ISOLATION OF MICROBIAL COMMUNITY AND EVALUATION OF PERFORMANCE EFFICIENCY OF PHYTOREMEDIATION PLANT

By

Abeerah Shahid

(NUST201362285MSCEE65213F)

A thesis submitted in partial fulfillment of requirements for the degree of

Master of Science

In

Environmental Sciences

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan

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Has been found satisfactory for the requirements of the degree of
Master of Science in Environmental Sciences

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This thesis is dedicated to Ami Abu, my brothers and adii 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: ***Say: Praised be Allah!*** *[27:59] and:* ***If you would count the blessings of Allah you would not be able to reckon them,*** *[14:34] and:* ***Of the blessings of your Lord, speak out,*** *[93:11] and:* ***Remember Me, and I will remember you, give thanks to Me,*** *[2:152]. 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. Heartfelt gratitude to* **Dr. Imran Hashmi***, Associate Dean, IESE, SCEE, NUST. His invaluable guidance and encouragement remained with me throughout this research and encouraged me in removing the obstacles that came in my way during this strenuous work. I would like to thank my Guidance and Examination Committee (GEC)* **Dr. Muhammad Arshad,** *Associate Professor, IESE, SCEE, NUST and* **Dr. Hamza Farooq Gabriel,** *Professor, NICE, SCEE, NUST who had always been very supportive in my research work. Their support and encouragement made me confident.*

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ABEERAH SHAHID

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LIST OF ABBREVIATIONS

mg/L	Milligram Per Liter
μS/cm	Microsiemens Per cm
MGD	Million Gallon Per Day
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine
EPA	Environmental Protection Agency
EC	Electrical Conductivity
DO	Dissolved Oxygen
TSS	Total Suspended Solids
TDS	Total Dissolved Solids
TP	Total Phosphates
COD	Chemical Oxygen Demand
rRNA	Ribosomal Ribonucleic Acid
EMB	Eosin Methylene Blue
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
WASA	Water and Sanitation Agency
CW	Constructed Wetland
CFU	Colony Forming Unit

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ABSTRACT

Water pollution has always been a matter of environmental concern around the sphere. With water shortage throughout world appropriate methods for water treatment are key objective for sustaining limited water resources. Constructed wetlands propose an effective mean for reducing environmental pollution by utilizing wetland vegetation. The present study was conducted at NUST H-12 to evaluate performance efficiency of constructed wetland and exploring the endophytes assisting phytoremediation. Selected macrophytes were *Pistia stratiotes* (water lettuce) and *Centella asiatica* (Pennywort). The evaluated water quality parameters were; Chemical oxygen demand (COD), Total suspended solids (TSS), Total dissolved solids (TDS), Electric conductivity (EC), pH, Total phosphates (TP), Dissolved oxygen (DO), Temperature, Total *coliforms* and Fecal *coliforms*. For isolation of endophytes roots and leaves of water lettuce and pennywort were explored. Water quality parameters were measured at four units (pilot scale unit, Lab scale unit, parallel unit and control unit. Results from 6 months studies depicted removal efficiency was high in summers due to improved plant growth. Highest rainfall was recorded for month of March as a result effluent concentration was considerably low. In all cases removal efficiency of parallel unit was found comparatively high due to more retention of wastewater. For both plants most significant removal efficiencies were recorded as TSS (62 and 83 %) DO (53 and 64 %) COD (74 and 87 %) TP (64 and 81 %) and Total *coliforms* (99 and 98 %). Results illustrated *Centella* was more effective phytoremediation agent. 10 microbial strains were isolated from *Pistia* and *Centella* and were identified by gene sequencing. Predominant genera reported were *Bacillus and Pseudomonas.* Results signified that a well-constructed and operated wetland is capable of enhancing water quality. Technical feasibility of CWs may be demonstrated by Pilot scale system making them suitable technology for Pakistan.

Chapter 1

INTRODUCTION

1.1 Background

Water is an irreplaceable and unique element. Everyone needs clean and fresh water to drink or for recreation. Polluted water loses its value aesthetically and economically, also it may become a hazard to health and environment. Water may renew naturally and cleanse itself, by permitting impurities to settle out (through sedimentation), or either by diluting the contaminants to a level where they are not in dangerous concentrations. Still, this natural progression demands time, and is challenging when large quantities of contaminants enter water bodies (Macedonioa *et al.,* 2012).

