CHAPTER II

MATERIAL AND METHODS

One of the major problems of water toxicology is to determine the exact impact of a perticular factor from the number of such factors existing in the environment on a given organism. There is no fixed criteria to differentiate between normalcy and pathology.

Due to the different habitats and niche the fishes belong to, it is difficult to asses the cumulative effect of the pollutants in natural environment, the task is further complicated by the individual degree of sensitivity of different fish species to pollutants and then range of adaptation to the changing environments.

The effect of pollutants on fish is studied by various methods like pathological, clinical, histological, anatomical, physiological, biochemical and biophysical etc. Developing methods fordeterminingtoxicity in aquatic environments and quick diagnosis of fish poisoning is important in studying indicator organisms, for early warning system, for different toxic substances.

In this investigation an attempt has therefore been made to study the impact of the commonest distillery effluent i.e. spent wash on two common and commercially important local fishes (i) Tilapia mossambica (Pet.) and (ii) Rasbora daniconius (Ham.)

The toxicity investigations were designed to study the LC 50 values to the toxicant in both the fish species. The histopathological changes on the vital body organs like Gills, Liver and Kidney at different concentrations of the effluent, was

given more importance in the studies.

(A) MATERIAL :

(i) Distillery effluent :

The distillery effluent i.e. spent wash was periodically collected from the premiges of Krishna Sahakari Sakhar Karkhana Ltd. Distillery, Rethare (Bk.), Tal. Karad during 1984-1985. The factory annually produces about 1,35,00,300 Litres of alcohol, for which around 10,000,00 gallons of water is used per day. 6,75,000 litres of water as a result of the process is discharged annually into Krishna river. The physio-chemical character of the distillery effluent are given in table no.1.

(ii) Selection of Fishes :

The two fish species used were selected keeping in mind their availability in local riverine and tank waters. Also it was expected that being sturdy these were considered appropriate species to study the influence of distillery effluent on their vital body organs like gills, liver and kidney.

(a) Tilapia mossambica (Peters) -

Grade	:	Pisces
Class	:	Osteichthyes
Order	:	Cypriniformes
Suborder	:	Cyprinoidei
Family	:	Cichlidae (Cichlids)
Genus	:	Tilapia
Species	:	mossambica

Colour	-	Dark brown.
Odour	-	Smell of burnt Sugar.
Temperature	-	90^{o} to 95^{o}C.
pH	-	4 to 4.5
BOD5 mg/L	-	35,000 to 45,000
COD mg/L	-	65,000 to 95,000
Total Solids mg/L	-	52,000 to 86,000
Suspended Solids mg/L	-	2,000 to 14,000
V.S.S. mg/L	-	40,000 to 60,000
Total Nitrogen PPM	-	1,000 to 1,200
BOD : N:P	-	100 : 2.75 : 0.8
Sodium PPM	-	150 to 200
Potassium PPM	-	8,000 to 10,000
Calcium PPM	-	500 to 600
Iron PPM	-	50 to 60
Sulphate PPM	-	2,000 to 5,000
Chloride PPM	-	5,000 to 6,000

Table No.1 : Showing Physico-chemical characteristics of distillery effluent (Spent wash). Figures supplied by All India Distiller's Association, New Delhi (Personal Communication, April, 85).

This is originally a South African fish and is known to thrive well in estuarine waters and therefore it is listed taxonomically in marine fishes. This fish was introduced in Maharashtra through Ceylon (Gazetteer of India, Maharashtra State, fauna, 1974). Tilapia is considered as one of the hardiest fishes known from fresh and estuarine waters. Though some of its varieties are herbivorous, known to prefer algae and plankton as a food, by and large it is an omnivorous fish. It grows upto 30 cms and weight upto 1 kg. It is a prolific breeder and therefore it has infested almost all fresh water bodies in the country. This 'mouth breeder' is known for its unique way of protecting young ones from predators. Today, this fish is considered as pest, due to its prolific breeding throughout the year and the small body size it reaches at the end as a result of food competition.

This fish species was selected for the experiments for the following reasons :-

1) It is considered as a hardy fish.
2) Though originally estuarine, it is commonly distributed in rivers and tanks in this area, and is available throughout the year.
3) It is a column feeder.
4) Being omnivorous it is found in all niches in freshwater.
5) It is an exotic fish variety.

(b) Rasbora daniconius (Ham.) :

Grade	:	Pisces
Class	:	Osteichthyes
Order	:	Cypriniformes
Suborder	:	Cyprinoidei
Family	:	Cyprinidae
Genus	:	Rasbora
Species	:	daniconius.

Rasbora is a small fish (10-15 cms.), it is cylindrical and slightly compressed fish, commonly found in all fresh waters in Indian sub-continent. This fish is very active and is a voraciuos feeder. Mostly omnivorous and therefore it is found near the water surface in search of food. Due to the dark band all along its body length it can be easily identified.

The reasons for selecting this fish variety for the experiment are as follows :

(1) It is a very common, available throughout the year.
(2) It is purely freshwater and local variety.
(3) Basically surface feeder.

