Material and Methods

3.1. Study Area

Lucknow, the capital of Uttar Pradesh ($26^{o}5$/N latitude, $80^{o}56$/E longitude, 128 m above the sea level), is spread over an area of 310 km^2 in the central plain of the Indian subcontinent, supporting a population of 36.50 lakh. In India, surface water and groundwater are the main sources of drinking water. Gomti river, a tributary of Ganga River, originates from Fulhar lake near Pilibhit flowing through the Lucknow city meandering for about 12 Km, is a major freshwater ecosystem in India.

The prime source of drinking water in Lucknow city is Gomti river. The water quality of the Gomti river is a major concern for the population of Lucknow. In recent years, Lucknow city has experienced an impressive population growth with expansion in all directions. Drains from all over the city carry and discharge huge loads of industrial effluents, domestic waste, agricultural runoff, sewage, medicinal wastes, washing of clothes and throwing of carcasses of half brunt dead bodies, etc. into river Gomti, resulting a variable increase in the forms and levels of inorganic pollutants such as nitrate, nitrite, phosphate, ammonium, Cd, Pb, Cr, Fe, Cu etc. in the river water and sediments. About 26 drains situated between Gaughat (upstream of Lucknow) and Pipraghat (downstream of Lucknow) discharge about 200-250 MLD of untreated sewage of urban effluent, including municipal and industrial wastewater into Gomti River (Shivani *et al.*, 2011).

Sewage treatment plants (STP), Mohan Meakins (Brewery), Hindustan Aeronautics, Cooperative Milk Dairy, Everyday Flash Light and Telco etc., are certain industrial establishments operating in Lucknow, effluents from these are carried by the drains and these drains dump huge quantities of untreated sewage/effluent into river water body. Sampling station at Gaughat of river Gomti which is the source of raw municipal water supply for Lucknow city along with seven residential sampling sites which are the

locations of municipal water supply for various residential areas of Lucknow were chosen for the monitoring of drinking water. Residential areas included Aminabad, Indira Nagar, Aishbagh, Nishatganj, Telibagh, Hazratganj and Charbagh. Further Gomti river at Lucknow city was divided into upstream and downstream sites. Six sites along with the bank of river Gomti were selected for water and plant samples collection from Gaughat (upstream) upto Pipraghat (downstream). Gaughat and Pucca Pull Sites are categorized as upstream sites, while as Hanuman Setu, Nishatganj, Gomti Barrage and Pipraghat sites are categorized as downstream sites.

Experiments under simulated conditions were conducted in NET House of Department.

