CHAPTER II

MATERIALS & METHODS
Chapter 2

The Rithwik biomass power plant, Rachagunneri, Yerpedu Mandal, Chittoor District, Andhra Pradesh, India. Tiupati town is located 30 km from this industrial area. The untreated effluent is released at outlets of the biomass power plant which reaches to an agriculture canal. The effluent was collected in the year 2005 at three points from outlets of the biomass power plant in a polythene can, with air tight lid. Appropriate dilutions were made with distilled water and used for experimental purpose.

2.1. Physico – chemical analysis of biomass power plant effluent

Effluent sample is collected from the outlets of biomass power plant. Before sampling the effluent, the polythene container was cleaned thoroughly using distilled water. Immediately after the effluent sampling the pH and EC were measured in the field and later taken to the laboratory and stored at 4°C for further analysis using standard methods (APHA, 2002).

**pH**

pH of the sample was noted using potentiometric method using pH meter already standardized by using buffer solutions of known value before analysis (Skoog et al., 1988).

**Electrical conductivity (EC)**

EC is the measure of the ability of an aqueous solution to convey an electric current. This ability depends upon the presence of ions, their total concentration, mobility, valance and temperature. EC was determined by conductivity meter (Richard, 1954).
Figure-3 Location of study area
Total solids

Total solids in the effluent were determined as per procedure outlined in standard method for the examination of water and waste water (APHA, 2002) is given below.

Apparatus: (a) Evaporating dishes. Dishes of 100 ml capacity made of one of the following materials.

1. Porcelain, 90 mm diameter.
2. Platinum – Generally Satisfactory for all purposes.
   a. Muffle furnace for operation at 500 ± 50°C
   b. Steam bath
   c. Desiccator
   d. Drying oven, for operation at 103 to 105°C.
   e. Analytical balance, capable of weighing to 0.1 mg.

Preparation on of evaporating dish: Clean porcelain dish was heated at 103 to 105°C for 1 hour. The dish was kept in desiccator until needed, immediately weighed before use.

Sample analysis

Measured volume of well-mixed sample was transferred to preweighed dish and evaporated to dryness on a steam bath or in a drying oven. If whenever a necessary, successive sample portion was added to the same dish after evaporation. When evaporating in a drying oven, temperature was lowered to approximately 2°C below boiling, to prevent splattering. The evaporated sample was dried for at lest 1 hour in an oven at 103 to 105°C, the dish was cooled in desiccator to balance temperature, and weighed. Drying, cooling desiccating and weighing were repeated until a constant weight was obtained.

\[
\text{Mg total solids / L} = \frac{(A - B) \times 1000}{\text{Sample volume, ml}}
\]

Where: \( A = \) Weight of dried residue + dish, mg, and
\( B = \) Weight of dish, mg.
Dissolved solids

Apparatus
a. Glass fiber filter disks (Whatman grade) without organic binder.
b. Filtration apparatus: one of the following, suitable for filter disk selected.
   1. Membrane filter funnel.
   2. Gooch crucible, 25 ml to 40 ml capacity, with Gooch crucible adapter.
   3. Filtration apparatus with reservoir and coarse (40 - to 60 μm) fritted disk as filter support.
c. Suction flask, of sufficient capacity for sample size selected.
d. Drying oven, for operation at 180 ± 2°C.

Sample analysis

Measured volume of well-mixed sample was filtered through glass fiber filter, and washed thrice with 10 ml of distilled water, allowing complete drainage between washings and suction was continued for about 3 min after filtration was complete. Filtrate was transferred to a weighed evaporating dish and evaporated to dryness on a steam bath. After having dried at least 1 h in an oven at 180 ± 2°C, it was cooled in a desiccator to balance temperature, and then weighed. Drying, desiccating and weighing was repeated until a constant weight was obtained.

\[
\frac{(A - B) \times 1000}{\text{Sample volume, ml}} = \text{mg total dissolved solids/L}
\]

Where: \( A = \text{Weight of dried residue + dish, mg, and} \)
\[ B = \text{Weight of dish, mg}. \]
Biochemical Oxygen Demand (BOD)

Apparatus
1. Incubation bottles, 250 – 300 ml capacity.
2. Air incubator or water bath, thermostatically controlled at 20 ± 1 °C. See the light excluded to prevent possibility of photosynthetic production of DO.

