Materials and methods

3.1 Study Area

3.1.1 Location

The study area is located in Panchgram adjoining a paper mill in Hailakandi district (latitude 24.68° N longitude 92.57° E) which lies in Southern part of the state of Assam, North-East India. The total geographical area of the district is 1327.00 sq. km. of which rural area is 1316.47 and urban area is 10.53 Sq. km. It is situated in the middle of Barak

valley and bounded by Cachar district in the north and east, Mizoram state in the south and Karimganj district in the west. The district consists of both plain and hilly areas. Dhaleshwari, the tributary of Barak is flowing in this district. The main rivers of this district are Kathakhal and Barak. Regarding administrative division, Hailakandi district consists of only one sub-division with 4 (four) revenue circles and 5 (five) development blocks. There are three towns viz. Hailakandi, Lala and Panchgram H.P.C. Township (C.T) in the district. In the present Ph.D. research work, different study sites around Cachar Paper Mill (CPM) have been undertaken in Panchgram area of Hailakandi district (Fig. 3.1).

3.1.2 Climate

The climate of the area is tropical, warm and humid with a heavy annual rainfall. The district accounts an average rainfall of 2329 mm/year but unevenly distributed throughout the year. During monsoon the relative humidity is about 91% and during pre-monsoon it is 54%. December and January are normally the driest months. In winter climate is cold and dry. Maximum temperature ranges from 30-40°C and minimum temperature is in between 6-12°C.

3.1.3 Study sites

In Barak valley, Cachar Paper Mill (CPM) at Panchgram is one of the most polluting industries in the region generating pollution both in the form of solid and liquid. The study sites are located in Panchgram area with a longitude of $29^{0}36^{/}$ E and latitude of $24^{0}52^{/}$ N which is 26 km away from Silchar town. **Table 3.1** shows the locations of the study sites in Panchgram in the Hailakandi district, Assam. For the present study two

distinct types of ecosystems viz. polluted and unpolluted ecosystem with a total of 8 study sites were selected around the papermill (CPM) area. **Plate 3.1-3.5** shows the overview of the study sites. River Barak is about 2 Km from the paper mill. Among the eight study sites, the four study sites (Site 1- 4) were selected at various sampling points of river Barak where the effluent of Cachar Paper Mill is released. The sampling site 1 was selected at river Barak, half km before the CPM effluent released point ie. Upstream zone and is considered as control site. Site 2 was selected near the effluent released point. Site 3 and 4 were selected at downstream river at a distance of 1km each after the effluent release point to see the dilution effect of the effluent. As the river flows through, it receives domestic wastes from the villages and as well as pesticides and fertilizers released from the fields during agricultural practices sited on its bank. 5 and 6 are the areas covered by solid waste deposits namely lime sludge and uncooked knot. These solid wastes are disposed in various areas within the mill campus and road sites and rich algal growth is found on these polluted substratums during rainy seasons which becomes scares during winter and pre-monsoon period due to scarcity of water. Side 7 and 8 include the tree bark ecosystems and areas around papermill.

Table 3.1: Geographical details of the study sites

Ecosystem types	Name of the sites		GPS coordinates	
River ecosystem	Site 1	Site 1	N 24°51 ‘42.3 ″	E 092°36 ‘34.9 ″
	Site 2	Site 2	N 24°51 ‘57.7 ″	E 092°36 ‘27.7 ″
	Site 3	Site 3	N 24°52 ‘04.2 ″	E 092°36 ‘19.7 ″
	Site 4	Site 4	N 24°52 ‘04.2 ″	E 092°35 ‘59.7 ″
Solid wastes	Lime Mud	Site 5a	N 24°51‘28.8 ″	E 092°35 ‘19.2 ″
		Site 5b	N 24°51‘27.8 ″	E 092°35 ‘14.0 ″
		Site 5c	N 24°51‘34.7 ″	E 092°35 ‘20.3 ″
		Site 5d	N 24°51 ‘48.8 ″	E 092°35 ‘51.1 ″
		Site 5e	N 24°51 ‘45.2 ″	E 092°35 ‘25.0 ″
	Uncooked Knot	Site 6a	N 24°51 ‘33.1 ″	E 092°36 ‘02.0 ″
		Site 6b	N 24°51 ‘33.0 ″	E 092°35 ‘07.0 ″
		Site c	N 24°51 ‘27.2 ″	E 092°35 ‘14.4 ″
Tree bark ecosystem	Tree bark	Site 7a	N 24°52 ‘01.8 ″	E 092°35 ‘44.6 ″
		Site 7b	N 24°51 ‘37.3 ″	E 092°36 ‘27.2 ″
		Site 7c	N 24°51 ‘40.9 ″	E 092°36 ‘00.0 ″
Terrestrial ecosystem	Around papermill	Site 8a	N 24°52 ‘10.1 ″	E 092°35 ‘46.0 ″
		Site 8b	N 24°51‘36.6 ″	E 092°35 ‘24.3 ″
		Site 8c	N 24°51 ‘47.0 ″	E 092°35 ‘52.0 ″
		Site 8d	N 24°51 ‘54.6 ″	E 092°36 ‘33.1 ″
		Site 8e	N 24°51 ‘03.1 ″	E 092°36 ‘03.5 ″

