MATERIALS & METHODS
Study Areas

Kashmir is a fertile valley at an average altitude of 1585m a.m.s.l, boarded to the South and West by the Pirpanch valley and to the North and East by the Himalayan foothills. The valley of Kashmir experiences a temperate-cum Mediterranean type of climate, with four distinct seasons (Raina, 1965).

Kashmir abounds in natural fresh-water lakes and springs, have been famous the worldover for its crystal clear waters. The lakes include a large number of glacial oligotrophic (Alapather, Shishnag, Tarsar, Marsar, Naranag, Kousarnag, Gangabal etc) mesotrophic (Manasbal, Nilnag, Nagin etc.) and eutrophic (Dal, Anchar, Wular etc.) categories. The lakes are mostly situated in rural Kashmir and have enormous effect on the socioeconomy of population. All the lakes are source of attraction and some also provide substantial quantity of fish, edible plants and also support some cottage industries. The rural population also depends on the lakes for washing, irrigation, and transport.

**Dal Lake:** It is situated on the north East of Srinagar at an altitude of 34.07 N and longitude of 74.52 E, on the right bank of the river Jhelum. According to the classification of trophic system of lakes by Thiemann (1929) Fritchman, (1931) and Ohle (1955), the Dal lake is eutrophic in character, being shallow in most parts with greenish water and high productivity. The lake has an area of 11.56 sq.km and the maximum depth is 6.5m in Nagin basin (Zutshi et.al, 1980). The lake is saucer-shaped, lying at the foot of zeberwan mountain, greater portion is covered by floating gardens which constitute about 35% of the whole lake and has two small islands on it.
The lake is principally fed by Arrah stream which arise on the western slope of Mahadev peak and enters the lake on its Northern side, near the village Telbal, while on east, it is fed by Chashma-shahi. Besides these, large number of springs occur spread over the lake. On the south, there is a sluice-gate called Dal gate, through which excess water from the lake leaves and after flowing through a circuitous canal falls into the Jhelum river.

The Dal forms an important ecological niche in the city but its condition is fast deteriorating. Instead of forming a great source of fish supply to provide the much needed portion to the large section of our population, it is turning more into a vegetable garden because of floating gardens. Lake has large number of macrophytes especially Slavina, Hydrilla, Wolfia, Nelumbium, Potamogeton etc. Common fishes in the lake are, Cyprinus carpio comminis, C.c. spicularis, Schizothorax niger, S.ecosinus, Labeo, Crossochilus latus, Botia birdi, Gambosia affinis, Nemachilus kashmiriensis etc.

Within this fast rate of unchecked eutrophication and reclamation, it is feared that within few years, this beautiful and important ecological niche will get vanished. The ecological importance of this lake has to be thoroughly understood and steps taken to improve and develop the lake not only into a healthy tourist resort but also into a big fishery resource.

For the collection of water samples and other research material for the present study, five sites were selected.

Station 1. Located on the north west of the lake, near Telbal
Map of Dal lake showing location of Study Sites.
Map of Anchar lake showing location of Study Sites.

- Study Sites
- Marsh Land
- Floating gardens

Study Site
- Marsh Land
- Floating gardens

To River Jehuma

Kushajjar Lake

Sind Hulla
Nallah, the main source of inflow into the lake.

Station II:-
Located on the eastern side in Nishat basin, in front of Nishat garden.

Station III:- Located in the South east, near the Ruplank island, Boulevard.

Station IV:- Located near the North, in front of Hazratbal Mosque.

Station V:- Nagin lake, which is an extension of Dal lake on the Western side.

Anchar lake:- It is (34°20'-34°26'N and 74°82'-74°85'E) a semi-urban lake, located near Soura at a distance of 14 kms. north west of Srinagar at an altitude of 1583, m a.m s.l. The surface area of lake is about 680, hectares, with a maximum depth 3.5 m. Lake is shallow, single basined, drainage type, fed by network of channels from Sindnallah on its Northern side and kushalsar lake on its southern side. Dal enters the Anchar on the southern extremity through "Amirkhan Nallah. The rich and varied macrophytic vegetation of lake comprises Trapa natata, Nymphoids pettata, Typha angustata, Ceratophyllum demericum and Myriophyllum. The important fish species that Anchar lake harbours are:- Schizothorax niger, S.esocinus, Crossochilus, Cyprinus carpio comminis, C.C spiculatis and carassius carassius.

Three sites were selected for the collection of water samples and fishes.

Station II:- This lies on the eastern side of the lake, adjacent to the kushalsar lake, lies close to the human habitation and is constantly being fed with sewage from Soura Medical Institute and
Anchar village.