Plate 3.1. Plant based treatment system designed in NET House of Department

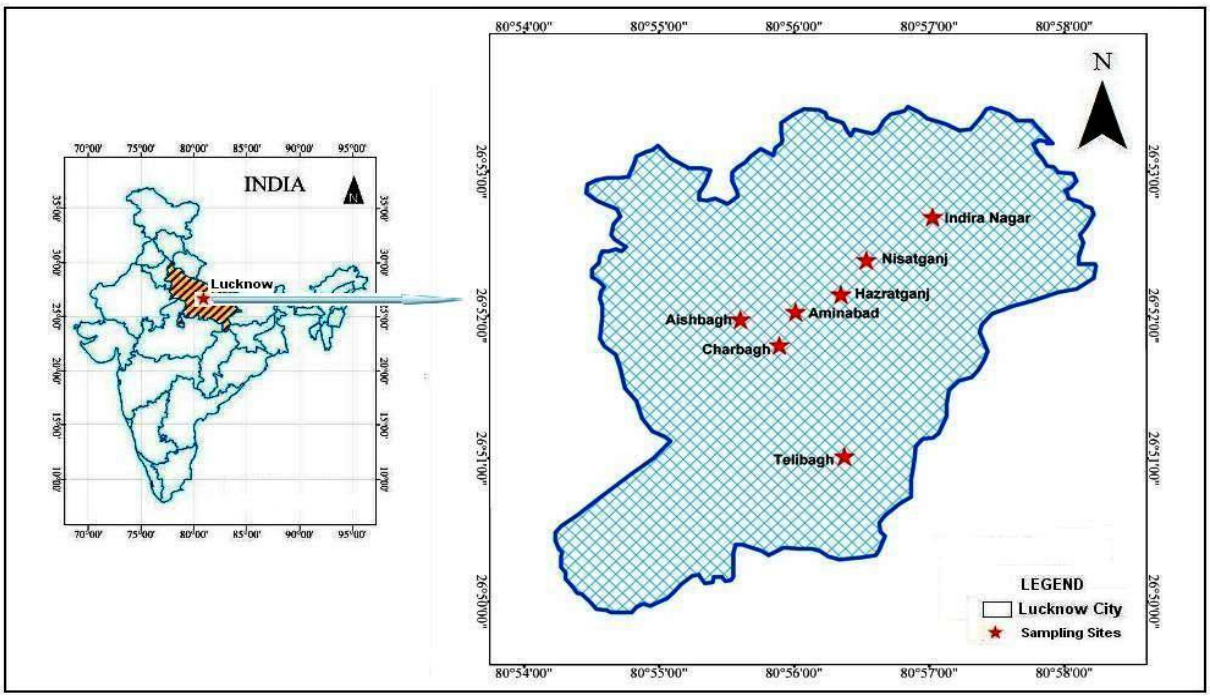

Plate3.2. Map showing seven residential sites of Lucknow city as drinking water sampling sites.

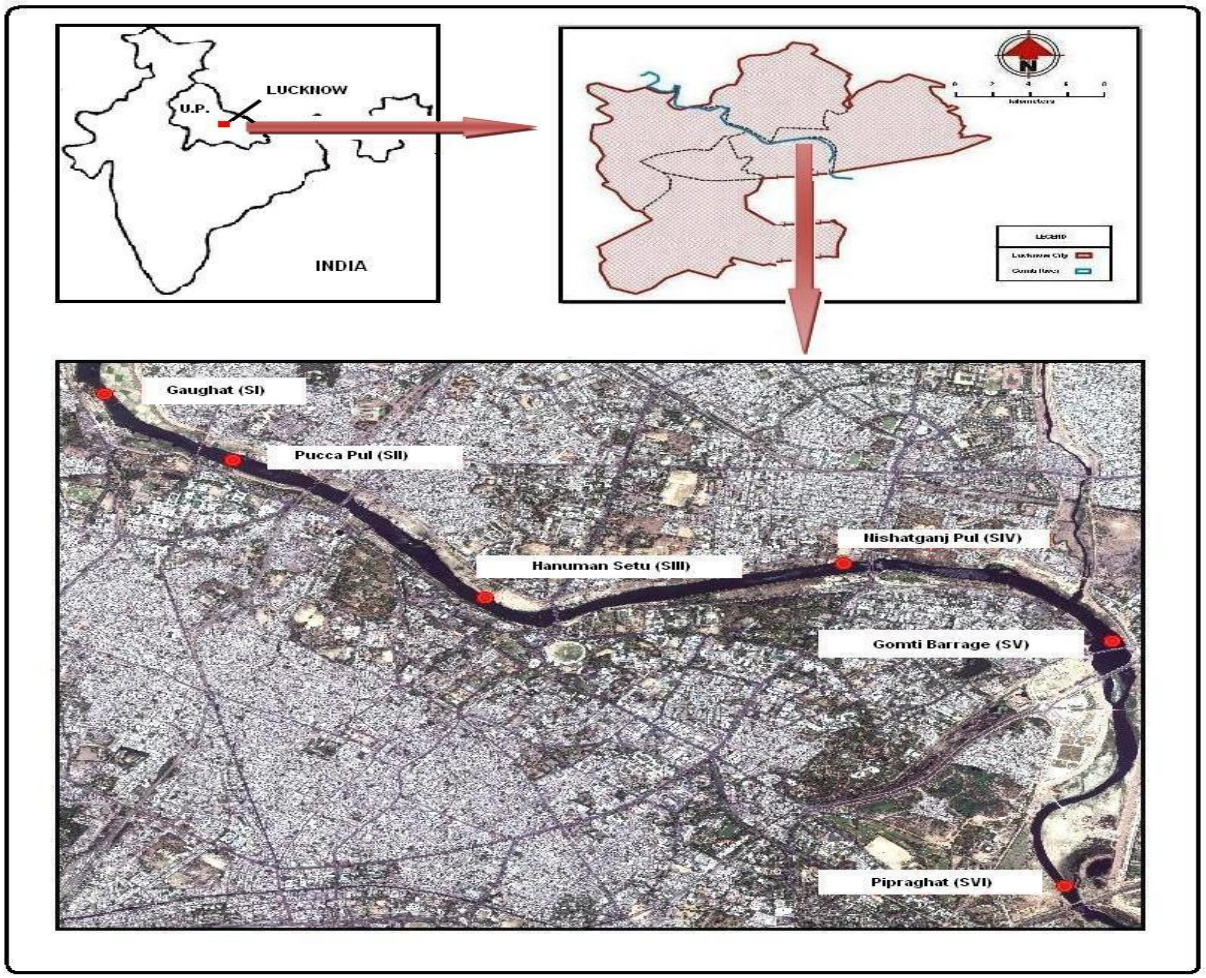

Plate3.3. Map showing river Gomti at Lucknow and location of different sampling sites (I to VI).