Fig. 3.1: Map of the study area showing the location of study sites

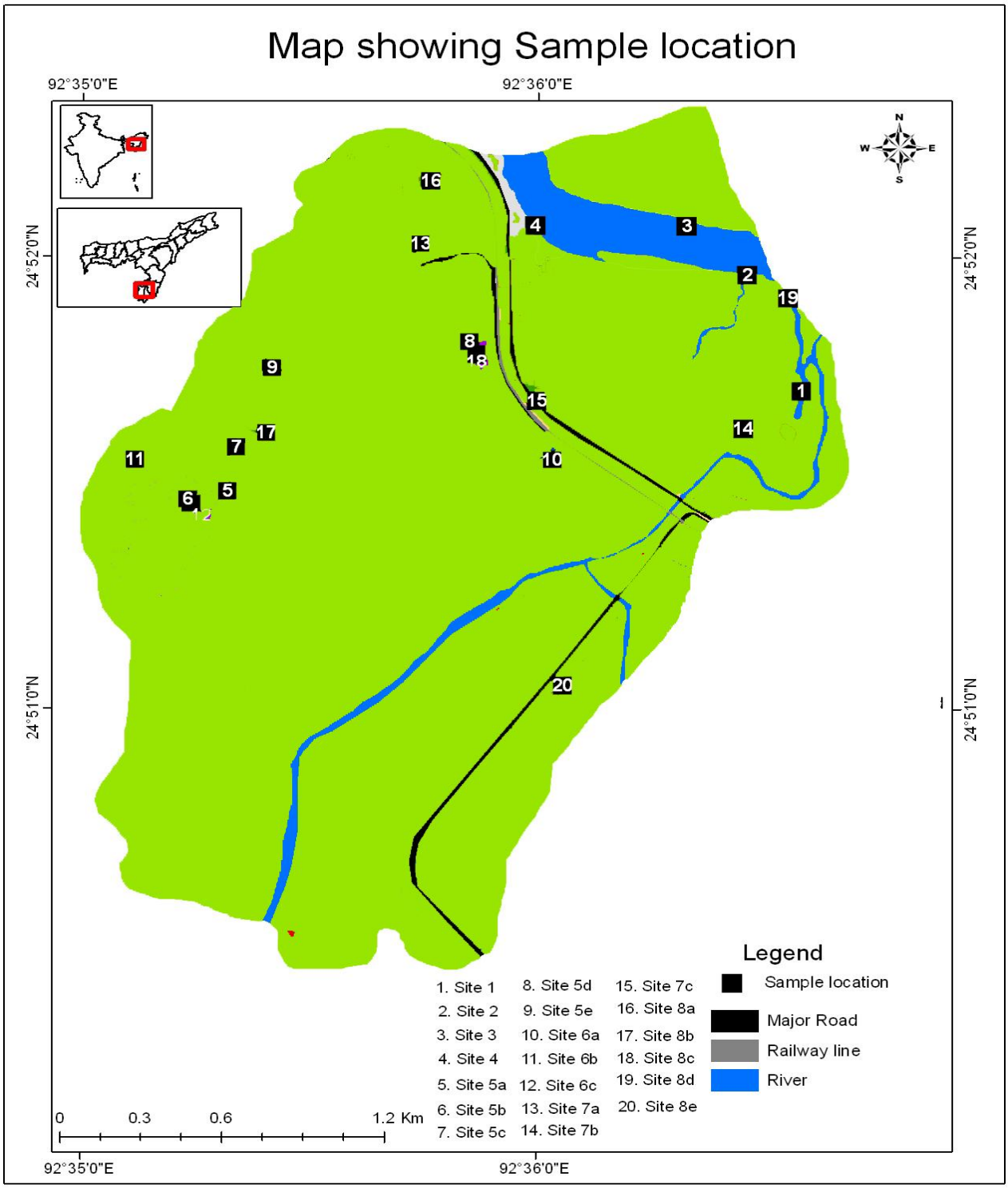

Plate 3.1: The overview of the study sites Site 1 (S1), Site 2 (S2), Site 3 (S3) and Site 4 (S4)

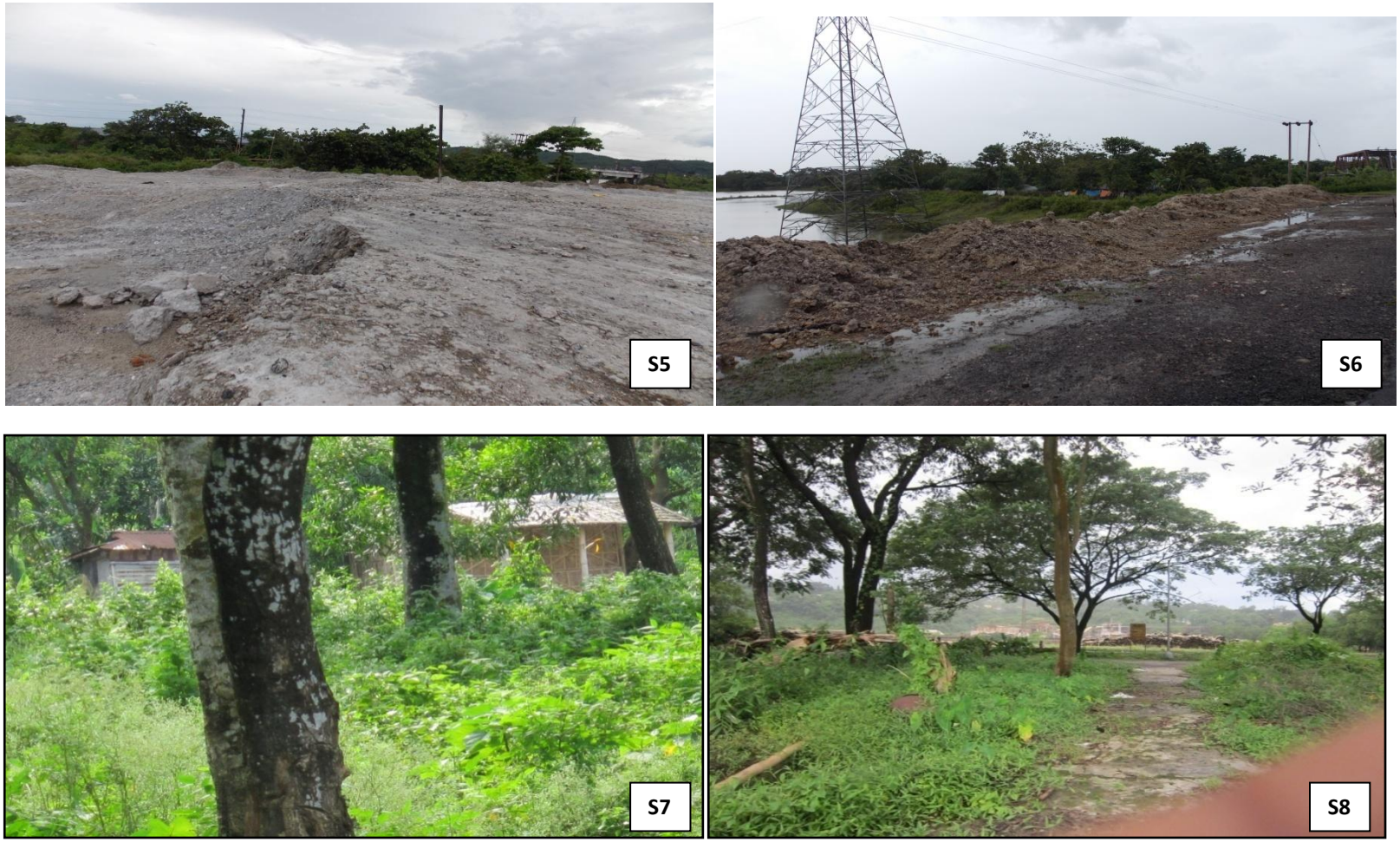

Plate 3.2: The overview of the study sites Site 5 (S5), Site 6 (S6), Site 7 (S7) and Site 8 (S8)

