CHAPTER-III
MATERIALS
AND
METHODS
Study Area

Anchar lake is a drainage type of urban valley lake situated near and within the city of Srinagar, the summer capital of Jammu and Kashmir State. The lake is mono-basined with its main catchment comprising Srinagar city and a number of semi-urban bordering villages. The lake receives main water supply through river Sind in the form of a network of channels. The run off from surrounding agricultural fields and the sewage and sewerage from the bordering human settlements including effluents from Sheri Kashmir Institute of Medical Sciences are also drained into the lake. The lake is heavily infested with thick macrophytic growth and the littorals of the lake are dominated by different species of trees especially *Phragmites* sp., *Typha* sp., *Sparganium* sp. and *Nelumbium nucifera* (lotus).

For physico-chemical analysis, six different sampling sites viz., site-I, II, III, IV, V and VI were selected from the Anchar lake established at different ecological regions under different environmental conditions and out of these the site IV is the drain exit coming out of SKIMS.
Map of the Anchar lake showing various sampling sites

- Site I (Eastern side)
- Site II (Centre of the Lake)
- Site III (Outlet on southern side)
- Site IV (Outlet from SKIMS) Drain
- Site V (Adjacent to SKIMS within the Lake)
- Site VI (Inlet on northern side)
Site-I: This site is situated near Jenab Sahib Soura on eastern side about 30 meters from the bank of the lake. This site is characterized by dense growth of big trees especially salix and poplar trees on either side on land patches and the site receives tremendous quantity of raw sewage and sewerage from adjacent human settlements deteriorating the quality of water.

Site-II: It is situated almost in the centre of the lake and is the deepest portion with standing water colonized by thick strands of macrophytes. Here water from site-VI, I and V mixes up and its flow is mild.

Site-III: This site lies at the exit of the lake on its southern extremity and this site receives slow moving waters from site-II, the centre of the lake. This site is characterized by macrophytes and dense growth of trees on the solid land patches on either side of the site.

Site-IV: This site is the drain exit of SKIMS which lies on the eastern side of the lake and all the sewage and effluents from the hospital are released into the lake.

Site-V: It represents the portion of the lake lying in front of SKIMS on the eastern side. This site is characterized by dark coloured water with foul odour, dense growth of macrophytes and solid waste dumps are seen scattered at this site.

Site-VI: This site represents the portion of the lake where the river Sind opens into the Anchar lake on its northern side via a network of channels. This site is characterized by fast flowing water which brings lot of sediments along with, thus the depth of the lake at this site is decreased.
Photograph showing Site – I (Littorals) of Anchar Lake

Photograph showing Site – II (Center) of Anchar Lake
Photograph showing Site – III (Exit) of Anchar Lake

Photograph showing Site – IV (Drain Exit of SKIMS) of Anchar Lake
Photograph showing Site – V (Adjacent to SKIMS) of Anchar Lake

Photograph showing Site – VI (Sind Inlet Channel) of Anchar Lake
Physico-chemical characteristics of water

For the physico-chemical analysis, standard methods of Welch (1948), Murphy and Riley (1962), Mackereth (1963), Golterman, Ohnsted and Clymo (1978), Trivedy and Goel (1986) and APHA (1989) were followed. The water samples were collected on monthly basis between 10.00 – 14.00h from each of the sampling sites in one litre polythene bottles for the laboratory investigation. The physico-chemical parameters analyzed for the present studies include the estimation of temperature, depth, transparency, dissolved oxygen, pH, specific conductivity, total dissolved solids (TDS), calcium and magnesium, total alkalinity, chlorides, orthophosphates, total phosphorus, ammonical-nitrogen, nitrate-nitrogen and free CO₂.

1. **Depth:** The depth of water at each sampling site (except at Site-IV i.e., Drain exit of Sheri-Kashmir Institute of Medical Sciences in Anchar lake) was recorded by sounding the lake bottom with a standard lead weight of one kg attached to the marked rope. The results were expressed in meter (m).

