MICROBIAL POPULATION IN MICROCOSMS AND NUTRIENT REMOVAL FROM CONSTRUCTED WETLANDS

BY

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"In the Name of Allah, the most

Beneficent, the most Merciful"

This thesis is dedicated to my maa and paa, my siblings who have meant and continue to mean so much to me, who have been so close to me that I found them with me whenever I needed

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Allah Most High has said: ***If you would count the blessings of Allah you would not be able to reckon them,*** *[14:34] I fully realize the blessings upon me by the most gracious and divine force of all forces that enabled me, and gave me sense and insight to accomplish this research objectively and successfully.*

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S. NIDA BATOOL

Contents

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
CWs	Constructed wetlands
HFCW	Horizontal flow constructed wetland
HSSFCWs	Horizontal subsurface flow constructed wetlands
MGD	Million gallon per day
NCBI	National Center for Biotechnology Information
OM	Organic Matter
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
rRNA	Ribosomal Ribonucleic Acid
SF	Surface flow
SFCWs	Surface flow constructed wetlands
SS	Suspended solids
SSF	Sub-surface flow
TDS	Total dissolved solids
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine I
TN	Total nitrogen
TP	Total phosphorous
VSSFCWs	Vertical subsurface flow constructed wetlands

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ABSTRACT

Water scarcity is an issue of global concern due to discharge of untreated effluent and many other reasons. For the treatment of wastewater, a cost effective and ecologically beneficial treatment system should be used like constructed wetlands especially in developing countries. Constructed wetlands are engineered system in which plants are grown which remove pollutants naturally. The aim of this study was to analyze the type of microbial population of wastewater present in different layers of constructed wetlands and to observe plant growth and nutrient removal through these plants. Water quality parameters were analyzed by using standard methods. Bacterial isolates were isolated and identified through sequencing analysis. Plant growth was observed and nutrient removal was calculated. Maximum removal of TSS and COD was observed at high temperature as removal efficiency for COD was 52 in pilot scale and 71.9% in lab scale while in surface and benthic layers 71% and 74 % removal was noted respectively. TSS removal in pilot and lab scale was 72.8% and 79.4% respectively. At different layers almost same amount of removal was observed. Fourteen isolates were retrieved out of which 10 isolates belongs to proteobacteria group while remaining 4 belongs to firmicutes. Observed growth rate of *Centella asiatica* and *Pistia stratiotes* was higher in summer season in higher temperatures. Similarly, high nutrient removal observed in summer. Orthophosphate removal observed was 27 and 43% in pilot and lab scale respectively. Removal of nitrates and ammonia was high in lab scale i.e. 84.5 and 79.5% respectively.

Chapter 1

INTRODUCTION

1.1 Background

Water is one of the prime elements on earth. About two third of the earth's surface is covered by water and about 75% of the human body comprised of it but only 2-3% of this water is fresh water resources. Life on earth depends on this irreplaceable and unique element. Water availability is increasingly becoming an issue of global concern as the population of the world increased. Similarly, due to increase in population along with other problem the quality of water is deteriorated day by day. Many countries of the world are facing water scarcity due to the discharge of untreated water into fresh water bodies which also caused depletion of water reserves. This scarcity of fresh water also raises the competition between different countries of the world (Kivasi, 2001).

Water is mainly contaminated through point source contaminants (e.g. landfills leaking septic tanks and accidental spills) and non-point source contaminants (e.g. infiltration of pesticides and fertilizers). Major problems that have affected the water quality of freshwater bodies are liquid waste sources including untreated or poorly treated domestic sewage and industrial wastewater that are discharged into these water bodies. Municipal sewage and industrial waste contained toxic and hazardous organic and inorganic substances that are readily biodegradable and harmful organisms or different pathogen in

them. These substances and pathogens were disease-causing agents which pose threats to the health of humans, wildlife and to aquatic life (Al Radif, 1999).

Increasingly stringent regulation were being implemented by the government bodies of different countries on the discharge of polluted water with a prime focus on reduction of waste to strive against the burden on an aquatic environment (Chan *et al*., 2009).

1.2 Wastewater and Its Composition

When the quality of water has been adversely affected by some anthropogenic activities and also contains liquid waste from industries, domestic residences, agricultural runoff and commercial properties then this water is called as wastewater and can encompass a wide range of contaminants.

From this definition of wastewater, we came to know different types of wastewater like domestic wastewater, wastewater from institutions, industrial wastewater, and infiltration into sewers, storm water, leachate and septic tank wastewater. Typically, the wastewater from domestic, municipal or industrial liquid waste is referred as sewage which was usually disposed of via pipe or sewer system. So the part of wastewater contaminated from wastewater like from laundry, personal washing, cleaning of kitchen utensils, food preparation, and the human body waste (feces urine and feces) is known as sewage. This untreated sewage mainly comprises of oils and greases; solids (including organic matter); nutrients (nitrogen and phosphorus); pathogens (including bacteria, viruses and protozoa); heavy metals (including mercury, cadmium, lead, chromium, copper); helminths (intestinal worms and worm-like parasites); and many toxic chemicals including PCBs, PAHs, dioxins, furans, pesticides, phenols and chlorinated organics and

runoff from streets, parking lots and roofs. So the discharge of untreated sewage is a great threat to humans and nature, especially in developing countries. This untreated wastewater transmits water-borne diseases and also causes eutrophication of surface waters and the condition is getting even worse without any proper sanitation system (Konnerup *et al*., 2009).

Treatment of wastewater is significant for both to public health and to the ecological system as a whole. High-quality effluent water is crucial for reducing the damage caused by releasing untreated wastewater into different water bodies. Also, the quality of the treated wastewater is important to countries that have limited water resources and use treated wastewater for irrigation. Although natural wetlands exist around the world, the use of constructed or artificial ones, built for the improvement of water quality, is a concept that has only been adopted in the last five decades (Kruh *et al.*, 2009).

1.3 Treatment Systems

The process in which the wastewater is converted or transformed into the form that can be no longer harmful to the environment and can be discharged into it is known as wastewater treatment. As the wastewater contains a high level of toxins, chemicals and microbial organisms in it so, the main aim of wastewater treatment is to reduce the number of contaminants either organic or inorganic to an acceptable level to discharge back the safe water into the environment. For treatment of wastewater, many different treatment techniques have been developed in the recent years. The reason for developing new techniques for the wastewater treatment was, to improve the quality of treated

effluent and to decrease the amount of wastewater that is produced in different commercial sectors and on a domestic scale.

The two most important ways to treat the wastewater are named as biological treatment system and physical or chemical treatment system. In physical treatment system physical processes and different chemical reactions are used to treat the wastewater. It is mostly used to treat the wastewater from industries, manufacturing firms and factories. For the treatment of domestic and commercial wastewater or sewage biological treatment system are used. Microbial activity and biological matter like activated sludge, is used to treat the sewage. These systems are not practical for widespread application in rural areas or for domestic scale and also are expensive. Furthermore, these systems are inadequate in completing the water and wastewater standards. So for developing countries it is important to select efficient alternative and low cost technologies for wastewater treatment. Constructed wetlands should be an equitable option for this reason, as this system is lower in development cost and requires lower operation and maintenance cost (Zhang *et al*.,2015).

1.4 Modernized Treatment System

For the protection of environment and restoration of water some ecological technologies are used such as constructed wetlands. This type of technology is represented as an innovative and emerging solution for wastewater treatment mostly for the developing countries as it is a cost effective and sustainable treatment system.

Constructed wetlands (CWs) similar to natural wetlands are engineered treatment systems that cover a variety of treatment modules (biological, chemical, and physical processes).

By removing the high levels of contaminants from wastewater including suspended solids, dissolved solids organic compounds, pathogens, and nutrients it's have been successfully used for mitigation of pollutants from the environment. CWs have become an increasingly popular for wastewater treatment due to its beneficial characteristics like high removal efficiency, cost effectiveness and great potential for nutrient and water reuse (Zhang *et al*., 2012).

1.5 Present Study

The present study was conducted to identify the predominant microbial species from the different microcosmic layers of constructed wetland established at National University of Sciences and Technology (NUST). Species were identified through biochemical analysis and gene sequencing of the isolated bacterial strains. Samples were collected both from pilot scale and lab scale setups for physicochemical and nutrient analysis. Seasonal plant growth was also evaluated.

1.6 Objectives

1. Isolation and identification of predominant microbial communities within the different layers of horizontal flow constructed wetland (HFCW)
2. Phylogenetic analysis of microbes through 16s RNA gene sequencing analysis
3. Evaluation of seasonal plant growth and nutrient analysis (phosphates, nitrates, ammonia)

Chapter 2

LITERATURE REVIEW

This chapter covers the literature about constructed wetlands including its type, water quality parameters, aquatic macrophytes used in the process of phytoremediation and microbial community present in constructed wetlands.

2.1 Overview of Constructed Wetland

Widely used conventional treatment plants for the treatment of industrial and domestic wastewater in developed countries are quit efficient but, require too much cost for their construction and trained personnel for their operation as well as are very intensive to maintain. While in developing countries the count of conventional treatment systems is very low, that can be found in working condition. For developing countries, possible alternatives should be used that should provide a sustainable way of wastewater treatment and are effective, reliable and cheap (Mburu *et al*., 2013).

2.1.1 Historical Perspectives

In past naturally occurring wetlands have been used by ancient Egyptians and Chinese for the treatment of liquid effluent, wetlands were formed naturally at any location where there was a frequent discharge of water occurred. First constructed wetland was recorded in 1904 in New South Wales, Australia which was used for the treatment of drainage effluent from residential houses (Price and Probert., 1997).

2.1.2 Constructed Wetland and Its Functioning

Wetlands are the specific type ecosystems which were distinguished by unique water and soil conditions. Water is either present within the root zone or at the surface and soil of wetlands is water saturated or poorly aerated. This system support type of vegetation which was adapted to wet conditions also known as hydrophytes contrarily categorized by the absence of vegetation that is flooding intolerant (Ansola *et al*., 2014). Constructed wetlands are the type of systems which are engineered ecosystems also known as artificial or manmade wetlands. Wetlands have been constructed and designed to operate through natural processes by utilizing wetland soils, vegetation and microbial communities associated with them in a more controlled environment in wastewater treatment (Vymazal, 2007). Demonstrated by Mulling and his coworkers, that significant removal of suspended particles, heavy metals, organic compounds and pathogens was occurred under ordinary operational conditions by CWs. Removal efficiencies for suspended particles and organic compounds were observed between 60 and 95%. While removal efficiencies of nutrients were below 60% even though high efficiencies had also been reported up to 90% (Mulling *et al*., 2013).

In developing countries, these constructed treatment systems were proved as a promising alternative treatment system as these systems have some beneficial economic and ecological characteristics. They have low maintenance and operational costs, low investment cost, as compared to conventional systems are simple to operate and produce high-quality effluent. The removal percentages of COD, SS and pathogens are comparatively high then removal percentages of nutrients (N and P) which are often low and variable (Song *et al*., 2006).

2.2 Types of Constructed Wetlands

Constructed wetlands are broadly classified into two major different types on the basis of level of water column with regard to its substrate bed which are following:

1. Surface flow (SF) wetlands, and
2. Sub-surface flow (SSF) wetlands.

Further, CWs also classified on type of growth shown by macrophytes in the system i.e.

1. Floating macrophytes systems;
2. Submerged macrophytes systems; or
3. Rooted emergent macrophytes systems (Sundaravadivel & Vigneswaran, 2001).

Following figure shows the flow chart of CWs types:

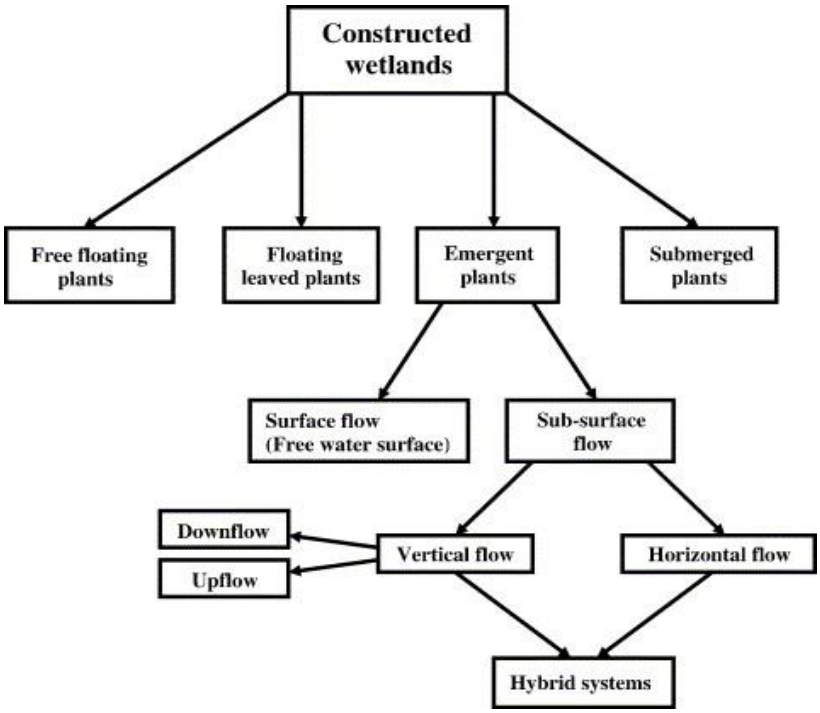

Fig. 2.1 Classification of constructed wetlands for wastewater treatment (Vymazal, 2009)

2.2.1 Surface Flow Constructed Wetlands

In surface flow wetlands shallow basins are present which is filled peat, soil or any other medium for support of macrophytes roots. Water is generally present or moves above the substrate or media while the bottom of the SF wetlands have soil in it for emergent vegetation. These types of wetlands are also known as aerobic wetlands or free water surface wetlands.

SF wetlands are sometimes called free water surface wetlands or aerobic wetlands. In SF wetlands the surface water layer is aerobic while substrate and shallower layer is anaerobic. Vegetation in SF wetlands may tolerate saturated soil conditions continuously which results in anaerobic soils (Halverson, 2004). Surface flow constructed wetlands

(SFCWs), were reported by Regueiro and his colleagues that this type of systems are used as tertiary treatment system and are also useful in the maximum removal of the vast diversity of organic microcontaminants (Regueiro *et al*., 2013).

2.2.2 Sub-Surface Flow Constructed Wetlands

One of the most common system and widespread eco-technology used for wastewater treatment in most of the developed countries is sub surface flow constructed wetlands (SSF) amongst all the natural treatment systems (Puigagut *et al*., 2007). In this type of CWs the flow system of water further divides it into two types i.e. either vertically (vertical subsurface flow constructed wetlands – VSSFCWs) or horizontally (horizontal subsurface flow constructed wetlands – HSSFCWs). The water passes through the porous media usually sand or gravel. SSFCWs design is based on hydraulically insulated filter beds in which may be planted by using different aquatic macrophytes (Truu *et al*., 2009). SSWCWs systems had proved to be efficient in removal of total suspended solids (TSS) and biochemical oxygen demand (BOD) in wastewater treatment processes (Merlin *et al*., 2002). Two types SSF constructed wetlands were defined as:

I. Horizontal flow constructed wetlands (HFCWs)

   The flow of wastewater in this system is slow through the porous media under the bed surface of the vegetated emergent plants; wastewater is fed at the inlet and moves towards the outlet.