3.2 Details of standard protocols used in the experimental analysis

3.2.1. pH: 20 ml of water sample was taken in a beaker. The pH was recorded by dipping the electrode in the sample using potable digital pH meter.

3.2.2. Electrical Conductivity: 20 ml of water sample was taken in a beaker. The EC was recorded by dipping the electrode in the sample using potable digital EC meter.

3.2.3. Nitrate: For nitrate estimation **Catalado method (Catalado et al., 1975)** is used.

Reagents prepared

a) 5% Salicylic Acid- Dissolve 5 gm salicylic acid in 100 ml conc. H_2SO_4.

b) 2 N NaOH- Dissolve 20 gm NaOH in 250 ml distilled water.

Procedure:

- Take 0.5 ml of water sample in a test tube.
- Add 0.5 ml of 5% salicylic acid to it.
- Finally add 9.0 ml of 2N NaOH.
- Orange- yellowish color will appear after 20 min.
- Take O.D. at 410 nm
- **Calculation-**
- Nitrate = K- factor × concentration

K- Factor = Absorbance/ Concentration

Fig. 3.1 Nitrate

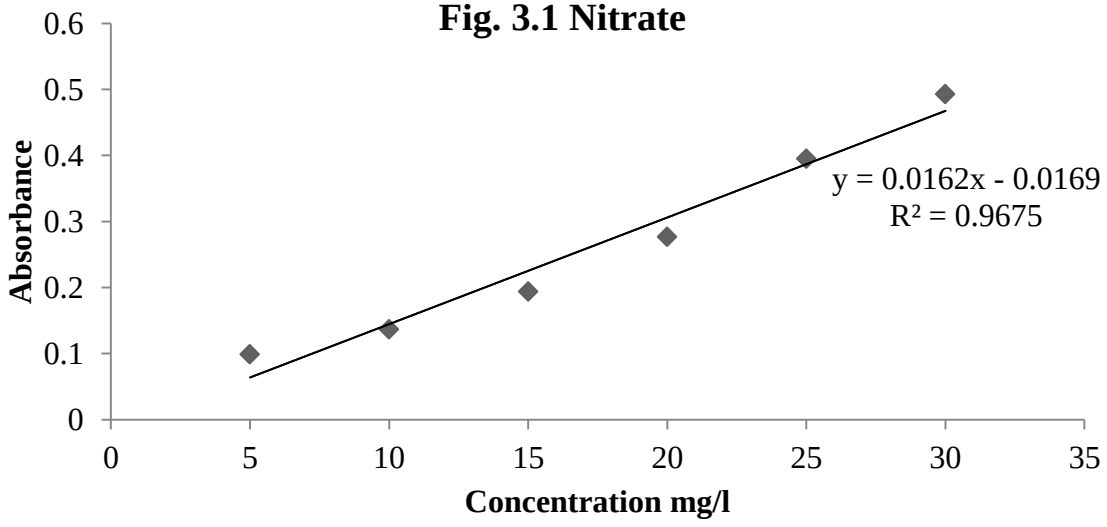

Fig. 3.1 Standard Curve of Nitrate

3.2.4. Nitrite- (Stevens and Oaks, 1973)

Reagents required:

a) NaNO2 stock solution- Dissolve 0.001 gm of NaNO2 in 100ml distilled water.

b) 1% Sulphanilamide (LR) solution: Dissolve 1 gm sulphanilamide in 100 gm of 1 N HCl.

c) 0.01%NED (N-1-napthyl ethyldiamine dihydrochloride): Dissolve 0.01 gm of NED in 100ml distilled water.

Procedure

- Take 1 ml of sample in a test tube.
- Add 1 ml of 1% sulphanilamide to it.
- Finally add 1 ml of 0.01% NED.
- After 10 minutes pink color will appear.
- Take O.D. at 540 nm.

