# Chapter – II

# Material and Methods

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Channa striatus commonly known as dekhu or Dokarya is predominantly found in river Krishna at Mahuli Station near Satara city. The fishes under study were collected from four different spots in the river Krishna and live fishes brought to the laboratory. The fishes were then acclimatized to laboratory conditions. Water samples were also collected from the same spots during morning hours (7.30 to 8.30 am) and were brought to the laboratory for further hydrobiological analysis.

Krishna water in which fishes were acclimatized was used for control conditions. The fishes were treated with various phosphate concentrations to study effects of phosphate concentration on the histological and histochemical architecture of their vital organs such as stomach, liver, kidney and gills.

The experimental live fishes subjected to various concentration of phosphate and various time intervals were sacrificed, dissected and different vital organs under study were removed and treated for histological and histochemical studies, described in the later part of this chapter.

**Hydrobiological Techniques :**

The present investigation has been conducted during the period of May 2007 to April 2008. This hydrobiological work involves studies on certain physico-chemical characteristics of water from river Krishna at Mahuli station near Satara city. Basic investigations on physico-chemical parameters were carried during out during the above mentioned period partly on the field and partly

in the laboratory. The different methods applied for various parameters were selected considering the field conditions and availability of material.

For physico-chemical parameters surface water samples from four different stations A, B. C and D were collected. The sampling stations were selected by considering the morphometry and entry of sewage in the river Krishna. The water samples were collected in the sterilized plastic cans of two liter capacity, brought to the laboratory and analysed immediately in order to obtain accurate results. This data was used for preparation of tables, graphs and comparison to make the fixed conclusion.

**Physical parameters :**

Physical parameters such as temperature, transparency, total dissolved solid and total solids were taken into consideration. The methodologies used for these parameters were as described by Trivedy et.al., (1987) and Saxena (1998)

**Temperature :**

The surface water temperature at all the four stations of the river Krishna was recorded in $^0$C by using standard mercury thermometer (0 – 100$^0$C) with 0.5$^0$C graduation. The temperature was recorded in the field during the water sample collection.

**Transparency :**

The transparency or light penetration capacity of the water in river Krishna was recorded by immersing section disk of cm diameter and by observing its visuality at a suitable station. The results were expressed in SDT cm by using following formula.

Secchi disc transparency in cm = A + B / 2

Where A = Depth at which Secchi disc disappears

B = Depth at which Secchi disck reappears.

**Electrical conductivity :**

It is measured with help of a conductivity meter at $25^0$C,

Electrical conductivity = (Observed conductance X Cell constant) in $\mu Mho/cm^{-1}$

**Total dissolved solids :**

The values of the total dissolved solids of the water samples were determined as a residue left after the evaporation of the known amount of filtered sample at $100^0$C. The results were expressed in mg/l by using following formula

$$TDS\ mg/l = (a - b)\ 1000 \times 1000 / V$$

Where,

a = Final weight of dish

b = Initial weight of dish

V = Volume of sample taken.

**Total solids :**

Total solids of the water sample of all four stations were determined as a residue left after the evaporation of unfiltered samples at $100^0$C and the results were expressed in mg/l by using following formula

$$Total\ solids = (a - b)\ 1000 \times 1000 / V$$

Where,

a = Final weight of dish

b = Initial weight of dish

V = Volume of sample taken.

**Chemical parameters :**

Important chemical parameters related with productivity such as pH (hydrogen ion concentration), dissolved oxygen, free carbon dioxide, total hardness, total alkalinity and phosphate phosphorus were studied for the water samples at all four stations of river Krishna. The standard methodologies for these parameters were applied as described by American Public Health Association APHA (1985), Trivedy et.al. (1987) and Saxena (1998).

**Hydrogen ion concentration (pH) :**

The pH values for the water samples were recorded in laboratory by using pocket sized pH meter (Hanna – model – Mauritins with a range from 0.0 to 14.0) as 7.0 being neutral, less than 7.0 acidic and above 7.0 as basic or alkaline.

**Dissolved oxygen :**

The dissolved oxygen content of the water samples was estimated by titrimetric unmodified Winkler's method and the results were expressed in mg/l. When Winkler's A ($MnSO_4$ and $MnCl_2$) and strong Winkler's B (Alkaline KI) were added to the samples. White precipitate of $Mn(OH)_2$ is formed which reacts with DO to form brown ppt ($Mn(OH)_3$). On acidification in presence of iodine, the iodine equivalent to the original DO in the sample liberated was titrated against N/80 Hypo ($Na_2S_2O_3$) with starch as an indication.

Dissolved oxygen is calculated by following formula

$$\text{D. O. mg/l} = \frac{\text{Burrett reading X N/80 X 8 X 100}}{\text{Volume of Sample}}$$

**Free Carbons dioxide :**

Free carbon dioxide content of the water samples were estimated and it is measured in mg/lit. by titrimetric method using (0.2272N) NaOH as a titrant and phenolphthalein as indicator. The result were expressed in mg/l. The samples containing free $CO_2$ in the form of $H_2CO_3$ was titrated against alkali (0.2272N.NaOH) and resultant change in pH from acidity to neutrality to alkalinity was detected by phenolphthalein one ml of 0.2272 N NaOH is equivalent to one mg of free $CO_2$.