Reagents
(a) Phosphate buffer solution
   About 8.5 g KH\(_2\)PO\(_4\), 21.75 g K\(_2\)HPO\(_4\), 33.4 g Na\(_2\) HPO\(_4\). 7H\(_2\)O and 1.7 g NH\(_4\)Cl was dissolved in 50ml distilled water and diluted to 1 L. The pH should be 7.2 without further adjustment. Reagent is discarded if there is any sign of biological growth in the stock bottle.
(b) Magnesium sulfate solution
   22.5 g Mg SO\(_4\).7H\(_2\)O was dissolved in distilled water and diluted to 1 L.
(c) Calcium chloride solution
   About 27.5 g CaCl\(_2\) was dissolved in distilled water and diluted to 1 L.
(d) Ferric chloride solution
   About 0.25 g FeCl\(_3\) 6 H\(_2\)O was dissolved in distilled water and diluted to 1 L.

Preparation of dilution water
Desired volume of water was placed in a suitable bottle and 1 ml each of phosphate buffer, MgSO\(_4\), CaCl\(_2\) and FeCl\(_3\) solutions / L were added.
Dilution water was siphoned into a 1-2 L-capacity graduated cylinder half full cylinder was filled without entraining air. Mixed sample of desired quantity was added and diluted to appropriate level with dilution water. Mixed well with a plunger-type mixing rod; entraining air was avoided. Mixed dilution sample was siphoned into BOD bottles. Initial DO was determined on one of these bottles. Second bottle was stoppered tightly, incubated for 5 days at 20\(^{0}\) C. Initial DO was determined immediately after filling BOD bottle. Final DO was determined after incubation of 5 days at 20\(^{0}\) C. Azide – modification of the iodometric method was followed for determination of DO. Procedure is given in determination of dissolved oxygen.
\[
\frac{D_1 - D_2}{P} = \text{BOD mg/L}
\]

\(D_1 = \) DO of diluted sample immediately after preparation, \(mg/L\)
\(D_2 = \) DO of diluted sample after 5 days incubation at \(20^\circ C\), \(mg/L\)
\(P = \) decimal volumetric fraction of sample used.

**Dissolved Oxygen (DO)**

**Reagents**

(a) Manganese sulfate solution: Dissolved 480 g \(\text{MnSO}_4 \cdot 4\text{H}_2\text{O}\), or 400 g \(\text{MnSO}_4 \cdot 2\text{H}_2\text{O}\), 364 g \(\text{MnSO}_4 \cdot \text{H}_2\text{O}\) in distilled water, filtered and diluted to 1 L. The \(\text{MnSO}_4\) solution should not give a color with starch when added to an acidified potassium iodide (KI) solution.

(b) Alkaline – iodide – azide reagent: 500 g NaOH (or 700 g KOH) and 135 g NaI (or 150 g KI) dissolved in distilled water diluted to 1 L. Then 10 g NaN₃ was dissolved in 40 ml distilled water and added.

(c) Sulfuric acid, \(\text{H}_2\text{SO}_4\), Conc. One Milliliter is equivalent to about 3 ml alkali iodide azide reagent.

(d) Starch: About 2 g of laboratory – grade starch and 0.2 g salicylic acid, as a preservative, was dissolved in 100 ml hot distilled water.

(e) Standard sodium thiosulfate titrant: About 6.205 g \(\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}\) was dissolved in distilled water. 1.5 ml of 6N NaOH or 0.4 g solid NaOH was added and diluted to 1000 ml. Then standardized with bi-iodate solution.

(f) Standard potassium bi-iodate solution, 0.0021M: About 812.4 mg KI (103)₂ was dissolved in distilled water and diluted to 1000 ml.

**Standardization**

Approximately 2g KI, free from iodate, was dissolved in an erlenmeyer flask with 100 to 150 ml distilled water. About 1ml of 6N \(\text{H}_2\text{SO}_4\) or a few drops of conc. \(\text{H}_2\text{SO}_4\) and 20 ml of standard bi-iodate solution was added. Diluted to 200 ml and titrated with the thiosulfate titrant, adding starch toward end of titration when a pale straw color is reached.
To the sample collected in a 250 to 300 ml bottle, 1 ml MnSO₄ solution was added followed by 1 ml alkali – iodide – azide reagent. While adding reagents pipette tips were held just above the liquid surface. Carefully stoppered to exclude air bubbles and mixed by inverting bottle for few times. When precipitate settled sufficiently 1.0 ml of Conc. H₂SO₄ was added. Restoppered and mixed by inverting several times until dissolution is complete. A few drops of starch solution was added to 200 ml sample and titrated with 0.025M Na₂S₂O₃ solution to a pale straw color.