Calculation

Nitrite (µg ml^{-1}) = K- factor × Absorbance

K-Factor = Absorbance/ Concentration

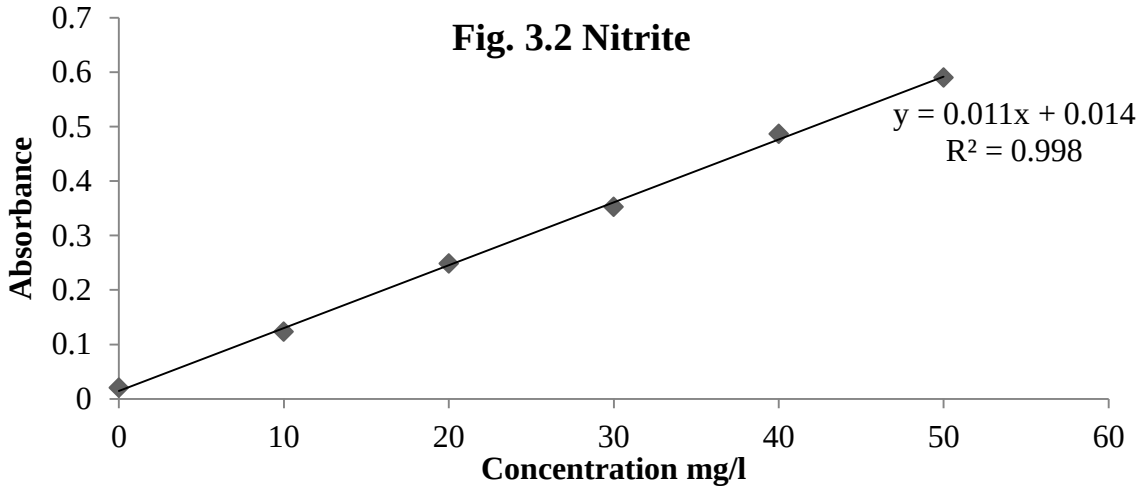

Fig. 3.2. Standard Curve of Nitrite

3.2.5. Ammonium: Ammonium was estimated using **Weather burn method, 1967 by using Nessler's Reagent.**

Reagents prepared

Nessler's Reagent: Dissolve 50 gm KI in 50 ml cold water. Add a saturated solution of mercuric chloride (22gms in 350 ml). Then add 200 ml of 5N NaOH and dilute to 1 L. Keep overnight and then filter.

Procedure-

- Take 2.5 ml of sample.
- Add 1.5 ml of Nessler's reagent to it
- Red to brown color appears, take O.D. at 420 nm.

Ammonium Standard Curve was also prepared separately.

Calculation

Ammonium (µg ml^{-1}) = K- factor × Absorbance

K-Factor = Absorbance/ Concentration

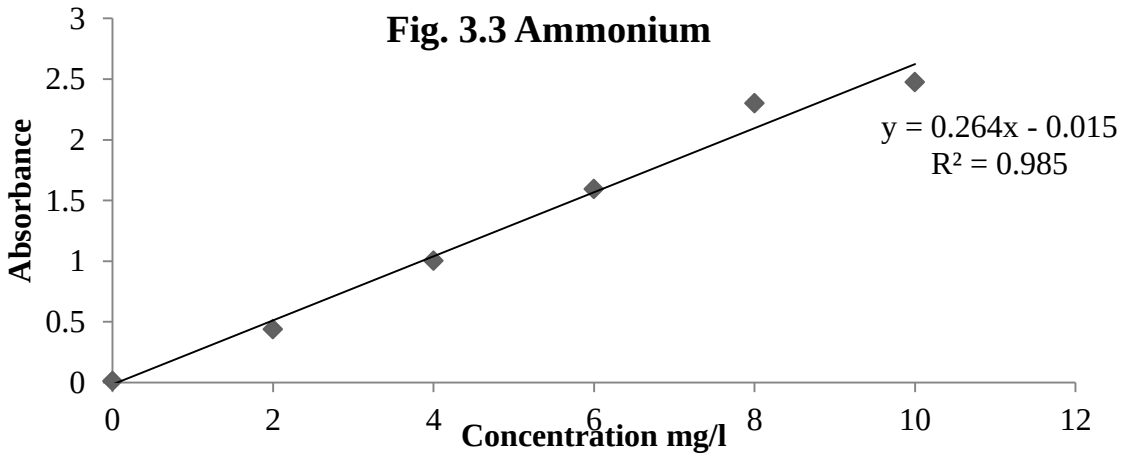

Fig. 3.3 Standard Curve of Ammonium

3.2.6. Phosphate

Phosphate content was estimated using **Ammonium molybdate and stannous chloride method**.

