardness 120 ± 7.0 mg/l as CaCO₃) was used as a diluent medium. Only healthy, mature and acclimatized specimens were selected at random from a single stock. Test organisms used in the present bioassay comprised of the adult worms with mean length 2.02 ± 0.72 cm; mean weight 2.05 ± 0.75 mg. Detail account of the bioassay followed in the present investigation is given below:

3.7.1. Acute toxicity test

Acute toxicity tests for worm were conducted in 500 ml Borosil glass beakers each containing 300 ml of water. A set of four beakers was exposed to one concentration of toxicant to make four replicates per concentration. Each set of test was accompanied by four replicates of control. Ten test organisms were used in each replicate. Test concentrations were prepared by diluting appropriate aliquots of stock solutions into the test medium.
Initially, rough range finding tests were conducted for toxicant to determine the dose range at which mortality occurs. The selected test concentrations of toxicants were finally used for the determination of 96h LC_{10,50,90} to \textit{B. sowerbyi (Table 3E)}.

The vitality of the worms was frequently checked using soft tweezers, and they were considered dead when there was no response to physical stimulation. The number of dead organisms was counted every 24h and removed immediately from the test medium to avoid any organic decomposition and oxygen depletion (Bhunia, 2000; Dhara et. al., 2013). The test medium was replaced every 24h by freshwater and the desired quantity of respective toxicant was immediately added to the water to assure a constant concentration of the toxicant in the solution and to avoid the interference of other abiotic factors with the animals’ performance (Al-Attar, 2005; Dhara et. al., 2013). Cumulative mortality of the test organisms after 96 h was used to estimate LC_{10,50,90} values with 95% confidence limits by a computer program (US EPA 1999).

Table 3E: Concentrations of Tannery effluent’s component, Chromium used during their 96 hrs acute toxicity tests to \textit{Branchiura sowerbyi}

<table>
<thead>
<tr>
<th>Name of toxicants</th>
<th>Concentrations used during acute toxicity test (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>0.0  40.0  41.0  42.0  43.0  44.0  45.0  46.0  47.0</td>
</tr>
<tr>
<td></td>
<td>48.0  49.0  50.0  51.0  -     -     -     -     -</td>
</tr>
</tbody>
</table>

3.7.2. Behavioural study

The behavioural changes of the test organisms exposed to various doses of toxicant like movement, clumping tendency and mucous secretion were recorded systematically by naked eye observation during the bioassay following the method of Rand (1985).

3.7.3. Statistical analysis

Mortality rate of \textit{B. sowerbyi} at different concentrations of each toxicant and at different times of exposure (24, 48, 72, 96h) was analyzed using the computer software R version 2.14.0 (US EPA, 1999) and probit analysis by Finney (1971) for determining 96h lethal concentrations (LC_{10,50,90}) with 95% confidence limits of different heavy metals to the test organism. The relation between mortality rate with exposure time and doses was determined by analysis of variance (ANOVA) followed by DMRT (Gomez and Gomez, 1984).