Studies on rhizospheric bacteria of *Phragmites* in constructed wetlands

A thesis submitted
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Contents

Certificate

Certified that the thesis "**Studies on rhizospheric bacteria of *Phragmites* in constructed wetlands**" which is submitted by Ms. Misha Bhatia, in fulfillment of the requirement for the award of Degree of Doctor of Philosophy in the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by her under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Candidate's Declaration

I, hereby declare that the work presented in the thesis entitled **"Studies on rhizospheric bacteria of *Phragmites* in constructed wetlands"** in fulfillment of the requirement for the award of Degree of Doctor of Philosophy in the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period from June, 2007 to December, 2013, under the supervision of Dr. Dinesh Goyal, Professor & Head, Department of Biotechnology & Environmental Sciences, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

Date: 9.1.14

(Misha Bhatia)

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(Misha Bhatia)

Synopsis

Contemporary world is facing problems with wide variety of pollutants and contaminants in water. Industrial effluent, sewage and agricultural runoff are the major source of surface water pollution. Discharging these effluents in agricultural land has become a common practice in India, as a result of which these contaminants (organic & inorganic) are transferred and concentrated into plant tissues from the soil. Their presence is known to have a potential impact on environmental quality and human health via ground water, surface water contamination and vegetables. In the last few decades, researchers have tried to adopt an eco- technological approach to clean up or remediate contaminated water with toxic pollutants using plants. The wide recognition to this approach is supported by fact that it is considered to be an environment friendly, safe and cost- effective technology.

The use of plants termed 'phytoremediation' ("phyto" meaning plant, and the latin suffix "remedium" means to clean or restore) actually refers to diverse collection of technologies that use plants (natural or genetically engineered) for cleaning contaminated environments. The processes through which plants perform this involve phytoaccumulation, phytoextraction, phytomining, phytostabilisation, phytovolatilization and rhizofilteration. Eventually a combination of biological and engineering strategies to improve applicability of phytoremediation has come up in the form of constructed wetlands. Vegetation, the principal component of constructed wetlands composed of plants having the ability to accumulate some compounds in a large concentration as compared to environment. In case of metals these are termed as 'hyperaccumulators'. Some common plants in wetlands are reed, cattail, rush and bulrush. However, the most common plant species is *Phragmites (*IWA, 2000; Scholz, 2006).

After the plants have been allowed to grow for some time, they are harvested from the wetlands and either incinerated or composted to recycle the metals. This procedure is practiced till the contaminant level in water comes down to allowable limit. Constructed either with horizontal or vertical flow these wetlands acts as a mechanism to treat non-point source pollution before it reaches lakes, rivers and oceans. Constructed Wetlands are gaining importance as effective and low-cost alternative for effluent treatment in small villages as these can be maintained by relatively untrained personals.

The development of mathematical models to explain and predict wetland's behavior is still in an early stage, especially in correlating predicted and experimental value. It is believed that the development of a model which can adequately describe flow conditions may represent an important contribution for modeling the overall performance of CW's. Keeping the above factors in mind a comprehensive study was undertaken on "Studies on rhizospheric bacteria of *Phragmites* in constructed wetlands". In this study, the treatment potential of a *Phragmite*- vegetated CW at lab scale was evaluated with inlet flow rate, outlet flow rate and retention time as the independent variables and response surface methodology was used to describe the treatment process to understand the effect of two or more independent variables on the dependent variable. The identification and role of rhizospheric bacteria associated with *Phragmites* in a constructed wetland was carried out. An attempt was made to understand the system in order to increase its potential by using microbial inoculants. The objectives of the study are as follows:

Objectives

1. Characterization of wastewater and development of *Phragmites*- vegetated wetland system for treatment of wastewater
2. Analysis of rhizospheric bacterial community of *Phragmites*
3. Monitoring survival of selected bacterial inoculants in rhizosphere
4. Impact of plant rhizosphere and bioaugmentation on remediation potential

Approach adopted to meet above objectives:

In a lab scale experiment, continuous horizontal flow of wastewater was maintained in a constructed wetland system vegetated with *Phragmites australis* in a tub with gravel stone bed on each side and along length of tub. A soil bed was formed above the gravel stone bed. In continuous system of horizontal subsurface flow constructed wetland

(HSSF-CW) range of inlet flow rate, outlet flow rate, retention time (all independent variables) was defined as 15-30ml/min, outlet flow 6-12ml/min, 1-24 hours respectively. All independent variables were standardized using different run orders where maximum remediation occur using Central Composite Design (CCD) matrix. The regression model was applied in all parameters to study their effect on output parameters

A culture independent approach *i.e.* DGGE was employed to study the root zone of *Phragmites* in constructed wetland. Also in culture dependent technique, some metal tolerant bacteria were isolated from various root zones (located at different depths). Isolates thus obtained were screened for their metal accumulation potential through mass balance study. Selected isolate (s) were tagged with a broad host range plasmid carrying antibiotic resistance gene (dual selection marker). The tagged bacteria were augmented into the lab scale constructed wetlands and allowed to acclimatize. The wetland was again assessed for remediation potential using metal spiked water. An effect on the remediation potential of wetland was noted in compared to the control system. Also augmented bacteria were periodically monitored for their survival potential in the wetland.

1. Characterization of waste water & Development of *Phragmites*- vegetated wetland system for treatment of wastewater

In this study, Design of Experiment was (DOE) was done through RSM which is based on a full quadratic equation where fit of the polynomial model is expressed by the coefficient of determination *i.e.* R^2. All the designed experiments were performed and results were analyzed through regression model and checked for significance. Model coefficients were estimated by using multiple regressions (Minitab) and model accuracy was checked using R^2, *F test* and *P value* for each parameter.

Experimental design of 3 factors using 20 runs with 6 centre points and software generated alpha value of 1.682 was used. A volume of 100 ml treated effluent was collected from outlet in each run and was analyzed for change in pH, electrical conductivity (EC), Dissolved oxygen (DO), Biochemical oxygen demand (BOD_5), Chemical oxygen demand (COD), total and suspended solids, Nitrate-N, Phosphates and metals (Fe, Cu, Mn, Zn, Ni, Pb, Cd). The samples for metal analysis were acid digested, volume made up using metal free water, filtered and analyzed for residual metal concentration by Atomic Absorption Spectrophotometer (GBC 932 AA; GBC Scientific Equipment Pvt. Ltd. Australia) using air–acetylene flame and single element hollow cathode lamp. Treatment of domestic wastewater using constructed wetland system vegetated by *Phragmites* for 6 months was studied for 20 days. On the basis of the experimental data a comparison was drawn between planted and unplanted system. Treatment of wastewater was best obtained as revealed by maximum reduction of BOD_5, COD, nitrate and metals with inlet flow rate of 22.5ml/min, outlet 9ml/min and retention time of 12.5 hrs. Significant reduction was obtained in parameters like BOD_5 66.7%, COD 60%, TDS 70% and Cd 50% as compared to unplanted control system where BOD_5, COD were reduced by 4.2%, 3% respectively and no significant removal of TDS and Cd was observed. Nitrates and Phosphates were also significantly reduced by 21% and 50% respectively. In planted system, Fe, Mn, Pb, removal rate obtained were 11.1%, 50%, 25% respectively as compared to non significant removal rates in unplanted system. As per ANOVA and P- value, the regression model was found significant in most of the parameters. The coefficients of the interactive effects between the variables did not appear to be very significant in comparison to the linear effects. The behavior of the model system was explained by quadratic equation Eq. (1)

$$Y = \beta + \sum \beta i\ Xi + \sum \beta ii\ (Xi)^2 + \sum \beta ij\ Xij$$

Where Y is the variable studied, β is the constant in regression model, X*i* is the *i*th independent factor, β*i* is linear coefficient value for variable X*i* and is the first order model concept, β*ii* is the square or quadratic coefficient for the factor *i* and β*ij* is the model coefficient for interaction between *i* and *j*.

2. Analysis of rhizospheric bacterial community of *Phragmites*

Rhizospheric bacterial community of *Phragmites* was monitored at different root zones *i.e* upper zone (0-15 cm), middle zone (15-30 cm), lower zone (30-45 cm) by four methods (a) Griffiths method (2000), (b) Glass bead-beating method (c) Soil DNA extraction kit (Himedia), (d) Chemical Lysis (CL) Method The nucleic acids were then

visualized on 0.7-0.8% agarose gel using U.V. transilluminator (312 nm). To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/230 nm (DNA / humic acids) and 260 nm/280 nm (DNA / protein) were determined. DNA extraction by Griffith's method yielded maximum purity. All crude DNA samples were purified using Sephadex G200 purification spin columns for further work. Amplification of soil DNA was carried out using 16S rDNA (8F, 1492R) & DGGE specific primers (PRBA 338F and PRUN 518R) with GC clamp added to the 5' end of the forward primer. The DGGE was carried out using a INGENYphor DGGE system as per the manual instructions. Briefly, PCR products were resolved on 8% (w/v) polyacrylamide gels in 0.5 X TAE using denaturing gradient ranging from 0 to 100% (where 100% denaturant contains 7 M urea and 40% formamide). Dice coefficient matrix was generated from the DGGE gel using *Bio-rad Quantity-One* software. Principal component analysis (PCA) was performed using *Quantity-One* where relative data of the DNA bands from the bacterial communities was analyzed to detect grouping patterns among the soil samples.
The bands excised from the gel, eluted, purified and reamplified using DGGE primers (without GC clamp) were sent for sequencing. 24 different bands were sequenced and a phylogenetic tree was constructed using MEGA5 software. Phylogenetic analysis of sequenced DGGE bands was performed. Partial bacterial 16S rDNA gene sequences were subjected to a NCBI BLASTN http://www.ncbi.nlm.gov/ blast/) search to identify sequences with highest similarity bacterial 16S rDNA sequences.

3. Monitoring survival of selected bacterial inoculants in rhizosphere

Physico-chemical characterization (Depth Collection, pH, Electrical conductivity (mS/cm), Organic C %, Ava P %, Ava S %), of various soil samples (Upper, Middle, Lower rhizosphere) was performed. Soil Dehydrogenase activity and bacterial enumeration on various media *i.e.* Nutrient Agar, Jensen media, Pikovskaya media and Cellulose minimal media was done. Pb and Cd tolerant strains were isolated from various root zones on minimal media i.e. BHB+1%glucose+ 25-100 (ppm) of metal. Three isolates UR-8, UR-10, UR-21, showing metal tolerance were checked for their antibiotic resistance and metal accumulation potential by growing in control and metal containing rich media for 24 hrs. Mass balance study was performed in UR-10 (with highest tolerance) by calculating metal accumulated inside cells and left in media for 48 hours at periodic intervals. UR-10 was transformed with broad host range plasmid pMMB277 using $CaCl_2$ method and transformed colonies were plated on X-gal+IPTG+ Chl^R for screening of transformants. Transformants were subjected to plasmid isolation to check the presence of pMMB277 plasmid.

4. Impact of plant rhizosphere and bioaugmentation on remediation potential

Transformed colonies were scaled up and inoculated in lab scale constructed wetland to study the impact of plant rhizosphere and bioaugmentation on remediation potential. In order to study metal uptake by the plant parts, roots, leaves and stem were separated, crushed and sieved through 0.2 mm sieved to obtain a fine powder form. The powder was acid digested metal analysis using atomic absorption spectrophotometry.

Salient Findings

1. *Phragmites* vegetated horizontal subsurface flow constructed wetland (HSSF-CW) at lab scale was constructed with dimensions of 48x 33x 20 cm in plastic tubs each having three sections (a) Inlet section (b) Vegetative section (c) Outlet section where treatment of domestic wastewater was studied over a period of 20 days. Treatment was best obtained with inlet flow rate of 22.5ml/min, outlet 9ml/min and retention time of 12.5 hrs as revealed by maximum reduction of BOD, COD, nitrate and metals. Significant reduction was obtained in parameters like BOD_5 66.7%, COD 60%, TDS 70% and Cd 50%, Nitrates 21% and Phosphates 50% as compared to unplanted control system where BOD_5, COD were reduced by 4.2%, 3% respectively and no significant removal of TDS and Cd was observed. In Fe, Mn, Pb, removal rate obtained were 11.1%, 50%, 25% respectively as compared to removal rates in unplanted system.

2. Response surface Methodology (RSM) was successfully applied to study the effect of independent variables (Inlet flow rate 15-30 ml/min, Outlet flow rate 6-12 ml/min, Retention time 1-24 hrs) on dependent variables (pH, EC, DO, BOD_5, COD, Total and Suspended Solids, Nitrate-N, Phosphates and metals like Fe, Cu, Mn, Zn, Ni, Pb, Cd). A second order polynomial response surface model was fitted to each of dependent variable with the help of statistical software MINITAB to generate a regression model with significant terms ($p< 0.05$). The behavior of the model system was explained by quadratic equation

   $$Y = \beta + \sum \beta i\, Xi + \sum \beta ii\, (Xi)^2 + \sum \beta ij\, Xij$$

   Where Y is the variable studied, β is the constant in regression model, X_i is the *i*th independent factor, β_i is linear coefficient value for variable X_i and is the first order model concept, β_{ii} is the square or quadratic coefficient for the factor *i* and β_{ij} is the model coefficient for interaction between *i* and *j*.

3. Physico-chemical characterization of a working wetland site at ACC Gagal Cement Works, at Barmana Distt Bilaspur, Himachal Pradesh, which has a

working *Phragmites* vegetated constructed wetland feeding on domestic wastewater from colony inhabited by population of about 500 persons, was done. The depth of collection of soil was 0-15 cm, 15-30 cm and 30-45 cm for upper, middle and lower rhizospheric zone respectively where pH, EC, Organic C, Ava P, Ava S and microbial count in terms of Soil Dehydrogenase activity and CFU (colony forming unit) count on various media (Nutrient agar, Jensen's media, Pikovskaya media, Cellulose minimal media) was studies. The depth of collection was 0-15 cm, 15-30 cm and 30-45 cm for upper, middle and lower rhizospheric zone respectively. The pH in zones varied from 8.11± 0.05 to 8.14 ± 0.12. The EC in zones varied from 0.83 ± 0.07 to 0.9 ± 0.01 mS/cm. The Organic carbon was present in 0.35 to 0.38%. Physico-chemical characteristics like pH, EC, Organic C, Ava P, Ava S (%) did not show much variation in three zones. A viable microbial count was also noted in terms of Soil Dehydrogenase activity (8.4 ± 0.06 µg TPF/g soil/day in upper rhizosphere, 7.9 ± 0.32 µg TPF/g soil/day in middle rhizosphere, 8.32 ± 0.02 µg TPF/g soil/day in lower rhizosphere). CFU (colony forming unit) count was done on various media *i.e* nutrient agar for total bacterial count ranged from 6 x 10^8 to 40 x 10^8 cfu g-1 soil, Jensen's media for nitrogen fixing bacteria ranged from 4 x 10^3 to 9 x 10^3 cfu g-1 soil, Pikovskaya media for P-solubilizers ranged from 2 x 10^3 to 30 x 10^3 cfu g-1 soil and Cellulose minimal media for cellulose degrading bacteria ranged from 6 x $10^{2\ \text{to}}$ 50 x 10^2 cfu g-1 soil by serial dilution and plating.

4. For study of soil microbial communities, total Soil DNA extraction was done at different soils depths (upper rhizospheric zone (0-15 cm), middle rhizospheric zone (15-30 cm), lower rhizospheric zone (30-45 cm)) by different methods. Of all four methods; (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method. DNA isolated by Griffiths *et al.* (2000) method showed less shearing, intactness and a good ratio of A260/A280.

5. As Sephadex G-200 spin column purification is found to be the best method (Miller *et al.*, 1999) for removing PCR-inhibiting substances while minimizing DNA loss, all the samples were subjected to Sephadex G-200 spin column purification for pure DNA recovery and removal of PCR inhibitors from crude extracts. The purity of the final purified rhizospheric soil DNA was also checked by the ability of PCR to amplify a region of the 16S rDNA with primers of 27F and 1492R (generating 1,500 bp amplicon) and re-amplification with PRBA 338F 5' and PRUN 518R to generate 180-200 bp amplicons.

6. Denaturing Gradient Gel Electrophoresis (DGGE) was successfully employed as culture- independent approach to study the soil diversity or community structure of a *Phragmites* vegetated wetland system. The bands in the DGGE profile corresponded to the 16S rDNA fragments; same sized but differed in their nucleotide sequence, reflecting the distinct numerically dominant microbial populations in the community. The diversity of the microbial population of each site was confirmed by the band profiling, with the highest appearing at the *Phragmites* upper rhizospheric soil sample. Whereas, the total bacterial community (band numbers) and DNA yield were high in *Phragmites* middle rhizospheric zone, but the microbial community diversity was low. A total of 24 non culturable starin sequences were obtained which belonged to different Phyla *i.e* Firmicutes, α-Proteobacterium, β- Proteobacteria, ϒ- Proteobacteria, δ-Proteobacteria, *Actinobacteria*, Bacteroidetes and Planctomycetes. Sulfate-reducing bacterium belonging to Phyla Firmicutes, Nitrogen metabolizing bacteria belonging to Phyla α-Proteobacterium (*Bradyrhizobium* sps), three strains belonging to ϒ-Proteobacterium, two strains belonging to *Actinobacteria* and three strains of Bacteroidetes which composed of Gram-negative, non-spore forming, anaerobic, and rod-shaped bacteria were observed. Principal Component Analysis (PCA) showed nearly the same profiles of DNA extracted from the same sample in duplicity *i.e.* two lanes of upper rhizospheric samples showed similarity and so with middle and lower rhizospheric samples. The results of cluster analysis also showed that the metabolic activities of Obs1 were more “closely” related to

those of Obs2 compared with Obs3 and Obs5. The metabolic activities of Obs6 and Obs 7 were in a single cluster. Similar results were obtained using dendrogram where clustering analysis of the DGGE profiles showed that bacteria in the six lanes of DGGE gel belonged to three clusters.

7. A number of Pb tolerating strains were isolated; 11 from upper rhizosphere zone, 6 from middle zone and 3 from lower zone from *Phragmites australis* rhizosphere zone. Also 8 Cd tolerating strains from upper rhizosphere and one from lower rhizosphere were isolated. Strain *Rhodobacter sphaeroides* (UR-10; isolated from upper rhizospheric zone) was found capacity to accumulate metal as shown by mass-balance study for 48 hours. At regular intervals, cell and supernantant from cukture inoculated with metal was taken, acid digested and analyzed for metal concentration using AAS. The strain UR-10 was found to accumulate about 25% of Cd and 30% of Pb inside the cells.

8. The *lac* marker alone has not been suitable for monitoring in bacteria in soil since 20% of cultured, aerobic, heterotrophic soil bacteria possess *lacZ*+ characteristic. The combination of induced *lacZ* expression and chloramphenicol resistance provided a stable, precise reporter system, which made its ecological monitoring easy on selective media. To check the survival and establishment of molecular tagged microbes in wetland system, the bacterial strains (UR-10; *Rhodobacter sphaeroides*) possessing Chl^R were molecularly tagged with pMMB277 plasmid and enumeration was done as blue colored lactose positive and chloramphenicol resistant colonies having low copy which showed a maximum growth in planted, non spiked system followed by a single metal spiked (Pb) constructed wetland system followed by dual metal spiked system.

9. For studying metal accumulation in *Phragmites* plant parts (roots, stem, leaves), tub based wetland systems was fed with single or dual metal spiked water and metal uptake by plant parts was analyzed, which showed significant differences but maximum metal was accumulated in Leaves > Roots> Stem for both Cd and

Pb in single and dual spiked metal system. Cd accumulation was found to be more maximum in leaves 0.32 ± 0.21 mg/kg per g dry wt (CW2) followed by 0.27 ± 0.22 mg/kg per g dry wt in roots and least 0.12 ± 0.06 mg/kg per g dry wt in stem. Pb accumulation was found to be more maximum in leaves 0.32 ± 0.03 mg/kg per g dry wt (CW2) followed by 0.27 ± 0.12 mg/kg per g dry wt in roots and 0.27 ± 0.14 mg/kg per g dry wt in stem.

List of Abbreviations

rpm	Revolution per minute
g	Gram
mg	Milligram
µg	Microgram
L	Litre
µS cm^{-1}	Microsiemens per cm
ml	Millilitre
µl	Microlitre
mg ml^{-1}	Milligrams per millilitre
µg ml^{-1}	Micrograms per millilitre
%	Percentage
mg L^{-1} (ppm)	Milligrams per litre (parts per million)
mg g^{-1}	Milligrams per gram
µg g^{-1}	Microgram per gram
cm min^{-1}	Centimeter per minute
g L^{-1}	Grams per litre
(x10^{3} cfu ml^{-1} wastewater)	x 10^{3} colony forming unit per millilitre of wastewater
EC	Electrical conductivity
TS	Total solids
TDS	Total dissolved solids
TSS	Total suspended solids
COD	Chemical oxygen demand
BOD	Biochemical oxygen demand
BOD$_5$	Five day Biochemical oxygen demand
DO	Dissolved oxygen
C_i	Initial Concentration of metal in aqueous solution
C_f	Final Concentration of metal in aqueous solution
V	Volume of culture media
Pb	Lead
Cd	Cadmium
Mn	Manganese
Fe	Iron
Zn	Zinc
Fe	Iron
Ni	Nickel
RSM	Response Surface Methodology
DGGE	Denaturing Gradient Gel Electropjoresis
NA	Nutrient Agar
JM	Jenson’s Media
HSSF-CW	Horizontal Subsurface Flow Constructed Wetland
VSSF-CW	Vertical Subsurface Flow Constructed Wetland
CCD	Central Composite Design

IPTG	Isopropyl β-D-1-thiogalactopyranoside
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Chl	Chloramphenicol
Kan	Kanamycin
Amp	Ampicillin
Srep	Streptomycin

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Chapter 1 Introduction

Comprising over 70% of the Earth's surface, water is the most precious natural resource that exists on our planet. Water reservoirs like rivers, lakes and oceans are no longer clean and pure; posing human health risks and results in an alarming increase in demand for potable water needed as well as an increase in generation of wastewater. The contemporary world is facing problems with a wide variety of pollutants and contaminants in water. Industrial effluent, sewage and agricultural runoff are the major sources of surface water pollution. Discharging these effluents in agricultural land has become a common practice in India, as a result of which these contaminants (organic and inorganic) are transferred and concentrated into plant tissues from the soil. Their presence is known to have a potential impact on environmental quality and human health via contamination of ground water, surface water and vegetables (Mishra and Pandey, 2005).

There are many causes for water pollution, but two general categories exist: direct and indirect. Former include effluent outfalls from factories, refineries and waste treatment plants etc. that emit fluid of varying quality directly into urban water supplies. Later include contaminants that enter the water supply from soil/ groundwater systems and from the atmosphere via rain water. Soil and ground water also contains the residue of human agricultural practices (fertilizers, pesticides, etc.) and improperly disposed industrial wastes (Pendias and Pendias, 1989). Atmospheric contaminants are also derived due to human practices (such as gaseous emissions from factories). The major pollutant categories found in contaminated waters belong to three major subgroups (a) heavy metals (b) xenobiotics and (c) acidifying gases. The discharge of effluent from domestic and industrial sources has detrimental effects on the aquatic ecosystem (Wakelin *et al.*, 2008) as this outfall can deposit large amount of organic matter, nutrients and pollutants leading to eutrophication (fertilization of surface water by nutrients that were previously scarce), temporary oxygen deficits and accumulation of pollutants into receiving waterways.

In the last few decades, researchers have tried to adopt an eco-technological approach to clean up or remediate wastewater using plants. This use of plants termed **"phytoremediation"** (*phyto* meaning plant and *remedium* meaning to clean or restore) actually refers to diverse collection of natural or genetically engineered plants for cleaning contaminated environments (Cunningham *et al.*, 1997). Also called green remediation, botano-remediation, agro-remediation, vegetative remediation, it involves a continuum of processes each occurring to differing degrees for different conditions, media, contaminants, and plants (Pivetz, 2001). Five main processes have been identified in remediation process: (a) Phytoextraction: plants remove metals from the soil and concentrate them in the harvestable parts of plants (Kumar *et al.* 1995), (b) Phytodegradation: plants and associated microbes degrade organic pollutants (Burken and Schnoor, 1997), (c) Rhizofiltration: plant roots absorb metals from waste streams (Dushenkov *et al.,* 1995), (d) Phytostabilisation: plants reduce the mobility and bioavailability of pollutants in the environment either by volatilization or by prevention of migration (Smith and Bradshaw 1972; Vangronsveld *et al.*, 1995) and (e) Phytovolatilisation: Volatilization of pollutants into the atmosphere via plants (Ban˜uelos *et al.*, 1997; Burken and Schnoor, 1999). However, it must be realized that all the processes described here tend to overlap to some degree and occur in varying proportions during phytoremediation (Pivetz, 2001).

Enormous quantities of organic and inorganic compounds are released into the environment as a result of human activities (Gavrilescu, 2004). The Major pollutant list includes organic and inorganic suspended/ dissolved solids, heavy metals, pesticides and other xenobiotics. The treatment process is costly and often segmented into primary, secondary and tertiary stages. Phytoremediation in the form of vegetated constructed wetlands (CW's) has emerged as a cheap and clean, eco-technological approach and its success lie in its ability to mimic the natural cleansing process (Begg *et al.*, 2000) by reducing pollutant level to a dischargeable limit. CWs comprise a substrate (e.g. Soil) supporting plant and microbial communities that work synergistically to treat wastewaters. CW's acts as a mechanism to treat non-point source pollution before it reach lakes, rivers and oceans. Dissolved metals can adsorb onto particles or exist as complexes to inorganic and organic ligands or be present in solution in the free-ion state.

Phragmites, commonly known as *Reeds,* is the universally used plant species in CW's (Scholz, 2006; IWA, 2000) which contribute to wastewater cleaning in microcosms (Wu *et al.,* 2011), hydroponics (Williams *et al.*, 2000) and outdoor wetlands (Lee and Scholz, 2007). Other plants such as *Typha angustifolia, Schoenoplectus, Cyperus* (Dunbabin and Bowmer, 1992), *Sparganium erectum, Phalaris arundinacea, Glyceria fluitans* and *Typha latifolia* (Chu *et al.*, 2006) have been used solely or in combination with *Phrgamites*. Many cases of treatment of different effluents through *Phragmites* have also been reported e.g. Dairy wastewater (Ibekwe *et al.*, 2003), dilute farm effluents (Moir *et al.*, 2005), textile effluents (Davies *et al.*, 2005) and urban runoff (Lee and Scholz, 2007) in horizontal as well as vertical reed-beds. A significant reduction in BOD, COD, suspended solids, metals, xenobiotics, other organic/ inorganic pollutants were observed in all cases. The behavior of metals in aquatic systems is complex and may include interactions among or between the major wetland compartments, above ground plant parts, roots, litter, biofilms, soil, and water (Kadlec and Knight, 1996). Several cases of metal tolerance of Cu, Ni, Zn, Cd, Pb, Se uptake and accumulation by *Phragmites* and bioaccumulation in roots and shoots as inferred by dry biomass analysis have been reported (Ye *et al.*, 1997; Yu *et al.*, 2005; Southichak *et al.*, 2006; Azaizeh *et al.*, 2006). Many plants extract nutrients from soil, water, environments and store large amounts of those elements in them which do not appear to be required for their functioning. These plants can take up various organic chemicals from the environment and degrade or otherwise process them for use in their physiological processes. In case of metals, these are termed as **'hyperaccumulators'**.

The diversity of microorganisms in the wetland environment is critical for the proper functioning and maintenance of the system. Bacteria are capable of a number of important functions ranging from nitrogen fixation, denitrification, iron and sulfate reduction, degraders of pollutants or heavy metal accumulation. Although bacteria and the extracellular enzymes that accompany their communities have been found to be useful as environmental bioindicators because of sensitivity to changes in soil/ water status as well as their role in soil biogeochemical processes, there has not been such a study that demonstrates the utility of microbial and functional status of soil communities as indicators of wetland and non-wetland land status. It may be hypothesized that the wetland soils bacterial communities would be ecologically distinct in their biotic and abiotic properties. Root-associated bacterial diversity can be higher in some plants when compared to others. Studying the root-associated bacterial communities of wetlands points to many species of bacteria involved in the methane-oxidizing, sulfur/ sulfate reducing, total nitrogen, phosphorus and organic matter removing bacteria. It's the presence of these bacteria which determine their nutrient removal capacity in a constructed wetland. The ecosystem functions of these bacterial species are needed to fully understand how effective *Phragmites* (common reed) dominated wetlands are for phytoremediation. (Li *et al.*, 2013). Attempts have also been made to study bacterial community structure and diversity in rhizospheric soils of *Phragmites australis, Scirpus mariqueter and Spartina alterniflora* in a river estuary by constructing 16S ribosomal DNA (rDNA) clone libraries (Wang *et al.*, 2007). This analysis indicated that changes in bacterial composition occur with the shift of species composition in plant communities.

The application of constructed wetland harboring macrophytes (*Phragmites, Typha, Juncus, Spartina* and *Scirpus)* has come up as a promising method for cleaning up varied types of effluents e.g. starting from domestic, agricultural and industrial sources. Its success can be revealed by its potential in terms of significant reduction in BOD, COD, suspended solids, total solids, total nitrogen, heavy metals along with remediation of xenobiotics, pesticides and polyaromatic hydrocarbons as the root zone of these plants serves as an active and dynamic zone for the microbial degradation of organic and sequestration of inorganic pollutant resulting in successful treatment of domestic, textile and other effluents. In many cases, a significant amount of metals and other organic pollutants are found to accumulate in plant parts *i.e.* stem leaves, roots. A progressive and novel approach when applied to such systems to overcome loading limits and improving removal efficiency by using techniques like bioaugmentation, biostimulation and genetically engineered plant/microbe can lead to successful and promising results. The large scale adoption of this technology needs many practical experiments to be done in lab and pilot scale, so that when this would be taken in real conjunction with actual remediation schemes; it will achieve the multipurpose of wastewater treatment, eco-friendly approach and biomass reuse.

The lacunae which have been identified are the lack of a sensible model for the wetland study and its successful implication. An attempt has to be made to understand the system in order to increase its potential by using microbial inoculants has never been carried out. Based on these lacunae, Response surface methodology (RSM), a collection of mathematical and statistical techniques for empirical model building was employed. The development of mathematical models to explain and predict wetland's behavior is still in an early stage, especially in correlating predicted and experimental value. It is believed that the development of a model which can adequately describe flow conditions may represent an important contribution for modeling the overall performance of CW's. In the present study treatment potential of a *Phragmites*- vegetated CW at lab scale was evaluated with the inlet flow rate, outlet flow rate and retention time as the independent variables and response surface methodology was used to describe the treatment process to understand the effect of two or more independent variables on the dependent variable. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. There are many instances from the literature which shows a successful implication of RSM. Using RSM in wetlands is the relatively new implication of this model. The effect of various independent variables on dependable variables is studied to reach at standardized flow rate equations. The present proposal is intended to phytoremediate the wastewater from the selected site in a *Phragmites* vegetated horizontal flow constructed wetland. Therefore, it is the objective of this observational study to characterize the soil bacterial communities in a functional wetland ecosystem to see the active microbial properties.

Chapter 2 Review of literature

2.1 Health impacts of polluted water

Water is referred to as polluted when it is impaired by anthropogenic contaminants and either does not support a human use (like serving as drinking water) or undergoes a marked shift in its ability to support its constituent biotic communities. The effects of water pollution are not only devastating to people, but also to animals, fish, and birds as it becomes unsuitable for drinking, recreation, agriculture, industry and also diminishes the aesthetic quality of lakes and rivers. More seriously, contaminated water destroys aquatic life, reduces its reproductive ability and eventually becomes a hazard to biota by entering in food chains (Rai, 2008). The effects of water pollution include poisoned drinking water and seafood animals (organism bio-accumulate toxins in their body from the environment over their life spans). The specific contaminants leading to pollution in water include a wide spectrum of chemicals, pathogens attributing to physical and sensory changes. Many of the chemical substances are toxic. Pathogens can produce waterborne diseases in either human or animal hosts. Alteration of water's physical chemistry includes changes in acidity, electrical conductivity, temperature and eutrophication.

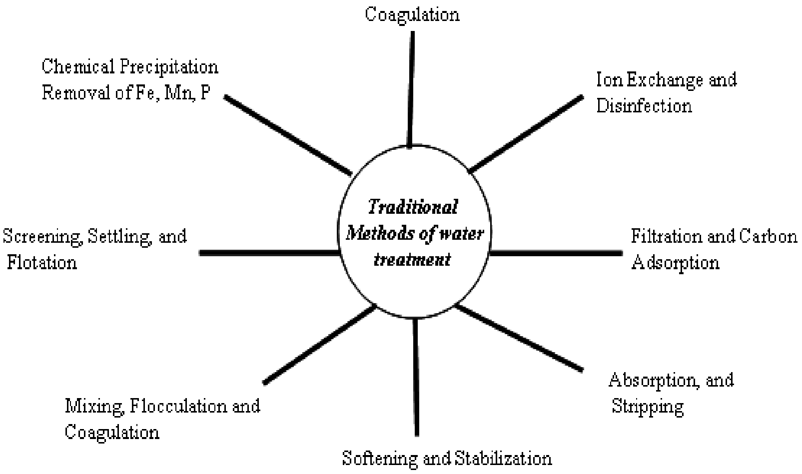


Fig. 1 Traditional treatment technologies

The traditional treatment process involves three stages, *primary, secondary* and *tertiary*. In primary treatment, solids are separated from the wastewater stream and in a secondary dissolved biological matter are progressively converted into a solid mass by using indigenous, waterborne micro-organisms. Finally, in tertiary, the biological solids are neutralized then disposed of or re-used, and the treated water may be disinfected chemically or physically (for example by lagoons and micro-filtration). Some of these methods are listed in Fig. 1.

2.2 Constructed Wetlands

The Ramsar convention brought wetlands to the attention of the world and proposed the following definition: *Wetlands are areas of marsh, fen, peat land or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed 6 m.* (Ramsar convention on wetlands 1997). Some common plants used in wetlands (natural or constructed) are listed in Table 1.

Constructed wetlands are a complex biological system that mimics natural self cleansing processes (Begg *et al.*, 2000) by reducing pollutant level to a dischargeable limit. In fact these can be treated as nature's kidneys. Root morphology and depth are important plant characteristics for phytoremediation. A fibrous root system, such as found in grasses (e.g., fescue), with numerous fine roots spreading throughout the soil, provide maximum contact with the soil due to the high surface area of the roots. A tap root system (such as in alfalfa) is dominated by one larger central root where root depth directly impacts the depth of soil that can be remediated or depth of ground water that can be influenced, as close contact is needed between the root and the contaminant or water (Pivetz, 2001). The universally used plant species are *Phragmites* (IWA 2000; Scholz, 2006) commonly called reeds which contribute to wastewater cleaning processes by increasing the permeability and porosity of substrate (Gampel, 2003), creating micro sites with reducing conditions by releasing oxygen from the roots termed as ROL (Radial oxygen loss) (Ivandi *et al.*, 1998; Tanner, 2000). Through these oxygenated and oxygen poor micro sites even resistant chemicals get affected (Gampel, 2003). The withered parts insulate the root zone during the cold period. So in the temperate climates the pollutant removal capacity is affected only slightly the seasons (Vymazal, 2000).

Table 1Common macrophytes used in constructed wetlands

Plant	Common name	Family	Reference
Schoenoplectus spp.	Club-rush	Cyperaceae	Dunbabin and Bowmer, 1992
Cyperus spp	Papyrus sedges	Cyperaceae	Dunbabin and Bowmer, 1992
Populus spp	Poplar Trees	Salicaceae	Reinartz and Warne, 1993
Salix spp	Willow Trees	Salicaceae	Reinartz and Warne, 1993
Kochia spp	Forage Kochia	Amaranthaceae	Vinton and Burke, 1995
Lemna minor	Duckweed	Lemnaceae	Debusk *et al.*, 1996; Prasad and Freitas, 2003
Poa pratensis	Kentucky Bluegrass	Poaceae	Rice *et al.*, 1997
Ceratophyllum spp.	Coontail	Ceratophyllaceae	Greenway, 1997
Medicago sativa	Alfalfa	Fabaceae	Rice *et al.*, 1997
Phragmites spp.	Reed	Poaceae	Ye *et al.*, 1997; Yu *et al.*, 2005; Lesage *et al.*, 2007
Sagittaria spp.	Common Arrowhead	Alismataceae	Emerson *et al.*, 1999
Carex spp.	Sedges	Cyperaceae	Wand *et al.*, 2002
Scirpus spp	Bullrush	Cyperaceae	Cynthia, 2002; Bhattacharya *et al.*, 2006
Potamogeton spp.	American pondweed	Potamogetonaceae	Wu and Guo, 2002
Juncus spp.	Rush	Junaceae	Marisa *et al.*, 2004
Paspalum distichum	Knotgrass	Poaceae	Yu *et al.*, 2005
Sparganium erectum	Bur-reed	*Typh*aceae	Blankenberg *et al.*, 2005
Phalaris arundinacea	Reed canary grass	Poaceae	Blankenberg *et al.*, 2005
Glyceria fluitans	Floating Sweet-grass	Poaceae	Blankenberg *et al.*, 2005
Typha latifolia	Cattail	*Typh*aceae	Xu and Jaffe, 2006
Spartina spp.	Coralgrass	Poaceae	Czako *et al.*, 2006
Oryza sativa	Common rice	Poaceae	Chu *et al.*, 2006
Eichhornia crassipes	Water Hyacinths	Pontederiaceae	Prasad and Freitas, 2003; Jayaweera *et al.*, 2007

Actually the plant forms a thick root-mass, which transports oxygen from the atmosphere to the root zone aiding microbial digestion. A complex microbial population develops in soil, which removes organic nutrients competes with and eliminates human pathogens. This biological filter also removes particulate solids, dissolved organics and heavy metals. A number of media (Gravel, soil, gravel/soil mixes and gravel/soil/organic fiber) can be used in the bed, depending on the situation and the quality of the effluents coming in. Apart from reducing the BOD (Biological Oxygen Demand) of the effluent to safe levels, horizontal reed beds are particularly useful in converting nitrates into nitrogen gas, which completes the N-Cycle. Horizontal reed beds are used for secondary or tertiary treatment systems and more often fall in a multistage systems.

Phragmites australis is a cosmopolitan, highly resilient and extremely productive emergent macrophyte which frequently forms dense, clonal stands in the littoral zones of lakes, freshwater swamps, wetlands, along rivers and canals with wide geographical distribution. It adapts to water level fluctuations and has been used for wastewater treatment worldwide (Brix, 1999). Its root system bears soil-penetrating rhizomes that firmly anchor the plant in water-saturated environments and oxygenates the soil creating an aerobic environment for rhizosphere microorganisms. Many reports have been published revealing contribution of macrophytes for removal of varied pollutants.

The scientific classification of *Phragmites australis* is given as:

Kingdon: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Poales
Family: Poaceae
Genus: *Phragmites*
Species: *australis*
Binomial name: *Phragmites australis*
(Cav.) trin ex steud.

Aquatic macrophytes (*Phragmites*) are adapted to waterlogged conditions (wetland conditions) by means of transporting oxygen (air) to the roots through their aerenchyma tissue (Armstrong *et al.*, 1992, 1996). The radiant flux/ channeling of oxygen from the roots to the rhizosphere called radial oxygen loss (ROL) is essential for respiration, creates partially aerobic condition in the waterlogged condition and causes the formation of an oxidative protective film, also called 'plaque', on the root surface. Oxidation of the rhizosphere is especially important in SSF (sub surface flow) CWs of the horizontal flow type as it creates aerobic microsites within the otherwise predominantly anaerobic substrate (Stottmeister *et al.*, 2003). The plaques are mainly composed of Fe- (Oxy-) hydroxides and other metals that are co-precipitated on the surface. The macrophytes transport approximately 90% of the oxygen available in the rhizosphere stimulating both aerobic decomposition of organic matter and the growth of nitrifying bacteria (Brix, 1997; Scholz, 2006). However, when compared to microorganisms, macrophytes only play a secondary role in the degradation of organic matter in wetland systems (Stottmeister *et al.*, 2003).

2.2.1 Design and construction of wetlands

Constructed wetlands are technologically developed system that is meant for treatment of wastewater utilizing natural processes, wetland vegetation, soil and associated rhizospheric bacteria (rhizoremediation). They differ from natural wetlands in the aspects that the functioning is in a controlled manner. The main parts of constructed wetlands are properly designed treatment tank, graded filling material, acclimatized microbial community and selected plants. A typical system can be divided into three sections: inlet, vegetation and outlet. Prior to inlet section is a pre-settlement tank with a continuous inflow. Vegetation section, the principal component of constructed wetlands is composed of plants having the ability to accumulate some compounds in large concentration as compared to environments and also removes nutrients in wetlands (Brix, 1997; Koottatep and Polpresert, 1997; Kivaisi, 2001; Clarke and Baldwin, 2002, Matheson *et al.,* 2002; Mbuligwe, 2005). It is composed of gravel bed on the sides and inside planted with macrophytes which lead to the outlet section connected to the collection tank. Various media sizes have been tried, but gravel bed with smaller diameter seems to work best as larger diameter results in turbulent flow. Smaller media also have the advantage of more surface area for microbial activity and adsorption. The length/ width ratio is also important for determining designs. Ratios around 4:1 are preferable as long beds have an inadequate hydraulic gradient and tend to result in water above the bed surface. The depth of the media is usually between 1-3 feet and most commonly 2 feet. The graphical model of a wetland system with different sections is shown in Fig. 2.

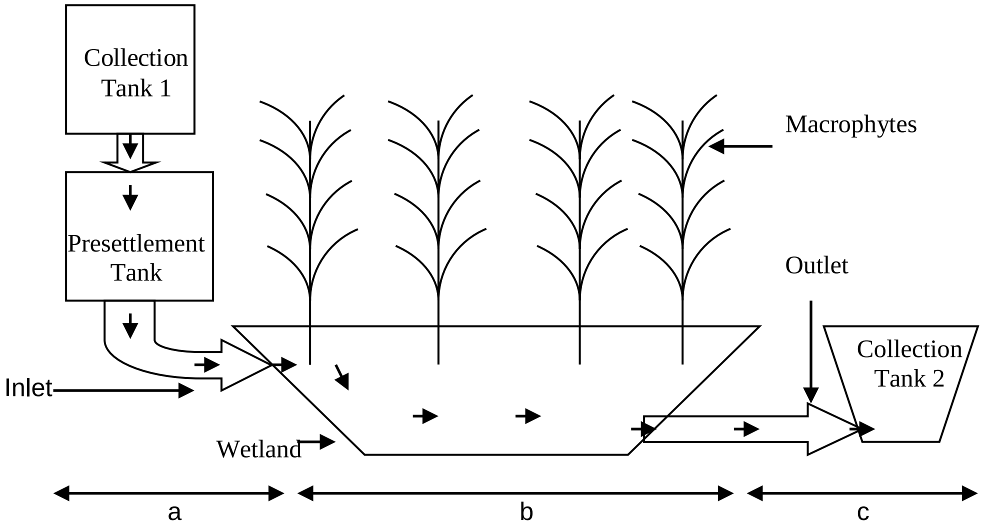


Fig. 2 Design of a wetland system (a) Inlet section (b) Vegetative section (c) Outlet section

Depending upon the design, CW's are categorized as free water surface and subsurface flow wetlands. Free water surface wetlands are like most natural wetlands where the water surface is exposed to the atmosphere, whereas in subsurface wetlands the water surface is below the ground level. The typical water depth of a surface flow constructed wetland is less than 0.4 m (Kadlec and Knight, 1996). The wetlands can also be categorized depending upon the direction of flow of water as: HSSF (Horizontal subsurface flow) and VSSF (Vertical subsurface flow). A hybrid constructed wetland is the combination of a vertical flow and a horizontal flow subsurface constructed wetland providing a means for more effective treatment efficiency. Two combination approaches; vertical flow-horizontal flow and horizontal flow-vertical flow has also been studied extensively in the literature (Vymazal, 2005).

2.2.2 Rhizoremediation

Bioremediation using plants has been naturally coupled with microbial remediation in the form of rhizospheric bacteria termed as ‘rhizoremediation’ where organic and nutrient removal is mostly performed by attaching micro biota (Tanner, 2000). Rhizoremediation involves interactions of plant roots and associated microbes to remediate elevated concentrations of some compounds; present as solid, liquid or gaseous substrates (Pilon-Smits, 2005; Salt *et al.*, 1998). Such interactions offer very useful means for treating water contaminated with recalcitrant organic compounds (Chaudhary *et al.*, 2005). Some co-metabolized (Cometabolism is defined as the oxidation of non growth substrates during the growth of an organism on another carbon or energy source) recalcitrant pollutants such as pesticides are only transformed and not effectively mineralized by microorganisms (Paul *et al.*, 2005). Microbes living in the rhizosphere termed rhizomicrobia also promote plant health by stimulating root growth (regulators), enhancing water and mineral uptake and inhibiting growth of pathogenic and non-pathogenic soil microbes (Kuiper *et al.*, 2004; Pilon-Smits, 2005). Rhizomicrobia may also accelerate remediation processes by volatilizing organics such as PAHs or by increasing the humification of organic pollutants (Salt *et al.*, 1998).

The success of a plant species as the spot of rhizoremediation depends on 1) highly branched root system to harbor large numbers of bacteria, 2) primary and secondary metabolism, and 3) establishment, survival, and ecological interactions with other organisms (Kuiper *et al.*, 2004). The rhizosphere of plants acts as a microcosm where microbial activity is enhanced leading to active degradation of recalcitrant compounds and reduction in parameters like BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand), TS (Total Solids) and salt level from various effluents like acid mine drainage, agricultural landfill and urban storm-water runoff. Some contaminants are also released into the environment as a result of spills (fuel, solvents), military (explosives, chemical weapons), agricultural (pesticides, herbicides), industrial (chemical, petrochemical) and wood treatment activities and get degraded in the root zone of plants or taken up, followed by sequestration or volatilization. Organic pollutants that have been successfully phytoremediated include organic solvents (trichloroethylene), herbicides (atrazine), explosives (trinitrotoluene), petroleum hydrocarbons (oil, gasoline, BTEX, monoaromatic hydrocarbons), and PAHs (polyaromatic hydrocarbons, MTBE, PCBs). Inorganic pollutants, mostly occur as natural elements and human activities such as mining and traffics promote their release into the environment, leading to toxicity. Inorganics like plant trace elements (Cr and Zn), non-essential elements (Cd and Hg) and radioactive isotopes cannot be degraded but are transformed via stabilization or sequestration in harvestable plant tissues (Pilon-Smits, 2005). The section ahead describes the literature related to remediation of such compounds in CW's.

2.2.3 Remediation process

From the earlier studies, it is deduced that combinations of the various phytoremediation processes may occur simultaneously or in sequence for a particular contaminant, or different processes may act on different. The identifying characteristics associated with wastewater are high BOD and COD value, suspended and dissolved solids, the presence of heavy metals and xenobiotics. Biological characteristics include coli-forms and other types of bacteria. When such water is subjected to a standardized retention time in a constructed wetland, the pollutant/ contaminant load comes down to allowable limits to be discharged into the environment. The major threats to human health are from heavy metals. Removal of heavy metals occurs mainly by binding to soils, sediments and particulate matter or precipitation as insoluble salts and uptake by bacteria, algae and plants. The major proportion of heavy metal removal is accounted for by binding processes within wetlands (Kadlec and Keoleian, 1986). Due to their positive charge, the heavy metals are readily absorbed, complexed and bound with suspended particles, which subsequently settle on the substrate. The precipitation of heavy metals as insoluble salts such as carbonates, bicarbonates, sulfides and hydroxides is another process that leads to their long term removal. These salts are formed as a result of reaction between heavy metals with other chemicals and lead to precipitation and settling of metal salts (Sheoran and Sheoran, 2006). Some wetland plant species have been found to have a property of heavy metal tolerance and accumulation *e.g. Typha latifolia*, *Glyceria fluitans* and *Phragmites australis* (Wu, 1990). Table 2, 3 and 4 depict the literature relating to heavy metal tolerance/ accumulation (Table 2); ability to treat different effluents (Table 3) and the capacity of remediation potential of different xenobiotics (Table 4).