Free $CO_2$ is calculated by following formula -

$$\text{Free } CO_2 \text{ mg/l} = \frac{\text{Burrett reading X N of NaOH X 44 X 1000}}{\text{Volume of Sample}}$$

**Total alkalinity :**

The carbonate and bicarbonate alkalinity of the surface water samples were estimated by titrimetric method and results were expressed as total alkalinity as $CaCO_3$ mg/l. The carbonate alkalinity was detected by using phenolphthalein indicator and bircarbonate by methyl orange.

The acid titrant (0.1 N HCl) converts carbonates into bicarbonates effectively reducing pH towards neutrality. The reduction in pH proportional to the content of $CO_3^-$ is detected by phenolphthalein.

pH range produced by bicarbonate ions is indicated by methyl orange. The sample containing $HCO_3^-$ when titrated against (0.1 N HCl) the

quantity of acid required to reduce pH from alkaline to acidic direction is proportional to the quantity of $HCO_3^-$.

$$A = \text{By phenolphthalein} = \frac{\text{Burrett reading X 0.1 X 1000}}{\text{Volume of Sample}}$$

$$B = \text{By Methyl orange} = \frac{\text{Burrett reading X 0.1 X 1000}}{\text{Volume of Sample}}$$

Total Alkalinity = A + B

**Hardness :**

The total hardness of the water samples at each station in the given river Krishna were estimated by titrating the water samples with standard EDTA (0.01 N) using ammonia buffer solution and eriochrome black T indicator The results were expressed is mg/l by using formula.

**Formula :** Total hardness of water ($CaCO_3$) mg/lit = T x 1000 / V

Where i) T = burette reading in ml.

ii) V = volume of water sample (50 ml)

**Phosphate / phosphorus :**

The orthrophosphate content in the water sample was estimated by employing stannous chloride method. The resultant blue colour intensities were measured on colorimeter (Erma – model) at 690 nm range. The optical density values of phosphate concentrations of the water samples were calculated referring to the standard graph of phosphate and results were expressed in mg/l.

**Determination of $LC_{50}$ :**

The well acclimatized fishes from the stock were divided into 7 batches containing 10 healthy fishes for each of the different concentration of phosphate. The fishes were then transferred to glass aquaria containing 0.006 M, 0.007 M, 0.01 M, 0.015 M phosphate. The fishes were exposed to the respective concentration for a definite period as 24 hrs, 48 hrs, 72 hrs and 96 hrs and the mortality was observed for 48 hrs. All the experiments were started in the morning. The fishes were keenly observed and the number of death of fishes were recorded after 24, 48, 72 and 96 hrs. for each concentration of the phosphate. $LC_{50}$ values were calculated by plotting the readings on the graph, where phosphate concentration was taken on X-axis and the percent mortality on Y-axis. $LC_{50}$ values were calculated by the method described by Lagler (1982).

**Histological (Histopathological) and Histochemical Techniques :**

The control and fishes exposed to various concentration of phosphate were taken out of aquaria. Each fish was sacrificed, dissected and important organs stomach, liver, kidney and gills were immediately removed. The small pieces of all these organs were immediately fixed in cold calcium acetate formalin (CAF – 2% calcium acetate in 10% formalin) for 24 hrs. The tissues were then washed thoroughly in running tap water for about 12 hrs. These tisses were then dehydrated through different grades of alcohol such as 30%, 50%, 70, 90% and absolute alcohol and cleared in xylene. The tissues were then embedded in paraffin wax (M.P. $58^0$ to $60^0$C) and blocks were prepared. The section were cut at 4 to 5 μm and spread on albuminized slides. The sections were

deparaffinezed, hydrated and brought to distilled water. Slides with sections of the control fish were stained with haematoxylin – eosin (H-E) for histological observations. The sections from experimental fishes were stained for haemotyxylin eosin (H-E) for histopathological observations and remaining sections were subjected to various histochemical techniques for characterization of mucosubstanced and to compare them with that of control fish.

**Haematoxylin – Eosin Method (H-E) :**

The following procedure was employed

1) Deparaffinized sections after hydration were brought to distilled water.
2) Treated with haemotoxylin (nuclear stain) for 2 to 3 minutes (here freshly prepapared Harri's haematoxylin was used as it gives best result within short time).
3) Kept in distilled water for about 10 to 15 minutes.
4) Dehydrated through different alcoholic grades upto 70% alcohol.
5) Stained with 70% alcohol eosin (cytoplasmic stain) for 10 minutes.
6) Followed by 90% and absolute alcohol grades and transferred to xylene for clearance for about 20 minutes.
7) Mounted in DPX.