2. **Water Temperature:** The temperature of surface water was recorded on the spot by using graduated Celsius thermometer. The bulb of the thermometer was dipped about 2 feet below the surface of water for at least two minutes. The results were expressed in °C.

3. **Transparency:** The water transparency at different sampling sites except at Site-IV was determined by using a standard secchi disc (diameter 20cm). The mean of the depths at which the secchi disc disappeared and reappeared was taken as water transparency. The results were expressed in meter (m).
4. **Hydrogen-ion Concentration:** The pH of water samples was determined by means of a composite digital device (EL Digital pH, Conductivity, Cum TDS meter, Model 181E). Before measuring the pH of water samples, the pH meter was standardized with known buffer solutions of pH 9.2 and 4.0.

5. **Dissolved Oxygen:** The dissolved oxygen content was determined by Winkler's method. Water samples were collected in 300ml dissolved oxygen bottles and fixed on the spot with 1ml each of alkaline Iodide (75g of KOH and 50g of KI in 200ml distilled water) and Manganese sulphate (100g of MnSO₄₂H₂O in 250ml distilled water). The bottle was tightly stoppered and inverted a few times to mix the reagents thoroughly and was brought to the laboratory. The precipitate formed was dissolved by adding 1ml of concentrated sulphuric acid to the sample. Then 50ml of the same sample was taken and titrated against 0.01N sodium thiosulphate solution (2.482g Na₂S₂O₃ 0.5H₂O in distilled water, 1.5ml of 6 N NaOH and final volume was made to 1 litre) using starch as indicator. The results were expressed in mg/l by using the following formula:

\[
\text{D.O. (mg/l)} = \frac{V_1 \times N \times e \times 1000}{V_2}
\]

- \(V_1\) = Volume of 0.01N sodium thiosulphate used.
- \(V_2\) = Volume of the sample taken for titration.
- \(N\) = Normality of sodium thiosulphate.
- \(e\) = Equivalent weight of oxygen.

6. **Total Dissolved Solids:** Total dissolved solids (TDS) were estimated using (EL Digital pH, Conductivity Cum TDS meter, Model 181E). The results were expressed in mg/l.

7. **Conductivity:** The conductivity of water samples was measured at 25°C with the help of a composite digital device (EL Digital pH, conductivity cum temperature meter, Model 1816). The conductivity meter was calibrated before use with standard potassium chloride solution (0.01M). The results were expressed in \(\mu\text{scm}^{-1}\) at 25°C.
Conductivity = observed conductance x cell constant x temperature factor at 25°C.

8. **Alkalinity**: 100 ml of water sample was titrated against 0.02N H$_2$SO$_4$ (0.55ml of concentrated H$_2$SO$_4$ in 100ml distilled water) using phenolphthalein (0.25g of pure phenolphthalein powder in 100ml of 60% ethanol) and methyl orange (0.5g dry methyl orange powder in 100ml of 95% ethanol) as indicators for carbonate and bicarbonate alkalinity respectively. The same sample tested for phenolphthalein or carbonate alkalinity was used and titrated for the estimation of bicarbonate alkalinity using methyl orange as indicator. The results were calculated using the following formulae and were expressed in mg/l.

\[
\text{Carbonate or bicarbonate (mg/l)} = \frac{V_2 - V_1 \times N \times e \times 1000}{V_3}
\]

- $V_1$ = Volume of titrant used for the sample
- $V_2$ = Volume of titrant used for the blank
- $V_3$ = Volume of the sample taken for titration
- $N$ = Normality of the H$_2$SO$_4$
- $e$ = Equivalent weight of carbonate or bicarbonate

9. **Chloride**: 100 ml of sample was titrated against 0.028N silver nitrate solution (4.7g of AgNO$_3$ in 100ml distilled water) using potassium chromate (5g of K$_2$CrO$_4$ in 100ml distilled water) as indicator till brick red colour appeared or the end point. Concentration in mg/l was determined by using the following formula

\[
\text{Cl (mg/l)} = \frac{V_1 - V_2 \times N \times e \times 1000}{V_3}
\]