Table 2: Remediation potential of different metals using wetlands

Metal	Vegetated plants	Removal mechanism	Removal statistics	Case study	References
Pb, Cd	*Typha domingensis*, *Lemma minor*	Bioaccumulation by plants	Potential metal removal rates are 3-8 mg Pb/m^2 day and 2-4 mg Cd/m^2 day	Wetland microcosms	Debusk *et al.*, 1996
Zn, Pb, Cd	*Phragmites*	Metal tolerance, uptake and accumulation	Zn in shoots: 47- 2049 µg g^{-1} d. wt Zn in roots: 100- 6684 µg g-1 d. wt Pb in shoots: 2.5- 80 µg g-1 d. wt Pb in roots: 8.4- 830 µg g-1 d. wt Cd in shoots: 0.3- 7.4 µg g-1 d. wt Cd in roots: 2.5- 49 µg g-1 d. wt	Metal accumulation in seedlings from two different sources under glass-house conditions	Ye *et al.*, 1997
Cu	*Phragmites australis*	Phytoextraction	Bioconcentration factor (BCF) increased from 349 to 1931 with an increasing Copper concentration from 7.85 to 78.5µm	Hydroponic experiment at different Cu concentrations	Ali *et al.*, 2001
Cu	*Scirpus californicus*	Copper immobilization in wetland	Shoots and roots of *S. californicus* sorbed 0.6% and 1.9%, respectively, of copper entering the system	Eight-acre constructed wetland treatment system receiving copper-contaminated water	Cynthia and John, 2002
N, P	*Phragmites*	N- reduction, P-immobilization, physical settlement of solids	51% reduction for total N, 13% total P, 84-90% for suspended solids and 49% for BOD	Constructed surface flow wetlands at Ireland	Healy and Cawley, 2002
Cd, Cu, Zn	*Juncus*	Phytoextraction, Phytostabilization	Plant bioaccumulation was only observed for Cd, Cu, and Zn, being similar for Cd at the two sites and significantly higher for Cu and Zn, 9	Estuarine environment	Marisa *et al.*, 2004

			and 4 times higher, respectively		
Pb, Zn	*Phragmites,Typha latifolia, Paspalum distichum*	Reduction in TSS, Pb, Zn, Cd, Cu %	99%, 98%, 75%, 83% and 68% reduction in Pb, Zn, Cd, Cu and TSS respectively	Reduction rates of contaminants in a treatment wetland, South China	Yu *et al.*, 2005
Cr	*Typha latifolia, Phragmites australis*	Abiotic reduction, precipitation and accumulation of Cr (III) in the sediments	Cr (VI) removal rates were 0.005 to 0.017 mg L^{-1} d^{-1}, 0.0003 to 0.08 mg L^{-1} d^{-1}, and 0.004 to 0.13 mg L^{-1} d^{-1} for the control, *T. latifolia*, and *P. australis* microcosms, respectively.	Greenhouse and bench-scale microcosm experiment	Xu and Jaffe, 2006
Se	*Phragmites,Typha*	Phytoextraction, Phytostabilization	25-74 µg l^{-1} reduction in treated water	Established outdoor SSF wetland	Azaizeh *et al.*, 2006
Cu, Cd, Ni, Pb, Zn	*Phragmites australis*	Adsorption	After 3 cycles of adsorption-elution, the adsorption capacity regained completely and desorption efficiency of metal around 90%	Reed biomass used as biosorbent from aqueous solution	Southichak *et al.*, 2006
Hg	*Transgenic Spartina alterniflora*	Converting ionic Hg into elementary Hg and volatilization from the plant	-	-	Czako *et al.*, 2006
Mn, Ni, Cu, Zn, Pb	*Scirpus littoralis*	Metal uptake by plant	Accumulation of Mn, Ni, Cu, Zn, Pb upto 494.92, 56.37, 144.98, 207.95 and 93.08 ppm dry wt in 90 days time	Metal accumulation studied under water-logged and field conditions for 90 days	Bhattacharya *et al.*, 2006
Pb, Mn, Cr	*Scirpus americanus, Typha latifolia*	Accumulation in plant parts	Nearly 100 %Removal of Pb, Cr and 71-100 % for Mn during 6-8 days of	Accumulation of metals by *in vitro* raised plants in	Santos-Díaz *et al.*, 2007

			experimentation	supplemented MS media	
Cu, Zn, Cd	*Typha*	Activity of the indigenous soil microflora and plant enzymes	-	Constructed wetland	Gurudeva *et al.*, 2007
Cd, Cu, Zn, Pb, Cr, Ni, Al, Fe, Mn	*Phragmites*	Accumulation in plant parts	12- 62 mg m-2 y-1 accumulation of metals in VSSF in leaves and 38-88 mg m-2 y-1 in stems 23-56 mg m-2 y-1 accumulation of metals in HSSF in leaves and 38-88 mg m-2 y-1 in stems	Combined CW in Belgium treating domestic wastewater	Lesage *et al.*, 2007
Cd, Cr, Cu, Hg, Mn, Ni, Pb, Zn	*Phragmites*	Accumulation in plant parts	Root Cd:1.13 ± 0.08, Cr: 6.97 ± 0.19, Cu:14.98 ± 0.93, Hg: 5.22 ± 0.38, Mn:475.80 ± 11.91, Ni:9.12 ± 0.20, Pb:16.54 ± 0.97, Zn :104.10 ± 9.28 Rhizome Cd:1.00 ± 0.08, Cr:1.52 ± 0.06, Cu:4.33 ± 0.32, Hg:3.19 ± 0.26, Mn:37.51 ± 2.82, Ni:1.67 ± 0.14, Pb:15.30 ± 0.93, Zn:32.67 ± 2.36 Stem Cd:0.68 ± 0.06, Cr:0.40 ± 0.04, Cu:2.31 ± 0.28, Hg:1.05 ± 0.12, Mn:27.92 ± 2.34, Ni:0.48 ± 0.08, Pb: 9.87 ± 0.80, Zn:10.04 ± 0.87	*Phragmites australis* and the corresponding water, sediment samples from the mouth area of the Imera Meridionale River (Sicily, Italy)	Bonanno and Giudice, 2010

			Leaf Cd:1.05 ± 0.10, Cr:0.69 ± 0.04, Cu:4.13 ± 0.19, Hg:1.73 ± 0.23, Mn:308.30 ± 11.47, Ni:1.69 ± 0.15, Pb:13.20 ± 0.74, Zn:28.40 ± 1.72		
Cd, Zn, Cr, Cu, Ni, Pb	*Phragmites*	Metal mobility and uptake	Plumes :19 to 117 µg kg−1 DM Cd, 98 to 408 µg kg−1 DM for Cr, 3.1 to 7.0 mg kg−1 DM for Cu, 0.5 to 2.3 mg kg−1 DM for Ni, 0.4 to 4.5 mg kg−1 DM for Pb, 36 to 132 mg kg−1 DM for Zn	Intertidal marshes in the Scheldt estuary	Du Laing *et al.*, 2009
Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Se, Sn, V, U, Zn	*Phragmites*	Accumulation in plant parts	Roots > Rhizomes > Leaves > Stems	our constructed wetlands with horizontal sub-surface flow (HF CWs) designed for treatment of municipal sewage in the Czech Republic	Vymazal *et al.*, 2009
Cd, Zn, Cr, Cu, Ni, Pb	*Phragmites australis*	Accumulation in plant parts	Rhizomes > Stems > Leaves	Experimental constructed wetland (CW) sited in Castelnovo Bariano	Bragato *et al.*, 2009
Pb, Cu, Zn	*Phragmites*	-	-	-	Liu *et al.*, 2010
Zn, Pb	*Typha*	Accumulation in tissues, precipitation as iron-hydroxides in root zones	0- 99% and 0-64% reduction for Zn and Pb respectively in pond 1 and 94-99% for Zn and 25-60% for Pb in pond 2, 69% removal rates of sulphate in each	Series of subsurface flow ponds filled with spent mushroom substrate constructed at Navan, Ireland	O'Sullivan *et al.*, 2001

Zn	*Phragmites australis, Acorns calamus, Scirpus tabernaemontani*	Accumulation in plant parts	*Scirpus tabernaemontani*: Removal effects were 31050.84 mg/kg (10206.67 mg/kg in above-ground parts and 20844.17 mg/kg in under-ground parts) *Acorus calamus* and *Phragmites australis*, the highest accumulation concentrations of zinc ion were 54130.67 mg/kg (16774.00 mg/kg in above-ground parts and 37356.67 mg/kg in under-ground parts) and 25423.34 mg/kg (4506.67 mg/kg in above-ground parts and 20916.67 mg/kg in under-ground parts)	Hydroponically cultured	Ren *et al.*, 2011
Cu, Zn, Cd, Pb	*Phragmites australis*	Phytoextraction	Cu, Zn, Cd: soil > *Phragmites australis* of aerial part> *Phragmites australis* of underground part Pb: Soil > *Phragmites australis* of aerial part≈ *Phragmites australis* of underground part	River wetland system	Hou *et al.*, 2011
Cu, Zn	*Typha latifolia, Phragmites australis*	Accumulation in plant parts	Cu, Zn: 80 and 91% for unplanted control, 83 and 92% for cattail, and 83 and 92% for reed wetland	River water contaminated by swine confined-housing operations	Yeh *et al.*, 2009
Hg	*Phragmites australis*	Accumulation in plant parts	Root (0.321±0.05 BCD) exhibited the highest Hg accumulation followed by rhizome (0.245±0.04	Hg-contaminated coastal lagoon	Anjum *et al.*, 2012

			BCD) and leaves		
Cd, Cr, Cu, Fe, Ni, Pb	*Phragmites cummunis, Typha angustifolia, Cyperus esculentus*	Accumulation in plant parts	P. cummunis was in the order of Fe (2813) > Mn (814.40) > Zn (265.80) > Pb (92.80) > Cr (75.75) > Cu (61.77) > Ni (45.69) > Cd (4.69) T. angustifolia:Fe > Mn > Zn > Cr > Pb > Cu > Ni > Cd C. esculentus:Fe > Mn > Zn > Pb > Ni > Cu > Cr > Cd	Plants grown in aqueous solution	Chandra and Yadav, 2011
Cr, Pb, Zn, Cu	*Spartina alterniflora, Phragmites australis*	Metal uptake	Cu: 300 microg/g in leaves, <200 microg/g in stems Zn: 500 microg/g in sediments, Cu: 200 microg/g Metal concentrations lower in stems than in leaves, and Cr, Pb, and Zn were lower in P. *australis* than in S. alterniflora	Metal-contaminated salt marshes study	Windham *et al.*, 2004
As, Cu, Fe, Mn, Pb, Zn	*Spartina maritima, Spartina densiflora*	Phytostabilization, Bioaccumulation	Zn in tissues: 27 to 1249 ppm and from 42 to 2326 ppm for S. densiflora and S. maritima Cu in tissues: 22 to 2546 ppm and from 27 to 4933 ppm for S. densiflora and S. maritima Pb: 0.1 to 217 ppm and from 0.1 to 292 ppm for S. densiflora and S.	to study metal accumulation by spartina species in two marshes with different levels of pollution	Cambrolle *et al.*, 2008

			maritime		
Cr, Cu, Pb, Fe, Zn	*Spartina alterniflora*	Phytoextraction	Cr- 3.0ppm, Cu- 7.0ppm, Fe-410ppm, Pb-0.5ppm, Zn-28 ppm in dry soil	Sediments in various locations in Bayou d'Inde in Southwest Louisiana a waterway (industrial and municipal waste streams)	Salla *et al.*, 2011
Cd	*Juncus subsecundus*	Metal accumulation	Cadmium accumulation and removal (except for Cd removal at 20 mg Cd kg−1) by plants was significantly higher in Cd treatments with than without PAH	Glasshouse experiment was conducted to investigate effects of Cd)without orwith PAHs on growth of Juncus subsecundus	Zhang *et al.*, 2012
Cd	*Typha angustifolia*	Hyperaccumulation in plants	Cd conc in Root- 1962.31±32.70 mg/L Cd conc in Leaf - 39.66±1.76 mg/L	Lab scale green house study	Xua, *et al.*, 2011
Al, Fe, Zn, Pb	*Typha domingensis*	Rhizofiltration	Pb2+ > Fe3+ > Al 3+ > Zn2+	Raising plants hydroponically and transplanting them into metal-polluted waters	Hegazy *et al.*, 2011
As, Cd, Pb	*Typha orientalis*	Tolerance Accumulation	-	Greenhouse study	Wang *et al.*, 2011
Cr, Cd, Pb	*Typha angustifolia*	Heavy metal uptake	-	Pot experiment	Bah *et al.*, 2011

Co, Cr, Ni	*Spartina*	Phytostabilization Bioaccumulation	Co in tissues: B0.1 to 35.8 and from B0.1 to 43.4 lg g-1 for S. densiflora and S. maritima Cr in tissues: 2 to 18.8 and from B0.1 to 25.2 lg g-1 for S. densiflora and S. maritima Tissue Ni: B0.5 to 11.1 and from B0.5 to 15.6 lg g-1 for S. densiflora and S. maritime	Metal contaminated site study	Cambrolle *et al.*, 2011
Cr, Ni, Zn	*Typha domingensis*	Heavy metal uptake	BFs: Cr 0.181, Ni 0.247, Zn 0.857 TFs: Cr 0.152, Ni 0.030, Zn 0.197	Primary treatment wetland (wastewater from industrial processes and sewage from the factory)	Hadad *et al.*, 2010
Zn	*Spartina densiflora*	Heavy metal uptake	100–4800 ppm Zn	Glasshouse experiment	Mateos-Naranjo *et al.*, 2008
Zn, Pb, Cd	*Typha latifolia*	Heavy metal uptake	Leaves: Zn: 22-122, Pb: 4-7-40 and Cd:0-2-0-8 //g g~^ d. wt Soil-sediments: Zn: 86-3009, Pb: 26-18894 and Cd: l-4-26//gg-i d. wt Roots: Zn: 46-946, Pb: 25-3628 and Cd: 10-17//g g"^ d. wt	Seedlings were grown in the metal treatment solutions or in the metal-contaminated media under laboratory conditions	Ye *et al.*, 1997
Hg	*Juncus maritimus, Scirpus maritimus*	Phytostabilization Phytoaccumulation	-	Hg-contaminated salt marsh sediment chemical environment	Marquesa *et al.*, 2011

Cr	*Spartina argentinensis*	Hyperaccumulation	-	Glasshouse experiment	Redondo-Gómeza *et al.*, 2011
Zn, Cd, Cu	*Phragmites australis*	Metal accumulation	-	Accumulation in reeds (*Phragmites australis*) in urban sediments from two stormwater infiltration basins	Bedell *et al.*, 2012
Al, Fe, Mn	*Phragmites australis*	Metal accumulation	Root: Al(OH)3 > Al2O3 > Fe3O4 > MnO2 > FeOOH	Heavy metals in the sediment of constructed wetlands	Wang and Jia, 2009
Al, Pb, Cd, Co, Ni, Cr, Fe, Mn, Zn, Cu	*Phragmites australis*	Metal accumulation	Al > Pb > Cd > Co > Ni > Cr micronutrients: Fe > Mn > Zn > Cu	*Phragmites australis* growing at 4 selected sites along the bank of the lower River	Ayeni *et al.*, 2012
Cu	*Phragmites australis*	Metal tolerance, Uptake, Accumulation	Cu concentrations in the PM shoots were higher than in the FS, WB and PB shoots, but lower than in the HK shoots	Two mine sites (Parys UK and Belgium)contaminated with Cu and three ‘clean’ sites (Felixstowe, UK; Wisbech, UK and Mai Po, Hong Kong)were studied under field and glasshouse conditions.	Ye *et al.*, 2003
Cu, Zn, Cd, Pb	*Phragmites karka*	Metal tolerance, Uptake, Accumulation	Cu> Zn> Cd> Pb	-	Yuka *et al.*, 2012

Table 3: Remediation potential of different wastewater using wetlands

Type of wastewater	Vegetated plants	Removal Mechanism	Removal Statistics	Case study	References
Industrial wastewater	*Phragmites, Schoenoplectus, Cyperus, Typha*	Uptake by plants	50% of influent metal load	Constructed wetlands	Dunbabin and Bowmer, 1992
Mineralization and pathogen containing wastewater	*Phragmites*	Treatment in wetland by adsorption to biofilms	2 to 3 log cycle reduction in counts of indicator bacteria	Field scale gravel bed hydroponics constructed wetland	Williams *et al.*, 2000
Mine effluent	*Typha*	Accumulation in tissues, precipitation as iron-hydroxides in root zones	0- 99% and 0-64% reduction for Zn and Pb in pond 1 and 94-99% for Zn and 25-60% for Pb in pond 2, 69% removal rates of sulphate in each pond	Series of subsurface flow ponds filled with spent mushroom substrate constructed at Navan, Ireland	O'Sullivan *et al.*, 2001
Dairy wastewater	*Phragmites australis, Scirpus validus*	Treatment by wetland plants and residing bacteria	Removal rate: TKN 25%, ammonium level 16%, BOD 73%, SS 91%, COD 38%, Faecal coliforms 99%	CW for dairy wastewater	Ibekwe *et al.*, 2003
Nitrogen and Bacterial contaminated water	*Phragmites, Typha*	Plant ammonia assimilation, nitrification, restitution of stored nitrogen in the vegetal tissues	Removal rate: 27% in Kjeldahl Nitrogen, 19% ammonia nitrogen, 4% nitrate-nitrite, 90% for bacteria	Two wetland combined system- one vertical and other horizontal	Keffala and Ghrabi, 2005

Textile effluent	*Phragmites*	Mineralization and degradation	70% removal efficacy	Degradation in VFCW	Davies *et al.*, 2005
Dilute farm effluent, dirty water	*Phragmites*	Biological treatment of wastewater in activated sludge and wetland conditions	Reduction in pH value from 10.9 to 7.6, BOD 821 to 65 mg/l, COD 2005 to 210 mg/l, and ammonium 0.3 to <0.1 for each wetland bed	Aerated sequencing batch reactor containing activated sludge followed by a series of constructed wetlands	Moir *et al.*, 2005
Removal of bacteria in sand columns	*Juncus effuses*, *Phragmites australis*	Predation and lysis	Efficiency of removal upto four orders of magnitude of cfu obtained	Removal of bacteria in planted and unplanted sand columns	Wand *et al.*, 2006
Domestic wastewater	*Phragmites*	Sediment accumulation	Concentrations of Cd, Cu, Pb and Zn in the sediment generally decreased along the treatment path of the CW	Combined CW: two VSSF reed beds followed by two HSSF reed beds	Lesage *et al.*, 2007
Urban runoff	*Phragmites*	Treatment in wetland	Removal performance of planted filters was more efficient and stable after the filters matured compared to that of unplanted filters	Experimental temporarily flooded vertical-flow wetland filters treating urban runoff	Lee and Scholz, 2007
Sludge stabilization	*Phragmites*	Reedbeds aeration	-	Reed bed pilot plant for sludge stabilisation	Bianchia *et al.*, 2011

Industrial wastewater	*Typha latifolia, Phragmites australis*	Uptake by plants and reedbed aeration	High removal of organics from tannery wastewater, up to 88% of BOD5 (from an inlet of 420 to 1000 mg L 1) and 92% of COD (from an inlet of 808 to 2449 mg L 1)	Two-stage constructed wetlands planted with *Typha latifolia* and *Phragmites australis*	Calheiros *et al.*, 2009
Domestic wastewater	*Phragmites australis, Phalaris arundinacea*	Aeration by plant roots	NH_4-N concentration of 29.9 mg/l was reduced to 6.5 mg/l average removal efficiency of 78.3%. Removal of BOD5 and COD amounted to 94.5% and 84.4% Phosphorus removal amounted to 65.4%	Three-stage experimental constructed wetland	Vymazal and Kröpfelováa, 2011
High-strength wastewater	*Typha angustifolia, Cyperus involucratus*	Aeration by plant roots	Average mass removal rates of COD, TKN and total-P at a HLR of 80mmd−1 were 17.8, 15.4 and 0.69 gm−2 d−1	Vertical flow (VF) constructed wetland systems to treat high-strength wastewater under tropical climatic conditions	Kantawanichkula *et al.*, 2 0 0 9

Nitrate-dominant wastewater	*Typha, Scirpus*	Enhanced biological denitrification by fueling heterotrophic microbial activity	Nitrate removal were around 500mgN/(m2 d). Areal removal rate 25% higher in cattail versus bulrush mesocosms. DO in bulrush between 0.5 and 2 mg/L, while DO in cattail mesocosms below 0.3 mg/L	Batch wetland mesocosms	Gebremariam and Beutel, 2 0 0 9
Domestic wastewater	*Phragmites australis*	Decontamination effect of *Phragmites australis*	Decontamination rate: 64.5% for BOD, 68% for COD, 79.7% for SS, 21.0% for Total Phosphorus, 20.7% for total nitrogen	Pilot subsurface horizontal flow constructed wetland	Kalipci, 2011
Nutrient Removal	*Phragmites mauritianus*	Remediation by plants	Conductivity values decreased by 24% and 28% in wetland 1 and wetland 2, TDS decreased by 32% and 28%, Ammonium nitrogen increased by 5% in wetland 1 and 12% in wetland 2, nitrate nitrogen	Horizontal Subsurface Flow Constructed Wetlands	Chale *et al.*, 2012

			decreased by 62% and 56%, Reactive phosphorus concentrations were reduced by 4% and 3%, in wetland 1 and wetland 2		
Municipal wastewater	*Canna*, *Phragmites*, *Cyprus*	Remediation by plants	Removal efficiency: COD 88%, BOD 90%, TSS 92%	Vertical flow constructed wetlands	Sohair *et al.*, 2012

Table 4: Remediation potential of Xenobiotics in various wetlands

Xenobiotics	**Vegetated plants**	**Removal mechanism**	**Removal Statistics**	**Case study**	**References**
Glycol-based deicing agent	*Scirpus spp, Medicago sativa, Poa pratensis*	Degradation	60.4%, 49.6%, and 24.4% of applied [14C]EG degraded to 14CO2 in the *Medicago sativa, Poa pratensis,* and nonvegetated soils, *Scirpus spp.* enhanced the mineralization of [14C]PG by 11% to 19% and [14C]EG by 6% to 20%.	Vegetated water incubation systems	Rice *et al.*, 1997
Atrazine	*Phragmites*	Retention in wetland	Between 17-42% of measured atrazine mass was obtained within 30-36 m of wetland	Amendment of atrazine in a constructed wetland	Moore *et al.*, 2000
Metalaxyl and Simazine (hydrocarbons)	*Typha*	Accumulation in plant parts	Metalaxyl and simazine activity in solution was reduced 34 and 65%	-	Wilson *et al.*, 2000
PAH-degradation	*Phragmites*	Degradation	-	-	Daane *et al.*, 2001
2,6-dimethylphenol, 4-chlorophenol, Naphthalene	*Carex gracilis Juncus effuses*	Degradation	Concentrations of 20 mg/l organic pollutant in the case of 4-chlorophenol, about 30 mg/l naphthalene and 50 mg/l 2,6-dimethylphenol were efficiently eliminated	Hydroponic cultures using sand-bed reactors planted under batch and flow-through conditions	Wand *et al.*, 2002
Oil Spill	*Spartina*	Degradation and accumulation	-	Constructed wetlands	Mendelssohn and Oianxin, 2003
Benzene, Toluene, Ethylbenzene, Xylenes (BTEX)	*Scripus cyperinus, Juncus effuses,*	Phytodegradation, Phytovolatilization	90 % of the BTEX removed	Constructed wetlands	O'Niell and Nzengung, 2004

	Carex lurida, Typha latifolia				
Fenpropimorph, Linuron, Metalaxyl, Metamitron, Metribuzin, Propachlor, Propiconazole	*Sparganium erectum, Phragmites australis, Phalaris arundinacea, Glyceria fluitans, Typha latifolia*	Retention of pesticides in arable soils	3-67 % retention of pesticides (Fenpropimorph, Linuron, Metalaxyl, Metamitron, Metribuzin, Propachlor, Propiconazole)	Constructed wetlands	Blankenberg *et al.*, 2005
DDT, PCBs	*Phragmites australis, Oryza sativa*	Accumulation in plant parts and transformation by reductive halogenation	92.0-95.0 ng DDT in roots and 70.5-78.0 ng in stem of reeds	Glasshouse experiments under hydroponic conditions	Chu *et al.*, 2006
Organic pollutant	*Phragmites*	Biodegradation and plant uptake	>90% removal of Lindane, Pentachlorophenol, Endosulphan and Pentachlorobenzene; 80-90% for Alachlor and Chlorpyriphos; 20% for Mecoprop and Simazine	HSSF pilot plant in Spain	Matamoros *et al.*, 2007
Textile azo dye acid orange 7	*Phragmites australis*	Degradation by plant	-	VFCW treatment	Carias *et al.*, 2008

Eenrofloxacin (ENR), Ceftiofur (CEF), Tetracycline (TET)	*Phragmites australis*	Accumulation in plant parts	Levels of 6 ± 2 lg/L for SWP and 43 ± 5 lg/L for control samples after 7 days,resulting in 94% and 57% of drug removal	Wastewater treatment plants (WWTPs)	Carvalho *et al.*, 2012
Bisphenol A, Bisphenol F	*Phragmites australis*	Biodegradation by rhizosphere bacteria	-	BPA and BPF degradation in the sediment	Tadashi *et al.*, 2009
PAH	*Juncus subsecundus*	PAH degradation	Cadmium accumulation and removal (except for Cd removal at 20 mg Cd kg−1) by plants was significantly higher in Cd treatments with than without PAHs,whereas accumulation of PAHs by plants (except for pyrene in roots at 0 added Cd)	Glasshouse experiment	Zhang *et al.*, 2012
Ibuprofen, Carbamazepine, Clofibric acid	*Typha*	Adsorption on LECA and phytodegradation	removal efficiencies of 96%, 97% and 75% for ibuprofen, carbamazepine and clofibric acid in summer, 26% in removal efficiency was observed for clofibric acid in winter	Microcosm constructed wetlands systems established with a matrix of light expanded clay aggregates (LECA) and planted with *Typha*	Dordio *et al.*, 2010
Terbuthylazine (TER)	*Typha latifolia*	Degradation by plant	-	Degradation pathways of terbuthylazine (TER) by *Typha* latifolia in constructed wetlands	Gikas *et al.*, 2012
4C-labeled 1,4-dichlorobenzene (DCB), 1,2,4-	*Phragmites*	Plant uptake	Plant uptake of DCB, TCB, γHCH was significant with bioconcentration factors reaching 14, 19 and 15	Hydroponic conditions	Miguel *et al.*, 2012

trichlorobenzene (TCB), γ-hexachlorocyclohexane (γHCH)					
Lindane (HCH), Monochlorobenzene (MCB), 1,4-dichlorobenzene (DCB), 1,2,4-trichlorobenzene (TCB)	*Phragmites*	Plant uptake	-	-	Faure *et al.*, 2012
Crude oil	*Typha latifolia*, *Typha angustifolia*, *Phragmites communis*, *Scirpus lacustris*, *Juncus*	Degradation, microbial dissimilatory sulphate reduction and biosorption	Oil content of the water after treatment was decreased to less than 0.2 mg/l from 2–10 mg/l, and the concentrations of heavy metals decreased below the relevant permissible levels	Passive system of the type of the constructed wetlands	Groudeva *et al.*, 2001
Azo dyes	*Phragmites*	Mineralization and degradation	Nearly 70% removal efficacy	Degradation in VFCW	Davies *et al.*, 2005

Experiments have been conducted using one or combinations of these plants planted at a particular site based on the type of pollutant treated (Table 2, 3, 4). A wetland composed of three plants *Phragmites australis, Typha latifolia* and *Paspalum distichum* was constructed to treat Pb/Zn mine drainage (Yu *et al.*, 2005). Tolerance and bioaccumulation of copper (Table 2) in *Phragmites australis* and *Zea mays* have been studied in hydroponic experiments at copper concentration ranging from 0.5 to 157 µM (Ali *et al.*, 2001). Zinc, Lead and Cadmium tolerance, uptake and accumulation have also been reported by *Phragmites* (Ye *et al.*, 1998). Depending upon the nature and quantity of pollutants, hybrid constructed wetlands are used sometimes. A combined constructed wetland designed for treatment of domestic wastewater consisting of two VSSF and two HSSF planted with *Phragmites* has been reported to accumulate Al, Cd, Cu, Fe, Mn, Ni, Pb, and Zn (Lesage *et al.*, 2007).

Studies have been carried out using *Phragmites* vegetated constructed wetland for the treatment of tannery effluent (Calheiros *et al.*, 2007) where significant reductions in BOD and COD was obtained. Wetlands have also been used for the removal of bacterial pathogens (Wand *et al.*, 2006) and as a biosorbent of many metals like $Cu^{2+,}$ $Cd^{2+,}$ $Ni^{2+,}$ Pb^{2+} and Zn^{2+} (Southichak *et al.*, 2006). The reduction obtained in BOD is 85.71%, COD 86.14%, TSS 87.58%, TS 87.64%, total N, 81.55% along with 90-98% of heavy metals like Fe, Pb, Ni, Zn, Mn (Prasad and Freitas, 2003; Chaturvedi *et al.*, 2006). Reed as biosorbent has shown a very high adsorption affinity value (Table 2), which predicts its ability to adsorb heavy metals at low concentration. Some experiments have also been carried out using vertical flow constructed wetland (VFCW). Davies *et al.* (2005) have demonstrated phytoremediation of textile effluents containing azo dyes in a VFCW. Chromium, a heavy metal present in tannery wastewater was removed using *Penisetum purpureum, Brachiaria decumbens, Phragmites australis* (Mant *et al.*, 2006).

A lot of work has been carried out for the treatment of domestic wastewater (Table 3) and its treatment performance was also assessed (Solano *et al.*, 2003; Lee *et al.*, 2007; Hamouri *et al.*, 2007). Nitrogen and bacterial removal in wetlands (Table 3) planted with *Phragmites* and *Typha* has been reported (Keffala *et al.*, 2005). Treatment of wastewater from a dairy parlor (Table 3) was performed (Moir *et al.*, 2005) where significant reductions in BOD, suspended solids, ammonium, nitrate, and phosphorus level has been observed. Gravel bed hydroponics (Table 3) in a constructed wetland system have also been used for the mineralization and pathogen removal (Williams *et al.*, 2000). Variation in endophytic fungal diversity closely associated with root, stem and leaves of *Phragmites australis* has also been reported (Wirsel *et al.*, 2001).

Macrophytes can assimilate pollutants in their tissue and provide a surface and an environment for microorganisms to grow (Vymazal, 2002). Moreover, they create good conditions for the sedimentation of suspended solids (SS) and prevent erosion by reducing the velocity of the water in the wetland. Results also indicate a significant role of macrophytes in heavy metal removal (Table 2).

Behavior of selected organic pollutants (Table 4) has been studied in horizontal flow constructed wetlands (HFCW) which include organochlorine, organophosphorus, phenols, chloroacetanilides, triazine, phenoxycarboxylic acid, phenylurea and pesticides (Matamoros *et al.*, 2007). A wetland composing *Sparganium erectum, Phragmites australis, Phalaris arundinacea, Myostis scorpioides, Auartica dioica* (Table 4) was used to check pesticide retention and 3 to 67% pesticide removal was reported. Accumulation, distribution and transformation of DDT and PCBs by *Phragmites australis* and *Oryza sativa* was also reported (Chu *et al.*, 2006). Biodegradation and plant uptake are postulated as the most likely elimination pathways considering the poor accumulation of these compounds in the wetland bed. Organic pollutants (Table 4) that have been successfully phytoremediated include organic solvents (trichloroethylene), herbicides (atrazine), explosives (trinitrotoluene), petroleum hydrocarbons (oil, gasoline, BTEX, monoaromatic hydrocarbons), and PAHs (polyaromatic hydrocarbons, MTBE, PCBs) (Pilon-Smits, 2005). Inorganic pollutants, mostly occur as natural elements and human activities such as mining and traffics promote their release into the environment, leading to toxicity (Pilon-Smits, 2005) and are successfully remediated through wetlands (Table 3). Inorganic cannot be degraded but are transformed via stabilization or sequestration in harvestable plant tissues (Pilon-Smits, 2005). Wetlands are usually utilized as secondary and even as tertiary treatment because of toxic effects on the aquatic plants due to the high organic loading of the influents (Solano *et al.*, 2003). Treatment mechanisms in wetlands involve naturally abiotic and biotic processes, and their interactions, which can all be mediated by local climate, hydrologic processes, and hydraulic processes. Inherent dynamic characteristics of these processes and interactions will eventually result in varying treatment effectiveness of wetlands.

2.2.4 Remediation statistics

From the review of data as complied in Table 2, an inference can be easily made about the trend of different metals being remediated by wetland plants. Major metals to be remediated in constructed wetlands are Pb (Debusk *et al.*, 1996; Ye *et al.*, 1997; Bonanno and Giudice, 2010), Zn (Ye *et al.*, 1997; Bonanno and Giudice, 2010), Cd (Debusk *et al.*, 1996; Ye *et al.*, 1997; Bonanno and Giudice, 2010), Cu (Bonanno and Giudice, 2010), Cr (Bonanno and Giudice, 2010). These metals can be termed as most common heavy metal pollutant present in wastewater/ soil followed by Al, As, B, BA, Co, Fe, Hg, Mn, Ni, P, Mo, N, As, Se, Sn, U, V. The graph below (Fig. 3) Shows the relative abundance of literature on remediation of heavy metals in last two decades.

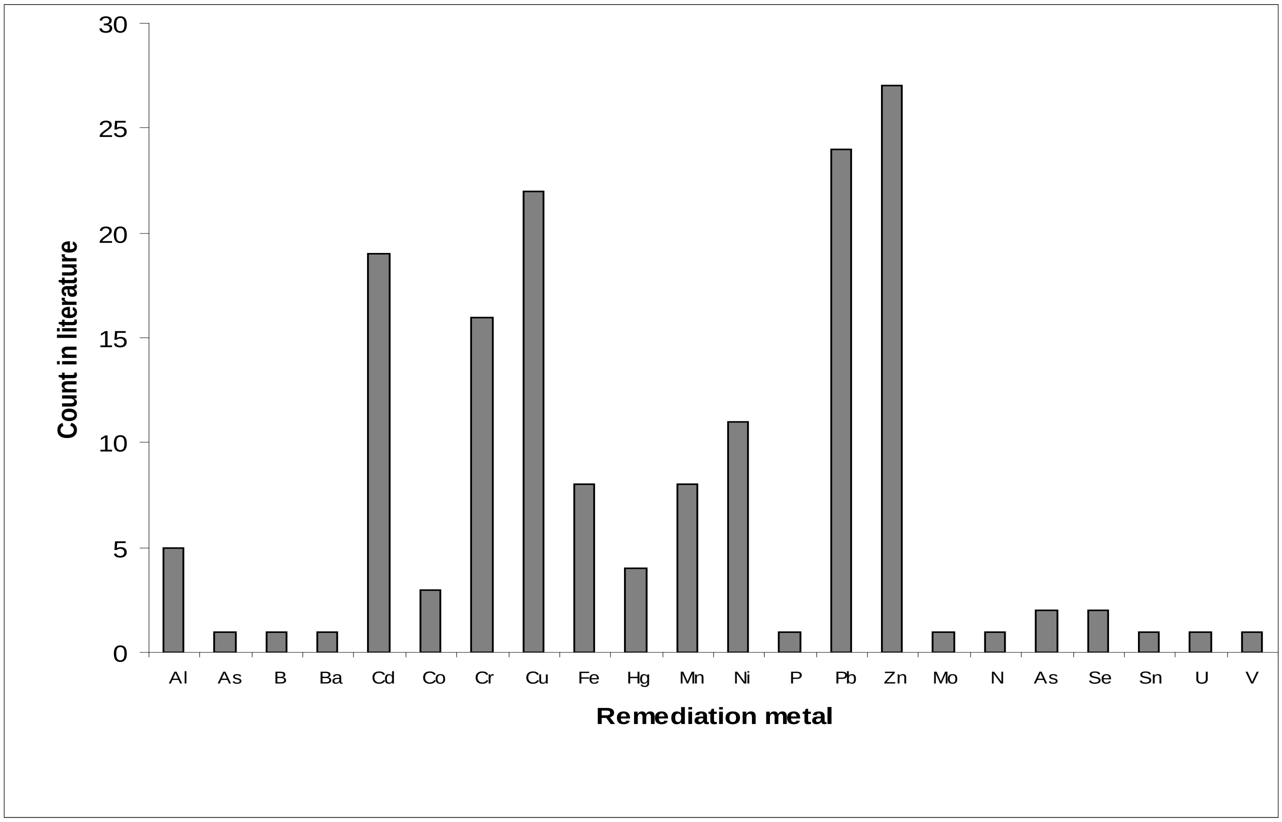


Fig 3: Literature count depicting metals remediate versus count in literature over last two decades

The presence of these metals can be attributed to discharge of industrial, gases, effluents and solid waste into the environment. The above case studies have been done in lab scale, pilot scale, glass house or hydroponically grown cultures later treated with metal spiked water. Most of the literature cited over time explains the data on metal accumulations in various plant parts (root, stem, leaves) and in soil/ sediments. So far there has been no focus on technology mechanisms/ microbe's role/ enzymatic processes involved in the remediation processes. The comparative analysis of remediation of different types of wastewater reveals that *Phragmites* are most commonly used plant species. Around 41% of CW's are vegetated with *Phragmites* solely or in combination with other plant *e.g. Acorns calamus, Scirpus tabernaemontani (*Ren *et al.*, 2011), *Typha latifolia*, (Yeh *et al.*, 2009), *Cyperus esculentus (*Chandra and Yadav, 2011), *Spartina alterniflora (*Windham *et al.*, 2004). *Typha* (23%), *Spartina* (15%), *Scirpus* (8%) and *Juncus* (5%) are other important plants after *Phragmites*. Plants less used include *Cyperus, Acorns, Lemna, Paspalam* (Fig. 4).

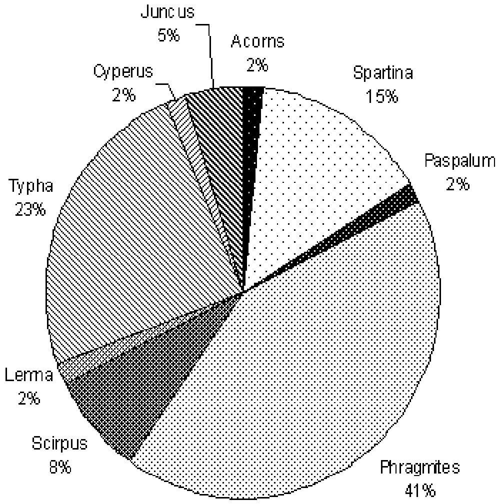


Fig 4: Various plants used for removal of heavy metals

For the treatment of xenobiotics, *Phragmites* are majorly used plants (32%), followed by *Typha* (22%), *Juncus* (11%). Other species less used are *Medicago, Glyceria, Phalaris, Oryza, Carex, Sparganium, Poa, Scirpus* and *Spartina* (Fig. 5). Major remediation processes are accumulation, degradation, mineralization and metabolism due to microbial activities. Xenobiotics (Table 4) largely include pesticides (Fenpropimorph, Linuron, Metalaxyl, Metamitron, Metribuzin, Propachlor, Propiconazole), PAH's (Benzene, Toluene, Ethylbenzene, Xylenes (BTEX) and dyes (Textile azo dye, acid orange 7). Along with degradation, some of these are also retained (Table 4) within soil/ sediments along the length of wetland (Blankenberg *et al.*, 2005; Dordio *et al.*, 2010).

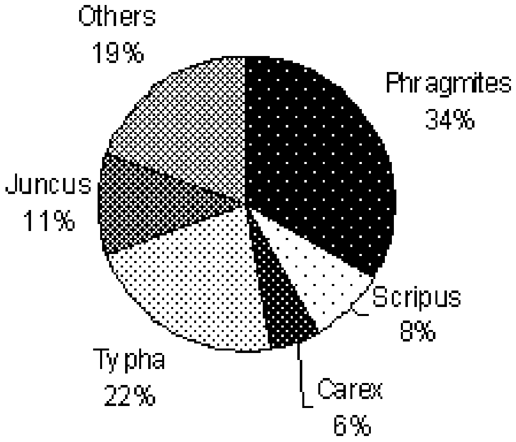


Fig 5: Various plants for treatment of xenobiotics

2.2.5 Applications and advantages of wetlands

The advantages of ecoflo reed bed system over the other water treatment technologies are their use in primary, secondary or tertiary wastewater treatment for private housing/communal/commercial developments because of easy integration in new and existing schemes. Being a living system, reed beds work in harmony with the environment blending visually with the natural landscape, this is particularly important in scenic areas (Reed et al., 1995; Gampel, 2003). These are extremely durable and provide a reliable, long-term solution for wastewater/sewage treatment as per their effectiveness in preventing fecal contamination from reaching wells, reservoirs and surface waters. A well-constructed wetland can effectively remove all suspended solids from wastewater. Reed beds require low-maintenance as its maturation with time essentially looks after itself. These systems are very competitively valued when compared to other treatment systems because unlike conventional systems, these improve with age as the roots mature and expand with time becoming more efficient in biologically filtering wastewater. After the plants have been allowed to grow for some time, they are harvested from the wetlands and either incinerated or composted to recycle the metals. This procedure is practiced till the contaminant level in water comes down to the allowable limit. The biomass generated can be used for biogas production, carrier in solid biofertilizers or compost preparation (Rai, 2008). The biomass devoid of harmful contaminants like heavy/ radioactive metals can be used as fodder.

Constructed either with horizontal or vertical flow these wetlands act as a mechanism to treat non-point source pollution before it reaches lakes, rivers and oceans. Experiments carried out with planted and unplanted reedbeds on same substrate have shown a significant influence on nutrient removal (Drizo *et al.*, 1996; Tanner, 2000). Moreover, the aquatic vegetation in wetlands plays an important role in removing nutrients (Brix, 1997; Koottatep and Polprasert, 1997; Kivaisi, 2001; Clarke and Baldwin, 2002; Matheson *et al.*, 2002; Mbuligwe, 2005). Plants take up nutrients such as N, P, and K from the wastewater, transport oxygen to the root area of the soil to enable aerobic microbes to decompose the pollutant (Allen *et al.*, 1997) and aid in the settling of suspended material by reducing the rate of sewage flow (Gopal, 1999).

Constructed wetlands have been increasingly used throughout the world for water treatment. Introducing certain novel amendments can meet the high standard of reclamation in a given environmental matrix. The microbial components in a wetland can degrade various pollutants and contaminants owing to their capacity to adapt to otherwise inhospitable climate. The rhizosphere of wetland plant is an elevated zone in terms of microbial presence and activity. Adding certain inoculants, not native and having higher remediation potential generates a biased rhizosphere termed as *Designer Rhizospheres* (Gentry *et al.*, 2004). For better performance, such novel amendments (bioaugmentation, biostimulation, pollutant availability, genetic alterations) are discussed below.

2.2.6 Bioaugmentation

Bioaugmentation, the addition of microbes to enhance a specific biological activity, has been practiced intentionally for years in wastewater treatment (Rittmann and Whiteman, 1994). In the constructed wetlands, the role of rhizospheric microbial population is quite active relative to the passive role of vegetation. Certain bacteria having the ability to degrade a particular pollutant/ contaminant based on their natural, non-engineered metabolic processes can be employed for the remediation purpose (Vogel, 1996). This use of rhizomicrobial populations present in the rhizosphere of plant for bioremediation is termed as rhizoremediation (Anderson *et al.*, 1993; Kuiper *et al.*, 2004) and when microbial populations are added from outside source, then it is known as bioaugmented rhizoremediation (Kuiper *et al.*, 2001; 2004; Ronchel and Ramos, 2001; Sriprang *et al.*, 2002). In most of the cases, the bioaugmentation impact on indigenous microbes is often overlooked keeping remediation as a primary goal. Addition of microbes to the soil can potentially result in establishment of new microbial population, shifts in microbial population or transfer of genetic material (like plasmids harboring metal/ antibiotic resistance genes) to indigenous population which is not its primary goal (Gentry *et al.*, 2004). The laboratory scale results of seeding microbes for degradation of soil pollutants have been ambiguous (Kuiper *et al.*, 2004).

2.2.7 Biostimulation

The intentional stimulation of resident xenobiotic degrading bacteria by the addition of electron acceptors, water, nutrients or electron donor termed biostimulation can also be employed to speed up remediation processes (Madsen *et al.*, 1991; Widada *et al.*, 2002). However, in many cases, the fertilization practice of contaminated site using compost, nitrogen, phosphorus and carbon has been unpredictable because it has been reported to enhance and not degrade the pollutants (Ramadan *et al.*, 1990; Wang *et al.*, 1990; Brodkorb and Legge, 1992; Namkoong *et al.*, 2002; Kuiper *et al.*, 2004). In case the degradative bacteria is absent in indigenous microbial population, bioaugmentation can be employed by introducing either wild type or genetically modified microbes into soil (Kuiper *et al.*, 2004).

2.2.8 Pollutant availability

The bioavailability of organic compounds is the most important factor that determines the overall success of a bioremediation process (Salt *et al.*, 1998; Chaudhry *et al.*, 2005). The availability of pollutants to the organisms or bio-availability depends upon the chemical nature of the pollutant (hydrophobicity, volatility, binding capacity, reactivity) and soil properties (particle size, water and organic content, cation exchange capacity, pH). Many chemicals, plant/ microbe exudates and secondary plant products are potential enhancers of pollutant bio-availability. Artificially this can be improved by adding soil amendments in the form of surfactants like Triton-X 100, SDS (Salt *et al.*, 1998; Kuiper *et al.*, 2004, Pilon Smits, 2005). In soil polluted with organic chemicals, a combined stress might enhance the degradation (Chaudhry *et al.*, 2005). In the field of root technology, certain strains of naturally occurring soil bacteria *Agrobacterium tumefaciens* has been used to induce root proliferation in order to increase the length and mass of plant roots and thus the degradation (Chaudhry *et al.*, 2005).

2.2.9 Genetic alterations

Genetic alterations of plants and transgenic plants for improved phytoremediation have already been developed and are spreading for field studies (Chaudhry *et al.*, 2005; Pilon Smits, 2005). It can be done by two methods (a) Gene introduction (b) Gene alteration. The most straightforward way is to add a broad host range plasmid having desired gene and a molecular marker which can be screened later on by a differentiable phenotypic trait.

Co-inoculation of a consortium of bacteria/ or with algae each with different parts of the catabolic degradation route, involved in the degradation of certain pollutant is often found to be more efficient than the inoculation of single strains with the complete pathway (Kuiper *et al.*, 2004). Usually several bacterial populations degrade pollutant more efficiently than a single species or strain due to presence of partners, which use the various intermediates of the degradative pathway more efficiently (joint metabolism) (Pelz *et al.*, 1999; Kuiper *et al.*, 2004). The close proximity of different strains and the formation of mixed micro colonies have been observed in the presence of pollutant naphthalene, illustrating the formation of communities where various activities fulfill each other (Kuiper *et al.*, 2001; 2004). However a few reports have been collected where the direct introduction of microbial strain or consortium for xenobiotic degradation activities is (bioaugmented rhizoremediation) which is able to efficiently colonize the root (Kuiper *et al.*, 2001; 2004; Ronchal and Ramos, 2001; Sriprang *et al.*, 2002).

Along with these techniques, sample pre-treatment before entering a constructed wetland can be done, e.g. aerobic or anaerobic digestion, filtration to remove suspended solids, pre-settlement tanks. For increasing the efficiency of model system, the concept of designer rhizospheres can be applied where plant-microbe–contaminant interaction has been improved by increasing quantity, quality of bacterial strains and enhancing pollutant availability. Some techniques like bioaugmentation, biostimulation and genetically engineered plant/microbe can be employed in this regard but on the ground of genetic manipulations many ethical issues get raised as the biggest challenge in their use will be the horizontal transfer of plasmids or genes in the environment. At all to increase the efficiency of constructed wetlands, there is a need for better knowledge of the biological processes involved in plant-microbe-contaminant interactions, novel genes for bioremediation in plant and bacteria, molecular and biochemical approaches in the degradation pathways and whole mobile genetic pool (consisting of plant and rhizospheric bacteria) in a constructed wetland.

