Methodology of present work is shown in the flow chart ( Fig. 10) which includes following Components.

**Biodiversity study**

- Morphological Studies (Cook, 1996)
- Herbarium Preparation (Bridson and Foreman, 1998)

**DNA Barcoding**

- DNA extraction using Macherey NAGEL kit
- Quantification of genomic DNA (Sambrook *et al.*,1982)
- Polymerize Chain Reaction (Levin,2003; Kress and Erikson, 2007)
- Agarose Gel Electrophoresis
- Purification of Amplified PCR Product using PCR Clean up kit
- Cycle sequencing (lvanova *et al.*, 2005)
- Sequence analysis using Basic Local Alignment Tool (BLAST) and submission of DNA Barcode in BOLD SYSTEM according to guideline provided on BOLD website (http://www.boldsystems.org)

**Selection of Plants for phytoremediation study**

- Culturing of selected plants (Hoagland, 1938)
- Determination of LC 50 Value (Patil, 2011) by exposing plants to various concentration of Zn, Cd and Ni.
- *In vitro* Metal ion Treatment and Accumulation of these metals in selected plant species (Soudek *et al.*, 2004)

**Effects of Heavy metals on Biochemical parameters of selected plant**

- Chlorophyll: (Arnon, 1949)
- Protein: (Thimmaiah, 1999)
- Proline: (Thimmaiah, 1999)
- Carbohydrate : Anthrone method (Sadasivam and Manikam, 1991)
- Catalase: (Thimmaiah, 1999)
- Peroxidase: (Sadasivam and Manikam, 1991)

**Anatomical observation on metal treated test plants**

Light Microscopy ( Johansen, 1940). The SEM study was accompanied by energy dispersive X-ray microanalyses (EDX) using an EDAX 711-type instrument which enables point analyses to be made of volumes as small as about 1 / $\mu m^3$.