- $V_1$ = Volume of silver nitrate used for sample
- $V_2$ = Volume of silver nitrate for blank
- $V_3$ = Volume of the sample taken for titration
- $N$ = Normality of silver nitrate solution
- $e$ = Equivalent weight of Chlorine
10. **Calcium:** To 25 ml of water sample 4 ml of IN sodium hydroxide (4g of NAOH in 100ml distilled water) and a pinch of murexide was added and then titrated against 0.01N EDTA (3.723g of disodium salt of EDTA in 1000ml distilled water) till colour changed from fine red to pink/purple. The concentration (mg/l) was determined by using the following formula:

\[
Ca^{++} \text{ (mg/l)} = \frac{V_1 - V_2 \times N \times e \times 1000}{V_3}
\]

- \(V_1\) = Volume of EDTA used for the sample
- \(V_2\) = Volume of EDTA used for the blank
- \(V_3\) = Volume of the sample taken for titration
- \(N\) = Normality of EDTA.
- \(e\) = Equivalent weight of calcium

11. **Magnesium:** For estimation of magnesium, a pinch of Erichrome Black T was added to 25 ml of water sample and then heated gently upto 70°C in a water bath. After this, 4ml of ammonia buffer (67.5g of ammonium chloride in 570ml of concentrated ammonia and diluted to 1000ml) was added to the sample and titrated against 0.01N EDTA (3.723g of disodium salt of EDTA in 1000ml distilled water) till blue colour appeared. As this titration would give total hardness due to calcium and magnesium together, so to get the magnesium hardness only, the volume of titrant used in calcium hardness was subtracted from that used in total hardness (i.e., Ca^{++} and Mg^{++} together). The results were expressed in mg/l by the following formula

\[
Mg^{++} \text{ (mg/l)} = \frac{(V_1 - V_2) - V_3 \times N \times e \times 1000}{V_4}
\]

- \(V_1\) = Volume of EDTA used for the sample
- \(V_2\) = Volume of EDTA used for the blank
- \(V_3\) = Volume of EDTA used for the Calcium hardness
- \(V_4\) = Volume of the sample taken for titration
- \(N\) = Normality of EDTA.
- \(e\) = Equivalent weight of magnesium
12. Orthophosphate-phosphorus (OPP): The orthophosphate phosphorus concentration was estimated by the addition of 1.5ml of Vogler's reagent (96.25g of ammonium molybdate and 5g of amino sulphonic acid in one portion of dilute $\text{H}_2\text{SO}_4$ (72ml conc.$\text{H}_2\text{SO}_4 + 350ml$ distilled water)}, and 0.17g of antimony potassium tartarate in another portion of dilute $\text{H}_2\text{SO}_4$ (72ml cone. $\text{H}_2\text{SO}_4 + 350ml$ distilled water) and mixing the two solutions} and 0.25ml of 10% ascorbic acid (10g of powdered ascorbic acid in 100ml of distilled water) to 25ml of water sample. The intensity of blue colour developed was measured after 20 minutes at 690nm spectrophotometrically (Model: Elico SL 150 UV/Visible). Potassium hydrogen phosphate was used for making various standards. The results were expressed in 'μg/l'.

13. Total Phosphate-phosphorus (TPP): For the estimation of total phosphate phosphorus 25 ml of water sample was digested to dryness. After this, 1ml of 70% perchloric acid was added and again digested. Then after cooling, added 10ml distilled water, 1 ml of acid molybedate (25g of ammonium molybdate dissolved in distilled water and mixed with a cool solution of 250ml of cone. $\text{H}_2\text{SO}_4$ and 400ml distilled water and raising final volume to 1000ml) and 1-3 drops of stannous chloride (2.5g stannous chloride dissolved in 100ml glycerol with heating and stirring) were added. The intensity of blue colour developed was measured at 690nm spectrophotometrically (Model: Elico SL 150 UV/Visible) after 20 minutes. The various standards were made from potassium hydrogen phosphate and the results were expressed in 'μg/l'.