The reasons for unanticipated failures of these techniques can be attributed to one or more of the following (Goldstein *et al.*, 1985; Kuiper *et al.*, 2004). First, the concentration of the contaminants at a site can be too low to support growth of the inoculums. This also includes the problem of low availability of certain pollutants. Second, the presence of certain compounds in the environment can inhibit the growth and activity of inoculums. Third, protozoan grazing rates on the inoculums can be higher than the growth rate of bacteria, resulting in the decline of the bacterial population. Fourth, the inoculums can prefer to use other carbon sources present in the soil, instead of the contaminant. Fifth, inocula may fail because of the unavailability of the microbes to spread through the soil and reach the pollutant.

2.3 Genetic Diversity associated with reeds

Wastewater treatment processes carried out on constructed wetlands are widely used due to their relative low cost and less maintenance requirements, minimum production of sludge and easy integration in the environment. The system is based on the degradation and uptake of organic matter by a microbial community under physically/ chemically disturbed conditions. Here, prokaryotes are among the most important contributors to the transformation of complex organic compounds like organic compounds or xenobiotics. Many studies have reported the importance of bacterial assemblages to the proper functioning and maintenance of treatment systems (Forster *et al.*, 2003), revealing that changes in the diversity of such communities can compromise the entire process. For example, the formation of micro-niches within the wetland system may support the survival and activity of sulfate-reducing bacteria, resulting in the production of H_2S and subsequent problems with sludge bulking or floc dissimulation (Schramm *et al.*, 1999). Microbial communities are essential for mineralization of organic matter and removal of nitrogen, sulfur and phosphorous (Glick, 2010). Adsorption of phosphorus and the abiotic oxidation of phenolics is enhanced in CWs where iron and/or manganese forms part of the substrate (Polubesova *et al.*, 2010). Changes in microbial community in response to acid mine drainage was also assessed (Weber *et al.*, 2008). Reports have been published on the isolation and characterization of rhizospheric bacteria for bioremediation of colored distillery effluent (Chaturvedi *et al.*, 2006). Microbial community has also been characterized in two-constructed wetlands designed to remove contaminants from dairy wastewater using DGGE and sequencing of PCR-amplified fragments of the gene carrying α subunit of the ammonia monooxygenase gene (Ibekwe *et al.*, 2003). Cultivation-based methods or metabolic fingerprinting methods involves community-level physiological profiling (CLPP) using BIOLOG microplates, and for plating onto different media (Borsodi *et al.* 2007), constructing 16S ribosomal DNA (rDNA) clone libraries (Wang *et al.*, 2007). Until now, studies were carried out using mainly traditional microbiological methods. The emergence of molecular techniques allowed to overcome the problems associated with culture-dependent methods that lead to an underestimation of the true diversity. Despite the knowledge that microbial communities evolving in CW play a key role on treatment processes, there are few reports concerning the study of bacterial communities in these areas (Yu and Mohn, 2001).

Miambi *et al.* (2003) showed that the use of PCR-DGGE for total microbial community DNA analysis and culture-dependent techniques gave different results concerning the microbial assemblages. Chen *et al.* (2008) showed that *Lactococcus lactis*, which was not detected in the culture-dependent method due to its scarcity, was found in culture-independent samples. Based on these studies, culture-independent methods in investigating microbial communities are likely to reveal a more comprehensive picture and assessment of community structure. The advent of modern molecular biology has made the study of bacterial community analysis without the need to culture the communities owing to its accuracy and robust results. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rDNA gene fragments fluorescent in situ hybridization (FISH), amplified (DNA) fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA) and clone libraries are culture independent techniques that have been widely used for studying environmental microbial communities. A comprehensive list of various techniques and their use in non-culturable diversity analysis in a wetland are discussed in Table 5.

Table 5: Various techniques used in diversity analysis in wetlands

Technique	Wetland detail	Reference
PCR-DGGE	Plant intercropping models in municipal sewage wastewater wetland	Chen *et al.*, 2011
	Horizontal subsurface-flow laboratory-scale constructed wetlands, one planted and the other one unplanted.	Baptista *et al.*, 2008
	Microbial community composition in two constructed wetlands designed to remove contaminants from dairy washwater.	Ibekwe *et al.*, 2003
	An effluent-dominated body of water that receives natural mountain runoff and tertiary wastewater from various municipalities in San Bernardino and Riverside Counties, and water from various creeks in the area that passes through a 10,000 ha. area with about 350 000 cattle.	Ibekwe *et al.*, 2007
	Wetland plants on the methanotrophic bacterial populations in the sediments of a full-scale constructed wetland	DeJournett *et al.*, 2007
	Horizontal subsurface flow planted sand filter treating domestic wastewater with molecular and culture-based methods.	Truu *et al.*, 2005
	Pilot-scale constructed wetland system consists of three types: subsurface-flow (SSF), surface-flow (SF) and a floating aquatic plant (FAP) system.	Jin and Kelley, 2007
	Characterize the microbial population present in a wetland that was receiving acid coal mine drainage (AMD).	Nicomrat *et al.*, 2006
	Nitrification/denitrification activity and the corresponding bacterial communities in landfill leachate that was treated in a compact constructed wetland	Sundberg *et al.*, 2007
Multiplex fluorogenic real-time PCR	Multiplex fluorogenic PCR assay to quantify E. coli O157:H7 in soil, manure, cow and calf feces, and dairy wastewater in an artificial wetland. Primers and probes were designed to amplify and quantify the Shiga-like toxin 1 (stx1) and 2 (stx2) genes and the intimin (eae) gene of E. coli O157:H7 in a single reaction	Ibekwe *et al.*, 2002
PLFA	Pilot-scale constructed wetland system consists of three types: subsurface-flow (SSF), surface-flow (SF) and a floating aquatic plant (FAP) system.	Jin and Kelley, 2007
Semi-quantitative PCR	Enumerating Ammonia oxidizing bacteria in a wetland	Silyn-Roberts and Lewis, 2001
In situ hybridisation, Semi-quantitative PCR	Subsurface flow dairy effluent treatment wetland was investigated using in situ hybridization and a crude semi-quantitative PCR.	Silyn-Robert and Lewis, 2001
FISH	Microbial populations identified by fluorescence in situ hybridization in a constructed wetland treating acid coal mine drainage	Nicomrat *et al.*, 2006
	Low cost CW formed by stabilization pond followed by in series free water surface and subsurface CW	Criado and Bécares, 2005
	Wetland to study the distribution of methanogens and methanotrophs in land units; Investigating CH_4 and N_2O emissions from eco-engineering wastewater treatment processes using constructed wetland microcosms	Inamori et al., 2007
	Constructed wetlands to treat sanitary landfill leachate containing high nitrogen	Polprasert and Sawaittayothin, 2006

	and bacterial contents	
	Microbial community structure of a trickling filter biofilm with a high anaerobic ammonium oxidation activity	Schmid *et al.*, 2000
	Pilot-scale model constructed to determine if a wetland treatment system (WTS) could effectively remove low-level mercury from an outfall located at the Department of Energy's Savannah River Site. Planted with giant bulrush, *Scirpus californicus*, and surface amended with gypsum ($CaSO_4$)	King *et al.*, 2002
	Savannah River Site's D-area harbours a 22-year-old exposed reject coal pile (RCP) from which acidic, metal rich, saline runoff has impacted an adjacent forested wetland.	Brofft *et al.*, 2002
ARDRA	Identification of bacterial populations in dairy wastewaters by use of 16S rDNA gene sequences and other genetic markers	McGarvey *et al.*, 2004
	Monitoring of potentially toxic cyanobacteria in water samples	Masseret and Sukenik, 2013
	Freshwater study of woopo wetland	Baik *et al.*, 2008
Barcode-tagged 16S rDNA V6 PCR	Ramsar intertidal wetland, intertidal, planted with mudflat and mangroves	Wang *et al.*, 2012
Tag-encoded FLX amplicon pyrosequencing (bTEFAP)	Wetland plante with loblolly pine, spruce pine, southern red oak, willow oak, ironwood, and hickory	Shange *et al.*, 2013

2.4 Modelling a wetland system

The main objective of numerical modeling of CW's is to obtain a better understanding of governing biological and chemical transformation and degradation processes, to provide insights into these "black box" systems, and last but not least, to evaluate and improve existing design criteria. Mathematical models can describe the main processes in CW's for a variety of different boundary conditions and therefore the main limitation of existing design guidelines and rules could be overcome (Langergraber, 2008, 2011). There is an urgent requirement for a modeled approach to understand the functioning of CW's in a simplified computer-based design tool for easy interpretation and analysis.

In the past, various models have been used for the study of wetland and to predict their behavior in various conditions. In a study by Wynn and Liehr (2001) a mechanistic, compartmental simulation model of subsurface-flow constructed wetlands has been studied. The model consists of six sub models, including the nitrogen and carbon cycles, both autotrophic and heterotrophic bacteria growth and metabolism, and water and oxygen balances. Model results reproduced seasonal trends, interactions between the carbon, nitrogen, and oxygen cycles, effluent biochemical oxygen demand, organic nitrogen, ammonium and nitrate concentrations. Wetland here was assumed to act as either a single continuously stirred tank reactor (CSTR) or a series of CSTR's. The model was developed using simulation software for Apple Macintosh called STELLA II.

In another study (Gao and Xie, 2014), a mathematical model for the prediction of phosphorus removal with total phosphorus (TP) as the check index in horizontal subsurface flow constructed wetlands was developed where the transient flow of an incompressible fluid in a heterogeneous anisotropic aquifer was derived by combining Darcy's Law with the continuity equation and a general two-dimensional flow equation was written in Cartesian coordinates.

In a study by Langergraber and Simunek (2012), the HYDRUS wetland module includes two biokinetic model formulations simulating reactive transport in CWs: CW2D and CWM1. In CW2D, aerobic and anoxic transformation and degradation processes for organic matter, N, and P are considered, whereas in CWM1, aerobic, anoxic, and anaerobic processes for organic matter, N, and S are taken into account. The result was of simulated horizontal flow CWs using both biokinetic models. Compared with the CWM1 implementation in the RETRASO code, the

HYDRUS implementation was able to simulate fixed biomass, which is of high importance for obtaining realistic predictions for the treatment efficiency of CWs. The same model was also employed by Fournel *et al.*, 2013 to study modeling the throttled outflow with a virtual porous layer having a particularly low saturated hydraulic conductivity K_s. Werner and Kadlec, 2000 modeled the non ideal flow of CW's with a network of an infinite number of small stirred tanks distributed along a set of main plug flow channels, Garcia *et al.* (2004) has modeled tracer tests performed at pilot HF CWs with different filter materials and different length/width ratios of 1:1, 1.5:1, and 2:1. Using the calibrated flow model, the effluent concentrations during summer has been simulated using the standard CW2D parameter set by Langergraber and Simunek, 2005. Chazarenc *et al.* (2003) applied mathematical tools from chemical engineering to model the hydraulic residence time (HRT) distribution of an HF CW. Mashauri and Kayombo (2002) developed a coupled model for a waste stabilization pond and HF CW based on the growth rate kinetics described by Monod kinetic equations. Polprasert *et al.* (1998), developed a model in WEST (Wastewater treatment plant Engine for Simulation and Training), in order to obtain a better insight in the role of the biofilms for wastewater treatment in reed beds.

Rousseau *et al.*, 2004 has also discussed many design models for horizontal subsurface flow constructed treatment wetlands, ranging from simple rules of thumb and regression equations, to the well-known first-order k-C^* models, Monod-type equations and more complex dynamic, compartmental models.

Design criteria and analysis methods for Constructed Wetlands have evolved from relationships derived from a technical database quantifying nutrient removal as per advanced models. The earlier design approach was based on empirical equations which were derived only from the relationship between input and output nutrient levels. There are many recent advance models which are useful tools for explaining the complexity of hydraulics in substrate coupled with many processes involved in contaminant removal, such as Monod kinetics model (Llorens et al, 2013) and ecosystem model (Xu et al, 2011). Still, there is the need to conduct studies on CWs to improve understanding of the system internal factors affecting contaminant removal processes and enhance the predictive ability of models as many times the model complexity does not produce proportional insight into the factors affecting contaminant removal due to the difficulty of estimating a large number of parameters inherent in the contaminant removal process.. Modelling has come up as a powerful tool for increasing understanding of water treatment systems such as CWs. This presents the first and the second step implementation of the two most significant arsenic retention processes that take place in CWs (aqueous complexation followed by arsenic precipitation, and arsenic sorption in the granular media) in RetrasoCodeBright (RCB) (giving the RCB-ARSENIC model), showing the first simulation results (on the physical and chemical processes). The aqueous complexation reactions included in the RCB-ARSENIC model are considered equilibrium reactions. Five theoretical case studies (total arsenic concentrations from 23.5 to 300 μg/L) have been simulated in order to test the model resulting from the first step implementation. (Llorens et al, 2013).

Małoszewski et al. (2006) used tracer experiments to determine hydraulic parameters in three parallel inhomogeneous gravel beds at a horizontal flow CW in Poland, where instantaneously injected bromide and tritium tracers were used to obtain RTDs. The model was successfully used to fit the experimental tracer breakthrough curves, to identify the different flow components, and to derive wastewater volumes, water-saturated porosity, mean wastewater travel times, longitudinal dispersivities, and hydraulic conductivity from model parameters. The multiflow dispersion model assumed the existence of several flow paths with different hydraulic properties; it was developed using the respective parallel combination of analytical solutions from the one-dimensional advection–dispersion equation. The application of the model, which describes possible diffusion of tracer into the zones with stagnant water, has demonstrated that the calibration of such a model is possible. Schwager and Boller (1997) simulated tracer experiments and oxygen transport in intermittent sand filters using an older version of HYDRUS-1D (Šimůnek et al., 1998) and MOFAT (Katyal et al., 1991), respectively.

Chen et al. (1999) has used the mixing cell method to model the BOD elimination process where the horizontal flow bed is subdivided into a number of same-sized cells that are assumed to be completely mixed. In each cell, the degradation is described by a first-order rate constant. The model is a simplification of the advection–dispersion partial differential equation and has yielded good results. The model presented by Chen et al. (1999) has indicated that the model is able to produce a better match to measured data than a plug flow model and can be also applied to transient conditions such as variable flow rates.

In a study by Mayo and Bigambo (2005) to predict nitrogen transformation in HF CWs, a mathematical model was developed considering the activities of biomass suspended in the water body and biofilm on aggregates and plant roots. The experimental CWs used for calibration and validation of the model operated with constant water flow, as the model was designed only for constant flow rates. The amount of biomass present had no influence on the removal of organic nitrogen; however, it significantly influenced ammonia nitrogen and nitrate nitrogen transformation. The developed CW model was also coupled with a high rate pond (Mayo and Mutamba, 2005). Marsili-Libelli and Checchi has (2005) proposed the combination of a set of ideal reactors with a robust identification method to approximate the dispersed flow and pollution reduction dynamics in HF CWs where the models are based on combinations of series and parallel CSTRs of unequal volumes and carbon removal is modeled using either first-order or Monod kinetics.

In an important study, Rousseau (2005) has developed a reaction model that is coupled with a network of CSTRs for describing the water flow where approach assumes a vertical uniform distribution of substrates, intermediates, products, and bacteria, which may not be the case for HF CWs. Vertical mixing between the CSTRs was therefore introduced to model vertical gradients in the filter bed. The Activated Sludge Model (ASM; Henze et al., 2000) has been used to model microbial conversions.

The multicomponent reactive transport module CW2D (Langergraber, 2001) was developed to describe the biochemical transformation and degradation processes in SSF CWs. CW2D was incorporated into the HYDRUS variably saturated water flow and solute transport program (Langergraber and Šimůnek, 2006; Šimůnek et al., 2006a, 2006b). The HYDRUS program numerically solves the Richards equation for saturated–unsaturated water flow and the convection–dispersion equation for heat and solute transport.

However, the properties/parameters, thus obtained from the modeled parameters have been unrealistic in some cases, which have intensified the significant gaps in the modeled based study approach of investigating wetland system.

2.5 Approach of the present study

The application of constructed wetland harboring macrophytes is a promising method for cleaning up. The versatility of this technology lies in its use for remediation of varied types of effluents starting from domestic, agricultural and industrial sources. In a study by Vymazal, 2011, in the last few years the technology of wetlands has been categorized as:

- Combinational of various types of constructed wetlands
- Treatment of specific compound c. Search for suitable media
- Identification of bacteria which assist in the treatment process
- Modeling of hydraulic and pollution removal.

On above aspects, the lacunae which have been identified are lack of a sensible model for the wetland study and its successful implication. It's also evident from the earlier studies that the identification and role of rhizospheric bacteria associated with *Phragmites* in a constructed wetland has not been established though some work has been carried out in this direction. An attempt has to be made to understand the system in order to increase its potential by using microbial inoculants has never been carried out. Based on these lacunae, Response surface methodology (RSM), a collection of mathematical and statistical techniques for empirical model building was employed. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment with a series of tests, called runs was designed, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Using RSM in wetlands is the relatively new implication of this model where the effect of various independent variables on dependable variables was studied to reach at standardized flow rate equations.

Based on the literature review, following objectives have been defined for the study:

1. **Characterization of wastewater and development of *Phragmites*-vegetated wetland system for treatment of wastewater**
2. **Analysis of rhizospheric bacterial community of *Phragmites***
3. **Monitoring survival of selected bacterial inoculants in rhizosphere**
4. **Impact of plant rhizosphere and bioaugmentation on remediation potential**

Chapter 3 Materials and Methods

Requirements:

Chemicals used for physico-chemical and biological analysis were procured from following:

1. HiMedia, Mumbai
2. Sdfine chemicals Ltd., Mumbai
3. CDH Laboratory Reagent, New Delhi
4. Ranbaxy, Mohali
5. SISCO (SRL), Mumbai
6. Merck, Mumbai
7. Sigma-Aldrich, USA
8. Bangalore Genei, Bangalore
9. Qiagen DNA Elution kits

Instruments used for analysis are as follows:

1. Atomic Absorption Spectrophotometer (GBC 932AA, Australia)
2. Orbital Shaker (Scigenics Biotech)
3. pH meter (Thermo Orion)
4. EC meter (Thermo Orion)
5. Gel Electrophoresis (Tarsons)
6. Hot plate (Tarsons)
7. Vortex Mixer, Spinix (Tarsons)
8. Shaker Incubator (Labcon)
9. BOD Incubator (Metrex Scientific Instruments)
10. U.V-Vis Spectrophotometer (Systronics)
11. Weighing Balance (Sartorius)
12. Spin-win (Tarsons)
13. Refrigerated Centrifuge (Plasto Crafts)

14. Hot air Oven (NSW, Narang Scientific Works)
15. Microwave Oven (LG)
16. Refrigerator (LG)
17.-20°C Deep Freezer (Vest Frost)
18. Autoclave (Equitron)
19. Laminar Air Flow Bench (Thermadyne)
20. PCR Machine (2720, Applied Biosystems)
21. DGGE (INGENYphor)
22. Serological water Bath (NSW, Narang Scientific Works)

Softwares used for analysis are as follows:

1. MiniTab
2. Gel Compar II
3. Mega 5
4. INGENYPhor DGGE gel software

3.1 Characterization of wastewater and development of *Phragmites*- vegetated wetland system for treatment of wastewater

3.1.1 Collection of Wastewater and Construction of wetland

Domestic wastewater sample was collected from facultative wastewater equalization tank (84 x 53 m) at Village Sanghol, Distt Fatehgarh Sahib, (Punjab) India receiving domestic effluent from the entire village. The site is located at 30°46'60" Northern latitude, 76°22'60" East departure, Altitude: 255. The water sampling was done in the month of February with average day temperatures of 12-18 degree C. Wastewater was characterized for various parameters pH, electrical conductivity (EC), Dissolved oxygen (DO), Biochemical oxygen demand (BOD_5), Chemical oxygen demand (COD), total, dissolved and suspended solids, nitrate-N, phosphates and metals (Fe, Cu, Mn, Zn, Ni, Pb, Cd) as per standard methods of wastewater analysis (APHA, 1999). A horizontal subsurface flow constructed wetland (HSSF-CW) at lab scale was constructed with dimensions of 48x 33x 20 cm in plastic tubs each having three sections (a) Inlet section (b) Vegetative section (c) Outlet section. The parts of wetlands are labeled as 1) Collection tank 2) Pre-settlement tank 3) Inlet pipe 4) Outlet pipe 5) Sample collection tank 6) Reed-beds. In vegetative section, a gravel stone bed was formed at the width of 7.5 cm on each side and all along the length of the tub to prevent soil leaching through the outlet and bottom to make water flow easier (Fig. 6). Above that a soil bed of 30 x 16.5 cm dimensions was laid. The system was attached with an inlet tap at one end on upward side and outlet tap on the opposite side at the lower end to regulate the flow rate. A total of six wetland systems were constructed and a control (unplanted system) was formed by vegetation deprived soil- gravel bed running at a standardized flow rate (discussed later). Root stocks of *Phragmites* were planted and grown for 6 months until they attained average height of 35-40 cm. A plant density of 8-10 was used in the soil bed of 30 x 16.5 cm. The system was kept in open and plants were allowed to acclimatize. A flow system was created using feeding wastewater from Collection tank (1) to Pre-settlement tank (2) and finally to Vegetation section (b) through the Inlet Pipe (3) and periodic samples was collected from Outlet pipe (4) to Sample collection tank (5). The design has been discussed in fig. 2. The flow rate was controlled each time by screw based taps maintained at inlet and outlet and the sample was collected through outlet. For experimentation the system was kept out-door, in natural conditions (average day temperature- 10-15°C, receiving adequate sunlight, with no rainfall) and the study was carried for 20 days.

Fig. 6: Experimental setup for standardization of domestic water treatment process

3.1.2 Experimental design using Response Surface Methodology (RSM) and Statistical analysis

Response Surface Methodology (RSM) is a statistical tool that explores the relationships between several explanatory variables and one or more response variables. The method was introduced by Box and Wilson in 1951. The main idea of RSM is to use a sequence of designing experiments to obtain an optimal response. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of *experiments*, the objective is to optimize a *response* (output variable) which is influenced by several *independent variables* (input variables). An experiment is a series of tests, called *runs*, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The response can be represented graphically, either in the three-dimensional space or as *contour plots* that help visualize the shape of the response surface. Contours are curves of constant response drawn in the *xi*, *xj* planes keeping all other variables fixed where each contour corresponds to a particular height of the response. An important aspect of RSM is the *design of experiments* (Box and Draper, 1987), usually abbreviated as DoE. The objective of DoE is the selection of the points where the response should be evaluated. A second-order model can be constructed efficiently with central composite design (CCD) (Montgomery, 1997). CCD is first-order ($2N$) designs augmented by additional centre and axial points to allow estimation of the tuning parameters of a second-order model. The design involves $2N$ factorial points, $2N$ axial points and 1 central point. CCD presents an alternative to $3N$ designs in the construction of second-order models because the number of experiments is reduced as compared to a full factorial design (15 in the case of CCD compared to 27 for a full-factorial design).

Here, three input variables with inlet flow rate ranging from 15-30 ml/min, the outlet at 6-12 ml/min and retention time varying from 1-24 hrs (Table 1) were employed for study. Hydraulic loading into the system has also been described in the same table. Design of Experiment (DOE) through Response surface methodology was developed and analyzed by MINITAB. A regression was performed on the data collected where the observed variable (response) is approximated based on a functional relationship between the estimated input variables (that can be changed independently of each other). The experimental design was defined as a specific set of 20 experiments illustrated by the central composite design matrix composed by the different level of combinations of variables studied.

Experimental design of 3 factors using 20 runs with 6 center points and software generated alpha value of 1.682 was used. A volume of 100 ml treated effluent was collected from outlet in each run and was analyzed for change in pH, electrical conductivity (EC), Dissolved oxygen (DO), Biochemical oxygen demand (BOD_5), Chemical oxygen demand (COD), total and suspended solids, nitrate-N, phosphates and metals (Fe, Cu, Mn, Zn, Ni, Pb, Cd). The samples for heavy metal analysis were acid digested, volume made up using metal free water, filtered and analyzed for residual metal concentration by atomic absorption spectrophotometer (GBC 932 AA; GBC Scientific Equipment Pvt. Ltd. Australia) using air–acetylene flame and single element hollow cathode lamp.

Second order polynomial response surface models were fitted to each of dependent variable with the help of statistical software MINITAB. A stepwise procedure was followed to generate a regression model with significant terms ($p < 0.05$). The RS plots were drawn around the central value in the design of experiments. The behavior of the model system was explained by quadratic equation Eq. (1)

$$Y = \beta + \sum \beta i \, Xi + \sum \beta ii \, (Xi)^{2} + \sum \beta ij \, Xij$$

Where Y is the variable studied, β is the constant in regression model, X*i* is the *i*th independent factor, β*i* is linear coefficient value for variable X*i* and is the first order model concept, β*ii* is the square or quadratic coefficient for the factor *i* and β*ij* is the model coefficient for interaction between *i* and *j*.

3.1.3 Wastewater and soil analysis

3.1.3.1 Collection of wastewater

The model object was a wastewater stabilization pond system at village Sanghol in tehsil Khamano located in Fatehgarh Sahib District of Punjab also known as Ucha Pind Sanghol with an approximate population 53,397, is located at a latitude of 30.7833, longitude 76.3833 and altitude (Feet) 843 which lie in the time zone (EST) of UTC+5: 30. The daily temperature ranges from 45°C in May -June to 4°C in December-January and has a sub-tropical continental monsoon climate with satisfactory rain fall. It is about 40 km from Chandigarh on the way to Ludhiana which holds a special position in the archaeological atlas of India as excavation at the site have yielded coins and seals related to Nomadic rulers.

The village demonstrates a unique way of treating domestic wastewater generated by the local population. The village is situated on a height so the drainage of domestic wastewater produced by whole village is collected in a common place by sewage pipe lines and treated in a wastewater stabilization pond system using microalgae. The system constituted an anaerobic pond (Length = 6.55 m x Breadth = 3.54 m), a facultative pond (Length = 84m x Breadth = 53m x Depth = 1.22 m) and a maturation pond (Length = 84m x Breadth = 50m x Depth = 1.83 m) in series. The wastewater generated was applied directly to the anaerobic pond where the settling of sludge and anaerobic treatment takes place with the help of anaerobic bacteria. Anaerobically treated water was passed to Pond 1 which is a facultative pond where the microalgae and duckweeds utilize inorganic nutrient for their growth and produce nascent oxygen during photosynthesis and thus augments complete oxidation of organic compounds lowering COD and BOD_5. After a residence time of 12-14 days the treated wastewater was passed to pond 2 which acted as a maturation pond in wastewater treatment system for final treatment of treated water.

Domestic wastewater was collected in the month of February 2009 from the inlet and outlet of the equalization pond in autoclaved plastic bottles, which were brought to the laboratory and characterized for Biochemical oxygen demand, Chemical oxygen demand, Total solids, Total suspended solids, Total dissolved solids and bacterial count. Parameters such as water temperature, conductivity, salinity and dissolved oxygen (DO) were recorded on-site using portable probes from Thermo Orion Model 150 and 125 respectively. Three input variables with inlet flow rate ranging from 15-30 ml/min, the outlet at 6-12 ml/min and retention time varying from 1-24 hours (Table 9) were employed to study. Hydraulic loading into the system has also been described in Table 9.

Design of Experiment (DOE) through Response surface methodology was developed by MINITAB (Minitab Inc. USA release version 11.12) and was also used to analyze the data collected. A regression was performed on the data collected where the observed variable (response) is approximated based on a functional relationship between the estimated input variables (that can be changed independently of each other). The experimental design was defined as a specific set of 20 experiments illustrated by the central composite design matrix composed by the different level of combinations of variables studied. Experimental design of 3 factors using 20 runs with 6 center points and software generated alpha value of 1.682 was used. In the present study, all the designed experiments were performed and results were analyzed through regression model and checked for significance. Model coefficients were estimated by using multiple regressions (MINITAB) and model accuracy was checked using R^2, *F test* and *P value* for each parameter.

3.1.3.2 Wastewater Analysis

pH

The pH of wastewater samples was measured using a portable water testing kit (Thermo Orion model 290, USA). For this, the standard buffer solution of pH 4.1, 7.0 and 9.18 was prepared to calibrate the electrode. This calibrated electrode then rinsed with distilled water, wiped dry the electrode and immersed into the sample to read the pH.

Electrical conductivity and salinity

Electrical conductivity and salinity were measured using a portable water testing kit (Thermo Orion model 125, USA). 100 ml of vigorously shaken sample was taken in a beaker and gave temperature compensation to the instrument. This electrode then rinsed with distilled water, wiped dry the electrode and immersed into the sample to read electrical conductivity and salinity.

Determination of TS, TDS, and TSS

Total solids, Total suspended solids and Total dissolved solids were determined as per the method given by APHA (1999)

Total solids

Procedure

1. 100 ml beakers were cleaned and heated to 103-105°C for 1 hour and stored in desiccators to get constant initial weight of beakers.
2. The beakers were weighed immediately after removing from desiccators to get initial weight of each beaker.
3. 50 ml of well mixed wastewater sample was transferred to pre-weighed beakers and kept in drying oven at 98°C till complete evaporation of water.
4. Evaporated samples were again dried for one hour in an oven at 103°C to 105°C and cooled in desiccators to balance temperature and weight.
5. The beakers were weighed as soon as they had cooled down for final weight.

Calculation

$$\text{Total solids (mg L}^{-1}\text{)} = \frac{(A - B) \times 100}{sample\ volume\ (ml)}$$

Where:

A is the final weight of beaker representing weight of residue and beaker (mg).

B is initial weight of beaker (mg).

Total dissolved solids

The total dissolved solids were determined in wastewater following same procedure and calculations as used for determination of total solids; where as a well mixed sample was filtered using whatman No. 42 filter paper (Whatman Pvt. Ltd., Mumbai) before transferring 50 ml sample to 100 ml pre-weighed beakers.

Total suspended solids

A well mixed wastewaters contained total solids, which are sum of total dissolved solids and total suspended solids, so total dissolved solids were calculated using calculation as follows:

Calculation

Total suspended solids (mg L^{-1}) = Total solids (mg L^{-1}) - Total dissolved solids (mg L^{-1})

Biochemical oxygen demand

Biochemical oxygen demand (BOD) of wastewater samples were determined as per the method given by APHA (1999)

Reagents

1. Phosphate buffer solution: 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $NaHPO_4$ .7H2O, and 1.7 g NH_4Cl were dissolved in about 500 ml double distilled water and diluted to 1 L.
2. Magnesium sulphate solution: 22.5 g $MgSO_4.7H_2O$ was dissolved per 1 L of double distilled water.
3. Calcium chloride solution: 27.5 g $CaCl_2$ was dissolved in 1 L double distilled water.
4. Ferric chloride solution: 0.25 g $FeCl_3$.6H2O was dissolved in 1L double distilled water.
5. Sodium sulphite solution: 1.575 g Na_2SO_3 was dissolved in 1L double distilled water. Solution was freshly prepared at each time of analysis.
6. Glucose-glutamic acid solution: Glucose and Glutamate of analytical grade were dried in oven at 103°C for 1 hour from which 150 mg of glucose and 150 mg glutamate were dissolved in 1 L of double distilled water.
7. Ammonium chloride solution: 1.15 g NH_4Cl was dissolved in about 500 ml double distilled water and pH was adjusted to 7.2 with 1N HCl and 1N NaOH solution, and final volume was made up to 1L with double distilled water.
8. Manganous sulphate solution: 480 g of $MnSO_4.4H_2O$, 400 g $MnSO_4.2H_2O$, were dissolved in double distilled water and filtered followed by volume made up to 1L with double distilled water.
9. Alkali-iodide-azide reagent: 500 g of NaOH and 135 g NaI were dissolved in 1L of double distilled water, followed by addition of 10 g NaN_3 previously dissolved in 40 ml double distilled water.
10. Sulfuric acid (H_2SO_4) Concentrated.
11. Starch: 2 g of laboratory-grade soluble starch and 0.2 g salicylic acid were dissolved in 100 ml of hot double distilled water.
12. Standard sodium thiosulphate: 6.205 g of $Na_2S_2O_3.5H_2O$ was dissolved in double distilled water followed by addition of 0.4 g solid NaOH and diluted to final volume of 1L.

Procedure

1. Dilution water was prepared by adding 1 ml L^{-1} of each phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions and saturating with dissolved oxygen by aerating with organic free filtered air for 4 hours.
2. 1ml previously acclimatized seed was also added to 1 L dilution water before use.
3. Wastewater sample was diluted appropriately with dilution water and filled in BOD bottles of 300 ml capacity by siphoning to avoid any air bubbles. The bottles were closed tightly with stopper and mixed well.
4. Initial DO of one bottle for each diluted sample, dilution water blank and standard (glucose-glutamic acid) was measured immediately after filling BOD bottles with azide modification method.
5. In azide modification method 1 ml of $MnSO_4$ solution was transferred to BOD bottle containing sample, followed by addition of 1 ml alkali-iodide-azide reagent with separate glass pipettes alternatively by dipping them into the sample.
6. BOD bottles were tightened with stopper and mixed by inverting to form precipitates.
7. 1 ml of concentrated sulfuric acid was added to the solution holding pipette tip just above liquid surface after settling of precipitates to half of the bottle.
8. Bottles were re-stoppered and mixed by inverting several times until dissolution is complete.
9. 20 ml of this sample was taken in titration flask and titrated with 0.025M $Na_2S_2O_3$ using starch solution as indicator to an endpoint of pale straw colour.
10. Initial D.O. of sample was calculated using following equation:

    For titration of 200 ml sample, 1 ml 0.025 M $Na_2S_2O_3$ = 1 mg DO L^{-1}
11. Continuing step 5, another bottle of each sample was tightly closed and incubated for 5 days at 20°C which was analyzed after 5 days of incubation for DO of samples following step 5-10. A blank and standard were also analyzed after 5 days.

Calculations

$$BOD\ (mg\ L^{-1}) = (S1-S2)-(B1-B2)\ F/P$$

Where: S1= initial DO of diluted sample/ standard (mg L^{-1})

S2= final DO of diluted sample /standard after 5 days incubation at 20°C (mg L^{-1})

B1= initial DO of seed control (blank) (mg L^{-1})

B2= final DO of seed control after 5 days incubation at 20°C (mg L^{-1})

F= ratio of seed control (% seed in diluted sample)

P= decimal volumetric fraction of sample used

Chemical oxygen demand

Chemical oxygen demand of wastewater samples were determined as per the method given by APHA (1999)

Reagents

1. Standard potassium dichromate solution: 12.259 g of previously dried $K_2Cr_2O_7$ of primary standard grade was dissolved in double distilled waster and diluted to 1L.
2. Sulfuric acid reagent: 5.5 g of Ag_2SO_4 of technical grade was added to 1 L of concentrated H_2SO_4 and kept for one to two days for complete solubilisation of Ag_2SO_4.
3. Ferroin indicator solution: Ferroin solution (o-Phenanthroline ferrous sulphate complex) was purchased from s. d. fine-chem. limited, Mumbai.
4. Standard ferrous ammonium sulphate (FAS) titrant (0.25 M): 98 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ was dissolved in double distilled water followed by addition of 20 ml concentrated H_2SO_4, cooled and final volume made up to 1L. This solution was standardized against standard $K_2Cr_2O_7$ solution before use.*

   **Standard $K_2Cr_2O_7$ was diluted to 100 ml with double distilled water followed by addition of 30 ml concentrated H_2SO_4, cooled and titrated with FAS titrant using 2 to 3 drops of ferroin indicator.*

$$Molarity\ of\ FAS\ solution = \frac{Volume\ \ 0.0417M\ \ K_2Cr_2O_7\ \ solution\ \ titrated\ \ (ml)}{volume\ of\ \ FAS\ used\ in\ titration\ \ (ml)} \times 0.25$$

5. Mercuric sulfate: $HgSO_4$ powder.
6. Potassium hydrogen phthalate (KHP) standard: 425 mg of previously dried (102°C) potassium hydrogen phthalate ($HCOOCC_6H_4COOK$) was diluted to 1L by double distilled water. This solution is stable and has theoretical COD of 1.176 mg O_2 mg^{-1}.

Procedure

1. Open reflux method was used to analyse chemical oxygen demand of wastewater samples.
2. 50 ml of appropriately diluted wastewater samples were suspended into 500 ml round bottom flasks of 500 ml followed by addition of 1 gm mercuric sulphate. Glass beads measuring 0.4 mm were added to prevent bumping.
3. 5 ml of concentrated sulfuric acid was added to reaction mixture very slowly and by proper mixing of solution to dissolve $HgSO_4$. The mixture was also cooled by water bath while mixing to avoid any possible loss to volatile materials.
4. 25 ml of 0.0417 M $K_2Cr_2O_7$ was also added to the solution followed by addition of remaining 70 ml of concentrated H_2SO_4 while continuing swirling and mixing of solution.
5. The solution was digested in digestion unit for 2 hr.
6. The digested solution was titrated against 0.25 M ferrous ammonium sulphate (FAS) using two to 3 drops of ferroin indicator to an end point of sharp colour change from blue-green to reddish brown.

Calculation

$$COD\ as\ mg\ O_2\ L^{-1} = [(A-B) \times M \times 8000]\ /ml\ sample$$

Where: A = ml FAS used in blank,

B = ml FAS used in sample,

M= Molarity of FAS,

8000 is milli equivalent weight of oxygen

Total Fe, Mn, Ni, Cr, Pb, Zn, Ca, Mg, Na

Estimation of total metals was done as per the method given by APHA (1999).

Reagents

1. Concentrated perchloric acid ($HClO_4$) and nitric acid (HNO_3).
2. Acid water solution containing HCl and water in a 1:1 ratio.

Procedure

1. 1g of soil/water sample/plant sample was placed in a 150 ml beaker.
2. HNO_3 and $HClO_4$ in a 3:1 ratio were added to the sample.
3. The sample was digested on a hot plate at 100°C for 3-4 hours until a whitish brown dry mass was obtained.
4. The samples after digestion were treated with acid water mixture and filtered through Whatman No.42 filter paper.
5. The filtrate was analyzed for total Fe, Mn, Ni, Cr, Pb and using an atomic absorption spectrophotometer (GBC 932A). The various metals along with their sensitivity limits are as follows.

Element	**Sensitivity** (µg ml-1)
Cr	0.05
Fe	0.05
Ni	0.04
Pb	0.06
Zn	0.008
Mn	0.02
Ca	0.02

Mg	0.003
Na	0.004

Available phosphorus

Available phosphorus in the soil/water sample was estimated as per the method given by Olsen *et al.* (1954).

Reagents

1. 0.5 M $NaHCO_3$ extracting solution: 84 g of sodium bicarbonate was added in distilled water and the volume was made up to 2 L. pH was adjusted to 8.5 with IM or 1N NaOH.
2. Reagent A: 12.0g of ammonium molybdate in 250 ml distilled water and 0.2908g of antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H_2SO_4, mixed thoroughly and volume made up to 2L with distilled water.
3. Reagent B (freshly prepared): 1.058g of ascorbic acid in 200 ml of reagent A and mixed.
4. Sulphuric acid (2.5 M): 140 ml of concentrated H_2SO_4 diluted to 1L.
5. Stock Standard P solution (50 ppm P): 0.2917 g KH_2PO_4 dissolved in water to a final volume of 1L.
6. Working Standard P solution (1 ppm): 20 ml of (50 ppm P) solution diluted to 1L.

Procedure

1. 2.5 g soil sample / 2.5ml water sample was placed in a 100 ml Erlenmeyer flask followed by the addition of 50 ml extracting solution.
2. The solution was kept on a shaker for 30 minutes and filtered through Whatman No. 42 filter paper.
3. 10 ml aliquot of the filtrate was transferred to a 100 ml beaker followed by addition of 1 ml of 2.5 M H_2SO_4, 15.5 ml of distilled water, 8 ml of Reagent B and another 15.5 ml of distilled water.
4. A blank was prepared as above.

For the standard curve: 0, 2, 5, 10, 15 and 20 ml of standard solution was placed in 50 ml volumetric flasks separately. Ten ml of extracting solution, 1.0 ml of 2.5 M H_2SO_4*, 8 ml Reagent B was added and the final volume was made up to 50 ml. The P concentrations of these solutions were 0.04, 0.1, 0.2, 0.3 and 0.4 ppm respectively. After 10 minutes, the P concentration was read at 882 nm.*

Calculation

P in soil (ppm) = P in extract (ppm) x 20 (the standard soil to solution ratio)

Total Nitrogen

Total nitrogen was estimated as per the Kjeldahl method given by Piper (1960).

Reagents

1. Concentrated H_2SO_4.
2. 0.02 N H_2SO_4.
3. Sulphuric-Salicylic acid: 1 g salicylic acid mixed with 30 ml sulphuric acid.
4. Sodium thiosulphate.
5. 4% boric acid.
6. Mixed indicator. 0.066 g of methyl red and 0.099 g of bromocresol green dissolved in 100 ml of ethyl alcohol.
5. 50% NaOH.
6. Digestion mixture: 10 g HgO, 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20).

Procedure

1. 5 g soil/5 ml water sample was mixed thoroughly with sulphuric-salicylic acid followed by 5g of sodium thiosulphate. Heating was carried out for 5 minutes followed by cooling and addition of 10g digestion mixture. The contents were mixed well in a Kjeldahl flask.

2. The flask was kept in the digestion chamber at 100°C for two hours.
3. The color change was monitored from dark brown to greenish white after which the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condensor attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator till the end-point color changes from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 until the endpoint colour changed from green to pink.

Calculation

$$\text{Total N \%} = \frac{(T-B) \times \text{Normality of } H_2SO_4 \times 1.4 \times 300}{\text{Weight of sample}}$$

Where T is the titre value for sample and B is for blank.

3.1.3.3 Soil Analysis

(a) Physical Analysis

pH

pH was determined as per the method given by Jackson (1967) in a soil-water suspension of 1:2 ratio. Ten g of soil/fly ash was placed in a 100 ml beaker and 20 ml of distilled water was added and the soil was stirred well for five minutes and kept undisturbed for some time followed by stirring again. pH was measured using a Thermo Orion Model 290 pH meter after calibration with buffers of pH 4.0, 7.0 and 9.2.

Electrical conductivity

Electrical conductivity was measured in µS cm-1 as per the method given by Jackson (1967). Ten g of soil was placed in a 100 ml beaker and 20 ml distilled water was added. The soil-water mixture was allowed to stand undisturbed until the soil settled completely. The conductivity meter (Orion Model 125) was calibrated with 0.01 M potassium chloride (1413 μS cm-1).

Organic carbon estimation

Total organic carbon was estimated as per the method given by Walkley and Black (1934).

Reagents

1. 1 N $K_2Cr_2O_7$: 49.04 g of potassium dichromate per litre of solution.
2. 0.5 N ferrous ammonium sulphate: 198 g salt per litre of solution.
3. Diphenylamine indicator: 0.5 g of diphenylamine in a mixture of 20 ml water and 100 ml concentrated sulphuric acid.
4. Concentrated sulphuric acid.
5. Orthophosphoric acid (85%) and sodium fluoride (NaF).

Procedure

1. 1 g of soil was taken in a 500 ml conical flask followed by the addition of 10 ml of 1N $K_2Cr_2O_7$. The flasks were swirled for mixing the soil and reagent.
2. 20 ml of H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200 ml of distilled water was added.
3. To the mixture, 10 ml of Orthophosphoric acid, 0.5 g of NaF and 1 ml diphenylamine indicator was added.
4. The contents were ultimately titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the end-point is observed from blue-violet to green. A blank was also run without soil.

Calculation

$$\text{Organic carbon (\%)} = 10\,(B-T) \times 0.003 \times 100 / B \times \text{Wt. of soil (g)}$$

Where B is the volume of ferrous ammonium sulphate solution required for blank titration.

T is the volume of ferrous ammonium sulphate solution required for soil sample titration.

Total Fe, Mn, Ni, Cr, Pb, Zn, Ca, Mg, Na

Estimation of total metals was done as per the method given by APHA (1999).

Reagents

1. Concentrated perchloric acid ($HClO_4$) and nitric acid (HNO_3).
2. Acid water solution containing HCl and water in a 1:1 ratio.

Procedure

1. 1g of soil/water sample/plant sample was placed in a 150 ml beaker.
2. HNO_3 and $HClO_4$ in a 3:1 ratio were added to the sample.
3. The sample was digested on a hot plate at 100°C for 3-4 hours until a whitish brown dry mass was obtained.
4. The samples after digestion were treated with acid water mixture and filtered through Whatman No.42 filter paper.
5. The filtrate was analyzed for total Fe, Mn, Ni, Cr, Pb and using an atomic absorption spectrophotometer (GBC 932A). The various metals along with their sensitivity limits are as follows.

Element	**Sensitivity** (µg ml-1)
Cr	0.05
Fe	0.05
Ni	0.04
Pb	0.06

Zn	0.008
Mn	0.02
Ca	0.02
Mg	0.003
Na	0.004

Available Sulphur

Estimation of available sulphur was done by the method given by Chesnin and Yien (1950).

Reagents

1. 0.15% calcium chloride.
2. 30-60 mesh barium chloride.
3. 0.25% gum acacia.
4. K_2SO_4 solution: 0.5434 g of reagent grade potassium sulphate in distilled water and diluted to 1l.
5. Whatman No.42 filter paper

Procedure

1. 1 g soil was digested with HNO_3 and $HClO_4$ in the ratio 3:1 on hot plate at 100°C until a whitish brown mass was obtained.
2. The sample was treated with HCl and water mixture and filtered through Whatman filter paper No. 42 and filtrate stored in bottles.
3. 10 ml of filtrate was taken and pH adjusted to 5.0 using 40% NaOH and volume was made up to 50 ml.
4. Then a 20 ml aliquot was taken 50 ml of 0.15 % calcium chloride was added to 10 g soil sample taken in a 150 ml conical flask.
5. The sample was agitated at 130 r.p.m. for 30 minutes followed by filtration through Whatman No. 42 filter paper.

6. 20 ml of filtrate was taken in a 25 ml volumetric flask and 1 g of 30-60 mesh barium chloride was added, swirled followed by 1 ml of gum acacia. The volume was made upto 25 ml with distilled water and absorbance read at 420 nm.
7. Standard curve: Different volumes 0.25, 0.5, 1.0, 2.5 and 5.0 ml of 100 ppm standard K_2SO_4 solution were taken in 25 ml volumetric flasks. Ten ml of 0.15% calcium chloride, 1.0 g barium chloride, and 1.0 ml gum acacia were added and volume made up with distilled water. The absorbance was read at 420 nm.

3.1.3.3 Soil Analysis

(a) Microbiological Analysis

Soil dehydrogenase activity

Soil dehydrogenase activity was measured as per the method given by Cassida (1977).

Reagents

1. Triphenyl tetrazolium formazan (1 mg ml-1 TPF).
2. 1, 3, 5 Triphenyl tetrazolium formazan (100 µg ml-1).
3. Methanol.
4. Calcium carbonate.
5. 0.1% and 0.2% yeast extract.
6. Sterile water.
7. Sterile glassware.
8. Whatman No 1Filter Paper.

Procedure

1. 10 g of soil sample was weighed and mixed with CaCO3 in the ratio 100:1.
2. 3g of each sample was dispensed in screw cap glass vials and 0.5 ml sterile water was added followed by 1hour incubation at 28°C.

3. 0.25 ml single strength substrate solution was added (0.1% yeast extract) followed by 0.25 ml sterile water. The vials were then incubated for 8 hours at 28°C.
4. 0.5 ml of 3% aqueous TTC (2, 3, 5 triphenyl tetrazolium chloride) and 0.25 ml double strength (0.2 %) yeast extract was added and mixed thoroughly with sterile glass rod.
5. This was followed by 6-hour incubation at 37°C and immediate extraction with 25 ml methanol and subsequent filtration through Whatman No. 1 Filter Paper.
6. The methanol extract containing red coloured formazan was read at 480 nm.
7. In 100 ml volumetric flasks 5, 10, 15 and 20 ml of working solution of 100 μg/ml 1, 3, 5 triphenyl tetrazolium formazan (diluted from stock solution of 1 mg ml-1 TPF) was added and final volume adjusted with methanol. The absorbance was read at 480 nm.

Bacterial enumeration on different growth media

Media

1. **Nutrient Agar**

Ingredients (gms / Litre)

Peptic digest of animal tissue 5.000
Sodium chloride 5.000
Beef extract 1.500
Yeast extract 1.500
Agar 15.000
Final pH (at 25°C) 7.4 ± 0.2 2.