When harmful constituents enter lakes, streams and other water bodies, they dissolve, remain suspended at time in water or even settle in the bed. This results in pollution triggering the water quality deterioration. Nearly 95 % of the industrial waste and approximately 90-95 % of domestic sewage come from the urban areas into the fresh water reserves without any prior treatment (Sharma *et al.,* 2012).

Water pollution is a major environmental and social concern. Wastewater can be defined as a combination of water-carried waste or liquid removed from, institutions, commercial formations and residencies or other related facilities, industrial wastewater that covers water from industries such as iron steel etc., infiltration/inflow water which is unnecessary water that passes into the sewer system either by direct or indirect means through cracks, porous walls or leaking joints, Inflow is storm water that passes the sewer system from, roof headers, storm drain connections, basement drains or foundation., and storm water that results in runoff due to rainfall flooding (Azizullah *et al*., 2011It largely encompasses a high load of disease-causing or pathogenic waste, oxygen demanding wastes, plant growth stimulating nutrients, organic materials, minerals and inorganic particles. Discharging of wastewater without proper treatment into the environment have adverse health and ecological impacts. Environmental Protection Agency (EPA) have set it mandatory to treat wastewater before discharging it into the environment. Industries are major polluters of environment. Disposal of treated wastewater below discharge standards from households or other units can result in adverse soil pollution and surface water contamination (Earnhart, 2013).The capacity of Water and Sanitation Agency (WASA) has limited number of wastewater treatment plants and need specialized input to enhance their capacities.

1.2 Domestic Wastewater and Its Composition

Wastewater used by community is acknowledged as Domestic wastewater as it encompasses all the constituents added to the water throughout its use. It is mainly composed of wastewater resulting from laundry, personal washing, cleaning of kitchen utensils, food preparation and the human body wastes (feces urine and feces). Such compounds are an excellent diet source for bacteria, micro-organisms whose insatiable appetite is exploited by public health engineers in the microbiological treatment of wastewater. In addition to multiple chemical compounds it also contains many masses of intestinal bacteria and other smaller numbers organisms. Domestic waste also contributes varied variety of chemicals such as soaps, fats and grease, detergents and pesticides, including anything that passes out from kitchen sink, such as vegetable peelings, sour milk, soil particles and sand etc. The list of variable chemicals in domestic wastewater is so extensive that it is not possible to quantify them (Micheal *et al.*, 2009).

1.2.1 Adverse Environmental and Health effects

Domestic wastewater contains suspended solids, pollutants in true solution as well as colloidal or non-settle able suspension. It has hazardous content and objectionable appearance. Human and animal fecal waste contains disease carrying pathogens that are responsible for number of diseases like cholera or typhoid. The existence of various solids and organic solvents as well as augmentation in algal growth results in decrease in dissolved oxygen (Mara, 2003). In few words urban runoff carry away a range of pollutants including sediments. Pathogenic liberation from the municipal plants and improperly treated sewage causes infected water sources, and adverse human health (Helmer, 1997).

1.3 Advances in Wastewater Treatment

Untreated wastewater is a major threat to human health and the environment. It is essential to treat wastewater in order to minimize the transmission of pathogenic diseases and to minimize water pollution and its resulting damage to biota. The elementary purpose of the wastewater treatment systems should be to accelerate the natural processes by which water filters itself. In past years, the natural process for treatment in lakes and stream was suitable to execute simple wastewater treatment. Early struggles in water pollution control prohibited human waste from discharging into water bodies or by minimizing floating debris. In recent year, industrial growth and population have amplified demands for natural resources (Ahuja, 2013).

Water pollution concerns now govern public concerns regarding monitoring healthy ecosystems and water quality. Although current investment in water pollution control has assisted in reducing the problem, a large portion of water bodies are still affected. In developing countries wastewater treatment methods are limited due to costly treatment processes, lack of awareness for environmental protection and lack of implementation of environmental laws.