14. Nitrate-nitrogen: The diphenylamine sulphonic acid method was used for the estimation of nitrate nitrogen. To 1 ml of filtered water sample, a drop of saturated NaCl solution and 4ml of nitrate reagent {2ml of stock solution (2.2g of diphenylamine in a mixture of 150ml $\text{H}_2\text{SO}_4$ and 50ml distilled water) in 380ml distilled water and 100ml $\text{H}_2\text{SO}_4$} were added.
After 70 minutes, the intensity of blue colour developed was measured at 690nm spectrophotometrically (Model: Elico SL 150 UV/Visible). The standards were prepared from potassium nitrate and the results were expressed in ‘μg/l’.

15. **Ammonical-nitrogen:** The ammonical nitrogen was estimated by Nesselerization method. To 25ml of water sample, 1.5ml of Nessler’s reagent (100g of mercuric iodide and 70g of potassium iodide dissolved in distilled water and mixed to a solution of 180g NaOH in 500ml distilled water and diluted to 1000ml) and 0.5ml of signette’s salt (20g of potassium antimony tartarate tetrahydrate in 200ml distilled water and traces of mercuric chloride for preservation) were added. After 20 minutes, the yellow colour developed was measured at 420nm with the help of spectrophotometer (Model: Elico SL 150 UV/Visible). Ammonium chloride was used for making various standards. The results were expressed in ‘μg/l’.

16. **Free Carbon Dioxide:** 50ml of water sample was taken in conical flask to which few drops of phenolphthalein indicator were added (Dissolve 0.5g of phenolphthalein in 50ml of 95% ethanol and add 50ml distilled water). To the sample 0.05 N CO₂ free NaOH solution was added drop wise, until the solution turned faintly pink). If the colour turned pink, free CO₂ was absent. If the sample remained colourless, it was titrated against 0.05 N NaOH (Prepare 1 N NaOH by dissolving 40g of NaOH in CO₂ free distilled water to make 1 litre of solution). Dilute 50ml of 1N NaOH to 1 litre to prepare 0.05 N NaOH.) and at the end point pink colour appeared.

\[
\text{Free CO}_2 \text{ (mg/l)} = \frac{(V \times N \times 1000 \times 44)}{\text{ml of water sample taken.}}
\]

Where \(V\) = Volume of NaOH used

\(N\) = Normality of NaOH
This part deals with the investigation of parasites in different selected hosts viz. fish, Amphibia (Frogs/toads) and birds (duck/Goose). The methodology adopted include:

a) **Collection of Fish Hosts**

**Collection:** During the present study the fish hosts examined for helminth parasites include *Cyprinus carpio communis* Linn., *Cyprinus carpio specularis* Linn., *Carassius carassius* Linn., *Schizothorax niger* Heckel, *Schizothorax curvifrons* Heckel, *Schizothorax esocinus* Heckel and *Schizothorax Plagiostomus* Heckel (Plate-I). Fishes were collected from the Anchar lake at different reference points with the help of local experienced fishermen at different collection centres like Soura, Eidhag, Sangam and Nagabal. The fishes were brought alive or fresh to the laboratory (Animal House Department of Zoology) and were identified using the Key provided by Sven o Kullander *et al.* (1999). The collection was done on monthly basis and on an average 30 specimens were dissected/month.

**Parasite Collection:** Before killing and dissection of fish the entire outer surface including gill chamber, operculum, mouth cavity, fins, scales etc were thoroughly examined for the presence of ectoparasites. After visual examination the fishes were killed by a method of a blow on head. Sometimes chloroform was mixed with water in sufficient quantity to effect anaesthesia or death. After death and before dissection, individual gill arch were removed and kept in separate petridishes containing normal saline {0.65% Nacl (Cable, 1977)}. The gills were teased with the help of brushes and needle so that the parasites got detached themselves and remained free in normal saline. The
parasites were later picked up and transferred to separate petridishes containing fresh saline water.

