CHAPTER 2: MATERIAL AND METHODS

2.1. Study Area

The metropolitan city Vadodara is bounded by 22°18′ N latitude and 73°16′ E longitude. Vadodara urban agglomeration covers an area of about 140 km^2. The region located NW to Vadodara city is the major chemical heavy industrial zone (Fig. 1). For the study purpose, we surveyed the entire industrial and adjoining non industrial zone.

Criteria for the study site selection were:

i. Polluted and non polluted sites,

ii. Reservoir capacity and sufficient water level during various seasons so that study can be carried out throughout the year.

The villages with perennial pond were surveyed as possible study sites. Since Koyali is located much within the industrial zone and prone to soil, water and air pollution from various industrial inputs; it was selected as polluted representative site. Dumad is away from the industrial pollution zone and has a good sized perennial water body; therefore, it was selected as non pollution representative site for present studies (Fig. 2). The sampling was done from different sub sites of each pond and polled for analysis to get a representative sample of the pond.

The Koyali pond (Lat 22°36.67"N and Long 73°11.67" E) has an area of 9.57.08 hectares and Dumad Pond (Lat 22°34.34" N and Long 73°11.21" E) has area of 8.80.20 hectares.

2.2. Study Protocol

The map of the study sites were collected from Survey of India topo sheets and from Google earth and sub categorized into equal divisions (Figs. 3, 4). These grid lines were kept in mind while sampling both for routine water analysis and plankton analysis. The peripheral points were samples routinely. The inner points at Koyali were sampled with the help of fisherman whenever they were available. At Dumad this was possible only twice with the help of local fisherman. The samples were polled for further analysis.

Initially, besides Dumad and Koyali other aquatic sites were also visited for comparison (Fig. 5). Sampling of water for routine and zooplankton analysis and of various macro fauna was also carried out initially for standardization of technique and comparisons. However, these findings are not incorporated in this report. The study sites Dumad and Koyali were studied for their usage pattern, ongoing activities and occurrence of macro fauna (Figs. 6, 7).

2.2.1. Pond Water Sampling for Physicochemical Analysis

Water samples from the study area were collected in 500 ml plastic bottles at a depth of about 0.3 m for routine physicochemical and pollution status analysis. The water samples were collected every month for the analysis of pH, Alkalinity, Total Hardness, Ca^{++} Hardness, Chlorides, BOD, COD, TDS, Conductivity and Turbidity using standard protocols (APHA, 2000).

2.2.2. Ground Water Sampling for Physicochemical Analysis

The entire industrial area was surveyed for location of bore wells and shallow dug wells. Since this area is covered under the drinking water facility of Vadodara, the use of bore wells or hand pumps is very much restricted. The agricultural fields in the outskirts still use bore wells for irrigation; though much restricted. Therefore, only few such sampling sites could be incorporated in present studies. The samples drawn by motorized pump were collected in 500 ml plastic bottles and brought to laboratory for further analysis of routine physicochemical parameters using standard protocols (APHA, 2000).

2.2.3. Pollutant Specific Analysis in Water

The surface and ground water thus collected were also used for the analysis of pollutants using AAS (Electronics Corporation of India, 4141) as per standard protocols. Metals like cadmium, chromium, copper, cobalt, iron, lead, nickel and zinc; total organicals, phenol compounds, oil and grease etc. were analyzed. The samples were filtered through membrane filter (0.45 µm) using suction pump, concentrated by evaporation in hot air oven at 100 °C, treated with conc. HNO_3 and analyzed by AAS. The minimum detection limits for heavy metals are as below:

Metal	Reading wave length (nm)	Minimum detection limit (µg/ml)
Cadmium	228.8	0.02
Chromium	357.9	0.1
Cobalt	240.7	0.1
Copper	324.7	0.04
Nickel	232	0.1
lead	217	0.25

pH (Electrometric): pH is defined as the intensity of the acidic or basic character of a solution at a given temperature. pH is the negative logarithm of hydrogen ion concentration. pH values from 0 to 7 are diminishingly acidic, whereas values of 7 to 14 are increasingly alkaline. At 25^0C, pH 7.0 is neutral, where the activities of the hydrogen and hydroxyl ions are equal and it corresponds to 10-7moles/l. The neutral point is temperature dependant and it is pH 7.5 at 0^0C and pH 6.5 at 60^0C. The pH of natural water usually lies in the range of 4.4 to 8.5. The basic principle of electrometric pH measurement is of the activity of hydrogen ions by potentiometric measurement using a standard hydrogen electrode and a reference electrode.