Reagents prepared-

a) Ammonium molybdate- Dissolve 1.25gm of ammonium molybdate in 50 ml conc. H_2SO_4.

b) Stannous chloride- Disslove 0.6 gm stannous chloride in 25 ml glycerol

Procedure-

- Take 10 ml of water sample in a test tube.
- Add 4 ml ammonium molybdate to it.
- Finally add 2-3 drops of stannous chloride to it.
- Take O.D. at 680 nm.

Calculation

Phosphate (µg ml^{-1}) = K- factor × Absorbance

K-Factor = Absorbance/ Concentration

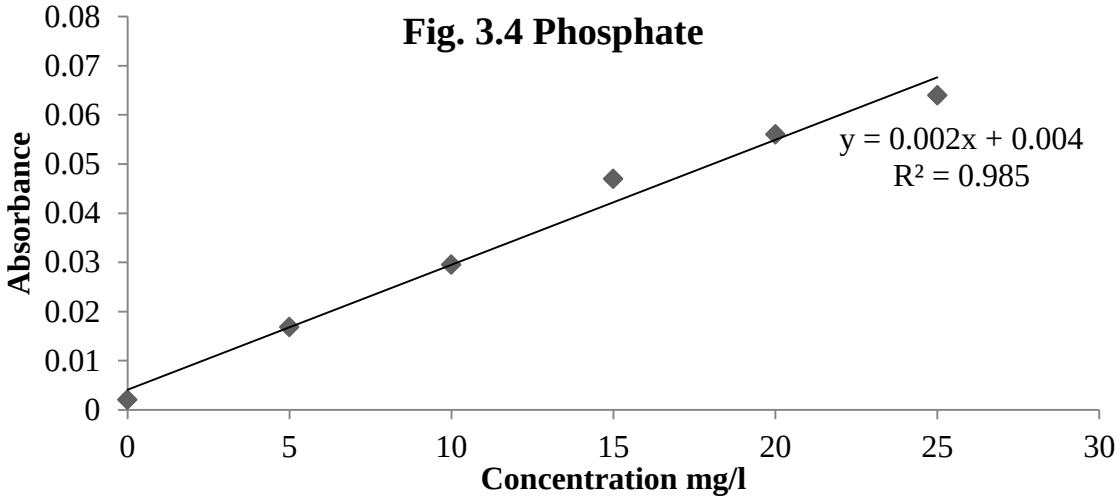

Fig. 3.4 Standard Curve of Phosphate

3.2.7. DO (Dissolved oxygen mg/l)

Reagents prepared

a) Manganous sulphate solution- Dissolve 36.4 gm of manganous sulphate in 100 ml of distilled water.

b) Alkaline Iodide Azide solution- 50 gm of NaOH and 15 gm of KI was dissolved and diluted to 95 ml, 1 gm of NaN3 was dissolved in 4 ml of distilled water. The two solutions were cooled and mixed and the solution was made up to 100ml.

c) Starch indicator- 1 gm of arrow root starch was added with continuous stirring in 200 ml distilled water.

d) Standard sodium thiosulphate solution (0.025 N)- 1.205 gm of sodium thiosulphate was dissolved in 200 ml distilled water which gave 0.025 N. Separately 0.1 gm NaOH was dissolved in 250 ml distilled water. 0.4 ml of this NaOH solution was taken and added in sodium thiosulphate solution.

Procedure-

The sample was collected in BOD bottle. The sample was filled to the neck of the bottle assuring that air bubbles have not been trapped under the stopper and maintained a water seal around the stopper, until ready for the next step and analysis.

- 1ml of manganous sulphate solution was added in the sample followed by 1 ml of alkali iodide azide solution.
- The stopper was placed carefully to avoid any air bubble and mixed by inverting the bottle repeatedly for 15 minutes.

- The precipitate was allowed to settle leaving the clear supernatant.
- The stopper was removed carefully and immediately 1ml of conc. H_2SO_4 was added and bottle was closed and mixed with gentle inversion the precipitate completely dissolves.
- Titrate 100ml content of the bottle with standard sodium thiosulphate solution using starch as indicator.

Calculation-

$$DO\ (mg/L) = \frac{\text{ml of titrant} \times \text{normality} \times 8 \times 1000}{V2\ (V1\text{-}V)/\ V1}$$

V= Volume of Manganous sulphate and iodide azide solution added.