For the collection of endoparasites, the fishes were dissected mid-ventrally and before removing the internal organs, the body cavity was thoroughly examined for any parasites. Various organs like liver, bladder, heart, etc. were removed and kept in separate petridishes containing normal saline (0.65% NaCl). The alimentary canal being the obvious part of entry for many kinds of parasitic infestations was removed completely and split open longitudinally and placed in petridishes containing saline water to which a few crystals of menthol were added so that the parasites which were firmly anchored to the lumen of the intestine were easily detached without causing any damage or distortion to the body of the parasite. Other organs were examined in the same manner by dissection and teasing by brushes. However, maximum numbers of parasites were collected from the alimentary canal of fish. Parasites collected from each fish were counted separately and regular record of the collection was maintained.

b) Collection of Amphibians

During the present study the amphibian hosts namely (Frogs/toads) were collected from littorals of Anchar lake at different reference points with the help of locals and personally by insect nets and were brought alive in large troughs containing water to the laboratory and later were subjected to chloroform treatment to effect anaesthesia or death (Plate-II). On an average 30 specimens were collected and dissected on monthly basis. Immediately after death the hosts were visually examined for any ectoparasite and then a thorough examination of the whole body of the host was done with the help of hand lens or dissecting microscope.

To search for endoparasites, the gut of the host including lungs, kidneys, urinary bladder, liver, heart etc were removed and placed in separate
petridishes containing physiological saline or plain distilled water. The heart, liver and other organs except alimentary canal were teased by the help of forceps and dissection needles for the search of parasites while as the alimentary canal was cut open longitudinally with the help of sharp pointed scissors from esophagus to the rectum so that there was no damage to the parasites. The cestodes, trematodes and nematodes were easily picked up from the gut with the help of brushes while as the acanthocephalans, which were anchored with the wall of the gut were removed by scrapping with the back of a scalpel or after adding menthol to the solution.

c) Collection of Bird Hosts

During the present study the duck/goose were collected from different littoral regions of Anchar lake namely Jenab Sb Soura, Iddgah, Sangam and Saidapora (Plate-III). Collection of hosts was also done on monthly basis and the hosts were then taken alive to the laboratory. The hosts were firstly examined for ectoparasites and the feathers of the hosts were searched and the ectoparasites were collected in petridishes with wet brush.

For the collection of endoparasites the body of the hosts was dissected open midventrally and different organs including alimentary canal were removed and kept in separate desired size petridishes where these organs were teased and cut open to search for parasites if any.

Processing of Parasites Collected from Different Hosts

The parasites collected from different hosts viz fish, amphibian and birds belong to different helminth groups like cestodes, trematodes, Acanthocephala, nematodes and ectoparasites. However, the processing of trematodes, cestodes and Acanthocephala is more or less same and was done as per the pattern given below:
Preparation of whole mount specimens of trematodes, cestodes and acanthocephala. It involves following steps

Fixation: It is the first step to process a parasite for a permanent microscopic examination. During the present study the parasites were fixed in Cornoy’s fixative. The composition of which according to Weesner (1968) is as under:

- Absolute alcohol 60 ml
- Chloroform 30 ml
- Glacial acetic acid 10 ml

> i.e. a ratio of 6:3:1

The worms were removed from the normal saline. The scolex of cestodes (especially Bothriocephalus) was fixed by placing a cover slip over it. Cornoy’s fixative was allowed to run between the slides with the help of a dropper or micropipette.

In case of large specimens of cestodes, the worms were cut into suitable size and some additional weight was placed so that proper fixing of the specimen takes place and again Cornoy’s fixative was run over the slides containing specimens. The scolex of cestodes was fixed separately on a clean glass slide by placing a cover slip on it.

Trematodes and acanthocephalans were placed on glass slide and covered by a cover slip and with the help of a dropper Cornoy’s fixative was introduced from one side so that the specimen were flooded.

Post-Fixation Treatment

After fixation the parasites were directly transferred from Cornoy’s fixative to 70% alcohol, to remove all traces of fixative. If the specimen were to be preserved, they were kept in 70% alcohol to which 5% glycerine or glycerol was added.