Alkalinity: Alkalinity of water is its acid neutralizing capacity. Alkalinity of surface water is primarily a function of carbonate and hydroxide content and also includes the contributions from borates, phosphates, silicates and other bases. Alkalinity is a measure of amount of strong acid needed to lower the pH of a sample to 8.3, which gives free alkalinity (phenolphthalein alkalinity) and to a pH 4.5 that gives total alkalinity. Total alkalinity is the sum of hydroxides, carbonates and bicarbonates.

Chlorides: Chloride anion is generally present in natural waters. The presence of chloride in natural waters can be attributed to the dissolution of salt deposits, irrigation drainage and sewage discharges. Human excreta, particularly urine also contributes to high amount of chlorides. In a neutral or slightly alkaline solution, potassium chromate indicates the endpoint of silver nitrate titration of chloride. Silver chloride is precipitated quantitatively before sufficient quantity of silver chromate is formed.

Turbidity: Suspension of particles in water interfering with the passage of light is called turbidity. Turbidity is caused by wide variety of suspended matter, which range in size from colloidal to coarse dispersion depending upon the degree of turbulence and also ranges from pure inorganic substances to those that are highly organic in nature. Turbidity measurement using turbidity tube method is based on the visual interpretation of the turbidity of water. The visual appearance of black cross mark at the bottom of the tube, through the open end is used for turbidity measurement.

Biochemical Oxygen Demand: Biochemical oxygen demand (BOD) is a measure of the amount of oxygen that bacteria will consume while decomposing organic matter under aerobic conditions. Biochemical oxygen demand is determined by incubating a sealed sample of water for five days and measuring the loss of oxygen from the beginning to the end of the test. Samples often must be diluted prior to incubation or the bacteria will deplete all of the oxygen in the bottle before the test is complete.

Chemical Oxygen Demand: Chemical oxygen demand (COD) does not differentiate between biologically available and inert organic matter, and it is a measure of the total quantity of oxygen required to oxidize all organic material into carbon dioxide and water. COD values are always greater than BOD values, but COD measurements can be made in a few hours while BOD measurements take five days.

Conductivity: Conductivity is a numerical expression of the ability of an aqueous solution to carry electric current. This ability depends on the presence of ions, their total concentration, mobility, valence and relative

concentrations and on the temperature of measurement. The instrument used for conductivity measurement consists of source for alternating current, a Wheatstone bridge, a null indicator and a conductivity cell. The conductivity cell measures the ratio of alternating current through the cell to the voltage across it.

Total Dissolved Solids (TDS): Total Dissolved Solids (TDS) are the infiltrable solids that remain as residue upon evaporation and subsequent drying at defined temperature. It gives the measure of ions dissolved in the water. In the electrometric measurement of Total Dissolved Solids, the conductivity measurements are used to calculate Total Dissolved Solids by multiplying conductivity by an empirical factor, which vary between 0.55 to 0.9, depending upon the soluble components and temperature of measurement.

Hardness: Calcium and magnesium are the principal cations that impart hardness. The total hardness of water therefore, reflects the sum total of alkaline metal cations present in it. Hardness caused by bicarbonates and carbonates of calcium and magnesium cations is called temporary hardness. Sulphates and chlorides of calcium and magnesium cause permanent hardness. Natural hardness of water depends upon the geological nature of the catchment area. Hardness plays an important role in the distribution of the aquatic biota and many species are identified as indicators for hard and soft waters. The degree of hardness of drinking water has been classified in terms of equivalent $CaCO_3$ concentration as follows:

Soft – 0 to 60 mg/L	Medium – 60 to 120 mg/L
Hard – 120 to 180 mg/L	Very hard – more than 180 mg/L

In an alkaline condition, EDTA reacts with Ca and Mg to form a soluble chelated complex. Ca and Mg ions develop wine red colour with Eriochrome Black T. When EDTA is added as a titrant, Ca and Mg divalent ions get complexes resulting in a sharp change from wine red to blue which indicates end point of the titration. At higher pH, about 12.0, Mg ions precipitate and only Ca ions remain in the solution. At this pH, murexide indicator forms a pink colour with Ca ions. When EDTA is added Ca ions form complex resulting in the change from pink to purple, which indicates end point of the reaction.

2.2.4. Sampling of Zooplankton

The Plankton Net: Fine nylon filament mesh of 20 micron size was used to prepare the plankton net of 30 cm diameter X 100 cm length. A bottle was attached to the end of the net cone for collection of the plankton.

Collection of Samples: The plankton net was drawn through the water and surface as well as sub-surface samples up to one meter depth were collected. The collected planktons were transferred to 100 ml containers for further processing. Alternatively, 20 l water was drained through the net and the plankton were collected and processed further.