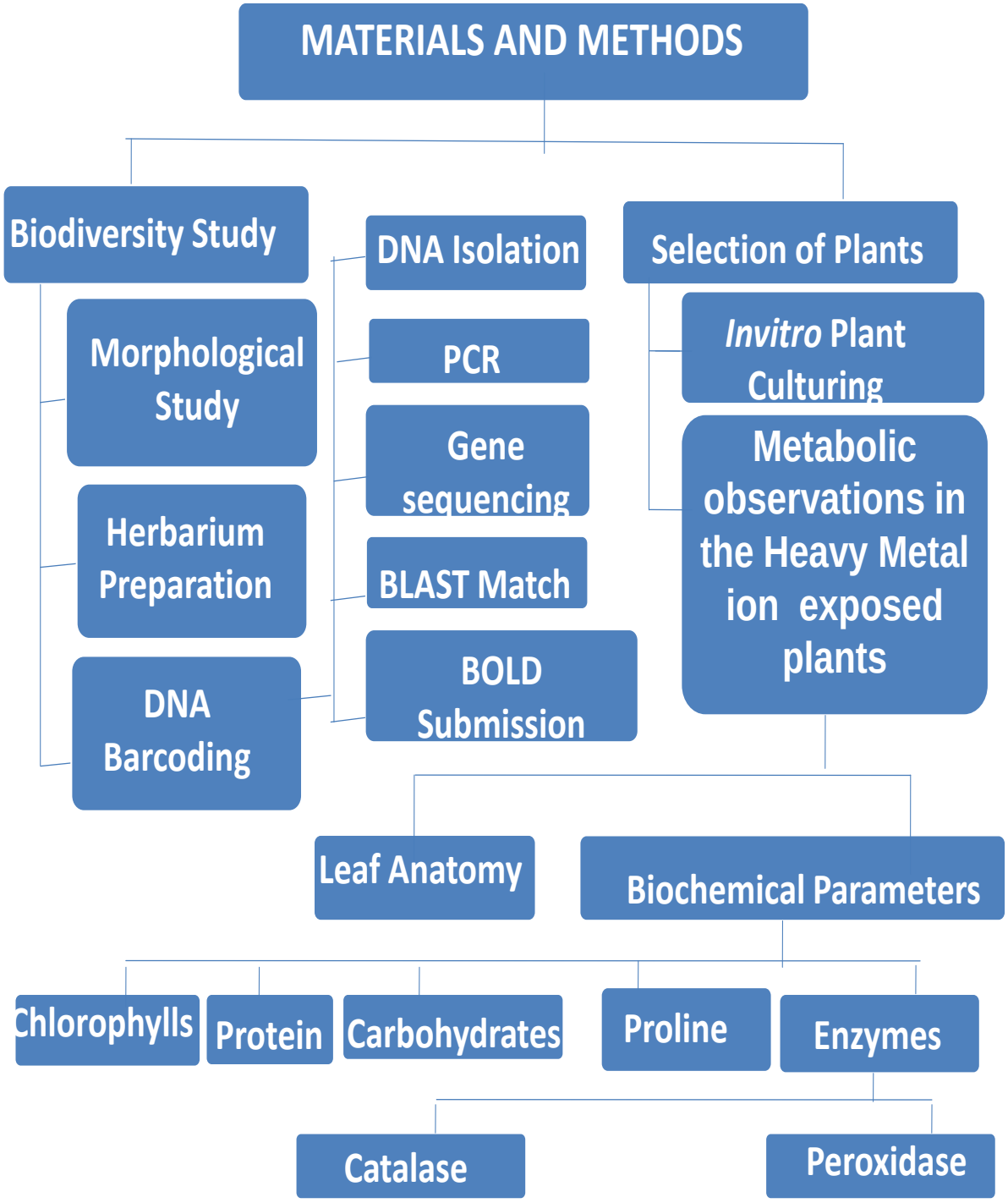


**Figure 10 : Flow Chart of Materials and Methods**

# 3.1 STUDY OF BIODIVERSITY

## 3.1.1 Morphological Study

### 3.1.1.1 Field collection

Plants were collected in plastic bag, pressed them and preserved as herbarium later.

**Free Floating Hydrophytes:** Some free floating plants like duckweeds do not make very satisfactory pressed and dried specimens, nor is it convenient to collect them into bag. Small 50 mL, cap bottle make good collection and preservation containers for this plant. Put a little water in to bottle to keep the plants moist. Later fill it with a solution of 10% formalin as permanent preservative.

**Submerged Hydrophytes:** Most submerged plants do not have any supporting tissue, they rely on water for support and they must be floated on to the card stock. Therefore, a paper which remains dimensionally stable under wetting so, subsequent drying is required. Most ordinary papers will wrinkle upon drying after being soaked, even if they are kept pressed during the drying process.

**Emergent Hydrophytes:** Emergent hydrophytes preserved as per procedure followed for submerged hydrophytes.

### 3.1.1.2 Herbarium preparation

Herbarium was prepared by following these steps.

**Collection**

Plants were collected including all available parts of the plant (i.e. all reproductive structures such as fruits, flowers and buds etc) as well as detailed notes about the plant and its surroundings.

**Pressing and Drying**

- Plants were preserved by pressing until dry. To construct a press open a sheet of newspaper, place plant on one side and fold over the top of the specimen. Newspaper or large sheets of blotting paper are used as they absorb moisture from the specimens. On top and below this plant/newspaper sandwich place a sheet of cardboard. Corrugated cardboard is better than solid cardboard as it allows the air to circulate within the press, helping the specimens to dry quickly.
- Cardboard is also important within the press as it provides flat surfaces to dry the specimens against. To complete the plant press, stack several plant/newspaper/cardboard sandwiches together and place a wooden boards or lattices on the top and bottom (pictured). Lattice is also preferred as it helps the specimens to dry faster.
- Aquatic plants usually need to be immersed in a tray of water to allow full expansion of branches and to show the habit of the plant. Within the tray of water, mounting card is slipped under the floating specimen and both are slowly dragged out of the water together using a paint brush to carefully arrange the specimen on the card.

**Poisoning**

Dried plants are treated with $HgCl_2$ prepared in 80% alcohol for the protection against the fungi attack.

**Mounting**

To mount a specimen means to adhere it onto a sheet of herbarium card. A well mounted specimen should display both artistic and botanical qualities.

It should be arranged on the card in a balanced, aesthetically pleasing way, paying attention to:

- Orientation and type of mounting card.
- Arranging and attaching the specimen.
- Position of label and accompanying annotations.
- Keeping loose parts in specimen bag.
- Official herbarium specimens are mounted on 29 x 43 cm archival quality white mounting card (250 GSM). The direction of the card is "portrait" (vertical).