II. Vertical flow constructed wetlands (VFCWs)

In this system as compared to HFCWs the wastewater is sporadically fed in large batches, which moves over the surface and infiltrates down through bed towards the drainage network at bottom (Vymazal, 2011).

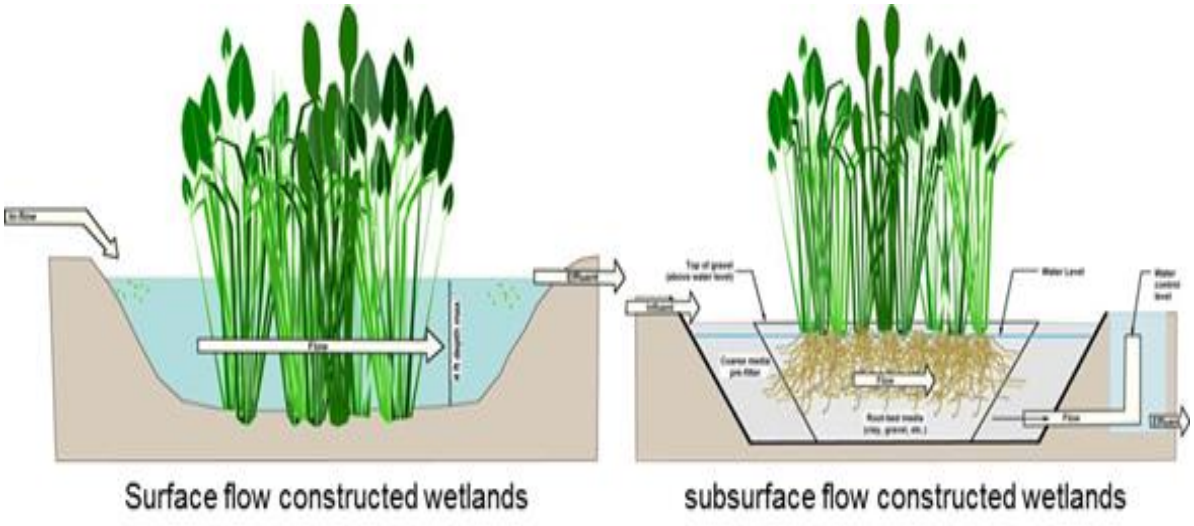

Fig 2.2 Types of constructed wetlands

2.3 Types of Vegetation in Constructed Wetlands

Macrophytes are the larger aquatic plants vegetated in wetlands for the process of phytoremediation. It included vascular plants, aquatic mosses and algae. Macrophytes used in wetlands have some important role in the treatment of wastewater and is an important element of wetland design (Yang *et al*., 2007; Brix, 1997). Presence of these macrophytes differentiates wetlands from other unplanted lagoons or soil filters. Plants that are grown in wetlands should have rich and roots and rhizomes potion, should be tolerant to nutrients and high organic loadings and should have extensive aboveground biomass for nutrient removal and for winter insulation in cold and temperate regions (Vymazal, 2011). Březinová & Vymazal studied the growth pattern of plants in HFCWs

and found that higher aboveground biomass occurred due to high nutrient removal from the system and concluded that vegetated systems may accelerate the nutrient removal if harvested from inflow and outflow zones (Březinová & Vymazal,2015).

Planted CWs showed a positive and substantial effect on removal of pollutants as compared to unplanted wetlands. These plants provide microorganism a vast surface area for their growth and also act as a source for rhizopsphere of reduced oxygen and carbon. The current velocity also decreased due to the presence of the aquatic macrophytes. Species selection of macrophytes foe CWs is based on facts that they should have fast growth rate, have large biomass, well developed below ground organs and tolerant to CWs conditions (Brisson & Chazarenc, 2009).

2.3.1 Typha and Phragmites

In European and Asian region, the most widely vegetated plants in HFCWs are common reed (*Phragmites australis*) and cattails (*Typha spp*.) (Calheiros *et al*., 2007). These plants are emergent macrophytes. Typha spp. is standing rhizomatous perennial plants which have extensive branches with horizontal rhizome system. The basal parts of the leaves of this plant are spongy while the leaves are flat. While the Phragmites spp. is a type of grass which is flood tolerant, perennial and with extensive rhizome system. The rhizomes of this plant penetrate to about 0.6-0.1m of depth. Stems of this plant have hollow internodes and are rigid (Vymazal, 2013). Tanner studied the comparison of eight emergent plants behavior towards the nutrient uptake and showed that phragmites had highest level nutrient uptake especially for nitrogen removal (Tanner, 1996).

2.3.2 Water Lettuce and Pennywort

Water lettuce (*Pistia stratiotes*) and pennywort (*Centella asiatica*) are the floating aquatic plants. These types of macrophytes have high productivity level of leaves, as compared to emergent plants have high nutritive value and they are easy to harvest and to stock (Sooknah & Wilkie, 2004). Water lettuce is a yellowish green leafy plant having a shell like shape (Coleman *et al*., 2001). Gupta and his coworkers reviewed treatment of water using water hyacinth, water lettuce and vetiver Grass and observed that 70% removal of TDS, 93% removal of BOD, 99% removal of fecal coliform, 59% of COD, 70% of nitrate, TP by 33% and 95% of ammonia removal by water lettuce (Gupta et al., 2012). Pennywort is also known as hydrocotyle asiatica Ponni shown that pennywort had removed about 97% BOD, 90% COD and 90% of pollutants (Ponni, 2014).

2.4 Physicochemical Parameters of Constructed Wetlands

Wetland, among different aquatic ecosystems plays a major role in improvement of water quality and in removal of nutrients loadings, not only of other water bodies but also of the water present within them. The water quality parameters include pH, electrical conductivity (EC), dissolved oxygen (DO), total suspended solids (TSS), total dissolved solids (TDS), chemical oxygen demand (COD), different forms of nitrogen, turbidity and temperature (Haidary et al., 2013).

Several studies reported the performance efficiency of CWs in enhancing the water quality and reducing the pollutant present in wastewater (Zedler and Kercher 2005; Shih *et al*., 2013; Mohammadpour *et al*., 2014). CWS have great capacity to reduce and absorb wastewater from municipal and agriculture sectors. Several processes occurred in

wetlands to remove nutrients and pollutants like filtration, settling, absorption and biological uptake to improve the water quality parameters (Mohammadpour *et al*., 2015).

2.4.1 Dissolved Oxygen

The oxygen which dissolves in water is known as dissolved oxygen. The amount of dissolved oxygen may depend on different factors like daily and seasonal patterns, temperature, elevation and salinity. In higher temperature ranges the amount of dissolved oxygen is lower which means that the effect of temperature on dissolved oxygen is negative. In wetlands dissolved oxygen is an important factor that plays a vital role in pollutants removal efficiency and also influences the microbial activities in constructed wetlands. it has been reportedly many times that the degradation of organic matter in CWs is fast if higher dissolved oxygen is present (Liu *et al*., 2016). Higher organic matter decomposition rate was observed in CWs when the oxygen level is high. This shows that dissolved oxygen may be a factor to determine the organic carbon supply in constructed wetlands (Chen *et al*., 2011).

2.4.2 pH

In treatment of wastewater, removal of organic compounds and of heavy metal is important. pH is an important factor in removal of these toxic substances. Water is composed of some ions that may be positively charged (hydrogen ions) or negatively charged (hydroxide ions). Amount of these ions shows the level of pH in water. In acidic conditions high concentration of positive ions is present while in basic conditions negative ions are present. The preferable range of wastewater pH is 6.5 - 9. Types of dissolved substances also change the pH of CWs.

2.4.3 Temperature

Temperature is a physical character of water from which the hotness and coldness is measured. In constructed wetlands temperature is an important parameter as it may also affect other parameters of the system. For sustainable operation of CWs the biggest challenge is seasonal variation from summer to winters. At lower temperatures (winters) apart from inhibition of microbial activities, the major reason for poor performance efficiency of the system is decay of plants present in ponds. Most of macrophytes decay in winter and show better performance in higher temperatures e.g. typha and phragmites spp. (Fan *et al*., 2016). Temperature also affects the intensity of nitrification process in constructed wetlands (Peng *et al*., 2014). Ling studied the effect of seasonal temperature on bacteria and nitrogen removal of constructed wetlands and concluded that at 14 ^{o}C temperature the total nitrogen removal varied within the range of 1.89-3.40 g N/m^{3}day (Wang & Li, 2015).

2.4.4 Chemical Oxygen Demand (COD)

Chemical oxygen demand in wastewater treatment is a useful measure in water quality analysis. It is basically indirectly used to measure the amount of organic pollutants present in wastewater. In COD removal constructed wetlands are very efficient. Removal efficiencies of COD was higher in ponds with emergent plants present in them studied by Ong (Ong *et al*., 2010). It has been reported earlier that by using hybrid CWs the removal efficiency of COD is about 97%. Ayaz with his colleagues observed effluent quality from hybrid CW system and showed the more than 95% of COD removal was occurred (Ayaz *et al*., 2015).

2.4.5 Total Solids

Measure of total dissolved solids and total suspended solids is known as total solids. Suspended solids are the type of solids that have ability to settle down or retained on the filter while dissolved solids pass through it. Suspended solids greatly affect the efficiency of constructed wetlands. It increases the turbidity level of water which is hazardous if present in effluents. Temperature of the water bodies also increases if higher level of suspended solids may present. Suspended solids may also affect the DO levels in water, their higher concentrations decrease the amount of dissolved oxygen.

2.5 Microbial Communities in Wetlands Microcosm

2.5.1 Surface and Benthic Layers in Wetlands

Surface layer of the ponds in HFCWs are the aerobic zone. As roots and rhizomes produces oxygen into substrate. Water when passes through this zone is cleaned due to physical or chemical processes and due to microbial degradation process (Vymazal *et al*., 1998). Benthic layer is basically oxygen deficient layer or anaerobic layer which includes water just above the sediment zone. In this zone nutrient availability is comparatively low as compared to the surface layer and temperature drops up to 2 ^{o}C.

2.5.2 Microbial Communities

In CWs many processes are occurring and these processes are linked with each other like phyto-degradation, sedimentation, sorption, plant uptake and microbial processes. Microcosms of microbial species are the surrounding conditions which are surrounded by these microorganisms. In CWs the microbial community is consisted of indigenous and foreign microorganisms. Indigenous microorganisms are known as autochthonous

while foreign microorganism as allochthones. Autochthonous microbes possess metabolic activities and are adaptive to wetland environment so these microorganisms are helpful in purification procedures while allochthones microbes don't have any operational importance on wetland system environment (Truu *et al*., 2009)

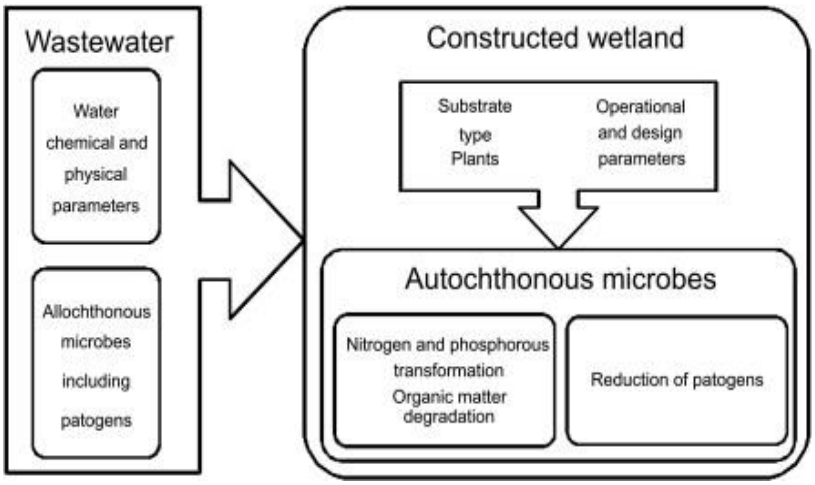

Fig 2.3 External and internal factors which may affect microbial community structure and activity in constructed wetlands

In many studies importance of microbial process in microbiologically mediated processes has been explained many times. Adrados studied the microbial communities in different types of natural treatment systems and showed that bacterial structures of microbes were linked with the oxygen conditions and also on organic matter present in the system. They observed members of actinobacteria were abundant in bio filter unit and also observed some representatives of α, β and δ-Proteobacteria, Acidobacteria and Chloroflex in VFCW and HFCW (Adrados *et al*., 2014).

Wang and He both analyzed the microbial diversity in CWs and observed that proteobacteria is the most abundant phylogenetic class of bacteria which is present in the

systems. Wang *et al* (2016) also observed that vegetation of CWs also affect microbial abundance and type of microbial communities present within the system. While He *et al* (2014) found out that levels of wetlands may affect microbial communities present in them (Wang *et al*., 2016; He *et al*., 2014).

2.5.3 Importance of Microbial Species in CWs

In ecosystem health of CWs microorganisms play an important role as these microbial species helps in the degradation of contaminants present in wetlands (Aroyyo et al., 2013). Many studies are evident that microbial activity in CWs helps in pollutant removal as well as cycling processes of carbon, nitrogen and Sulphur. Numbers of microbial activities are occurring in wetland treatment system including enzymatic activities, nitrification and denitrification etc. (Faulwetter *et al*., 2009). So microorganisms are considered as a major driving factor in treatment processes of CWs. Because they have ability to degrade OM either in aerobic or anaerobic conditions (Stottmeister *et al*., 2003; Chen *et al*., 2015).

2.6 Nutrient Removal in Wetlands

Nutrient removal is one of the major functions of CWs. Nutrient removal efficiencies through constructed wetlands are affected by seasonal temperature and types of plants present in them. Average nitrogen and phosphorous removal through CWs is about 50% (Bateganya *et al*., 2016; Picard *et al*., 2005).

2.6.1 Removal of Nitrogen

The two major techniques through which nitrogen removal is achieved are biological treatment processes and physicochemical treatment techniques. Biological removal of

niterogen in wastewater occurred through aerobic processes of nitrification and denitrification. In SSFCWs the removal processes of nitrogen are dynamic and quite complex. Forms of nitrogen found in waste water are Nitrate-Nitrogen (NO_3-N), Nitrite-Nitrogen (NO_2-N), ammonia (NH_3) etc. (Lee *et al*., 2009).

Keffala and Ghrabi studied the nitrogen removal through domestic wastewater using CWs and concluded that in planted system removal of nitrite-nitrogen is less i.e. 4-13 % while nitrogen ammonia (19-6%) and nitrogen kjeldhal (27-5%) is greater in planted wetland (Keffala & Gharabi, 2005). Annual nitrogen removal cycle from SSFCW is also observed by Kuschk and his fellows and they found that removal efficiencies vary greatly in summer and winters. 53% removal is observed in August while only 11% removal is calculated in January (Kuschk *et al*., 2003).