Narcotizing, Relaxing and Preservation: The samples were subjected to low and gradual narcotization using menthol crystals and alcohol over a period of around six hours to permit relaxation of organisms. Then gradually formalin was added to the samples. After 24 h, the samples were

centrifuged at low rate for 5 min and then transferred to fresh water and preserved with formalin.

Microscopic Observations: The preserved samples without any further dilutions or concentration were used for qualitative and quantitative studies of zooplanktons. Larger animals were observed under simple microscope while all other analyses were carried out using compound microscope at 45X. The unstained preparation of zooplankton was studied. The identification of zooplankton was done using descriptive or illustrative keys (Needham and Needham, 1962; Edmondson, 1963; Posthuma, 1971; Westpal, 1974; Tonapi, 1982; Adoni, 1985). Attempts were made to identify all the zooplankton up to genus and species level. However, in some cases it was not possible due to either insufficient literature or due to ambiguity of identification character, therefore, such specimens were identified at least up to family/genus level. Recent taxonomic keys were utilized to classify the plankton.

Quantification Studies: Quantitative analysis was carried out from same samples to study the density of various zooplanktons. This is finally represented as no of zooplankton per litre of water samples. The Sedzwick-Rafter (SR) counter was used for quantification. The sample was gently, but thoroughly shaken and was placed immediately on the SR counter and covered with the cover glass. One end of the cover glass was focused under microscope and after careful scanning of species and its number, the SR counter was shifted to next field and proceeded parallel to first observation in reverse direction. Depending on the density, further dilution was made and zooplanktons were counted to note density and percentage composition.

Number of organisms per litre = crude count of plankton X standard factor.

Standard factor = Net towing distance (length) X net towing instances X area of net operation (cylinder of water corresponding to the net).

Alternatively, where samples were also collected by measured volume of water, drop count method was employed as below:

Number of organisms per litre = A x 1/L x N/V

Where A= number of organisms per drop, L= Volume of original sample

N= Volume of concentrated sample, V= Volume of one drop

In all the cases the first method was used. The other method was used for comparison only and it was found that the error rate was low.

Data Analysis: The data were subjected to statistical analysis using various population indices.

Species density, D (No./ l) = Total number of animals of a species / Volume of sample.

Group wise relative density, RDG =Species density / total density of a phylum x100.

Total relative density, RDT = Species density / total density at a site x 100.

Population Indices: Several population indices were calculated for comparison of the diversity at the study sites; e.g., Shannon, Simpson, Menhinick, Mergalef and Burger and Parker (Shannon and Weaver, 1949; Simpson, 1949; Menhinick, 1964; Margalef, 1968; Berger and Parker, 1970).

2.3. Fish Studies

2.3.1. *In situ* Studies

For in situ studies, samples were collected from the study ponds and without any further exposure or experimentation tissue samples were processed for various analyses. Fishing was done during early morning hours by professional local fisherman using wooden boat and fishing nets (locally made from nylon threads; about 40 feet long and 6 feet wide with a cork line at the top rope and metal line with a ground rope). Four to six adult Tilapia *(Oreochromus mossambicus,* Peters 1852) measuring 15-18 cm (weight 26-30 g) were collected each time, seasonally from Koyali and Dumad ponds and brought live to the laboratory (Fig. 8). The liver, muscle and gills were dissected out and stored at -80°C till further biochemical analysis. A portion of tissues was fixed in 10% neutral formalin for histological studies. A portion of the tissues was also processed for heavy metal analysis. The tissues were digested and assessed for the heavy metal levels using AAS as per standard protocols.

2.3.2. Experimental studies

The fishes (15-18 cm length, 26-30 g weight) were collected from Dumad pond, non polluted site, brought to the lab and transferred to bath tub of 50 l water capacity in group of 10 per tub for acclimatization. After 10 days to 2 weeks, the fishes were divided into several groups of six each for further studies.

To determine the doses, the fishes were treated with the freshly collected heterogeneous industrial effluent at different concentration for

durations ranging from 1 day to 30 days. Primarily this was set as 96 hrs (4 days) toxicity testing experiment. Later the same was estimated for the longer duration up to 30 days. Fishes were exposed to 5 to 50 % dose of the effluent and observed for 96 hrs toxicity test. The water and toxicants were changed daily to avoid any residue formation or any effect that cannot be correlated with known conditions. Up to 24 % of the effluent concentration mortality was not recorded till 30 days and up to 28% of dosages, no mortality was seen till 4 days. Gradual increase in mortality was noted in 4 day toxicity assessment schedule. Increase in mortality was seen at 30% both by 15 and 30 days, which resulted into 100% mortality at 40% doses by 15 days. Based on these findings 10% and 20% doses were selected for exposure duration of 30 days in experimental set up. The fishes were fed standard feed for exotic fishes consisting of rice bran and oil cakes.

