CHAPTER - 4

MATERIALS AND METHODS

4.1 FIELD METHODS

Systematic samplings of water, surface and core sediments at different locations along the downstream were done for the River Chambal.

The sampling locations were chosen carefully in order to get maximum representation of the diverse environments. The water, surface and core sediment samples were collected in three seasons namely monsoon (August, 2003), post-monsoon (October, 2003) and pre-monsoon (April, 2004) from different locations along the downstream. Samples were collected from the main course of river and its tributaries (Shipra, Chhoti Kalisindh, Kalisindh, Parbati and Banas), before and after their confluence to get the complete picture of their influence on the river. Various locations on the river are Nagda 1, Nagda 2 (after GRASIM drainage), Cipawra Chambal 1 (before confluence with Shipra), Cipawra Chambal 2 (after confluence), Shipra, Parapeepli Chambal 1 (before confluence with Chhoti Kalisindh), Parapeepli Chambal 2 (after confluence), Chhoti Kalisindh, Gandhi Sagar 1 (before dam), Gandhi Sagar 2 (after dam), RAPP 1 (before dam), RAPP 2 (after dam), Kota Barrage 1 (before barrage), Kota Barrage 2 (after barrage), Laban Chambal 1 (before confluence with Kalisindh), Laban Chambal 2 (after confluence), Kalisindh, Pali Chambal 1 (before confluence with Parbati), Pali Chambal 2 (after confluence), Parbati, Rameshwar Chambal 1 (before confluence with Banas), Rameshwar Chambal 2 (after confluence), Banas, Sabalgarh, Dhaulpur, Jaithpur, Udi, Etawah (Yamuna 1) (before confluence with Chambal) and Yamuna 2 (after confluence) (Figure 4.1, 4.2).

4.1.1 Water Samples

Water samples were collected from about 10 cm below the surface in cleaned polypropylene bottles. Bottles were completely filled with water samples and capped airtight. For cation, 50 ml of sample was filtered with 0.22 μ filter paper and
Figure 4.1 Map of sampling locations and drainage area of the Chambal River basin

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Figure 4.2 Overview of sampling locations and tributaries of river Chambal
preserved on site with ultra pure HNO₃. These water samples were brought to laboratory for various physico-chemical analyses and stored at 4°C.

4.1.2 Surface Sediment Samples
Surface sediment samples were collected from the sampling locations by scooping the top 5-10 cm layer using a small plastic scoop along the riverbed, at sediment water interface. The bed sediment samples were then transferred to a polyethylene bag, which was sealed to air by fastening the mouth immediately. These samples were brought to the laboratory for studying its physico-chemical properties and mineralogy.

4.1.3 Core Sediment Samples
Core sediment samples were obtained by drilling in 6.25 cm diameter PVC pipe into the sediment at certain locations in the river and the estuary region and pulling out the pipe with care as not to disturb the sediment profile in the core. The pipe was sealed from both the ends and marked appropriately and brought to the laboratory for further analysis.

4.2 LABORATORY METHODS

4.2.2 WATER ANALYSIS

pH and Conductivity
The pH and conductivity were measured in unfiltered water samples. "Rachho" (Model No. 123) pH meter was used to measure the pH. The glass electrode was conditioned and calibrated with buffer solutions of pH 4, 7 and 9. The temperature of the instrument was set according to the temperature of the samples. The samples were stirred continuously to maintain homogeneity before noting the pH. Conductivity was measured in micro mS/cm using Systronics Conductivity Meter 306. The instrument was calibrated with 0.01 M KCl standard (1413 in mhos/cm at 25°C).

Bicarbonate
The bicarbonate content was determined by potentiometric titration method (APHA, 1985). 20 ml of samples and a series of bicarbonate standards ranging from 100 mg/l to 1000 mg/l were titrated against 0.02 N HCl. The end-point was noted at pH 4.5. A
graph between bicarbonate standards and volume of HCl consumed was plotted. The readings for the samples were found out from this graph.