2.6.2 Removal of Phosphorous

Removal of phosphorous is dependent on type of media used in construction of wetlands. Phosphorous or phosphate removal in SSF constructed wetlands occurred through precipitation or adsorption in filter media used in constructed wetlands. So for maximum removal efficiency of phosphorous it is important that the filter media used have high binding capacity for phosphorous. Saturation of filter media also occurred during phosphorous removal so the solution for the problem is that a separate filter unit having replaceable material in it would be used (Vohla *et al*., 2010). Drizo studied the phosphorous and ammonia removal in HFCW and found that throughout the investigation 98-100% removal was observed both in planted and unplanted treatment systems (Drizo *et al*., 1997).

Chapter 3

MATERIALS AND METHODS

3.1 Study Site

The purpose of this study was to identify the microbial species present in different layers of wetlands and also to evaluate the nutrient removal of waste water through bioremediation plant at pilot-scale and lab scale setup. The pilot scale setup established at NUST H-12 sector having the capacity to treat one sewerage line of 0.1 Million gallons per day (MGD). This sector was initiated as a public residential area in 1991. NUST covers the area of 800 acres and comprises of 20 departments with hostel facilities for both male and female students. According to the recent survey over 15,000 students were enrolled and over 1,280 academic faculty staff is present in NUST.

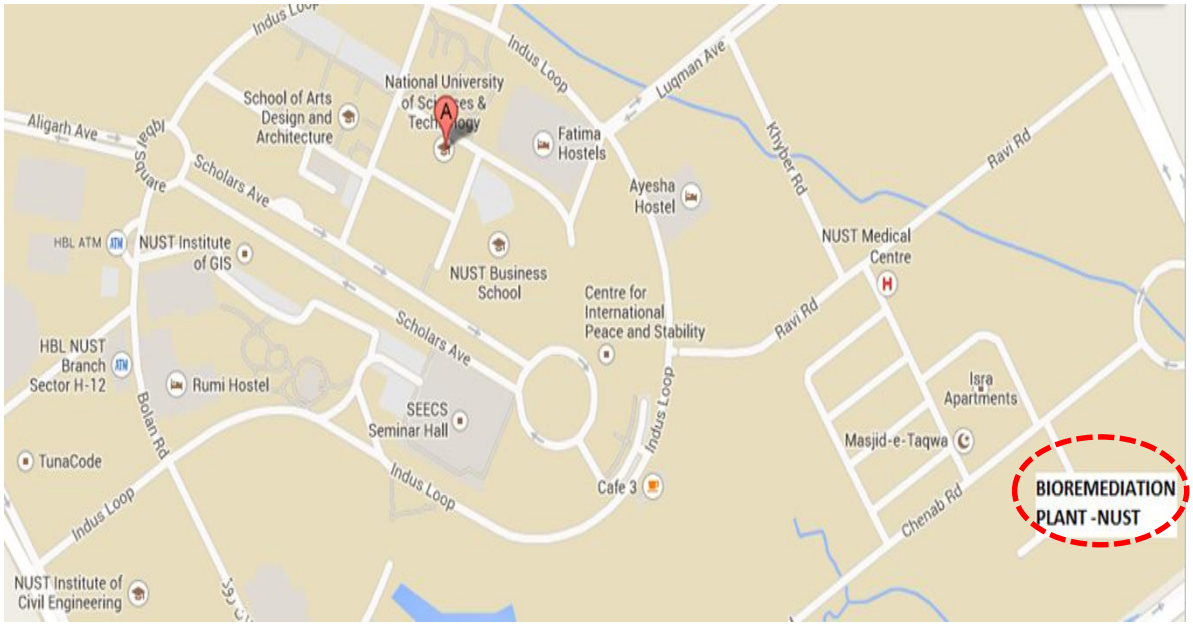

Fig 3.1 Layout of NUST (Encircled point indicates study site)

3.2 Establishment of Pilot and Lab Scale Setup

3.2.1 Pilot Scale Setup

The pilot scale setup of HF constructed wetland was established in NUST sector H-12. It treats the wastewater of NUST campus sewage line, which receives wastewater of faculty residential area, offices, and student hostels. Wastewater from sewage line directed towards the sedimentation tank where sludge is settled down and waste water flows towards eight ponds for treatment. Plants were cultivated in ponds used for bioremediation. All these eight ponds were interconnected and coated with plastic sheets (geo-membrane) to avoid infiltration of waste water into underlying acquifer. Gravels and organic sand were used in the beds of the ponds and are connected in series. The plants used for bioremediation in all ponds are as follow:

- In constructed wetland **1st pond** was inlet pond and was cultivated with *Typha latifola* (Bulrush), provided with support through gravel and soil.
- **2nd pond** contained *Pistia stratiotes* (water lettuce). This plant does not require any additional support for its growth.
- In **3rd pond and 4th pond,** *Centella asiatica* (Pennywort) were cultivated which further sustained by thermocol sheet to support roots and organic soil.
- **5th and 6th pond** contained Duckweed in them.
- *Pistia stratiotes* (water lettuce) was cultivated **in the 7th pond.**
- **The 8th pond** was the outlet to the system with aerators in it.

The treated water from this constructed wetland is used for horticulture purposes.

3.2.2 Lab Scale Setup

To analyze the performance efficiency of wetlands on lab scale this setup was established. Lab scale setup consisted of three units in it i.e. pilot scale replica, parallel scale unit and control unit.

- Pilot scale replica

Replica of pilot scale setup was established in the laboratory to evaluate the performance efficiency different physicochemical parameters of constructed wetland were examined. Impermeable plastic tubs were used to form this setup to ensure no leakage; tubs were further interconnected with 1ft steel pipes. The setup was placed in an open space so that it receives the maximum sunlight. The order of plants grown in this setup was the same as of the pilot scale unit.

- Parallel scale unit

Parallel scale unit was the individual units for pennywort and water lettuce to measure their efficiencies individually. Same plastic tubs were used as in replica unit. Waste water was provided through steel pipes attached to the sedimentation tank. After certain retention time, effluent from the tubs was discharged into the ground through plastic pipes connected at another end of tubs.

- Control unit

In control unit plants were not grown in the plastic tubs. The main purpose of this unit was to confirm either the treatment process is exclusively due to phytoremediation or there is an impact of other physical processes.

3.3 Sampling

For microbial analysis, samples were collected in Schott (glass) leak proof and sterile bottles. Bottles were first properly washed with detergent and further rinsed with distilled water. Then they were autoclaved at 121^{o}C temperature at a cycle of 15 minutes and dried in an oven at 105^{o}C for 1 hour. For physicochemical and nutrient analysis polyethylene bottles were used. The samples were collected generally thrice a month from the surface and benthic layers of the horizontal flow constructed wetland (Pilot scale). Collected samples were preserved according to the standard sample collection method at 4^{o}C in the refrigerator. For microbial analysis, samples were collected from the inlet and outlet as well as from the second pond to show the relation present between microcosms of different ponds.

3.4 Physicochemical Analysis

For physicochemical analysis, water samples were taken 3 times in a month from all the 8 ponds including inlet and outlet from pilot scale unit and lab-scale unit. Physicochemical parameters which were analyzed include Dissolved oxygen, Electrical conductivity, pH, temperature, Chemical oxygen demand, Total suspended solids and Total dissolved solids. APHA standard methods for wastewater analysis, has been used (APHA, 2012). The methods and instruments used for the analysis of these parameters are illustrated in table 3.1.

Table 3.1: Methods and Instruments for Physicochemical Parameters

Sr No.	Method of Analysis	Equipment Used	Units
1.	pH	HACH 156 pH meter	--------
2.	Temperature	Laboratory Method HACH Session 1	°C
3.	Electrical Conductivity (EC)	Potentiometric Method Conductivity Meter	(µS/cm)
4.	Total Dissolved Solids (TDS)	Potentiometric Method Conductivity Meter	(mg/L)
5.	Total Suspended Solids (TSS)	Gravimetric Dried Method Analytical Mass Balance	(mg/L)
6.	Chemical Oxygen Demand (COD)	The Closed Reflux Method Through Titration	(mg/L)
7.	Dissolved Oxygen (DO)	Crison Oxi 45 DO meter	(mg/L)

The parameters like DO, temperature and pH were measured on site by using DO meter (Crison Oxi 45), HACH session 1 and HACH 156 respectively. All the analyses were performed according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). While the parameters i.e. COD, TSS, EC, and TDS were measured in the laboratory. COD was measured by using the closed reflux titrimetric method. For titration, sample was prepared according to the standard method and was titrated with ferrous ammonium sulfate to measure the COD. Total suspended solids (TSS) were analyzed by using gravimetric dried method. EC and TDS were measured through portable conductivity meter.

3.5 Microbiological Analysis

3.5.1 Isolation Through Spread Plate Technique

For the isolation of bacterial species, serial dilution technique was used. Serial dilutions from 10^{-1} to 10^{-9} were prepared in this method. Nine mL of autoclaved distilled water was taken in 9 test tubes for this purpose. One ml of the sample was transferred to the tube labeled as blank dilution 10^{-1} by using a sterile pipette and vortexed it. Then 1 mL from this dilution is transferred to the 10^{-2} tube. The same procedure was repeated for further dilutions up to 10^{-9}. After preparation of dilution, 0.1 mL of the dilution fluid from each dilution was transferred to already prepared nutrient agar plate with the help of micropipette and was spread with the help of sterilized spreader. This agar plate is incubated for 24 hours at 37 ^{o}C.

3.5.2 Heterotrophic Plate Count

The plates that were incubated were observed after 24 hours. Plates showing the countable range i.e. 30- 300 of bacterial colonies were placed on the colony counter and colonies were counted. The method used for heterotrophic plate count per 1 mL is as follow:

Number of colonies / mL = number of colonies x dilution factor

3.5.3 Pure Culture Isolation

Fourteen different bacterial colonies were picked after observing them on colony counter from 3 different ponds i.e. inlet, outlet, and pond no.2. Further, these colonies were streaked on nutrient agar plates through streak plate technique for their pure cultures. In this process, the whir loop was first sterilized by holding the loop in the flame of Bunsen burner until the loop became red hot. Then the loop was allowed to cool down by holding

it still. An isolated colony from the agar plate was picked and streaked n the new agar plate. The Same process was repeated for remaining colonies. After 4- 5 cycles of streaking the pure cultures were obtained. These pure cultures were stored in the refrigerator for further use.

3.6 Bacterial Identification

Identification of unknown isolated strains was done by their morphological characteristics and biochemical analysis which are described below:

3.6.1 Morphological Characteristics

The first step in most of the identification schemes is a colony or cellular morphology. Colony morphology was observed to identify and characterize the selected isolated strains. Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) was used to perform the morphological analysis. Table 3.2 shows morphological characteristics which were usually observed (Table 3.2):

Table 3.2 Morphological Characteristics and Description

Sr. No.	Morphological Characteristics	Description
1	Size	Small, large, punctiform
2	Margins	Entire, curled, lobate, undulate, filiform
3	Texture	Creamy, dry, mucoid
4	Color	Yellow, orange, off white, pale yellow
5	Form	Rhizoid, circular, filamentous, irregular

3.6.2 Gram Staining

The most important technique used for the identification of gram positive and gram negative bacteria is gram staining and was first developed by Danish Physician Hans Christian Gram in 1884. On the basis of distinct and consistent differences in the cell wall, bacteria were identified. In this practice first, the tiny drop of distilled water was placed on the surface of the slide. Then the colony was transferred to the slide with the help of sterilized cool whir loop. The colony was mixed in water either in clockwise or anti-clockwise rotation to form a smear. The smear was allowed to dry in air. Then heat fixed the dried smear by passing the slide through the flame of Bunsen burner for 3 times without exposing the smear directly to flame. The slide was touched with skin to check that it was not as hot as it uncomfortable to touch.

Poured the crystal violet solution which is a primary stain on the smear, kept it for 1 minute and rinse it with distilled water. Then smear was flooded with gram's iodine as it acts as a mordant for 1 minute and washed with distilled water. After this smear was washed with 95% alcohol for 10-20 seconds for decolorization process and rinsed with distilled water. At the end, safranin was added on the slide and allowed to counterstain for 30 seconds, rinsed with distilled water and air dried. A drop of immersion oil was poured on the cover slip and observed the slide in the microscope.

3.6.3 Biochemical Characterization

3.6.3.1 Catalase Test

Catalase test was performed to confirm the presence of catalase enzyme. In most bacteria, hydrogen peroxide is produced through metabolic reaction in the presence of oxygen and water, which is toxic to cells. Catalase enzyme is an enzyme which converts hydrogen peroxide H_2O_2 in oxygen and water. Catalase enzyme produced by bacteria to neutralizes the toxic forms of oxygen.

$$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$$

For catalase test 24 hour, fresh culture inoculum was placed on the slide by the help of sterilized wire loop. Then a drop of 3% hydrogen peroxide was added to it. Bubble formation confirmed the presence of catalase enzyme and showed that test was positive.

3.6.3.2 Oxidase Test

An oxygen reductase enzyme Cytochrome Oxidase C is present in most of the aerobic bacteria. It transfers an electron to oxygen on electron transfer chain to form water in the system. N, N, N', N'-tetramethyl-p-phenylenediamine I (TMPD) chemical was used in this test, comprises of an artificial electron acceptor. Strips of filter paper were cut and loop full of inoculum of 24-hours fresh culture was placed on the strip. One drop of TMPD reagent was applied on the inoculum. The appearance of blue or purple color on the strips within few seconds confirmed the presence of cytochrome oxidase enzyme.

3.6.3.3 Macconkey Agar Test

MacConkey agar test is a test to differentiate gram-negative lactose fermenting bacteria from non-fermenting bacteria or gram-positive bacteria, as macConkey agar is a selective

and differential media for their growth. For macConkey agar test after preparation of agar plates, isolates were streaked on the plates. Plates were incubated for 24 hours at 37^{o}C temperature. Isolates which were lactose fermenting gram-negative changed into pink.

3.6.3.4 Simmon Citrate Test

To differentiate the gram-negative bacilli isolates Simmons citrate test was performed. Isolates which belongs to Enterobacteriaceae family were distinguished using this test. The medium used for this test contains citrate as a sole carbon sole in it. This agar was used to test the ability of isolates to utilize citrate as a carbon source. Agar plates or slants were prepared and streaked. After incubation of 24 hours at 37^{o}C change of color showed the results. Isolates which used citrate as a carbon source and were of Enterobacteriaceae family changed the green color of agar into blue.

3.6.3.5 Mannitol Salt Agar Test

Mannitol salt agar is also a selective and differential media used for identification of isolates belongs to the genus staphylococcus and are pathogenic. Non-pathogenic bacteria will not show growth on it. Plates or slants of mannitol salt agar were prepared and streaked and then incubated for 24 hours at 37oC temperature. The change of color from red to yellow showed that the isolated strain belongs to pathogenic staphylococcus genus and fermented mannitol salt.