2. Jensen’s Media

Ingredients (gms / Litre)
Sucrose 20.000
Dipotassium phosphate 1.000
Magnesium sulphate 0.500
Sodium chloride 0.500
Ferrous sulphate 0.100

Sodium molybdate 0.005

Calcium carbonate 2.000

Agar 15.000

3. Pikovskaya Media

Ingredients (gms / Litre)

Yeast extract 0.500

Dextrose 10.000

Calcium phosphate 5.000

Ammonium sulphate 0.500

Potassium chloride 0.200

Magnesium sulphate 0.100

Manganese sulphate 0.0001

Ferrous sulphate 0.0001

4. Saline test tubes (0.9% NaCl in ddH_2O)

Procedure

1. 10g of soil sample was dissolved in 100ml of saline solution and was kept on shaker for 30 mins. 1 ml aliquot of this suspension was serially diluted and 100 µl was plated on different media plates such as Nutrient agar, Jensen's media, Pikovskaya media.
2. After 24 hrs of incubation, CFU (Colony Forming Unit) count was done.

3.2 Analysis of rhizospheric bacterial community of *Phragmites*

3.2.1 Soil sampling

Rhizospheric bacterial community of *Phragmites* was monitored at different root zones *i.e* upper rhizosphere, middle zone and lower zone. This gives an insight to the community structure present at each zone. Plants were then uprooted from ACC Gagal Cement Works and rhizospheric soil samples were acquired from different zones: upper zone (0-15 cm), middle zone (15-30 cm), lower zone (30-45 cm). Soil samples were collected from five different points in each system; samples from the same zone were combined and mixed completely to produce one homogeneous sample. All samples were maintained on ice until arrival in the laboratory and then stored at 4°C.

3.2.2 Soil DNA Extraction

Soil DNA was extracted from soil samples using four methods discussed as below:

3.2.2.1 Griffiths method (Griffiths *et al,* 2000)

Reagents

1. 0.1 mm glass beads and 0.5 mm zirconia/silica beads (0.5 g each) in 2 ml screw-top microcentrifuge tubes
2. Modified CTAB extraction buffer: Equal volumes of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer (pH8)
3. Phenol (pH>7.8):chloroform:isoamylalcohol (25:24:1)
4. Chloroform:isoamylalcohol (24:1)
5. PEG/NaCl solution: 1.6 M NaCl and 30% (wt/vol) polyethylene glycol 6000
6. Sterile MilliQ H_2O
7. 70% EtOH
8. Tris-EDTA pH7.4

Procedure

1. To 0.5 g soil, 0.5 g each of 0.1mm glass and 0.5mm zirconium/silica beads were added in 2 ml eppendorf. 0.5 ml of modified CTAB (hexadecyltrimethylammonium bromide) extraction buffer was added and vortexed.
2. To this homogenate, 0.5 ml phenol: chloroform: isoamylalcohol (25:24:1), followed by bead-beating, at 16000*g for 5 min at 4°C.

3. To aqueous layer equal volume of chloroform: isoamylalcohol (24:1) was added, mixed and centrifuged at 16000*g for 5 min at room temperature.
4. To aqueous layer 2 volumes of PEG/NaCl precipitate solution added and incubate at room temp for 2 hours.
5. DNA pellet obtained was thus washed and resuspended in 50µl DNAse/ RNAse-free TE and stored at -20°C (pH 7.4 or 8.0).

3.2.2.2 Glass bead-beating method

Reagents

1. Lysis buffer (0.1 M Tris–Cl [pH 8.0], 0.1 M EDTA [pH 8.0], 0.1 M NaCl, 0.2% hexadecyltrimethylammonium bromide [CTAB] [w/v])
2. Glass beads (0.01mm)
3. 3 M NaAc (pH 5.2)
4. 70% Ethanol
5. TE Buffer
6. Phenol/ chloroform (1:1)

Procedure

1. Two grams of sediment sample (wet weight) were suspended in 500 µl of lysis buffer in a 10 ml centrifuge tube, and an equal weight of glass beads was added.
2. The tube was vortexed vigorously for 60s and then centrifuged at 8000*g for 15 min at 4°C in a centrifuge. The supernatant was transferred to afresh tube and extracted by the equal volume of phenol/ chloroform (1:1, v/v).
3. The upper aqueous phase was added 1/10 volume of 3 M NaAc (pH 5.2) and 2.5 volumes of ethanol and precipitated at room temperature for 30 min. Then the tube was centrifuged at 8000*g for 15 min at 4°C.
4. The supernatant was removed and the pellet was rinsed with 500 µl of 70% ethanol (v/v). The tube was centrifuged at 8000*g for 15 min at 4°C. The supernatant was removed and the pellet was allowed to dry in the air for 15 min.

5. The pellet was dissolved in 50 µl of TE (pH 8.0) and the DNA solution was stored at -20°C.

3.2.2.3 Soil DNA extraction kit (Himedia)

Reagents

As per kit instructions.

Procedure

1. DNA extraction kit for environmental samples was purchased from Himedia Laboratories, India. The procedure was performed according to the manufacturer's instructions.
2. The DNA was dissolved in 50 µl of TE buffer and stored at -20°C.

3.2.2.4 Chemical Lysis (CL) Method

Reagents

1. 0.5 M NaH_2PO4 (pH 8.0)
2. Lysis solution (3 M NaCl, 0.2 M Tris–Cl, 0.2 M EDTA, pH 8.0)
3. 12% SDS solution
4. 3 M NaAc
5. Phenol
6. TE buffer

Procedure

1. Two grams of sediment sample (wet weight) were suspended in 1 ml of 0.5 M NaH_2PO4 (pH 8.0), 1.4 ml of lysis solution (3 M NaCl, 0.2 M Tris–Cl, 0.2 M EDTA, pH 8.0) in a 10 ml centrifuge tube, and 0.5 ml of 12% SDS (w/v) was added.

2. The tube was incubated at 65°C for 30 min and inverted up and down every 15 min. The mixture was extracted with an equal volume of chloroform.
3. The upper aqueous phase was transferred to a fresh tube and 1/10 volume of 3 M NaAc (pH 5.2) and 2.5 volumes of ethanol was added.
4. The tube was incubated at room temperature for 30 min. Then the tube was centrifuged at 8000*g for 15 min at 4°C in a centrifuge.
5. The pellet was rinsed with 500 µl of 70% ethanol (v/v) and the tube was centrifuged at 8000*g for 15 min at 4°C.
6. The supernatant was removed and the pellet was allowed to dry in the air for 15 min. The pellet was dissolved in 50 µl of TE (pH 8.0) and the DNA solution was stored at -20°C.

3.2.3 Electrophoresis of nucleic acids on Agarose gels

Nucleic acids were loaded on agarose gels (0.7) prepared in 0.5X TBE buffer pH 8.0 using a 6X loading buffer. Ethidium bromide (EtBr) (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then migrated and visualized on a U.V. transilluminator (312 nm).

3.2.4 DNA Quantification and purity

Co-extracted humic acids are the major contaminant when DNA is extracted from soil. These compounds absorb at 230 nm whereas DNA absorbs at 260 nm and protein at 280 nm. To evaluate the purity of the extracted DNA, absorbance ratios at 260 nm/230 nm (DNA / humic acids) and 260 nm/280 nm (DNA / protein) were determined. The yield of the crude DNA was calculated by assuming that an O.D. of 1 at 260 nm corresponds to 50 µg/ml for double stranded DNA. All crude DNA was purified using Sephadex G200 purification spin columns for further PCR amplification.

3.2.5 Amplification using 16S rDNA and DGGE (GC Clamp) specific primers

The amplification mixture for each sample DNA contained 100 pmol of each primer, 200 µM dNTPs, 0.2% BSA, 1X PCR buffer, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase

(Fermentas) in a 100 µl reaction volume. Template DNA which is soil DNA product used was in the range of 1 pg to 100 µg in a total of 100 µl final reaction volume. PCR was performed in an automated thermal cycler (Applied Biosystems 2720) with an initial 94°C denaturation for 9 min, followed by 30 cycles of 94°C for 30 sec, 55ºC for 30 sec, 72°C for 30 sec and a final extension at 72°C for 7 min using primers listed in Table 6.

Table 6: Primer details for amplification of 16S rDNA gene

Primer	Sequence	Target region	References
8F	AGA GTT TGA TCC TGG CTC AG	16S rDNA	Eden *et al.*, 1991
1492R	GGT TAC CTT GTT ACG ACT T	16S rDNA	Eden *et al.*, 1991

The reamplification was carried out by taking 1 µl of the first PCR product as a template and the PCR mixture contained 100 pmol of each primer, 200 µM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase (Fermentas) in a 100 µl reaction volume. The primers used in the present study were the universal primers conserved for the V3 region of bacterial 16S rDNA gene, PRBA 338F 5' and PRUN 518R with a GC clamp added to the 5' end of the forward primer (as tabulated in Table 7).

Table 7: Primer details for amplification of V3 region of bacterial 16S rDNA gene

Primer	Sequence	Target region	References
PRBA 338F	5' AC TCC TAC GGG AGG CAG CAG 3'	V3 region of 16S rDNA	Lane, 1991
PRUN 518R	5' ATT ACC GCG GCT GCT GG 3'	V3 region of 16S rDNA	Muyzer *et al.* 1993
GC clamp	5'CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3'	-	Muyzer *et al.* 1993

Initial denaturation was done at 94ºC for 9 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 7 min was also performed. Crude DNA extracts were loaded on 1.5 % agarose gel prepared in 1 X TBE buffer containing ethidium bromide (0.5 µg/ml) at constant voltage of 75 volts, 30-40 min and visualized using gel documentation system and then digitized.

3.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

3.2.6.1 Gel preparation and Sample loading

Denaturing Gradient Gel Electrophoresis (DGGE) is such a technique that attempts to monitor of microbial populations, where culture dependant methods fall short. This molecular biology approach is a fingerprinting methodology that has led to revolutionary changes in many of the traditional routines used in assessing microbial ecology (McAuliffe *et al.*, 2005). Microbial ecology aims at studying interactions among microorganisms and between microorganisms and their environment. This involves long-term study, which includes various and numerous environmental sample analysis (Muyzer and Smalla, 1998). However, conventional cloning, hybridization and culture methods as mentioned above are not always practical for such investigations. Moreover, these techniques do not provide any information on the dynamics of the microbial populations in complex ecosystems and potential effects of environmental changes on such populations (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998; Sheffield *et al.*, 1989.). DGGE is a particular type of gel electrophoresis in which a constant heat (about 60ºC) and an increasing concentration of denaturing chemicals are used to force DNA molecules to unwind. A quick glimpse at electrophoresis tells us that this is a separation technique based on the electrical charge, shape and the molecular weight of particulates such as DNA, proteins and RNA (Creighton, 1999). In DGGE, DNA, which is negatively charged, is attracted by the positive electrode and forced to migrate through the pores of a polyacrylamide gel. Once it reaches the concentration of denaturing reagents at which it unwinds, it is said to have melted. This determines the melting domains (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998), which are defined as stretches of base pairs with an identical melting temperature (Muyzer *et al.*, 1993). In other words, base pairs formed by nucleotides A (adenine) and T (thymine), and those formed by C (cytosine) and G (guanine) are chemically melted apart. Basically, what happens is that hydrogen bonding between the base pairs is broken by the temperature and the increasing gradient of denaturing chemicals (urea and formamide) (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998). Any variation of DNA sequences within these domains will result in different melting temperatures, thus causing different sequences to migrate at different positions in the gel (Muyzer *et al.*, 1993). This provides DGGE with the power to distinguish between mutated and wild type sequences without prior knowledge of what these sequences are, justifying why this method is used to detect mutations/diversity within closely related organisms (McAuliffe *et al.*, 2005, Walter *et al.*, 2000). The protocol and reagents for DGGE gel are mentioned below.

Reagents

1. 40% Acrylamide/Bis (37.5:1)

Acrylamide 38.93 g; Bis-acrylamide 1.07 g

Volume made with 100.0 ml; Filter through a 0.45 µ filter and store at 4°C.

2. 50x TAE Buffer

Tris base 242.0 g 2 M; Acetic acid, glacial 57.1 ml 1 M; 0.5 M EDTA, pH 8.0 100.0 ml 50 mM

Volume made to 1L; Autoclaved and stored at room temperature.

3. 0% Denaturing Solution

	6% Gel	8% Gel	10% Gel	12% Gel
40% Acrylamide/Bis	15 ml	20 ml	25 ml	30 ml
50x TAE buffer	2 ml	2 ml	2 ml	2 ml
dH2O	83 ml	78 ml	73 ml	68 ml
Total volume	100 ml	100 ml	100 ml	100 ml

Store at 4°C in a brown bottle for approximately 1 month.

4. 100% Denaturing Solution

	6% Gel	8% Gel	10% Gel	12% Gel
40% Acrylamide/Bis	15 ml	20 ml	25 ml	30 ml
50x TAE buffer	2 ml	2 ml	2 ml	2 ml
Formamide (deionized)	40 ml	40 ml	40 ml	40 ml
Urea	42 g	42 g	42 g	42 g
dH2O	100 ml	100 ml	100 ml	100 ml

Store at 4°C in a brown bottle for approximately 1 month.

5. Gel Percentage

Gel Percentage	Base Pair Separation
6%	300–1,000 bp
8%	200–400 bp
10%	100–300 bp

6. 10% Ammonium Persulfate

Ammonium persulfate 0.1 g in 1ml of dH2O (Store at -20°C for about a week)

Procedure

The DGGE was carried out using a DGGE system (Ingenyphor) as per the manual. Briefly, PCR products were resolved on 8% (w/v) polyacrylamide gels in 0.5 X TAE using denaturing gradients ranging from 0 to 100% (where 100% denaturant contains 7 M urea and 40% formamide). The gels were prepared by using 8% (w/v) acrylamide stock solutions (acrylamide/bisacrylamide ratio 37.5:1). For each sample, 10 µl PCR product was loaded in the bottom of the well. Electrophoresis was carried out at a low voltage (20 V) for 20 min and then at 150 volts for 15-18 hours at a constant temperature of 60ºC. The gels were stained for 20 min with ethidium bromide and washed twice for 5 min with Milli-Q water prior to UV transillumination.

3.2.6.2 Statistical Analysis

The dice coefficient matrix was generated using *Bio-rad Quantity-One* software. The relative data of the DNA bands from the bacterial communities were analyzed to detect grouping patterns among the soil samples.

3.2.6.3 DNA elution from DGGE Gel

Individual bands were cut from the DGGE gel using new razor blades, placed in 20 µl of sterile distilled water, and allowed to incubate overnight at 37°C. The water containing the eluted DGGE band was recovered and placed in a new sterile microcentrifuge tube. Bands were eluted using QIAquick Gel Extraction Kit. Five microlitres of the eluted band was reamplified with primer 1 and primer 2 (Table 7) without GC clamp. The products were ethanol precipitated to remove excess primers and then resuspended in 10 µl of sterile distilled water.

3.2.6.4 Phylogenetic tree Analysis

Phylogenetic analysis of sequenced DGGE bands Partial bacterial 16S rDNA gene sequences were subjected to a NCBI BLASTN http://www.ncbi.nlm.gov/ blast/) search to identify sequences with highest similarity. A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) version 5 software.

3.3 Monitoring survival of selected bacterial inoculants in rhizosphere

3.3.1 Strain selection

Isolation of rhizospheric bacteria was done by following method: Roots were cut in three parts: upper zone (0-15 cm), middle zone (15-30 cm), lower zone (30-45 cm) and were suspended in 100 ml saline water. 1ml of aliquot was taken and spread on nutrient agar plates. Colonies obtained were repeatedly streaked to obtain pure isolates. The isolates thus obtained were screened for heavy metal tolerance (Cd, Pb) in minimal media. Isolates obtained from above study will be selected based on their growth curve study and metal accumulation potential.

Growth Curve

The growth curve of metal tolerating bacterial isolates UR-10, UR-8, UR-21 was studied by plotting the absorbance of cultures at hourly intervals as a function of time (Cappuccino, 1987).

Requirement

1. Overnight grown bacterial cultures S2, S3 and S5.
2. Test tubes containing nutrient broth.

3. Mechanical shaker
4. Cuvettes
5. Autopippetes
6. Spectrophotometer

Procedure

1. The overnight grown bacterial culture each of UR-10, UR-8, UR-21 was checked for absorbance at 600nm and a zero hour reading was taken. A set up for monitoring growth along with metal (Media + 25ppm Cd/ Pb) was also set up.
2. The culture was inoculated in the test tubes containing approximately 10 ml nutrient broth and the tubes were placed on the shaker.
3. The absorbance was measured after every 1 hour and the process continued upto 25 hours.
4. The growth of isolates was measured as a function of time by plotting the absorbance against each hour.

Antibiotic profiling of bacterial isolates

Antibiotic profiling of bacterial isolates UR-10, UR-8, UR-21 was carried out by using standard antibiotics (Cappuccino, 1987).

Requirements/ Reagents

Pure bacterial cultures UR-10, UR-8, UR-21, kanamycin, streptomycin, ampicillin and chloramphenicol.

Stock solutions

Antibiotics: 10 mg ml^{-1}

Working solution

1.Streptomycin 50 µg ml^{-1}
2.Kanamycin 50 µg ml^{-1}
3. Ampicillin 50 µg ml^{-1}
4.Chloramphenicol 10 µg ml^{-1}

Procedure

1. 50 ml media was dispensed in 10 ml flasks (250 ml) and broth in test tubes (10 ml each).
2. Working solutions of antibiotics were made and filter-sterilized in the laminar flow cabin.
3. Two plates each of control, Str 50, Kan 50, Nal 50 were prepared.
4. The five selected cultures UR-10, UR-8, UR-21 were inoculated in 5ml broth and kept for overnight growth at 30°C.
5. The absorbance of the cultures was checked at 600nm the following day after which each culture was spotted on antibiotic-containing medium by dividing each petriplate into five sections.
6. The plates were left undisturbed for an hour followed by incubation at 30°C for 24-48 hours.

Metal Accumulation Potential

The isolate showing best growth along with metal was selected for metal accumulation study. Half strength nutrient broth was inoculated with selected culture (inoculum $O.D_{600}$ - 2.173) and supplemented with 25 ppm of Cd/Pb as an active ingredient. Samples were collected after each 12 hrs and subjected to metal analysis on Flame–AAS.

3.3.2 Molecular tagging of selected microbes

Molecular tagging of selected microbes will be done by introducing reporter gene system like lacZ and antibiotic resistance due to easy detectability, sensitivity and rapidity (Bronstein *et al.*, 1994). The isolates were checked for antibiotic profiling. A wide host range vector pMMB277 (Fig. 7) having antibiotic resistance with added capability of direct screening by *lacZ* expression was transformed in isolates meant for bioaugmentation. Transformed isolates were checked for β- galactosidase expression on antibiotic+ IPTG+ X-gal plates. Reporter gene system harbored in wide host range plasmid will be transformed in isolates previously checked for antibiotic profiling. Inoculation of transformed strains to soil will be done using suitable carrier to maximize the survival. Ecological monitoring in terms of population buildup will be studied by enumeration on antibiotic+ IPTG+ X-gal nutrient agar plates.

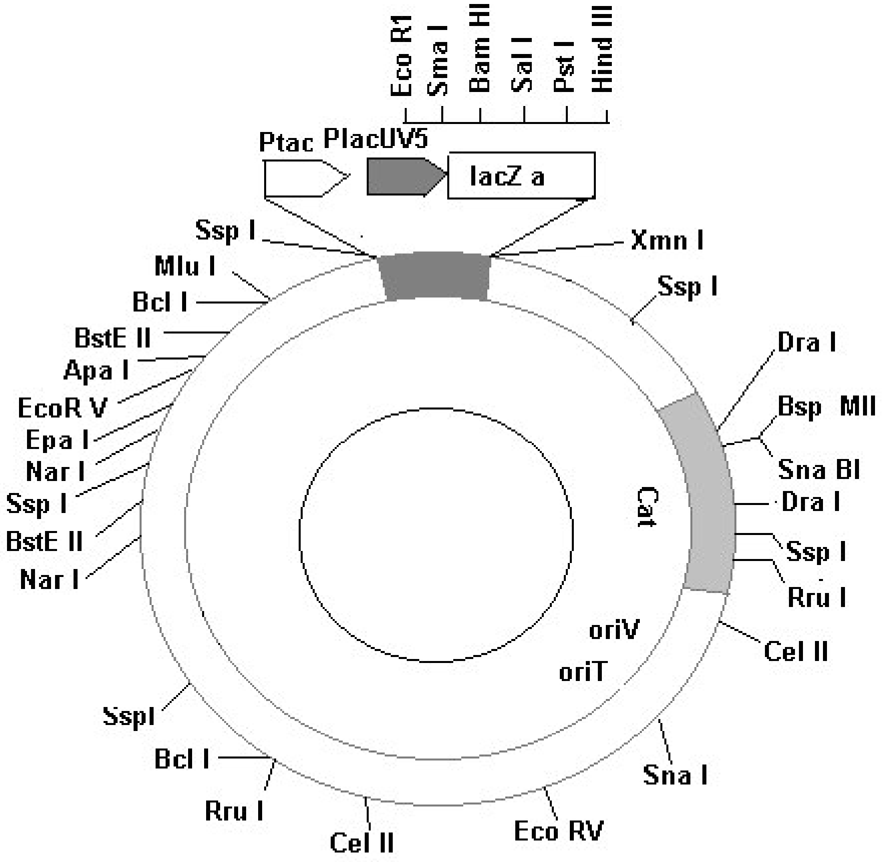


Fig. 7 Map of pMMB277 plasmid broad host range, artificial cloning vector.

3.3.2.1 Gram Staining

Gram staining of the selected bacterial isolate was done as per the standard method using crystal violet and Iodine solution and counter staining with safranin.

3.3.2.2 DNA isolation from bacterial isolate

Reagents

1. CTAB (Dissolve 4.1 g NaCl in 80 ml of water and slowly add 10 g CTAB while heating (≈65°C) and stirring. This takes more than 3 hrs to dissolve CTAB. Adjust final volume to 100 ml and sterilize by filter or autoclave)
2. TE buffer (10mM Tris; 1 mM EDTA, pH 8.0)
3. Lysozyme
4. Proteinase K
5. 5M NaCl
6. 10% SDS
7. Chloroform
8. Isoamyl alcohol
9. Phenol
10. Isopropanol
11. Ethanol
12. DNase-free RNAse I (100 mg/mL)
13. Molecular biology grade DNase-free water

Equipment

1. Hot Plate
2. 250 mL glass beaker
3. Magnetic stir rod
4. Thermometer
5. Automatic pipette dispenser
6. Centrifuge

7. 65°C water bath
8. 37°C incubator/heat block
9. 56°C heat block

Procedure

1. Taken overnight grown culture and transfer bacterial suspension to the appropriate centrifuge tube and spin down cells in microfuge or centrifuge at 10,000 rpm for 5 minute.
2. Discarded the supernatant and re-suspend cells in TE and added 20µl of lysozyme (conc. 100 mg/ml). Mix well and incubate for 30 min. at 37°C.
3. Added 10% SDS. Mix well.
4. Added Proteinase K (10 mg/ml). Mix well
5. Incubated for 1-3 hr at 56°C. If cells are not lysed (as seen by cleared solution with increased viscosity) incubation can proceed overnight (16 hrs).
6. Added 5 M NaCl. Mix well
7. Added CTAB/ NaCl (heated to 65°C), mixed well and incubated at 65°C for 10 min.
8. Added chloroform: isoamyl alcohol (24:1) and microbes were spinned for 10 min at room temperature
9. Aqueous phase was transferred to clean micro centrifuge tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged.
10. Transferred aqueous phase to clean micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24:1). Mixed well.
11. Centrifuged to obtained aqueous phase and added 0.6 volume isopropanol (-20°C).
12. Incubated at -20°C for 2 hrs and centrifuged for 15 min at 4°C.
13. Washed the pellet with cold 70% ethanol (directly from -20°C freezer), spin at max speed for 5 min.
14. Discarded the supernatant and pellet was dried at room temp. Re-suspend in ~50 µl of TE Buffer.

3.3.2.3 16S rDNA amplification and sequencing

To identify the isolate, 16S rDNA was amplified and sequenced. The amplification mixture for each sample DNA contained 100 pmol of each primer, 200 µM dNTPs, 0.2% BSA, 1X PCR buffer, 1.5 mM $MgCl_2$ and 2.5 units of Taq DNA polymerase (Fermentas) in a 100 µl reaction volume. PCR was performed in an automated thermal cycler (Applied Biosystems 2720) with an initial 94°C denaturation for 9 min, followed by 30 cycles of 94°C for 30 sec, 55ºC for 30 sec, 72°C for 30 sec and a final extension at 72°C for 7 min using primers listed in Table 8. The amplicon was sequenced and the sequence obtained was BLAST analyzed to obtained maximum percentage homology with the database.

Table 8: Primer sequence for 16S rDNA amplification

Primer	Sequence	Target region	References
8F	AGA GTT TGA TCC TGG CTC AG	16S rDNA	Eden *et al.*, 1991
1492R	GGT TAC CTT GTT ACG ACT T	16S rDNA	Eden *et al.*, 1991

3.3.2.4 Plasmid isolation

Plasmid pMMB277 was isolated from *E.coli* 2842 by the alkali lysis method (Brinboim and Doly, 1979).

Reagents

1. Solution I: 50mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8.0.
2. Solution II: 0.2 N NaOH, 1% w/v SDS.
3. Solution III: 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml water and stored at 4ºC.
4. Lysozyme: 10 mg ml-1.
5. 1X TE Buffer 10mM Tris-Cl, 1mM EDTA.

Procedure

1. Cells were harvested from 1.5 ml of exponentially grown bacterial culture by centrifugation at 12000 rpm. for 2 minutes after measuring the absorbance (O.D. 600nm =1.0). The supernatant was discarded and the bacterial pellet air-dried.
2. 200 µl of ice-cold solution I was added immediately to the dried pellet followed by 50 µl lysozyme.
3. The culture was kept on an ice bath for five minutes and 400 µl of freshly prepared solution II was added and mixed gently.
4. 300 µl of ice-cold solution III was added followed by reincubation in ice for 20-60 minutes with intermittent mixing.
5. Centrifugation was coducted further at 12000 rpm. for 10-15 minutes and 800 µl of supernatant was collected in a fresh eppendorf tube. Equal volumes of isopropanol and supernatant were mixed.
6. The mixture was incubated at 4°C for 10-15 minutes followed by centrifugation at 10,000 rpm. for 15 minutes.
7. The supernatant was discarded and the pellet air-dried followed by addition of 30 µl TE buffer and stored at 4°C until further use.

Solvent extraction

1. RNAse was activated for 8-10 minutes at 100°C and slowly cooled to room temperature after which 1-2 µl was added to the eppendorf tube and mixed by vortexing. The culture was centrifuged at the rate of 30 seconds /100 rpm followed by incubation at 37°C for one hour.
2. Equal volumes of phenol and chloroform (250 µl: 250µl) were added to the eppendorf tube and mixed well. Centrifugation was carried out at 10000 g for 10 minutes.
3. The aqueous layer was separated in a fresh eppendorf tube and equal volume of chloroform was added followed by centrifugation at 10000 rpm for 10 minutes.
4. The aqueous layer was separated and 50 µl of 0.3 M CH_3COONa (1/10 of the volume) was added followed by mixing of an equal volume of isopropanol. The culture was stored at 4°C for 20-25 minutes.

5. Centrifugation was carried out at 10000 rpm. for 10 minutes and the supernatant was removed. The pellet was rinsed with 1 ml of 70% ethanol at 4°C and air dried.
6. The pellet was re-dissolved in 50µl of TE (pH 8.0) and stored at -20°C followed by gel electrophoresis using 0.7% agarose.

3.3.2.5 Transformation

Transformation of phosphate-solubilizing bacterial isolates with plasmid pMMB277 of E.coli 2842 was carried out by the calcium chloride method (Mendal and Higa, 1970).

Procedure

1. Overnight grown bacteria were inoculated in 25 ml of fresh nutrient broth and incubated at 37°C for two and half hours followed by incubation in ice.
2. The cells were harvested by centrifugation at 7000 rpm. for 8 minutes at -4°C and re-suspended in 100 mM CaCl$_2$ and kept for 10-20 minutes in ice.
3. The step was repeated and cells were re-suspended in ice chilled CaCl$_2$ and kept at -4°C for two and half hours to make the competent cells.
4. 100 µl of competent cells were transferred to a sterile eppendorf tube and 10 µl of plasmid DNA was added, mixed and kept on ice for 30 minutes.
5. Heat shock treatment was given next in the water bath at 42$^{\square C}$ for 2 minutes and 1.0ml of fresh nutrient broth was added followed by incubation for one hour at 37°C.
6. The cells were harvested by centrifugation at 6000 rpm. for 6 minutes.
7. 100µl of concentrated cell suspension was plated on chloramphenicol (10µg ml-1) plus IPTG and X-gal containing nutrient agar plates and the transformant checked for their β-galactosidase expression.

3.3.3 Enumeration of pMMB277 transformant from wetland soil

Bacterial counts in soil with and without selected transformant were carried out as per the standard plate at regular intervals. Bacterial count was on NA media containing 10µg ml^{-1} chloramphenicol (Cappuccino, 1987).

Requirements

Chloramphenicol + IPTG+X-gal containing nutrient agar plates

Preparation of stock solutions

1. IPTG *(Isopropyl-β-D-thiogalactopyranoside)*: 100 mg ml^{-1} of sterile water and filter sterilized by 0.22µl disposable filter.
2. X-gal *(5-Bromo-4-chloro-3 indolyl-β-D-thiogalactopyranoside)*: 20 mg ml^{-1} of dimethylformamide wrapped in aluminium foil and stored at - 20°C.
3. Chloramphenicol Stock solution (10 mg ml^{-1}) and working solution (10µg ml^{-1})

Procedure

1. Transformant was grown in nutrient broth and absorbance of the cultures was measured the following day at 600 nm followed by centrifugation at 7000 rpm. for 15 minutes.
2. The supernatant was removed and the pellet was washed with 0.85 N saline followed by vortexing.
3. The suspension was washed thrice with saline repeatedly and finally the pellet was suspended in sterile water and inoculated in the soil in wetland system. A control system was maintained without transformant inoculation.
4. For monitoring the survivals of transformants, soil was sampled on regular intervals and enumerations were carried out on NA + Chl^R plates with IPTG (8 µl) and X-gal (30 µl).

3. 4 Impact of plant rhizosphere and bioaugmentation on remediation potential

The wetland established plants of *Phragmites australis* were gently uprooted at regular intervals and washed in normal tap water to remove any adhering soil particles. Thereafter the plants were dried with blotting sheets. For dry weight the plants were kept overnight in an oven at 80-90°C and the weight was taken the following day. In order to study metal uptake by the plants, they were oven-dried overnight at 80-90°C. Roots, leaves and stem were separated and crushed in a mixer grinder after which they were sieved through 0.2 mm sieve to obtain a fine powder form.

The powder was digested using concentrated nitric and perchloric acid for metal analysis using atomic absorption spectrophotometry as discussed in earlier.

Chapter 4 Results & Discussion

4.1 Development of *Phragmites*-vegetated wetland system for treatment of wastewater

A horizontal subsurface flow constructed wetland (HSSF-CW) at lab scale was constructed with dimensions of 48x 33x 20 cm in plastic tubs each having three sections (a) Inlet section (b) Vegetative section (c) Outlet section (Fig. 7). The system was attached with an inlet tap at one end on upward side and outlet tap on the opposite side at the lower end to regulate the flow rate. A volume of 100 ml treated effluent was collected from outlet in each run and was analyzed for change in pH, electrical conductivity (EC), Dissolved oxygen (DO), Biochemical oxygen demand (BOD_5), Chemical oxygen demand (COD), total and suspended solids, nitrate-N, phosphates and metals (Fe, Cu, Mn, Zn, Ni, Pb, Cd). The samples for heavy metal analysis were acid digested, volume made up using metal free water, filtered and analyzed for residual metal concentration by atomic absorption spectrophotometer (GBC 932 AA; GBC Scientific Equipment Pvt. Ltd. Australia) using air–acetylene flame and single element hollow cathode lamp.

Second order polynomial response surface models were fitted to each of dependent variable with the help of statistical software MINITAB. A stepwise procedure was followed to generate a regression model with significant terms ($p < 0.05$). The RS plots were drawn around the central value in the design of experiment.

Table 9. Independent variables and their range of center composite design

Independent variables		Minimum Value	Maximum Value	Hydraulic load
Inlet flow Rate (ml/min)	X_1	15	30	0.0104- 0.0208 cm^3/day
Outlet flow Rate (ml/min)	X_2	6	12	0.0042- 0.0083 cm^3/day
Retention time (hrs)	X_3	1	24	_

Treatment of domestic wastewater using constructed wetland system (Fig. 7) vegetated by *Phragmites* for 6 months was studied over a period of 20 days. On the basis of the experimental data a comparison on % remediation was drawn between planted and unplanted system (Table 10). Treatment of wastewater was best obtained as revealed by maximum reduction of BOD, COD, nitrate and metals with inlet flow rate of 22.5ml/min, outlet 9ml/min and retention time of 12.5 hrs. Significant reduction was obtained in parameters like BOD_5 66.7%, COD 60%, TDS 70% and Cd 50% (Table 10) as compared to unplanted control system where BOD_5, COD were reduced by 4.2%, 3% respectively and no significant removal of TDS and Cd was observed. Nitrates and Phosphates were also significantly reduced by 21% and 50% (Table 10) respectively. In Fe, Mn, Pb, removal rate obtained were 11.1%, 50%, 25% respectively as compared to non significant removal rates in unplanted system (Table 10).

Table 10. Treatment of domestic wastewater in CW vegetated system with *Phragmites* (Planted) and Unplanted (Control)

Parameter	Initial Value (before treatment)	Outlet Value (Unplanted)	Outlet Value (Planted)	% Reduction (Unplanted-control)	% Reduction (Planted)
pH	7.7	8.1	8.3	-	-
EC (uS)	136.9	133.4	135.8	2.6	0.8
TS (g/100ml)	0.1	0.1	0.04	-	60
TDS (g/100ml)	0.1	0.1	0.03	-	70
SS (g/100 ml)	0.05	0.1	0.04	-	20
DO (mg/L)	9.5	8.9	7.5	6.3	21.1
BOD_5 (mg/L)	240	230	80	4.2	66.7
COD (mg/L)	2000	1940	800	3	60
Nitrates (mg/L)	1.9	1.8	1.5	5.3	21.1
Phosphate (mg/L)	0.6	0.6	0.3	BDL	50
Fe (ppm)	0.9	1.1	0.8	BDL	11.1
Cu (ppm)	0.02	0.02	0.02	BDL	0
Mn (ppm)	0.6	0.6	0.3	BDL	50
Zn (ppm)	0.2	0.3	0.2	BDL	0
Ni (ppm)	0.1	0.1	0.1	BDL	0
Pb (ppm)	0.4	0.4	0.3	BDL	25
Cd (ppm)	0.02	0.02	0.01	BDL	50

* BDL- Below Detection Limit

A lab scale horizontal flow sub surface wetland (HSSF-CW) was constructed with dimensions of 48x 33x 20 cm in plastic tubs each having three sections (a) Inlet section (b) Vegetative section (c) Outlet section. In vegetative section, a gravel stone bed was formed at the width of 7.5 cm on each side and above that a soil bed of 30 x 16.5 cm dimensions was laid. The system was attached with an inlet tap at one end on upward side and outlet tap on the opposite side at the lower end to regulate the flow rate. Treatment of domestic wastewater using constructed wetland system vegetated with *Phragmites australis* was studied using Response surface methodology (RSM), a mathematical model was employed for analyzing the effect of independent factors (inlet flow rate, outlet flow rate, retention time) on dependent variables (wastewater parameters e.g. electrical conductivity, BOD_5, COD, nitrates, phosphates, total solids, suspended solids, heavy metals) in order to maximally treat wastewater. All independent variables were standardized using central composite design matrix (Montgomery, 1997) utilizing different run orders. Treatment of wastewater was best obtained with 22.5ml/min of inlet and 9ml/min of outlet and 12.5 hrs of retention time. A reduction of 60- 67% was obtained in some parameters like total solids, BOD_5, COD, and heavy metal concentration as compared to the control unplanted system (Table 10). Similar study has been carried out in a pilot scale constructed wetlands in treating a mild domestic wastewater where a removal of about 56–77% of COD, 50–88% of TSS, 20–88% of TP (total Phosphorus), 27–96% of NH4 + and 99% of total coliforms numbers was observed (Katayon *et al,* 2008). In a wetland microcosms planted with *Typha domingensis*, *Lemma minor*, potential metal removal rates are 3-8 mg Pb/m^2 day and 2-4 mg Cd/m^2 day are observed (Debusk *et al.*, 1996) (Table 2). In case of *Phragmites* planted wetlands also, 99%, 98%, 75%, 83% and 68% reduction in Pb, Zn, Cd, Cu and TSS respectively have been observed in contaminants in a treatment wetland, South China (Yu *et al.*, 2005).

4.1.1 Response Surface Methodology (RSM) approach to study effect of variables on treatment parameters

Three input variables with inlet flow rate ranging from 15-30 ml/min, the outlet at 6-12 ml/min and retention time varying from 1-24 hours (Table 9) were employed for study. Hydraulic loading into the system has also been described in Table 9. In the present study, all the designed experiments were performed and results were analyzed through regression model and checked for significance. Model coefficients were estimated by using multiple regressions (MINITAB) and model accuracy was checked using R^2, *F test* and *P value* for each parameter.

As per ANOVA and *P-value,* the regression model was studied in pH, Electrical Conductivity (EC), Dissolved oxygen (DO), Biochemical oxygen demand (BOD_5), Chemical oxygen demand (COD), Total and Suspended Solids, Nitrate-N, Phosphates (Table 11), Metals ((Table 12) *i.e* Fe, Cu, Mn, Zn, Ni, Pb, Cd and found significant in most of the parameters. Amongst the three independent variables *i.e.* Inlet flow rate, Outlet flow rate, Retention time, the coefficients of the interactive effects between the variables did not appear to be very significant in comparison to the linear effects. However, the interaction effect between inlet and outlet flow rate $X_1 * X_2$ and inlet and retention time $X_1 * X_3$ were found to be significant (Table 11). These results are in close agreement with those obtained from the response surface analysis, confirming that the RSM could be effectively used to optimize the process parameters in complex processes using the statistical design of experiments. The behavior of various variables studied for treatment process is discussed in Table 11.

Table 11 Coefficients of final models obtained for wastewater parameters

Variables	**pH**	**EC (uS)**	**TS (g/100ml)**	**TDS (g/100ml)**	**SS (g/100 ml)**	**DO (mg/L)**	**BOD_5 (mg/L)**	**COD (mg/L)**	**Nitrates (mg/L)**	**Phosphate (mg/L)**
Constant β	8.2637	135.804	0.043639	0.064173	0.0401	7.5082	78.98	804.6	1.4527	0.36895
X_1	-0.0716	0.557	0.002143	0.00213	-0.0052	0.0313	22.27	195.3	-0.0137	-0.0119
X_2	-0.1043	0.093	0.003621	0.008507	0.00314	0.1528	11.52	314.5	0.0242	0.09587
X_3	-0.0085	0.17	-0.00146	-0.00324	-0.0024	0.1228	-25.58	61.1	-0.0305	0.2324
$X_1{}^2$	0.029	0.167	-0.00825	-0.00113	-0.0084	0.1231	26.13	309.6	-0.1457	0.00394
$X_2{}^2$	0.029	-0.045	0.000945	-0.00325	-0.0077	0.3529	24.36	-196	-0.0159	-0.04226
$X_3{}^2$	-0.0488	-0.311	-0.0033	-0.00466	0.0022	0.5297	12.87	210.6	-0.0061	0.30853
$X_1 * X_2$	-0.07	0.012	0.0015	0.004	0.009	-0.2375	-21.25	67.5	-0.1663	0.08954
$X_1 * X_3$	-0.06	-0.637	-0.0055	0.001	-0.004	0.2375	2.5	127.5	0.136	0.03025
$X_2 * X_3$	-0.0125	-0.137	-0.0055	-0.004	-0.002	-0.2625	-11.25	-147.5	-0.1571	-0.04719
R^2	0.814	0.772	0.763	0.737	0.868	0.863	0.904	0.845	0.867	0.824
F test	9.04	5.28	6.35	3.7	12.71	15.3	14	9.9	13.72	2.22
P	0.003	0.019	0.011	0.001	0.001	0	0.001	0.002	0.001	0.019

- R^2 and F-test are also reported for each parameter, * P value given is relative to give p-values only.

pH

The effect of all three independent variables was studied on the pH of wastewater. A regression model was found highly significant as predicted from R^2, F test and P value (Table 11). The smaller the magnitude of P, the more significant is the corresponding coefficient. Fig. 8 Graph A shows the RS (response surface) plots of pH with outlet flow rate and retention time. In each graph, the independent variable not present is kept constant at the central value of design of experiment e.g. in graph A the inlet flow rate is kept constant at 22.5ml/min. Apart from the regression studies, Response surface methodology (RSM) also provided an insight into quadratic and interaction effects of independent variables however linear model ($X_1 * X_3$) was found most significant in this study. The regression equation for the output response of pH for any value of inlet and outlet flow rate and constant retention time can be explained by Eq. (2).

$$pH = (8.2637) + (-0.0716\ X_1) + (-0.1043\ X_2) + (-0.0085\ X_3) + (0.029\ X_1^2) + (0.029\ X_2^2) + (-0.0488\ X_3^2) + (-0.07\ X_1X_2) + (-0.06\ X_1X_3) + (-0.0125X_2\ X_3) \qquad (2)$$

Apart from the linear effect of the parameter for the variation studies, the RSM also gives an insight into the quadratic and interaction effect of the parameters. These analyses were done by means of Fisher's 'F'-test and Student 't'-test. The student 't'-test was used to determine the significance of the regression coefficients of the parameters (Table 11).

The P-values were used as a tool to check the significance of each of the interactions among the variables, which in turn may indicate the patterns of the interactions among the variables. In general, larger the magnitude of t and smaller the value of P, the more significant is the corresponding coefficient term (Montgomery, 1991). The regression coefficient, t and P values for all the linear, quadratic and interaction effects of the parameter are given in Table 11. It was observed that the coefficients for the linear effect of inlet flow rate, outlet flow rate (P = 0.015, 0.002 respectively) were highly significant (Table 11) and coefficient for the linear effect of time was the least significant.

Electrical Conductivity (EC)

It was observed that the coefficients for the linear effect of inlet flow rate (P = 0.004) was highly significant and coefficient for the linear effect of outlet flow rate was the least significant (Table 11). The coefficients of the square effects among the variables did not appear to be very significant in comparison to the linear effects. However, the interaction effect between inlet and retention time (P = 0.008) were found to be significant. Fig. 8 Graph B shows the RS (response surface) plots of EC with outlet flow rate and retention time (Table 11). The regression equation for the output response of electrical conductivity (EC) can be explained by Eq. (3).

$$EC = (135.804) + (0.557\ X_1) + (0.093X_2) + (0.17\ X_3) + (0.167\ X_1^{2)} + (-0.045\ X_2^{2)} + (-0.311\ X_3^{2)} + (0.012\ X_1\ X_2) + (-0.637\ X_1\ X_3) + (-0.137X_2\ X_3) \qquad (3)$$

TS (Total solids)

It was observed that the coefficients for the square effect of inlet flow rate (P = 0.002) was highly significant and coefficient for the square effect of outlet flow rate was the least significant (Table 11). The coefficients of the linear effects between the variables did not appear to be very significant in comparison to the square effects. However, the interaction effect between inlet and retention time (P = 0.071) and outlet and retention time (P = 0.071) were found to be significant (Table 11). Fig. 8 Graph C shows the RS (response surface) plots of TS with inlet flow rate and retention time. The regression equation for the output response of total solids (TS) can be explained by Eq. (4)

$$TS = (0.043639) + (0.002143\ X_1) + (0.003621\ X_2) + (-0.001464\ X_3) + (-0.008248\ X_1^{2)} + (0.000945\ X_2^{2)} + (-0.003298\ X_3^{2)} + (0.0015\ X_1X_2) + (-0.0055\ X_1X_3) + (-0.0055\ X_2X_3) \qquad (4)$$

TDS (Total dissolved solids)

It was observed that the coefficients for the linear effect of outlet flow rate (P = 0.013) was significant and coefficient for the linear effect of inlet flow rate was the least significant (Table 11). The coefficients of the interaction effects among the variables did not appear to be very significant in comparison to the linear effects. However, the square effect of retention time (P = 0.118) was found to be significant. Fig. 8 Graph D shows the RS (response surface) plots of TDS with outlet flow rate and retention time (Table 11). The regression equation for the output response of total dissolved solids (TDS) can be explained by Eq. (5)

$$TDS = (0.064173) + (0.00213\ X_1) + (0.008507\ X_2) + (-0.003235\ X_3) + (-0.001128\ X_1^{2)} + (-0.003249\ X_2^{2)} + (-0.004663\ X_3^{2)} + (0.004\ X_1X_2) + (0.001\ X_1X_3) + (-0.004\ X_2X_3) \quad (5)$$

Suspended solids (SS)

It was observed that the coefficients for the square effect of inlet and outlet flow rate (P = 0.000, P = 0.002 respectively) was highly significant (Table 11) and coefficient for the square effect of retention time was the least significant. The coefficients of the linear effects between the variables were less significant in comparison to the square effects (Table 11). However, the interaction effect between inlet and outlet flow rate (P = 0.005) and inlet and retention time (P = 0.140) were found to be significant. Fig. 8 Graph E shows the RS (response surface) plots of pH with inlet flow rate and inlet flow rate. The regression equation for the output response of suspended solids (SS) can be explained by Eq. (6)

$$SS = (0.040096) + (-0.005179\ X_1) + (0.003142\ X_2) + (-0.00237\ X_3) + (-0.008407\ X_1^2) + (-0.0077\ X_2^2) + (0.0022\ X_3^2) + (0.009\ X_1X_2) + (-0.004\ X_1\ X_3) + (-0.002\ X_2X_3) \quad (6)$$

In such systems, TSS is removed mainly by physical processes such as sedimentation and filtration (Kadlec and Knight, 1996) followed by aerobic or anaerobic microbial degradation inside the substrate (Manios *et al.*, 2003).

Dissolved Oxygen (DO)

It was observed that the coefficients for the square effect of outlet flow rate and retention time (P = 0.003, P = 0.000 respectively) was highly significant (Table 11) and coefficient for the square effect of inlet flow rate was the least significant. The coefficients of the linear and interaction effects between the variables were not significant in comparison to the square effects (Table 11). Fig.8 Graph F shows the RS (response surface) plots of DO with inlet flow rate and retention time. The regression equation for the output response of dissolved oxygen (DO) can be explained by Eq. (7).

$$DO= (7.5082) + (0.0313\ X_1) + (0.1528\ X_2) + (0.1228\ X_3) + (0.1231\ X_1^2) + (0.3529\ X_2^2) + (0.5297\ X_3^2) + (-0.2375\ X_1X_2) + (0.2375\ X_1X_3) + (-0.2625\ X_2X_3) \qquad (7)$$

Biochemical Oxygen Demand (BOD_5)

It was observed that the coefficients for the linear and square effect of inlet and outlet flow rate were highly significant. In linear effects (Table 11), inlet flow rate and retention time (P=0. 003, P=0. 000 respectively) were found more significant than the outlet flow rate (P= 0.065). In square effects, all three independent variables inlet, outlet flow rate and retention time (P= 0.000, P= 0.000, P= 0.039 respectively). The coefficients of the interaction effects between the variables were less significant (Table 11) in comparison to the linear and square effects. Fig. 8 Graph G shows the RS (response surface) plots of BOD_5 with outlet flow rate and retention time. The regression equation for the output response of Biochemical Oxygen Demand (BOD_5) can be explained by Eq. (8).

$$BOD_5 = (78.98) + (22.27\ X_1) + (11.52\ X_2) + (-25.58\ X_3) + (26.13\ X_1^2) + (24.36\ X_2^2) + (12.87\ X_3^2) + (-21.25\ X_1X_2) + (2.5\ X_1X_3) + (-11.25\ X_2X_3) \qquad (8)$$

The removal efficiencies observed in our system wetlands fall within the range of results found in the literature for similar systems and they can be considered as moderately high performers (Kadlec and Knight, 1996; Klomjek and Nitisoravut, 2005; Vymazal, 2002; Vymazal, 2005).