**Labeling**

At a minimum your label should include:

- Scientific name of the specimen, including the authority.
- Collector's name/s.
- Date of collection.
- Locality where the plant was collected, including latitude and longitude.
- Habitat or type of plant community
- Habit

## 3.2 DNA Barcoding

### 3.2.1 Sampling strategies for DNA Isolation

Sampling strategies includes following methods

**Processing of fresh material**

Plant material was washed before proceeding for DNA isolation using 70% ethanol and again rinsed with sterile distilled water. Approximately 50 mg of fresh leaf tissue was chopped into small (1mm) pieces.

**Fresh tissue storage in liquid Nitrogen**

Approximately 50 mg of fresh leaf tissue was chopped into small (1mm) pieces. Chopped Plant tissue was transferred into each well in 8-well tube strips containing two 3mm diameter tungsten beads. Plant tissue samples were freezed in liquid nitrogen.

**Dry tissue storage under controlled condition**

The leaf samples were then placed into a Labconco freeze dryer and lyophilized for two days. The dried leaf samples were used directly for DNA extraction.

**Silica gel drying**

Plant material was dried with silica gel at time of plant collection. The dried leaf samples were used directly for DNA extraction.

### 3.2.2 DNA Extraction:

Genomic DNA was extracted using fresh leaves and seeds of plants in an initial weight of approximately 60 mg for leaves and 30 mg for seeds. Extraction was carried out using Macherey NAGEL kit. Protocol for the DNA isolation is as follows.

- Plant material was washed before proceeding for DNA isolation using 70% ethanol and again rinsed with sterile distilled water.
- Approximately 50 mg of fresh leaf tissue and 20 mg of dried plant tissue was chopped into small (1mm) pieces. Plant tissue was transferred into each well in 8-well tube strips containing two 3mm diameter tungsten beads. Plant tissue samples were freezed in liquid nitrogen. The cells were disrupted by crushing for 20 seconds at the frequency of 30 Hz/S in TissuLyser-II (Qiagen).
- In mechanically crushed plant tissue 500 µl PL 1 and 10 µl RNase A was added and mixed thoroughly by vortexing. Sample was incubated at 65º C in waterbath for 30 min.
- The lysate was cleared by centrifugation at 20 min at full speed (5600-6,000 X g)
- To this 400 µl clear lysate, 450 µl binding buffer PC was added and mixed by repeated pipetting.
- A mixture was transferred to the nucleospin plant II binding plate. Vaccum of 0.2-0.4 bar was applied to allow samples to pass through the membrane.
- The binding plate was washed with 400 µl buffer PW1. Vaccum of 0.2-0.4 bar was applied to allow buffer to pass through the membrane.
- The binding plate was washed with 700 µl buffer PW2 and Vaccum of 0.2-0.4 bar was applied to allow buffer to pass through the membrane.
- The binding plate was then again washed with 700 µl buffer PW2 and Vaccum of 0.2-0.4 bar was applied to allow buffer to pass through the membrane.
- The MN wash plate and the waste tray was removed and the rack of tube strips was placed into the manifold base. The binding plate was rearranged in to the manifold top and 100 µl of pre-heated buffer PE (70ºC) was added directly into each well of Nucleospin plant II binding plate. Vaccum of 0.4 bar was applied until the elution buffer was passed through the membrane completely.
- DNA was stored at -20º C.

**Reagents supplied with kit**

- Lysis buffer – Buffer PL1 and Buffer PL2
- Wash Buffer – Buffer PW1 and Buffer PW2
- Elution Buffer- Buffer PE
- RNase A

**Quantification of genomic DNA:**

After extraction of genomic DNA, quantification was done according to Sambrook et al., 1982. 10 µL of extracted DNA was dissolved in 30 µl of Tris buffer (pH 8) and O.D. was taken at 260 and 280 nm (PowerWave HT Microplate Spectrophotometer, BioTek). Quantity of DNA was calculated by using following formula:

- Amount of DNA (ng/µL) = O.D. at 260 X dilution factor X 50 (extension coefficient)

Quality was assessed by taking the (O.D. at 260nm)/ (O.D. at 280nm). Samples which showed the O.D. between 1.6-1.8 were taken for further work.