**Phosphate**

Phosphate was determined by the Ascorbic Acid method (APHA, 1985). Phosphate standards ranging from 0.1 to 3 mg/l were prepared from KH$_2$PO$_4$. 40 ml of each sample and standard solution was pipetted out in 50 ml volumetric flask and 5 ml of Molybdate Antimony solution (prepared by dissolving 4.8 gm of Ammonium Molybdate and 0.1 gm of Potassium antimony tartarate in 400 ml 4N H$_2$SO$_4$ and making the total volume to 500 ml with same acid) and 2 ml of Ascorbic Acid solution (2.0% w/v) was added and mixed well. The mixture was diluted to 50 ml and optical density was measured at 650 nm using Cecil Spectrometer (Model No. 594). A graph was plotted between concentrations of the standards; and optical density and sample concentrations were obtained from this graph.

**Dissolved Silica**

The dissolved silica content was determined by the Molybdate Silicate Method (APHA, 1985). Silica standards were prepared, ranging from 0.1 to 10 mg/l from Sodium metasilicate nanohydrate. 20 ml of each sample and standard were pipetted out in 50 ml volumetric flask and 10 ml of ammonium molybdate solution (prepared by dissolving 2 gm of ammonium molybdate in 10 ml distilled water, 6 ml concentrated HCl was added and final volume was made upto 100 ml by distilled water) and 15 ml of Reducing Reagent (prepared by mixing 100 ml metol sulphite solution, 60 ml 10% oxalic acid and 120 ml 25% Sulphuric acid and making the final volume to 300 ml by adding distilled water) was added and mixed well. Metol sulphite was prepared by dissolving 5 gm metol in 210 ml distilled water and 3 gm sodium sulphate was added and the volume was made upto 280 ml with distilled water. The samples were stirred properly and kept for 3 hr to complete the reaction. The optical density was measured at 650 nm using Cecil Spectrometer-594. Graph between concentration of the standards and optical density was drawn and concentration of samples were recorded from it.
Chloride, Fluoride, Nitrate and Sulphate
These were analysed using Metrohm Ion Chromatograph with 709 IC Pump, 733.1 IC Separation Centre and 732 IC conductivity detector. The column used for anion analysis was IC Anion Column Metrosep Anion Dual 2 (6.1006.100). The eluent used was 5 mmol/L phthalic acid, 2% acetonitrile, pH 4.5 (adjusted with NaOH) and a conductivity of approx. 400 \( \mu S/cm \). The flow rate maintained during the analysis was 0.8 mL/min. The anions were eluted out in the order of fluoride, chloride, nitrate and sulphate. It took 20 min for each run.

Sodium, Potassium, Calcium and Magnesium
These were analysed using Metrohm Ion Chromatograph with 709 IC Pump, 733.1 IC Separation Centre and 732 IC conductivity detector. The column used for cation analysis was IC Anion Column Metrosep Cation 1-2 (6.1010.000). The eluent used was 4 mmol/L tartaric acid, 1 mmol/L dipicolinic acid and a conductivity of approx. 700 \( \mu S/cm \). The flow rate maintained during the analysis was 1.0 mL/min. The cations were eluted out in the order of sodium, potassium, calcium and magnesium. It took 15 min for each run.

Trace Metals
Trace metals (Iron, Manganese, Copper, Cadmium, Chromium, Nickel, Zinc, Lead, and Arsenic) were analysed by using Atomic Absorption Spectrophotometer (AAS), Schimadzu-AA-6800. First, the instrument was calibrated by using known concentration of metal samples and standard curve was drawn between absorption and concentration; from this graph concentration of water samples were calculated. Flame used for metal analysis was air-acetylene.

Dissolved Organic Carbon (DOC)
The 100-ppm stock solution was prepared from potassium perpthalate \( (C_8H_4O_4) \) salt. Then standards ranging between 5-25 mg/l were prepared by serial dilution of stock solution. 10 ml of samples and standards were added to Erlenmeyer flask, 0.4ml of buffer solution was added and stirred at a moderate speed for 10 min. Persulphate powder pillow was added to sample and reagent blank vial. 0.3 ml deionized water was added to reagent and 0.3 ml prepared sample was added to sample vial and stirred
to mix. Blue indicator ample was inserted in each vial. The vial assemblies were capped tightly and placed in the COD reactor for 2 hours at 103-105°C. The vial assemblies were removed carefully from the reactor and placed in a test tube rack. The vials were allowed to cool for 1 hr. The optical density of these vials was taken at 430 nm using Cecil Spectrophotometer (model no. 594).