Plate 3.3: The overview of the study sites during flooded period of Site 1 (S1), Site 2 (S2) and dry period of Site 5 (S5) and Site 6 (S6)

3.2 Methodology

3.2.1 Analysis of physico –chemical properties of water and microclimatic conditions of samples

A total of 9 physico-chemical parameters of water samples and 2 microclimatic conditions were determined using the standard methods. The samples were collected and analysed according to the seasons, monsoon (June to August), post monsoon (September to November), winter (December to February) and pre monsoon (March to May). Monsoon, post monsoon, winter and pre monsoon are represented as M, PO, W and PM. Air and water temperatures were measured using a mercury thermometer in unit °C. Water analysis was performed in triplicate following standard procedures (APHA, 2005; Wetzel and Likens, 1979; Suess, 1982). Transparency of water was measured by Secchi Disc method (Wetzel and Likens, 1979). Water pH was measured using a digital pH meter. Free CO2 and total alkalinity were determined using the standard method (APHA, 2005). Dissolved oxygen of water samples were determined using Winkler's method (Wetzel and Likens, 1979). Biological oxygen demand (BOD) of water sample was determined by Winkler's method (Wetzel and Likens 1979). COD was determined by Reflux method. TSS of the water samples were measured by was Gravimetric method. TDS was determined by the method following Tripathi and Govil (2001). Soluble reactive phosphorous (SRP) was estimated by Ascorbic acid method and dissolved silica was obtained by Molybdate blue method following Wetzel and Likens (1979). Chloride was determined by Argenometric method. Nitrate was determined by Brucine method (Suess, 1982).

3.2.1.1 Heavy metal analysis

The water samples were collected in triplicates from different sites in cleaned PVC bottle and filtered using Whatman paper No. 42. The samples were fixed with concentrated hydrochloric acid (0.5ml) in order to avoid metal loss. An amount of 300ml of the water samples were taken from the collected water and were concentrated to 30ml (Gupta, 1996). Analysis of heavy metal was performed by AAS (Atomic Absorption Spectrophotometer) Model: Perkin Elmer 3110.

3.2.2 Soil analyses

A total of 6 soil parameters were analyzed following the standard methods. Soil pH was measured using pH meter. The conductivity of soil samples were measured by direct reading using a conductivity meter. Soil bulk density was estimated by soil core method (Brady and Weil, 2004). The moisture content of the soil was determined by oven drying method (Soil Survey Standard Test Method) (Gupta, 1999). Soil organic carbon was analyzed by Walkey and Black's rapid titration method (Jackson, 1958). The texture of the soil was analyzed by Bouyoucos Soil Hydrometer Method (Allen, 1989).

3.2.3 Algal Analysis

3.2.3.1 Collection and identification of algal samples

Algal samples growing on the different study sites were collected randomly and were scrapped and preserved in formalin (4.5%) solution for further identification and quantitative analysis. Three replicates of each algal sample were collected in every time during the collection. The algal population was studied by optical microscopy and

photomicrographs were obtained by Leica application suit (LM1000 LED). Algal identification was performed with the help of relevant taxonomic literature (Desikachary, 1959; Smith, 1950; Prescott, 1954; Sarode and Kamat, 1984). Algal enumeration was done by "Lackey's drop method" (Lackey, 1938). To estimate chlorophyll *a*, samples from 1cm2 area were scraped. To the fresh homogenized sample 90% acetone was added to make the volume up to 5ml. Freezing and thawing technique was followed to extract the Chlorophyll *a* using Trichromatic equation mentioned by Strickland & Parson (1968).