### 3.2.3 Polymerase Chain Reaction (PCR)

PCR was carried out using 1X final concentration of ReadyMix™ Taq PCR Reaction Mix (Sigma) and, template DNA (50 ng/µl). The reaction was carried out in Thermal cycler (Applied Biosystems Veriti®)

Final concentration of PCR reagents in reaction mixture (20 µl)

1x ReadyMix™ Taq PCR Reaction Mix (10 µl)

10 pmole of each Primer

50-100 ng Template DNA

**ribulose-bisphosphate carboxylase gene 1 (rbcL-A) amplification using primer set rbcLaF and rbcLaR**

rbcL gene in plants was amplified in a volume of 20 µl containing 10 µl Taq PCR reaction mix, 10pmol forward primer (rbcLa F), 10 pmol reverse primer (rbcLa R), 50 ng template DNA and sterile ion-free water(to make up the final desired volume). Amplification was carried out in Thermal cycler (Applied Biosystems Veriti®).

Reactions were amplified through 35 cycles with the following conditions (Levin, 2003 and Kress & Erickson, 2007):

Denturation: 30 seconds at 95°C

Annealing: 40 seconds 55°C

Extension: Two minutes at 72°C

This was followed by a final extension step at 72°C for seven minutes. Initial denturation was carried out at 95°C for five minutes.

| Sr. No. | Primer | Sequence |
|---|---|---|
| 1 | rbcLaF | ATGTCACCACAAACAGAGACTAAAGC |
| 2 | rbcLaR | GTAAAATCAAGTCCACCRCG |

**Table 2: Primer list with sequence**

### 3.2.4 Agarose gel electrophoresis

**Solutions for agarose gel electrophoresis**

Running buffer: 10X Tris borate EDTA (TBE) buffer

| | |
|---|---|
| Tris base | 108 g |
| Boric acid | 55 g |
| 0.5 M EDTA (pH 8) | 40 ml |

**Table 3: Chemicals used in Agarose gel electrophoresis**

Above mentioned chemicals were mixed in a volumetric flask and solution was made up to 1 liter by adding distilled water. Buffer was subjected to autoclave treatment as mentioned above. 10X TBE were diluted to 1X prior to use.

**Tris HCl EDTA buffer:**

200 ml buffer solution of pH 8.0 was prepared by dissolving chemicals as Table 7;

| Chemical | Required Strength in buffer | Amount added in 200 ml |
|---|---|---|
| Tris-HCl | 10 mM | 0.24g |
| EDTA | 1 mM | 0.06g |

**Table 4: Tris EDTA Buffer list**

**Ethidium bromide:**

Stock solution of having concentration of 10 mg/ml was prepared using double distilled water and stored in an amber colored bottle.

**DNA loading dye:**

10 ml loading dye was prepared by mixing sucrose 5.0 g (50 %), bromophenol blue 0.025 g (0.25 %), xylene cyanol 0.025 g (0.25 %).

**Plate preparation and casting the gels:**

Cleaned agarose gel casting cassette and comb were wiped with methanol and open sides of the tray were sealed with gel sealing tape. The comb was placed in the given slits of the plate. Calculated amount of agarose in TBE buffer was mixed to prepare 2 % solution. The agarose was dissolved completely in the buffer by heating the mixture at 80-85$^{o}$C in microwave oven and was cooled to 50$^{o}$C. Ethidium bromide was added in a final concentration of 0.6 mg/ml and mixed well. Liquid was gently poured into the casting tray before it gets solidified. The combs and sealed tape were removed slowly after complete solidification of the agarose gel.

**Preparation of samples and scanning of gels:**

The amplified DNA samples having approximately 5 µl volume were mixed with 2 µl gel loading dye and were carefully loaded in the wells using gel-loading tips. 100 bp Marker Electrophoresis was carried out at 150V. The gel images were recorded in JPEG or TIF formats using gel documentation system (Biorad, USA). The gels were analyzed by using the software Image lab version 3.0 (Biorad, USA).