3.7 Molecular Characterization

3.7.1 16s rRNA Gene Sequencing

Isolated strains (14 in number) were freshly cultured on nutrient agar plates. After 24 hours of incubation when the growth was obtained these strain were sprayed by 1 mL of

autoclaved distilled water. Inoculums of strains were mixed with glass rod and were collected with the help of micropipette in 2 mL Eppendorf tubes. Tubes were centrifuged at 2000 rpm for 10 minutes to separate supernatant from bacterial culture pellet. Then 1 mL of 50% glycerol solution following 1 mL of 30% nutrient broth was added in tubes containing pellets. These preserved isolates were sent to Genome Analysis Department Macrogen Inc., South Korea for 16s rRNA gene sequencing.

3.7.2 Phylogenetic Analysis

Phylogenetic analysis shows the relationship of ancestors and descendants of the bacterial species. In this an evolutionary branched tree was formed that shows the genetic linkages among the isolated organisms. Sequences obtained from Macrogen were first aligned and trim using Software CLUSTALW Once the junk data was removed and sequences were completely trimmed they were analyzed using BLAST at National Center for Biotechnology Information (NCBI) databases. Accession numbers of highest matched isolate sequences were selected and FASTA. FASTA sequences were run in MEGA 7 Software to develop a phylogenetic tree which showed the linkage between isolated strains and of GenBank (NCBI).

3.8 Seasonal Growth Evaluation and Nutrient Analysis

3.8.1 Growth Evaluation

The biomass of the plants (Water lettuce and pennywort) planted in lab scale setup was sampled in beginning, at the peak standing periods of plants and in the end of the research period. Plant growth was monitored in harvesting the water lettuce plants in months of March, May and in July while the growth of pennywort was monitored in months of

February, April and July. Three replicates were collected of each plant during the harvesting. Stems and roots were cut from the apex. Plant's root length and shoot length of all the samples were measured and the number of leaves was counted. For dry weight the shoots and roots of the harvested plant were dried in oven at 105 $^{\circ}$C for 24 hours and measured (Vymazal & Kropfelov, 2005).

3.8.2 Nutrient Analysis

Nutrient analysis of the constructed wetlands was performed twice a month. Analysis of both the pilot scale setup and lab scale setup was performed. Nutrient parameters that were examined were listed in table 3.3 along with their methods. Standard APHA methods were used for analysis of nutrients (APHA, 2012).

Table 3.3 Methods for Nutrient Analysis

Sr no.	Parameters	Methods	Units
1.	**Nitrite-N (NO_2-N)**	Sulphanilamide Spectrophotometric Method	mg/L
2.	Nitrate-N (NO_3-N)	Phenol Sulphanilamide Spectrophotometric Method	mg/L
3.	Ammonia-N (NH_3-N)	Phenate Method	mg/L
4.	Orthophosphate	Ammonium Molybdate	mg/L

3.8.2.1 Nitrate-N (NO_3-N) Analysis

Reagent preparation

Reagent (a): Phenol disulphonic acid

Reagent (b): Add 168.2 g of Potassium hydroxide (KOH) in small quantity of distilled water and dissolve then the volume was made up to 250 mL.

Standard stock solution: 3.61 g of potassium nitrate was added in distilled water and filled the volumetric flask up to the mark and made volume 500 mL (1000 mg/L). then from this solution 1 mL was taken in a flask and filled the flask up to the mark and made the volume 1000 mL which gave 1 mg/L.

Procedure

0.5 ml of reagent (a) i.e. Phenol disulphonic acid was added in 25 mL of filtered wastewater sample and dried it on water bath. When the sample was about to dry reagent (b) was added and mixed well. Yellow color was observed and absorbance was taken at wavelength of 410 nm through UV- Visible spectrophotometer. Same process was repeated for standards and for blank. Standards were prepared to obtain a calibration curve.

3.8.2.2 Nitrite-Nitrogen NO_2-N Analysis

Reagent Preparation

Reagent (a): sulphanilamide reagent: 1 g of sulphanilamide was dissolve in 100 mL of 10% HCL.

Reagent (b): N-1 naphthyethyleneamine dihydrochloride reagent: 0.1 g of aromatic amine reagent was dissolved in 100 mL of distilled water.

Standard Stock Solution: 2.46 g of anhydrous sodium nitrite was dissolved in 1 L of distilled water (50 mg/L).

Procedure

50 mL of water sample was filtered using Whatman filter paper No. 1. 1 mL of reagent (a) was added to the filtered sample. After 3 minutes 1 mL of reagent (b) was added in it and shaken thoroughly. Same process was repeated for standard solutions and blank. Absorbance was measured at 543 nm using UV- Visible spectrophotometer. Calibration curve was obtained from standard solutions that were prepared.

3.8.2.3 Orthophosphate Analysis

Reagent Preparation

Reagent (A): (a) Ammonium Molybdate Strong Acid Solution: 5 g of ammonium molybdate $(NH_4)_6$ $MO_7O_2.4H_2O$) was dissolved in 35 mL of distilled water.

(b) 62 mL of conc. H_2SO_4 was added to 80 mL of distilled water. Both the solutions (a) and (b) were mixed and cooled to the room temperature and then volume was made up to 200 mL by adding distilled water into it.

Reagent (B): Stannous Chloride Solution: 0.5 g of stannous chloride was added in 2 mL of conc. HCL and the volume was made up to 20 mL by adding distilled water into it.

Standard stock solution: 21.95 mg of anhydrous KH_2PO_4 was added in 1 L distilled water and dissolved. Then 10 mL of prepared solution was taken in flask and distilled water was further added to make the volume up to 500 mL.

Procedure

25 mL of filtered sample was taken in 100 mL conical flask. 1 mL of reagent (A) was added to the sample and 3 drops of reagent (B) was added to the solution, and then mixed thoroughly. Blue color was appeared after 10 minutes. After the color appeared absorbance was measured at 690 nm. Same procedure was repeated for blank and standard solution for a calibrated curve.

3.8.2.4 Ammonia (NH_4) Analysis

Reagents Preparation

Ammonia free distilled water was used to prepare all the reagents.

Reagent (A): Mixed Indicator Solution: (a) 200 mg of methyl indicator was dissolved in 100 mL of 95% ethyl or isopropyl alcohol. (b) 100 mg of methylene blue was dissolved in 50 ml of 95% ethyl or isopropyl alcohol. Both the solution (a) and (b) were combined together shelf life of this reagent was up to 1 month.

Reagent (B): Indicating Boric Acid Solution: 20 g H_3BO_3 was dissolved in distilled water and 10 mL of mixed indicator was added to it and made the volume up to 1000 mL. Shelf life of this reagent was also of 1 month**.**

Reagent (C): Standard sulfuric acid titrant, 0.02N: standard solution of sulfuric acid titrant was prepared.

Procedure

a. Preparation of equipment: 500 mL of distilled water was added to 20 mL of borate buffer solution. pH of the solution was adjusted to 9.5 with 6N NaOH solution and then added to distillation flask. Few glass beads were added in the flask and used this mixture to steam out the distillation apparatus until distillate showed no traces of ammonia.

b. Sample preparation: 50 mL of the sample was taken and 25 mL of borate buffer solution was added into it. pH was adjusted to 9.5 by using 6N NaOH using pH meter.

c. Ammonia determination: digestion reagent was cooled down and added into the distillation flask. Few glass beads were added into it. Boiled it until the volume was greatly reduced and boiled for 30 minutes. Color of the sample changed into pale green after digestion. After digestion sample was diluted upto 300ml with distilled water and then sodium hydroxide thiosulphate reagent was added and connected to flask to steamed distillation apparatus and swirl flask and asure complete mixing of sample.

d. Distillation: 200 mL of distillate was collected after distillation. 50 mL of indicating boric acid was added before the titration process which act like an absorbent.

e. Then titration was done to determine ammonia.

Ammonia was calculated by using the following formula;

$$\text{mg } NH_3\text{-N} = \frac{(\mathbf{A-B}) \times \mathbf{280}}{\mathbf{mL\ of\ Sample}}$$

A = volume of H_2SO_4 titrated for sample in mL,

B = volume of H_2SO_4 titrated for blank, mL.

3.9 Statistical Analysis

To analyze the physicochemical analysis, root lengths, shoot lengths and dry weights Microsoft Excel data analysis tools were used. Standard deviations and means of all parameters were calculated.

Chapter 4

RESULTS AND DISCUSSION

4.1 Physicochemical Analysis

4.1.1 Comparison of Monthly Average Physicochemical Profile of Pilot and Lab Scale

4.1.1.1 Temperature Profile of Pilot and Lab Scale Setup

In both pilot and lab scale the change in temperature plays a major role in removal efficiencies of pollutants. Both system shows high removal efficiencies when the temperature is high i.e. in summer (Moortel *et al*., 2010). Increase in temperature at pilot scale and lab scale was recorded in the month of June. Fig 4.1 shows the monthly temperature profiles of pilot and lab scale respectively

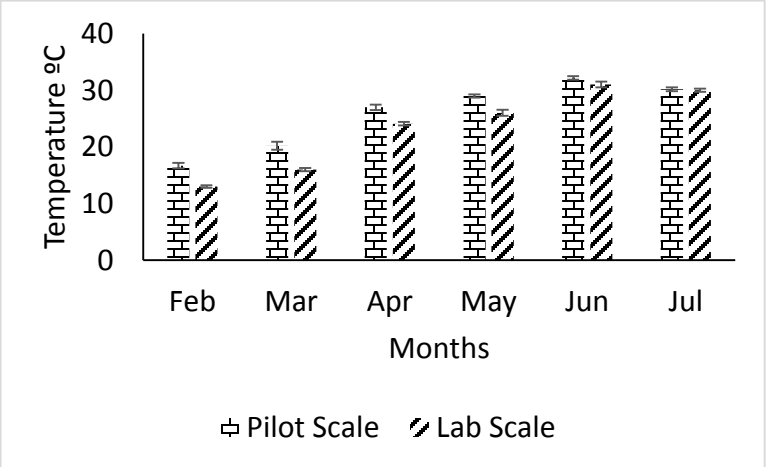

Fig 4.1 Temperature profile of pilot and lab scale

4.1.1.2 pH of Pilot and Lab Scale

In all six months no significance difference was observed in pH range of both setups. Fig 4.2 shows the comparison of mean average pH of inlet and outlet zone of pilot and lab scale setup. In outlet maximum pH value was observed in the month of February both for lab and pilot scale setups i.e. 7.62 for pilot setup and 8.8 for lab scale. While the minimum pH value of outlet was observed in month of June that is 7.07 for pilot scale and 7.56 for lab scale respectively.

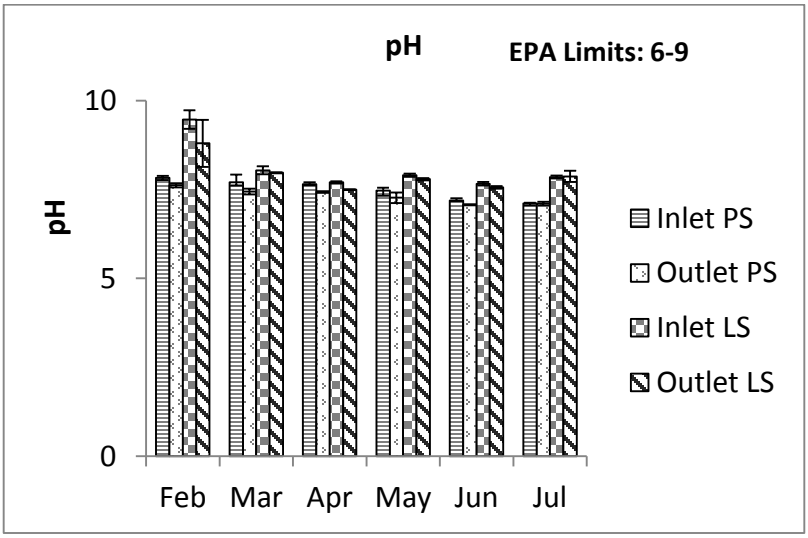

Fig 4.2 Monthly pH comparison of pilot and lab scale setup

4.1.1.3 Dissolved Oxygen (DO)

It is observed that dissolved oxygen of treatment system at both pilot scale and lab scale has increased in lower temperatures and has decreased in higher temperature. At pilot scale the maximum DO was measured in month of March due to heavy rainfall as the rainwater continuously came in contact with atmospheric oxygen and when this rain water dissolved in wetlands water it increases the DO levels. Maximum DO in winter were observed as the propensity of cold water to hold the oxygen content in it is high as

compared to warm water. Similar increased value of DO was observed in lab scale setup at temperature of 11 ºC in month of March. According to EPA dissolved oxygen at 14 ºC is 10.2 mg/l (EPA, 2012) but due to presence of degraded organic matter and high level of suspended solids decreases the oxygen contents. Lowest DO was observed in month of July (Mitsch *et al*., 2005).

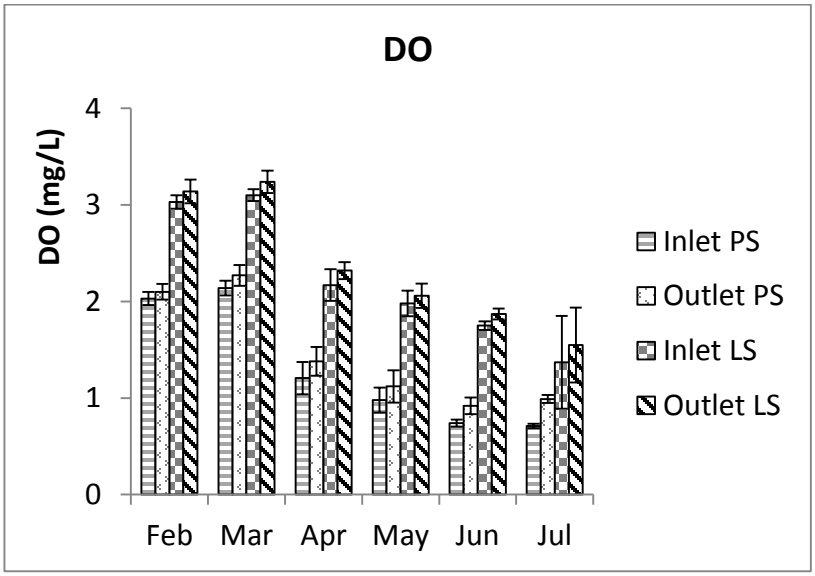

Fig 4.3 Monthly DO comparison of pilot and lab scale setup

4.1.1.4 Electrical Conductivity and Total Dissolved Solids

Electrical conductivity of is measure of ionic conductivity occurring in water and as these ions are dissolved solids so total dissolved solids directly related to electrical conductivity. Fig 4.4 and 4.5 shows the change in EC and TDS level of pilot and lab scale. Which shows that EC and TDS level were within the EPA limits i.e. 3500 mg/L (EPA, 2012: Morrison *et al*., 2001).