3.2.3.2 Isolation and characterization of cyanobacteria

In order to initiate the isolation and cyanobacterial culture, "plating technique" was followed as this method was found advantageous in providing both qualitative and quantitative assessment of cyanobacterial abundance from soil simultaneously. Representative randomized 25 surface soil samples from the whole demarcated plots were collected after removing upper 1 cm soil crust. To assess cyanobacterial abundance, fresh soil samples (10g) were shaken with sterile water (90ml) for 2 h, according to Lukešoúa (1993). Dilutions (10-2, 10-3) were prepared from the soil suspensions obtained and (1ml) were spread on solidified (1.5%) BG-11 media (Rippka *et al*., 1979). Plates were incubated at a temperature of 22°c±1 under a 16:8 (light: dark) photoperiod at a light intensity of 2000-3000 lux. Total abundance of the species was assessed by direct counts of visible algal colonies after 3 weeks and made unialgal by repeated streaking on agar plates with or without combined nitrogen. The morphological parameters such as shape of the cells and tips, length and breadth of intercalary cells as well as heterocysts and akinetes, presence and absence of constriction of the cross wall, sheath and its colour

were taken into account. For each biometrical character repeated measurements were obtained from different cells, heterocysts and filaments (Singh *et al*., 2008). The taxonomic identification of the cyanobacteria was done based on the cell or colony morphology (Prescott, 1954 and Desikachary, 1959). Numbers of individuals were expressed per 1 g of soil by counting the colonies developed on the agar plate.

3.2.3.3 Growth measurement of cyanobacteria and photosynthetic pigment analysis

The cyanobacterial growth was estimated by analyzing biomass of the species in terms of chlorophyll *a* concentration. 1ml of the inoculum was added to 100ml of BG-11 medium with or without combined nitrogen for heterocystous and non heterocystous isolates respectively. Chlorophyll *a* estimation was done in each alternate day. Specific growth rate and generation time was estimated using the equations suggested by Levasseur *et al*., (1993). Chlorophyll *a* and carotenoid pigments were extracted by "cold extraction method" using 90% acetone (Parsons and Strickland, 1965; Parsons *et al*., 1984). Phycobiliproteins of the dry biomass were extracted in 0.05M phosphate buffer (pH 6.8) using the equation mentioned by Bennet and Bogorad (1973). The biomass was subjected to repeated freezing and thawing for 48 hours with 24 hours interval (Moraes *et al*., 2011).

3.2.3.4 Biochemical estimation of the cyanobacterial isolates

The biochemical attributes of the isolates were measured in terms of total carbohydrate, soluble protein and phycobilliproteins. For estimation of total carbohydrate, Anthrone method (Spiro, 1966) was used. Soluble protein was measured by modified Lowry

method (Herbert, 1971). Photosynthetic pigments and biochemical parameters were analyzed in stationary phase of the growth period.

3.2.3.4 Bioremediation studies

3.2.3.4.1 Biomass production of test species

The biomass of the test isolates selected for the analysis are maintained in the same medium and under similar laboratory conditions mentioned earlier, before use as inoculum for experiments. The biomass was harvested in between the early stationary phase of the growth period by filtration, washed with deionized water and finally separated by centrifugation at 1,000 × g for 5 minutes at 4°C. These cells were used in the experiments thereafter. All the experiments were conducted in triplicate.

3.2.3.4.2 Stock solution preparation of metals and serial dilution of test solution

A stock solution of the aqueous adsorbate Cu (1000 mg l^{-1}) and Cr was prepared separately by dissolving analytical grade (AR grade) salts of 100 milligram of CuSO4 and $K_2C_rO_4$ in 100 ml of distilled water and then filtered at 0.2 μm. From these stock solutions, desired concentrations of the metal were obtained by further dilutions. The effluent of paper mill was considered as 100% concentration. From this 100% concentration of effluent, 10, 30, 50, 70 and 90% level of effluent concentrations were prepared in BG-11 media to study efficiency of organism in media.

3.2.3.4.3 Remediation bioassay

Exponentially growing culture was inoculated in to the medium containing different doses of effluent. BG11 media without inoculation of algal species was considered as control. The experiment was undertaken for a total period of 24 days under laboratory conditions to allow the species to get a culture of exponential growth treated under the stressed conditions. Growth was monitored in terms of chlorophyll *a* for every 3 days to determine the stimulatory or inhibitory effect of pollutants on the tested cyanobacteria (their resistance or sensitivity) in order to define the most resistant and promising bioremediation species. Color removal of the samples was noted by visual observation. Periodic (weekly) monitoring of the effluent samples was done for investigating the biochemical attributes and physiochemical characteristics of the effluents. The enzymatic antioxidant activity of Catalase was determined by Aebi (1984). The glutathione reductase was assayed according to Scaedle and Bassham (1977). Heavy metal concentrations of the metal treated solution and wastewater were determined before and after the experiment using Graphite Furnace (Analytik Jena AG Vario 6) and metal uptake were calculated from the difference in metal concentration in the aqueous phase before and after absorption according to Shaik *et al*. (2006).