Station II:— It is located on the western side of the lake, have comparatively clear water with low vegetation. The lake receives several feeding channels from Sindh nallah and was noticed to be the main centre of the operation of the professional fishermen.

Station III:— Situated towards the eastern bank of the lake. It is surrounded by floating gardens bearing rich vegetation.

The present investigation on the two lakes Dal and Anchar, was carried out from May 1992, to April 1994, on monthly basis, for the different limnological features and the Microbial and Helminthlogical infections of the fishes inhabiting them.

After surveying the two lakes (Dal and Anchar) for the selection of collection sites during April 1992, Methodology for the analysis of physical and chemical features were adapted. Collection of water samples was started from May 1992, to April 1994, on monthly basis.

The surface samples of the water were collected from the different study sites of the two lakes in polyethylene bottles of one litre of capacity and were transported to the laboratory for the detailed analysis. The water samples for determining the dissolved oxygen were collected in corning glass bottles of 125 ml capacity and fixed on the spot.

Physical parameters.

Temperature:— The atmospheric temperature and water temperature at each sampling site was recorded every month.
Chemical parameters:

Hydrogen-ion-concentration:
This was recorded by manual and digital pH meter which was calibrated by using buffers of known pH strength.

Dissolved oxygen:
This was determined by Wrinklers method. Separate water samples were collected in well stoppered glass bottles with caution to avoid trapping of the air bubbles. The dissolved oxygen was fixed at the spot by adding two ml of each of Magnous sulphate and alkaline iodide to the water sample. This was allowed to stand for some time till the precipitate was dissolved by adding 2 ml of concentrated sulphuric acid in the laboratory. 50 ml of this sample was titrated against N/50, sodium thiosulphate, using starch solution as an indicator. End point was indicated by the disappearance of the blue colour.

Free carbon dioxide:
To 50ml of water sample, 5 drops of phenolphthaleine indicator were added and then titrated against sodium hydroxide till the faint pink colour developed.

Alkalinity:
Few drops of phenolphthaleine added to 50 ml of water sample, if produces no change in colour of the sample indicating the absence of phenolphthaleine alkalinity, then few drops of methylorange were added to the same sample and titrated against 0.02N/H so till the colour changed from yellow to orange. Total alkalinity was calculated by the formula devised by Welch (1948).
Chloride:—
Two ml of pot. chromate were added as indicator to 50 ml of water sample which was then titrated against silver nitrate till color changed from yellow to brick red. The amount of silver nitrate used in the titration was multiplied by a factor of 20.

Collection of Hosts:—
The fishes were collected with the aid of local fisherman from Dal lake and Anchar lake. These were brought alive to the laboratory in buckets or polythene bags containing water. The fishes were identified, weighed and their size was recorded. The different species of fish examined for Microbial and Helminth infections were:—
**Schizothorax niger, S. esocinus, S. curvifrons, Labeo, Cyprinus carpio comminis, Cyprinus carpio spiculalis, Carassius carassius, Nemachilus kashmirensis.**

Collection of parasites:—
The fish were pithed and were examined for parasitic infection. A fairly accurate investigation of diseased fish was possible only on live or quite recently dead fish.

Bacteria:—
First of all small portions of mucus or epithelial tissues were scraped off from those regions of the body which indicated a suspicious whitish coating, red colouration and other abnormal characteristics. After the smears were taken all conspicuous features of the skin, fins and gills were recorded and exactly designated, larger parasites were examined under the magnifying
After completing the external examination, the necessary bacteriological investigation was initiated. The bacteriological investigation was extended to abscesses, ulcers, skin, gut and so on, as it appears advisable on the basis of the characteristic features of the disease. In the case of acute bacteriological infections (Furunculosis) the pathogens were mostly detected in all the organs while in the case of chronic course of the disease, bacteria were usually encountered as foci in the abscesses, ulcers and the organs.

The first observation about the presence of bacteria was made by microscopic examination of unstained or stained smears of body fluids, scrapings and teased or squash preparations of organs. The exact detection of bacterial infections, stipulated the bacterial inoculations and culture of micro-organisms.

Inocula from fish were taken from the infected foci (liver, kidney, swellings, skin lesions) under the aseptic conditions. The sample of the material was generally taken by means of swabbing and swabs were quickly used to inoculate the bacteriological media.

The media used for the present work included Bloodagar, MacConkey agar and Brain Heart infusion agar.

Bloodagar was widely used as it is not only an enriched medium but it is also an indicator medium.

Composition:-

Peptone = 20 gms.