Chemical Oxygen Demand (COD)

It was observed that the coefficients for the linear and square effects (P = 0.008, P = 0.002 respectively) was highly significant. In linear effects, the coefficients of inlet and outlet flow rate (P = 0.038, P = 0.003 respectively) were highly significant (Table 11) and in square effects all parameters inlet, outlet flow rate and retention time (P= 0.003, P= 0.034, P= 0.025 respectively) were found significant. The coefficients of the interaction effects between the variables were less significant in comparison to the linear and square effects. Fig. 8 Graph H shows the RS (response surface) plots of COD with inlet flow rate and retention time. The regression equation for the output response of Chemical Oxygen Demand (COD) can be explained by Eq. (9)

$$COD = (804.6) + (195.3\ X_1) + (314.5\ X_2) + (61.1\ X_3) + (309.6\ X_1^2) + (-196\ X_2^2) + (210.6\ X_3^2) + (67.5\ X_1X_2) + (127.5\ X_1X_3) + (-147.5\ X_2X_3) \qquad (9)$$

These results were expected since the BOD_5 and the COD measures the organic matter present in wastewater and the same principles of behavior and removal inside the CW's are applied to them (Kadlec and Knight, 1996).

Nitrates

It was observed that the coefficients for the square and interaction effects (P = 0.007, P = 0.001 respectively) was highly significant. In square model, the coefficients for inlet flow rate (P= 0.000) were most significant and in interaction effect, the coefficients of all parameters inlet, outlet and retention time (P = 0.002, P = 0.004, P = 0.008 respectively) were found significant (Table 11). The coefficients of the linear effects between the variables were less significant in comparison to the square effects (Table 11). However, the interaction effect between inlet and outlet flow rate (P = 0.005) and inlet and retention time (P = 0.140) were found to be significant. Fig. 8 Graph I shows the RS (response surface) plots of nitrates with inlet flow rate and retention time. The regression equation for the output response of nitrates can be explained by Eq. (10).

$$Nitrates = (1.4527) + (-0.0137\ X_1) + (0.0242\ X_2) + (-0.0305\ X_3) + (-0.1457\ X_1^2) + (-0.0159\ X_2^2) + (-0.0061\ X_3^2) + (-0.1663\ X_1X_2) + (0.136\ X_1\ X_3) + (-0.1571\ X_2X_3) \qquad (10)$$

Phosphates

It was observed that the no model was found significant (Table 11) among linear, square and interaction (P = 0.323, P = 0.149, P = 0.936 respectively). Fig. 8 Graph J shows the RS (response surface) plots of phosphates with inlet flow rate and outlet flow rate. The regression equation for the output response of phosphates can be explained by Eq. (11).

$$Phosphates = (0.36895) + (-0.0119\ X_1) + (0.09587\ X_2) + (0.2324\ X_3) + (0.00394\ X_1^2) + (-0.04226\ X_2^2) + (0.30853\ X_3^2) + (0.08954\ X_1X_2) + (0.03025\ X_1X_3) + (-0.04719\ X_2X_3) \qquad (11)$$

These results are similar to the removal rate of 45% found by Prochaska and Zouboulis (2006) who used a mixture of river sand and dolomitic limestone. Such results confirm the good capacity of the selected substrate for phosphorous removal (Zurita *et al.*, 2006). The performance can be further improved by reducing the substrate size as this would increase the contact surface and allow the wastewater to move slower through the substrate providing more time and surface area for chemical adsorption (Prochaska *et al.*, 2007).

Table 12: Coefficients of final models obtained for metals (ppm) in wastewater

Variables	Fe	Cu	Mn	Zn	Ni	Cd	Pb
Constant β	0.7304	0.01499	0.33672	0.208	0.08533	0.00813	0.26453
X_1	-0.225	-0.0024	0.29257	0.0733	-0.0088	-0.0016	0.00044
X_2	-0.3798	0.01614	-0.0127	0.0935	0.00768	0.00177	0.04019
X_3	0.1445	-0.011	0.19172	-0.0913	-0.0062	-0.0048	-0.049
X_1^2	0.2871	0.00834	0.1848	0.0356	0.01272	0.00215	0.00695
X_2^2	0.2018	0.00127	0.14945	0.0321	0.00812	0.00427	-0.0118
X_3^2	0.3421	0.00658	0.10331	0.0933	0.00211	0.00621	0.03364
$X_1 * X_2$	0.7523	-0.0185	-0.053	0.1052	-0.0035	-0.0071	-0.0249
$X_1 * X_3$	-0.8447	0.01625	0.122	-0.1018	0.00925	0.00538	0.02563
$X_2 * X_3$	-0.8783	-0.029	0.0715	-0.1185	-0.01	-0.0054	-0.0581
R^2	0.719	0.743	0.926	0.83	0.787	0.79	0.763
F test	3.98	2.49	26.21	6.81	7.17	3.17	3.21
P	0.042	0.012	0	0.009	0.013	0.032	0.03

* R^2 and F-test are also reported for each parameter, * P value given is relative to given only.

Metals (Fe, Cu, Mn, Zn, Ni, Cd, Pb)

In the case of Fe, no model was found significant among linear, square and interaction (Table 12). From the p-value, the coefficients of interaction model were found significant but none of the interaction models of three variables was much significant in this study (P= 0.100, P= 0.069, P= 0.060 respectively). For Fe Eq. (12) is derived as below.

$$Fe = (0.7304) + (-0.225\ X_1) + (-0.3798\ X_2) + (0.1445\ X_3) + (0.2871\ X_1^2) + (0.2018\ X_2^2) + (0.3421\ X_3^2) + (0.7523\ X_1X_2) + (-0.8447\ X_1X_3) + (-0.8783\ X_2X_3) \quad (12)$$

In the case of Cu (Table 12), no model was found significant among linear, square and interaction (P = 0.379, P = 0.811, P = 0.120 respectively). For Cu, Eq. (12) is derived.

$$Cu = (0.01499) + (-0.0024X_1) + (0.01614\ X_2) + (-0.011X_3) + (0.00834\ X_1^2) + (0.00127\ X_2^2) + (0.00658\ X_3^2) + (-0.0185\ X_1X_2) + (0.01625\ X_1X_3) + (-0.029\ X_2X_3) \quad (13)$$

For Mn, coefficients for both linear and square model were found significant (P = 0.000, P = 0.001 respectively). Among linear effects, inlet flow rate and retention time were significant (P = 0.000, P = 0.000 respectively). In square effects all three parameter coefficients were significant (Table 12) for inlet flow rate, outlet flow rate and retention time (P = 0.000, P = 0.003, P = 0.023 respectively). Eq (14) for Mn is derived as follows:

$$Mn = (0.33672) + (0.29257\ X_1) + (-0.01274\ X_2) + (0.19172\ X_3) + (0.1848\ X_1^{\ 2}) + (0.14945\ X_2^2) + (0.10331\ X_3^2) + (-0.053\ X_1X_2) + (0.122\ X_1X_3) + (0.0715\ X_2X_3) \quad (14)$$

In the study of Zn (Eq. 15), coefficients of linear and interaction model were found significant (P = 0.009, P = 0.011 respectively). In linear effects, outlet flow rate and retention time were more significant (P = 0.018, P = 0.020 respectively) than the inlet flow rate (P = 0.051). In interaction effects of Zn (Table 12), all the parameters *i.e.* Inlet and outlet flow rate, inlet and retention time, outlet and retention time were significant (P = 0.036, P = 0.041, P = 0.021 respectively).

$$Zn = (0.208) + (0.0733\ X_1) + (0.0935\ X_2) + (-0.0913\ X_3) + (0.0356\ X_1^2) + (0.0321\ X_2^2) + (0.0933\ X_3^2) + (0.1052\ X_1X_2) + (-0.1018\ X_1X_3) + (-0.1185\ X_2X_3) \quad (15)$$

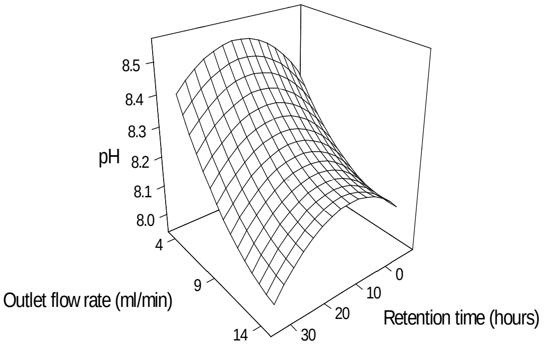
In the case of Ni (Eq. 16), Cd (Eq. 17), Pb (Eq. 18), no model was found significant (Table 12) among linear, square and interaction (P = 0.119, P = NA, P = 0.238 for Ni, P = 0.289, P = 0.094, P = 0.072 for Cd, and P = 0.070, P = 0.369, P = 0.153 for Pb respectively).

$$Ni = (0.08533) + (-0.00877\ X_1) + (0.00768\ X_2) + (-0.0062\ X_3) + (0.01272\ X_1^2) + (0.00812\ X_2^2) + (0.00211\ X_3^2) + (-0.0035\ X_1X_2) + (0.00925\ X_1X_3) + (-\ 0.01X_2X_3) \qquad (16)$$

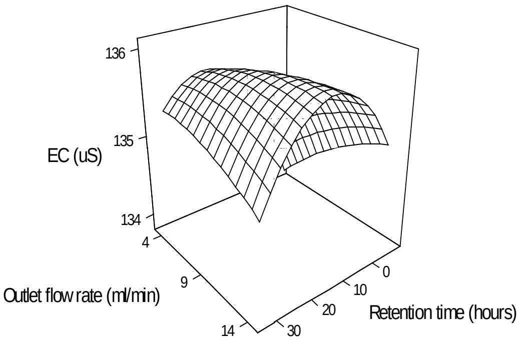
$$Cd= (0.008132) + (-0.001648\ X_1) + (0.001767\ X_2) + (-0.004799\ X_3) + (0.002145\ X_1^2) + (0.004266\ X_2^2) + (0.006211\ X_3^2) + (-0.007125\ X_1X_2) + (0.005375\ X_1X_3) + (-0.005375\ X_2X_3) \qquad (17)$$

$$Pb = (0.26453) + (0.00044\ X_1) + (0.04019\ X_2) + (-0.04895\ X_3) + (0.00695\ X_1^{\ 2}) + (-0.01179\ X_2^2) + (0.03364\ X_3^2) + (-0.02488\ X_1X_2) + (0.02563\ X_1X_3) + (-0.05813\ X_2X_3) \qquad (18)$$

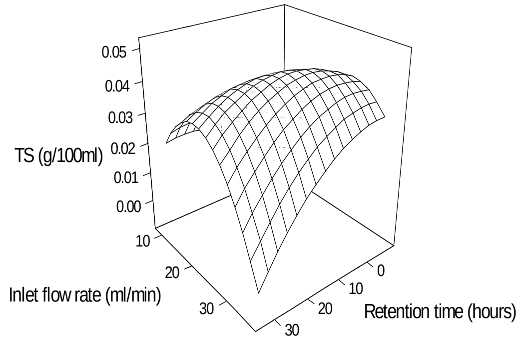
Fig. 8 Graph K, L, M, N, O, P, Q shows the RS (response surface) plots of Fe, Cu, Mn, Zn, Ni, Cd, Pb. In some metals Fe, Cu, Zn, the initial values in wastewater were higher than those obtained after treatment. This can be attributed to their higher concentration in the soil samples (data not shown). The regression model was found highly significant in some important parameters like BOD$_5$, COD, and nitrate reduction.



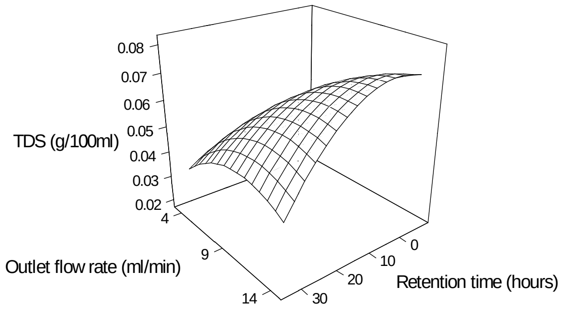
(A)



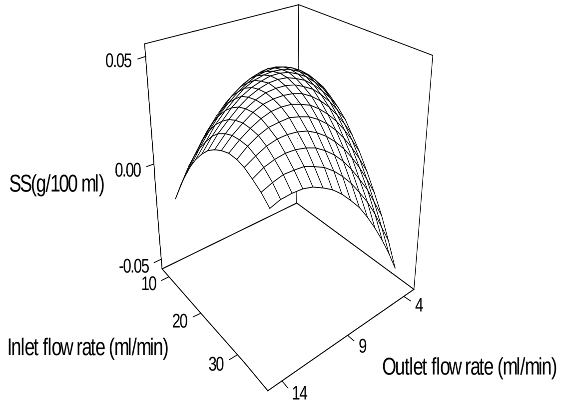
(B)


0.05
0.04
0.03
TS (g/100ml)
0.02
0.01
0.00
10
20
Inlet flow rate (ml/min)
30
0
10
20
30
Retention time (hours)

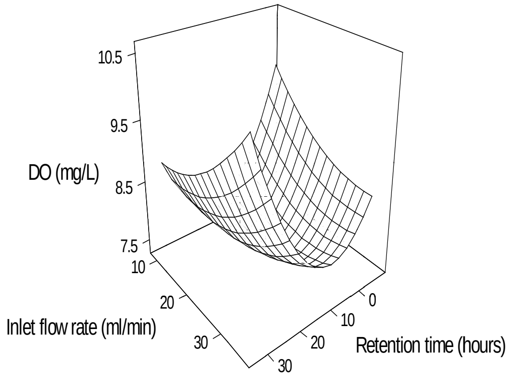
(C)


0.08
0.07
0.06
TDS (g/100ml)
0.05
0.04
0.03
0.02
4
9
Outlet flow rate (ml/min)
14
0
10
20
30
Retention time (hours)

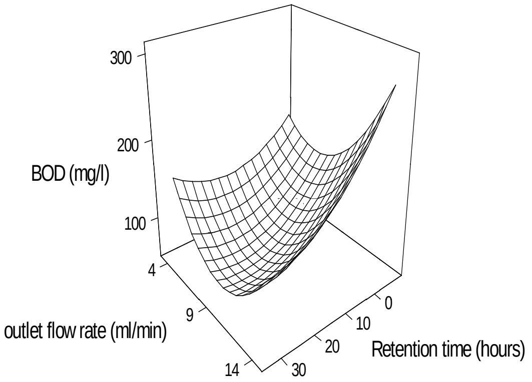
(D)


0.05
SS(g/100 ml)
0.00
-0.05
10
20
Inlet flow rate (ml/min)
30
4
9
14
Outlet flow rate (ml/min)

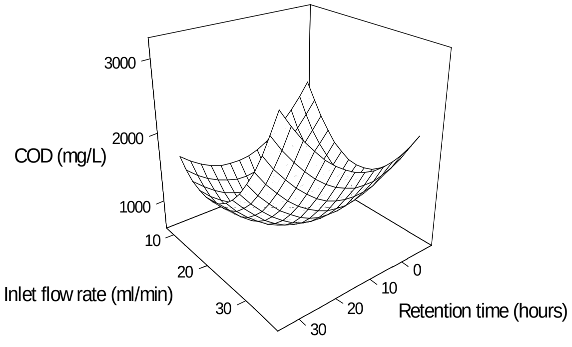
(E)


10.5
9.5
DO (mg/L)
8.5
7.5
10
20
Inlet flow rate (ml/min)
30
0
10
20
30
Retention time (hours)

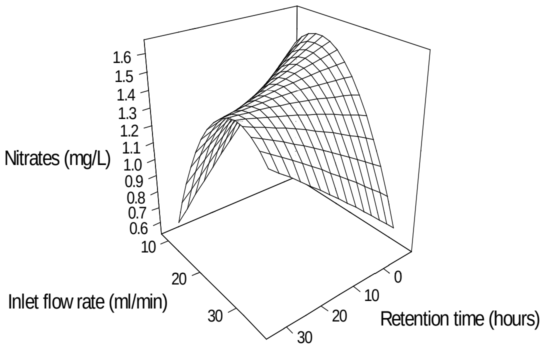
(F)


300
200
BOD (mg/l)
100
4
9
outlet flow rate (ml/min)
14
0
10
20
30
Retention time (hours)

(G)

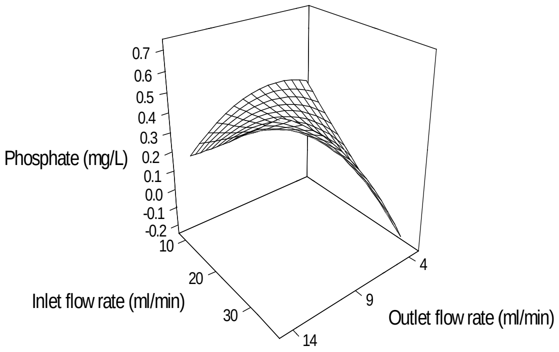

3000
2000
COD (mg/L)
1000
10
20
Inlet flow rate (ml/min)
30
0
10
20
30
Retention time (hours)

(H)



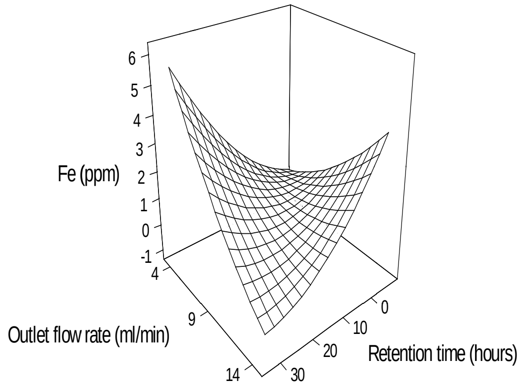
Nitrates (mg/L)
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
Inlet flow rate (ml/min)
10
20
30
Retention time (hours)
0
10
20
30

(I)



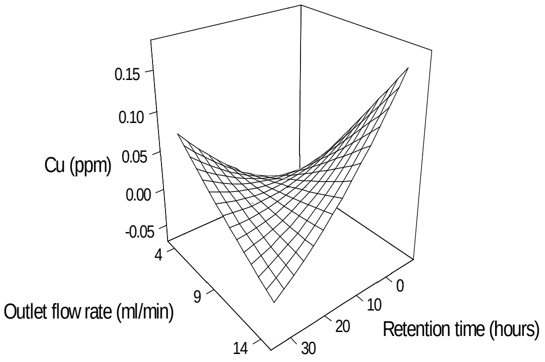
Phosphate (mg/L)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Inlet flow rate (ml/min)
10
20
30
Outlet flow rate (ml/min)
4
9
14

(J)



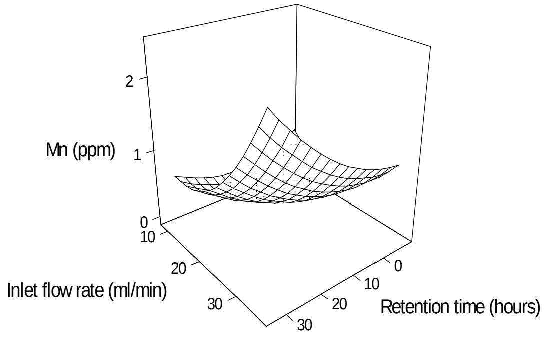
Fe (ppm)
6
5
4
3
2
1
0
-1
Outlet flow rate (ml/min)
4
9
14
Retention time (hours)
0
10
20
30

(K)



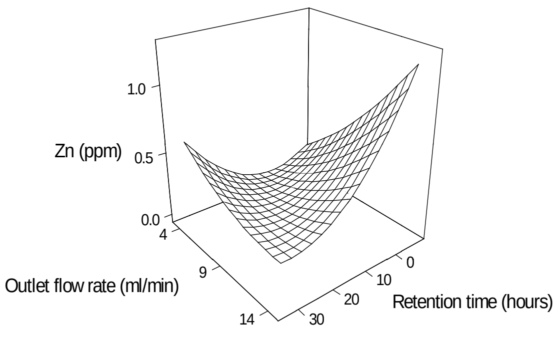
Cu (ppm)
0.15
0.10
0.05
0.00
-0.05
Outlet flow rate (ml/min)
4
9
14
Retention time (hours)
0
10
20
30

(L)



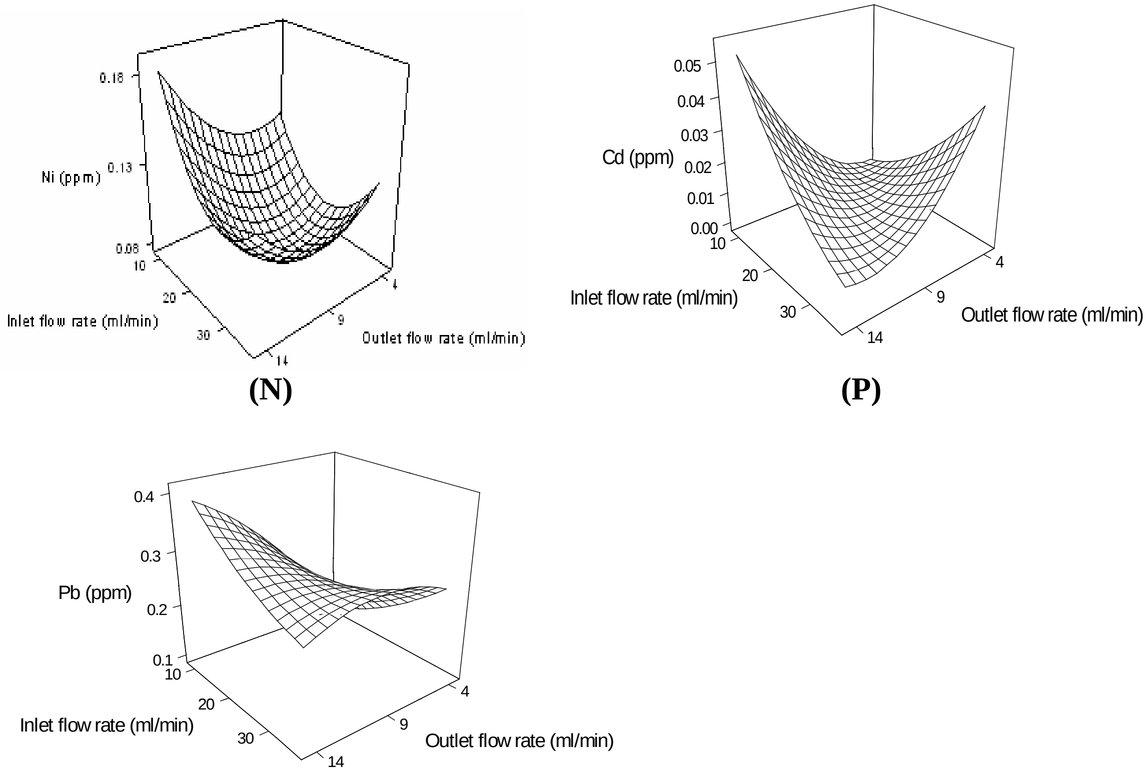
Mn (ppm)
2
1
0
Inlet flow rate (ml/min)
10
20
30
Retention time (hours)
0
10
20
30

(M)



Zn (ppm)
1.0
0.5
0.0
Outlet flow rate (ml/min)
4
9
14
Retention time (hours)
0
10
20
30

(N)



(N) **(P)**

(Q)

Fig. 8 RS graphs: (A) Corresponding to *pH; (*B) Corresponding to EC; (C) Corresponding to TS; (D) Corresponding to TDS; (E) Corresponding to SS; (F) Corresponding to DO; (G) Corresponding to BOD$_5$; (H) Corresponding to COD; (I) Corresponding to Nitrates; (J)Corresponding to Phosphates; (K) Corresponding to Fe; (L) Corresponding to Cu; (M) Corresponding to Mn; (N) Corresponding to Zn; (O) Corresponding to Ni; (P) Corresponding to Cd; (Q) Corresponding to Pb

Contrary to traditional methods, where determining the optimum operation conditions is by monitoring the influence of each variable individually on the response, RSM provides a better approach where, just one variable is changed and the others remain at a constant level. Here, the interactive effect between the variables is also considered. RSM model used in this experimental methodology is commonly a full quadratic equation or the diminished form of this equation. Here, the quality of the fit of the polynomial model is expressed by the coefficient of determination *i.e.* R^2 (Thomas *et al.*, 1997). While studying wastewater treatment in a wetland, there are many factors influencing the process, treating each factor separately would be very time consuming; furthermore, if several factors play a role, their interactions would not be discernable even if they were dominant. Hence, the use of experimental factorial design (Fannin *et al.*, 1981) and of the response surface methodology (Korbahti *et al.*, 2007) already successfully applied. Not only for domestic effluent, RSM has been successfully applied to various treatment processes such as textile dye wastewater (Korbahti and Tanyolac, 2008), industrial paint wastewater (Korbahti *et al.*, 2007) and palm oil mill effluent (Zinatizadeh *et al.*, 2006).

In the present study, treatment of wastewater was best obtained with 22.5ml/min of inlet and 9ml/min of outlet and 12.5 hrs of retention time with average hydraulic loading of 0.0104-0.0208 cm^3/day. Under the optimal flow rate and retention time, a significant reduction in BOD_5 of up to 67% was obtained in planted in contrast to 4.2% in case of non planted wetlands (Table 10). In case of COD, a reduction of 60% was found in planted soil beds as compared to 3% in non-planted (Table 10). In case of Fe, Cu, Zn the regression model was not found much significant as the concentration of the metals (Table 12) in the wastewater was initially low, which lead to its leaching from the soil into the wastewater samples collected from time to time but studies have been reported where a significant/ reduction and accumulation of metal is done by *Phragmites (*Ayeni *et al.*, 2012; Bonanno and Giudice, 2010; Dunbabin and Bowmer, 1992; Hou *et al.*, 2011)

ANOVA analysis reflects the significance of a model, in our study it reflected (Table 11) with a confidence level greater than 99% (P <0.01) in treatment process, the F-value and P-value for BOD and COD were 14.0, 9.9 and 0.001, 0.002 respectively, as shown. The *F-value* was also found significant in case of pH 9.4, EC 5.28, TS 6.35, TDS 3.7, SS 12.71, DO 15.3 and nitrates 13.72. Also P-value was found significant in all the cases (Table 11). Thus, the estimated models fit the experimental data adequately.

In a study by Lesage *et al.*, 2007, the accumulation of Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn in the sediment and biomass of *P. australis* was assessed in a combined constructed wetland (CW) consisting of two vertical subsurface flow (VSSF) reed beds followed by two horizontal subsurface flow (HSSF) reed beds (Table 12). The sediment in the VSSF reed bed was contaminated with Cu (201 ± 27 mg kg^{-1} DM) and Zn (662 ± 94 mg kg^{-1} DM) after 4 years of operation and the sediment was the main pool for metal accumulation in the CW.

Eke and Scholz (2008) studied the performance of constructed wetlands which remove benzene 85%, chemical oxygen demand (COD), 70%; ammonia-nitrogen, 83%; nitrate-nitrogen, 88%; orthophosphate-phosphorus, 58%. Katayon *et al.* (2008) studied the design and performance of pilot scale constructed wetlands in treating a mild domestic wastewater. The results showed that the constructed wetlands were capable of removing about 56–77% of COD, 50–88% of TSS, 20–88% of TP, 27–96% of NH4 + and 99% of total coliforms numbers. Different hydraulic retention times caused significant effect on removal rate of COD, TP and NH4 +, but not to TSS and total coliforms removal. The total number of coliforms was positively correlated with concentration of COD, TP and NH4 + in effluents. Planted wetland cells were superior in both NH4 + and TP removal to unplanted wetland cells.

This study further concludes, response surface methodology (RSM) was successfully applied to a wetland system for optimizing treatment process in constructed wetlands where the effect of independent factors (inlet flow rate, outlet flow rate, retention time) on wastewater parameters was studied to standardize flow rates and retention time to maximally treat wastewater. The *F*-value obtained through RSM, is the ratio of mean square due to regression to the mean square due to error and indicates the influence (significant or not) of each controlled factor on tested models. Also, the *P*-value corresponding to the *F*-value indicates the probability that differences between calculated and tabulated statistics are due to random experimental error. These statistical optimization methods have been used for the treatment of wastewaters (Wu *et al.*, 2008), where the role macrophyte *Phragmites australis* was assessed in experimental, mature and temporarily flooded vertical-flow wetland filters treating simulated urban runoff rich in organic matter.

RSM has several advantages compared to the traditional method that can be summarized as (i) RSM gives a large amount of knowledge from a small number of experimental runs (ii) The interaction effect of the independent parameters on the response can be observed and investigated via RSM. Many attempts have been carried out on numerical modeling of constructed wetlands (Langergraber *et al.*, 2009; Toscano *et al.*, 2009). Various approaches like using regression models, first-order k-C* models, Monod-type equations and more complex dynamic, compartmental models have also been used (Rousseau *et al.*, 2004).

4.2 Analysis of rhizospheric bacterial community of *Phragmites*

4.2.1 Site study

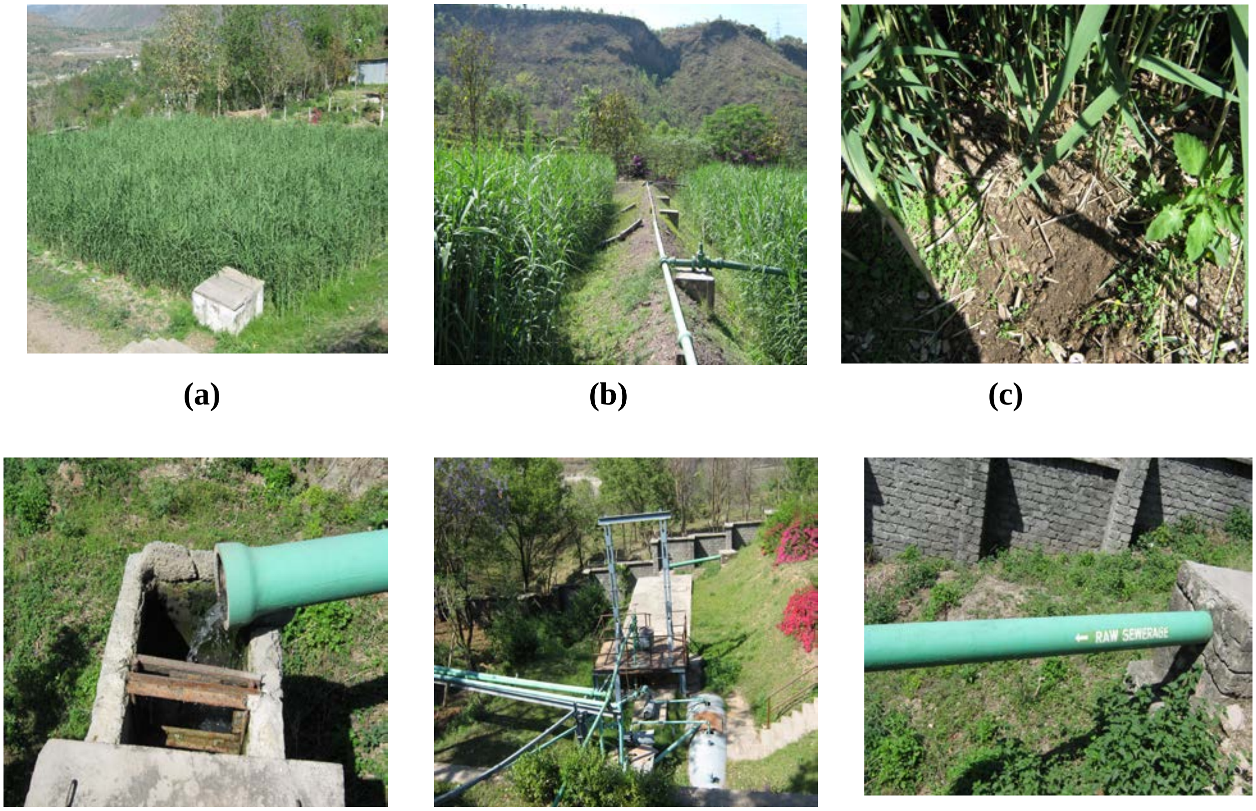
To study rhizospheric bacterial diversity, a working wetland site was selected at ACC Gagal Cement Works, a cement production plant located at Barmana Distt Bilaspur, Himachal Pradesh in (India) coordinates: 31°24'41"N 76°50'15"E (Fig. 9). It has a working *Phragmites* vegetated constructed wetland which feeds on domestic wastewater from colony inhabited by about 500 persons.

The wastewater collected was fed to wetland through an inlet pipe (Fig. 9d, f), which enters into an aeration/ equalization tank (Fig. 9e). Timely aeration of wastewater was done with the help of aerator in the equalization tank, so partial aerobic conditions are maintained inside the tank (Fig. 9e). There are two horizontal wetlands maintained at the site (Fig. 9b) and water was fed to these through extensive networks of pipes and pumps.

This wetland plant is operational for the whole year except rainy season. Some of the pictures of wetland plant are given in Fig. 9 (a-f). (a) shows wetland system (b) shows two wetlands located on each side, which is fed through many small pipelets through main pipe which receives wastewater from equalization tank (c) shows the site of soil sampling (d) inlet pipe which collects wastewater and enters to equalization tank (e) shows the equalization tank (located underground) and extensive network of pies and pumps (f) pipe receiving raw and delivers it in equalization tank. Dimensional wetland parameters are given in Table 13.

S.No	Wetland Parameters	Value
1	Dimension	45 x 32 m
2	Average length of plant	100 cms
3	Average number of plant per square feet	6
4	Depth of root	40-50 cms

Table 13: Various wetland parameters



(a) (b) (c)

(d) (e) (f)

Fig. 9 Site study Details at ACC Gagal Cement Works, at Barmana Distt Bilaspur, Himachal Pradesh (a) Wetland System (b) Two wetland systems fed through network of pipes (c) Site of Sample collection (d) Underground Equilization tank (e) Network of pipes and pumps for supplying wastewater to wetlands (e) Raw sewage pipe entering Underground Equilization tank

4.2.2 Soil sampling and analysis

Physico-chemical analysis of soil from different depth zones *i.e.* upper zone (0-15 cm), middle zone (15-30 cm), and lower zone (30-45 cm) was done (Table 14). Soil samples were collected from five different points in each system; samples from the same zone were combined and mixed completely to produce one homogeneous sample. All samples were maintained on ice until arrival in the laboratory and then stored at 4°C.

Table 14: Physico-chemical characterization of various soil samples

Parameters	**Soil Samples**		
Sampling site	**Upper rhizosphere**	**Middle rhizosphere**	**Lower rhizosphere**
Sample No.	1, 2	3, 5	6, 7
Depth Collection	0-15 cm	15-30 cm	30-45 cm
pH	8.13 ± 0.24	8.11± 0.05	8.14 ± 0.12
Electrical conductivity (mS/cm)	0.85 ± 0.03	0.9 ± 0.01	0.83 ± 0.07
Organic C %	0.35± 01	0.35 ± 0.09	0.38 ± 0.14
Ava P %	0.71± 0.05	0.74 ± 0.81	0.71± 0.22
Ava S %	1.07 ± 0.16	0.95 ± 0.24	1.12 ± 0.13
Soil Dehydrogenase activity (µg TPF/g soil/day)	8.4 ± 0.06	7.9 ± 0.32	8.32 ± 0.02
Bacterial count: Nutrient Agar (cfu g-1 soil)	6 x 10^8	40 x 10^8	7 x 10^8
Bacterial count: Jensen media (cfu g-1 soil)	90 x 10^4	6 x 10^3	4 x 10^3
Bacterial count: Pikovskaya media (cfu g-1 soil)	30 x 10^3	2 x 10^3	4 x 10^3
Bacterial count: Cellulose minimal media (cfu g-1 soil)	50 x 10^2	7 x 10^2	6 x 10^2

In total 6 samples were collected (Table 14), two from each zone, sample 1, 2 from upper rhizospheric zone, 3, 5 from middle rhizospheric zone, 6,7 from lower rhizospheric zone. The depth of collection was 0-15 cm, 15-30 cm and 30-45 cm for upper, middle and lower rhizospheric zone respectively. The pH in zones varied from 8.11± 0.05 to 8.14 ± 0.12. The EC in zones varied from 0.83 ± 0.07 to 0.9 ± 0.01 mS/cm. The Organic carbon was present in 0.35 to

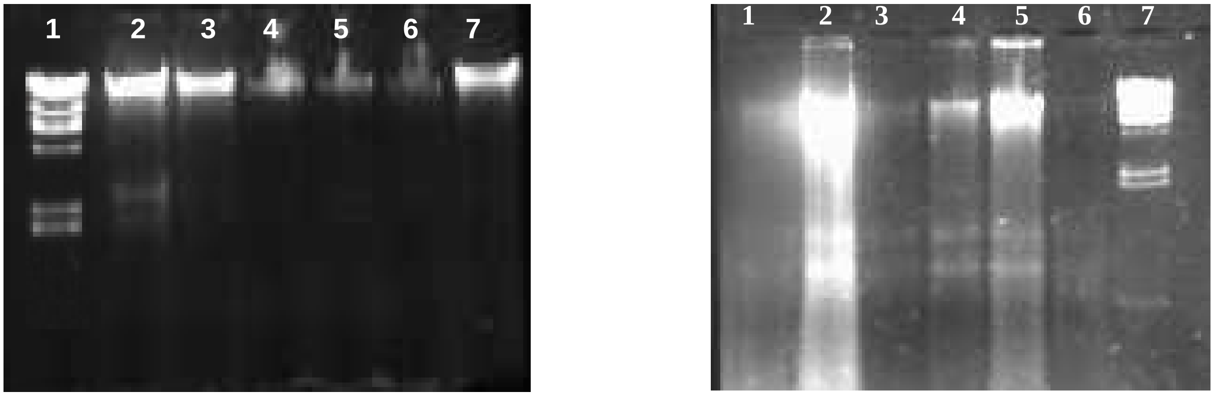
0.38%. Physico-chemical characteristics like pH, EC, Organic C, Ava P, Ava S (%) did not show much variation in three zones. A viable microbial count was also noted in terms of Soil Dehydrogenase activity (8.4 ± 0.06 µg TPF/g soil/day in upper rhizosphere, 7.9 ± 0.32 µg TPF/g soil/day in middle rhizosphere, 8.32 ± 0.02 µg TPF/g soil/day in lower rhizosphere). CFU (colony forming unit) count was done on various media *i.e* nutrient agar for total bacterial count ranged from 6 x 10^8 to 40 x 10^8 cfu g-1 soil, Jensen's media for nitrogen fixing bacteria ranged from 4 x 10^3 to 9 x 10^3 cfu g-1 soil, Pikovskaya media for P-solubilizers ranged from 2 x 10^3 to 30 x 10^3 cfu g-1 soil and Cellulose minimal media for cellulose degrading bacteria ranged from 6 x $10^{2\text{ to}}$ 50 x 10^2 cfu g-1 soil by serial dilution and plating.

4.2.3 Soil DNA extraction by different methods

Molecular analyses for the study of soil microbial communities often depend on the direct extraction of DNA from soils. So to increase the DNA yield, four different methods were employed for extracting total DNA from three different soils samples *i.e* 1, 2 from upper rhizospheric zone (0-15 cm), 3, 5 from middle rhizospheric zone (15-30 cm), 6, 7 from lower rhizospheric zone (30-45 cm). 4 µl of each DNA sample was mixed with 2 µl of 6X Gel loading dye (10mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60mM EDTA) and sample was loaded onto 0.7% agarose gel with ethidium bromide (EtBr). After running for 30 mins at 60Volts, the gel was visualized under U.V transilluminator. The gel pictures for the four methods for Soil DNA extraction are placed in Fig. 10. Of all four methods; (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method; DNA isolated by Griffiths *et al.* (2000) method showed less shearing, intactness and a good ratio of A260/A280 (Fig 10).

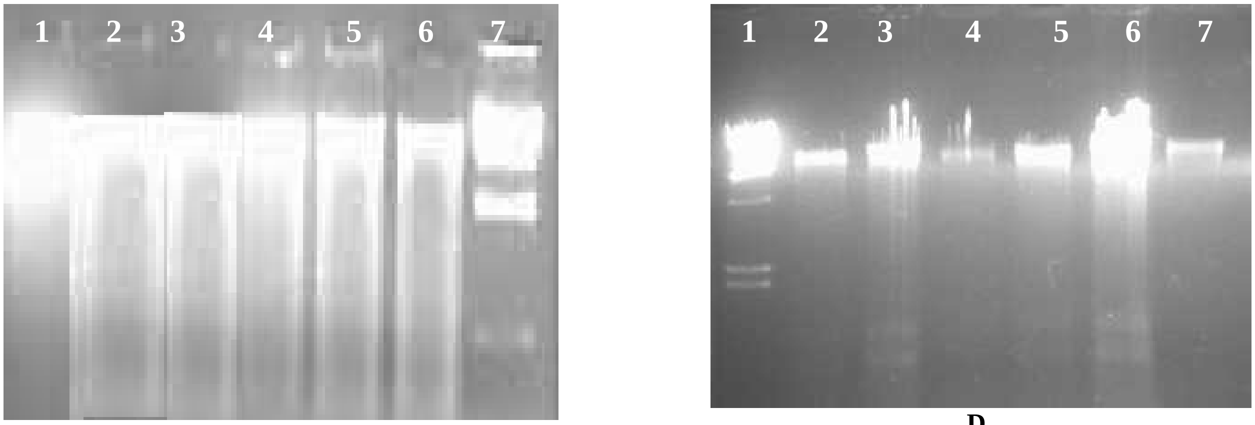
For molecular ecological analysis of a microbial community in wetland, efficient and unbiased DNA extraction and purification methods were used. DNA, isolated by different methods was subjected to purity comparison. DNA samples were diluted with TE buffer until an absorbance at 340 nm between 0.01 and 0.1 was obtained (Humic acid amounts absorbance is read at 340 nm), the A260/280 ratios and the concentrations of DNA were quantified on a U.V-Vis Spectrophotometer (Systronics). As Sephadex G-200 spin column purification is found to be the best method (Miller *et al.,* 1999) for removing PCR-inhibiting substances while minimizing

DNA loss, all the samples were subjected to Sephadex G-200 spin column purification for pure DNA recovery and removal of PCR inhibitors from crude extracts.



A
Lane 1- Lambda Hind III ladder
Lane 2, 3- *Phragmites* upper rhizo
Lane 4, 5 - *Phragmites* middle rhizo
Lane 6, 7- *Phragmites* lower rhizo

B
Lane 1, 2- *Phragmites* upper rhizo
Lane 3,4 - *Phragmites* middle rhizo
Lane 5,6- *Phragmites* lower rhizo
Lane 7- Lambda Hind III ladder



C
Lane 1, 2- *Phragmites* upper rhizo
Lane 3,4 - *Phragmites* middle rhizo
Lane 5,6- *Phragmites* lower rhizo
Lane 7- Lambda Hind III ladder

D
Lane 1- Lambda Hind III ladder
Lane 2, 3- *Phragmites* upper rhizo
Lane 4, 5- *Phragmites* middle rhizo
Lane 6, 7- *Phragmites* lower rhizo

Fig. 10. DNA extraction from soil by four different methods (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method

For purity comparison a graphical representation is given below for A260/A230 (Fig. 11 a) and A260/A280 (Fig. 11 b) of DNA isolated by four different methods. For A260/A280, ratio of

~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is "pure" for RNA. For 260/230 ratio – a low ratio may be the result of a contaminant absorbing at 230 nm or less such as carbohydrate carryover, residual phenol, residual guanidine, glycogen. Abnormal 260/280 ratios usually indicate that the sample is either contaminated by protein or a reagent such as phenol or that there was an issue with the measurement. Of all the methods, DNA isolated by Griffiths *et al.* (2000) method showed less shearing, intactness and a good ratio of A260/A280 (Fig 10).

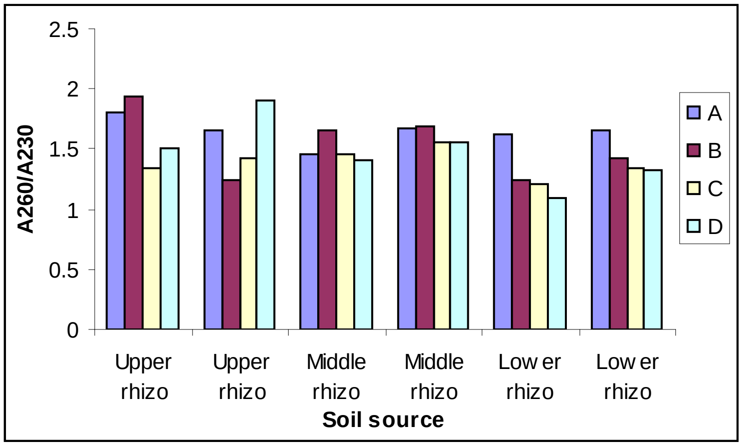


Fig. 11 (a) Purity comparison (A260/A230) of DNA isolated by four different methods (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method

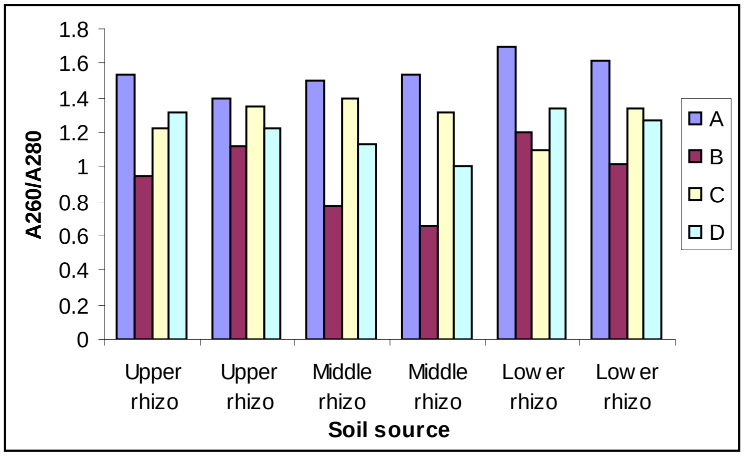


Fig. 11 (b) Purity comparison (A260/A280) of DNA isolated by four different methods (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method

Direct extraction of DNA from environmental soil yield more DNA, requires shorter time, and introduces less bias than methods in which cells are separated from the sample matrix before DNA extraction (von Wintzingerode *et al.* 1997). A large number of methods have been published for the extraction and purification of total community DNA from environmental samples such as soils and sediments for molecular ecological analysis of microbial communities (Burgmann *et al.* 2001; Jiang *et al.* 2005; Martin-Laurent *et al.* 2001). A comprehensive listing of technologies used in unculturable diversity analysis has been mentioned in Table 5. PCR-DGGE (Denaturing gradient gel electrophoresis) and FISH (Fluorescence In Situ Hybridization) are most popular techniques in diversity analysis owing to their redundant and reliable results.

In the present study four different DNA extraction methods from soil were tried *i.e* (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method. Griffiths method was found to yield high quality DNA among all which has less shearing and less amount of contaminants. The purity comparison of all DNA extraction methods by A260/A230 and A260/A280 also supported this. 260/230 ratio – a low ratio may be the result of a contaminant absorbing at 230 nm or less. 260/280 ratio – a low ratio may be the result of a contaminant absorbing at 280 nm or less.

From literature, among the four different DNA purification methods (silica-based DNA binding, agarose gel electrophoresis, ammonium acetate precipitation, and Sephadex G-200 gel filtration) for DNA recovery and removal of PCR inhibitors from crude extracts, Sephadex G-200 spin column purification was found to be the best method for removing PCR-inhibiting substances, while minimizing DNA loss during purification (Miller *et al.*, 1999). Adopting this concept, DNA extracted (Fig. 10) was purified from humic acid and PCR was performed by adding BSA which revealed a band of appox. 1,500 bp (Fig.12). A further amplification for V3 region (Fig. 13) of 16S gene was done by specific primer (Table 7). For optimal DGGE separation (Fig. 14), a 40 bp GC clamp was incorporated into the 5' primer (Muyzer *et al.* 1993), which was necessary for the optimal resolution of the fragments in the denaturing gradient gel (Table 7), resulting in generation of appox. 180-200 bp amplicon (Fig. 13). There has been concern regarding chimera formation during the PCR amplification process (Suzuki and Giovannoni, 1996), so to avoid this problem, the shorter and highly variable V3 region of 16S rDNA was chosen for further amplification.

