MATERIAL AND METHODS

WATER SAMPLING

Water samples were collected every month during 7 to 8 a.m. in glass jars at certain depth as suggested by APHA from all (four) sites.

1. Glass containers of five liters capacity, to avoid the solution action, were used for the collection and storage of water samples.
2. Containers were thoroughly cleaned, washed and rinsed before every collection.
3. For each site separate containers were used.
4. The collected samples were labeled properly indicating the location and date of sampling. For DO estimation, water samples were collected in 300 ml capacity BOD bottles. These bottles were corked tightly under the surface of water to prevent air bubble formation.
5. Until estimation, all the samples were protected from direct sunlight and heat.
6. Well water sample collected with the help of metallic bucket, and transferred it to glass containers which were tightly corked.

ANALYSIS OF SAMPLE

Samples were analyzed periodically for physico-chemical characteristics. During the course of present study, 14 of physicochemical parameters were considered for analyses for each sample.


In view of the desirability, the analyses of some parameters done immediately after collection and the visual parameters such as colour are recorded on the field.

**Physical parameter**

1. **Colour**

   Colour of the samples was noticed by visual observation.

2. **pH (Sorenson, 1909)**

   **Electrometric method**

   pH of the sample was measured on the digital pH meter using glass electrode. The pH of spent wash and ground water measured immediately after collection of the sample.

3. **Total Solids (TS) (Haward, 1933)**

   **Gravimetric method**

   The 50ml unfiltered sample evaporated in an evaporation dish on water bath having temperature not more than 98°C. Then treat the residue at 104-105°C in oven for one hour and take the final weight after cooling in dessicater.

4. **Total Dissolved Solids (TDS) (Haward, 1933)**

   **Gravimetric method**

   In 50ml of capacity an evaporating dish, ignite sample at 550± 50°C in a muffle furnace for about a hour in desiccator and weight. Filter the sample in the preweighted evaporating dish on water bath or hot plate. Treat the residue at 103°C-105°C in oven for 1 hour and take the final weight.
5. Total Suspended Solids (TSS) (Haward, 1933)

**Gravimetric method**

Total suspended solids were calculated by deducting the value of dissolved solids from total solids.

**Chemical parameters**

1. **Dissolved Oxygen (DO)** (Welsh and Smith, 1963)

**Iodometric method**

Water samples were collected in 300ml of BOD bottles with all necessary precautions removing air bubbles. To this add 2ml of Winkler’s ‘A’ and 2ml of Winkler’s ‘B’ solution to fix oxygen in water. After returning to the laboratory 2ml of conc. Sulphuric acid was added to dissolve the precipitate, from this 50ml solution was takes into conical flask and titrated against N/40 sodium thiosulphate solution using 1% starch as an indicator.

2. **Biochemical oxygen demand (BOD)** (Welsh and Smith, 1963)

**Titrametric method**

Two sets of suitable dilutions of samples were taken in 250ml BOD bottles and 2ml phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride solution were added to both sets and aerated it well and allowed to precipitate to settle. From the stock solution 50ml sample was pipette out in conical flask and titrated against N/40 sodium thiosulphate using 1% starch as an indicator till the colour changes blue to colourless.

The second set was kept in BOD incubator at 20⁰c for five days. After 5 days take into 50ml sample in conical flask and titrated against N/40 sodium thiosulphate using starch as an indicator, till the colour changes blue to colourless.
3. Chemical oxygen demand (COD) (Moore et al., 1949)

**Redox method**

To 20 ml of sample, add 10 ml of 0.25N potassium dichromate solution, a pinch of HgSO₄ and Ag₂SO₄ and 30 ml H₂SO₄ were added. This was refluxed 2 hrs at reflux assembly and cool it in water bath and then make up the volume 130 to adding it distilled water and titrated with 0.1 N ferrous ammonium sulphate using 2-3 drops of ferrion as an indicator wine red colour indicate an end point.