Meat extract = 5 gms.
NaCl = 5 gms.
Agar = 12 gms.
Sheep's blood = 10%
Distilled water = 1000 ml.

MacConkey agar was used for differentiating lactose fermenting organism from non-lactose fermenters. It is also called differentiated agar as it differentiate one organism from another.

Composition:
Peptone = 20 gms.
NaCl = 5 gms.
SOD. Taurocholate = 5 gms.
Lactose = 10 gms.
1% aqueous neutral red = 5.7 ml.
Agar = 15 gm.
Distilled water = 1000 ml.

Brain Heart infusion agar used for the routine examination.

Composition:
Calf brain, infusion form = 200 gms.
Beef heart, infusion form = 250 gms.
Proteose peptone = 10 gms.
Glucose = 2 gms.
NaCl = 5 gms.
Na HPO₄·12H₂O = 2.5 gms.
Agar = 15 gms.

After incubation of the inoculated culture at 37°C in an incubator, the positive cultures of pathogenic micro-organisms become recognisable on the culture plates. Starting with the
individual colonies, reinoculation was done in the same manner on
the fresh culture plates, thus pure cultures were obtained from
the same streak and were verified further. Characterisation of
pure cultured organisms was done by applying morphological and
biochemical tests.

Morphological:–

Motility:– The motility of the bacteria was verified by
investigation by the hanging-drop method.

Gram Staining:–

This test divided the bacteria into two groups. Gram-
positive bacteria and Gram-negative bacteria. For this test young
colonies were taken for smear preparation. Heat fixed smear was
stained for one minute with crystal violet, washed in tap water,
covered with Gram’s iodine solution for one minute rewashed in tap
water, decolourized by acetone for 30 seconds and counter stained
for 30 seconds in safranin. The smear was washed thoroughly and
gently blotted dry prior to microscopic examination.

The composition of the different reagents used are as follows:–

Crystal violet:–

Crystal violet = 1 gm.
Distilled water = 100 ml.

Gram’s Iodine:– Pot. iodide = 1gms. Iodine Oxidation

Safranin:– Safranin = 0.5 gms.

Distilled water = 100 ml.

Biochemical Tests:–

Oxidase Production:– In order to detect oxidase productivity a
piece of filter paper was moistened with freshly prepared 1%
tetramethyl-p-phenylenediamine, dilydrochloride and a bacterial colony was smeared over it by means of platinum loop. A positive result was indicated by a purple colouration within 30 seconds.

**Catalase Production:**
This was recorded by effervescence within one minute from 3% hydrogen peroxide, following application of a bacterial colony.

**Indole Production:** This was recorded after seven days incubation in peptone water. A positive response was indicated by a red colouration, following the addition of few drops of Kovacs reagent.

**Methyl red (MR) and Voges proskaver (VP) reaction:**
For MR test the bacteria were inoculated on MR and VP medium and incubated for 2-5 days. Then 2 drops of methyl red solution was added. Appearance of red colour indicated the positive reaction.

After completion of MR test, napthal solution and aq. KOH solution was added. Appearance of red colour indicated the positive reaction.
Carbohydrate decomposition.
The capacity of bacteria for the oxidation and enzymatic decomposition of carbohydrates to acids (shown by using indicators) and gas (seen in Durham's tubes) was examined by adding various carbohydrates (Glucose, lactose, sucrose, manitol) to the nutrient medium.

Decarboxylase test.
Four media (arginin, lysine, ornithine and control) were inoculated and incubated for seven days. The inoculated media were examined daily. The medium 1st became yellow due to acid production from glucose and later on it changed to violet due to decarboxylation. The control medium remained yellow.

Degradation of blood.
This was recorded as clear zones around the colonies on basal medium supplemented with 5-10% defibrinated sheep's blood.

Protozoa:
Smear scrapings from the skin, fins and gills were mounted in saline for examining ectoparasitic protozoans. Myxosporidian spores, when obtained were smeared directly on the slides. For collecting endoparasites the hosts were dissected and various organs were systematically examined. The alimentary canal and other organs removed from the host, were cutopen and kept in different petridishes containing saline (0.65%). The gut contents were then smeared on the slides.

Fixation and Staining:
Prior to fixation and staining protozoan parasites were studied
alive. Rapid fixation and staining techniques were used to show nuclei and other structures of the protozoans in temporary preparations, given as under:-

Lugol's iodine solution:

Has the following (cf. Mackinnon and Hawes, 1961).
Composition:-
Iodine 4 gms.
Potassium iodide 6 gms.
Distilled water 100 ml.