3.2.3.4.4 Toxicity analysis

Probit analysis is a method that estimates biological activities by regression analysis. In this study, the chlorophyll *a* measurement for the cyanobacteria was calculated, and the results were converted into percent inhibition rates with Abbott's formula. Probit analysis was performed by using the percent inhibition results obtained on day 15 of the

experiment. The lethal concentration values (LC50) of the metals and wastewater for the selected cyanobacteria were calculated (Finney, 1952). Resistant strains were compared with one another and the strain with the highest resistance was identified.

Analysis of the effect of metals on the cell morphology by SEM:

Surface morphology of the control and metal treated samples was studied by scanning electron microscope (JEOL-JSM-6360). Samples were prepared for SEM using standard protocol (Dey *et al*., 1989) for biological samples.

3.2.3.5 Molecular characterization

3.2.3.5.1 DNA extraction

Genomic DNA of the isolated cyanobacterial strain was extracted following the method described by Smoker and Barnum (1988). Briefly 1ml aliquot of mid to late log phase culture was pelleted by centrifugation. The pellet was washed and suspended in 500µl STE buffer. To the pellet 20 µl of lysozyme (10mg/ml) was added and incubated at 55°C for 30 minutes. After incubation, 10 µl proteinase K (10mg/ml) and 20 µl of 10% SDS was added and incubated in water bath at 55°C for 30 minutes. The mixture was cooled in ice and extracted with equal volume of phenol: chloroform: isoamayl alcohol mixture (25:24:1). The supernatant was added equal volume of 4M ammonium acetate and DNA was precipitated by the addition of two volumes of isopropanol by centrifugation at 14,000 rpm for 15 minutes. The pellet was washed with 70% ethanol, dried and finally dissolved in TE buffer and stored at -20°C.

3.2.3.5.2 Oligonucleotide primers and PCR amplification of 16S rDNA

For amplification of 16SrRNA gene from the isolated cyanobacterial strain, CYA 106f (5/CGGACGGGTGAGTAACGCGTGA-3/) and CYA781r (a) (5/GACTACTGGGGTATCTAATCCCATT-3/) were used as forward and reverse primers respectively (Nübel *et al*., 1997). Each 50µl reaction mixture contained 2x Genet Bio premix (Prime Taq TM DNA Polymerase 1 unit/10 µl, 20mM Tris –HCl, 80mM KCl, 4mM MgCl2, enzyme stabilizer sediment, loading dye, pH 9.0, 0.5mM of each dATP, dCTP, dGTP, dTTP)25 µl, 1µl of each forward and reverse primers, 2µl DNA template,21µl MQ water. The PCR reaction was performed in My Gene TM series Peltier Thermal Cycler (Model MG96G). Thermo-cycling condition for the PCR was followed as initial denaturation at 94°C for 5min; 30cycles of 94°C for 1min, 58°C for 45s, 72°C for 1min and final extension at 72°C for 7min. The obtained sequences were checked for the homology to other sequences deposited in the available databases using Basic Local Alignment Search Tool (BLAST) search (http://www.ncbi.nim.nih.gov/BLAST). The gene sequences were submitted to NCBI Genbank under the respective accession numbers.

3.2.3.5.3 Phylogenetic analysis

Phylogenetic analysis of the DNA sequence data was performed with MEGA5 (Tamura *et al*., 2004) analysis platform and aligned with other sequences obtained from Genbank (www.ncbi.nim.nih.gov) using ClustalW aligning utility. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).

3.3 Statistical analysis

All the data were given as mean with standard deviation to have an essence of the whole dataset. Quantitative characterization of algal community (species density, abundance and frequency) was done following Dash and Dash (2009). The algal community structure was analyzed using different diversity indices. Shannon-Wiener diversity index_H, Simpson's dominance index_D, Pielou's evenness index_J were calculated with the help of statistical software, PAST V-2.13. All other statistical analyses like Analysis of variance (ANOVA), Tukey multiple comparisons, bivariate correlation, Principal Component Analyses (PCA), and hierarchical cluster analysis were carried out by SPSS V-19.