The experimental design was as follows:

Experimental group	Dose	Exposure duration (number of animals exposed)		
		7 days	15 days	30 days
Control	None	06	06	06
Low dose	10 % Effluent	06	06	06
High dose	20 % effluent	06	06	06

Next day of the exposure duration, the fishes were dissected out to collect liver, muscle and gills and stored at -80°C till further biochemical analysis. A portion of tissues was fixed in 10% neutral formalin for histological studies. A portion of the tissues was also processed for heavy metal analysis. The tissues were digested and assessed for the heavy metal levels using AAS as per standard protocols.

2.3.2.1. Histological Studies

Small pieces of tissues were preserved in 10 % neutral formalin for 24h, rinsed under running tape water for 3-4 h, dehydrated in ethyl alcohol grade, cleared in xylene and clove oil and the paraffin blocks were made. 5µm sections were stained with hematoxylin and eosin and observed under microscope for structural details. Microphotographs were taken at 20X, 40X or 100X for documentation of details.

2.3.2.2. Biochemical Studies

The tissues stored at -80 °C were thawed and then processed to prepare homogenate as required for different parameters to be evaluated. Following analyses were carried out in liver, muscle and gills:

Total protein, Ascorbic acid, Glutathione, Acid and Alkaline phosphatases, Super oxide dismutase, Glutathione peroxidase.

The protocols are briefly described below:

i. **Protein:** (Lowry et al., 1951)

Principle: Protein reacts with folin-phenol reagent to develop a blue colored complex due to reduction of phosphomolybdic and phosotungustic component in folin reagent. This reaction is given by the amino acid tyrosine and tryptophan present in the protein, color develops by biuret reaction, of the proteins with alkaline cupric tartarate. The intensity of the color deepens on the amount of these aromatic amino acid residues present and thus varies for different proteins.

Reagents	Standard	Sample	Blank
Homogenate	-	0.1 ml	-
Distilled water	1 ml	0.9 ml	1 ml
Reagent C	5 ml	5ml	5ml
Keep for 10 mins			
Folin phenol reagent	0.5 ml	0.5 ml	0.5 ml
Keep for 30 mins in dark			
Read at 660nm			

ii. Superoxide Dismutase (Marklund and Marklund, 1974)

Principle: Pyrogallol is autooxidised at 420 nm at pH 8. SOD inhibits this auto oxidation of pyogallol in rate limiting fashon. 50% inhibition of pyrogallol auto oxidation is equivalent to 1 IU of enzyme.

Procedure:

Reagents	Test	Control
Buffer	1.0 ml	1.0 ml
Homogenate	0.01 ml	-
Pyrogallol	0.05 ml	-

Read at 420 nm in kinetic mode for 180 sec at every 30 sec.

iii. Glutathione Peroxidase (Rotruck et al., 1973)

Principle: GPx catalyses the reduction of hydrogen peroxide by reduced glutathione, resulting in H_2O and oxidized glutathione which is then instantly and continuously converted into GSH by an excess of GR using NADPH providing for constant level of GSH . Reduced glutathione acts as a reductant. The estimation is based on the oxidation of GSH by 5, 5'- dithio bis 2 nitro benzoic acid.

DTNB used to measure the total glutathione content of biological samples.

	Reagent	Sample	Blank
1)	PO_4 Buffer	0.4ml	0.4ml
2)	Na azide	0.1ml	0.1ml
3)	GSH	0.2ml	0.2ml
4)	Homogenate	0.1ml	-
5)	DW	-	0.1ml
Mix well			
6)	H_2O_2	0.1ml	0.1ml
7)	DW	1.1ml	1.1ml
Incubate at 37^0 C for 10 min.			
8	10% TCA	0.5ml	0.5ml
10 min. at 4000 rpm			
9	Na_2HPO_4	3ml	3ml
10	DTNB	1ml	1ml
read at 412 nm			

iv. **Reduced Glutathione** (Beutler et al., 1969)

Principle: Glutathione is a major endogenous thiol present in the tissue. The sulphydryl groups in glutathione reduces the 5,5' –dithio bis -2- nitro benzoic acid [ellemans reagent, DTNB] to form 1 mole of 5-thio-2-nitro benzoate [TNB] per mole of SH. The 5-thio-2-nitro benzoate [TNB] anion has an intense yellow color with an absorbance maximum at 412nm and can used to measure –SH group.

The test system contains tissue extract [100µl], 1ml precipitating solution, 3ml phosphate buffer, and 0.5ml DTNB. GSH was taken as standard. Absorbance was recorded at 412nm within 1 minute of adding DTNB.

v. **Ascorbic Acid:** (Roe et al., 1954)

Principle: Ascorbic acid is converted to dehydro ascorbic acid by shaking it with norit. It is then coupled with 2, 4-DNPH in presence of thiourea as mild

reducing agent, then converted into a red coloured compound which is assayed colorimetrically.