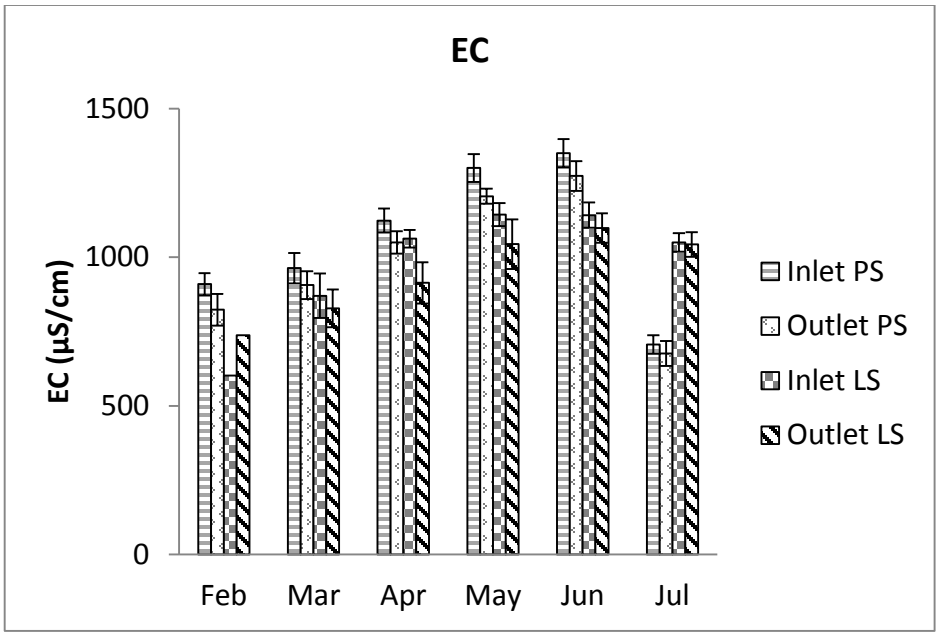

Fig 4.4 Monthly EC comparison of pilot and lab scale setup

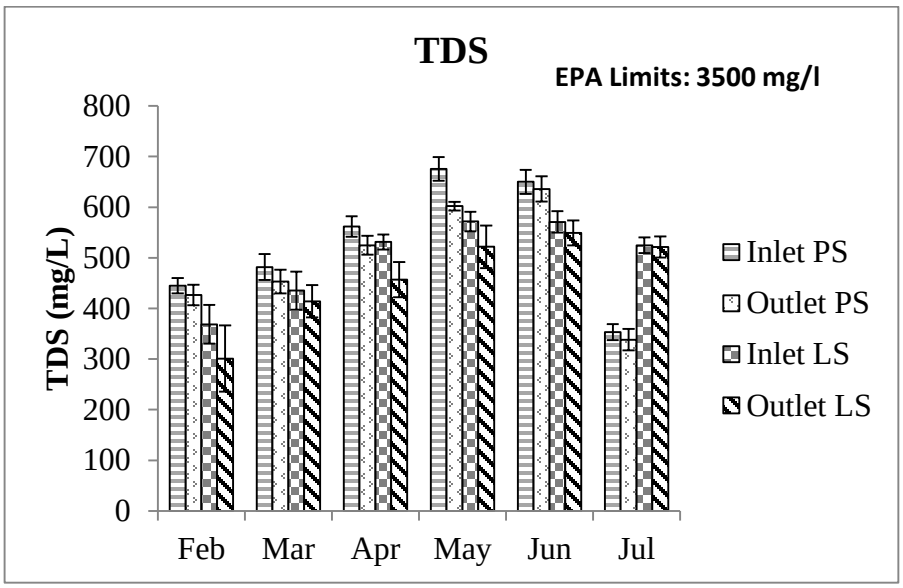

Fig 4.5 Monthly TDS comparison of pilot and lab scale setup

Electrical conductivity of CWs also depends on temperature and evapotranspiration level (Caselles-Osorio & Garcia, 2007). So, electrical conductivity in effluent was slightly higher due to the evapotranspiration. The lowest EC and DO was measured in month of

February while highest values were observed in summer season which shows that temperature also effects the conductivity level of the systems. At lab scale maximum EC and TDS values observed were 1098 and 549.2 µS/cm respectively. As in month of June temperature was 32 ^{o}C so the EC value observed was highest during the whole study i.e. 1273 µS/cm as the warm water have more viscosity and dissolved solids due to which electrical current moves freely (Todd *et al.,* 2005).

4.1.1.5 Total Suspended Solids

Both setups were shown the maximum removal of TSS in month of June and July. In winter as the degradation of plants were occurred so the removal efficiency of the setup was low. Maximum removal efficiency of lab scale setup was calculated in month of June i.e. 72.8% while of pilot scale setup maximum efficiency was 79.4% in July. Fig 4.6 (a) shows the monthly comparison of lab scale and pilot scale setup while Fig 4.6 (b) shows the removal efficiencies between the two setups.

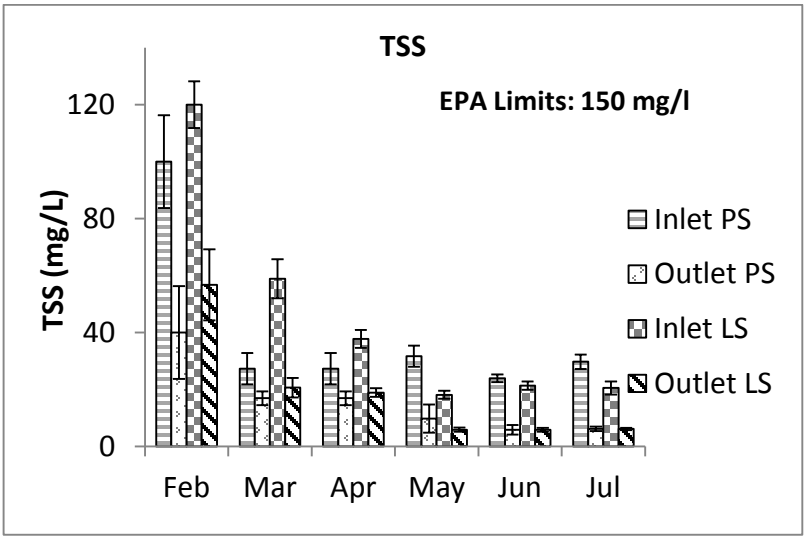

Fig 4.6 (a) Monthly TSS comparison of pilot and lab scale setup

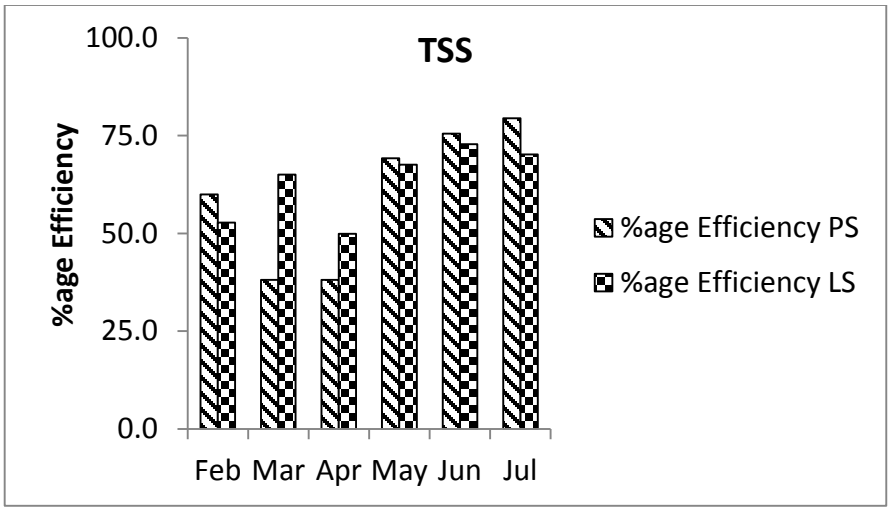

Fig 4.6 (b) Monthly TSS removal efficiency comparison of pilot and lab scale setup

4.1.1.6 COD

COD values were calculated for the six months of the year i.e. from February to July and the highest values was recorded in the month of February when the temperature was 13-16 ^{o}C. Lowest values were observed in months of June and July in summers when the temperature was high. Both setups show the same trend that COD level decreases with the increase in temperature (Taylor *et al*., 2011). Highest value of COD was 552.5 and 267.7 mg/L for pilot and lab scale. This is because the plant growth is slowed down in high temperature due to which oxygen content decreases and chemical oxygen demand increases. Lowest values were recorded in month of July i.e. 49 and 65.6 mg/L in pilot and lab scale setup respectively. Both the temperature and precipitation factors are the reason in lower COD levels of treatment system. Fig 4.7 (a) shows the monthly comparison of pilot and lab scale setup while Fig 4.7 (b) shows the percentage removal efficiencies of COD in both setups.

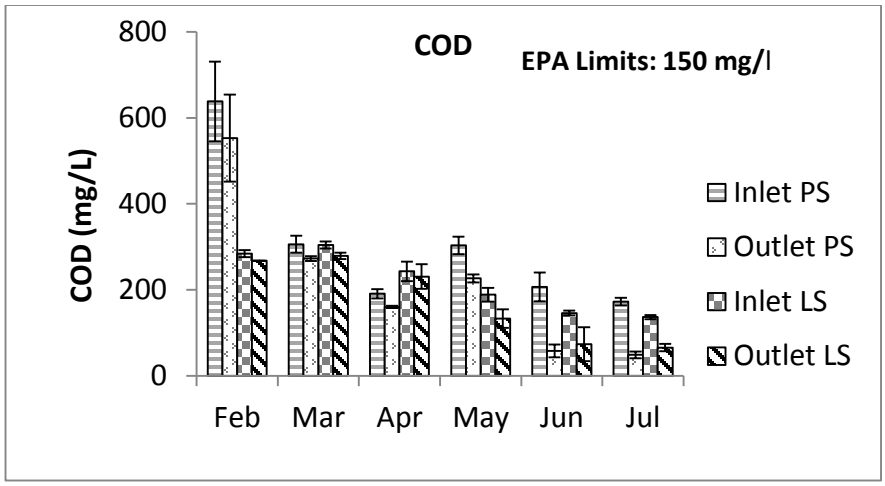

Fig 4.7 (a) Monthly COD comparison of pilot and lab scale setup

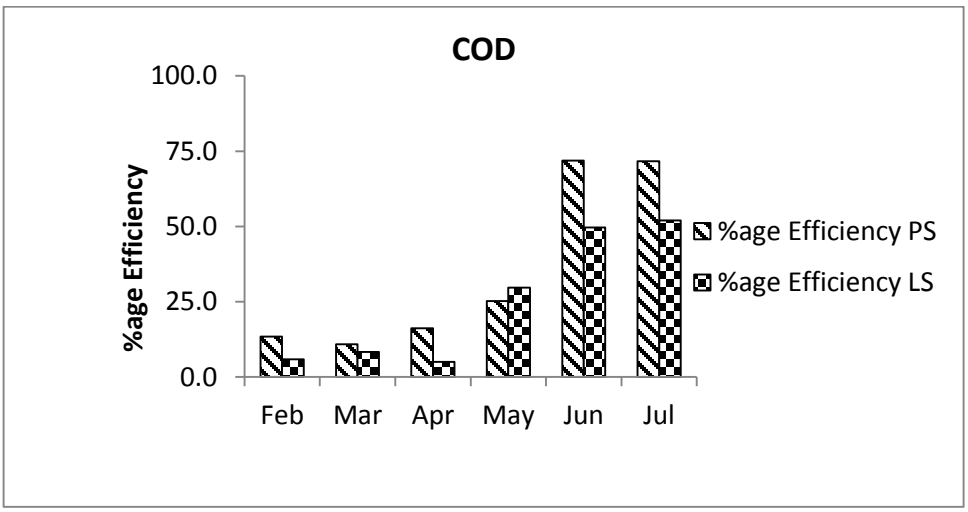

Fig 4.7 (b) Monthly COD removal efficiency comparison of pilot and lab scale setup

Maximum removal of lab scale was observed in July i.e. 52% while in pilot scale maximum removal has occurred in the month of June that 71.9%. Maximum COD removal was observed in high temperatures. From results it was observed that percentage removal of COD by pilot scale is high as compared to lab scale

4.1.2 Comparison of Monthly Average Physicochemical Profile of Surface and Benthic Layer of Pilot Scale Setup

4.1.2.1 pH

pH layers of the system (surface and benthic layer) has shown no change throughout the experiment. Minimum values of pH of both layers were observed in month of June. pH of surface layer was 7.08 and of benthic layer was 7.07. From this it was interpreted that no significant change was observed in pH of different layer of the pilot scale system.

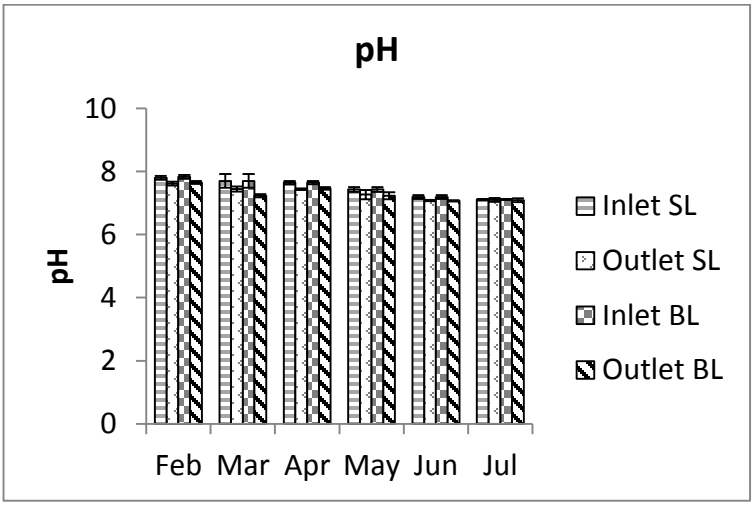

Fig 4.8 Monthly pH comparison of surface and benthic layer of pilot scale setup

4.1.2.2 DO

Dissolved oxygen of the benthic layer was low as compared to the surface layer, as amount of oxygen in depths of the pond is low and that's why that zone is known as anaerobic zone. Both layers showed same trends with respect to temperature change i.e. both have higher values in month February and March as the temperature is low and in

March heavy rainfall was observed. Maximum DO level of surface layer was 2.27 mg/L and 2.05 in benthic layer (Akratos & Tsihrintzis, 2007).

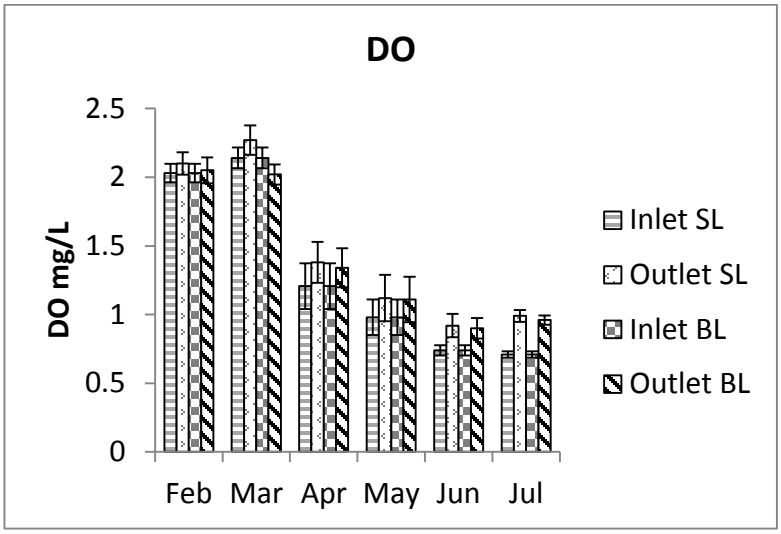

Fig 4.9 Monthly DO comparison of surface and benthic layer of pilot scale setup

4.1.2.3 EC and TDS

EC and TDS are the parameters which were inter linked with each other. Due to the presence of dissolved solids the electrical conductivity was produced in the wetland system. Electrical conductivity between the layers of the ponds were slightly different as the surface layer was in contact with the atmosphere and have greater ionic exchange through evapotranspiration. The maximum value of EC was measured in month of May both for surface and benthic layer i.e.1273 and 1251 µS/cm respectively. TDS values in month of May were 636.7 and 625.5 mg/L. Fig 4.10 (a) and (b) shows the comparison between EC and TDS of both layers respectively.