4.2.3 SEDIMENT ANALYSIS

Grain size analysis
Mechanical analysis for determining the grain size of surface sediments was done upto 37 μm using the standard method of sieving (using ASTAM Standard Sieve).

Surface Sediment Mineralogy
The surface sediments were oven dried and X-ray diffractogram (XRD) (Model: PW 1140) were recorded using Cu (Kα radiation) source, proportional detector and Ni filter. The chart drives was 1 cm/min, goniometer 1°/min, range 400 cps and voltage 16 mV/35KV were maintained. The primary peaks of various minerals were identified by comparing 20 values for highest intensity peak, as described by Lindholm, (1987).

Elemental Analysis
The sediments were analyzed for Total Carbon, Total Nitrogen and Total Phosphorus. Samples were taken out from cold room and were oven dried at 60°C for 24 hrs. (Raaphosstt, 1994). Homogenization was done by quartering. The analyses done on sediments are:

Carbon Analysis
The mass percentages of carbon, hydrogen, nitrogen and sulphur in all the samples were analysed by EURO EA Elemental Analyzer. Before analysis, sediments were made free of halogens by washing with distilled water. Untreated samples were used for determining total carbon.

Phosphorus Analysis
Total phosphorus in sediment was determined by Ascorbic Acid Method. Standard phosphate solution ranging from 0.2 to 2 ppm was prepared using KH₂PO₄. 5 ml of
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digested samples and standard solution was pipetted out in 50 ml volumetric flask and 20 ml distilled water was added, then 10 ml of reducing agent (prepared by dissolving 2.108 gm of ascorbic acid in 400 ml of ammonium molybdate-ammonium potassium tartarate solution, adding 12 gm of ammonium molybdate in 250 ml of warm distilled water, 0.291 gm antimony potassium tartarate was added in 100 ml distilled water and both solution were added to 1000 ml of 5 N H2SO4, final volume made up to 2000 ml and mixed well) was added. The mixture was diluted to 50 ml and optical density was measured at 880 nm using "Cecil Spectrophotometer (model No.594)". A graph was plotted between concentrations of the standards and optical density and sample concentrations were obtained from this graph.

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\% P = \frac{C \times 0.025}{W}
\]

C = Corrected concentration (sample- blank)

W = Weight of soil sample taken.

Analysis for the major and minor elements

Finely ground homogenized sediments were taken in the beaker and boiled with 30% (v/v) H2O2 (Jackson, 1973) for removal of organic matter. The treatment is repeated until the emission of CO2 stopped.

Analyses of major and minor elements were carried out by the two-solution method (Shapiro, 1975). Solution 'A' was used for silica and phosphorus analysis and Solution 'B' was used for metal analysis.

Solution 'A'

0.025 gm of finely powdered sediment was taken in 50 ml Nickel crucible and 3-4 pellets of NaOH were added to it. The crucible was gently heated on a furnace in order to mix sediment and melt pellets, and then the crucible was constantly heated to dull redness for 30 min. After that, the crucible was allowed to cool down to room temperature. 10 ml of distilled water was added in the crucible and kept overnight. The solution was transferred to 250 ml volumetric flask with the help of a policeman. 5 ml of (1:1) HCl and 25 ml water was also added. This solution was boiled till it was clear and the total volume was made upto 250 ml by adding distilled water.
(i) Silica Analysis
0.8 ml of solution ‘A’, standard solution (Canadian soil standards i.e. SO1, SO2, SO3, SO4) and reagent blank were transferred to 50 ml Teflon beaker. 20 ml of distilled water was added to each beaker. 0.2 ml of ammonium molybdate solution was added and kept for 10 mts. 4 ml of 10% (w/v) tartaric acid was added by swirling the beaker. 0.1 ml of reducing reagent was added and samples properly stirred and kept for 30 mts. Optical density was measured by Cecil Spectrophotometer-594 at 650 nm.