For titration of 200ml sample, 1ml 0.025 M Na₂S₂O₃ 1 mg DO / L.

Chemical Oxygen Demand (COD)

Apparatus

(1) Reflux apparatus consisting of 500 or 250 ml erlenmeyer flasks.
(2) Condenser
(3) Hot Plate

Reagents

(a) Standard Potassium Dichromate Solution (0.0417 M)

Dissolved 12.259 g K₂Cr₂O₇, primary standard grade, previously dried at 103°C for 2h, in distilled water and diluted to 1000 ml.

(b) Sulfuric Acid Reagent

Added Ag₂SO₄ reagent or technical grade, crystals or powder, to conc. H₂SO₄ at the rate of 5.5 g Ag₂SO₄ / Kg H₂SO₄. Allowed to stand for 1 to 2 days to let dissolve Ag₂SO₄.

(c) Ferroin Indicator Solution

Dissolved 1.485g 1,10 – phenanthroline monohydrate and 695 mg FeSO₄.7H₂O in distilled water and diluted to 100 ml. This indicator solution was purchased already prepared.

(d) Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25M

Dissolved 98 g Fe(NH₄)₂(SO₄)₂ 6H₂O in distilled water. Added 20 ml conc. H₂SO₄, cooled, and diluted to 1000 ml. Standardized this solution daily against standard K₂Cr₂O₇ solution as follows.
10.0 ml standard \( \text{K}_2\text{Cr}_2\text{O}_7 \) diluted to about 100 ml. Added 30 ml conc. \( \text{H}_2\text{SO}_4 \) and cooled titrated with FAS titrant using 0.10 to 0.15 ml (2 to 3 drops) ferroin indicator.

Molarity of FAS solution

\[
\text{Volume } 0.0417 \text{ M } \text{K}_2\text{Cr}_2\text{O}_7
\]

\[
\text{Solution titrated, ml} = \frac{\text{Volume FAS used in titration, ml}}{0.25}
\]

(e) Mercuric sulfate, \( \text{HgSO}_4 \) crystals or powder.

20 ml sample was taken in 250 ml refluxing flask. About 1 gm \( \text{HgSO}_4 \), several glass beads, were added. To this 10 ml dichromate and 30 ml of sulfuric acid reagent were added. Flask was attached to condenser and turned on cooling water. Refluxed for 2h cooled and condenser was washed with distilled water. Disconnect reflux condenser and mixture was diluted to about twice its volume with distilled water. Cooled to room temperature and titrated excess dichromate with FAS, using 0.10 to 0.15 ml (2 or 3 drops) ferroin indicator. The end point of the titration was sharp color change from blue – green to reddish brown. In the same manner, blank was titrated a blank containing the reagents and a volume of distilled water equal to that of sample.

\[
(A - B) \times M \times 8000
\]

COD as \( \text{mg} \ \text{O}_2 / \text{L} \) = \[
\frac{(A - B) \times M \times 8000}{\text{ml sample}}
\]

where

\[
A = \text{ml FAS used for blank.}
\]

\[
B = \text{ml FAS used for sample, and}
\]

\[
C = \text{molarity of FAS.}
\]
2.2. Pot culture studies

Sandy Clay Loam soil near biomass power plant collected and used for pot culture. The seeds of *Arachis hypogaea* L var TCGS-320, *Phaseolus aureus* Roxb var LGG-420 were obtained from Agriculture Research Station, N.G.Ranga University, Tirupati and treated with 0.2 N mercuric chloride for two minutes and washed with running water to remove contamination of seed coat, prior to germination studies. This experiment carried out with *Arachis hypogaea* L. *Phaseolus aureus* Roxb. using different concentrations of effluent 25, 50, 75, 100% and tap water which served as control. Each treatment including control was performed in triplicate and in every pot 10 seeds were used. A measured volume (50ml) of different effluent concentrations were applied to pots everyday. Seedlings were maintained under natural day light and night temperature of 30°C ± 2°C. Seedlings were removed at regular intervals (10, 15, 20, 25, and 30) DAS and used for experiments.