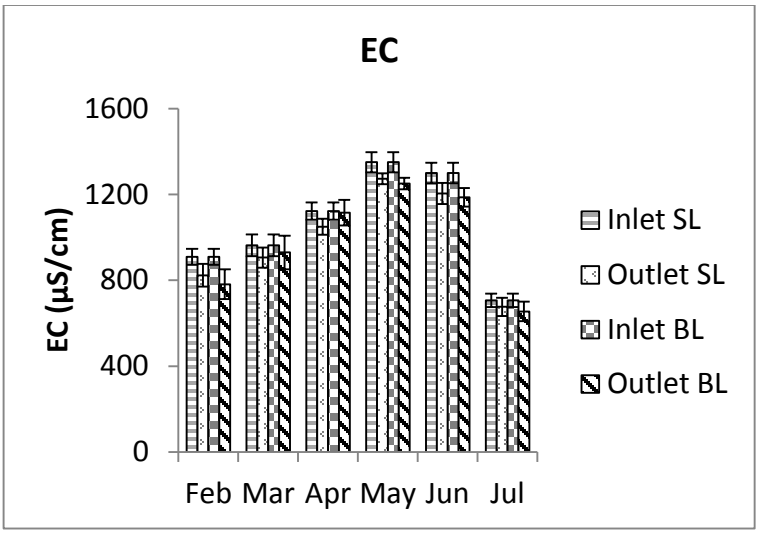

Fig 4.10 (a) Monthly EC comparison of surface and benthic layer of pilot scale setup

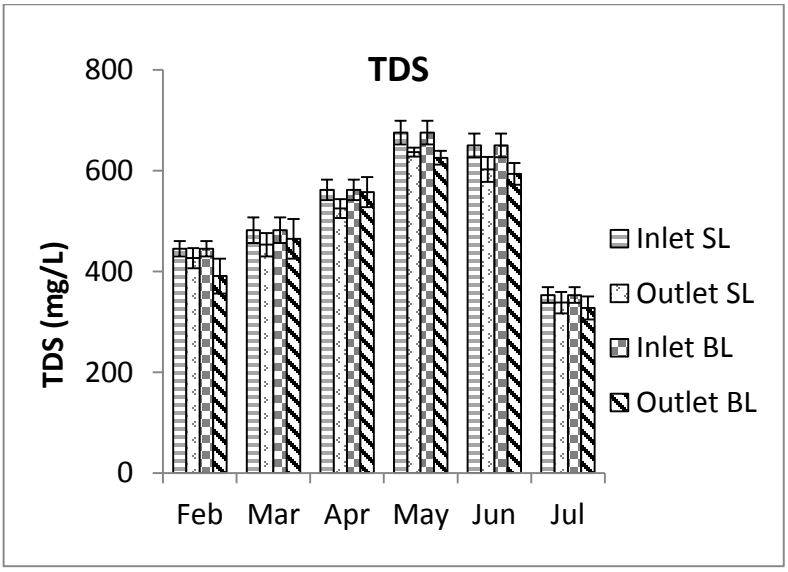

Fig 4.10 (b) Monthly TDS comparison of surface and benthic layer of pilot scale setup

4.1.2.4 TSS

Suspended solids observed in benthic layer were higher than in surface layer as in benthic layer sedimentation occurred. The maximum removal was measured in summers while the minimum removal was observed in February as the temperature in this month was low and degradation of plants has happened in this season. Maximum removal efficiency of surface layer was 79.2% with the removal amount of 5.86 mg/L in outlet. In benthic layer maximum removal percentage was 56.1% with amount of TSS of 10.6 mg/L in outlet. Fig 4.11 (a) and (b) shows the monthly average comparison and percentage removal of both surface and benthic layer.

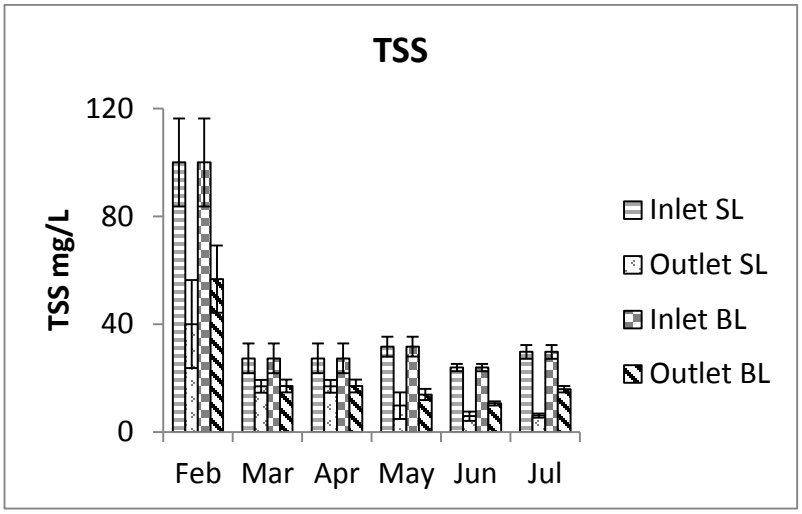

Fig 4.11 (a) Monthly TSS comparison of surface and benthic layer of pilot scale setup

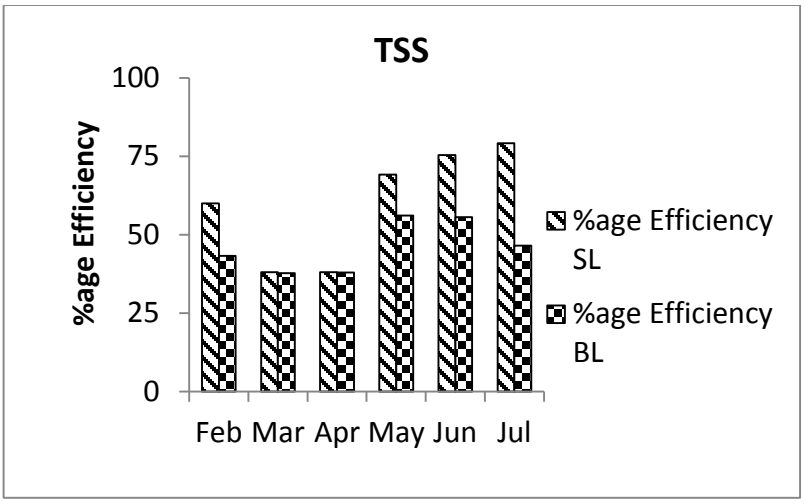

Fig 4.11 (b) Monthly TSS removal efficiency comparison of surface and benthic layer of pilot scale setup

4.1.2.5 COD

The COD level in benthic layer was observed to be high as compared to surface layer, as the suspended solids level is high in anaerobic zone and due to less oxygen content present in this layer. In February COD value observed was high in both layers (552.7 in surface layer and 558.3 mg /L in benthic layer) while minimum (49 in surface layer and 51 mg/L in benthic layer) in July. The removal percentage of surface layer was 71.9 while in benthic layer was 74.2 % in month of June as the suspended solids were comparatively low than in February. Fig 4.12 (a) shows monthly average of surface and benthic layer and Fig 4.12 (b) show the monthly percentage removal of COD.

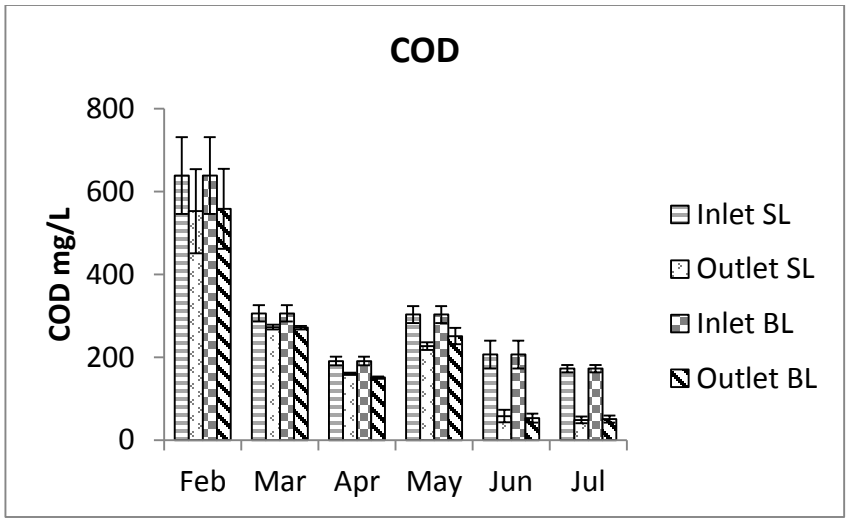

Fig 4.12 (a) Monthly COD comparison of surface and benthic layer of pilot scale setup

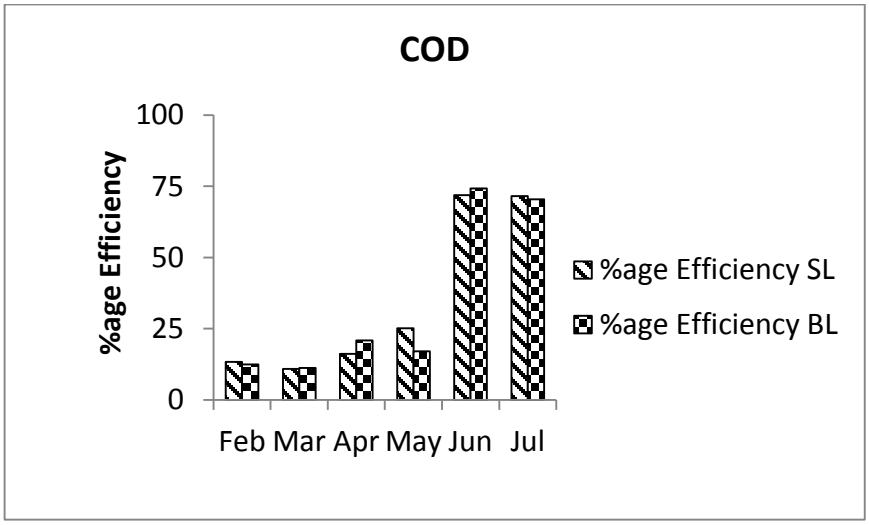

Fig 4.12 (b) Monthly COD removal efficiency comparison of surface and benthic layer of pilot scale setup

4.2 Identification of Microbial Species from Different Layers of Wetlands

Predominant microbial species were isolated through serial dilution and spread plate technique and then identified from the microcosm of surface and benthic layer of the constructed wetlands by using 16S rRNA gene sequencing. 14 strains were selected for identification on the basis of their dominancy and cells and colonies morphological and biochemical characteristics. These 14 strains were named from NB-S1 to NB-S14.

4.2.1 Heterotrophic Plate Count

In constructed wetlands both obliging and non-obliging bacterial communities were present, which may increase or decrease the removal efficiency respectively. Thus, from this it is widely accepted that the bacterial diversity in microcosm of wetland is an important element in performance of the system (Samsó& García 2013). The population and types bacterial communities were proved to be important for the extent and nature of the activity found in the wetland system. The CFU/ ml, was determined by using spread plate technique and shows high bacterial count in the system as shown in table 4.1.

Table 4.1: CFU/mL of the Sample

Sample	CFU/0.1mL
Inlet	2.06×10^6
Pond 2 (surface layer)	1.27×10^5
Pond 2 (benthic layer)	1.56×10^5
Outlet (surface layer)	3.3×10^4
Outlet (benthic layer)	9×10^4

Highest bacterial count was found in the inlet pond i.e. 2.06×10^6 per 1mL of the sample while in the outlet pond 3.3×10^4 and 9×10^4 bacterial count were observed in surface and benthic layer respectively. This shows decreasing trend in bacterial removal in the wetland system and high diversity of bacterial communities were found.

4.2.2 Morphological Characterization

The predominant identified strains were first observed morphologically which includes shape, elevation, color, size, and surface. Table 4.2 shows the morphological characteristics of the identified strains colonies from surface and benthic layers of the constructed wetland.

Table 4.2: Colony Morphology of Isolated Bacterial Strains

Strains	Shape	Elevation	Color	Size	Surface
NB01	Round	Flat	Cream white	Medium	Smooth
NB02	Circular	Raised	Whitish	Small	Smooth
NB03	Circular	Raised	Off white	Small	Smooth/ shiny
NB04	Circular	Raised	Brown centered	Small	Smooth
NB05	Circular	Raised	Off white	Small	Smooth
NB06	Circular	Raised	White	Small	Smooth
NB07	Circular	Concaved	Transparent	Small	Smooth
NB08	Round	Concaved	White	Pinpoint	Smooth
NB09	Round	Concaved	White	Medium	Smooth
NB10	Round	Concaved	Pink centered	Medium	Smooth
NB11	Round	Concaved	White	Small	Smooth
NB12	Round	Raised	Cream white	Medium	Smooth
NB13	Round	Raised	White	Small	Smooth
NB14	Irregular	Flat	White	Large	Rough

4.2.3 Biochemical Characterization

Biochemical tests of the isolated strains revealed the shape of their cells, different biochemical characteristics and gram staining showed that either the strains were gram negative or gram positive. Table 4.3 shows the cell morphology and biochemical characteristics of the isolated strain due to which they were further selected for identification through 16s rRNA gene sequencing.

Table 4.3: Cell Morphology and Biochemical Characteristics of Isolated Bacterial Strains

Strains	Catalase Test	Oxidase Test	Simmon's Test	MSA Test	MacConkey Test	Cell Shape	Gram Staining
NB01	+	-	-	-	+	Bacillus	Negative
NB02	+	-	-	+	-	Bacillus	Positive
NB03	+	+	+	+	+	Bacillus	Negative
NB04	+	+	-	+	+	Bacillus	Negative
NB05	-	-	-	-	-	Cocco-Bacillus	Positive
NB06	+	-	-	+	+	Bacillus	Negative
NB07	+	-	-	+	+	Bacillus	Negative
NB08	+	-	-	+	+	Bacillus	Negative
NB09	+	-	-	+	-	Bacillus	Positive
NB10	-	+	-	-	+	Bacillus	Negative
NB11	+	+	+	+	+	Bacillus	Negative
NB12	+	+	+	+	+	Bacillus	Negative
NB13	+	-	-	+	+	Bacillus	Negative
NB14	+	-	+	-	-	Bacillus	Positive

From biochemical tests of isolated strains, it was shown that all of them were rod shaped. Strains NB-S2, NB-S5, NB-S9 and NB-S14 were gram positive while the remaining

strains were gram negative (Kumar *et al*., 2011). On the basis of these biochemical and morphological characteristics these 14 strains (NB-S1 to NB-S14) were further send for 16s rRNA gene sequencing to Genome Analysis Department Macrogen Inc. Korea.