Ammonium molybdate solution was prepared by dissolving 1.875 gm ammonium molybdate in 19 ml distilled water and adding 6 ml 20% (v/v) H2SO4. The reducing reagent was prepared by dissolving 0.07 gm sodium sulphite in 1.0 ml distilled water and then 0.015 gm 1-amino-2-naphthol-4-sulphonic acid was added and stirred well until dissolved. 9 ml of 10% (w/v) sodium bisulphite solution was added to it and mixed well.

(ii) Alumina Analysis
15 ml of solution ‘A’ (sample, standard and blank) were taken in 100 ml volumetric flasks. 2 ml of Calcium chloride solution (prepared by dissolving 7 gm CaCO3, and adding 100 ml of water and 15 ml of concentrated HCl and diluted to 500 ml) was added. 1 ml of hydroxylamine hydrochloride (prepared by dissolving 5 gm of hydroxylamine hydrochloride in 50 ml) was added by swelling the flask. 1 ml of potassium ferricyanide (0.75%) was added to each flask and mixed. 2 ml of thioglycolic acid solution (4%) was added and kept for five minutes. 10 ml of buffer solution (prepared by dissolving 100 gm of sodium acetate in water, 30 ml of glacial acetic acid was added and then diluted to 500 ml) was added and kept for 10 minutes. 10 ml of Alizarin Red-S solution (0.05%) was added to each flask and volume was made up to 100 ml by adding distilled water. The samples were properly stirred and kept for 50 min. The absorbance was measured at 470 nm using Cecil Spectrophotometer-594.

Solution ‘B’
0.10 gm of finely grounded samples were transferred to the Teflon crucibles and 2 ml of aqua regia (HNO3 and HCl ratio 1:3) and 5 ml HF were added to each crucible, these were then sealed in metallic cases. The crucibles were heated for one and half
hours at 100°C and allowed to cool down to room temperature. 5.6 gm of boric acid crystal (H₃BO₃) was dissolved in 20 ml distilled water and then added to the bomb contents which was made upto 100 ml. The solution was transferred to polypropylene bottles for storage. The samples were left undisturbed overnight to allow the formation and settling of borosilicate from the solution. This gelatinous precipitate is separated by centrifugation. The solution was then filtered using 0.45 μm Millipore filter papers and the solution obtained was used for analysis of major and minor elements using AA-6800 Atomic Absorption Spectrophotometer (Shimadzu).

Sequential Extraction of Heavy Metals

It is now generally agreed that the total metal concentration in sediments does not provide a good estimate of the potentially available element to the aquatic biota (Keelay et al., 1974; Loring, 1981; Luoma et al., 1983 and Illera et al., 2000). The bioavailability and recycling of trace elements depends on the geochemical fractions with which the metal is associated in the sediment (Jenne and Luoma, 1977). Much work on heavy metal fractionation using sequential extraction techniques on various types of sediments has been reported (Chester et al., 1985; Kersten and Forstner, 1986; Saeki et al., 1993; Mat et al., 1994; Lim and Kiu, 1995 and Lopez-Sanchez et al., 1996).

Although, several limitations of sequential extraction procedures (Tessier et al., 1979) have been reported. For example, due to the limited selectivity of the extraction reagents, often more than one metal species is released in each extraction step (Rapin et al., 1986; Kersten and Forstner, 1986 and Tack and Verloo, 1995). The method still remains as one of the most widely used approaches to distinguish between different geochemical association of many trace metals and to gain a better insight of geochemical processes occurring in sediments.

For sequential extraction of heavy metals from sediments, 1 gm of the soil was taken and after each step the sample was separated by centrifugation. The sample was washed with distilled water and dried at 40°C for further analysis. The following procedure was adopted for the extraction.
Fraction 1: Really Exchangeable
The samples were extracted at room temperature for 1 hr with 8 ml of magnesium chloride solution (1M MgCl₂, pH 7.0) with continuous agitation.

Fraction 2: Bound to Carbonate
The residue from fraction 1 was leached at room temperature with 8 ml of 1 M NaOAc adjusted to pH 5.0 with acetic acid (HOAc). Continuous agitation was maintained, and the time necessary for complete extraction was evaluated based on the 2000 ppm lead carbonate sample prior to experimental trials and was determined to be 3 hr.

Fraction 3: Bound to Iron and Manganese Oxides
The residue from fraction 2 was extracted with 20 ml of 0.04 M NH₂OH.HCl in 25% (v/v) HOAc. This fraction experiment was performed at 96±3 °C with occasional agitation for 6 hr.