The major problem in diversity analysis is because of the variety of microbes' species and the enzyme inhibitors such as humic acids which is a potential PCR inhibitor. These have been overcome by purifying DNA prior to amplification and adding BSA to PCR reaction mixture. In studies, relating to high-quality microbial DNA extraction and purification from compost than that from other environmental samples, only very few methods have been developed for extracting and purifying total DNA because of presence of huge amount of humic acids (Howeler *et al.* 2003; LaMontagne *et al.* 2002).

4.2.4 PCR Amplification of soil DNA samples

The purity of the final purified rhizospheric soil DNA was also checked by the ability of PCR to amplify a region of the 16S rDNA with primers of 27F and 1492R (sequence mentioned in Table 6). The EtBr (Ethidium Bromide) stained gel (0.8% agarose gel in 0.5X TBE Buffer) profile showed the PCR amplified 16S rDNA to be between 1,430 and 1,500 bp in size, consistent with the expected PCR products of 1,500 bp in size (appox.). Additionally, nonspecific PCR amplification was not detected in any lanes. Below is the agarose gel (Fig. 12) depicting 1.5 kb (appox.) amplified band in all lanes (1-6).

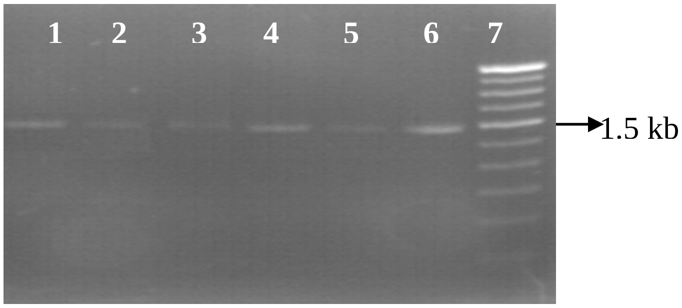


Fig. 12: PCR Amplification by 16S rDNA primers (Lane 1, 2- V3 amplicon of *Phragmites* upper rhizo; Lane 3, 4- V3 amplicon of *Phragmites* middle rhizo; Lane 5, 6- V3 amplicon of *Phragmites* lower rhizo; Lane 7- 1Kb ladder)

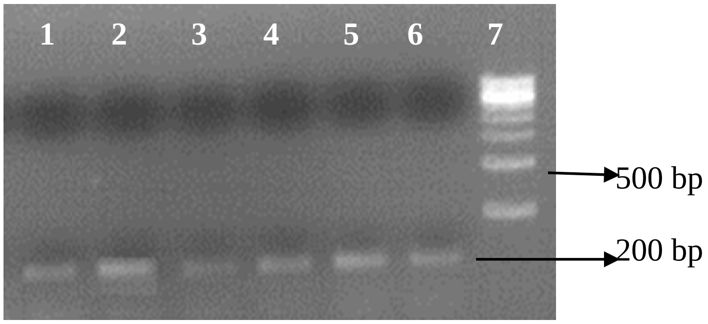


Fig. 13: PCR amplification by GC clamp primers (Lane 1, 2- V3 amplicon of *Phragmites* upper rhizo; Lane 3, 4- V3 amplicon of *Phragmites* middle rhizo; Lane 5, 6- V3 amplicon of *Phragmites* lower rhizo; Lane 7- 500 bp ladder)

The re-amplification was carried out by taking 1 µl of the first PCR product as a template and the PCR mixture contained 100 pmol of each primer, 200 µM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$ and 2.5 units of *Taq DNA polymerase* (Fermentas) in a 100 µl reaction volume. The primers used were the universal primers conserved for the V3 region of bacterial 16S rDNA gene, PRBA 338F 5' and PRUN 518R with a GC clamp added to the 5' end of the forward primer (Table 7). The Ethidium Bromide (EtBr) stained gel (1.5 % agarose gel prepared in 1 X TBE Buffer) profile showed the PCR amplified 16S rDNA to be between 180-200 bp in size, consistent with the expected PCR products of appox. 200 bp in size. Additionally, no nonspecific PCR amplification was detected in any lanes. Fig. 13 shows the agarose gel depicting 180-200 bp (appox.) band (DGGE amplicon) in all lanes (1-6).

4.2.5 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE was carried out using a DGGE system (Ingenyphor) as per the manual. Briefly, PCR products were resolved on 8% (w/v) polyacrylamide gels in 0.5 X TAE using denaturing gradients ranging from 0 to 100% (where 100% denaturant contains 7 M urea and 40% formamide). Fig. 14 shows the DGGE analysis pattern of PCR products obtained after amplification of the genomic DNA of three samples Lane1, 2 *Phragmites* upper rhizospheric sample, Lane 3, 5 *Phragmites* middle rhizospheric sample, Lane 6, 7 *Phragmites* lower rhizospheric sample. Substantial separation of the 16S rDNA fragments derived from different soil DNA samples was observed when they underwent separate electrophoresis. When same

DNA sample was loaded in duplicity, a similar separation pattern was observed, and the DGGE found to provide an immediate display of the constituents of the rhizospheric bacterial population. When16S rDNA amplification (GC clamped) of the rhizospheric soil genomic DNA, directly extracted from each site, were applied to the DGGE, the 200 bp sized (appox.) PCR products were obtained (Fig. 13). The bands in the DGGE profile corresponded to the 16S rDNA fragments; same sized but differed in their nucleotide sequence, reflecting the distinct numerically dominant microbial populations in the community. The intensity of the PCR product was believed to be proportional to the abundance of the template, and therefore, the abundance of each population (which corresponds to a single band located at same loci on different lanes). Therefore, band, which was common to all sites, suggests this is the major dominant population, and bands which had high intensities were abundant in each soil sample. That the diversity of the microbial population of each site was confirmed by the band profiling, with the highest appearing at the *Phragmites* upper rhizospheric soil sample. Whereas, the total bacterial counting and DNA yield were high in *Phragmites* middle rhizospheric zone, but the microbial community diversity was low. Therefore, middle rhizospheric site was characterized by the dominance of a few organisms and consequently, a low diversity.

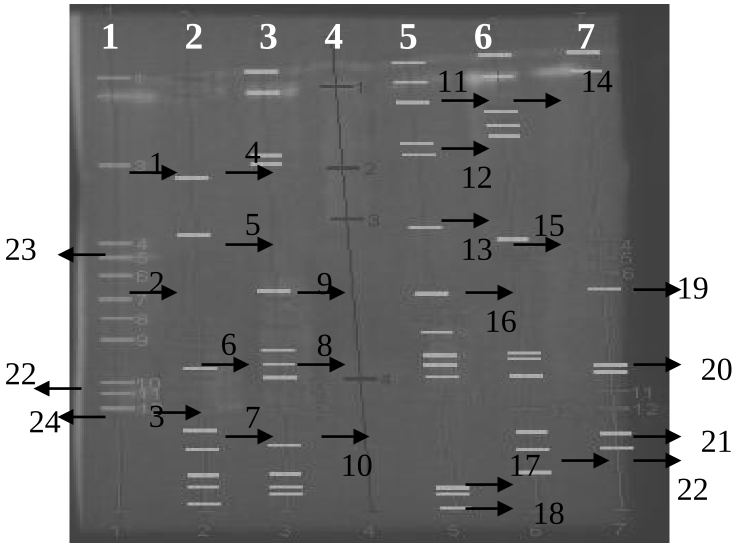


Fig. 14: DGGE banding pattern (Lane 1, 2-Upper rhizospheric zone DNA, Lane 3, 5-Middle rhizospheric zone DNA, Lane6, 7- Lower rhizospheric zone DNA, Lane 4- Marker- mixture of amplicons of known samples, Band numbering 1-24)

A dendrogram was constructed from the distance matrix, using then weighted pair group method, with the arithmetic means. Fig. 15 defines a dendrogram of genetic similarity value of 16S rDNA of soil DNA samples analyses by DGGE. Lane 1 and 2, depicts the maximum homology as it is same soil DNA sample (isolated in duplex). Similarly lane 3, 5 and Lane 6, 7 shows maximum homology in between the lanes.

4.2.6 Sequencing of DGGE fragment and Analysis

Individual bands were cut from the DGGE gel and placed in 20 µl of sterile distilled water which allows passive movement of DNA out of gel and allowed to incubate overnight at 37°C. The DNA from the DGGE band was recovered and re-amplified with primer 1 and primer 2 (sequence mentioned in Table 7 without GC Clamp). For the determination of the more specific community structure traits, a sequencing analysis of the specific bands was performed. The results of the alignment to the 16S rDNA sequence, using the BLAST network service, are shown in Table 15.

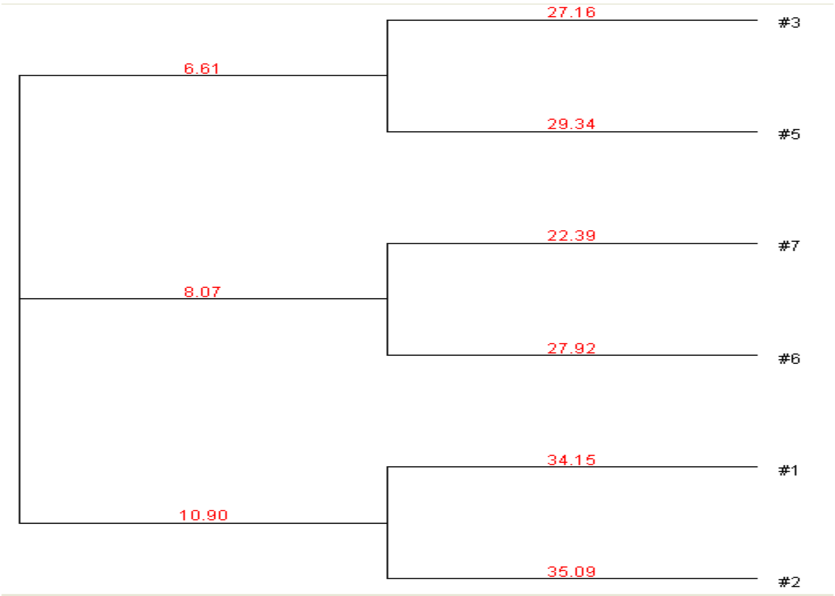


Fig. 15 Dendrogram of genetic similarity matrix value of 16S rDNA analyzed by PCR-DGGE fragments from rhizospheric bacterial community, Lane 1, 2- V3 amplicon of upper rhizo; Lane 3, 5- V3 amplicon of *Phragmites* middle rhizo; Lane 6, 7- V3 amplicon of *Phragmites* lower rhizo

Table 15: BLAST analysis of sequences obtained from DGGE

Representative sequence	(GenBank accession number)	Best match database	Similarity	Division
Unculturable bacteria 01	KF973165	*Pseudomonas* sp. JC4 16S ribosomal RNA gene, partial sequence KC294042.1	100%	Proteobacteria
Unculturable bacteria 02	KF973166	*Arthrobacter* sp. JB199 16S ribosomal RNA gene, partial sequence KF669525.1	100%	Actinobacteria
Unculturable bacteria 03	KF973167	*Bacillus megaterium* strain UF07 16S ribosomal RNA gene, partial sequence KF717520.1	100%	Firmicutes
Unculturable bacteria 04	KF973168	*Flavobacterium segetis* strain AT1048 16S ribosomal RNA gene, partial sequence AY581115.3	100%	Bacteroidetes
Unculturable bacteria 05	KF973169	*Janthinobacterium lividum* gene for 16S ribosomal RNA, partial sequence AB428446.1	100%	β- Proteobacteria
Unculturable bacteria 06	KF973170	*Arthrobacter* sp. JB199 16S ribosomal RNA gene, partial sequence KF669525.1	99%	Actinobacteria
Unculturable bacteria 07	KF973171	*Acinetobacter calcoaceticus* strain c20 16S ribosomal RNA gene, partial sequence JQ781519.1	100%	ϒ- Proteobacteria
Unculturable bacteria 08	KF973172	*Bacillus amyloliquefaciens* strain NLP245 16S ribosomal RNA gene, partial sequence KF710033.1	100%	Firmicutes
Unculturable bacteria 09	KF973173	*Bacillus niacini* gene for 16S ribosomal RNA, partial sequence, strain: JAM-F8 AB889607.1	100%	ϒ- Proteobacteria
Unculturable bacteria 10	KF973174	*Exiguobacterium acetylicum* strain CL12 16S ribosomal RNA gene, partial sequence KF574924.1	100%	Firmicutes
Unculturable bacteria 11	KF973175	*Flavobacterium columnare* strain ARS-1 clone GIA-4 16S ribosomal RNA gene, partial sequence KC912677.1	100%	α- Proteobacteria
Unculturable bacteria 12	KF973176	*Rhodobacter changlensis* strain WL-71s 16S ribosomal RNA gene, partial sequence KF580870.1	100%	α- Proteobacteria
Unculturable bacteria 13	KF973177	Sulfate-reducing bacterium enrichment culture clone 1-3-5-otu2-2 16S ribosomal RNA gene, partial sequence KF220596.1	100%	Firmicutes
Unculturable bacteria 14	KF973178	*Hymenobacter* sp. KJ035 16S ribosomal RNA gene, partial sequence JX272928.1	98%	Bacteroidetes
Unculturable bacteria 15	KF973179	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: 20JB29 AB826016.1	100%	Planctomycetes
Unculturable bacteria 16	KF973180	*Rhodobacter changlensis* strain WL-71s 16S ribosomal RNA gene, partial sequence KF580870.1	100%	Proteobacteria,
Unculturable bacteria 17	KF973181	*Burkholderia pseudomallei* strain DRDEBPS1017 16S ribosomal RNA gene, partial sequence KF586861.1	100%	Proteobacteria

Unculturable bacteria 18	KF973182	*Bradyrhizobium* sp. RITF806 16S ribosomal RNA gene, partial sequence JQ796661.2	100%	α- Proteobacteria
Unculturable bacteria 19	KF973183	*Pseudomonas aeruginosa* strain PAF14 16S ribosomal RNA gene, partial sequence KF813066.1	100%	Proteobacteria
Unculturable bacteria 20	KF973184	Uncultured bacterium clone SDSF_8F_h08 16S ribosomal RNA gene, partial sequence KC713044.1	100%	ϒ- proteobacteria
Unculturable bacteria 21	KF973185	*Enterobacter* sp. XJJC-134-5RF7 16S ribosomal RNA gene, partial sequence KF828866.1	100%	ϒ- Proteobacteria
Unculturable bacteria 22	KF973186	Aeromonas bivalvium strain D15 16S ribosomal RNA gene, partial sequence KF500920.1	100%	ϒ- Proteobacteria
Unculturable bacteria 23	KF973187	*Agrobacterium tumefaciens* strain TA-AT-6 16S ribosomal RNA gene, partial sequence KF673154.1	100%	α- Proteobacteria
Unculturable bacteria 24	KF973188	*Beggiatoa* sp. B15LD partial 16S RDNA gene and ITS1, strain B15LD FR690999.1	100%	δ- Proteobacteria

A separation was achieved on DGGE gel (Fig. 14) ranging from 0 to 100 % denaturation. The bands obtained were eluted and sequenced to know the rhizospheric bacterial diversity. The sequences obtained in this study were compared to those available in the GenBank database and the sequence similarities are presented in Table 15. Sulfate reduction and nitrogen metabolism are the major bacterial processes in marine sediments (Ravenschlag *et al.* 2000; Parkes *et al.* 2000). Similar findings are revealed where Sulfate-reducing bacterium belonging to Phyla Firmicutes has been observed. Nitrogen metabolizing bacteria belonging to Phyla α-Proteobacterium have been observed like *Bradyrhizobium* sps. Three strains belonging to ϒ-Proteobacterium have been observed band no 7, 20, 21 (Fig 14). Two strains belonging to *Actinobacteria* has been observed band 2, 6 (Fig 14). Three strains of *Bacteroidetes* which composed of Gram-negative, non-spore forming, anaerobic, and rod-shaped bacteria corresponding to band 4, 14 (Fig 14). The sequence bands detected were related to NCBI database and all conclusions were based on sequence information.

Similarities between banding patterns in the DGGE profile were calculated based on the presence and absence of bands and expressed as a similarity coefficient. In this study, the Dice similarity coefficient was used to calculate pairwise comparisons of the DGGE fingerprint profiles obtained from six soil DNA samples. This similarity coefficient is calculated based on the following formula:

$$\mathbf{D_{sc} = [2j/(a+b)]}$$

Where a = number of DGGE bands in lane 1,
b = number of DGGE bands in lane 2,
j = number of common DGGE bands in lane 1 and lane 2,
and D_{sc} = 1 indicates identical profiles

Dendrograms showing clustering according to the similarity of banding patterns between samples were constructed by the Unweighted Pair Group Method of Arithmetic Averages (UPGMA) using Quantity one software (Bio-rad Inc). Principal Component Analysis (PCA) Fig.16 showed nearly the same profiles of DNA extracted from the same sample in duplicity *i.e.* two lanes of upper rhizospheric samples (Fig. 14) showed similarity and so with middle and lower rhizospheric samples. In Fig.16, the results of cluster analysis also showed that the metabolic activities of Obs 1 were more “closely” related to those of Obs 2 compared with Obs 3 and Obs 5. The metabolic activities of Obs 6 and Obs 7 were in a single cluster. Similar results were obtained using dendrogram where clustering analysis of the DGGE profiles showed that bacteria in the six lanes of DGGE gel belonged to three clusters (Fig. 15).

On the basis of BLAST analysis, a phylogenetic tree has been constructed by NJ method by MEGA (Molecular Evolutionary Genetic Analysis) version 5 software (Fig. 17).

A range of DNA fingerprinting’ techniques for diversity in environmental samples are available for analysis of amplification products of both types of gene, including denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998; Ovreas *et al.*, 1997), temperature gradient gel electrophoresis (TGGE) (Muyzer, 1993), terminal restriction fragment length polymorphism analysis (T-RFLP) (Smalla *et al.*, 2007) and single strand conformation polymorphism analysis (SSCP) (Smalla *et al.*, 2007). These techniques allow more rapid analysis than cloning and sequencing of amplification products. They also enable quantitative or semi-quantitative analysis of relative abundances of different community members e.g. the intensity of the PCR product is believed to be proportional to the abundance of the template, and therefore, the abundance of each population (Muyzer *et al.*, 1993).

An optimal separation was achieved on DGGE gel ranging from 0 to 100 % denaturation. The bands obtained were eluted and sequenced to know the rhizospheric diversity. The sequences obtained in this study were compared to those available in the GenBank database and the sequence similarities are presented in Table 15. studies in literature have revealed that in a two-stage, HSSF-CW planted with *Phragmites* and *Typha*, for tannery wastewater treatment, strains as revealed by DGGE analysis were affiliated with ϒ-Proteobacteria, Firmicutes, α-Proteobacteria, Sphingobacteria, Actinobacteria and Bacteroidetes (Calheiros *et al*, 2009). A study by Dong *et al*, 2010, revealed that total bacterial community composition was dominated by *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp., *Anammox* (anaerobic ammonium oxidation) and some unculturable denitrification bacteria. Sulfate reduction and nitrogen metabolism are the major bacterial processes in marine sediments (Ravenschlag *et al.* 2000; Parkes *et al.* 2000). Similar findings are revealed in our study in Table 15 where Sulfate-reducing bacterium belonging to Phyla *Firmicutes* has been observed. Nitrogen metabolizing bacteria belonging to Phyla α-Proteobacterium have been observed like *Bradyrhizobium sps* (Table 15). Three strains belonging to ϒ- proteobacteria have been observed band no 7, 20, 21 (Table 15) (Fig 14). Two strains belonging to *Actinobacteria* has been observed band 2, 6 (Fig 14). Three strains of Bacteroidetes which composed of Gram-negative, non-spore forming, anaerobic, and rod-shaped bacteria corresponding to band 4, 14 (Table 15) (Fig 14). The sequence bands detected were related to NCBI database and all conclusions were based on sequence information. In this study, 15 sequences corresponding to Proteobacteria were detected (Table 15) out of which 3 belonged to α-Proteobacterium, 1 belonged to β- Proteobacteria, 5 belonged to ϒ- proteobacteria and one belonged to δ-Proteobacteria (Table 15). In the diversity, 4 sequences corresponding to Firmicutes were detected which are *Bacillus megaterium, Bacillus amyloliquefaciens, Exiguobacterium acetylicum and a* Sulfate-reducing bacterium (Table 15). 2 sequences corresponding to *Actinobacteria* were detected. Bacteroides which composed of a group of Gram-negative, obligately anaerobic bacteria were also detected (Table 15).

DNA sequences that show lower similarity to those in the database may be when they are derived from previously uncultivated or unknown bacterial species. The phylogenetic analysis showed that the sequences retrieved in this study formed eight major phylogenetic clusters: α-*proteobacteria*, β-*proteobacteria*, γ- *proteobacteria*, δ- Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Planctomycetes (Table 15). The unknown bacteria belonged to Planctomycetes and ϒ- proteobacteria.

Based on the intensity of each band, Dice coefficient was calculated using Quantity one software. Diversity indices are useful as a first approach to estimate the diversity of microbial communities, *i.e.*, the higher *H* is, the greater is the diversity of the microbial community. A diversity index consists of two components: (1) the total numbers of species present or species richness and (2) the distribution of the number of individuals among those different species, called species evenness, or species equability (Hu *et al.*, 2007). The relationship between the microbial community patterns was further analyzed using hierarchical clustering. In Fig.16, the results of cluster analysis also showed that the metabolic activities of Obs1 were more “closely” related to those of Obs2 compared with Obs3 and Obs5. The metabolic activities of Obs6 and Obs 7 were in a single cluster. The Principal Component Analysis (PCA) illustrated that the microbial communities in different depth of *Phragmites* rhizospheric soils were distinctive (Fig. 16). Similar results were obtained using dendrogram where clustering analysis of the DGGE profiles showed that bacteria in the six lanes of DGGE gel belonged to three clusters (Fig. 15).

Bacterial diversity as analyzed by culture independent approaches tends to reveal a more comprehensive assessment than culture dependent approaches. Chen et al. (2008) showed that *Lactococcus lactis,* which was not detected in the culture-dependent method due to its scarcity, was found in culture-independent samples. Diversity analysis also gives a direct picture of the microbiological processes occurring in a system. Some studies have shown that shifts in the structure of bacterial communities can be associated with changes in a number of soil properties including soil texture and soil nitrogen availability (Frey et al., 2004; Lauber et al., 2008). On the other hand, Calheiros et al. (2009) indicated that the diverse community of bacteria in the constructed wetland might influence the final effluent quality. Based on these studies, combining culture-dependent and culture-independent methods in investigating microbial communities would likely reveal a more comprehensive assessment.

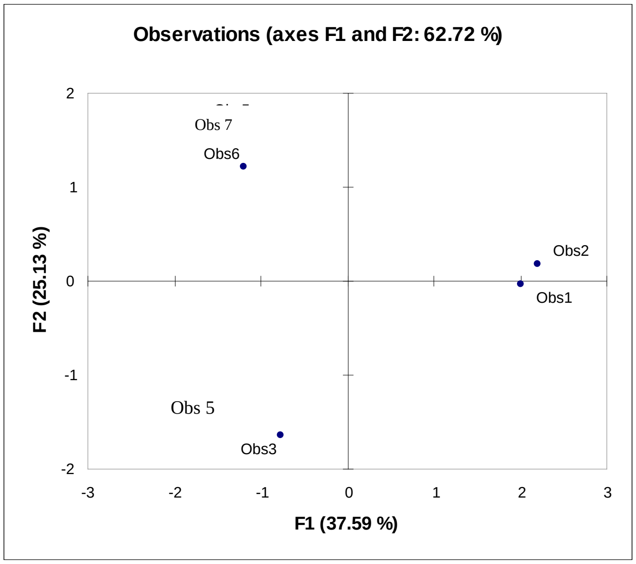


Fig. 16: Statistical Analysis: Principal Component Analysis of DGGE pattern

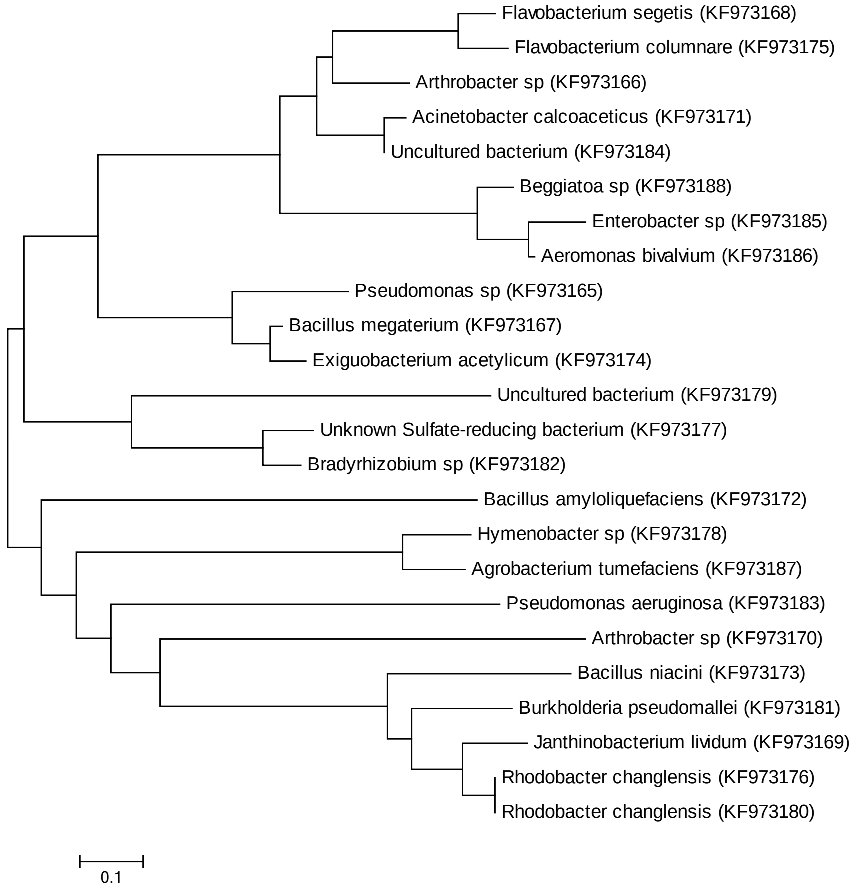


Fig. 17: Phylogenetic tree analysis by sequencing DGGE bands (Neighbor-hood Joining Method)

4.3 Monitoring survival of selected bacterial inoculants in rhizosphere

4.3.1 Strain isolation and metal tolerance screening

Isolates obtained by serial dilution, plating and repeated streaking were screened for heavy metal tolerance (Cd and Pb) in minimal media Bushnell Hass Broth (Himedia Laboratories) supplemented with 1% Glucose (as carbon source) and 25, 50, 75, 100 ppm of Pb. Similar metal tolerance was done with 5, 10, 25 ppm of Cd in minimal media supplemented with 1% Glucose. Table 16 (a) and 16 (b) represents the heavy metal tolerance of different isolates

Table 16 (a): Pb tolerance of bacterial isolates

	Pb tolerance			
Isolate	**BHB+ 1%glucose+ 25 (ppm) Pb**	**BHB+ 1%glucose+ 50 (ppm) Pb**	**BHB+ 1%glucose+ 75 (ppm) Pb**	**BHB+ 1%glucose+ 100 (ppm) Pb**
Upper rhizosphere				
UR-21	+	+	+	+
UR-10	+	+	+	+
UR-13	+	+	+	-
UR-14	+	+	+	+
UR-20	+	+	+	-
UR-23	+	+	+	+
UR-8	+	++	++	++
UR-33	+	+	+	-
UR-35	+	+	+	+
UR-36	+	+	-	-
UR-30	+	+	+	-
Middle rhizosphere				
MR-7	+	+	+	-
MR-7	+	+	+	+
MR-5	+	++	++	+
MR-9	+	+	+	-
MR-14	+	++	++	-
MR-8	+	+	+	-
Lower rhizosphere				
LR-5	+	+	+	-
LR-1	+	+	+	-
LR-12	+	+	+	+

Table 16 (b): Cd tolerance of bacterial isolates

	Cd-tolerance		
Isolate	**BHB+ 1%glucose+ 5 (ppm)Cd**	**BHB+ 1%glucose+ 10 (ppm) Cd**	**BHB+ 1%glucose+ 25 (ppm) Cd**
Upper rhizosphere			
UR-21	+	+	+
UR-8	+	+	+
UR-40	+	+	-
UR-33	+	+	-
UR-2	+	+	-
UR-30	+	+	-
UR-10	+	+	+
UR-36	+	+	-
Middle rhizosphere			
MR-5	+	+	+

4.3.2 Growth curve and antibiotic resistance study

Three bacterial strains UR-8, UR-10, UR-21, showing higher tolerance of Cd and Pb, were examined for growth curve. The lag phase of bacterial isolate UR-10 lasted for 5 hours followed by 6 hours lag phase for UR-8, UR-21 (Fig 18, 19) beginning of exponential phase (Fig.18). In log phase all three isolates showed exponential growth where maximum growth was observed for UR-10 in both Cd and Pb containing media followed by UR-8 and UR-21. The cell division occurred exponentially until 13 hours in Pb (Fig. 18) and 15 hours in Cd (Fig. 19) hours, after which it continued linearly until 24 hours.

All three isolates were studied for their antibiotic profile (Table 17). Strains UR-8 and UR-21 were resistant to streptomycin (50 µg ml^{-1}). All bacterial strains were sensitive to kanamycin (50µg ml-1), chloramphenicol (10µg ml^{-1}) and ampicillin (50µg ml^{-1}) in addition to the lac Z-ve nature of all five strains. Based on this study strain UR-10 was selected for transformation with plasmid pMMB277 (Fig.6) containing the *lacZ.*

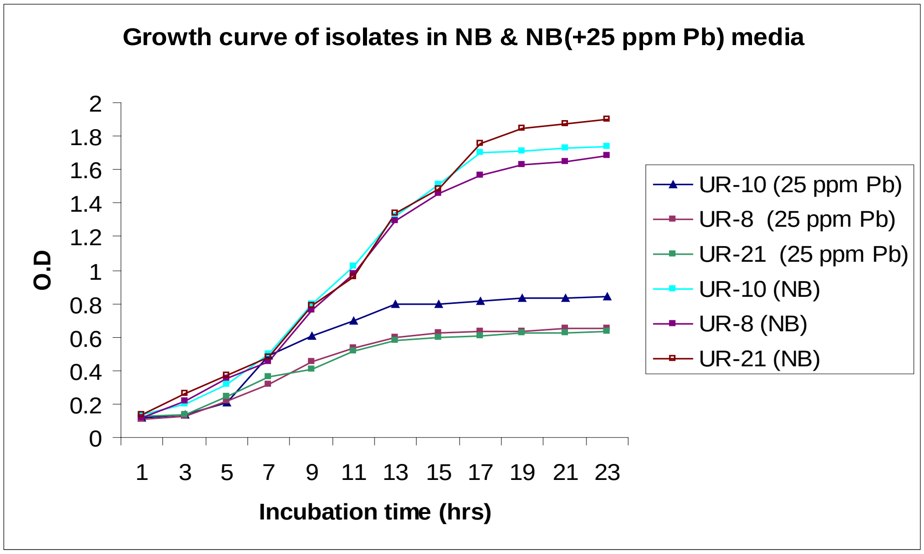


Fig 18: Growth curve of bacterial isolate in Pb containing media

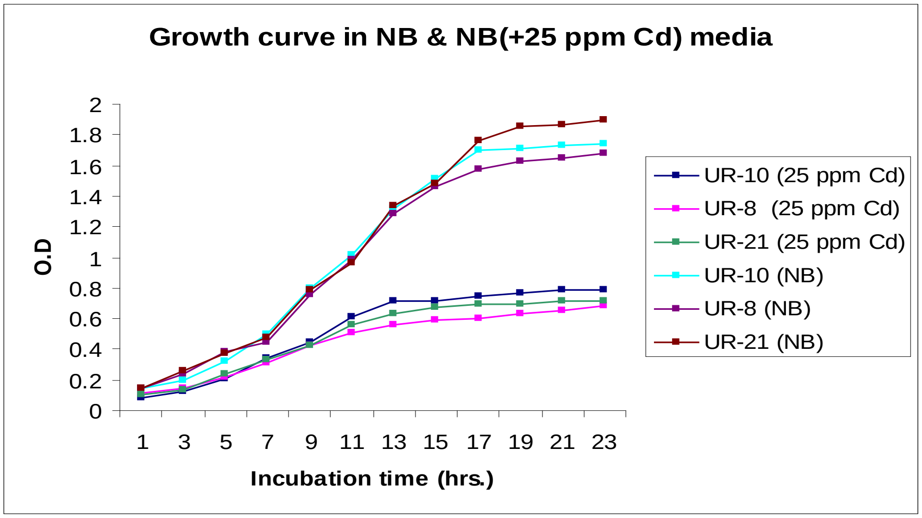


Fig 19: Growth curve of bacterial isolate in Cd containing media

Table 17: Antibiotic resistance study in bacterial isolates

Antibiotics		UR-10	UR-8	UR-21
Streptomycin	Str50	-	+	+
Kanamycin	Kan50	-	-	-
Ampicillin	Amp50	-	-	-
Chloramphenicol	Chl10	-	-	-

4.3.3 Metal accumulation study (Cd, Pb) by isolate UR-10

Half strength nutrient broth was inoculated with UR-10 (inoculum $O.D_{600}$ - 2.173) and supplemented with 25 ppm of Cd, as an active ingredient. Samples were collected after each 12 hrs and subjected to metal analysis on Flame–AAS in supernatant and cell pellet. Fig. 20 describes the accumulation of Pb inside cells and in the supernatant. Although a reduction in growth has been observed, it has been noted that cell were highly tolerant for Pb and accumulated (Fig. 20) around 30% of total metal Pb inside the cells. A trendline defines the increase in metal concentration inside the cells.

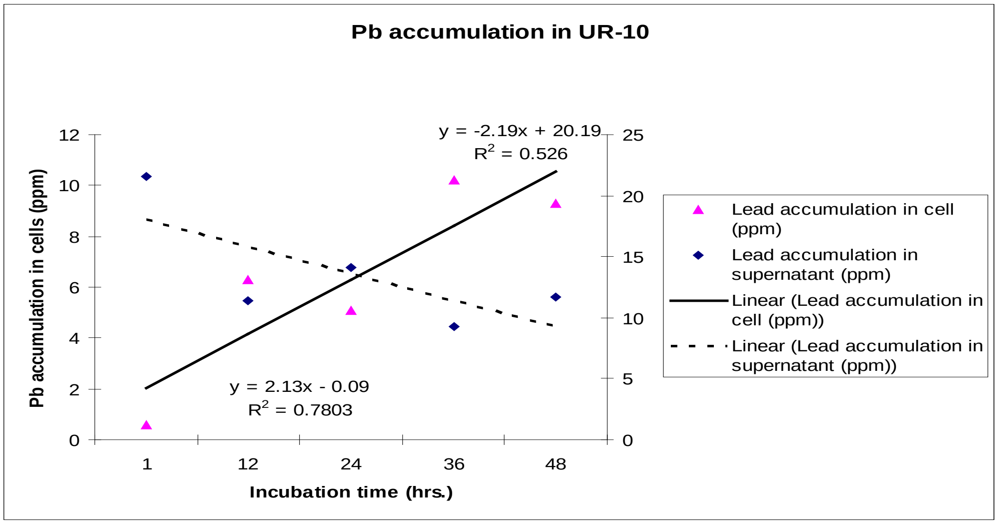


Fig 20: Pb accumulation study in bacterial isolate UR-10

Similar experiment was performed with 25 ppm of Cd and UR-10 culture at 37°C. Half strength nutrient broth was inoculated with UR-10 (inoculum $O.D_{600}$ - 2.173) and supplemented with 25 ppm of Cd, as an active ingredient Samples were collected after each 12 hrs and subjected to metal analysis on Flame–AAS in supernatant and cell pellet. Fig. 21 describes the accumulation of Cd inside cells and in the supernatant. It has been noted that cell were highly tolerant for Cd and accumulated (Fig. 21) around 25% of total metal Cd inside the cells. A trend line defines the increase in metal concentration inside the cells and decrease in supernantant.

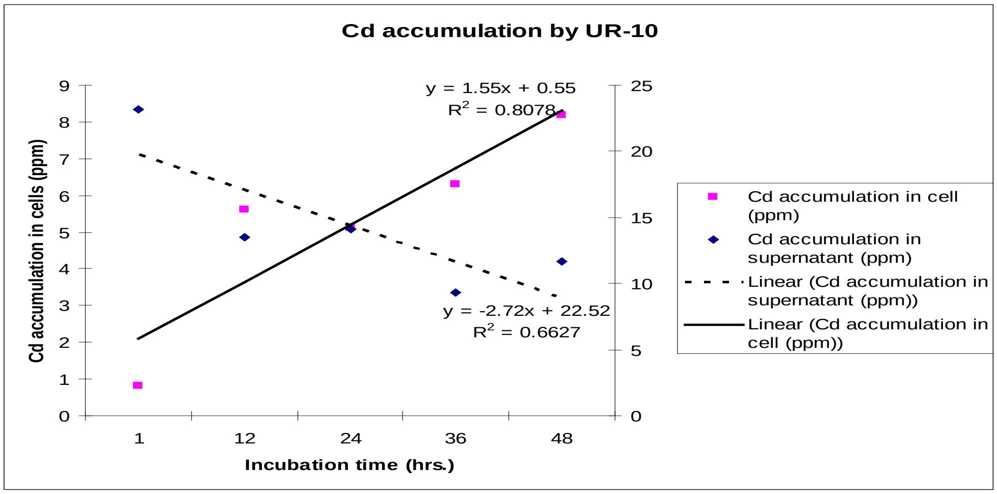


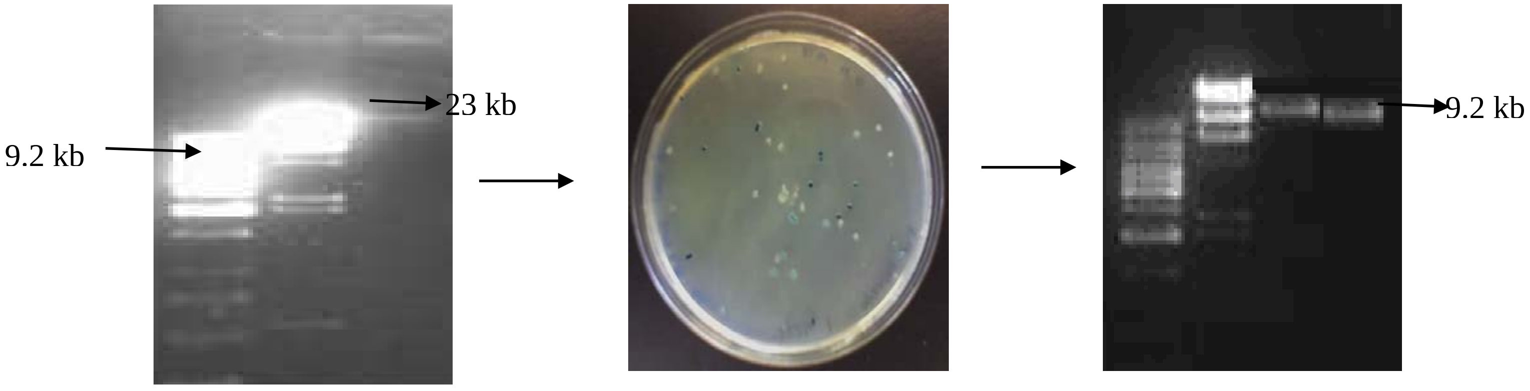
Fig. 21: Cd accumulation study in bacterial isolate UR-10

Bacteria, and other microorganisms, exhibit a number of metabolism-dependent and-independent processes or the uptake and accumulation of heavy metals and radionuclides. The removal of such harmful substances from effluents and waste waters by microbe-based technologies may provide an alternative or additional means of metal/radionuclide recovery for economic reasons and/or environmental protection (Gadd, 1990). The uptake and solubilization of several metals by microbes in the soil are largely unknown (Benka-Coker, 1991. According to Christensen *et al.* (1979), the relationship between the toxicity of heavy metals and soil microbes in an ecosystem could be synergistic or antagonistic. In our study, to know the potential of selected bacterial isolate UR-10, a metal accumulation study using mass balance was carried out where rich media was supplemented with metal (Cd, Pb) as active ingredient. Here, half strength nutrient broth was inoculated with UR-10 (inoculum O.D600 - 2.173) and supplemented with 25 ppm of Cd, as an active ingredient at 37°C, 120 rpm. Samples were collected after each 12 hrs and subjected to metal analysis on Flame–AAS in supernatant and cell pellet. 1 ml of supernatant and cell pellet of 1 ml culture was subjected to acid digestion and metal analysis. Fig. 20 describes the accumulation of Pb inside cells and in the supernatant. Although a reduction in growth has been observed, it has been noted that cell were highly tolerant for Pb and accumulated (Fig. 20) around 30% of total metal Pb inside the cells. A trend line defines the increase in metal concentration inside the cells. Though after 36 hrs, the metal concentration in supernatant increased slightly, this can be attributed to the cell death and lysis imparted by heavy metal toxicity. There are many studies in literature reporting heavy metal accumulation in bacterial cells. Bioaccumulation of several metals have been reported by a *Pseudomonas* sp., strain MGF-48, isolated from the effluent of a metal melting factory (Malekzadeh *et al*, 2002). Kang et al (2007) reported a recombinant *E. coli* strain which has 25-fold higher Cd accumulation than in the control strain. In our study, similar experiment when performed with Cd (Fig. 21) showed 25% of accumulation inside cells

Taking advantage of this beneficial property of metal accumulation inside bacterial cell, several studies have reported successful use of microorganisms in elimination of organic contaminants from different environments (soil, aquatic ecosystems, sediments etc.). For instance, Ueno *et al.* (2006) by introduction of *Pseudomonas aeruginosa* strain WatG into diesel-oil-contaminated soil microcosms achieved a higher degradation rate (64% ± 4.2%) of pollutants during the first two weeks, than in only Luria-Bertani medium amended microcosms (49.5% ± 12%) (Ueno *et al.*, 2006). Beside bioaugmentation (introduction of microbial strains into contaminated environments in order to enhance the bioremediation process) with single strains, Jacques *et al.* (2008) studied the capacity of a microbial consortium (*Mycobacterium fortuitum, Bacillus cereus, Microbacterium sp., Gordonia polyisoprenivorans, Microbacteriaceae bacterium* and *Fusarium oxysporum*) to degrade and mineralize PAH compounds. The Cd tolerant *Pseudomonas sp*. H1, by intracellular sequestration, was able to decrease the level of soluble toxic Cd ions enhancing thus the degradation of 2,4-D by the less heavy-metal resistant *Ralstonia eutropha* (Roane *et al.*, 2001) in a similar study.

4.3.4 Transformation of isolate UR-10 by Plasmid pMMB277

Plasmid pMMB277 (Gene, 1997) is a low copy number with wide host range and controlled expression vector which was isolated from *E.coli* 2842 by the alkali lysis method. Fig.22 shows electrophoresis on 0.8% agarose gel of the plasmid having size 9.237 Kb. The plasmid was purified from low melting agarose and used for transformation studies. The isolate UR-10 was transformed with pMMB277 plasmid and the transformant was obtained was checked for β-galactosidase expression on chloramphenicol +IPTG+X-gal plates (Fig. 22 b) where broad host range plasmid pMMB277 was found in blue colonies. Transformants were subjected to plasmid isolation for the presence of pMMB277 plasmid by agarose gel electrophoresis (Fig. 22 c). The plasmid was purified from low melting agarose. UR-10 was successfully transformed with purified plasmid and was checked for galactosodase expression on chloramphenicol +IPTG + X-gal plates and for plasmid by agarose gel electrophoresis. Fig 22 describes the process of isolation of plasmid, transformation and its isolation from transformed cells.



(a) (b) (c)

Fig 22: Molecular tagging of selected microbe UR-10 (a) Gel picture shows Isolated plasmid pMMB277 (9.2 kb) with Lambda Hind III ladder; (b) Plasmid transformed in UR-10 plated; (c) Plasmid isolation of transformed UR-10

4.3.5 Identification of isolate UR-10

Fig. 23: Gram staining of UR-10 (Gram negative)

The Gram character of the isolate was Gram negative (Fig. 23). 16S rDNA region was amplified, sequence was obtained and BLAST analysis was performed to identify the isolate UR-10. The sequence has been submitted to NCBI Accession no KF973189 shows 100% sequence similarity with *Rhodobacter sphaeroides* based on partial sequence analysis of 16S rDNA.

Isolates as obtained by serial dilution, plating and streaking were screened for heavy metal tolerance (Cd and Pb) in Bushnell Hass Broth supplemented with 25, 50, 75, 100 ppm of metal which revealed three heavy metal tolerant isolates UR-10, UR-8, UR-21. The growth curves of all isolates when studied in rich media supplemented with metals (Cd and Pb 25 ppm showed active growth phase, establishing that these isolates are metal tolerant upto 25 ppm of metal concentrations (Cd, Pb) (Table 16a, 16b).

The *lac* marker alone has not been suitable for monitoring of bacteria in soil since 20% of cultured, aerobic, heterotrophic soil bacteria possess *lacZ+* characteristic. The combination of induced lacZ expression and chloramphenicol resistance provided a stable, precise reporter system, which made its ecological monitoring easy on selective media. So, antibiotic profiling (Table 17) revealed that UR-8 and UR-21 were resistant to streptomycin (50 µg ml-1) and all bacterial strains were sensitive to kanamycin (50µg ml-1), chloramphenicol (10µg ml-1) and ampicillin (50µg ml-1) in addition to the lac Z-ve nature of all five strains. Based on this study strain UR-10 was selected for transformation with plasmid pMMB277 (Fig.6) containing the *lacZ* and chloramphenicol molecular marker. Based on the higher growth rate of UR-10, it was selected for further transformation.

Antibiotic resistance has been widely used as genetic marker in microbial eceology and for monitoring studies in soil (Williams and Davies 1965, Lindow *et al.*, 1988; Nikaido 1994; Jacques *et al.*, 1995). The Gram character of the isolate was Gram negative (Fig. 23). 16S rDNA region was amplified, sequence was obtained and BLAST analysis was performed to identify the isolate UR-10 which showed 100% sequence similarity with *Rhodobacter sphaeroides* based on partial sequence analysis of 16S rDNA (NCBI Accession no KF973189).

4.4 Impact of plant rhizosphere and bioaugmentation on remediation potential

4.4.1 Genetic monitoring of microbial inoculants

For monitoring the genetic viability of transformed cells in the environment, a wetland system study was done to mimic the environment conditions. In total, 13 wetland systems were created, where one was kept as non-spiked planted control and all other were spiked with metal containing water. Four wetlands CW1, CW2, CW3, CW4 (Table 18) were spiked with Cd metal (1L of 5ppm metal containing water), CW5, CW6, CW7, CW8 (Table 18) were spiked with Pb metal solution (1L of 5ppm metal containing water), and next four wetlands CW9, CW10, CW11, CW12 spiked with both the metals (dual metal system) containing Cd and Pb (each 1L of 5ppm metal containing water). A study over the bacterial count was studied over 28 days after 7 day interval.

Ecological monitoring of tagged bacterial strain UR-10 was done to study survival and establishment of molecular tagged microbes in wetland soil planted with *Phragmites australis.* Strain UR-10 was mass cultivated and inoculated in the wetlands system constructed. The ecological monitoring in terms of population build up was studied by enumeration on chloramphenicol, IPTG and X-gal containing nutrient agar plates. For analyzing the total microbial population enumeration was done on Nutrient agar plate and Soil Dehydrogenase activity was noted. 500 ml of culture inoculum was added in each wetland system containing about 6 kg of soil (dry soil weight).