4. Chloride (Caldwell and Mayer, 1935)

**Silver nitrate method**

To 50 ml sample was titrated with 0.02N silver nitrate solution and add 5% potassium chromate as an indicator till the samples colour turns brick red.

5. Hardness (Goetz and Smith, 1959)

**EDTA Titrametric method**

To 2 ml of ammonia buffer solution was added in 50 ml of samples and then titrated with 0.01 m EDTA using Eriochrome Black T as an indicator till the sample colour turns blue.

6. Carbon dioxide (CO₂) (Moore, 1939)

**Titrametric method**

To 50 ml of sample in a conical flask add few drops of phenolphthalein indicator if the colour turns pink, free carbon dioxide absent. If the sample remain colourless titrate it against 0.05N NaOH till the pink colour appear.

7. Sulphate (Kolthoff et al., 1969)

**Turbidimetric method**

To 100 ml of sample, add 50 ml of conditioning reagent stir the sample and add spoonful of Barium chloride (BaCl₂) crystals, and again stir for a minutes and read on a spectrophotometer at 420 nm before 4 minutes.
8. Phosphate (Kitson and Mellon, 1944)

**Stannous chloride method**

To 50 ml filtered sample, add 2 ml of ammonium molybdate solution and 5 drops of stannous chloride after developing a blue colour, read at 690nm on a spectrophotometer. Note down the reading after 5 minute but before 12 minutes of the addition of the last reagent.

9. Nitrate (Wagner, 1940)

**Phenol disulfonic method**

To 50 ml sample, add an equivalent amount of silver sulphate solution. Heat it and filter the precipitation of AgCl₂. Evaporate the precipitation and cool it. Dissolve the residue in 2ml phenol disulfonic acid and dilute up to 50ml to this add 6ml of liquid ammonia to develop a yellow colour.

**TOXICITY TEST**

Freshwater fish, *Puntius ticto* (Ham) are selected for bioassay studies to evaluate the toxicity of distillery and dairy effluents. *Puntius ticto* were collected from Kham River nearby at Aurangabad and brought to the laboratory. *Puntius ticto* is periodic breeder, easily available throughout year and moderately maintained in laboratory aquaria.

The fish shows distinct sexual dimorphism. The male are comparatively smaller than females having silver colour on side while females are reddish in colour.

The fishes were allowed to acclimatize to the laboratory conditions for four weeks before being used for the test. During the period of acclimatization, the fishes were fed on small pieces of live earthworms. Feeding was done after every 24hr.and stopped twenty four hours before the toxicity test. Filtered and aged water was used, during acclimatization of fishes.
The fishes were maintained in large aquaria so as to prevent over crowding. For the first week, the acclimatized fish were supplied with artificial air. All necessary precautions were taken to keep the aquaria clean and away from any mechanical disturbances. Glass troughs of size (3×1×1 fit) were used as test containers. Artificial aeration during test was avoided. The fishes were selected for the tests ranging from 3.5 to 4.6g in weight 5.8-7.5 in length.

The effluent was collected from the distillery and dairy industry, for the selection of lethal concentrations; some pilot tests were carried out essential to determine the range of toxicity of a particular effluent. The range of concentrations was selected so as to get 0 to 100% mortality, order to maintain the level of the toxicant; the water was changed at every 24 hr during the short term or 96 hr exposure.

Toxicity can also be determined by static bioassay method. Ten healthy fishes of uniform size and weight were exposed to each concentration. Simultaneously a control group was also maintained with no toxicant concentration under analogous condition.

The physico-chemical parameters of the distillery and dairy effluent have been analyzed regularly during the test period following the standard methods described in APHA (2000).