Fraction 4: Bound to organic matter
3 ml of 0.002 M HNO₃ along with 5 ml of 30% H₂O₂ (pH adjusted at 2 with HNO₃) was added to the residue from fraction 4 and heated to 85±3°C for 2 hrs with occasional agitation. 12 ml of 30% H₂O₂ was added and the heating was continued for another 3 hrs. On cooling, 4 ml of Distilled water was added and was shaken for 30 min.

Fraction 5: Residual Fraction
The sample was extracted as solution B. The metal content was determined using AA-800 Atomic Absorption Spectrophotometer (AAS).

4.2.4 HUMIC SUBSTANCES
Extraction
Humic Substances were isolated from the river sediments, collected from the whole stretch of the Chambal River basin in monsoon and two samples from Gandhi Sagar in post-monsoon and pre-monsoon to get an overall estimation of the seasonal variation. Samples were stored at 4 °C before analysis. Wet sediment samples were
air-dried and passed through 2.0 mm sieve. 20 g of samples were equilibrated to a pH value 1 to 2 with 1 M HCl at room temperature. Solution volumes were adjusted to 100 ml with MQ water. The suspensions were kept on shaker for 1 hr and then the supernatant were separated from the residue by centrifugation at 8000 g for 30 min followed by decantation. Supernatant were saved for the isolation of fulvic Acid.

Soil residues were neutralized with 1 M NaOH to pH 7, then 200 ml of 0.1 M NaOH was added. The suspensions were kept for intermittent shaking for 24 hr. The alkaline suspension was allowed to settle overnight and supernatant was collected by centrifugation and decantation. Supernatant was acidified with 6 M HCl with constant stirring to pH 1 and then the suspension was allowed to stand for 12 to 16 hr. To separate the humic acid (precipitate) and fulvic acid fractions (supernatant-FA Extract 2) suspension was centrifuged.

Humic acid fraction was redissolved by adding a minimum volume of 0.1 M KOH under N₂. Solid KCl was added to attain a concentration of 0.3 M [K⁺] and then centrifuged at high speed to remove the suspended solids. The humic acid was reprecipitated by adding 6 M HCl, with constant stirring to adjust pH at 1 and the suspension was allowed to stand again for 24 hr. The supernatant was centrifuged and discarded. The humic acid precipitate was suspended in 0.1 M HCl/0.3 M HF solution in a plastic container and kept overnight for shaking at room temperature. Suspension was centrifuged and the HCl/HF treatment was repeated so that the ash content is below 1%. Humic acid was then freeze dried and kept at 4°C. Thus obtained Fulvic acid was scanned by spectrophotometer without purification.

**Characterisation of Humic Substances**

**Elemental Analysis**

The mass percentages of carbon, hydrogen, nitrogen and sulphur in each humic acid sample were analysed by EURO EA Elemental analyzer. Humic acids are composed of carbon, hydrogen, nitrogen and oxygen as major elements, along with sulphur and halogens present in practically negligible quantities. Therefore, the approximate percentage of oxygen was calculated by subtracting the sum of mass percentages of carbon, hydrogen and nitrogen from hundred.
UV-VIS Spectrophotometric Measurements
All UV-VIS spectrophotometric absorbance measurements were performed by recording UV-Vis spectra within the 800-200 nm range against blanks consisting of 0.1M NaOH for HA and FA determination. A rise in pH leads to an increased absorbance because of the increased electron density of chromophores. To compare all the spectra of HA and FA, pH of all the samples were adjusted to 7. Since the maxima of the spectra can vary for different sediment samples, the UV-Vis spectrum for each sample was recorded rather than measurement at single wavelength (Moreda-Pineiro et al., 2004). The E2/E3 (absorbance at 250 nm divided by absorbance at 365 nm) and E4/E6 ratios (the absorbance at 465 nm divided by the absorbance at 665 nm) were calculated. (Chen et al., 1977; Malcom, 1989; Lassen et al., 1994)

Infrared Spectrometry
The pellets were preheated to 65°C for 2 hr to eliminate the moisture interference, 1 mg of the pre dried sample was kept at 45°C for about 18 hr, mixed with 300 mg of similarly pre dried KBr and grounded for 15 sec. A control pellet is also formed in the same manner, following which both the pellets are placed in vaccum oven at 100°C for 2 hr. The pellet containing HA is scanned in double beam mode against the KBr blank (Stevenson and Goh, 1974). The spectra were recorded using Perkin Elmer RX1 FTIR spectrophotometer.