Staining Methods

Prior to staining process all traces of fixing reagents / preservatives were removed by washing the specimens in distilled water.
Following stain was generally used to study the specimens for better results under microscope.

**Aceto-alum carmine**

**Composition**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carmine Powder</td>
<td>5 gm</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

To prepare aceto-alum carmine 5 grams of carmine powder and 5 ml of glacial acetic acid were added to 100 ml of distilled water and boiled till the carmine powder gets dissolved and then following ingredients were added.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potash alum</td>
<td>5 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The mixture was again boiled, cooled and filtered. For preservation, thymol crystals were added.

The specimens were properly stained and were kept in the diluted stain for about 20-30 minutes (1 part of aceto-alum carmine and 9 parts of distilled water).

However, the time duration for staining varies with the size and thickness of the specimens.

**Post-Staining Treatment**

The stained specimens were passed through various grades of ethyl alcohol in an ascending order from 30% to 100%. In each grade the specimens were kept for 10-20 minutes except for 70% where we can keep them overnight. In 100% alcohol two washes were given. Again the duration of time in each alcohol grade depends upon the length, size and thickness of the specimen.

For clearing the specimens, xylene was used as a clearing agent. Due to this the specimens are rendered transparent and miscible with mountant.
Mounting: The cleared specimens were properly placed on the glass slide and a sufficient amount of mountant was added. After that a proper sized cover slip was placed on the specimens carefully so as to avoid any air bubbles to be trapped in.

Preparation for whole mount specimens of Nematodes

It involves following steps:

Fixing: Immediately after collection, the nematodes were washed in normal saline. Fixing and killing was done either in Berland's fluid (1982) (i.e., 70% ethanol and 5% glycerol) or the specimens were treated with hot 70% alcohol.

Composition of Berland's Fluid

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>10 ml</td>
</tr>
<tr>
<td>5% glycerol</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Clearing: After killing and fixing, the specimens were cleared in lactophenol. Smaller specimens were cleared in glycerine but the larger specimens in Lactophenol for 2 to 5 hours depending upon the size and width of the specimens.

Composition of Lactophenol

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol melted crystals</td>
<td>20 ml</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>20 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20 ml</td>
</tr>
<tr>
<td>Glycerine</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

For temporary mounting simple glycerine was used. But for permanent mounting glycerine Jelly was used.

Composition of Glycerine Jelly

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>10 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>60 ml</td>
</tr>
<tr>
<td>Glycerine</td>
<td>70 ml</td>
</tr>
<tr>
<td>Phenol</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
For the preparation of glycerine Jelly Gelatin was dissolved in water, then heated. To this phenol and glycerine were added and this mixture was allowed to cool.

When used the jelly was slightly warmed and one or two drops of warm glycerine jelly was placed in the centre of the slide and the worms were carefully placed in the medium and a cover slip was slowly and carefully placed over it avoiding any air bubble. The slides were sealed with nail polish after a day or two till the jelly was coagulated.

**Whole mount preparation for Ectoparasites**

The ectoparasites collected in petridishes were subjected to chloroform or ethyl acetate treatment for killing. These were fixed in 95% ethanol and preserved in 80% alcohol till processing. The whole mount processing involves the steps given below:

**Maceration:** In this case the ectoparasites were boiled in 5% potassium hydroxide solution.

**5% Potassium hydroxide:**

<table>
<thead>
<tr>
<th>Potassium hydroxide</th>
<th>5 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Dehydration:** The specimens were treated with different grades of alcohol so as to remove water. The protocol for dehydration is as:

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100% alcohol I</td>
<td>10 minutes</td>
</tr>
<tr>
<td>100% alcohol II</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>
Clearing: In this case specimens were cleared in xylene as:

- Xylene I: 15 minutes
- Xylene II: 15 minutes

Mounting: After clearing specimens in xylene, they were mounted in Canada balsam or DPX. Here a drop of mountant was put in the centre of the glass slide. The specimens were properly oriented over it and then covered with a cover slip of suitable size. After mounting the slide was rinsed with varnish. Finally specimens were dried and labelled.