4.2.4 Gene Sequencing and Phylogenetic Tree

For identification of strains, first sequences of the strains were screened and noise was removed manually from them. Then the strains were identified through BLAST search (Nanda *et al*., 2016) that is available at National Center for Biotechnology Information (NCBI), the data bases from this revealing upto 99% match to different bacterial species. CLUSTALW was used for the complete genome alignment. After all this a phylogenetic tree was assembled by using software called MEGA 7 which showed the phylogenetic linkage and similarity among identified strains showed by Fig 4.13.

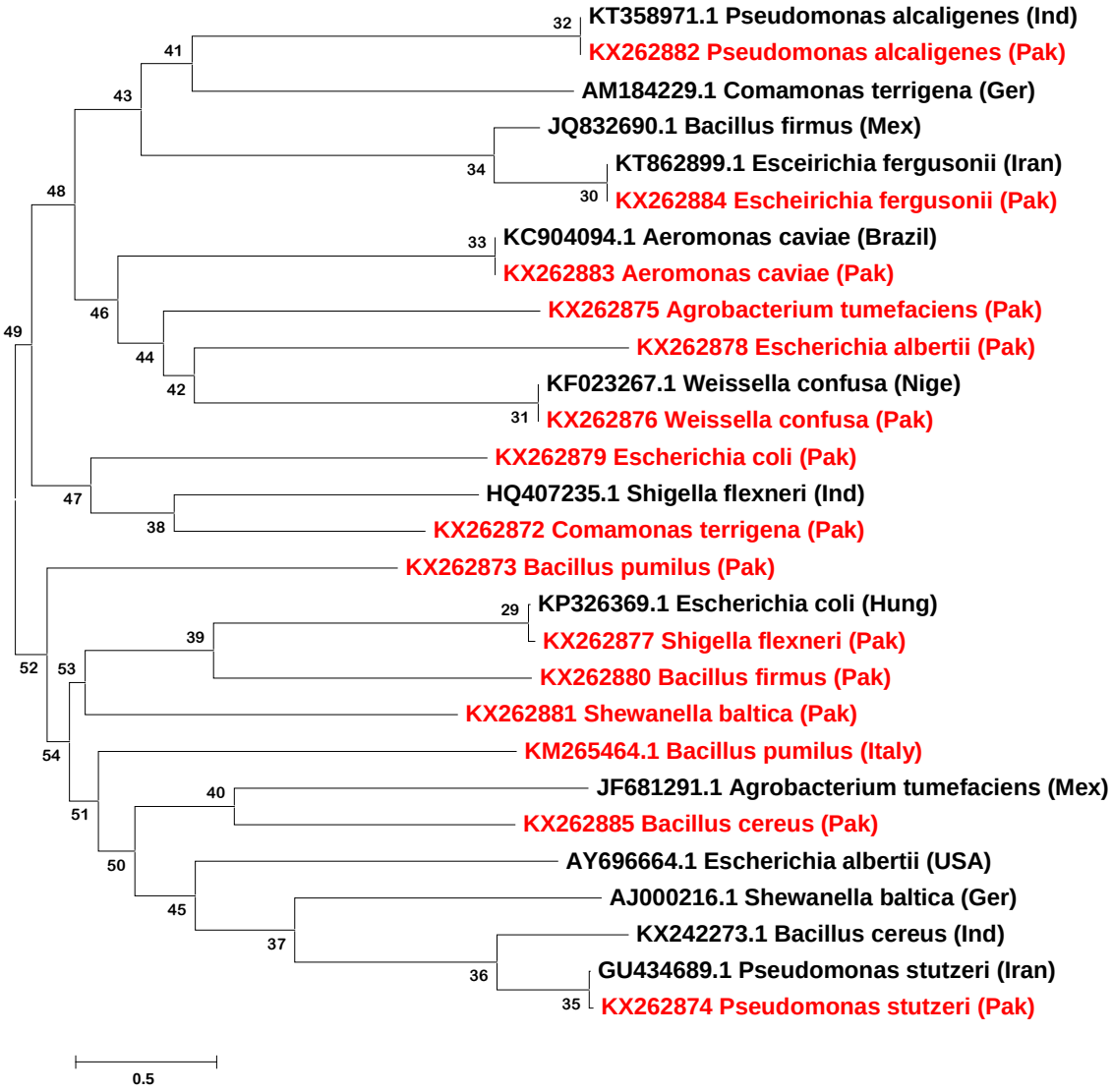

Fig. 4.13 Phylogenetic tree demonstrating the relatedness and linkage of bacterial strains

The identification of strains from 16s rRNA gene sequencing selected from surface and benthic layers of constructed wetland, had showed the presence of microbial species mostly belonging to the phyla proteobacteria (i.e. α, β, γ-proteobacteria) along with other phyla like Firmicutes as reported earlier by Zhong and He (Zhong *et al*., 2015; He *et al*., 2014). Out of 14 strains 10 strains NB01, NB03, NB04, NB06, NB07, NB08, NB10,

NB11, NB12, and NB13 belonged to proteobacteria phyla (1 from β-proteobacteria, 1 from α-proteobacteria, and 8 from γ-proteobacteria). While strains NB02, NB05, NB09, and NB14 belonged to the phyla Firmicutes. Thus, proteobacteria is most the abundant phyla which is present in constructed wetlands (Wang *et al*., 2016).

Bacterial species which were identified from constructed wetlands are also useful as most of them are nitrifying and denitrifying bacteria which reduce nitrates into nitrites and nitrites into nitrogen like the strain NB01 *Comamonas terrigena* which belongs to to β-proteobacteria class and are helpful to convert nitrate into nitrite (Chang *et al*., 2002). Similarly, strain NB03 *Pseudomonas stutzeri* which belongs to γ- proteobacteria also act like denitrifying bacteria, releases enzymes which convert nitrate into nitrite, and after several processes of conversion finally de-nitrogen this nitrate under anaerobic, microaerophillic and sometime in aerobic conditions (Lalucat *et al*., 2006). Some bacterial species from *Pseudomonas spp* like strain NB11 *Pseudomonas alcaligenes* may be able to degrade the organic pollutants and hydrocarbon compounds like petroleum hydrocarbon and helpful in carbon removal from the wastewater (Tu *et al*., 2014). So along with harmful bacterial species useful ones are also present in constructed wetlands. Table 4.4 shows the phylogeny of the identified strains.

Table 4.4 Phylogeny of identified strains

Strains ID	Accession No.	Names of strains	Phylogenetic Affiliation
NB01	KX262872	*Comamonas terrigena*	β-proteobacteria
NB02	KX262873	*Bacillus pumilus*	Firmicutes
NB03	KX262874	*Pseudomonas stutzeri*	γ- proteobacteria
NB04	KX262875	*Agrobacterium tumefaciens*	α-proteobacteria
NB05	KX262876	*Weissella confuse*	Firmicutes
NB06	KX262877	*Shigella flexneri*	γ- proteobacteria
NB07	KX262878	*Escherichia albertii*	γ- proteobacteria
NB08	KX262879	*Escherichia coli*	γ- proteobacteria
NB09	KX262880	*Bacillus firmus*	Firmicutes
NB10	KX262881	*Shewanella baltica*	γ- proteobacteria
NB11	KX262882	*Pseudomonas alcaligenes*	γ- proteobacteria
NB12	KX262883	*Aeromonas caviae*	γ- proteobacteria
NB13	KX262884	*Escherichia fergusonii*	γ- proteobacteria
NB14	KX262885	*Bacillus cereus*	Firmicutes

4.3 Seasonal Plant Growth and Nutrient removal from Constructed wetlands

4.3.1 Seasonal Growth Pattern of *Pistia stratiotes* (water lettuce)

The seasonal growth of *Pistia stratiotes* was calculated by measuring the length and biomass of root and shoots of the plant from March – July at lab scale level as it grows at high rate in summer season. Fig 4.14 (a) shows the shoot length of the plant. Initially when the plants were planted in month of March their initial shoot length was 2.8 cm

after 2 months the shoot length increases to about 6.2 cm. The maximum shoot length of the plant was measured in months of July which is 11.2 cm and the maximum no. of leaves present in this month was 21 leaves per plant.

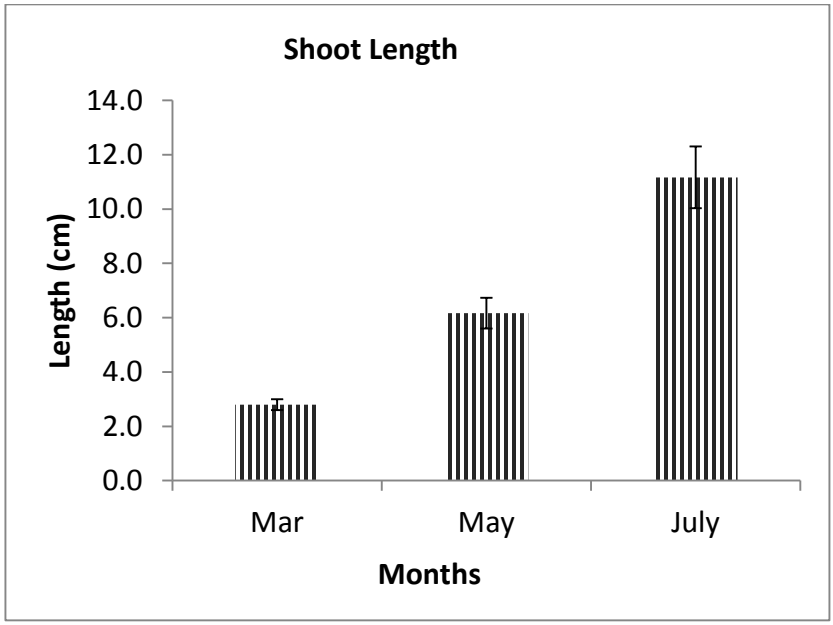

Fig 4.14 (a) Shoot Length of *Pistia stratiotes* from March to July

Root length of *Pistia stratiotes* showed the high uptake of nutrient and high level of pollutant removal through it. The maximum root length was measured in month of July i.e. 23.1 cm while the initial root length at the time of planting in March was 2.8 cm with the 8 no. of leave. Comparison of both root and shoot length at the time of planting and harvesting it was noticed that water lettuce showed high nutrient uptake and same trend of metal uptake. Roots of the plant showed that most of the nutrient removal was done by the roots of the plant. Fig 4.14 (b) shows the growth trend of roots of the plant. The trend of shoot and root growth was same as discussed in the earlier studies (Gupta *et al*., 2012; Polomski *et al*., 2009).

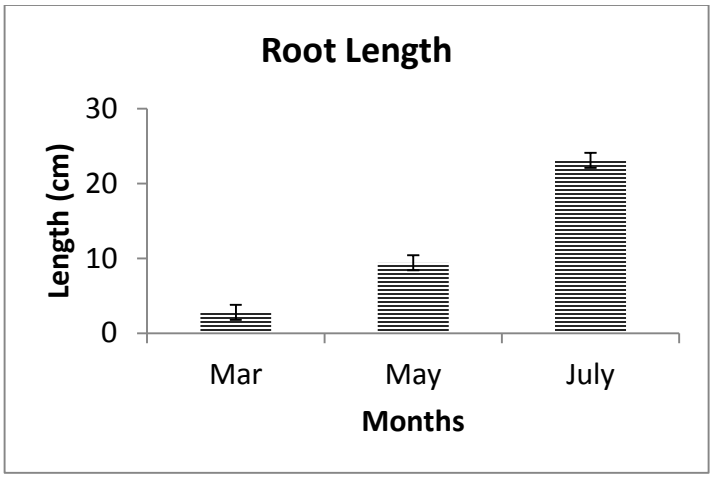

Figure 4.14 (b) Root Length of *Pistia stratiotes* from March to July

Dry weight of both root and shoot was measure. Results show the maximum nutrient removal was achieved by both the root and shoot of the plant. Fig. 4.15 (a) has showed that initially the dry weight of roots was very low i.e. 14 mg while when the plant was harvested in the month of July its dry weight was 197.7 mg which shows the maximum growth of plant occurred during the 6 months and high nutrient removal through plant was observed.

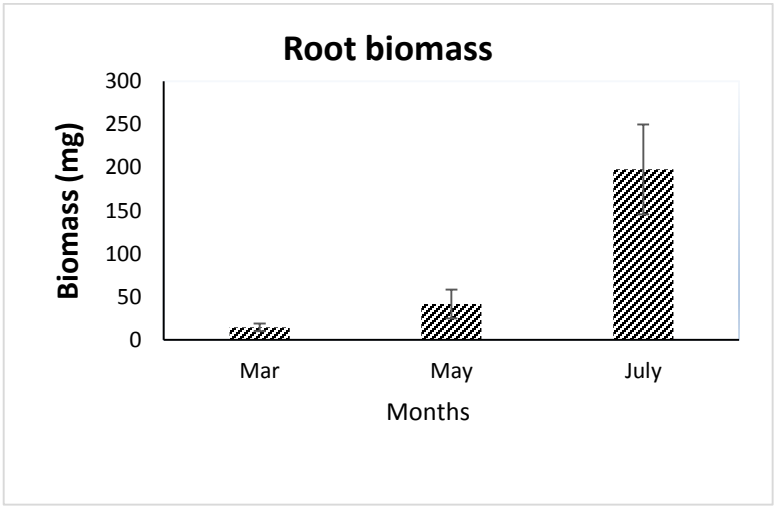

Fig 4.15 (a) Root biomass of *Pistia stratiotes* from Mar to July

Similar results were observed for the dry weight of shoots of water lettuce at initial reading and very low dry weight was measured for smaller plant while for the fully grown plant when harvested has dry weight of 1090 mg was observed in month of July. The comparison of root and shoot dry weights have shown that shoot had more weight and may have ability to remove more nutrients from wastewater. Fig 4.15 (b) shows the shoot dry weight of water lettuce.