Altering the situation in which development for abating pollution requires multiple technologies. Past methods used to reduce water pollution must be altered to resolve current and evolving issues (EPA, 2012). Considerable advances have been made in the field of wastewater purification technology, and presently it encompasses a range of low tech as well as high tech solutions. Basic wastewater systems include a combination of chemical, biological and physical operations and processes to remove organic matter, solids, and rarely nutrients from wastewater.

1.4 The Present Study

In the present study, water samples were collected from selected plants ponds constructed wetland and lab scale setup established at National University of Sciences and Technology (NUST) and analyzed for changes in the physicochemical and microbiological parameters to measure the performance efficiency of plants in series system and parallel system. Roots and leaves of selected plants from wetland were extensively studied for isolation of endophytes that contribute in phytoremediation process. Biochemical analysis and gene sequencing were executed to identify predominant microorganisms.

1.5 Aims and Objectives

Aim of study was to investigate the possibilities of using CWs for secondary and tertiary treatment of wastewater. By exploring endophytic diversity and role of plants this study can contribute in developing a vibrant understanding of phases involve in CWs. Objectives of study were:

1) Evaluating performance efficiency of selected plants in series and parallel system

2) Investigating comparative efficiencies of selected plants

3) Isolation of endophytes from roots and leaves of selected plants

Chapter 2

LITERATURE REVIEW

2.1 Conventional Methods for Wastewater Treatment

For several years, the central goal for treatment of domestic wastewater was merely to lessen its content of oxygen-demanding substances, suspended solids, dissolved inorganic mixes, and pathogenic bacteria. In previous years, trend has been shifted towards improving methods for the municipal treatment practices. Conventional wastewater treatment consists of a combination of physical, chemical and biological processes and operations to remove solids, organic matter and, sometimes, nutrients from wastewater.

General terms used to describe different degrees of treatment, in order of increasing treatment level are preliminary, primary, secondary, and tertiary and/or advanced wastewater treatment (Sonune and Rupali, 2004). A wide range of other treatment methods including stabilization pond systems, septic tanks, activated sludge, trickling filters, anaerobic systems, activated carbon absorption and land application systems are used in developing countries (Rashidi and Ali, 2015). In comparison to basic techniques to achieve advance treatment for further removal of nutrients demands a very high constructional and operational cost. Exploration for a viable cost-effective approach for absolute removal of effluent and nutrients has triggered renewed concern for natural systems such as microbial application for treatment of wastewater that cannot be attained by conventional wastewater treatment facilities (Sperling and Marcos, 2008).

2.2 Biological Treatment of Wastewater

Bioremediation is a natural process that employs microorganisms for elimination and reduction of harmful or hazardous waste at contaminated or affected site. One significant aspect of bioremediation is, it does not require sterile environment and can be carried out in open settings with variety of microorganisms (Surajit and Hirak, 2014).

2.2.1 Phytoremediation

The direct use of living green plants for the detoxification or immobilization of pollutants from environment such as water or soil or sediments is called phytoremediation. Number of process are mediated by plants that are helpful in contaminant degradation and removal; rhizodegradation is the process in which microorganisms enhances biodegradation in below ground root region, phytodegradation involves uptake and breakdown of contaminants below or above ground within the root, stems, or leaves, phytoextraction allows removal of contaminants by uptake and accumulation., rhizofiltration involve adsorption of contaminants on roots that leads to its removal, phytovolatilization of contaminants after degradation uptake and volatilization of contaminants (Morikawa and Hiromichi, 2003). Figure 2.1 depicts multiple phases of phytoremediation process.