After thorough washing of the plant tissues like leaf and root with double distilled water various physiological and biochemical compositions were determined in experimental plants. Fresh leaves and roots are used for estimation of total chlorophyll content (chlorophyll a and b), total carbohydrates, total free amino acids, total proteins, proline content, starch, nitrate and nitrite reductase enzymatic activities.

**Plant growth parameters**

**Germination**

The number of seeds germinated in each treatment was recorded on 10, 15, 20, 25, 30 DAS of the experiment and germination percentage was calculated.

**Total plant length (Shoot + Root)**

Plant length measures were taken from all the plants (grown in plants of both control and treated) at the end of one month in plants grown in pots. The plants were removed carefully with root system and washed thoroughly. The length of the shoot and root were measured by meter scale.
2.3. Biochemical parameters

Estimation of chlorophyll levels

The chlorophyll content was estimated according to the method of Arnon (1949). About one gram of leaf sample was cut into small pieces and homogenized in a pre-cooled mortar and pestle using 80% (V/V) acetone. The extract was centrifuged at 3000 rpm for 15 minutes and made up to 25 ml with 80% (V/V) acetone. The clear solution was transferred to a colorimeter tube and the optical density was measured at 645 nm and 663 nm, against and 80% acetone blank in Shimadzu Double Beam spectrophotometer (UV 240).

Total chlorophyll (µg/ml) = (20.2 X O.D. at 645 nm) + (8.02 X O.D. at 663 nm).

The levels of chlorophyll ‘a’ and chlorophyll ‘b’ were determined using the equation given below:

Chlorophyll ‘a’ (µg/ml) = (12.7 X O.D. at 663 nm) - (2.69 X O.D. at 645 nm).

Chlorophyll ‘b’ (µg/ml) = (22.9 X O.D. at 645 nm) - (4.68 X O.D. at 663 nm).

The chlorophyll ‘a’ and chlorophyll ‘b’ content was expressed as mg chlorophyll per gram fresh weight of the leaf.

Estimation of total carbohydrates

Total carbohydrates were measured by the Anthrone method (Hedge et al., 1962). Weigh 100 mg of the sample into a boiling tube. Hydrolyze by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N-HCl and cool to room temperature. Neutralize it with solid sodium carbonate until the effervescence ceases, make up the volume to 100 ml and centrifuge. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis. Prepare the standards by taking 0, 0.2 to 1 ml of the working standard. ‘0’ serve as blank. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water and add 4 ml of anthrone reagent. Heat for eight minutes in a boiling water bath. Cool rapidly and read the green to dark green colour at 630 nm. Draw the standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph calculate the amount of carbohydrate present in the sample tube.
Amount of carbohydrate present in 100 mg of the sample

\[
\text{mg of glucose} = X \times 100
\]

Volume of test sample

**Estimation of starch**

Estimation of starch by anthrone method McCredy et al., (1950). Homogenize 0.1 to 0.5 g of the sample in hot 80% ethanol to remove sugars. Centrifuge and retain residue. Wash the residue repeatedly with hot 80% ethanol till the washings do not give colour anthrone reagent. Dry the residue well over a water bath.

To the residue add 5.0 ml of water and 6.5 ml of 52% perchloric acid. Extract at 0°C for 20 min. Centrifuge and save the supernatant. Repeat the extraction using fresh perchloric acid and centrifuge and pool the supernatants and make up to 100 ml. Pipette out 0.1 or 0.2 ml of the supernatant and make up the volume to 1 ml with water. Prepare the standards by taking 0.2 to 1 ml of the working standard and make up the volume to 1 ml in each tube with water and 4 ml anthrone reagent add to each tube. Heat for eight minutes in a boiling water bath. Cool rapidly and read the intensity of green to dark green color at 630 nm. Standard curve was prepared with known amounts of glucose equivalent present in the sample with 0.9.

**Preparation of reagents**

**Anthrone reagent:** 200 mg of anthrone was dissolved in 95% cold conc. \( \text{H}_2\text{SO}_4 \) and made up to 100 ml.