It stained nuclei and other structures including glycogen. Lugol's iodine was also used to demonstrate iodinophilous vacoules in myxosporidian spores. (Kudo, 1933).

For making permanent preparations following steps were taken:-

Adhesion:-
Myxosporidian spores were directly smeared on grease free slides. However, other protozoans were adhered to the slides using Mayers albumen (Weesner, 1960), having the following composition:-
White of an egg 50 ml.
Glycerine 50 ml.
Sodium salicylate 1 gm.

Fixation:
The semidried smears were fixed in the acetone free absolute methanol (Price and Reed, 1970) This was suitable for smears.
Staining - Giemsa's stain- This stain was used for staining myxosporidian spores. However, prior to staining the spores were
treated with saturated aqueous solution of urea or 4% KOH solution for extrusion of polar filaments.

Silver impregnation:

For exact species identification, especially ciliates, the preparations were silver impregnated. The fresh preparations was left to dry rapidly and then covered with 2% aqueous solution of silver nitrate and kept for 7-10 minutes in dark place. Then it was thoroughly washed with distilled water, immersed into distilled water in a petridish and irradiated with ultraviolet rays for 10-20 minutes or it was placed for several hours in the direct sunlight. After intensive blackening of the film on the slide, the preparation was dried and examined by oil immersion.

Helminths:

Before killing, fishes were thoroughly examined for cysts or ectoparasites. Then their viscera was removed and placed in different petridishes containing normal saline, to allow parasites to be released from its lumen. Its inner surface was scraped with the help of a brush to remove any adhering parasite usually Cestodes and Acanthocephala. Gills and fins were also examined. Other organs were also examined in the same manner. However, the parasites were mostly recovered from the intestine.

Further processing of trematodes, cestodes and acanthocephala is more or less on the same pattern and is as follows:
Fixation:

Generally they were fixed in Carnoy's fixative.

The composition of which is:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute alcohol</td>
<td>60 mls.</td>
</tr>
<tr>
<td>Chloroform</td>
<td>30 mls.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 mls.</td>
</tr>
</tbody>
</table>

For fixation, the parasites were placed between two glass slides and with the help of dropper, the Carnoy's fixative was introduced from one side. But for large specimens of acanthocephala and cestodes some pressure was put on the slide to ensure proper fixation. Cercarial cysts were first ruptured, generally pressing the cysts under a cover slip on a slide and then fixed in carnoy's.

Washing:

After fixation, specimens were thoroughly washed in tap water to remove all traces of fixative.

Staining:

Only acetoalum carmine was used as it gave better results. Its composition is:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid</td>
<td>= 45 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>= 55 ml.</td>
</tr>
<tr>
<td>Dry carmine stain</td>
<td>= 1 gm.</td>
</tr>
</tbody>
</table>

Differentiation:

Specimens were washed in acid water to remove excess of stain.
Dehydration:–

Parasites were dehydrated in 70 %, 90 % and 100 % ethyl alcohol. 2-3 washes were given in absolute alcohol to ensure proper dehydration.

Clearing and mounting:–

Xylene was used as a clearing agent. The specimens were mounted in D.P.X. Every care was taken to avoid direct exposure of the stained parasites to air or dirt.

Nematodes:–

The nematode parasites collected from the intestine of the hosts, were thoroughly washed and placed in normal saline which was then removed and the worms were killed and fixed in hot 70 % alcohol and preserved in 70 % alcohol to which small amount of glycerine (5 %) was added.

Clearing:–

The nematodes were cleared in glycerine. Smaller worms were completely cleared within a day or so. But larger worms were cleared in lactophenol. Lactophenol was preserved in brown bottles because exposure to light causes it to turn yellow.

Composition of lactophenol:–

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</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>

In case of over clearing in lactophenol or glycerine, a few drops of 70% alcohol were allowed to run under the coverslip to help
the study of smaller papillae and other details.

Mounting:
For making temporary slides, the nematodes were mounted in glycerine. For permanent mounting, glycerine jelly was used which was slightly warmed before use and allowed to cool later. The slides were sealed with nail polish.

Histopathology, Microscopy, Micrometry and Photomicrography:
For histopathological studies, pieces of tissues from kidney were fixed in 10% formaline immediately after necropsy. Tissues were dehydrated in an ethanol series, infiltrated and embedded in paraffin wax and sectioned on a rotatory microtome. Tissues were stained by haematoxylin and eosin. Study of stained specimens was conducted under olympus research microscope. Drawings were made to scale with the help of prism type camera lucida. Measurements made directly from camera lucida drawings by drawing scale of stage micrometer under the same magnification. Photomicrography was done by the help of PM-6 camera.