**Purification of amplified PCR products**

Purification of rbcl gene amplified products were done using GenElute™ PCR Clean-up kit (cat no. NA 1020-1kt) using following protocol

- Column preparation: GenElute plasmid mini spin column was assembled in 2.0 ml collection tube provided with kit. 0.5 ml of column preparation solution was added to the columns and centrifuged at 12,000 g for 30 seconds.
- To 20 µl of PCR product 100 µl of binding solution. After proper mixing solution was transferred to the binding column. Columns were centrifuged at 16,000 g for 1 minute. Flow-through was discarded.
- Binding column was placed in a collection tube and 0.5 ml of wash solution was added to the binding column and centrifuged at maximum speed for 1 minute. This step was repeated in order to remove the impurities.
- To dry the binding membrane completely and in order to prevent the alcohol contamination in preceding reactions columns were centrifuged at maximum speed for 3 minutes.
- Columns were transferred to the fresh 2 ml collection tube and 50 µl of Elution solution was applied to the center of each column.
- DNA elution was carried out at maximum speed for 1 minute.
- Eluted DNA (PCR product) was stored at -20º C.

### 3.2.5 Cycle Sequencing

- Sequencing was carried out using BigDye® Terminator v 3.1 Cycle sequencing kit. The BigDye Terminator v3.1 Cycle Sequencing Kit provides the required reagent components for the sequencing reaction in a ready reaction, pre-mixed format.
- Cycle sequencing was performed in 10 µl volume. Reaction mixture was prepared as follows. Two reaction tubes were prepared for forward sequencing primers and reverse sequencing primers. In this experiment RbcL A gene amplification primers (Rbcla F and rbcla R) served as sequencing primers.

| Reagent | Quantity |
|---|---|
| Terminator Ready Reaction mix v 3.1 | 4 µl |
| Bigdye Sequencing buffer | 1 µl |
| Template | 150 - 300 ng |
| Primer | 10 pmole |
| Deionized water | To make the volume up to 10 µl |

**Table 5: Cycle sequencing table**

- Before going to amplification reaction mixture was mixed well in an individual tube and spun down briefly.
- Amplification was carried out in Thermal cycler (Applied Biosystems Veriti®). Reactions were amplified through 35 cycles with the following conditions:
- Initial denaturation at 96°C for 1 min, followed by 30 cycles of 96°C for 10 sec, annealing at 55°C for 5 sec, and extension at 60°C for 4 min, followed by indefinite hold at 4°C (Ivanova *et al*., 2005).

**Purification of Cycle sequencing Products**

In order to achieve optimal results, complete removal of unincorporated dye terminators before performing capillary electrophoresis necessary. Excess dye terminators in sequencing products can obscure data in the early part of the sequence and interfere with base calling. Purification was done using BigDye XTerminator® Purification Kit. Kit contains SAM™ Solution and BigDye® XTerminator™ Solution. Following purification protocol was followed:

- Cycle sequencing reaction plate was spin at 100 g for 1 minute.
- Purification reaction premix was prepared by adding 10 µl of BigDye XTerminator® to 45 µl of SAM™ solution. Total reaction was 55 µl for each well.
- After removing seal of the 96-well plate 55 µL of SAM™ Solution/XTerminator® Solution premix was added to each well.
- Plate was sealed using MicroAmp® Clear Adhesive Films and subjected to vortex for 30 minutes at 2000 rpm on IKA® Vortex 4 digital.
- After vortexing plate was centrifuged at 1000 rpm for 2 minutes.
- Plate kept at room temperature (25ºC) before going to capillary electrophoresis.

**Capillary electrophoresis**

Capillary electrophoresis of cycle sequenced products was Performed on 3500 XL platform (Applied biosystems)

- Instrument software 3500 was used to give commands to instrument. Dye Set Z and the Sequencing Install Standard, BigDye® Terminator v3.1 Kit was used to create the BigDye® Direct spectral calibration information to apply to the data.
- BigDye® mobility and calibration files were used for optimal basecalling with the BigDye® Cycle Sequencing Kit v 3.1.
- Plate containing cycle sequenced products was loaded on Position A/ Position B.
- Sample information was loaded on plate preparation mode.
- Capillaries were filled with POP-7™ polymer.

Selecting following parameters capillary electrophoresis was performed.

| Polymer | Array | Run Module | Mobility file |
|---|---|---|---|
| POP-7™ polymer | 50 cm | BDX_StdSeq50_POP7xl | KB_3500_POP7xl_BDTv3direct. |

Run time for 24 samples was 2 hours and 17 minutes.