V1= Volume of BOD bottle (ml)

V2= Volume of the content titrated in ml

3.2.8. BOD (Biochemical Oxygen Demand)

Reagents prepared

a) Manganous sulphate solution- Dissolve 36.4 gm of manganous sulphate in 100 ml of distilled water.

b) Alkaline Iodide Azide solution- 50 gm of NaOH and 15 gm of KI was dissolved and diluted to 95 ml, 1 gm of NaN_3 was dissolved in 4 ml of distilled water. The two solutions were cooled and mixed and the solution was made up to 100ml.

c) Starch indicator- 1 gm of arrow root starch was added with continuous stirring in 200 ml distilled water.

d) Standard sodium thiosulphate solution (0.025 N)- 1.205 gm of sodium thiosulphate was dissolved in 200 ml distilled water which gave 0.025 N. Separately 0.1 gm NaOH was dissolved in 250 ml distilled water. 0.4 ml of this NaOH solution was taken and added in sodium thiosulphate solution.

Procedure: The sample was collected in BOD bottle. The sample was filled to the neck of the bottle assuring that air bubbles have not been trapped under the stopper and maintained a water seal around the stopper, until ready for the next step and analysis.

- 1ml of manganous sulphate solution was added in the sample followed by 1 ml of alkali iodide azide solution
- The stopper was placed carefully to avoid any air bubble and mixed by inverting the bottle repeatedly for 15 minutes.

- The precipitate was allowed to settle leaving the clear supernatant.
- The stopper was removed carefully and immediately 1ml of conc. H_2SO_4 was added and bottle was closed and mixed with gentle inversion the precipitate completely dissolves.
- Titrate 100ml content of the bottle with standard sodium thiosulphate solution using starch as indicator.

Calculation-

$$BOD\ (mg/L) = \frac{\text{ml of titrant} \times \text{normality} \times 8 \times 1000}{V2\ (V1\text{-}V)/\ V1}$$

V= Volume of Manganous sulphate and iodide azide solution added.

V1= Volume of BOD bottle (ml)

V2= Volume of the content titrated in ml

BOD = initial DO – final DO

3.2.9. Chemical Oxygen Demand (COD)

Reagents prepared-

1) Standard Potassium dichromate solution- Dissolve 12.259gm of potassium dichromate in 1000 ml distilled water.

2) Standard FAS- Dissolve 98gm of ferrous ammonium sulphate in distilled water. Add 30 ml conc. H_2SO_4 to it and maintain it to 1000ml by adding distilled water.

Procedure-

- Take 30 ml of sample and add 10 ml of standard potassium dichromate to it.
- Then add 30 ml of conc. H_2SO_4 and pinch add Ag_2SO_4 to it.
- Keep it for reflux for 2 hours.
- Finally titrate the content with standard FAS solution

Calculation: $$COD\ (mg/L) = \frac{(A\text{-}B) \times M \times 8000}{\text{ml of sample}}$$

Where, A= ml of FAS used for blank

B= ml of FAS used for sample

M = molarity of FAS

3.2.9. Metal Estimation

Heavy metals viz. Fe, Cu, Cd, Pb and Cr were determined after acid digestion of samples (100ml for water samples and 1gm for soil) with an acid mixture (9 parts nitric acid: 4 parts perchloric acid) at about 100º C. The solution was allowed to evaporate to dryness, and temperature was further raised to 105 º C to reduce the volume to 0.5-1.0 ml. The solution was filtered through Whatman filter paper No. 40 in a volumetric flask. The residue was re-dissolved and diluted to 15 ml with distilled water. Blanks were also run simultaneously and analyzed to correct for possible external contributions of the metals. Analytical data quality of metals was ensured through repeated analysis (n=3) of EPA quality control in samples. Metal concentration was determined by atomic absorption spectrophotometer (AAS 240 FS, Varian).

Metal Concentration ($\mu g\ g^{-1}dw$) = $\frac{XV}{W}$

Where,

X = Reading in ppm on AAS,

V = Final volume of digested samples (ml)

W = Dry weight of sample/ ml of sample taken

3.2.10. Calculation of Bioconcentration factor (BCF), Translocation factor and Metals removal efficiency

Bioconcentration factor (BCF), expressed as the ratio of metal concentration in plant tissue to that of the water was calculated by:

$$\mathrm{BCF} = \frac{\text{Metal content in plant tissue}}{\text{Initial metal content in water column}}$$

Translocation factor (TF), the ratio of metals in shoot versus root of plants was calculated by the formula of Padmavathiamma and Li (2007).

$$\mathrm{TF} = \frac{\text{Metal content in plant shoot}}{\text{Metal content in plant root}}$$

Metal removal efficiency was calculated on percentage basis according to the equation (Chaudhuri et al., 2011)

$$R\ \% = \frac{Co - Ct}{Co} \times 100$$

Whereas, Co is initial metal concentration in water and Ct is final metal concentration in water at the end of experiment.