Proton NMR Spectrometry
PMR spectra of 10 mg/ml solution of humic acid in D2O solvent were recorded in Bruker DRX-300 (300 MHz FT NMR) spectrophotometer. The signal for D2O was used as reference and set to 4.8 ppm chemical shift.

C13 NMR Spectroscopy
Liquid-state C13 NMR spectra were recorded in 0.5 M NaOD (50mg in 0.5 ml) on a Bruker 250 MHz instrument. Spectral width was set to 17 KHz, and pulse width 3.5 μs (45°C). The spectra were referenced to external TMS (tri-methyl silane) (Thomsen et al., 2002).
Gel Electrophoresis

SDS-PAGE

SDS–PAGE is a well-established technique for the separation of proteins. Polyacrylamide gels can also be used for the separation of humic acids on the basis of their molecular wt. (Evans et al., 2000; Cavani et al., 2003).

Method

Purified humic acid samples were separated on 18% Tris-CI SDS-PAGE. PAGE was run in vertical electrophoresis unit (Amasham Pharmacia 600V), 20cm x 20cm. Glass gel plates were assembled according to manufacturer’s instruction and acrylamide solution was poured into the gaps between the glass plates leaving sufficient space for stacking gel. 18% resolving gel was casted mixing 18%(v/v) acrylamide : bisacrylamide (29:1), 375mM Tris-CI pH 8.8, and 0.1% SDS v/v. 0.1% APS and 6-7 μl of TEMED was used for the polymerization of the gel. After the polymerization was completed overlay was poured off and top was washed with deionised water. Stack gel was prepared mixing 5%(v/v) acrylamide : bisacrylamide (29:1), 125 mM Tris-CI pH 6.8, 0.1% SDS. 0.1%APS and 4-5μl of TEMED was used for the polymerization of the gel also. Stack gel was poured directly onto the surface of resolving gel, and immediately clean Teflon comb was inserted into the stacking gel solution between the glass plates. Comb was removed after the polymerization of the stack gel; wells were properly washed with deionised water and gel was mounted in the electrophoresis apparatus.

Sample Preparation

Purified humic acid samples were dissolved in sample buffer (50 mM Tris-cl pH 6.8, 2% SDS (v/v), 10% glycerol (v/v). Approximately 1mg humic acid was dissolved in 50 μl sample buffer. 20 μl of each sample was loaded into the individual wells in predetermined order. Pre stained protein molecular wt. marker was run along with humic acid samples. Unused wells were filled with 1X sample buffer. Top and bottom reservoirs were filled with Tris-glycine electrophoresis buffer (25 mM Tris-base, 250 mM glycerin 0.1% SDS. Electrophoresis apparatus was attached to electric power supply (Amasham Pharmacia). Gel was run at 25 mA for 3 hours at constant volts. Gel was run until marker dye reached at the bottom of the resolving gel. The glass
plates were removed from the electrophoresis apparatus; gel was placed onto a transparent sheet, orientation was marked and it was scanned. The significant portion of humic acid is coloured and can be visualized on the gel without staining.

4.3 GEOCHEMICAL PLOTS

Mineral Stability and Gibbs Diagram
Mineral stability and Gibbs diagrams were plotted using “Microsoft Excel (Window XP)” and Waterclast software (DOS based).

Saturation Index (SIc and SIId)
This was calculated using Phreeqc software.

Grain Size Analysis
Grain size analysis was done by using a statistical program “GRADISTAT”.

4.4 STATISTICAL ANALYSIS

Correlation Matrix
Correlation matrix between various water and sediment quality parameters was constructed using “Microsoft Excel (Window XP)”.

Factor Analysis
Factor analysis for water and sediment samples were computed using “Statistical Package SPSS-10.3”. The extraction method for factor analysis was “Principle Component Analysis” and “Varimax Rotation” was used for deriving factors.