**Table 6: Parameter for capillary gel elctrophoresis**

### 3.2.6 Sequence analysis and submission of barcodes to Barcode of Life Database Systems (BOLD)

Sequence analysis was using sequencing analysis version 5.4 (Apllied Biosystems) and BioEdit, biological sequence alignment editor (Ibis Biosciences). Consensus sequences generated after aligning gene sequences from forward and reverse primers. These sequences were subjected to Sequence match analysis using Basic Local Alignment Search Tool (BLAST) on NCBI.

Consensus sequences which showed significant match with the earlier identified data on NCBI were submitted to BOLDSYSTEMS according to the guidelines provided onto BOLD website (http://www.boldsystems.org/). For few species where NCBI data was not available were subjected to detailed and thorough morphological analysis and submitted to BOLD.

### 3.2.7 Tree-based analysis

Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007), and the phylogenetic trees were inferred with the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). In phylogenetic analyses, genus identification was considered successful when the unknown sample formed a monophyletic group together with all members of a single genus, with a bootstrap support of >70%. An equal strategy was applied for species-level identification (de Groot *et al*., 2011). For generating phylogenetic tree total 9 plant species were selected and based on their DNA barcodes tree analysis was performed.

## 3.3 SELECTION OF PLANTS FOR BIOACCUMULATION OF HEAVY METAL IONS (Zn, Ni AND Cd)

The plant species selected for the metal removal process in the present research, were: *Lemna polyrrhiza* L., *and Lemna Triscula* L. The study was aimed at the accumulation of various concentrations of Zinc (Zn), Nickel (Ni) and Cadmium (Cd) at different intervals of time. This study involves three main parts:

- Culturing of the plants *invitro*
- Determination of $LC_{50}$ value
- Heavy metal determination in the plant cells and metabolic observations in the Cd, Ni and Zn exposed plants at various experimental periods.

### 3.3.1 Culturing of the plants *invitro*

The fresh water hydrophytes was collected from a pond located at Harni, Gotri M.S. University of Baroda. It was thoroughly washed under running tap water to remove any epiphytic algae attached to it. It was then transferred to a plastic tub. The media preparation and the culturing methods were carried out following standard method (APHA-AWWA-AWPC, 1989).

Different ingredients used in the preparation of the medium are presented in Tables 7 and 8. Reagent grade micro and macronutrients were added to double distilled water to obtain final concentration of the medium. The medium was made aseptic and free from interference of bacterial growth by adding 1 ml of each macronutrient stock solution to 800 ml double distilled water in the order listed, and thoroughly mixed after each addition.

It was later filtered, autoclaved and then added 1 ml filtered and sterilized micronutrient solution. The final volume was made to 1000 ml with double distilled water.

**Table 7: Macronutrients used in the stock solution**

| Compound | Concentration mg/l |
|---|---|
| $NaNO_3$ | 25.5 |
| $NaHCO_3$ | 15.0 |
| $K_2HPO_4$ | 1.04 |
| $MgSO_4.7H_2O$ | 14.7 |
| $MgCl_2$ | 5.70 |
| $CaCl_2.2H_2O$ | 4.41 |

**Table 8: Micronutrients used in the stock solution**

| Compound | Concentration µg/l |
|---|---|
| $H_3BO_3$ | 186 |
| $MnCl_2$ | 264 |
| $ZnCl_2$ | 3.27 |
| $CoCl_2$ | 0.780 |
| $CuCl_2$ | 0.009 |
| $Na_2MoO_4.2H_20$ | 7.26 |
| $FeCl_3$ | 96.0 |

### 3.3.2 Determination of $LC_{50}$ value

In the pilot scale experiment, the test plants were exposed to wide range of the metal ion concentration i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppm. The plants were regularly observed and the data was recorded. It was noticed that the plants were unable to survive in the concentration range between 10-20 ppm Cd, Zn and Ni ions. In the subsequent experiments it was revealed that the concentration mortality ($LC_{50}$) of Cadmium chloride on exposed plants were 0.5 ppm during 144 hrs. Therefore, the trace element under study was supplied at 0.1, 0.2, 0.3, 0.4 and 0.5 ppm for 2, 4 and 6 days. Similarly, the concentration mortality ($LC_{50}$) of Zinc sulphate exposed plants were 9 ppm during 216 hrs. Therefore, the trace element under study was supplied at 1, 3, 5, 7 and 9 ppm for 3, 6 and 9 days. Similarly, the concentration mortality ($LC_{50}$) of Nickel chloride exposed plants were 9 ppm during 216 hrs. Therefore, the trace element under study was supplied at 1, 3, 5, 7 and 9 ppm for 3, 6 and 9 days.