The results showed that maximum CFU count was noted in non spiked control, planted system 430 x 10^7 cfu on Nutrient agar, 22.06 TPF g^{-1} soil day^{-1} Soil Dehydrogenase activity and 120 x 10^7 cfu on 28th day (Table 18) by enumeration on chloramphenicol, IPTG and X-gal containing nutrient agar plates. The enumeration on chloramphenicol, IPTG and X-gal containing nutrient agar plates gives the count of bioaugmented strain UR-10 in the system. The increase in number of molecular tagged bacteria was 38 x 10^4 cfu to 120 x 10^4 cfu from 7th day to 28th day in case of control. While in case of metal spiked system it ranged from 9 x 10^4 cfu in CW1 to 48 x 10^4 cfu in CW3 (Table 18). In case of Pb spiked system, the count was 14 x 10^4 cfu in CW8 to 97 x 10^4 cfu in CW6. Thus Pb appeared to impart less toxicity to molecular tagged bacteria than Cd spike system. In case of dual spiked system the count of molecular tagged bacteria carrying plasmid ranged from 7 x 10^4 cfu in CW12 to 50 x 10^4 cfu in CW11.

The minimum microbial activity was observed in CW2 spiked with Cd + Pb (5 ppm each) where 56 x 10^7 cfu on Nutrient agar, 4. 12 TPF g^{-1} soil day^{-1} Soil Dehydrogenase activity and 42 x 10^7 cfu on 28th day by enumeration on chloramphenicol, IPTG and X-gal containing nutrient agar plates. Blue colored lactose positive and chloramphenical resistant colonies having low copy number plasmid depicts the tagged isolates which showed a maximum growth in planted, non spiked system followed by a single metal spiked (Pb) constructed wetland system *i.e* 140, 127, 134, 93 cfu on 28th day of inoculation. In case of Cd, it was further reduced to 56, 74, 64 and 69 cfu in 1st, 2nd, 3rd and fourth CW system respectively at 28th day.

Augmentation of natural bacterial populations with highly efficient laboratory strains is attractive in maximizing bioprocess performance where laboratory strains compete with indigenous species for common substrates. This competition often results in a replacement of the laboratory strain by wild-type populations more adapted to that particular environment. However, retention of the inoculated population is not guaranteed, and thorough monitoring is thus required to evaluate the bioaugmentation efficiency (Tartakovsky, 1999).

To evaluate the bioaugmentation efficieny, a check on the survival and establishment of molecular tagged microbes in wetland system, *pmmb277* plasmid bearing bacterial strains possessing antibiotic resistance marker were done, which showed blue colonies on X-gal/ IPTG plates. For monitoring the viability of bioaugmented cells in the environment, a wetland system study was done to mimic the environment conditions. In total, 13 wetland systems were created, where one was kept as non-spiked planted control and all other were spiked with metal containing water. Four wetlands CW1, CW2, CW3, CW4 (Table 18) were spiked with Cd metal (1L of 5ppm metal containing water), CW5, CW6, CW7, CW8 (Table 18) were spiked with Pb metal solution (1L of 5ppm metal containing water), and next four wetlands CW9, CW10, CW11, CW12 spiked with both the metals (dual metal system) containing Cd and Pb (each 1L of 5ppm metal containing water). A study over the bacterial count was studied over 28 days after 7 day interval. For inoculation, strain UR-10 was mass cultivated and inoculated in the wetlands system constructed. The ecological monitoring in terms of population build up was studied by enumeration on chloramphenicol, IPTG and X-gal containing nutrient agar plates. For analyzing the total microbial population enumeration was done on Nutrient agar plate and Soil

Dehydrogenase activity was noted. 500 ml of culture inoculum was added in each wetland system containing about 6 kg of soil (dry soil weight).

The results showed that maximum CFU count was noted in non spiked control, planted system (Table 18) 46 x 10^7 cfu on 7th day, 120 x 10^7 cfu on 14th day, 247 x 10^7 cfu on 21st day, 430 x 10^7 cfu on 28th day on Nutrient agar plates. The Soil Dehydrogenase activity (SDA) measured as TPF g^{-1} soil day^{-1} also increased exponentially from 38.83 to 56.06 TPF g^{-1} soil day^{-1} (Table 18). To see the viability of bioaugmented molecular tagged bacteria, enumeration was done on chloramphenicol, IPTG and X-gal containing nutrient agar plates which increased from 38 x 10^4 cfu to 120 x 10^4 cfu from 7th day to 28th day in case of control. While in case of metal spiked system it ranged from 9 x 10^4 cfu in CW1 to 48 x 10^4 cfu in CW3 (Table 18). In case of Pb, the count was 14 x 10^4 cfu in CW8 to 97 x 10^4 cfu in CW6. Thus Pb appeared to impart less toxicity to molecular tagged bacteria than Cd spike system. In case of dual spiked system the count of molecular tagged bacteria carrying plasmid ranged from 7 x 10^4 cfu in CW12 to 50 x 10^4 cfu in CW11.

In literature, many successful bioaugmentation studies have been carried out targeting a specific pollutant in a wetland. In a study by Shao *et al* (2013), two subsurface flow constructed wetlands planted with *Typha orientalis* (CCW) and *Phragmites* (RCW) were constructed to study the effect of the addition of *Paenibacillus* sp. XP1 on nitrogen removal from rural domestic wastewater in autumn (15–21°C) where bioaugmented system had higher ammonia and nitrogen removal efficiency than control. Another study by Runes et al, 2001, bioaugmentation promoted pesticide degradation in nursery run-off channeled through constructed wetlands. In our study, minimum microbial activity was observed in dual metal spiked system. Though the metabolism of accumulation and tolerance of one metal is separate from the other, but still the cells are in more stress conditions in dual spiked systems. Blue colored lactose positive and chloramphenical resistant colonies having low copy number plasmid depicts the tagged isolates which showed a maximum growth in planted, non spiked system followed by a single metal spiked (Pb) constructed wetland system *i.e* 140, 127, 134, 93 cfu on 28th day of inoculation. In case of Cd, it was further reduced to 56, 74, 64 and 69 cfu in 1st, 2nd, 3rd and 4th CW system respectively at 28th day.

Table 18: Genetic monitoring of microbial inoculants

S.No	Type of effluent	Metal Spiked	Monitoring Parameters	7 days	14 days	21 days	28 days
C	Non-spiked	Control	CFU Count (1 x 10^7 cfu)	46	120	247	430
			SDA (TPF g^{-1} soil day^{-1})	38.83	44.44	45.06	56.06
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	38	56	78	120
CW1	Spiked	Cd	CFU Count (1 x 10^7 cfu)	6	24	41	56
			SDA (TPF g^{-1} soil day^{-1})	22.67	14.02	11.34	16.66
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	9	17	22	37
CW2	Spiked	Cd	CFU Count (1 x 10^7 cfu)	9	27	52	74
			SDA (TPF g^{-1} soil day^{-1})	24.22	13.60	9.90	11.13
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	12	18	29	44
CW3	Spiked	Cd	CFU Count (1 x 10^7 cfu)	16	32	39	64
			SDA (TPF g^{-1} soil day^{-1})	24.07	15.90	10.30	12.59
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	14	26	34	48
CW4	Spiked	Cd	CFU Count (1 x 10^7 cfu)	13	29	39	69
			SDA (TPF g^{-1} soil day^{-1})	26.18	15.46	14.02	17.11
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	11	21	26	37
CW5	Spiked	Pb	CFU Count (1 x 10^7 cfu)	20	55	87	140
			SDA (TPF g^{-1} soil day^{-1})	30.61	22.67	20.23	16.66
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	24	43	56	81
CW6	Spiked	Pb	CFU Count (1 x 10^7 cfu)	15	48	94	127
			SDA (TPF g^{-1} soil day^{-1})	24.07	10.30	6.59	7.83
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	16	43	78	97
CW7	Spiked	Pb	CFU Count (1 x 10^7 cfu)	19	51	88	134
			SDA (TPF g^{-1} soil day^{-1})	19.43	14.02	10.09	12.61
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	21	42	67	85
CW8	Spiked	Pb	CFU Count (1 x 10^7 cfu)	18	46	77	93
			SDA (TPF g^{-1} soil day^{-1})	21.99	11.34	13.16	19.38
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	14	33	58	76
CW9	Spiked	Cd+Pb	CFU Count (1 x 10^7 cfu)	14	23	48	66
			SDA (TPF g^{-1} soil day^{-1})	9.69	4.73	5.24	9.48
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	8	17	26	47
CW10	Spiked	Cd+Pb	CFU Count (1 x 10^7 cfu)	29	48	48	56
			SDA (TPF g^{-1} soil day^{-1})	8.25	6.40	6.59	4.12

			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	12	25	35	42
CW11	Spiked	Cd+Pb	CFU Count (1 x 10^7 cfu)	19	46	54	65
			SDA (TPF g^{-1} soil day^{-1})	10.10	4.73	9.48	5.14
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	8	18	31	50
CW12	Spiked	Cd+Pb	CFU Count (1 x 10^7 cfu)	24	39	58	73
			SDA (TPF g^{-1} soil day^{-1})	14.02	9.90	15.46	6.38
			Chl^R +X-gal/ IPTG Enumeration (1 x 10^4 cfu)	7	14	21	39

4.4.2 Metal uptake by *Phragmites* parts

For studying metal accumulation in *Phragmites* plant parts (roots, stem, leaves) a wetland system study was done to mimic the environment conditions. In total, 13 wetland systems were created, where one was kept as non-spiked planted control and all other were spiked with metal containing water. Four wetlands CW1, CW2, CW3, CW4 (Table 19) were spiked with Cd metal (1L of 5ppm metal containing water), CW5, CW6, CW7, CW8 (Table 19) were spiked with Pb metal solution (1L of 5ppm metal containing water), and next four wetlands CW9, CW10, CW11, CW12 spiked with both the metals (dual metal system) containing Cd and Pb (each 1L of 5ppm metal containing water). Study was done in four systems and metal spiked water (5ppm Cd or 5 ppm lead or a combination having 5 ppm concentration of Cd and Pb, was treated in a wetland. The spiked water was retained in the tub for 30 days and the accumulation study was done in root, stem, leaves and soil bed. In order to study metal uptake by the plants, roots, leaves and stem were separated and crushed in a mixer grinder after which they were sieved through 0.2 mm sieve to obtain a fine powder form and one gram per sample was taken for further study. The powder was digested using concentrated nitric and perchloric acid for metal analysis using atomic absorption spectrophotometer as discussed earlier. The result was statistically tested for its significance.

Roots: Cd accumulation was maximum in CW3 0.27 ± 0.22 mg/kg per g dry wt. and ranged from 0.18 ± 0.02 (CW4) to 0.27 ± 0.22 (CW3) mg/kg per g dry wt in single metal spiked system. While in dual metal spiked system (Cd + Pb), the Cd concentration in roots ranged from 0.17 ± 0.11 (CW 11) to 0.25 ± 0.13 (CW10) mg/kg per g dry wt. For Pb, the accumulation ranged from 0.11 ± 0.02 (CW7) to 0.27 ± 0.12 (CW6) mg/kg per g dry wt. after 30 days of incubation (Table 19). In dual metal system, the concentration of Pb ranged from 0.20 ± 0.11(CW12) mg/kg per g dry wt. to 0.27 ± 0.16 (CW11) mg/kg per g dry wt.

Stem: Cd accumulation was maximum in CW4 0.20 ± 0.15 mg/kg per g dry wt. and ranged to 0.1 ± 0.04 (CW2) mg/kg per g dry wt in single metal spiked system. While in dual metal spiked system (Cd + Pb), the Cd concentration in stem ranged from 0.13 ± 0.08 (CW12) to 0.16 ± 0.03 (CW 10, 11) mg/kg per g dry wt. For Pb, the accumulation ranged from 0.13 ± 0.08 (CW7) to

0.27 ± 0.14 (CW8) mg/kg per g dry wt. after 30 days of incubation in dual metal system (Table 19). In dual metal system, the concentration of Pb ranged from 0.19 ± 0.11 (CW11) mg/kg per g dry wt. to 0.28 ± 0.10 (CW12) mg/kg per g dry wt.

Leaves: Cd accumulation was maximum in CW2 0.32 ± 0.21mg/kg per g dry wt. and ranged to 0.22 ± 0.01(CW3) mg/kg per g dry wt in single metal spiked system. While in dual metal spiked system (Cd + Pb), the Cd concentration in leaves ranged from 0.18 ± 0.08 (CW12) to 0.38 ± 0.11 (CW11) mg/kg per g dry wt. For Pb, the accumulation ranged from 0.16 ± 0.23 (CW8) to 0.32 ± 0.03 (CW7) mg/kg per g dry wt. after 30 days of incubation in dual metal system (Table 19). In dual metal system, the concentration of Pb ranged from 0.13 ± 0.10 (CW12) mg/kg per g dry wt. to 0.31 ± 0.14 (CW11) mg/kg per g dry wt.

Sediment Bed/ Soil: In non spiked, control, non planted system**,** Cd was found to be 0.07 ± 0.02 mg/kg per g dry wt. and Pb as - 0.12 ± 0.10 mg/kg per g dry wt. Cd accumulation was maximum in CW3 0.19 ± 0.11 mg/kg per g dry wt. in single metal spiked system. While in dual metal spiked system (Cd + Pb), the Cd concentration ranged from 0.12 ± 0.03 (CW10) to 0.30 ± 0.17 (CW12) mg/kg per g dry wt. For Pb, the accumulation ranged from 0.26 ± 0.11 (CW8) to 0.40 ± 0.21 (CW7) mg/kg per g dry wt. after 30 days of incubation in single metal system (Table 19). In Pb, dual metal spiked system, the metal concentration ranged from 0.21 ± 0.12 (CW12) to 0.33 ± 0.11 (CW11) mg/kg per g dry wt. after 30 days of incubation.

Polluted soil or wastewaters may contain different types of heavy metals. Of these metals, Cadmium (Cd) can be absorbed more easily and quickly (Izadiyar and Yargholi, 2010). Once Cadmium accumulates in plants in quantities greater than the standard values, it can cause a variety of symptoms in living cells. In a study, for each plant type, results showed that Cadmium absorption by the plants are greater in the samples containing 50 and 100 ppm Cadmium, compared with the control treatment and are various in different plant parts (Izadiyar and Yargholi, 2010). Wetland macrophytes, compared with other plant and animal species, have been reported to have a larger or comparable capacity for metal accumulation (Jana, 1988). Because of their fibrous root systems with large contact areas, aquatic plants generally have the ability to accumulate larger metal concentrations in plant organs than those in the surrounding water (Wang *et al.*, 1997). Aquatic macrophytes can have a large capacity for metal accumulation, can tolerate large metal concentrations in water, and can be ideal in situ biomonitors for metal contamination (Bonanno and Giudice, 2010)

Study was done in four systems as single and dual metal spiked experiments where metal spiked water (5ppm Cd or 5 ppm lead or a combination having 5 ppm concentration of each) was treated in a wetland. The spiked water was retained in the tub for 30 days and the accumulation study was done in root, stem, leaves and soil bed. In order to study metal uptake by the plants, roots, leaves and stem were separated and crushed in a mixer grinder after which they were sieved through 0.2 mm sieve to obtain a fine powder form and one gram per sample was taken for further study. The powder was digested using concentrated nitric and perchloric acid for metal analysis using atomic absorption spectrophotometer.

Cd accumulation in roots was maximum in CW3 0.27 ± 0.22 mg/kg per g dry wt. and ranged from 0.18 ± 0.02 (CW4) to 0.27 ± 0.22 (CW3) mg/kg per g dry wt in single metal spiked system. Cd accumulation in stem was maximum in CW4 0.20 ± 0.15 mg/kg per g dry wt. and ranged to 0.1 ± 0.04 (CW2) mg/kg per g dry wt in single metal spiked system. In non spiked soil, control, non planted system, Cd was found to be 0.07 ± 0.02 mg/kg per g dry wt. In soil, Cd accumulation was maximum in CW3 0.19 ± 0.11 mg/kg per g dry wt. in single metal spiked system. In leaves, Cd accumulation was maximum in CW2 0.32 ± 0.21mg/kg per g dry wt. and ranged to 0.22 ± 0.01(CW3) mg/kg per g dry wt in single metal spiked system (Table 19). Similar findings are revealed from literature, where study of *Phragmites australis* and the corresponding water, sediment samples from the mouth area of the Imera Meridionale River revealed Cd considerable accumulation in plant parts *i.e.* Root Cd:1.13 ± 0.08, Rhizome Cd:1.00 ± 0.08, Stem Cd:0.68 ± 0.06, Leaf Cd:1.05 ± 0.10. Ye *et al.*, 1997 reported an accumulation of 0.3- 7.4 µg g-1 d. wt Cd in shoots and 2.5- 49 µg g-1 d. wt in roots.

Pb accumulation in roots, ranged from 0.11 ± 0.02 (CW7) to 0.27 ± 0.12 (CW6) mg/kg per g dry wt. after 30 days of incubation (Table 19). In Stem Pb, the accumulation ranged from 0.13 ± 0.08 (CW7) to 0.27 ± 0.14 (CW8) mg/kg per g dry wt. after 30 days of incubation (Table 19). In Leaves, Pb accumulation ranged from 0.16 ± 0.23 (CW8) to 0.32 ± 0.03 (CW7) mg/kg per g dry wt. after 30 days of incubation in dual metal system (Table 19). In soil, Pb accumulation ranged from 0.26 ± 0.11 (CW8) to 0.40 ± 0.21 (CW7) mg/kg per g dry wt. after 30 days of incubation in single metal system (Table 19). In non spiked soil, control, non planted system, Cd was found to be 0.07 ± 0.02 mg/kg per g dry wt (Table 19). Ye *et al.*, 1997 reported Pb accumulation of 2.5-

80 µg g-1 d. wt in shoots and 8.4- 830 µg g-1 d. wt in roots. Hou *et al.*, 2011 reported statistics as: Cu, Zn, Cd in soil > *Phragmites australis* of aerial part> *Phragmites australis* of underground part Pb: Soil > *Phragmites australis* of aerial part≈ *Phragmites australis* of underground part in River wetland system.

In dual metal spiked system (Cd + Pb), the Cd concentration in roots ranged from 0.17 ± 0.11 (CW 11) to 0.25 ± 0.13 (CW10) mg/kg per g dry wt and Pb ranged from Pb- 0.20 ± 0.11(CW12) mg/kg per g dry wt. to 0.27 ± 0.16 (CW11) mg/kg per g dry wt. In stem, (Cd + Pb system), Cd concentration ranged from 0.13 ± 0.08 (CW12) to 0.16 ± 0.03 (CW 10, 11) mg/kg per g dry wt. and Pb ranged from 0.19 ± 0.11 (CW11) mg/kg per g dry wt. to 0.28 ± 0.10 (CW12) mg/kg per g dry wt. In leaves, (Cd + Pb system), Cd concentration in leaves ranged from 0.18 ± 0.08 (CW12) to 0.38 ± 0.11 (CW11) mg/kg per g dry wt and Pb ranged from 0.13 ± 0.10 (CW12) mg/kg per g dry wt. to 0.31 ± 0.14 (CW11) mg/kg per g dry wt. In soil, (Cd + Pb system), Cd concentration ranged from 0.12 ± 0.03 (CW10) to 0.30 ± 0.17 (CW12) mg/kg per g dry wt. and Pb, ranged from 0.21 ± 0.12 (CW12) to 0.33 ± 0.11 (CW11) mg/kg per g dry wt. after 30 days of incubation (Table 19).

In similar study by Vymazal *et al.*, 2009, concentrations of 19 trace elements were evaluated in biomass of P. *australis* growing in four HF constructed wetlands, highest concentrations were found for Al, Fe, Mn, Zn and Ba, while the lowest concentrations were found for Hg and Cd and U in the aboveground biomass and Hg, Cd and Sn in the belowground biomass. In general, concentrations found in *Phragmites* biomass are comparable to those found in *Phragmites* growing in natural stands. The concentrations decreased in the order roots > rhizomes > leaves > stems (Vymazal *et al.*, 2009). In the present study roots, stem, leaves and soil showed significant differences in concentration for Cd, Pb, but maximum concentration for both metals was accumulated in Leaves > Roots> Stem in both single and dual spiked metal system.

Table 19: Metal accumulation in *Phragmite* plant parts and sediment bed/ soil

S.No	Type of effluent	Metal Spiked	Treatment Days	Vegetation	Roots (mg/kg per g dry wt.)	Stem (mg/kg per g dry wt.)	Leaves (mg/kg per g dry wt.)	Sediment bed (mg/kg per g dry wt.)
C	Non-spiked	Control	30days	Non planted	-	-	-	Cd- 0.07 ± 0.02 Pb- 0.12 ± 0.10
CW1	Spiked	Cd	30 days	Non planted	-	-	-	0.74 ± 0.02
CW2	Spiked	Cd	30 days	Planted	0.24 ± 0.01	0.1 ± 0.04	0.32 ± 0.21	0.12 ± 0.04
CW3	Spiked	Cd	30 days	Planted	0.27 ± 0.22	0.12 ± 0.06	0.22 ± 0.01	0.19 ± 0.11
CW4	Spiked	Cd	30 days	Planted	0.18 ± 0.02	0.20 ± 0.15	0.29 ± 0.14	0.16 ± 0.02
CW5	Spiked	Pb	30 days	Non planted	-	-	-	0.88 ± 0.84
CW6	Spiked	Pb	30 days	Planted	0.27 ± 0.12	0.19 ± 0.12	0.21 ± 0.44	0.3 ± 0.12
CW7	Spiked	Pb	30 days	Planted	0.11 ± 0.02	0.13 ± 0.08	0.32 ± 0.03	0.40 ± 0.21
CW8	Spiked	Pb	30 days	Planted	0.21 ± 0.11	0.27 ± 0.14	0.16 ± 0.23	0.26 ± 0.11
CW9	Spiked	Cd+Pb	30 days	Non planted	-	-	-	Cd- 0.65 ± 0.13 Pb- 0.73 ± 0.15
CW10	Spiked	Cd+Pb	30 days	Planted	Cd- 0.25 ± 0.13 Pb- 0.23 ± 0.15	Cd- 0.16 ± 0.03 Pb- 0.19 ± 0.11	Cd- 0.35 ± 0.04 Pb- 0.25 ± 0.07	Cd- 0.12 ± 0.03 Pb- 0.23 ± 0.11
CW11	Spiked	Cd+Pb	30 days	Planted	Cd- 0.17 ± 0.11 Pb- 0.27 ± 0.16	Cd- 0.16 ± 0.09 Pb- 0.19 ± 0.12	Cd- 0.38 ± 0.11 Pb- 0.31 ± 0.14	Cd- 0.17 ± 0.08 Pb- 0.33 ± 0.11
CW12	Spiked	Cd+Pb	30 days	Planted	Cd- 0.22 ± 0.17 Pb- 0.20 ± 0.11	Cd- 0.13 ± 0.08 Pb- 0.28 ± 0.10	Cd- 0.18 ± 0.08 Pb- 0.13 ± 0.10	Cd- 0.30 ± 0.17 Pb- 0.21 ± 0.12

Chapter 5 Conclusion

5.1 Impact of work

The present study was intended to phytoremediate the wastewater from the selected site in a *Phragmites* vegetated horizontal flow constructed wetland. An attempt has been made to study the bacterial diversity and physico-chemical characteristics of wetland during the remediation after regular intervals to monitor the process and to enhance efficiency through bioaugmentation. The aim of underlying study was to construct a wetland system which can be employed for the primary, secondary or tertiary level of water treatment for the wastewater generated from domestic/ agricultural activities. The study has given some very useful data on the bioremediation potential of wetland system before and after the amendments. Contrary to traditional methods, where determining the optimum operating conditions has been monitoring the influence of each variable individually of the response, RSM has undoubtedly provided a better approach where, just one variable is changed and the others remain at a constant level and the interactive effect between the variables is also considered. While studying wastewater treatment in a wetland, there are many factors influencing the process, treating each factor separately would be very time consuming; furthermore, if several factors play a role, their interactions would not be discernable even if they were dominant. Hence, the use of experimental factorial design and of the response surface methodology has been quite successfull to study a wetland system.

The findings have been very much significant as a good reduction has been obtained in parameters like BOD_5 66.7%, COD 60%, TDS 70% and Cd 50%, Nitrates 21% and Phosphates 50% as compared to an unplanted control system where BOD_5, COD were reduced by 4.2%, 3% respectively. Also study has successfully implemented the RSM approach for treatment/ remediation of wastewater in a lab scale constructed wetland.

This approach can thus be successfully utilized for the primary, secondary and tertiary treatment of wastewater as this study has implemented phytoremediation in the form of vegetated constructed wetlands (CW's) as a cheap and clean, eco-technological approach and its success in its ability to mimic the natural cleansing process by reducing pollutant level to a dischargeable limit. Here, plant and microbial communities have worked synergistically to treat non-point source pollution before it reaches lakes, rivers and oceans.

5.2 Salient findings of this study

1. *Phragmites* vegetated horizontal subsurface flow constructed wetland (HSSF-CW) at lab scale was constructed with dimensions of 48x 33x 20 cm in plastic tubs each having three sections (a) Inlet section (b) Vegetative section (c) Outlet section where treatment of domestic wastewater was studied over a period of 20 days. Treatment was best obtained with inlet flow rate of 22.5ml/min, outlet 9ml/min and retention time of 12.5 hrs as revealed by maximum reduction of BOD, COD, nitrate and metals. Significant reduction was obtained in parameters like BOD_5 66.7%, COD 60%, TDS 70% and Cd 50%, Nitrates 21% and Phosphates 50% as compared to unplanted control system where BOD_5, COD were reduced by 4.2%, 3% respectively and no significant removal of TDS and Cd was observed. In Fe, Mn, Pb, removal rate obtained were 11.1%, 50%, 25% respectively as compared to removal rates in unplanted system.

2. Response surface Methodology (RSM) was successfully applied to study the effect of independent variables (Inlet flow rate 15-30 ml/min, Outlet flow rate 6-12 ml/min, Retention time 1-24 hrs) on dependent variables (pH, EC, DO, BOD_5, COD, Total and Suspended Solids, Nitrate-N, Phosphates and metals like Fe, Cu, Mn, Zn, Ni, Pb, Cd). A second order polynomial response surface model was fitted to each of dependent variable with the help of statistical software MINITAB to generate a regression model with significant terms ($p< 0.05$). The behavior of the model system was explained by quadratic equation

$$Y = \beta + \sum \beta i \, Xi + \sum \beta ii \, (Xi)^2 + \sum \beta ij \, Xij$$

   Where Y is the variable studied, β is the constant in regression model, Xi is the ith independent factor, βi is linear coefficient value for variable Xi and is the first order model concept, βii is the square or quadratic coefficient for the factor i and βij is the model coefficient for interaction between i and j.

3. Physico-chemical characterization of a working wetland site at ACC Gagal Cement Works, at Barmana Distt Bilaspur, Himachal Pradesh, which has a working *Phragmites* vegetated constructed wetland feeding on domestic wastewater from colony inhabited by population of about 500 persons, was done. The depth of collection of soil was 0-15 cm, 15-30 cm and 30-45 cm for upper, middle and lower rhizospheric zone respectively where pH, EC, Organic C, Ava P, Ava S and microbial count in terms of Soil Dehydrogenase activity and CFU (colony forming unit) count on various media (Nutrient agar, Jensen's media, Pikovskaya media, Cellulose minimal media) was studies. The depth of collection was 0-15 cm, 15-30 cm and 30-45 cm for upper, middle and lower rhizospheric zone respectively. The pH in zones varied from 8.11± 0.05 to 8.14 ± 0.12. The EC in zones varied from 0.83 ± 0.07 to 0.9 ± 0.01 mS/cm. The Organic carbon was present in 0.35 to 0.38%. Physico-chemical characteristics like pH, EC, Organic C, Ava P, Ava S (%) did not show much variation in three zones. A viable microbial count was also noted in terms of Soil Dehydrogenase activity (8.4 ± 0.06 µg TPF/g soil/day in upper rhizosphere, 7.9 ± 0.32 µg TPF/g soil/day in middle rhizosphere, 8.32 ± 0.02 µg TPF/g soil/day in lower rhizosphere). CFU (colony forming unit) count was done on various media *i.e* nutrient agar for total bacterial count ranged from 6 x 10^8 to 40 x 10^8 cfu g-1 soil, Jensen's media for nitrogen fixing bacteria ranged from 4 x 10^3 to 9 x 10^3 cfu g-1 soil, Pikovskaya media for P-solubilizers ranged from 2 x 10^3 to 30 x 10^3 cfu g-1 soil and Cellulose minimal media for cellulose degrading bacteria ranged from 6 x $10^{2\text{ to}}$ 50 x 10^2 cfu g-1 soil by serial dilution and plating.

4. For study of soil microbial communities, total Soil DNA extraction was done at different soils depths (upper rhizospheric zone (0-15 cm), middle rhizospheric zone (15-30 cm), lower rhizospheric zone (30-45 cm)) by different methods. Of all four methods; (A) Griffiths method (B) Glass bead-beating method (C) Soil DNA extraction kit (Himedia) (D) Chemical lysis (CL) method. DNA isolated by Griffiths *et al.* (2000) method showed less shearing, intactness and a good ratio of A260/A280.

5. As Sephadex G-200 spin column purification is found to be the best method (Miller *et al.*, 1999) for removing PCR-inhibiting substances while minimizing DNA loss, all the

samples were subjected to Sephadex G-200 spin column purification for pure DNA recovery and removal of PCR inhibitors from crude extracts. The purity of the final purified rhizospheric soil DNA was also checked by the ability of PCR to amplify a region of the 16S rDNA with primers of 27F and 1492R (generating 1,500 bp amplicon) and re-amplification with PRBA 338F 5' and PRUN 518R to generate 180-200 bp amplicons.

6. Denaturing Gradient Gel Electrophoresis (DGGE) was successfully employed as culture-independent approach to study the soil diversity or community structure of a *Phragmites* vegetated wetland system. The bands in the DGGE profile corresponded to the 16S rDNA fragments; same sized but differed in their nucleotide sequence, reflecting the distinct numerically dominant microbial populations in the community. The diversity of the microbial population of each site was confirmed by the band profiling, with the highest appearing at the *Phragmites* upper rhizospheric soil sample. Whereas, the total bacterial community (band numbers) and DNA yield were high in *Phragmites* middle rhizospheric zone, but the microbial community diversity was low. A total of 24 non culturable starin sequences were obtained which belonged to different Phyla *i.e* Firmicutes, α-Proteobacterium, β- Proteobacteria, ϒ- Proteobacteria, δ- Proteobacteria, *Actinobacteria*, Bacteroidetes and Planctomycetes. Sulfate-reducing bacterium belonging to Phyla Firmicutes, Nitrogen metabolizing bacteria belonging to Phyla α-Proteobacterium (*Bradyrhizobium* sps), three strains belonging to ϒ-Proteobacterium, two strains belonging to *Actinobacteria* and three strains of Bacteroidetes which composed of Gram-negative, non-spore forming, anaerobic, and rod-shaped bacteria were observed. Principal Component Analysis (PCA) showed nearly the same profiles of DNA extracted from the same sample in duplicity *i.e.* two lanes of upper rhizospheric samples showed similarity and so with middle and lower rhizospheric samples. The results of cluster analysis also showed that the metabolic activities of Obs1 were more “closely” related to those of Obs2 compared with Obs3 and Obs5. The metabolic activities of Obs6 and Obs 7 were in a single cluster. Similar results were obtained using dendrogram where clustering analysis of the DGGE profiles showed that bacteria in the six lanes of DGGE gel belonged to three clusters.

7. A number of Pb tolerating strains were isolated; 11 from upper rhizosphere zone, 6 from middle zone and 3 from lower zone from *Phragmites australis* rhizosphere zone. Also 8 Cd tolerating strains from upper rhizosphere and one from lower rhizosphere were isolated. Strain *Rhodobacter sphaeroides* (UR-10; isolated from upper rhizospheric zone) was found capacity to accumulate metal as shown by mass-balance study for 48 hours. At regular intervals, cell and supernantant from cukture inoculated with metal was taken, acid digested and analyzed for metal concentration using AAS. The strain UR-10 was found to accumulate about 25% of Cd and 30% of Pb inside the cells.

8. The *lac* marker alone has not been suitable for monitoring in bacteria in soil since 20% of cultured, aerobic, heterotrophic soil bacteria possess *lacZ+* characteristic. The combination of induced *lacZ* expression and chloramphenicol resistance provided a stable, precise reporter system, which made its ecological monitoring easy on selective media. To check the survival and establishment of molecular tagged microbes in wetland system, the bacterial strains (UR-10; *Rhodobacter sphaeroides*) possessing Chl^R were molecularly tagged with pMMB277 plasmid and enumeration was done as blue colored lactose positive and chloramphenicol resistant colonies having low copy which showed a maximum growth in planted, non spiked system followed by a single metal spiked (Pb) constructed wetland system followed by dual metal spiked system.

9. For studying metal accumulation in *Phragmites* plant parts (roots, stem, leaves), tub based wetland systems was fed with single or dual metal spiked water and metal uptake by plant parts was analyzed, which showed significant differences but maximum metal was accumulated in Leaves > Roots> Stem for both Cd and Pb in single and dual spiked metal system. Cd accumulation was found to be more maximum in leaves 0.32 ± 0.21 mg/kg per g dry wt (CW2) followed by 0.27 ± 0.22 mg/kg per g dry wt in roots and least 0.12 ± 0.06 mg/kg per g dry wt in stem. Pb accumulation was found to be more maximum in leaves 0.32 ± 0.03 mg/kg per g dry wt (CW2) followed by 0.27 ± 0.12 mg/kg per g dry wt in roots and 0.27 ± 0.14 mg/kg per g dry wt in stem.

5.3 Deliverables of the study

An increase in the activities of indigenous microbial species in a rhizosphere is likely to enhance the degradation of soil contaminants due to co-metabolic processes of each other. Thus, provision of appropriate nutrients and growth conditions may have a significant impact on microbial numbers in the rhizosphere and consequently the remediation of contaminated soils. In the era of direct DNA analysis, culture independent approaches have provided us with good and accurate analysis of the community structure. The deliverables of this study are:

- Characterization of wastewater from selected sites
- Isolation of bacterial strains from *Phragmites* rhizospheric soil
- Data on bacterial community analysis in *Phragmites* root zone
- Bioaugmentation to increase remediation potential of wetlands
- Data on survival of inoculated microbes

5.4 Future Prospects

Molecular bio-monitoring for both the bacteria (amount and community composition) and their degradation genes (type, amount, expression, and transfer) are of great value in the way understanding molecular microbial ecology behind bioremediation processes in a constructed wetland and thus, forming the basis for environmental applications. These methods when applied for several purposes within the environmental biotechnology can be used to make a good diagnosis of the status of contaminated soil, to map the genetic machinery of intrinsic microbes for degradation, to evaluate the intrinsic biodegradation potential and to predict the development of the contaminated soil; to be able to control and enhance the bioremediation process and; to estimate and monitor the success of bioremediation. In the long run, ameliorating the use of wetlands will be equivalent to protecting by using nature's own resources would be both economically and environmentally beneficial for society.

Microbial ecology studies today struggles with the interactions between microorganisms and disturbed environment e.g. encountering recalcitrant compounds, xenobiotics, toxic substances, where only microbes with different degradation capabilities could bring benefit. Therefore, the study of bacterial consortia as active bioremediation units and as possible inoculants is also important in the future. In addition, there is too little information on plasmid dynamics in soil to estimate the significance of horizontal gene transfer for the efficiency of bioremediation. Therefore, as the next step to understand more about the molecular microbial ecology behind bioremediation, we should pay attention to and study the whole gene pool, consisting of e.g. plasmids and transposons in wetland system. This also highlights the gaps that have not been addressed within this study.

Chapter 6 Bibliography

1. Ali, N.A., Bernal, M.P., Ater, M. (2001). Tolerance and bioaccumulation of copper in *Phragmites australis* and Zea mays. *Plant Soil.* 239(1):103-111.
2. Allen, L.H. (1997). Mechanics and rate of O_2 transfer to and through submerged rhizomes and roots via aerenchyma. *Soil Crop Sci. Soc.* Fl. 56: 47-54.
3. Anderson, T.A., Guthrie, E.A., Walton, B.T. (1993). Bioremediation in the rhizosphere: plant roots and associated microbes clean contaminated soil. *Environ. Sci. Technol.* 27(13):2630-2636.
4. Anjum, N.A., Ahmad, I., Válega, M., Pacheco, M., Figueira, E., Duarte, A.C., Pereira, E. (2012). Salt marsh macrophyte *Phragmites australis* strategies assessment for its dominance in mercury-contaminated coastal lagoon (Ria de Aveiro, Portugal). *Environ. Sci. Pollut. Res.* 19:2879–2888.
5. APHA. (1999). *Standard methods for examination of water and wastewater,* 19th edition, American Public Health Association, Washington DC, USA.
6. Armstrong, J., Armstrong, W., Beckett, P.M. (1992). *Phragmites australis*: venture- and humidity-induced pressure flows enhance rhizome aeration and rhizosphere oxidation. *New phytol.*120:197-207.
7. Armstrong, J., Armstrong, W., Beckett, P.M., Halder, J.E., Lythe, S., Holt, R., Sinclair, A. (1996). Pathways of aeration and the mechanisms and beneficial effect of humidity- and venture-induced convections in *Phragmites australis* (Cav.) Trin. Ex. Steud. *Aquat. Bot*. 54:177-197.
8. Ayeni, O., Ndakidemi, P., Snyman. R., Odendaa, J. (2012). Assessment of Metal Concentrations, Chlorophyll Content and Photosynthesis in *Phragmites australis* along the Lower Diep River, CapeTown, South Africa. *Energ. Environ. Res*. 2(1):128-139.
9. Azaizeh H., Salhani N., Sebesvari Z., Emons H. (2006). Phytoremediation of Selenium using sub-surfcae flow constructed wetlands. *Int. J. Phytorem.* 8:187-198.

10. Bah, A.M., Dai, H., Zhao, J., Sun, H., Cao, F., Zhang, G., Wu, F. (2011). Effects of Cadmium, Chromium and Lead on Growth, Metal Uptake and Antioxidative Capacity in *Typha* angustifolia. *Biol. Trace. Elem. Res.* 142: 77–92.
11. Baik, K.S., Park, S.C., Kim, E.M., Sook Bae, K., Ann, Jae-Hyung., Ka, Jong-Ok, Chun, J., Seong, C.N. (2008). Diversity of bacterial community in freshwater of Woopo wetland. *J Microbiol.* 46(6): 647-655.
12. Ban˜uelos, G.S., Ajwa, H.A., Mackey, L.L., Wu, C., Cook, S., Akohoue, S. (1997). Evaluation of different plant species used for phytoremediation of high soil selenium. *J. Environ. Qual.* 26 :639– 46.
13. Baptista, J.D., Davenport, R.J., Donnelly, T., Curtis, T.R. (2008) The microbial diversity of laboratory-scale wetlands appears to be randomly assembled. *Water Res.* 42: 3182-3190.
14. Bedell, J.P., Saulais, S., Delolme, C. (2012). Zinc, cadmium, and copper mobility and accumulation in reeds (*Phragmites australis*) in urban sediments from two stormwater infiltration basins. Geophysical Research Abstracts. Vol. 14, 7480.
15. Begg, J.S., Lavigne, R.L., Veneman, P.L.M. (2000). *Phragmites reed beds: Constructed wetlands for municipal wastewater treatment – European perspective.* In 7th Int. Conf. on Wetland Systems for Water Pollution Control; University of Florida, 399-405.
16. Benka-Coker, M. O. (1991). ‘Studies of the Effect of Crude Oil and Heavy Metal Contamination of Soil and Water on the Microbial Populations at the Kokori Flare Site, Bendel State, Nigeria, Ph.D. Thesis, University of Benin, 165 pp.
17. Bhattacharya, T., Banerjee, D., Gopal, B. (2006). Heavy metal uptake by Scirpus littoralis Schrad. from fly ash dosed and metal spiked soils. *Environ. Monit. Ass.* 121(1-3), 361-378.
18. Bianchia, V., Peruzzib, E., Masciandaro, G., Ceccanti, B., Raveloc, S.M., Iannelli, R. (2011). Efficiency assessment of a reed bed pilot plant (*Phragmites australis*) for sludge stabilisation in Tuscany (Italy). *Ecol. Eng.* 37:779–785.
19. Birnboim, H.C., Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 24; 7(6):1513-23.

20. Blankenberg, Anne-Grete B., Braskerud, B., Haarstad, K. (2005). Pesticide retention in two small constructed wetlands: treating non-point source pollution from agricultural runoff. *Int. J. Environ. Anal. Chem.* 86(3-4), 225-231.
21. Bonanno, G., Giudice, R. Lo. (2010). Heavy metal bioaccumulation by the organs of *Phragmites australis* (common reed) and their potential use as contamination indicators. *Ecol. Indic.* 10:639–645.
22. Borsodi, Andrea K., Rusznyák, A., Molnár, P., Vladár, P., Mária, N., Reskóné, T., Erika, M., Sipos, R., Gedeon, G., Márialigeti, K. (2007). Metabolic Activity and Phylogenetic Diversity of Reed (*Phragmites australis*) Periphyton Bacterial Communities in a Hungarian Shallow Soda Lake. *Microb. Ecol.* 53(4):612-620.
23. Box, G. E. P., Wilson, K.B. (1951). On the Experimental Attainment of Optimum Conditions (with discussion). *J. R. Stat. Soc.* Series B13(1):1–45.
24. Box, G.E.P., Draper, N.R. (1987). Empirical Model-Building and Response Surfaces. John Wiley & Sons, New York.
25. Bragato, C., Schiavon, M., Polese, R., Ertani, A., Pittarello, M., Malagoli, M. (2009). Seasonal variations of Cu, Zn, Ni and Cr concentration in *Phragmites australis* (Cav.) Trin ex steudel in a constructed wetland of North Italy. (1–3), 35–44.
26. Brix, H. (1997). Do macrophytes play a role in constructed treatment wetland. *Water Sci. Technol.* 35:11-17.
27. Brix, H. (1999). Genetic diversity, ecophysiology and growth dynamics of reed (*Phragmites australis*). *Aquat. Bot.* 64:179-184.
28. Brodkorb, T.S., Legge, R.L. (1992). Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with Phanerochaete chrysosphorium. *Appl. Environ. Microbiol.* 58(9):3117-3121.
29. Brofft, J.E., McArthur, J.V., Shimkets, L.J. (2002). Recovery of novel bacterial diversity from a forested wetland impacted by reject coal. *Environ. Microbiol.* 4(11):764-9.
30. Bronstein, I., Fortin, J., Stanley, P.E., Stewart, G.S.A.B., Kricka, L.J. (1994). Chemiluminescent and bioluminescent reporter gene assays. *Anal. Biochem.* 219: 169-181.
31. Burgmann, H., Pesaro, M., Widmer, F., Zeyer, J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. *J. Microbiol. Methods* 45:7–20.

32. Burken, J.G., Schnoor, J.L. (1997) Uptake and metabolism of atrazine by poplar trees. *Environ. Sci. Technol.* 31:1399– 406.
33. Burken, J.G., Schnoor, J.L. (1999). Distribution and volatilization of organic compounds following uptake by hybrid poplar trees. *Int. J. Phytorem.* 1:139– 51.
34. Calheiros, C.S.C., Rangel, A.O.S.S., Castro, P.M.L. (2007). Constructed wetland systems vegetated with different plants applied to the treatment of tannery wastewater. *Water Res.* 41 (8):1790–1798.
35. Calheiros, C.S., Rangel, A.O., Castro, P.M. (2009). Treatment of industrial wastewater with two-stage constructed wetlands planted with *Typha* latifolia and *Phragmites australis*. *Bioresour Technol.* 100(13):3205-13.
36. Cambrolle, J., Mateos-Naranjo, M., Redondo-Go´mez, S., Luque, T., Figueroa, M.E. (2011). The role of two Spartina species in phytostabilization and bioaccumulation of Co, Cr, and Ni in the Tinto–Odiel estuary (SW Spain). *Hydrobiologia*. 671:95–103.
37. Cambrollé, J., Redondo-Gómez, S., Mateos-Naranjo, E., Figueroa, M.E. (2008). Comparison of the role of two *Spartina* species in terms of phytostabilization and bioaccumulation of metals in the estuarine sediment. *Mar. Pollut. Bull*. 56:2037–2042.
38. Cappuccino, J.G., Sherman, N. (1987). Microbiology: A laboratory manual. Benjamin Cummings Publications.
39. Carias, C.C., Novais, J.M., Martins-Dias, S. (2008). Are *Phragmites australis* enzymes involved in the degradation of the textile azo dye acid orange 7? *Bioresour. Technol*. 99(2):243-51.
40. Carvalho, P.N., Basto, M.C., Almeida, C.M. (2012). Potential of *Phragmites australis* for the removal of veterinary pharmaceuticals from aquatic media. *Bioresour. Technol.* 116:497-501.
41. Cassida, L.E., Jr. (1977). Microbial metabolic activity in soil as measured by dehydrogenase determination. *Appl. Environ. Microbiol.* 34:630-636.
42. Chale, F.M.M. (2012). Nutrient Removal in Domestic Wastewater Using Common Reed (*Phragmites* mauritianus) in Horizontal Subsurface Flow Constructed Wetlands. *TaJONAS*. 3(1):495-499.

43. Chandra, R., Yadav, S. (2011). Phytoremediation of Cd, Cr, Cu, Mn, Fe, Ni, Pb and Zn from Aqueous Solution Using *Phragmites* Cummunis, *Typha* Angustifolia and *Cyperus Esculentus*. *Int. J. Phytorem.* 13:580–591.
44. Chaturvedi, S., Chandra, R., Rai, V. (2006). Isolation and characterization of *Phragmites australis* (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. *Ecol. Eng.* 27(3):202-207.
45. Chaudhry, Q., Blom-Zandstra, M., Gupta, S., Joner, E.J. (2005). Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. *Environ. Sci. Pollut. Res.* 12:34-48.
46. Chazarenc, F., Merlin, G., Gonthier, Y. (2003). Hydrodynamics of horizontal subsurface flow constructed wetlands. *Ecol. Eng.* 21:165–173.
47. Chen, S., G.T. Wang, and S.K. Xue. 1999. Modeling BOD removal in constructed wetlands with mixing cell method. *J. Environ. Eng.* 125:64–71.
48. Chen YH, Wu XF, Chen ML, Zhang ZN, Li KL, Wang ZC, Lei D. (2011). Analysis of microorganism species diversity in plant intercropping models in a wetland system constructed for treatment of municipal sewage. *Huan Jing Ke Xue*. 32(8):2397-402.
49. Chen, H., Wang, S., Chen, M. (2008). Microbiological study of lactic acid bacteria in kefir grains by culture-dependent and culture-independent methods. *Food Microbiol.* 25:492–501.
50. Chesnin, L., Yien, C.H., (1950). Turbidimetric determination of available sulphates. *Proc. Soil Sci. Soc. Am*. 15 :149-151.
51. Christensen, E. R., Scherfig, J., Dixon, P. S. (1979). ‘Effects of manganese, copper and lead on *Selenastrum capricornutum* and *Chlorella stigmatophora*, *Water Res.* 13:79–92.
52. Chu, W.K., Wong, M.H., Zhang J. (2006). Accumulation, distribution and transformation of DDT and PCBs by *Phragmites australis* and *Oryza sativa* L:1. Whole plant study. *Environ. Geochem. Health.* 28:159-168.
53. Clarke, E., Baldwin, A.H. (2002). Responses of wetland plants ammonia and water level. *Ecol. Eng.* 18:257-264.
54. Creighton, Thomas C. (1999). Encyclopedia of Molecular Biology, Volumes 1-4. John Wiley and Sons.