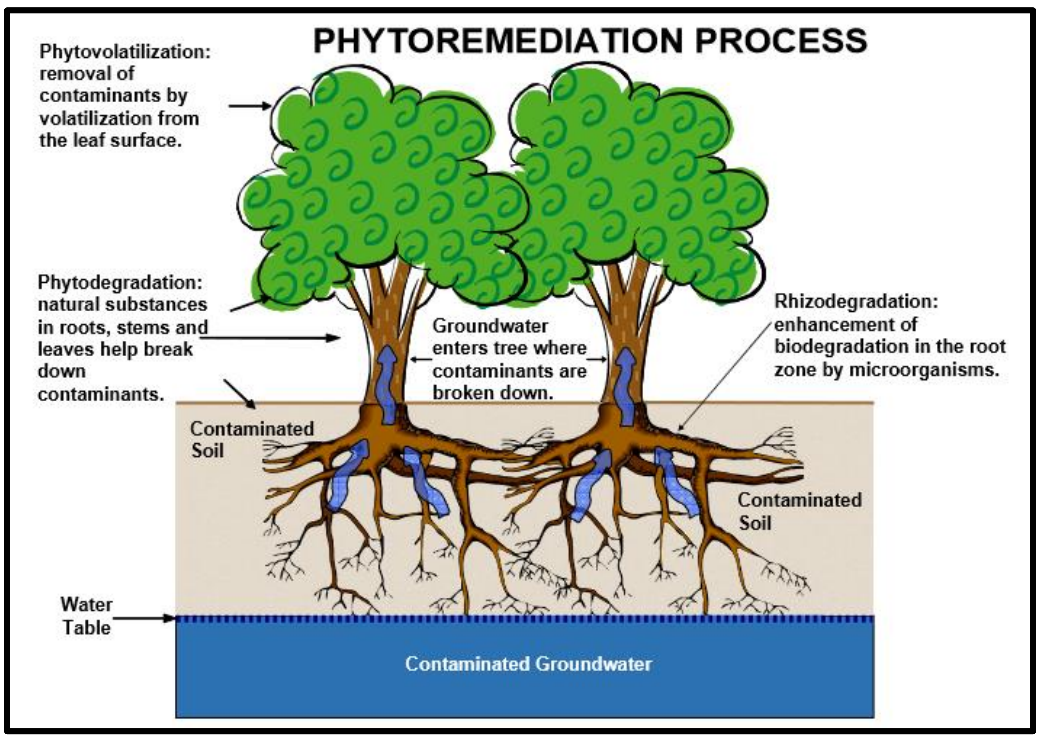


Figure 2.1: Schematic diagram depicting multiple phytoremediation process (Zhang *et al*., 2014)

At present most common engineered system that forms its bases from phytoremediation are constructed wetlands (Paul *et al.*, 2003).

2.3 Natural Systems: Constructed Wetlands

Natural systems depends on naturally biological transformation mechanisms for pollutants. These systems are assisted by solar radiation, mixing, natural aeration and potential energy in soil and biomass for storage (Selma and Ayaza, 2001). The most significant dynamic of natural systems is resource conservation as compared to conventional treatment process that demands high cost of operation and energy consumption. Some of natural treatment methods can be listed as composting, aerated lagoons, oxidation ponds and constructed wetlands. However with respect to comparative preference the most effective and demanding natural treatment systems, is constructed wetlands (Zhanga *et al*., 2010). Constructed wetland (CW) are engineered treatment systems that have been constructed and designed to take advantage of natural processes comprising wetland, soils, vegetation and their linked microbial community to assist in wastewater treatment. They are engineered to utilize several processes that take place in natural wetlands, but perform so within added controlled environment (Vimal *et al*., 2015).

Wetlands signify a type of natural system that uses the ability of variety of flora and fauna, and a very small energy input, to stabilize and remove pollutants from water. The U.S. Fish and Wildlife Service defines wetlands as "lands transitional between terrestrial and an aquatic system where the water table is usually at or near the surface or the land is covered by shallow water with attributes at least periodically, like the land supports predominantly hydrophytes; the substrate is predominantly un drained hydric soils; or the substrate is non soil (organic matter) with water or covered by shallow water for some time during the growing season each year" (US Fish and Wildlife Service, 2000). Few advantages of these systems include relatively untrained personnel for maintenance, lower or zero energy requirements, easily established at site of waste water production and low cost system (Chorng *et al.*, 2011). Plants are most significant element of wetland systems. The mechanisms and processes by which macrophytes assists in water treatment in CWs are still under debate (Miklas and Shcolz, 2006).

Plant contribution in enhancing CW performance depends on number of factors such as CW type (e.g., horizontal, vertical, surface flow, subsurface flow, with or without recirculation), wastewater load quantity and quality, plant species and with their combinations, medium type, climate as well as plant management (Brisson and Florent, 2009).