Photomicrography

Photography was conducted by using a PM-6 Olympus camera, which was fitted on Olympus research microscope.

Data Analysis

The most common measurements of parasite population levels in hosts are prevalence, mean intensity and mean abundance Bush et al. (1997). Prevalence refers to the percentages of organisms infected by a particular species of parasite. Mean intensity is the number of parasites of a given species per infected host. Mean abundance refers to the number of parasites of a given species per host examined, infected and uninfected. The nomenclature used to define ecological parameters is in consistency with that of Margolis et al. (1982).

\[
\text{Mean intensity} = \frac{\text{Total number of particular parasite species}}{\text{Total number of hosts infected with that particular parasite species}}
\]

\[
\text{Prevalence} = \frac{\text{Total number of hosts infected with a parasite species}}{\text{Total number of hosts examined for that particular parasite species}} \times 100
\]

\[
\text{Mean Abundance} = \frac{\text{Total number of particular parasite species}}{\text{Total number of hosts examined}}
\]

Correlation

Literally correlation means association of two or more facts. In statistics correlation may be defined as "the tendency of simultaneous variation
between two variables.” According to Guilford “A coefficient of correlation is a single number that tells us to what extent two or more things are related and to what extent variations in one go with variations in other.”

Pearson's product moment method is used to study the correlation among various variables during the present study. The coefficient of correlation ($r$) gives an idea about the degree of linear relationship between two variables. Formula to obtain coefficient of correlation ($r$) is used as follows:

$$r = \frac{\sum X \cdot Y}{\sqrt{\sum X^2 \cdot Y^2}}$$

where $X$ is the independent variable normally represented by the abscissa and $Y$ is the dependent variable represented by the ordinate. $x$ and $y$ are the deviations from the respective means.

In language we say that ‘$r$’ can be calculated by dividing the sum of products of deviations from their respective means by the square root of the products of the sums of squares of deviations from the respective means of the two variables.

Here

- $r$ = coefficient of correlation
- $x$ = deviations of $X$ variable
- $y$ = deviations of $Y$ variable
- $x \cdot y$ = sum of multiplication of deviations $x$ and $y$

The standard error was obtained by using following formula:

$$SE_d = \sqrt{SEX_1^2 + SEX_2^2}$$

where,

- $SEX_1$ = Standard error of the first mean
- $SEX_2$ = Standard error of the second mean
Besides parasitism, the fish hosts have direct impact of pollutants therefore they are subjected to various hematological alterations. Thus a few blood parameters like Haemoglobin (Hb), Total Erythrocyte Count (TEC), Total Leucocyte Count (TLC) and Differential Leucocyte Count (DLC) were taken into consideration in case of fish hosts.

Collection of Blood Samples

Various methods of taking blood samples from fish were employed depending upon the size of the fish. In case of small fish, blood was obtained by cutting off the tail. For this purpose, the fish was suspended on a hook by its mouth. The place for incision was wiped with cloth or cotton to remove the adherent water and then the tail was cut off with a pair of scissors. The first few drops of blood were rejected. The dripping blood was collected in clean test tubes.

In case of larger fishes blood was drawn out, with a lumbar puncture (LP) needle syringe, from the heart by stabbing the needle through the ventral body wall exactly in the mid line from the posterior margin of the opercular cover directed dorsocaudally at an angle of $45^0$ (Lucky, 1977). This was the most commonly used method. If no definite puncturing was possible, then the thoracic cavity of fish was opened carefully by a longitudinal incision medially between the pectoral fin and after the blood and body fluids were removed by wiping, the needle was introduced into the heart. The blood taken was kept in glass vials containing EDTA as anticoagulant. For preparing smears on clean slides for staining, fresh blood without anticoagulant was used.
Haematological Techniques

Estimation of Haemoglobin Concentration

The haemoglobin estimation was done by Sahli’s method.