3.2.11. Protein estimation: The protein content was then determined by the method described by Lowry et al., (1951). For protein estimation, 500 mg leaves of the samples were homogenized in 3 ml of 10% tricholoroacetic acid (TCA) and centrifuged at 10000rpm for 10 mins. After decanting the supernatants, the pellets were washed and heated for 7 min with 3 ml of 1N NaOH (sodium hydroxide), cooled and centrifuged again at 10,000 rpm for 10 min. The 0.5 ml of extracted sample was taken in 2.5 ml of 0.5% $CuSO_4$ (copper sulphate in 1% potassium sodium tartarate), 48 ml of 5% Na_2CO_3 (sodium carbonate) was added. 0.5 ml (1N) of folin-phenol reagent was added after 10 min. 30-min incubation developed a blue color complex in the mixture. Absorbance was taken at 700nm against a blank without sample. Protein was calculated by a standard curve using bovine serum albumin as the standard.

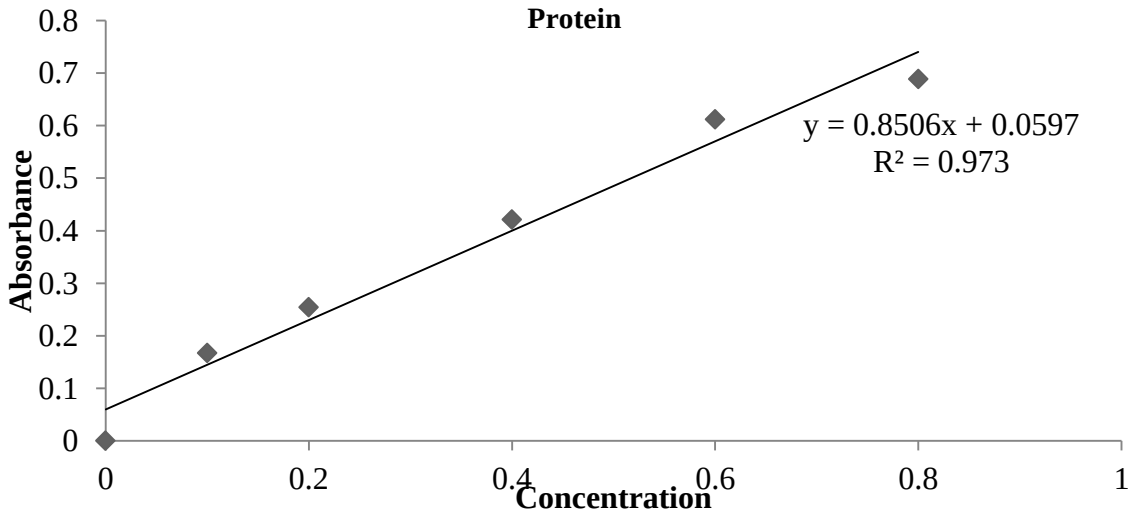

Fig. 3.5. Standard curve for Protein

3.2.12. Proline Estimation: For proline estimation, 500 mg of leaf samples were extracted with 10 ml of 3% sulphosalicylic acid and centrifuged at 5000 rpm to remove cell debris. Supernatant was used to assay the proline content following Bates et al., (1973).

3.2.13. Chlorophyll and carotenoid estimation

Chlorophyll content was estimated as per the protocol of Machalochan & Zalik (1963).

1.0g fresh plant sample was homogenized in 5ml of 80% acetone and centrifuged at 5000rpm for 15 minutes at 4ºC. Optical density of the supernatant was taken on spectrophotometer at 480, 510, 645 and 663nm.

Calculation

Total Chlorophyll (mg/g):20.2(OD645) +4.68(OD663) ×V/1000×W

Total carotenoids (mg/g): [4.695×OD480-0.268] ×chla + chl b

Where

V=volume of 80% acetone; W=weight of sample and OD=optical density.

3.2.14. Scanning Electron Microscopic (SEM) and Fourier Transform Infrared Spectroscopic (FTIR) analysis

Thin cross sections plant samples of 2mm are excised for the sample preparation. The samples were fixed in 2.5% Glutaraldehyde/ Karnovsky's fluid (David et al., 1973), buffered with 0.1M sodium phosphate buffer at pH 7.4. Fixation of the samples was done for 6-8 hours at 4^0C. After this, samples were washed thrice with 0.1M fresh phosphate buffer for 15min each at 4^0C. Post fixation for 2hours in 1% Osmium tetraoxide was performed for the samples. After this, samples were dehydrated using increasing concentration of Acetone 30%, 50%, 70%, 90% and 100%. The duration of treatment was 30 mins at 4^0C for each concentration. Finally, the species were air dried and mounted on

stubs, then coated with a thin layer of gold for examining in Scanning Electron Microscope (JEOL, JSM 6490 LV-Japan).