(B) METHODS :

This study was carried out from Jan. 1985 - Dec. 1985. The fishes, in live form, were collected from lotic and lentic ecosystems around Kolhapur city. After acclimatizing them for 15 days in the laboratory conditions and then they were used for the actual toxicological experiments. During the experiments

they were not given any food. Glass aquaria of the dimensions 45 cm X 22 cm X 30 cm and capacity of 25 litres each were used for acclimatization of the fishes. Ordinary chlorine free tap water was used during the experimentation. The acclimatized fishes of the different experimental groups were exposed to the sublethal and lethal concentrations of distillery effluent, i.e. 1%, 2%, 3%, 4% and 5% V/v. in the respective aquaria of 25 litres capacity. The aquaria were maintained without cover in good laboratory conditions. The room temperatures during the experiments fluctuated from 27°C to 32°C. The water temperature range in the aquaria was from 25°C to 30°C. The standard methods for above experimentation as given by Metelev and et.al. (1971) were adopted.

During the laboratory investigations various concentrations in the control and test aquaria were monitored for temperature, pH, $CaCO_3$ hardness and Dissolved Oxygen (DO) values and the results were expressed in graphical form.

(i) Determination of LC 50 :

The acclimatized fishes of both varieties were divided into 5 subgroups seperately containing 10 fish each. They were transferred to the glass aquaria containing 1%, 2%, 3%, 4% and 5% V/v, concentration of distillery effluent (spent wash). Tap water was analysed for temperature, pH, Hardness and Dissolved oxygen, before beginning of the experiment. The fishes were exposed to the respective concentration for a definite period in the laboratory environment and mortality was observed for

48 hours. Before transfering the fishes to the respective aquaria, the media therein were aspirated to attain the DO level not less than 5 mg/l. The behaviour of the fishes was keenly observed and the number of dead fishes was recorded in each aquarium. The forked lengths in mm. and weights in gms., of the fishes subjected to experiments were also measured. The LC 50 values were calculated by plotting the readings on the graph paper, where percent mortality was taken on Y-axis and the percent concentration of distillery effluent on X-axis. The interpolation at 50% mortality thus calculated gave the LC 50 values.

(ii) Experimental set up for Histopathological studies :

For histopathological study, preaspirated glass aquaria mixed with desired concentration of 1%, 2%, 3%, 4% and 5% V/v. of undiluted distillery effluent, each containing 10 fishes of given variety, were prepared. The exposed and controlled fishes were sacrificed after reaching asphyxiation (over turned fishes).

(a) Fixation : The abdomen of live fish was opened quickly and the gill, liver and kidney were removed immediately and fixed in Aqueous Bouin's fixative.

Bouins Fixative :

i) Saturated aqueous picric acid	...	75 ml.
ii) Formaldehyde	...	25 ml.
iii) Glacial acetic acid	..	5 ml.

(b) Histological technique : Standard method for histological and histopathological study as given by Thompson (1966) was employed.

Processing of the tissue :

After fixation for 24 hrs. the tissue blocks were washed in running tap water. Subsequently these tissue blocks were dehydrated through graded alcohol by keeping them in different grades for not less than 30 minutes, giving one or two changes, by this the tissues were dehydrated completely. After dehydration the tissue blocks were transferred to xylene, keeping in it for about 5-10 minutes. Then they were kept in one part of xylene and one part of wax for 30 minutes for cold impregnation. The tissues were taken out and were put in the melted wax (58° - 60°C Paraffin wax, BDH) in the oven adjusted to 60°C. After keeping them for 1.30 to 2.0 hrs. paraffin blocks were prepared. After hardening of the wax, the blocks were trimmed and sections of 5-7 µ were cut on the rotary microtome (Spencer-20). The paraffin sections were spread on the albuminised slides and sections were double stained with Haematoxylin-Eosin technique for histological and histopathological observations.

(c) Staining procedure : For paraffin embedded tissue sections following procedure was carried out :

i) Xylene . . . . . dewaxing
ii) Ethyl alcohol Ab .. . . . . rinse
iii) Ethyl alcohol 90% . . . . . rinse
iv) Ethyl alcohol 70%, 50%, 30% . . rinse
v) Distilled water . . . . . rinse
vi) Haematoxylin (Harris) 30 sec. to 1 min.

vii)	Distilled water	wash under running
	tap water . . .	10-15 minutes.
viii)	Acid water	rinse (differentiation)
ix)	Running tap water	1-2 minutes
x)	Ethyl alcohol 30%, 50%, 70% .	rinse
xi)	Alcoholic Eosin 70%	5 minutes
xii)	Ethyl alcohol 90%	5 minutes
xiii)	Ethyl alcohol Ab	10 minutes
xiv)	Xylene	30 minutes
xv)	Mounted in DPX.	

The permanent slides of the exposed and controlled fishes were observed under light microscope at 100-450 magnification. Histological and histopathological observations in gill, liver and kidney were carefully recorded with simultaneous comparison with controlled one. The notable histopathological changes were photomicrographically recorded. The results were analysed and compared with the available data of other workers.