Reagent	Standard	Sample	Blank
6% TCA	-	-	4ml
Homogenate	-	4ml	-
Standard ascorbic acid	4ml	-	-
2, 4 – DNPH	1ml	1ml	1ml
Thiourea	3 drops	3 drops	3 drops
Keep in water bath for 15 mins.			
H_2SO_4	5ml	5ml	5ml
Mix well, wait for 30 mins and Read at 540 min.			

vi. **Acid Phosphatase** (Bessey et al., 1946)

Principle: The enzyme hydrolyses esters of phosphoric acid at pH 4-7. Tissue homogenate is incubated with substance which is a colorless compound which is p-Nitro-phenol phosphate. Upon enzymatic hydrolysis of phosphate group the Yellow salt of PNP is liberated. The substance that is liberated is an indicator of phosphatase alkalinity. The reaction is stopped by adding alkali and without further treatment the quantity of yellow colour substance is assayed spectrophotometrically.

Reagent	Sample	Blank
Acid Buffer	0.5ml	0.5ml
PNP	0.5ml	0.5ml
Homogenate	0.O2ml	-
Incubate at 37 ^{0} C for 2 minutes		
0.1 NaoH	4ml	4ml
Incubate for 30 min and read at 420 nm		

vii. **Alkaline Phosphatase** (Bessey et al., 1946)

Principle: The enzyme hydrolyses esters of phosphoric acid at pH 9-11. Tissue homogenate is incubated with substance which is a colorless compound which is p-Nitro-phenol phosphate. Upon enzymatic hydrolysis of phosphate group the Yellow salt of PNP is liberated. The substance that is liberated is an indicator of phosphatase alkalinity. The reaction is stopped by adding alkali and without further treatment the quantity of yellow colored substance is assayed spectrophotometrically.

Reagents	Control	Test
Working buffered substrate	0.4 ml	0.4 ml
Place in water bath at 37 C for 5 minutes.		
Tissue homogenate	-	0.02 ml
0.05 NaOH	4 ml	4 ml
Absorbance read at 405 nm		

Statistical analysis: The data are presented as mean ± S.D. values. Numbers of animals per group are stated in the table or figure legends. The statistical analysis of data was done using one-way analysis of variance (ANOVA). The significance of the results was ascertained at $p<0.05$. Population indices were compared for community analysis.

2.4. Molluscan Diversity Studies

The peripheral areas of the ponds were regularly surveyed on foot and keenly observed for the presence of various animals. During summer when the water level was low, the shallow sub merged regions pf the pond were also checked for the presence of animals. Live molluscs or empty shells were collected from the study sites and brought to lab for taxonomic identification using descriptive and illustrative keys and confirmed using Zoological Survey of India identification key and documentation records (Rao, 1989). The specimen collection was carried out throughout the study period.

2.5. Avifauna Diversity Studies

The direct citing of birds was considered to record the avifaunal diversity at both the ponds and its surroundings. These studies were carried out during different time periods of the day throughout the study period. Taxonomic identification was confirmed using standard keys (Ali, 2002). Avifauna was categorized into resident/migrant with their conservation status.

2.6. Other Faunal Diversity Studies

Several invertebrates and few vertebrates, fishes, amphibians and reptiles, were found in and around the ponds under study. The specimen were collected from the sites and brought to laboratory for identification. Where it was possible, the specimens were identified at the site only and representative photographs were taken for further identification in the lab. The animals were not brought to the lab and after identification were released