For FTIR analysis of plants samples, dry root and shoot samples were grind properly to form a uniform powder. Then FTIR spectra of the samples prepared as KBr discs were taken by Infrared Spectrophotometer (Nikolet^{TM} 6700) in the wave number of 4000-400 cm^{-1} (Teng et al., 2013).

3.2.15. Organic Carbon and Organic Matter: Organic carbon was determined by method described by Walkley and Black (1934).

Reagents

1N Potassium dichromate$K_2Cr_2O_7$: Dissolve 49.04 gm of $K_2Cr_2O_7$ in distill water and make the volume upto 1 lt

0.5 N Ferrous ammonium sulphates (FAS)-Dissolve 196 gm of FAS in distill water and make volume upto 1 lt.

Concentrated Sulphuric acid (H_2SO_4)

Ortho phosphoric acid (85%)

Diphenyl amine indicator-Dissolve 0.5 gm of Diphenyl amine in a mixture of 40 ml distill water and 20 ml conc.H_2SO_4

Procedure- Take 0.1 gm of soil in a dry conical flask of 500 ml.

- Add 10 ml of 1N $K_2Cr_2O_7$ with the help of Pipette and swirled a little.
- Add 20 ml of conc. H_2SO_4.
- Allow the flask to stand for 30 min and then add 200 ml of distilled water.
- Now add 10 ml of O phosphoric acid and 1 ml of diphenyl amine indicator.
- The colour of the solution changes to Blue.
- Now Titrate with 0.5 N FAS till colour changes Blue to Green.
- Simultaneously run a Blank sample with the same procedure as above.

$$\text{Organic Carbon (OC)} = \frac{N(B-C)\times 0.003\times 100}{\text{Wt of soil (gm)}}$$

N= Normality of FAS; B = Volume of FAS for Blank; C= Volume of FAS required for titration of sample

Actual Organic Carbon = Organic Carbon×1.3

%Organic Matter = Actual Organic Carbon×1.724

3.2.16. Total Phosphorus

Reagents

1. Conc. Sulphuric acid (H_2S0_4)
2. Phenolphthalein indicator
3. Conc. Nitric acid (HNO_3)
4. Sodium hydroxide (NaOH), 1 N: Dissolve 40 g NaOH in 1 L of distilled water.

Procedure

- Take a suitable volume 0.2 gm of sample in a Kjeldahl flask.
- Add 1 ml H_2S0_4 and 5 ml HNO_3
- Digest the sample on a hot plate till the volume becomes nearly 5 ml and Continue the heating further until the solution becomes colorless after complete removal of HNO_3
- Cool and transfer completely to a 100 ml volumetric flask.
- Add 1 drop of phenolphthalein indicator.
- Neutralize the acidity by adding 1 N NaOH. At the end the solution turns pink. Make up the final volume to 100 ml. Read absorbance at 730 nm.

3.2.17. Sequential Extraction of metals: Tessier sequential extraction of metals was employed for metal speciation. The detailed protocol is given in Table 3.1.

Table 3.1 Tessier Sequential Extraction of Metals.

Speciation	Extractant	Environmental Conditions
(1) Exchangeable	8ml of 1.0M $MgCl_2$ (pH=7)	With agitation at 220 rpm for 1 h at 25^0C
(2) Carbonate	8 ml 1.0 M NaOAc (pH=5, adjusted with conc.HOAc),)	With continuous agitation for 5 h at 25^0C
(3) Reducible	20 ml 0.04 M $Nh_2OH.HCl$ in 25% HOAc (v/v)	6h at 96 C in water bath with occasional agitation
(4) Organically bound	3ml 0.02 M HNO_3 and 5 ml 30% H_2O_2 (pH=2, adjusted with conc. HNO_3).	Heated at 85^0C for 2 h
	After 2 h, 3 ml 30% H_2O_2 was added.	Heated at 85^0C for 3 h with occasional agitation.
	After cooling, 5 mL of 3.2 M NH_4OAc in 20% (v/v) HNO_3 was added.	Agitated for 0.5 at 25^0C
(5) Residual	10 ml of H_2SO_4 and $HCIO_4$ (5:1) mixture.	Heated at 300^0C