In this method the haemoglobin in red cells was converted into acid haematin. The brown colour so developed was matched against standard brown tinted glass in a comparator by direct vision. Reading was taken directly as $g^{Hb}/100$ ml blood.

Reagent used was N/10 HCl (0.1N HCl)

Method

The tube was filled with N/10HCl upto mark 20. Blood was drawn into the pipette upto the mark 0.02ml and expelled into the tube. The contents were mixed gently but well and after waiting for at least 10 minutes brown colour developed. Distilled water was added to the solution by gentle mixing till the colour of the solution matched the standard. The upper meniscus was read as the reading.

Estimation of Blood Cells

White Blood Corpuscles

A white cell count (TLC) estimates the total number of white cells in a cubic millimeter of blood.

In this case a diluting fluid was used which contained a weak acid to lyse the red blood cells and a stain for staining the nucleus of white blood cells e.g., Turks fluid.

<table>
<thead>
<tr>
<th>Glacial acetic acid</th>
<th>1.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% aqueous solution of gentian violet</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>98.0 ml</td>
</tr>
</tbody>
</table>

(A pinch of thymol may be added to the diluting fluid to prevent growth of molds).
The chamber used for cell counts was Neubauer’s chamber, which had an area of 9sq/mm and a depth of 0.1mm. using white cell pipette, the blood was drawn upto 0.5 mark and the diluting fluid to 11.0 mark thus the dilution was 1:20.

Transferred the fluid blood mixture to the counting chamber (called charging the chamber). Allowed the cells to settle to the bottom of the chamber for two minutes. Cleaned the under part of the chamber and placed it on the stage of the microscope using 10x or low power objective for counting WBC’s uniformly in the four larger corner squares. Cells present on the outer most lines were counted and those present on the opposite side were not counted.

The number of cells per cubic millimeter of blood calculated are as follows:

\[
= \text{number of cells counted} \times \frac{20 \times 10 \text{(depth factor)}}{4} = \text{number of cells counted} \times 50
\]

**Red Blood Corpuscles**

Total RBC counts were performed in the same fashion as in TLC but using RBC pipette and isotonic diluting fluid so that RBC’s did not get haemolyzed.

In this case Hayem’s fluid was used, the composition of which is as under:

- Sodium chloride - 0.5 mg
- Sodium sulphate - 2.5 g
- Mercuric chloride - 0.25 g
- Distilled water - 100 ml

Mercuric chloride is antifungal and anti bacterial and at the same time Hayem’s fluid is isotonic with blood.

The blood was drawn upto 0.5 mark in the RBC pipette and diluted fluid to 101 mark. Charged the chamber. Counted RBC’s using 40x objective in the 80 smallest squares of the chamber.
\[
\text{RBC Count} = \frac{\text{No. of cells counted} \times \text{Dilution factor} \times \text{depth factor}}{\text{area counted}}
\]
Where dilution is 1 in 200, depth is 1/10mm.
Area counted is \(\frac{80}{400} = \frac{1}{5}\) sq.mm
\[
\frac{\text{Number counted} \times 200 \times 10}{1/5} = \text{Number counted} \times 10000
\]

Differential Leucocyte count of White blood Corpucles

This method was used to determine the relative proportions of white blood corpuscles.

Procedure

A drop of blood was placed on the end of a clean slide.

The slide was placed on a smooth surface. A second slide was held at an angle of \(45^\circ\) just infront of the drop of blood and was drawn against the slide in such a way that the blood spread along its edges and was pushed forwards in such a way on the first slide without exerting any pressure so that a blood smear was prepared which was allowed to dry in air.

The film was stained with Lesishman’s Stain. A few drops of distilled water were poured over the stain so that the stain was drained and finally the slide was dried and examined under microscope.

\[
\frac{\text{No. of type cells}}{\text{Total number of WBCs}} \times 100
\]
PLATE I

FISH HOSTS
PLATE - II

AMPHIBIAN HOSTS
PLATE III

BIRD HOSTS