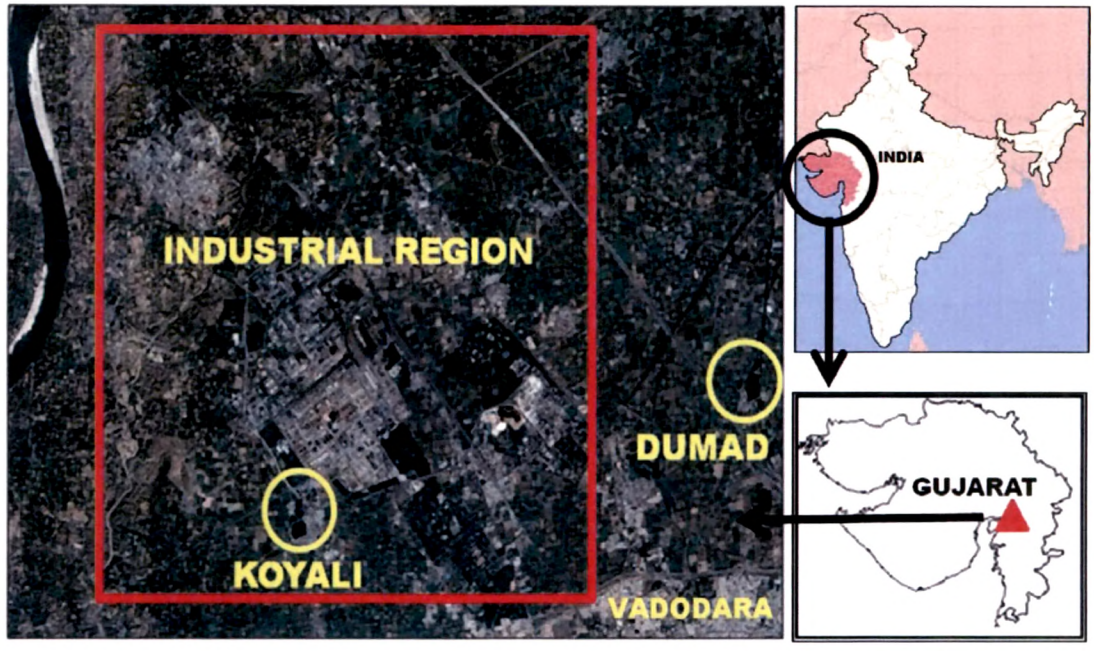

Fig. 1: Map showing location of the study area with reference to Gujarat and India.

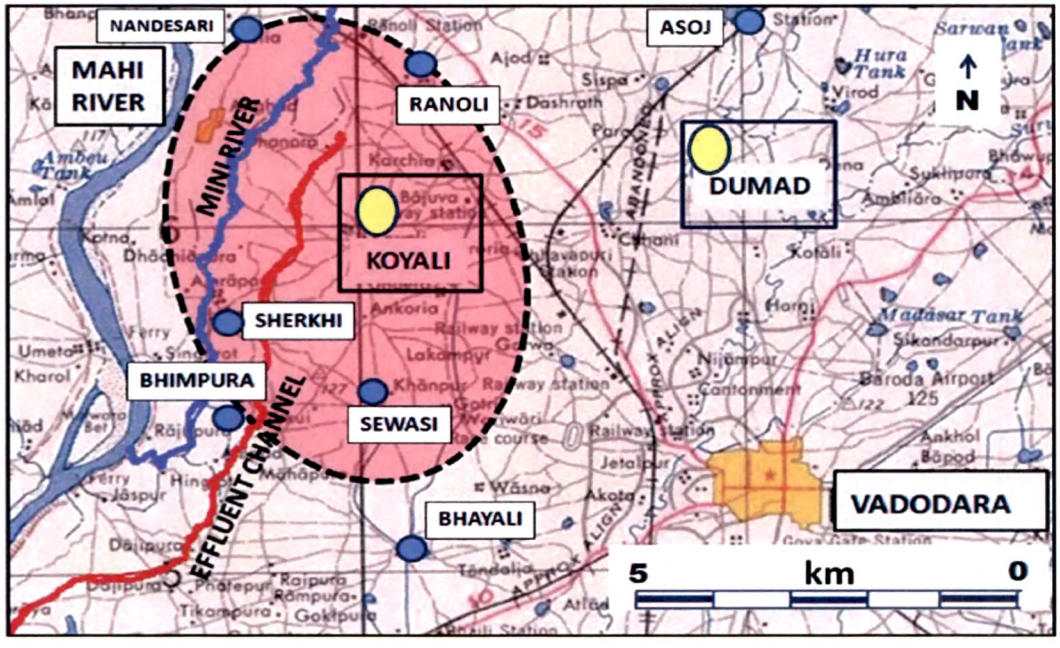

Fig. 2: Map showing location of several villages surveyed in the study region and also the selected ponds.