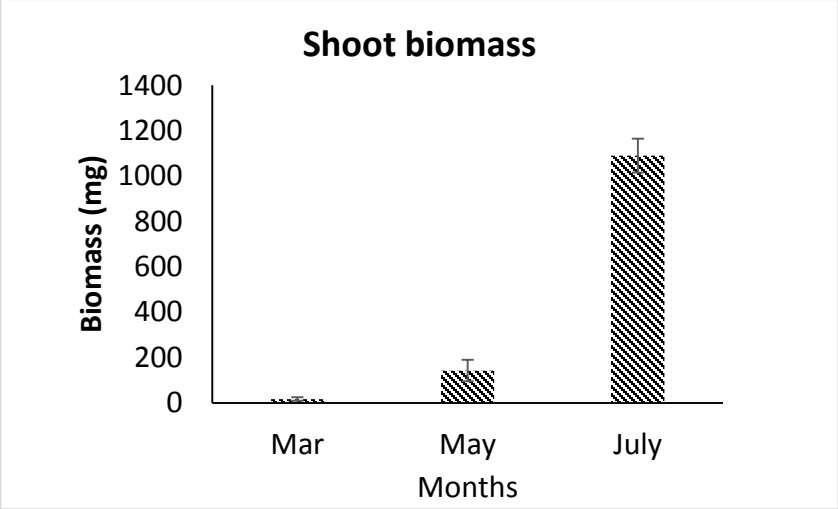

Fig 4.15 (b) Shoot Biomass of *Pistia stratiotes* from March to July

4.3.2 Seasonal Growth Pattern of *Centella asiatica* (pennywort):

Centella asiatica is a type of plant that may grow throughout the year it may neither be affected by winters nor by summers. The plant was planted in tubs at lab scale level in month of February and the initial shoot value was 2.11 cm. In month of July the plant was harvested after six months and maximum shoot value was measured i.e. 165 cm. No. of leaves also increased from 2 to 14 leaves per plant. Fig 4.16 (a) shows the shoot variation between the six month of *Centella asiatica*.

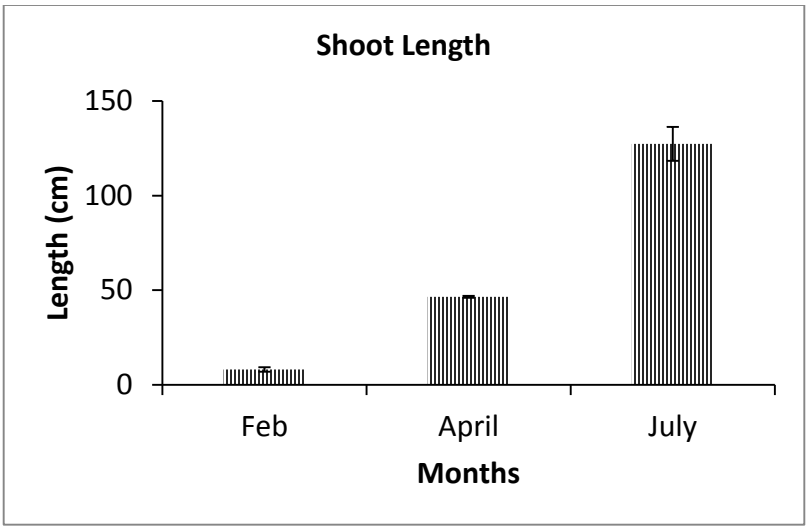

Fig 4.16 (a) Shoot Length of *Centella asiatica* from Feb to July

Centella asiatica is a type of plant with very fine and delicate roots that are white in color. Roots are present at under the joint of stem. Initial reading of roots at the time of acclimatization of plant in month of February was 3.7 cm while when the plant was harvested in month of July the root length was 6.5 cm. This data may show that the nutrient uptake by the roots of the *Centella asiatica* is very low.

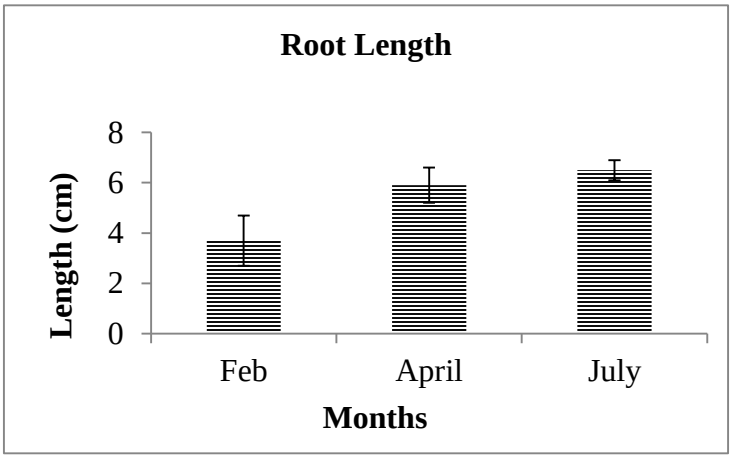

Fig 4.16 (b) Root Length of *Centella asiatica* from Feb to July

Dry weight pattern of WL and PW were almost same. In pennywort the shoot dry weight was high as compared to root weight. The roots of pennywort are quite delicate. Maximum growth by the species observed when the plant was harvested in the month July i.e. 207 mg. Fig 14.17 (a) shows the dry weight of root and (b) shows the dry weight of shoots in mg.

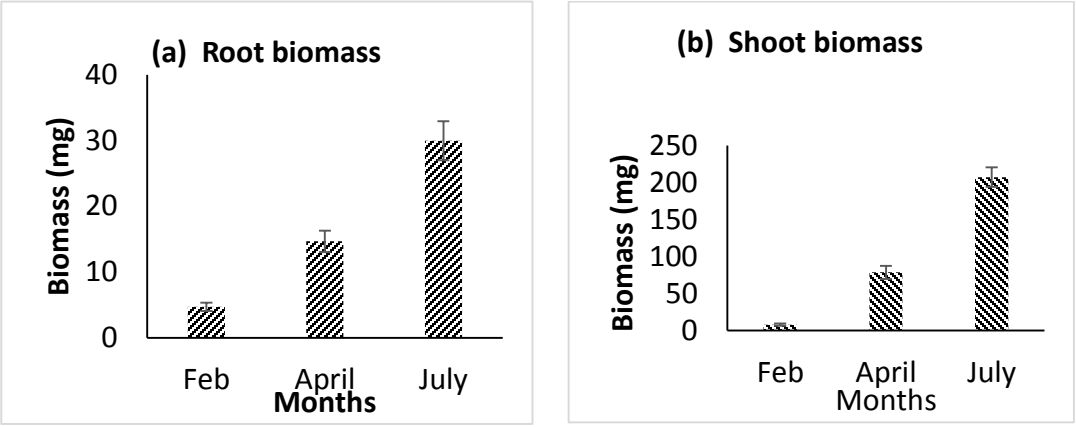

Fig 4.17 (a) Root Biomass (b) Shoot biomass of *Centella asiatica* from Feb to July

4.3.3 Nutrient Removal Analysis

4.3.3.1 Orthophosphate Removal by Pilot and Lab Scale Setup

During inactive growth season, orthophosphate is a type of nutrient which is directly consumed by macrophytes present in the CWs. Phosphate removal may also be done by sorption of phosphate into substrate of constructed wetlands used (Prochaska & Zouboulis, 2006). The removal percentage of orthophosphate has observed in both from lab and pilot scale setup. It is interpreted from the results that the overall removal efficiency of both system was quite low. Highest removal efficiency was measured in the month of July. 27% removal of phosphate was observed in pilot scale setup and 43% in lab scale setup. Probable reason of high removal of phosphorous in summers is that the

growth of macrophytes is maximum in this season. The lowest removal was observed in April i.e. 21.12 at pilot scale and 11.97 in lab scale (Chung *et al*., 2008). Fig 4.18 shows the removal efficiencies of both pilot and lab scale setup.

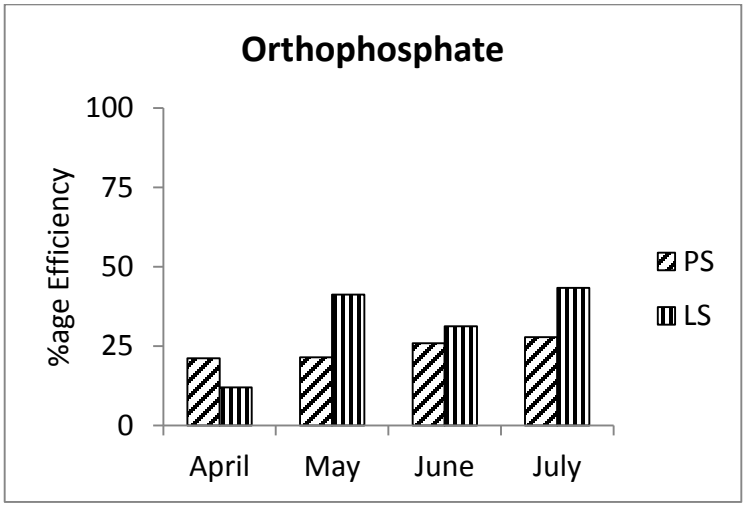

Fig 4.18 Monthly Orthophosphate removal efficiency comparison of pilot and lab scale setup

4.3.3.2 Nitrate-Nitrogen removal

Nitrate in constructed wetland also losses due to the denitrification process of bacterial species but most of the nitrate removal occurred due to the vegetation present in wetlands (Bachand, & Horne 1999). In pilot and lab scale setup the maximum nitrate removal was observed in the month of May. This is because in summers the growth of plants was maximum in summer season. In Fig 4.19 the maximum removal efficiency of pilot scale was 84.58% whereas for lab scale it was 77.7% (Tong & Sikora, 1995).

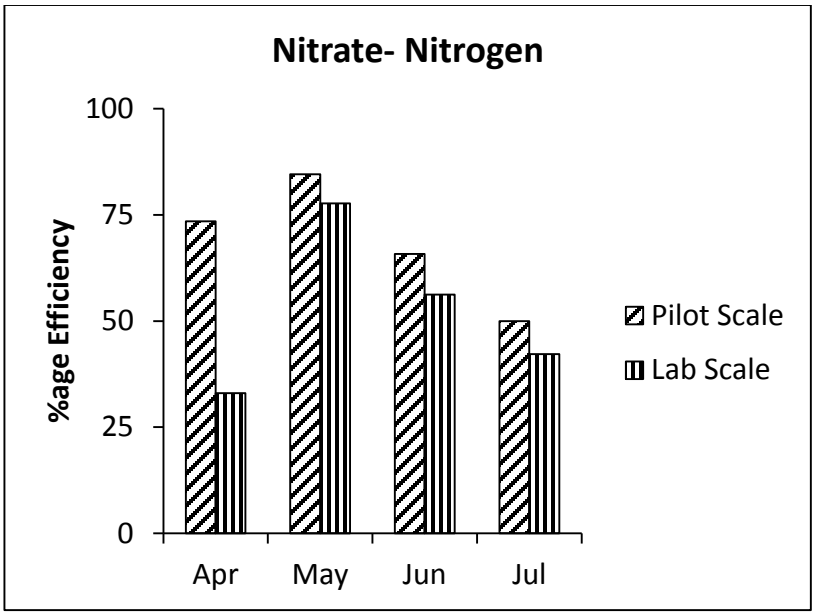

Fig 4.19 Monthly nitrate-nitrogen removal efficiency comparison of pilot and lab scale setup

4.3.3.3 Nitrite-Nitrogen Removal

Nitrite removal from wetlands has been occurring through number of processes either physical or biological. Nitrite in pilot scale setup is not observed or measured as the setup has a direct contact with the environment and no. of bacterial processes including nitrification and de-nitrification. As due to these factors were involved in conversion of nitrite into nitrogen. At lab scale setup very low quantity of nitrite was measured and maximum removal of nitrite was investigated. Upto 93.63% of removal efficiency was measured. This shows that the maximum nitrite is converted into useful nitrogen or atmospheric nitrogen. The following graph shows the nitrite removal efficiency of lab scale.

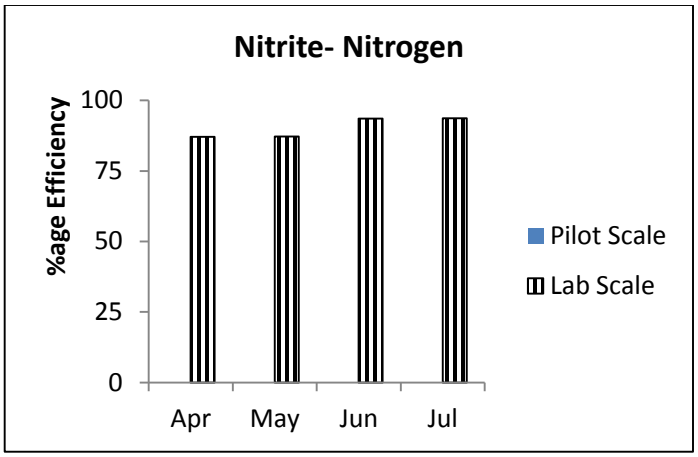

Fig 4.20 Monthly nitrite-nitrogen removal efficiency comparison of pilot and lab scale setup

Ammonia Removal

Removal percentages of both systems showed that, in month of May maximum removal was obtained from both setups. The maximum percentage removal by pilot scale was 74% whereas for lab scale setup it was 79.5%.

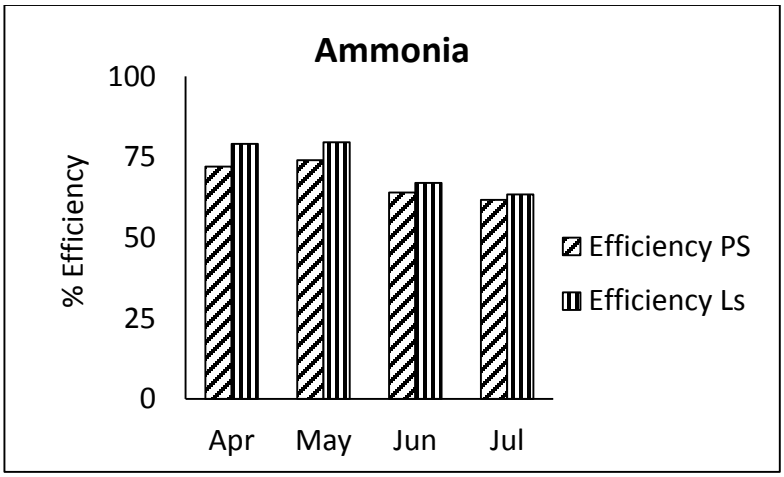

Fig 4.21 Monthly ammonia removal efficiency comparison of pilot and lab scale setup

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Following conclusions were drawn from current study:

Performance efficiency of both pilot and lab scale setup and surface and benthic layer of pilot scale setup was observed.

- Efficiency of treatment system was high during summers as compared to low temperature winters and spring. In pilot scale setup 79.4% removal of TSS and 71.9% removal of COD was observed which is higher than the removal efficiency of lab scale setup. Similarly, in comparison of surface and benthic layer, high removal efficiency was observed in surface layer of the HFCWs at pilot scale.
- Out of 14 strains 10 strains NB01, NB03, NB04, NB06, NB07, NB08, NB10, NB11, NB12, and NB13 belonged to proteobacteria phyla (1 from β-proteobacteria, 1 from α-proteobacteria, and 8 from γ-proteobacteria). While strains NB02, NB05, NB09, and NB14 belonged to the phyla Firmicutes. Thus, proteobacteria is the most abundant phyla which is present in constructed wetlands.
- Growth pattern in plants (water lettuce, pennywort) were high in summers and removal percentage for nutrient parameters (ammonia, orthophosphate, and

nitrate) were higher at pilot scale than that of lab scale because of ideal lab conditions.

Recommendations

- Detailed study on beneficial microorganism may be carried out
- Comparative study may be analyzed with other wastewater treatment system
- For better understanding of degradation process of contaminants in CWs degradation pathways may be studied.

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