The permanent slides of the control and exposed fishes were observed simultaneously under light microscope at different magnification for histological and histopathological observations in vital organs under study.

The histological structure in control fish and well marked histopathological changes occurred in the structure of various organs after

intoxication were recorded photomicrographically. The results were analysed and compared with the available data of other workers.

**Histochemical methods :**

**I) Histochemical techniques :**

The small pieces of the organs were fixed in the ice cold calcium acetate formalin (CAF – 2% calcium acetate in 10% formalin) fixative for 24 hrs. The fixation of the tissues was followed by washing in chilled distilled water, in running tap water, dehydration in alcohol, clearing in xylene and paraffin embedment. The sections were cut at 5 to 6μ. Some sections were routinely stained by haematoxylene – eosin technique for histological observations.

**II) Histochemical techniques :**

For visualization of mucosubstance there are series of histochemical methods evolved by different workers in this field. The various histochemical techniques with their merits and demerits for the mucosubstance localization, have been reviewed by Spicer (1965), Curron (1964), Barka and Anderson (1965), Lillie (1965), Thompson (1966), Spicer and Henson (1967), Spicer (1967) and Pearse (1968).

For the present study the following series of techniques for visualization of mucosubstnaces in the different organs of C. striatus were employed.

**Fixation and post – fixation procedure :**

The different tissues of the organs were quickly cut into smaller pieces and immediately immersed in ice-cold solution ($4^0$C) of 2% of calcium acetate in 10% formalin (CAF). After prolonged fixation (24 hrs), the tissues were

well washed in chilled distilled water, followed by washing in running tap water. After dehydration in alcohol, clearing in xylene and paraffin embedment, the sections were cut at 5 to 6 μ. The section were subjected to various histochemical techniques hereafter described for the detection of mucosubstances

**I) Neutral Mucosubstances :**

**A) Periodic Acid – Schiff Reaction (PAS)**

**(McManus, 1946; Hotchikiss, 1948)**

1) After dewaxing and hydration, section were brought to distilled water.
2) Oxidized with 0.5% periodic acid for 10 minutes.
3) Washed with distilled water.
4) Treated with Schiff's reagent for 10 minutes.
5) Rinsed three times (total 6 minutes) with 0.5% sodium meta-bisulphate.
6) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in Canada balsm.

**Result :** Periodate reactive, hexose containing mucosubstnaces stain pink magenta.

**II) Acid Mucosubstances :**

**A) Alcian Blue (AB) at pH 2.5 (Mowry, 1956)**

1) After dewatering and hydration, section were brought to distilled water.
2) Rinses in 3% acetic acid.
3) Stained with AB (1% AB in 3% acetic acid pH 2.5) for 30 minutes.
4) Rinsed in 3% acetic acid.
5) Washed in running water for 5 minutes.
6) Dehydrated, cleared and mounted as usual.

**Result :** Weakly acidic sulfated mucosubstances, hyaluronic acis and sialomucins stain dark blue. Strongly acidic sulfated mucins are stained weakly or not at all.

**B) Alcian Blue (AB) at pH 1.0 (Lev and Spicer, 1964)**

1) After dewaxing and hydration sections were brought to distilled water.
2) Stained for 30 minutes in 1% AB in 0.1 N HCl (pH 1.0).
3) Blotted on a puffless filter papter
4) Dehydrated quickly, cleared and mounted as usual.

**Result :** Only sulfomulins stain intense blue.

**III) Distinction between neutral and acidic mucosubstances :**

**A) AB pH 2.5 – PA Sequential staining techniques :**

1) After dewatering and hydration, section were brought to distilled water.
2) Rinsed briefly in 3% acetic acid.
3) Stained with 1% AB in 3% acetic acid (pH 2.5) for 30 minutes.
4) Rinsed in 3% acetic acid.
5) Washed in distilled water for 5 minutes.
6) Processed as in I-A for PAS staining technique.

**Result :**

Alcian blue reactive periodate unreactive acid mucosubstnaces stain blue, and PAS reactive mucosubstances stain blue purple and PAS reactive but Allian blue unreactive mucosubstances colour magenta.

**B) AB pH 1.0 – PAS Sequential staining technique :**

(Spicer, 1965; Spicer et. al., 1967)

1) After dewaxing and hydration, sections were brought to distilled water.
2) Stained with 1% AB in 0.1 N HCl (pH 1.0) for 30 minutes.

3) Sections were blotted on a puffless filter paper.

4) Processed as in I-A for PAS, staining technique.

**Result :** Only sulfomucins are stained blue or blue-purple. Nonsulfated and only periodate reactive mucosubstnaces are stained pink magenta.

## PLATE 'S'

**Photographs show site of stations in the river Krishna at Mahuli near Satara City.**

**A)** Photograph showing river water prior to sewage entry.

**B)** Photography showing river water where sewage enters.

**C) & D)** Small ducts carrying sewage from nearby area to the sewage entry point.

**A1)** Landscape photoscope showing river Krishna at Mahuli Station near Satara city.

# PLATE NO. 'S'