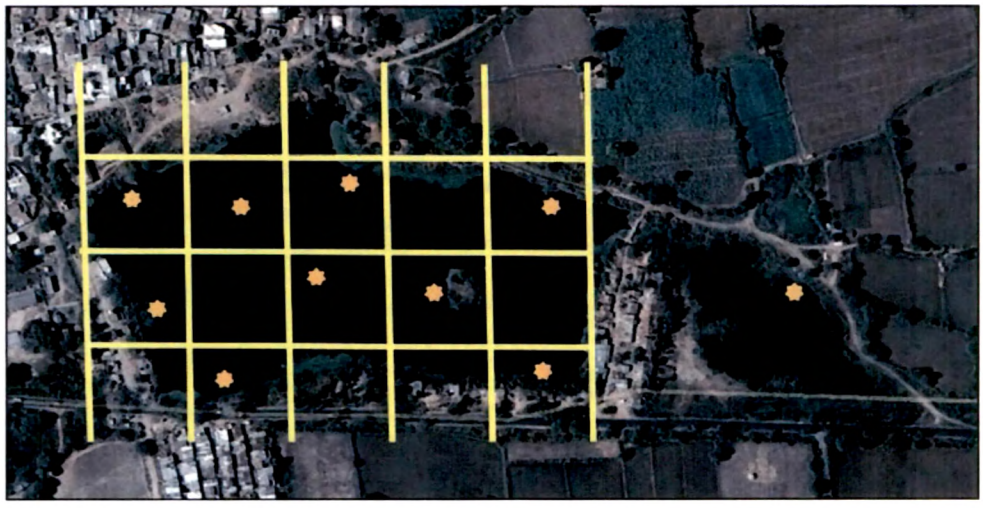

Fig. 3: Grid sub divisions of Dumad study site. * indicate sampling locations.

Fig. 4: Grid sub divisions of Koyali study site. * indicate sampling locations.

Fig. 5: Other aquatic bodies surveyed: a: Bhimpura, b: Rampura, c: Ranoli.

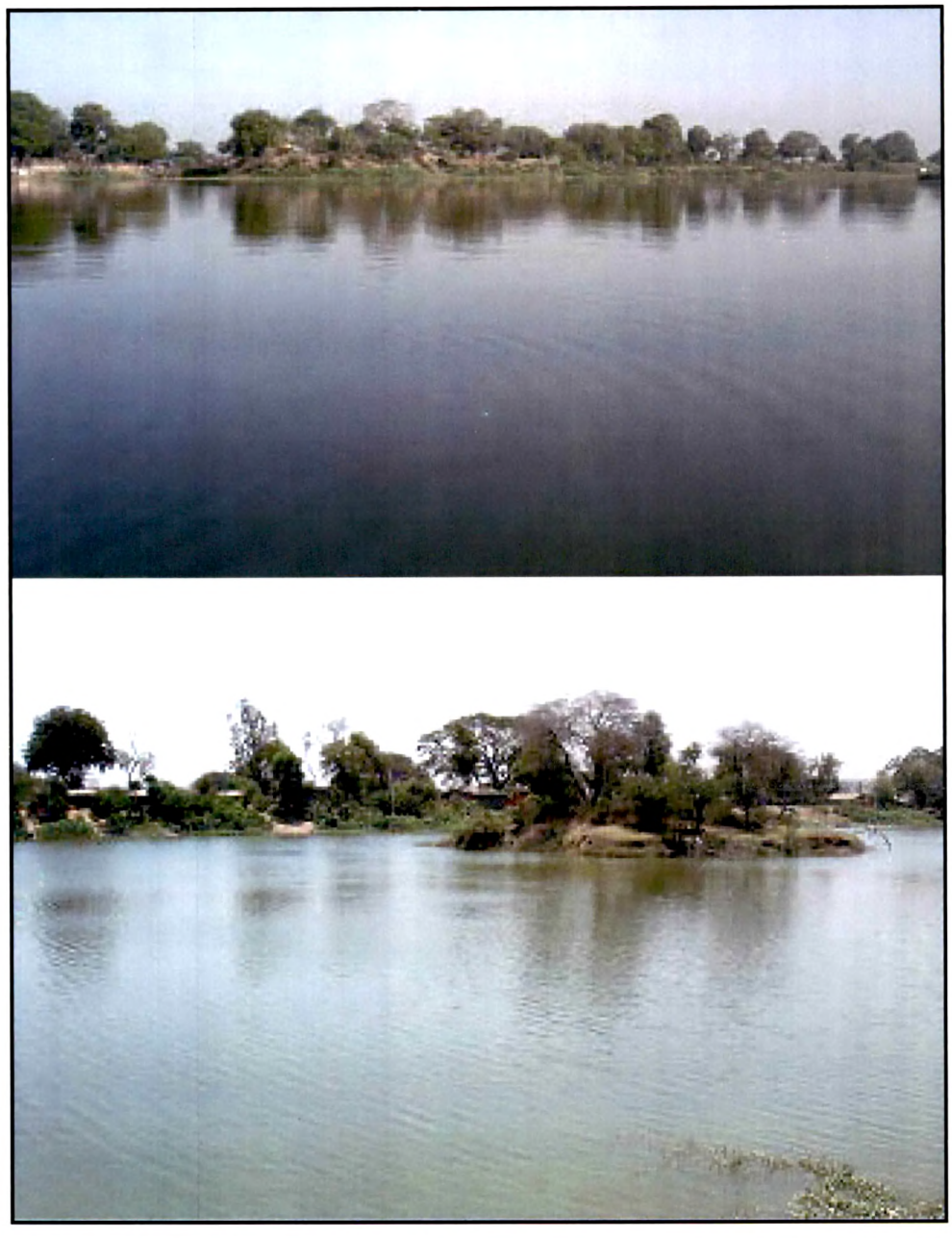

Fig. 6: Showing the views of Dumad pond. Note that the pond retained large volume of water throughout the year.