80% ethanol

52% perchloric acid

Standard Glucose: Stock -100 mg in 100 ml water. Working standard 10 ml of stock diluted to 100 ml with water.
**Estimation of total free amino acids**

The activity was measured by the method of Moore and Sand Stein, (1948). Weigh 500 mg of the plant sample and grind it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, add 5 to 10 ml of 80% ethanol. Filter or centrifuge. Save the filtrate or the supernatant. Repeat the extraction twice with the residue and pool all the supernatants. Reduce the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acids. If the tissue is tough, use boiling 80% ethanol for extraction. To 0.1ml of extract, add 1ml of ninhydrin solution. Make up the volume to 2ml with distilled water. Heat the tube in a boiling water bath for 20 min. add 5ml of the diluents and mix the contents. After 15 min read the intensity of the purple colour against a reagent blank in Systronic (UV-VIS, 118) double beam spectrophotometer 570nm. The colour is stable for 1h. Prepare the reagent blank as above by taking 0.1ml of 80% ethanol instead of the extract.

Dissolve 50 mg leucine in 50ml of distilled water in a volumetric flask. Take 10ml of this stock standard and dilute to 100ml in another volumetric flask for working standard solution. A series of volume from 0.1 to 1ml of this standard solution gives a concentration range 10µg to 100µg. Proceed as that of the sample and read the color.

**Reagents**

Ninhydrin: Dissolve 0.8 g Stannous chloride (Sncl₂ , 2H₂O) in 500 ml of 0.2 citrate buffer (pH 5.0). Add this solution to 20 g ninhydrin in 500 ml methyl cellosolve (2 methoxy ethanol)

0.2 M citrate buffer pH 5.0

Diluent solvent- Mixed equal volumes of water and n-propanol, and use.

**Estimation total protein content**

Total protein content was measured by the method of Lowry et al., (1951) using Folin-Ciocalteau reagent. Extraction is usually carried out with buffers used for
the enzyme assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation. Pipette out 0.2 to 1 ml of working standard in to series of test tubes. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Makeup the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank. Add 5 ml Alkaline Copper reagent to each tubes including the blank. Mix well and allow standing for 10 min. Then add 0.5 ml of Folin-Ciocalteau reagent, mix well and incubate at room temperature in the dark for 30 min. Blue colour is developed and read at 660 nm.

Preparation of reagents

Reagent A 2% Sodium Carbonate in 0.1 N Sodium Hydroxide.

Reagent B 0.5% Copper Sulphate (CuSO4.5H2O) in 1% potassium sodium tartrate.

Reagent C Alkaline Copper solution mix 50 ml of A and 1 ml of B prior to use.

Reagent D Folin-Ciocalteau – Reflux gently for 10 hours a mixture consisting of 100 mg sodium tungstate (Na2WO4.2H2O), 25 g sodium molybdate (Na2MoO4.2H2O), 700 ml water, 50 ml of 85% phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5 L flask. Add 150 g lithium sulphate, 50 ml of water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 L and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1 N NaOH to a phenolphthalein end-point).

Protein Solution (Stock Standard) Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make upto 50 ml in a standard flask.

Working Standard Dilute 10 ml of the stock solution to 50 ml with distilled water in standard flask. One ml of this solution contains 200 μg protein. Express the amount of protein mg/g or 100 g sample.

Determination of proline content

Proline was estimated according to the procedure of Bates et al., (1973). One gram of freshly harvested leaves were taken and washed thoroughly with tap and distilled water. Leaves were blotted dry and cut into small bits.
ground in 10 ml of 3% aqueous sulfosalicylic acid and filtered through Whatman No.1 filter paper. For two ml of filtrate 2 ml of acid ninhydrin reagent (1.25g ninhydrin, 20 ml of glacial acetic acid, 20 ml of 6M phosphoric acid) and 2 ml of glacial acetic acid were added and samples were kept in boiling water bath for one hr. After one hour heated the samples were kept in ice bath to terminate the reaction. Four ml of toluene was added to each tube and mixed vigorously. The chromphore was aspirated for the aqueous phase and warmed the samples to room temperature. The samples were read to 520 nm in systronic UV-VIS, 118 spectrophotometer. Proline concentration in the samples was computed based on a similarly prepared standard curve for proline.

Reagents
Acid ninhydrin Warm 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation until dissolved store at 4°C and use within 24 hrs. 3% aqueous sulphosalicylic acid, Glacial acetic acid, Toluene, Proline.

Nitrate reductase (EC: 1.6.6.1)

The vacuum infiltration method Lin and Kao (1980) was followed for estimation of nitrate reductase activity. Enzyme preparation was carried out by taking three hundred mg of leaf and root material which was cut into pieces of 0.5 cm length. They were taken into a 50 ml of Erlenmeyer flask containing 15 ml of infiltration medium consisting of 15mM KNO₃ in 50 ml of 25mM potassium phosphate buffer pH 7.5. The contents were infiltrated under low pressure for 3 min using a vacuum pump. The process was repeated so that all the leaf material was visibly wetted and got submerged into the infiltration medium. The flasks were then wrapped with aluminium foil and were incubated in dark at 35°C with occasional gentle shaking.