The resulting mortalities were noted for each concentration for of 24, 48, 72 and 96hrs. No aeration was provided during the exposure period. Each experiment was repeated twice to secure constant results. The data collected were then extrapolated statistically by means of probit analysis methods by plotting curve (% mortality/ concentration) into regression lines (mortality in probit/ concentration i.e. probit kill concentration) according to the Finney (1971) allows the calculation of average lethal concentration (LC$_{50}$) for 24, 48 72 and 96hrs.
HISTOLOGY

Histology is the study of tissue at cellular level, and has been successfully employed as a diagnostic tool employed in medical and veterinary sciences as the first investigation sight in mid nineteenth century. Even today, no methodology has been either reported or developed, except some modification in the technique which is being applied to clinical studies.

In the present study, pathological lesions produced after acute exposure of dairy and distillery effluent have been studied in detail.

In the present study, freshwater fish, *Puntius ticto* were collected from Kham River. The fishes were maintained in glass aquaria and were acclimatized for four weeks. During the acclimatization, healthy fishes showing normal activity were selected for then histopathological studies.

For the histopathological studies, the fishes were exposed to Dairy and Distillery effluents at different concentrations. The LC$_{50}$ values for 24 to 96 hrs are then justified by probit analysis method. A batch of 10 fishes for control was also maintained simultaneously. The tissues like gill, kidney and liver were taken out for their pathological observation on termination experiments of respective toxicants. The tissues were immediately fixed in Bouins fluid for 24hrs and processed according to standard procedure. The blocks were prepared in paraffin wax and sections were cut on rotatory microtome to a thickness of 6 µ and stained using haematoxyline and eosin and mounted in DPX.

**FIXATIVE**

*Bouin’s fixative:*

- Saturated aqueous picric acid: 75 ml
- 40% formaldehyde: 25 ml
- Acetic acid glacial: 05 ml
Fixed the tissue for 18 to 24 hour’

**PREPARATION OF STAINS:**

**Harris Alum Haematoxylin:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Haematoxylin</td>
<td>1 gms.</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>200 ml.</td>
</tr>
<tr>
<td>Ammonium or Potassium Alum</td>
<td>20 gms.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200 ml.</td>
</tr>
<tr>
<td>Mercuric Oxide</td>
<td>0.5 gms</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>8 ml.</td>
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</table>

(Add after cooling)

Dissolve the haematoxylin in absolute alcohol and add alum solution previously dissolved in hot distilled water. Heat the mixture up to boiling point and add the mercuric oxide. Cool rapidly; filter the stain ready for use.

**Eosin:**

1% stock alcoholic solution

1) Eosin 1 gm
2) Distilled water 20 ml
3) Dissolve and add alcohol 95% 80 ml

**Working solution (1:3):**- One part of eosin stock solution and 3 parts of 80% alcohol, to this add 0.5 ml glacial acetic acid to each 100 ml of stain and stir just before the use.

**MICROBIOLOGICAL STUDY**

Following techniques have been followed for estimation of most probable number (MPN) of total coliform bacteria in the water sample.

**Apparatus:** Fermentation tubes, (or test tubes of 25 ml and 50 ml), Inoculation loops, Durham’s vials, Water bath etc.
**Presumptive Test**

1. Prepare dilution as per requirements, generally 5 tubes of 10ml double strength medium and 5 tubes of 1ml and 0.1ml of sample with 10ml of single strength medium. Put 1 Durham’s vial inverted in each tube and sterilize it at 121°C for 15 min.

2. McConkey’s Broth media used for this test.

3. Shake the entire water sample vigorously immediately before inoculating.

4. Add sample to the test tubes, using sterilized pipette and mix thoroughly separate pipette used for different samples as well as dilution.

5. Within 30 min. put all these test tubes in an incubator at 35°C to 37°C.

6. Examine each tube carefully after 48 hrs and gas should be recorded as positive and those without gas negative test.

**McConkey’s Broth**

1. Single strength
   - Peptone - 20gm
   - Lactose- 10gm
   - 1% Neutral methyl red - 1ml
   - Distilled water- 1000ml

2. Double strength
   - Peptone - 40gm
   - Lactose- 20gm
   - 1% Neutral methyl red- 1ml
   - Sodium taurocholate- 10gm
   - Sodium chloride - 10gm
   - Distilled water- 1000ml