55. Criado, C., Bécares, E. (2005). Characterization of bacterial communities of a constructed wetland in cold conditions. *J. Gen. Appl. Microbiol.* 51(3):197-201.
56. Cunningham, S.D., Shann, J.R., Crowley, D.E., Anderson, T.A. (1997). Phytoremediation of contaminated water and soil. In: Kruger, E.L., Anderson, T.A. and Coats, J.R. eds. Phytoremediation of soil and water contaminants. ACS symposium series 664. Washington, DC, American Chemical Society, p. 2-19.
57. Cynthia, M., John, R. (2002). Contributions of Scirpus californicus in a constructed wetland system receiving copper contaminated wastewater. (P261) SETAC-(2002), Salt lake city, Utah. 16-20 Nov.
58. Czako, M., Feng, X., He, Y., Liang, D., Marton, L. (2006). Transgenic Spartina alterniflora for phytoremediation. *Environ. Geochem. Health*. 28(1-2): 95-102.
59. Daane, L.L., Harjono, I., Zylstra, G.J., Haggblom, M.M. (2001). Isolation and characterization of polycyclic aromatic hydrocarbon- degrading associated bacteria with the rhizosphere of salt marsh plants. *Appl. Environ. Microbiol.* 67:2683-2691.
60. Davies, L.C., Carias, C.C., Novais, J.M., Martins-Dias, S. (2005). Phytoremediation of textile effluents containing azo dye by using *Phragmites australis* in a vertical flow intermittent feeding constructed wetland. *Ecol. Eng.* 25, 594-605.
61. Debusk, T.A., Laughlin, R.B., Schwartz, L.N. (1996). Retention and compartmentalization of lead and cadmium in wetland microcosms. *Water Res.* 30 (11):2707-2716.
62. DeJournett, T.D., Arnold, W.A., LaPara, T.M. (2007). The characterization and quantification of methanotrophic bacterial populations in constructed wetland sediments using PCR targeting 16S rDNA gene fragments. *Appl. Soil Ecol.* 35: 648-659.
63. Dong, X., Reddy, G.B. (2010). Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique. Bioresource Technology 101:1175–1182.
64. Dordio A., Carvalho, A.J.P., Teixeira, D.M., Dias, C.B., Pinto, A.P. (2010). Removal of pharmaceuticals in microcosm constructed wetlands using *Typha* spp. and LECA. *Bioresour Technol.* 101:886–892.

65. Drizo, A., Frost, C.A., Smith, K.A., Grace, J. (1996). Phosphate and ammonium removal by constructed wetlands with horizontal subsurface flow, using shale as a substrate. In 5th Int. Conf. on Wetland Systems for Water Pollution Control, Vienna, I/10-1 - 8.
66. Du Laing, G., Vandecasteele, B., De Grauwe, P., Moors, W., Lesage, E., Meers, E., Tack, F.M., Verloo, M.G. (2009). Factors affecting metal concentrations in reed plants (*Phragmites australis*) of intertidal marshes in the Scheldt estuary. *Ecol. Eng.* 35(2):310–318.
67. Dunbabin, J.S., Bowmer, K.H. (1992). Potential use of constructed wetlands for treatment of industrial wastewater containing metals *Sci. Total Environ.*111:151-168.
68. Dushenkov, V., Kumar, P.B.A.N., Motto, H., Raskin I. (1995). Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environ. Sci. Technol.* 29:1239–45.
69. Eden, P.A., Schmidt, T.M., Blakemore, R.P., Pace, N.R. (1991). "Phylogenetic Analysis of Aquaspirillum magnetotacticum Using Polymerase Chain Reaction-Amplified 16S rDNA-Specific DNA". *Int. J. Syst. Bacteriol.* 41 (2): 324–325.PMID 1854644.
70. Eke, Paul E., Scholz, M. (2008). Benzene removal with vertical-flow constructed treatment wetlands. *J Chem Technol Biotechnol.* 83:55–63.
71. Emerson, D., Weiss, J.V., Megonigal, J.P. (1999). Iron-Oxidizing Bacteria Are Associated with Ferric Hydroxide Precipitates (Fe-Plaque) on the Roots of Wetland Plants. *Appl. Environ. Microbiol.* 65(6):2758-2761.
72. Fannin, T.E., Marcus, M.D., Anderson, D.A., Bergman, H.L. (1981). Use of a fractional factorial design to evaluate interactions of environmental factors affecting biodegradation rates. *Appl Environ Microbiol.* 42(6):936–943.
73. Faure, M., Miguel, A.S., Ravanel, P., Raveton, M. (2012). Concentration responses to organochlorines in *Phragmites australis*. *Environ. Pollut.* 164:188–194.
74. Forster, S., Lappin-Scott, H., Snape, J., Porter, J. (2003). Rains, drains and active strains: towards online assessment of wastewater bacterial communities. *J. Microbiol. Methods.* 55:859–64.
75. Fournel, J., Forqueta, N., Mollea, P., Grasmick, A. (2013). Modeling constructed wetlands with variably saturated vertical subsurface-flow for urban stormwater treatment. *Ecol. Eng.* 55:1–8.

76. Frey, S.D., Knorr, M., Parrent, J.L., Simpson, R.T. (2004). Chronic nitrogen enrichment affects thee structure and function of the soil microbial community in temperate hardwood and pine forests. *Forest Ecol. Manage.* 196:159–171.
77. Gadd, G. M. (1990). Heavy metal accumulation by bacteria and other microorganisms. *Experientia*. 46(8):834-840.
78. Gampel, T. (2003). Kistelepülések szennyvíztisztítása vízinövényes talajszûrôvel. Vízellátás, csatornázás VI. Évf., pp. 70-72.
79. Gao, L., Xie, L. (2014). Modeling Phosphorus Removal in Horizontal Subsurface Flow Constructed Wetlands. *JOCET*. 2(2) April 2014.
80. Garcia, J., Chiva, J., Aguirre, P., Alvarez, E., Sierra, J., Mujeriego, R. (2004). Hydraulic behaviour of horizontal subsurface flow constructed wetlands with different aspect ratio and granular medium size. *Ecol. Eng.* 23(3): 177-187.
81. Gavrilescu, M., (2004). Removal of heavy metals from the environment by biosorption – a review. *Eng. Life Sci.* 4:219–232.
82. Gebremariam, S.Y., Beutel, M.Y. (2009). Nitrate removal and DO levels in batch wetland mesocosms: Cattail (*Typha* spp.) versus bulrush (*Scirpus spp.). Ecol. Eng.* 34:1–6.
83. Gentry, T.J., Rensing, C., Pepper, I.L. (2004). New Approaches for Bioaugmentation as a Remediation Technology. *Crit. Rev. Environ. Sci. Tech***.** 34:447–494.
84. Gikas, E., Papadopoulos, N.G., Bazoti, F.N., Zalidis, G., Tsarbopoulos, A. (2012). Use of liquid chromatography/electrospray ionization tandem mass spectrometry to study the degradation pathways of terbuthylazine (TER) by *Typha* latifolia in constructed wetlands: identification of a new TER metabolite. Rapid Commun. *Mass Spectrom.* 26:181–188.
85. Glick, B.R., (2010). Using bacteria to facilitate phytoremediation. Biotechnol. Adv. 28:367-374.
86. Goldstein, R.M., Mallory, L.M., Alexander, M. (1985). Reasons for possible failure of inocualtion to enhance biodegradation. *Appl. Environ. Microbiol*. 50(4):977-983.
87. Gopal, B.B. (1999). Natural and constructed wetlands for wastewater treatment: potentials and problems. *Water Sci. Tech.* 40(3):27-35.
88. Greenway, M. (1997). Nutrient content of wetland plants in constructed wetlands receiving municipal effluent in tropical Australia. *Water Sci. Tech*. 35(5):135-142.

89. Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey M.J. (2000). Rapid Method for Co-extraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rDNA-Based Microbial Community Composition. *Appl. Environ. Microbiol.* 66:125488-5491.
90. Groudeva, V.I., Groudev, S.N., Doycheva, A.S. (2001). Bioremediation of waters contaminated with crude oil and toxic heavy metals. *Int. J. Miner. Process*. 62 (1-4):293-299.
91. Gurudeva, V., Doycheva, A., Krumova, K., Groudev, S. (2007). Bioremediation in situ of an alkaline soil polluted with heavy metals. *Adv. Mater. Res.* 20(21):287-290.
92. Hadad, H.R., Mufarrege, M.M., Pinciroli, M., Di Luca, G.A., Maine, M.A. (2010). Morphological Response of *Typha domingensis* to an Industrial Effluent Containing Heavy Metals in a Constructed Wetland. *Arch. Environ. Contam. Toxicol.* 58:666–675.
93. Hamouri, B., El Nazih, J., Lahjouj, J. (2007). Subsurface-horizontal flow constructed wetland for sewage treatment under Moroccan climate conditions. *Desalination*. 215:153–58.
94. Healy, M., Cawley, A. M. (2002). Nutrient processing capacity of constructed wetland in western Ireland. *J. Environ. Qual.* 31: 1739-1747.
95. Hegazy, A. K., Abdel-Ghani, N. T., El-Chaghaby, G. A. (2011). Phytoremediation of industrial wastewater potentiality by *Typha domingensis*. *Int. J. Environ. Sci. Technol.* 8(3):639-648.
96. Henze, M., Gujer, W., Mino, T., van Loosdrecht, M.C.M. (2000). Activated sludge models ASM1, ASM2, ASM2D and ASM3. IWA Scientific and Technical Rep. 9. Int. Water Assn., London.
97. Hou, Y.X., Wang, Y., Li, H.Y., Li, X.X., Hu, X.J. (2011). Accumulation and Distribution of Heavy Metals in *Phragmites australis* in the Wetland of Liaohe River Estuary. *Adv. Mater. Res.* 356 – 360:994-997.
98. Howeler, M., Ghiorse, W.C., Walker, L.P. (2003). A quantitative analysis of DNA extraction and purification from compost. *J. Microbiol. Methods.* 54:37–45.
99. Hu, Q., Qi, H.Y., Zeng, J.H., Zhang, H.X. (2007). Bacterial diversity in soils around a lead and zinc mine. *J. Environ. Sci.* (China).19(1):74-9.

100. Ibekwe, A.M., Grieve, C.M., Lyon, S.R. (2003). Characterization of microbial communities and composition in constructed dairy wetland wastewater effluent. *Appl. Environ. Microbiol.* 69:5060-5069.

101. Ibekwe, A.M., Lyon, S.R., Leddy, M., Jacobson-Meyers, M. (2007). Impact of plant density and microbial composition on water quality from a free water surface constructed wetland. *J Appl. Microbiol.* 102: 921-936.

102. Ibekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K., Lyons, S.R. (2002). Multiplex fluorogenic real-time PCR for detection and quantification of Escherichia coli O157:H7 in dairy wastewater wetlands. *Appl. Environ. Microbiol.* 68: 4853-4862.

103. Inamori, R., Gui, P., Dass, P., Matsumura, M., Xu, K.Q., Ebie, Y., Inamori, Y. (2007). Investigating CH4 and N2O emissions from eco-engineering wastewater treatment processes using constructed wetland microcosms. *Process Biochem.* 42: 363-373.

104. Ivandi, L., Major, B., Szilágyi, F. (1998). Az eleveniszapos és a gyökérmezôs szennyvíztisztítás összehasonlítása. XVI MHT *Vándorgyûlés*. 8-9: 414-428.

105. IWA Specialist Group on Use of Macrophytes in Water Pollution Control. (2000). *Constructed Wetlands for Pollution Control,* Scientific and Technical Report Number 8, IWA Publishing, London, UK.

106. Izadiyar, M.H., Yargholi, B. (2010). Study of Cadmium Absorption and Accumulation in Different Parts of Four Forages. *American-Eurasian J. Agric. and Environ. Sci*. 9(3): 231-238, 2010.

107. Jackson, M.L., 1967. Soil Chemical Analysis. Asia Publishing House. Bombay.

108. Jacques, M., Kinkel, L., Morris, C.E. (1995). Population Sizes, Immigration, and Growth of Epiphytic Bacteria on Leaves of Different Ages and Positions of Field-Grown Endive (*Cichorium endivia var. latifolia*). *Appl. Environ. Microbiol*. 899–906.

109. Jacques, R.J.S., Okeke, B.C., Bento, F.M., Teixeira, A.S., Peralba, M.C.R., Camargo, F.A.O. (2008). Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. *Bioresour. Technol.* 99:2637–2643.

110. Jana, S. (1988). Accumulation of Hg and Cr by three aquatic species and subsequent changes in several physiological and biochemical plant parameters. *Water Air Soil Pollut.* 38:105–109.

111.Jayaweera, M.W., Kasturiarachchi, J.C., Kularatne, R.K.A., Wijeyekoon, S.L.J. (2007). Removal of aluminium by constructed wetlands with water hyacinth (Eichhornia crassipes (Mart.) Solms) grown under different nutritional conditions. *J. Environ. Sci.* 42(2):185-193.

112.Jiang, J., Alderisio, K.A., Singh, A., Xiao, L. (2005) Development of procedures for direct extraction of Cryptosporidium DNA from water concentrates and for relief of PCR inhibitors. *Appl Environ Microbiol.* 71:1135–1141.

113.Jin, G., Kelley, T.R. (2007). Characterization of microbial communities in a pilot-scale constructed wetland using PLFA and PCR-DGGE analyses. *J Environ Sci Health A Tox Hazard Subst Environ Eng.* 42(11):1639-47.

114.Kadlec, R.H., Keoleian, G.A. (1986). Metal ion exchange on peat. In: Fuchsman, C.H. (Ed.), Peat and Water pp. 61-93. Elsevier , Amsterdam.

115.Kadlec, R.H., Knight, R.L. (1996). *Treatment Wetlands.* CRC Press, Lewis Publishers, Boca Raton, FL.

116.Kalipci, E. (2011). Investigation of decontamination effect of *Phragmites australis* for Konya domestic wastewater treatment. *J. Med. Arom. Plants*. 5(29):6571-6577.

117.Kang, S.H., Singh, S., Kim, J.Y., Lee, W.,Mulchandani, A., Chen, W. (2007). Bacteria Metabolically Engineered for Enhanced Phytochelatin Production and Cadmium Accumulation. *Appl. Environ. Microbiol.* 73(19): 6317-6320.

118.Kantawanichkula, S., Kladpraserta, S., Brix, H. (2009). Treatment of high-strength wastewater in tropical vertical flow constructed wetlands planted with *Typha* angustifolia and *Cyperus involucratus*. *Ecol. Eng.* 35:238–247.

119.Katayon, S., Fiona, Z., Noor, M.J., Megat, M.H., Abdul, G., Ahmad, J. (2008). Treatment of mild domestic wastewater using subsurface constructed wetlands in Malaysia'. *Int. J. Environ. Stud***.** 65(1):87 — 102.

120. Katyal, A.K., Kaluarachchi, J.J., Parker, J.C. (1991). MOFAT: A two-dimensional finite element program for multiphase fl ow and multi-component transport: Program documentation and user’s guide. Center for Environ. and Hazardous Material Studies, Virginia Polytechnic Inst. and State Univ., Blacksburg.

121.Keffala, C., Ghrabi, A. (2005). Nitrogen and bacterial removal in constructed wetlands treating domestic wastewater. *Desalination*. 185(1-3):383-389.

122.King, J.K., Harmon, S.M., Fu, T.T., Gladden, J.B. (2002). Mercury removal, methylmercury formation, and sulfate-reducing bacteria profiles in wetland mesocosms. *Chemosphere.* 46: 859-870.

123.Kivaisi, A.K. (2001). The potential for constructed wetlands for wastewater treatment and reuse in developed countries: A Review. *Ecol. Eng.* 16:545-560.

124.Klomjek, P., Nitisoravut, S. (2005). Constructed treatment wetland: a study of eight plant species under saline conditions. *Chemosphere.* 58:585–593.

125.Koottatep, T., Polprasert, C. (1997). Role of plant uptake on nitrogen removal in constructed wetlands located in the tropics. *Water Sci. Tech*. 36(12):1-8.

126. Korbahti, B.K., Aktas, N., Tanyolac, A. (2007). Optimization of electrochemical treatment of industrial paint wastewater with response surface methodology. *J. Hazard. Mater*. 148: 83-90.

127.Korbahti, B.K., Tanyolac, A., (2008). Electrochemical treatment of simulated textile wastewater with industrial components and Levafix Blue CA reactive dye: Optimization through response surface methodology. *J. Hazard. Mater.* 151:422–431.

128.Kuiper, I., Bloemberg, G.V., Lugtenberg, B.J.J. (2001). Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Mol. Plant Microbe Interact.* 14:1197-1205.

129.Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., Lugtenberg, B.J.J. (2004). Rhizoremediation: A beneficial plant microbe interaction. *Mol. Plant Microbe Interact.* 17:6-15.

130. Kumar, P.B.A.N., Dushenkov, V., Motto, H., Rasakin, I. (1995). Phytoextraction: the use of plants to remove heavy metals from soils. *Environ. Sci. Technol.*29:1232– 8.

131.LaMontagne, M.G., Michel, F.C.Jr, Holden, P.A., Reddy, C.A. (2002). Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J Microbio. Methods.* 49:255–264.

132. Lane, D.J. (1991). 16S/23S rRNA sequencing. In: Goodfellow, M.C. (Ed.), Nucleic acid Techniques in Bacterial Systematics. Wiley, UK, pp. 115–148.

133.Langergraber, G. (2008). Modeling of Processes in Subsurface Flow Constructed Wetlands: A Review. *Vadose Zone J.* 7(2).

134. Langergraber, G. (2011). Numerical modelling: a tool for better constructed wetland design? *Water Sci Technol*. 64(1): 14-21.

135. Langergraber, G., Girald, D., Mena, J., Meyer, D., Peña M., Toscano, A., Brovelli A., Korkusuz E.A. (2009) Recent developments in numerical modelling of subsurface flow constructed wetlands. *Sci. Total Environ*. 407(13):3931-3943.

136.Langergraber, G., Šimůnek, J. (2005). Modeling variably saturated water flow and multicomponent reactive transport in constructed wetlands. *Vadose Zone J*. 4:924–938.

137. Langergraber, G., Šimůnek, J. (2006). The multi-component reactive transport module CW2D for constructed wetlands for the HYDRUS software package. Hydrus Software Series 2. Dep. of Environ. Sciences, Univ. of California, Riverside.

138.Langergraber, G., Šimunek, J. (2012). Reactive transport modeling of subsurface flow constructed wetlands using the HYDRUS wetland module. *Vaodose J*. 11(2).

139.Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40 (9):2407–2415.

140.Lee B., Scholz M. (2007). What is the role of *Phragmites australis* in experimental constructed wetland filters treating urban runoff?, *Ecol. Eng.* 29:87-95.

141.Lesage, E., Rousseau, D.P.L., Meers, E., Tack, F.M.G., De Pauw, N. (2007). Accumulation of metals in the sediments and reed biomass of a combined constructed wetland treating domestic wastewater. *Water, Air Soil Pollut.* 183:253-264.

142. Li, Y.H., Zhu, J.N., Liu, Q.F., Liu, Y., Liu, M., Liu, L., Zhang, Q. (2013) comparison of the diversity of root associated bacteria in *Phragmites australis* and *Typha* augustifolia L. in artificial wetlands. *World J. Microbiol. Biotechnol.* 29(8):1499-1508.

143.Lindow, S. E. (1988). Lack of evidence of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. *Phytopathology*. 78:444-450.

144.Liu, Y., Chen, H., Wu, G., Wu, X. (2010). Feasibility of estimating heavy metal concentrations in *Phragmites australis* using laboratory-based hyperspectral data—A case study along Le’an River, China. *Int. J. Appl. Earth Obs. Geoinf.* 12(2):166–S170.

145. Llorens, E., Obradors, J., Alarcón-Herrera, M. T. (2013). “Modelling the non-biogenic steps of arsenic retention in horizontal subsurface flow constructed wetlands,” *Chem. Engg. J.* 223:657-664.

146. Madsen, E.L. (1991). Determining in situ biodegradation: facts and challenges. *Environ. Sci. Technol.* 25:1663- 1673.

147. Małoszewski, P., Wachniew, P., Czupryński, P. (2006). Study of hydraulic parameters in heterogeneous gravel beds: Constructed wetland in Nowa Słupia (Poland). *J. Hydrol.* 331:630–642.

148. Malekzadeh, F., Farazmand, A., Ghafourian, H., Shahamat, M., Levin, M., Colwell, M.M. (2002). Uranium accumulation by a bacterium isolated from electroplating effluent. *World J. Microbiol. Biotechnol*. 18(4):295-302.

149. Manios, T., Stentiford, E.I., Millner, P. (2003). Removal of total suspended solids from wastewater in constructed horizontal flow subsurface wetlands. *J. Environ. Sci. Health.* A38:1073–1085.

150. Mant, C., Costa, S., Williams, J. (2006). Phytoremediation of chromium by model constructed wetland. *Bioresour. Technol.* 97(15):1767-72.

151. Marisa, C., Almeida, R., Mucha, A.P., Teresa, M., Vasconcelos, S.D. (2004). Influence of sea rush Juncus maritimus on metal concentration and speciation in Esturine sediment colonized by plant. *Environ. Sci. Technol.* 38(11):3112-3118.

152. Marquesa, B., Lillebøb, A.I., Pereiraa, E., Duartea, A.C. (2011). Mercury cycling and sequestration in salt marshes sediments: An ecosystem service provided by *Juncus maritimus* and *Scirpus maritimus*. *Environ. Pollut.* 159(7):1869–1876.

153. Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G. (2001). DNA extraction from soils: old bias for new microbial diversity analyze methods. *Appl Environ. Microbiol*. 67:2354–2359.

154. Marsili-Libelli, S., Checchi, N. (2005). Identification of dynamic models for horizontal subsurface constructed wetlands. *Ecol. Model.* 187:201–218.

155. Mashauri, D.A., Kayombo, S. (2002). Application of the two coupled models for water quality management: Facultative pond cum constructed wetland models. *Phys. Chem. Earth.* 27:773–781.

156. Masseret, E. Sukenik, A. (2008). Amplified rDNA Restriction Analysis (ARDRA) for monitoring of potentially toxic cyanobacteria in water samples. *Isr. J. Plant Sci.* 56(1-2): 75-82.

157. Matamoros, V., Puigagut, J., Garcia, J., Bayona, J.M. (2007). Behaviour of selected priority organic pollutants in horizontal subsurface flow constructed wetlands: A preliminary screening. *Chemosphere*. 69(9):1374-1380.

158. Mateos-Naranjo, E., Redondo-Go´ mez, S., Cambrolle, J., Luque, T., Figueroa, M. E. (2008). Growth and photosynthetic responses to zinc stress of an invasive cordgrass, *Spartina densiflora*. *Plant Biol.* 10:754–762.

159. Matheson, F.E., Nguyen, M.L., Cooper, A.B., Burt, T.P., Bull, D.C. (2002). Fate of 15N-nitrate in unplanted, planted and harvested riparian wetland soil microcosms. *Ecol. Eng.* 19:249-264.

160. Mayo, A.W., Mutamba, J. (2005). Modelling nitrogen removal in a coupled HRP and unplanted horizontal flow subsurface gravel bed constructed wetland. *Phys. Chem. Earth* 30:673–679.

161. Mbuligwe, S.E. (2005). Comparative treatment of dye rich wastewater in engineered wetland system (EWS's) vegetated with different plants. *Water Res.* 39:271-280.

162. McAuliffe, L., Ellis, R.J., Lawes, J.R., Ayling, R.D., Nicholas, R.A. (2005). 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma species*. *J. Med. Microbiol.* 54(8):731-9.

163. McGarvey, J.A., Miller, W.G., Sanchez, S., Stanker, L., (2004). Identification of bacteria populations in diary wastewaters by use of 16S rDNA gene sequences and other genetic markers. *Appl. Environ. Microbiol.* 70 :4267–4275.

164. Mendal, M., Higa, A. (1970). Calcium chloride method for bacterial transformation. *J. Mol. Biol*. 53:159-162.

165. Mendelssohn, I. A., Oianxin, L. (2003). The development of bioremediation for oil spill cleanup in coastal wetlands. U.S. Dept. of the Interior, Minerals Management Service, Gulf of Mexico OCS Region, New Orleans, LA. OCS Study MMS (2002)-048. 84 pp.

166. Miambi, E., Guyot, J.P., Ampe, F. (2003). Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 82:111–120.

167.Miguel, A.S., Ravanel, P., Raveton, M. (2013). A comparative study on the uptake and translocation of organochlorines by *Phragmites australis*. *J. Hazard. Mater.* (244–245)60–69.

168.Miller, D. N., Bryant, J. E., Madsen, E. L., Ghiorse, W. C. (1999). Evaluation and Optimization of DNA Extraction and Purification Procedures for Soil and Sediment Samples. *Appl Environ Microbiol.* 65(11): 4715–4724.

170.Mishra, V., Pandey, S.D. (2005). Immobilization of heavy metals in contaminated soil using non-humous soil and hydroxy apetite. *Bull. Environ. Contam. Toxicol.* 74:725-731.

171.Moir, S.E., Svoboda, I., Sym, G., Clark, J., Mcgechan, M.B., Castle K. (2005). An experimental plant for testing methods of treating dilute farm effluents and dirty water. *Biosyst. Eng.* 90:349-355.

172.Montgomery, D.C. (1991). Design and analysis of experiments. New York: Wiley.

173.Moore, M.T., Rodgers Jr, J.H., Cooper, C.M., Smith Jr, S. (2000). Constructed wetland for mitigation of atrazine associated agricultural runoff. *Environ. Pollut.* 110(3):393-399.

174.Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rDNA. *Appl Environ Microbiol*. 59(3):695-700.

175.Muyzer. G., Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek.* 73: 127–141.

176.Namkoong, W., Hwang, E.Y., Park, J.S., Choi, J.Y. (2002). Bioremediation of diesel-contaminated soil with composting. *Environ. Pollut.* 119:23-31.

177. Nicomrat, D., Dick, W.A., Tuovinen, O.H. (2006) Microbial populations identified by fluorescence in situ hybridization in a constructed wetland treating acid coal mine drainage. *J. Environ. Qual.* 35:1329-1337.

178.Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science.* 264(5157):382-8.

179. Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A. (1954). Estimation of available phosphorous by extraction with sodium bicarbonate. USDA Circ., 939**.**

180. O’Niell, Walter L., Nzengung, V.A. (2004). In-Situ Bioremediation and Phytoremediation of Contaminated Soils and Water: Three Case Studies. *J. Environ. Eng. Manage., JEEAM.* 4(30):49-54.

181.O’Sullivan, A.D., Murray, A.D., Otte, M.L. (2001). Bioremediation of alkaline mine effluent using treatment wetlands. In: Land Reclamation- a different approach: Proceedings of 18^{th} annual meeting of the American Society for Surface Mining and Reclamation, (Ed.) Richard Vincent *et al.*, Albuquerque, NM, USA, June 3-7, pp. 292-293.

182.Ovreas, L., Forney, L., Daae, F. L., Torsvik, V. (1997). Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR amplified gene fragments coding for 16S rDNA. *Appl. Environ. Microbiol.* 63:3367-3373.

183. Parkes, R.J., Cragg, B.A., Wellsbury, P. (2000). Recent studies on bacterial populations and processes in subseafloor sediments: a review. *Hydrogeol J* 8:11–28.

184.Paul, D., Pandey, G., Pandey, J., Jain, R.K. (2005). Accessing microbial diversity for bioremediation and environmental restoration. *Trends Biotechnol.* 3(23):135-142.

185.Pelz, O., Tesar, M., Wittich, R.M., Moore, E.R.B., Timmis, K.N., Abraham, W.R. (1999). Towards elucidation of microbial community metabolic pathways; unraveling the network of carbon sharing in a pollutant degrading bacterial consortium by immunocapture and isotopic ratio mass spectrometry. *Environ. Microbiol.* 1:167-174.

186.Pendias, H., Pendias, K. (1989). Trace elements in soil and plants. Bota Racon, FL, CRC.

187.Pilon-Smits, E. (2005). Phytoremediation. *Annu. Rev. Plant Biol.* 56:15-39.

188. Piper, C.S., (1960). Soil and Plant Analysis. Hans Publishers, Bombay.

189.Pivetz, B.E. (2001). Phytoremediation of Contaminated Soil and Ground Water at Hazardous Waste sites, United States Environmental Protection Agency, EPA/540/S-01/500.

190.Polprasert, C., Khatiwada, N.R., Bhurtel, J. (1998). A model for organic matter removal in free water surface constructed wetlands. In: Proceedings 19th IAWQ Biennial International Conference, Vancouver, Canada, 21-26 June 1998, 354-361.

191. Polprasert, C., Sawaittayothin, V. (2006) Nitrogen mass balance and microbial analysis of constructed wetlands treating municipal landfill leachate. *Water Sci. Technol.* 54:147-154.

192. Polubesova, T., Eldad, S., Chefetz, B. (2010). Adsorption and oxidative transformation of phenolic acids by Fe(III)-Montmorillonite. *Environ. Sci. Technol.* 44:4203-4209.

193. Prasad, M.N.V., Freitas, H. (2003). Metal hyperaccumulation in plants- Biodiversity prospecting for phytoremediation technology. *Electron. J. Biotechnol*. 6(3):285-321.

194. Prochaska, C.A., Zouboulis, A.I. (2006). Removal of phosphates by pilot vertical-flow constructed wetlands using a mixture of sand and dolomite as substrate. *Ecol. Eng.* 26:293–303.

195. Prochaska, C.A., Zouboulis, A.I., Eskridge, K.M. (2007). Performance of pilot-scale vertical-flow constructed wetlands, as affected by season, substrate, hydraulic load and frequency of application of simulated urban sewage. *Ecol. Eng.* 31:57-66.

196. Rai, P.K. (2008). Heavy metal pollution in aquatic ecosystems and its phytoremediation using wetland plants: An ecosustainable approach. *Int. J. Phytoremed.* 10(2):133-160.

197. Ramadan, M.A., Tayeb, O.M., Alexander, M. (1990). Inoculation size as a factor limiting success of inoculation for biodegradation. *Appl. Environ. Microbiol.* 56:1392-1396.

198. Ramsar Convention on Wetlands. (1997). Convention on Wetlands of International Importance Especially as Waterfowl Habitat, Ramsar, Iran. Available at: http://www.ramsar.org/[Accessed 16 August (2007)].

199. Ravenschlag, K., Sahm, K., Knoblauch, C., J/rgensen, B.B., Amann, R. (2000) Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments. *Appl Environ Microbiol* 66:3592–3602

200. Redondo-Gómeza, S., Mateos-Naranjoa, E., Vecino-Buenoa, I., Feldmanb, S.R. (2011). Accumulation and tolerance characteristics of chromium in a cordgrass Cr-hyperaccumulator, Spartina argentinensis. *J. Hazard. Mater.* 185(2–3):862–869.

201. Reed, S.C., Middlebrooks, E.J., Crites, R.W. (1995). Natural Systems for Waste Management and Treatment. McGraw-Hill Book Co., New York, NY, Inc. USA.

202. Reinartz, J.A., Warne, E.L. (1993). Development of vegetation in small created wetlands in south eastern Wisconsin. *Wetlands*. 13(3):153-164.

203.Ren, J., Fu, Z.W., Tao, L., Yang, Q. (2011). Accumulation Effect of *Phragmites australis,* Acorns calamus and Scirpus tabernaemontani on Zn super(2+) in Water Body. *Wetland Sci.* 9(9):322-327.

204.Rice, P.J., Anderson, T.A., Anhalt, J.C., Coats, J.R. (1997). Phytoremediation of surface water and soil contaminated with aircraft deicing agents. Posters Shawnee A, P-53, Clemson University, Pendleton, SC 29670.

205.Rittman, B.E., Whiteman, R. (1994). Bioaugmentation: a coming of age.*Water Qual. Int.* 1:12-16.

206.Roane, T. M., Josephson, K. L., Pepper, I. L. (2001). Dual-Bioaugmentation Strategy To Enhance Remediation of Cocontaminated Soil. *Appl**.** Environ**.** Microbiol**.*** 3208–3215.

207.Ronchel, M.C., Ramos, J.L. (2001). Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl. Environ. Microbiol.* 67:2649-2656.

208. Rousseau, D.P.L. (2005). Performance of constructed treatment wetlands: Model based evaluation and impact of operation and maintenance. Ph.D. diss., Ghent Univ., Belgium.

209. Rousseau D.P., Vanrolleghem P.A., De Pauw N., (2004), Model-based design of horizontal subsurface flow constructed treatment wetlands: a review. *Water Res.* 38(6):1484-1493.

210.Runes, H.B., Jenkins, J.J., Bottomley, P.J. (2001). Atrazine degradation by bioaugmented sediment from constructed wetlands. *Appl Microbiol Biotechnol.* 57(3):427-32.

211.Salla, V., Hardaway, C.J., Sneddon, J. (2011). Preliminary investigation of Spartina alterniflora for phytoextraction of selected heavy metals in soils from Southwest Louisiana. *Microchem. J.* 97:207–212.

212.Salt, D.E., Smith, R.D., Raskin, I. (1998). Phytoremediation. *Annu. Rev. Plant Physiol.* 49:643-668.

213.Santos-Díaz, M.S., Barrón-Cruz, M.C., Torre, M.C.A. (2007). Induction of in vitro roots cultures of *Typha latifolia* and *Scirpus americanus* and study of their capacity to remove heavy metals. *Electron. J. Biotechnol.* 10(3):417-424.

214.Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer KH, Wagner M. (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Sys.t Appl. Microbiol.* 23: 93-106.

215.Scholz M. (2006). Wetland Systems to Control Urban Runoff. Elsevier, Amsterdam, The Netherlands.

216.Schramm, A., Santegoeds, C., Nielsen, H., Ploug, H., Wagner, Pribyl, M., Wanner, J., Amann, R., de Beer, D. (1999). On the occurrence of anoxic micro- niches, denitrification, and sulfate reduction in aerated activated sludge. *Appl. Environ. Microbiol*. 65:4189–96.

217. Schwager, A., Boller, M. (1997). Transport phenomena in intermittent filters. *Water Sci. Technol.* 35(6):13–20.

218.Shange, R., Haugabrooks, E., Ankumah, R., Ibekwe, A.M., Smith, R.C., Dowd, S. (2013). Assessing the Diversity and Composition of Bacterial Communities across a Wetland, Transition, Upland Gradient in Macon County Alabama. *Diversity. 5:*461-478.

219.Shaoa, Y., Peiab, H., Huab, W. (2013). Nitrogen removal by bioaugmentation in constructed wetlands for rural domestic wastewater in autumn. *Desalin. Water. Treat.* 51:34-36.

220.Sheffield, V.C., Cox, D.R., Lerman, L.S., Myers, R.M. (1989). Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci U S A.* 86(1):232-6.

221.Sheoran, A.S., Sheoran, V. (2006). Heavy metal removal mechanism of acid mine drainage in wetlands: A critical review. *Minerals Eng.* 19:105–116.

222. Silyn-Roberts, G., Lewis, G. (2001). In situ analysis of Nitrosomonas spp. in wastewater treatment wetland biofilms. *Water Res.* 35:2731-2739.

223. Šimůnek, J., Šejna, M., van Genuchten, M.Th. (2006a). The HYDRUS software package for simulating the two- and three-dimensional movement of water, heat, and multiple solutes in variably-saturated media: Technical manual. Version 1.0. PC-Progress, Prague, Czech Republic.

224. Šimůnek, J., Šejna, M., van Genuchten, M.Th. (2006b). The HYDRUS software package for simulating the two- and three-dimensional movement of water, heat, and

multiple solutes in variably-saturated media: User manual. Version 1.0. PC-Progress, Prague, Czech Republic.

225. Smalla, K., Oros-Sichler, M., Milling, A., Heuer, H., Baumgarte, S., Becker, R., Neuber, G., Kropf, S., Ulrich, A., Tebbe, C.C. (2007). Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rDNA gene fragments: do the different methods provide similar results? *J Microbiol Methods.* 69(3):470-9.

226. Smith, R.A.H., Bradshaw, A.D. (1972). Stabilization of toxic mine wastes by the use of tolerant plant populations. Transactions of the Institution of Mining and Metallurgy, Section A. 81, 230– 7.

227. Sohair, I., Abou-Elela, Hella, M.S. (2012). Municipal wastewater treatment using vertical flow constructed wetlands planted with *Canna, Phragmites* and *Cyprus*. *Ecol. Eng.* 47:209–213.

228. Solano, M.L., Soriano, P., Ciria, M.P. (2003). Constructed wetlands as a sustainable solution for wastewater treatment in small villages. *Biosyst. Eng.* 87:109–118.

229. Southichak, B., Nakano, K., Nomura, M., Chiba, N., Nishimura, O. (2006). *Phragmites australis*: a novel biosorbent for the removal of heavy metals from aqueous solution. *Water Res.* 40:2295–2302.

230. Sriprang, R., Hayashi, M., Yamashita, M., Ono, H., Saeki, K., Murooka, Y. (2002). A novel bioremediation system for heavy metals using the symbiosis between leguminous plant and genetically engineered rhizobia. *J. Biotechnol.* 99(3):279-293.

231. Stottmeister, U., Wieβner, A., Kuschk, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A., Moormann H. (2003). Effect of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnol. Adv.* 22:93-117.

232. Sundberg, C., Tonderski, K., Lindgren, P., Novais, J. (2007) Potential nitrification and denitrification and the corresponding composition of the bacterial communities in a compact constructed wetland treating landfill leachates. *Water Sci. Technol.*56:159-166.

233. Suzuki, M.T., Giovannoni, S.J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rDNA genes by PCR. *Appl. Environ. Microbiol.* 62:625-630.

234.Tadashi, T., Yusuke, S., Daisuke; I., Kazunari, S., Young-Cheol, C., Shintaro, K., Michihiko, K. (2009). Biodegradation of bisphenol A and bisphenol F in the rhizosphere sediment of *Phragmites australis. J. Biosci. Bioeng.* 108(2):147-150.

235.Tanner, C.C. (2000) Plants as ecosystem engineers in subsurface-flow treatment wetlands. In 7th Int. Conf. on Wetland Systems for Water Pollution Control; University of Florida, pp. 13-22.

236.Tartakovsky, B., Levesque, M.-J., Dumortier, R., Beaudet, R., Guiot, S. R. (1999). Biodegradation of Pentachlorophenol in a Continuous Anaerobic Reactor Augmented with Desulfitobacterium frappieriPCP-1. *Appl. Environ. Microbiol*. 65(10): 4357-4362.

237.Thomas, M., Beaucbamp, Y., Youssef, Y.A., Masounave, I. (1997). An experimental design for surface roughness and built-up edge formation in lathe dry turning. *J. Environ. Qual.* 2:167–180.

238. Toscano, A., Langergraber, G., Consolia, S., Cirelli, G.L. (2009). Modelling pollutant removal in a pilot-scale two-stage subsurface flow constructed wetlands. *Ecol. Eng.* 35(2):281-289.

239.Truu, J., Nurk, K., Juhanson, J., Mander, U. (2005). Variation of microbiological parameters within planted soil filter for domestic wastewater treatment. *J. Environ. Sci. Health A Tox.* 40:1191-1200.

240.Ueno, A., Hasanuzzaman, M., Yumoyo, L., Okuyama, H. (2006). Verification of Degradation of n-Alkanes in Diesel Oil by *Pseudomonas aeruginosa* Strain WatG in Soil Microcosms. *Curr. Microbiol.* 2006:52, 182-185.

241.Vangronsveld, J., Van Assche, F., Clijsters, H.(1995). Reclamation of a bare industrial area contaminated by non-ferrous metals: in situ metal immobilization and revegetation. *Environ. Pollut.* 87:51 – 59.

242.Vinton, M.A., Burke, I.C. (1995). Interactions between individual plant species and soil nutrient status in shortgrass steppe. *Ecology*. 76:1116–33.

243.Vogel, T.M. (1996). Bioaugmentation as a soil bioremediation approach. *Curr. Opin. Biotechnol.* 7:311-316.

244.von Wintzingerode, F., Göbel, U.B., Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rDNA analysis. *FEMS Microbiol Rev*. 21:213–229.

245.Vymazal, J. (2000). Constructed wetlands for wastewater treatment in the Czech Republic. In 7th Int. Conf. on Wetland Systems for Water Pollution Control; University of Florida, pp. 955-961.

246. Vymazal, J. (2002). The use of sub-surface constructed wetlands for wastewater treatment in the Czech Republic: 10 years experience. *Ecol. Eng.*18:632–646.

247.Vymazal, J. (2005). Horizontal sub-surface flow and hybrid constructed wetland systems for wastewater treatment, *Ecol. Eng*. 25:478–490.

248.Vymazal, J., Kropfelova, L., vehla, Jaroslav S., Chrastny, V., Tıchova, Jana S. (2009). Trace elements in *Phragmites australis* growing in constructed wetlands for treatment of municipal wastewater. *Ecol. Eng*. 3 5:303–309.

249.Vymazal, J., Kröpfelováa, L. (2011). A three-stage experimental constructed wetland for treatment of domestic sewage: First 2 years of operation. *Ecol. Eng.* 37, 90–98.

250. Walkley, A., Black, C.A., (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Sci*. 37:29-38.

251.Wakelin, S.A., Colloff, M.J., Kookana, R.S. (2008). Effect of wastewater treatment plant effluent on microbial function and community structure in the sediment of a freshwater stream with variable season flow. *Appl. Environ**.** Microbiol**.*** 74(9):2659-2668.

252.Walter, J., Tannock, G.W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D.M., Munro, K., Alatossava, T. (2000). Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol.* 66(1):297-303.

253.Wand, H., Kuschk, P., Soltmann, U., Stottmeister, U. (2002). Enhanced Removal of Xenobiotics by Helophytes. *Acta Biotechnol.* 22:175 – 181.

254.Wand, H., Vacca, G., Kuschk, P., Kruger, M., Kastner, M. (2006). Removal of bacteria by filtration in planted and non-planted sand columns. *Water Res*. 41(1):159-167.

255. Wang, F.Y., Guo, Z.H., Miao, X.F., Xiao, X.Y. (2011). Tolerance and Accumulation Characteristics of *Typha* orientalis Presl for As, Cd and Pb in Heavily Contaminated Soils. *J. Agro-Environ. Sci.* 30(10):1966-1971.
256. Wang, H., Jia, Y. (2009). Bioaccumulation of heavy metals by *Phragmites australis* cultivated in synthesized substrates. *J Environ Sci.* 21(10):1409-14.
257. Wang, M., Chen, Jia-K., Bo, L.I. (2007). Characterization of Bacterial Community Structure and Diversity in Rhizosphere Soils of Three Plants in Rapidly Changing Salt Marshes Using 16S rDNA. *Pedosphere.* 17(5): 545–556.
258. Wang, W., Gorsuch, J.W., Hughes, J.S., (1997). Plants for Environmental Studies. CRC Press, New York, 563 pp.
259. Wang, X., Yu, X., Bartha, R. (1990). Effect of bioremediation on polycyclic aromatic hydrocarbon residues in soil. *Environ. Sci. Technol.* 24:1086-1089.
260. Wang, Y., Sheng, H.F., He, Y., Wu, J.Y., Jiang, Y.X., Tam, N.F., Zhou, H.W. (2012). Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl. Environ. Microbiol.* 78(23):8264-71.
261. Weber, K.P., Gehder, M., Legge, R.L. (2008). Assessment of changes in the microbial community of constructed wetland mesocosms in response to acid mine drainage exposure. *Water Res.* 42:180–188.
262. Werner, T.M., Kadlec, R.H. (2000). Wetland residence time modeling. *Ecol. Eng.* 15:77–90.
263. Widada, J., Nojiri, H., Omori, T. (2002). Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. *Appl. Environ. Microbiol.* 60:45-59.
264. Williams, J., Bhagat, M., May, E., Ford, M., Butler, J. (2000). Mineralisation and pathogen removal in gravel bed hydroponic constructed wetlands for wastewater treatment, *Water Sci. Technol.* 32:49-58.
265. Williams, S. T., Davies, F. L. (1965). Use of Antibiotics for Selective Isolation and Enumeration of Actinomycetes in Soil. Hartley Botanical Laboratories, Univer8ity of Liverpool. *J. Microbiol.* 38:251-261.

266.Wilson, P.C., Whitwell, T., Klaine, S.J. (2000). Metalaxyl and Simazine toxicity and uptake by *Typha* latifolia. *Archives Environ. Contam. Toxicol.* 39(3):282-288.

267.Windham, L., Weis, J.S., Weis, P. (2004). Metal dynamics of plant litter of *Spartina alterniflora* and *Phragmites australis* in metal-contaminated salt marshes. Part 1: Patterns of decomposition and metal uptake. *Environ Toxicol Chem.* 23(6):1520-8.

268.Wirsel, Stefan, G.R., Leibinger, W., Ernst, M., Mendgen, K. (2001). Genetic diversity of fungi closely associated with common reed. *New Phytol.* 149: 589-598.

269.Wu, H., Zhang, J., Li, P., Zhang, J., Xiec, H., Zhang, B. (2011). Nutrient removal in constructed microcosm wetlands for treating polluted river water in northern China, *Ecol. Eng.* 37:560–568.

270.Wu, L. (1990). Colonization and establishment of plants in contaminated sites. In: Heavy Metal Tolerance in Plants: Evolutionary Aspects, pp. 269–284. (Shaw, A.J., Ed.). Boca Raton, FL, CRC.

271.Wu, L., Guo, X. (2002). Selenium accumulation in submerged aquatic macrophytes *Potamogeton pectinatus* L. and *Ruppia maritima* L. from water with elevated chloride and sulfate salinity. *Ecotoxicol. Environ. Saf.* 51:22-27.

272.Wu, X., Scholz, M. Rao, L. (2008). Constructed Wetlands treating urban runoff contaminated with nitrogen, 978-1-4244-1748-3/08. 2008 IEEE.

273.Wynn, T.M. Liehr, S.K. (2001). Development of a constructed subsurface-flow wetland simulation model. *Ecol. Eng.* 16:519–536.

274.Xu, S., Jaffe, P.R. (2006). Effect of plant on the removal of hexavalent chromium in wetland sediment. *J. Environ. Qual*. 35:334-341.

275.Xu, X. F., Tian, H. Q., Pan, Z. J., Thomas, C. R. (2011). “Modeling ecosystem responses to prescribed fires in a phosphorus-enriched Everglades wetland: II. Phosphorus dynamics and community shift in response to hydrological and seasonal scenarios,” *Ecol. Mod.* 222: 3942-3956.

276.Xua, W., Shia, W., Yanc, F., Zhang, B., Liang, L. (2011). Mechanisms of cadmium detoxification in cattail (*Typha* angustifolia L.). *Aquat. Bot.* 94:37–43.

277.Ye, Z.H., Baker, A.J.M., Wong, M.H., Willis, A.J. (1997). Zinc, lead and cadmium tolerance, uptake and accumulation by the common reed, *Phragmites australis* (Cav) Trin Ex. Steudel. *Ann. Bot.* 80:363–370.

278.Ye, Z.H., Baker, A.J.M., Wong, M.H., Willis, A.J. (2003). Copper tolerance, uptake and accumulation by *Phragmites australis*. *Chemosphere.* 50:795–800.

279. Ye, Z.H., Baker, A.J.M., Wong, M.H., Willis, A.J (1998). Zinc, Lead and Cadmium Tolerance, Uptake and Accumulation by the Common Reed, *Phragmites australis* (Cav.) Trin. ex Steudel. *Ann. Bot.* 80: 360-363

280.Yeh, T.Y., Chou, C.C., Pan, C.T. (2009). Heavy metal removal within pilot-scale constructed wetlands receiving river water contaminated by confined swine operations. *Desalination*. 249:368–373.

281. Yu, Y.H., Feng, W.S., Shen, Y.F. (2005). Use of microbial community to evaluate performance of a wetland system in treating Pb/ Zn mine drainage. *Environ. Manage.* 36:842-848.

282.Yu, Z., Mohn, W. (2001). Bacterial diversity and community structure in anaerated lagoon revealed by ribosomal intergenic spacer analysis and 16SrDNA. *Appl. Environ. Microbiol.* 67:1565–74.

283.Yuka, U.N., Mohammed, H.A., Aina, E. (2012). Preliminary Studies on the Phytoremediation Potential of *Phragmites* karka (Retz.) in Asa River. *J. Fish. Aquat. Sci.* 8 (1):87-93.

284.Zhang, Z., Rengel, Z., Chang, H., Meney, K., Pantelic, L., Tomanovic, R. (2012). Phytoremediation potential of Juncus subsecundus in soils contaminated with cadmium and polynuclear aromatic hydrocarbons (PAHs). *Geoderma* 175–176:1–8.

285. Zinatizadeh, A.A.L., Mohamed, A.R., Abdullah, A.Z., Mashitah, M.D., Isa, M.H., Najafpour, G. (2006). Process modeling and analysis of palm oil mill effluent treatment in an up-flow anaerobic sludge fixed film bioreactor using response surface methodology (RSM), *Water Res.* 40:3193-3208.

286. Zurita, F., De Anda, J., Belmont, M.A. (2006). Performance of laboratory-scale wetlands planted with tropical ornamental plants to treat domestic wastewater. *Water Qual. Res. J. Can.* 41:410–417.