After 3 hours incubation, an aliquot of 1.5 ml (infiltration medium) was taken in a test tube and to which 0.75 ml of 0.02% (W/V) sulphanylamide and 0.75 ml of 0.025% (W/V) N-(1-napthyl) ethylene diamine hydrochloride were added and was then kept in dark for 30 min. It was then centrifuged to remove turbidity due to oozing out of cell sap and thus obtained clear solution was transferred to a cuvette and scanned for absorbance at 540 nm in systronic UV-VIS, 118 spectrophotometer. A
blank reaction was conducted using all components and distilled water instead of infiltration medium.

**Nitrite reductase (EC 1.6.6.4)**

The activity was measured by the method of Losada and Panique (1971). Sliced leaf and root material (300 mg) was taken in a 50 ml flask containing 10 ml of infiltration medium, 50 mM Tris-HCl buffer, pH 8.0, 0.5 mM methyl viologen, 2 mM KNO$_2$ and the flask was evacuated at 6 mm Hg for 30 sec. The reaction was initiated by adding 0.3 ml of 2.5% (W/V) sodium dithionate and the flask was kept in dark for 30 min. The reaction was then terminated by vigorous shaking to oxidize the dithionate. An aliquot (1.5 ml) was then added to 0.75 ml of 0.2% sulphanilamide and 0.75 ml of 0.02% N-(1-naphthyl) ethylene diamine hydrochloride was incubated in dark for 30 min and the unreduced NO$_2$ was measured at 540 nm against blank using systronic double beam spectrophotometer (UV-VIS, 118).

**2.4. Effluent analysis**

Hydrochloric acid, HCl, redistilled.
Methyl isobutyl ketone (MIBK), redistilled
Ammonium pyrrolidine dithiocarbamate (APDC) solution, 1% (w/v) in distilled, deionized water, Prepare the ADPC solution fresh daily and purify as follows: shake the ADPC solution with an equal volume of MIBK, allow the phases to separate and retain the aqueous (lower) phase (Brooks et al., 1967).

**Sample preparation**

Filter the effluent sample through a 0.45 µ Millipore filter and acidify with HCl to a -ml pH of-. Place a 750-ml aliquot of the filtered, acidified effluent into a 1-liter polypropylene flask. Add 35 ml of MIBK followed by 7 ml of 1% ADPC solution. Equilibrate for 30 min on a mechanical shaker. Separate the organic layer in a separator funnel and store in polypropylene bottle. The extracts should be analyzed within 3 hr, save the aqueous layer for the preparation of standard solutions.

**2.5. Soil analysis**

The collected of soil samples at different concentrations (25, 50, 75, 100% and control) and different time intervals (10, 15, 20, 25 and 30 DAS). Soil samples were
dried at 70°C in the laboratory oven. To 1 gm sample, 8 ml conc. HCl and 2 ml conc. HNO₃ were added and kept for over night at 35°C. Digestion was done according to the method of McGrath and Cunliffe (1985). After dilution and filtration the digested solution was analyzed for determination of Cr, Cu, Mn, Fe, Co, Ni, Cd, Pb and Zn by AAS (Perkin-Elmer, 2380).

2.6. Plant analysis

Plants (Arachis hypogaea L. and Phaseolus aureus Roxb.) collected at different concentrations (25, 50, 75, 100% and control) and different time intervals (10, 15, 20, 25 and 30 DAS). The plants were carefully washed with deionized water and oven-dried at 70°C in the laboratory. Powdered plant samples (whole plant, 1 gm) were digested with 5ml diacid mixture, nitric acid (HNO₃) perchloric acid (HClO₄) in the ratio of 3:2 at 110°C for 8 hours. Then distilled water was added to the digested samples to make up the volume to 50 ml and then filtered by Whatman-42 filter paper (Institute Agronomico de S. Paulo, 1978). The samples were ready for elemental analysis by AAS (Perkin-Elmer, 2380).

2.7. Data analysis

Data were expressed as Mean ±Standard error of mean (S.E.M.S). Results were statistically analyzed by student's test (Pillai and Sinha 1968).