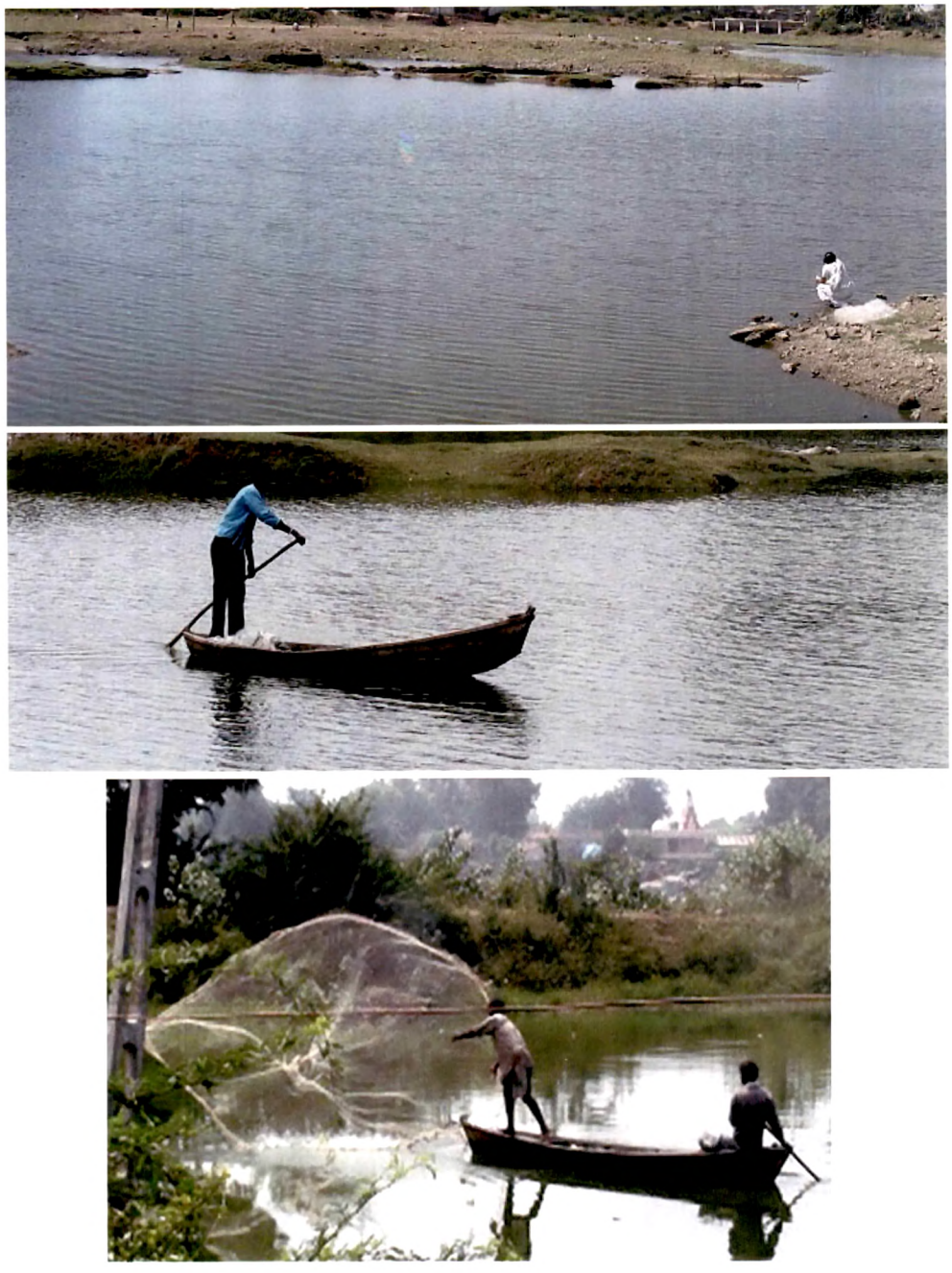

Fig. 7: Showing the view of Koyali pond. Note that the culture fishery activity is a routine here.

Fig. 8: a: The experimental model, adult Tilapia *Oreochromus mossambicus,*

b: Fishermen harvested the fishes from Dumad pond for experimental purposes,

c: Onsite examination and morphometric studies of the experimental model.