### 3.3.3 Determination of metal uptake rate:

The stock solutions of 1000 ppm for Cd, Ni and Zn ions were prepared in double distilled water by dissolving the salt of $CdCl_2$, $NiCl_2$ and $ZnSO_4$. The experiment was carried out for 10 days after determining Lc50 value. The experiment was conducted in 250 ml pre-sterilized erlenmeyer flasks and the final volume in each flask was made 200 ml containing desired concentration of metal. To each flask 20 ml of the cultured media was added and the pH was adjusted to 6.5. The media was autoclaved for 15 minutes at 15kg/$cm^2$/pressure before adding it to the metal concentration.

After the culture has attained exponential growth, healthy plants were dried and 1 gms of the plants were introduced in each flask. The flasks were tightly plugged with presterilized cotton plugs and then kept at 25 $\pm$ 2°C having 14 lux light intensity. A total of 3 sets in duplicate along with one control (without any metal concentration) for each set were run simultaneously. The flasks were shaken manually to allow rapid and continuous contact between the filaments and metal ion solution.

After 3 days the contents of each flask of first set was filtered through whatman filter paper. The plants remained on the filter paper were carefully removed with pre-sterilized forceps and immediately transferred to 100 ml beaker. The plants were then digested on hot plate with 10 ml mixture of concentrated $HNO_3$, $HClO_4$ and $H_2SO_4$ into 250 ml flask until brown fumes no longer generated. Five ml of acid mixture was added to the digested sample carefully and was further digested at 80°c. Ten ml of double distilled water was added to the digested sample, mixed well and the mixture was filtered into an acid washed container. The preparation of digested sample, which is the prerequisite for metal concentration determination in the plant cells, was carried out following the method.

The digested filtered sample was transferred into 25ml/10 ml volumetric flask and diluted up to the mark with double distilled water and kept in polyethylene bottle and then subjected to metal ion determination on atomic absorption spectrophotometer (GBC 911). Same procedure was followed for second and third experimental sets.

**The metal uptake rate of plant cells in mg/gm was calculated using the formula**:

Metal uptake (mg/gm) = X x final volume of a sample (ml) / gms of tissues used

Where,

X = concentration of metal ion in digested algal cells (ppm)

**3.3.4 Metabolic observations (Associated Biochemical changes)** on Cd, Ni, Zn exposed plant cells include:

Chlorophyll: (Thimmaiah, 1949)

Protein: (Thimmaiah, 1951)

Proline: (Thimmaiah, 1999)

Carbohydrate : Anthrone method (Sadasivam and Manikam, 1991)

Catalase: (Thimmaiah, 1999)

Peroxidase: (Sadasivam and Manikam, 1991)

### *3.3.5* Light microscopy (LM*)*

For anatomy investigations plants were cut in appropriate pieces. The plant samples were fixed in 5 ml formalin, 5 ml Acetic Acid and 90 ml 50% alcohol. Then dehydrated with graded TBA series ( 10,20,30,40,50, 60, 70, 80, 90 and 100) and 100% ethanol. The dehydrated tissues were embedded in paraffin wax thin sections were cut on microtome (Leica RM 2155) and stained with Toluidine blue in benzoate buffer, pH = 4.4 (Feder and O´Brien, 1968).

## 3.3.6 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX)

For the surface morphology determination of control and cadmium treated plants SEM, SEM-EDX were performed. The fine sample was dried in oven at 105$^{o}$C for 24 hour. Then, the sample was analyzed by SEM instrument (JEOL Japan-6490). EDS was also analyzed by EDS (EDS-133) instrument.

### 3.3.7 Statistical methods

All statistical analyses were conducted using the SPSS statistical software and $p < 0.05$ was used to determine significance in all tests. A number of statistics such as the mean, range, and standard deviation were used to describe the distribution of chlorophyll data. The Pearson correlation with 2-tailed significance tests were used to characterize the relationship between chlorophyll concentrations in after treatment period (3,6 aned 9 Days) and the analysis of variation was also calculated as the ratio of the standard deviation to the mean. Multiple stepwise regressions were used to build and assess the chlorophyll prediction models with vegetation indices.