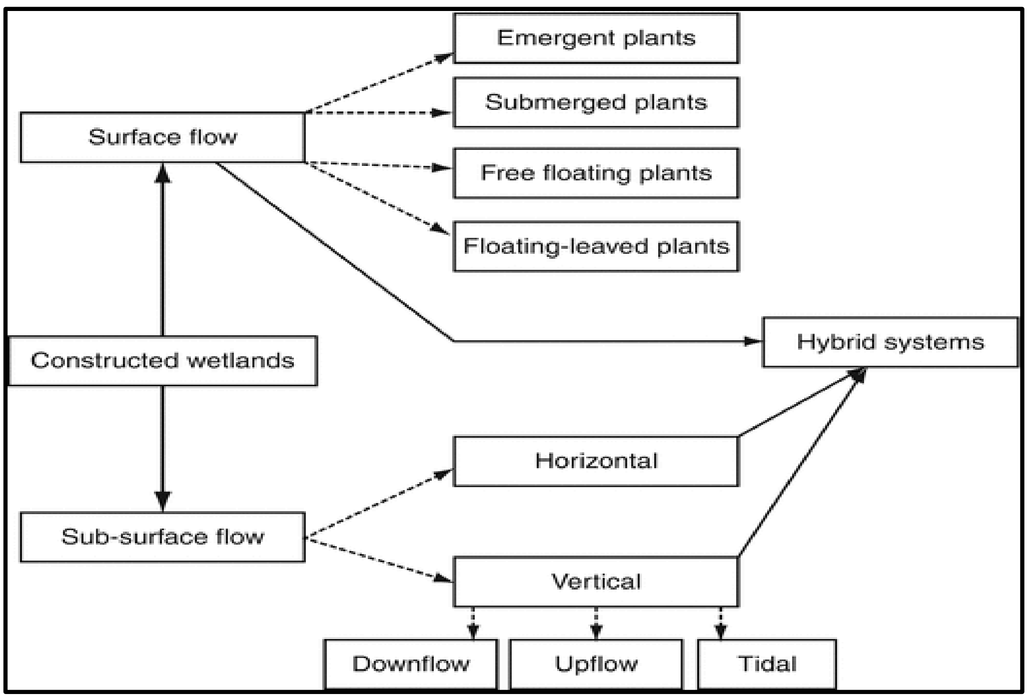


Figure 2.2: Classification of constructed wetland for wastewater treatment (Vymazal, 2007)

2.3.1 Types of Constructed Wetlands

Constructed wetlands are intended to take advantage of variety of processes in natural wetlands but in presence of controlled environment. The most basic type of classification for these natural systems is on basis of water flow types.

Water Surface Systems (FWS)

These systems normally consist of channels or basins, provided with additional subsurface barrier to avoid leaching. It consists of a suitable medium such as soil to support the growing vegetation. Water flows at comparatively shallow depth. These characters control water flow in narrow channels (Vymazal, 2009).

Subsurface Flow Systems (SFS)

These systems mainly use rock medium provided with emergent plants and an extensive root system embedded in the media. Media may also include sand or soil media. Wastewater treatment subsurface flow constructed wetlands having gravel as media and emergent macrophytes treat wastewater with complex biological, physical and chemical reactions. Subsurface flow wetlands are categorized as horizontal flow and vertical flow constructed wetlands.

I. Horizontal Flow Systems (HFS)

In this system wastewater is fed at the inlet and it moves horizontally through the bed parallel to surface into the outlet.

II. Vertical Flow Systems (VFS)

In vertical flow system, the effluent flows vertically through planted layer down from substrate and moves out (Vymazal, 2007).

2.3.2 Potential for Application of Wetlands in Developing Countries

Constructed wetlands are extensively accepted and used due to their cost effectiveness throughout world. As it is an exceptional technology it may be applied in industries or communities that have access to vast land. So far limited information has evolved regarding application of wetland in developing countries. Research on evaluation of efficiency of wetland has also helped in obtaining engineering data related to physicochemical and biological constrains. The observed benefits of Constructed Wetlands (CW) in developing countries include economical maintenance and operational capital cost and discharge standards established by environmental regulation bodies can be achieved (Kivaisi and Amelia, 2001). Table 2.1 represent status of CWS around the world:

Region	Status of Constructed Wetlands (CWs)
United states	Phytoremediation is given the name of green revolution. According to US EPA It has potential to decontaminate an estimated 30,000 sites polluted by mining companies, electroplating and battery manufacturers.
Europe	Limited Application of phytotechnologies. Mostly private companies have been established to use plant resources for pollution control. European Commission has initiated funded projects for keeping interest in this field
Asia	In **India** Pariyej resrvior a "Wetland of international importance" has facilitated in heavy metals degradation through accumulation in certain macrophytes. Species such as *Ipomoea aquatica* and *Typha angustata* have been planted at multiple sites as bioremediants. In **Pakistan** heavy metal pollution is of highest concern for this CWs has been established on small scale near multiple sites. In **China** water hycanth is extensively used in CWs and for biogas production.
Australia	Major concern is Organic pollutants degradation and immobility of contaminants. Plants are successfully used as biopumps. A number of Universities and organizations such as Phytolink Australia are dedicated to restore environment.
South Africa	Industrialization has created an alarming situation. According to a study recently about half million trees have been planted recently based on plant technologies. Since 2000 number of studies have focused on different vegetation for effective remediation of ground water.

Table 2.1: Status of CWs around the world (Zhang *et al.*, 2014)

2.4 Plants Selection Criteria

Plants used in constructed wetland are acknowledged as macrophytes and emergent hydrophytes. Large portions of macrophytes such as leaves and flowers emerge above the medium and are wide-open to air, however their rhizomes and roots remain immersed below the media and water. Selection of plants is based on pollutants of concern, the extent to which they can exploit the environment and on requirements of treatment level (Buhpindr *et al.,* 2009). Native species are always preferred in order to avoid invasive species. Other than this, vegetation should be resistant to changing weather, fast growing and easy to maintain. Selected plants should have extensive root system throughout contaminated area (Stottmeister *et al*., 2003). Most of the plant functions are well-defined while others are extremely debatable.

One feature that is past any argument is the aesthetic appeal that macrophytes provide by covering and screening the wetland bed and through monitoring odors. Macrophyte cover also bounds the amount of surfacing water on the bed that provide breeding environment for insects such as mosquitoes. The second unsettled part of plants in constructed wetlands is their capacity to stabilize and increase the hydraulic conductivity of medium (Anjuli *et al*., 2012). This is of special significance. Few of common species of macrophytes are listed below:

2.4.1 Typha and Phragmites

Most commonly used macrophytes are typha and phragmites. These plants provide additional surfaces to bacteria in roots and rhizomes for discharge of gaseous byproducts. These plants have fast growth and have the potential to thrive in extreme temperatures. They provide proper insulation and shading of surface and help in evapotranspiration. *Phragmites australis* (Common Reed) and *Typha latifolia* (Common Cattail) are large persistent grasses that are native to wetland sites in tropical and temperate areas of the world. They produce a large annual biomass usually provide small potential for Nitrogen and Phosphorus removal (Vymazal, 2007). Calherios *et al* treated industrial wastewater in CW planted with phragmites and typha. COD was removed by 92% at outlet and BOD was removed by 88% (Calheiros *et al.,* 2009).

2.4.2 Duckweed, Water lettuce and Pennywort

Lemnoideae (Duckweed) are widely distributed small free floating plants. Duckweed falls among most fast emergent plants. If provided with optimum condition may grow 2-3 days. Duckweed show a decrease in growth at temperature below 17 ºC but can tolerate a varied range of pH (3.0-10.0). They lack deep root system therefore provide less surface attachment for microbes. Duckweed has tendency to accumulate wide range of mineral nutrients. *Pistia stratiotes* (water lettuce) is light greenish-yellow shell like plant (Jerry *et al*., 2001). A cluster of long fibrous unbranched roots outspreads from underwater stolon in form of central extension. They grow better in normal or still water and is frost sensitive. *Centella asiatica* (Pennywort) are considered effective for phosphorus removal in summer however the removal potential can drop to even 50 % winters. It is considered to have high assimilative capacity for nutrients (Gabriela *et al*., 2005).

2.4.3 Other Macrophytes

Some of the other macrophytes used are: *Eichhornia crassipes* (Water hyacinth) which is an aquatic weed plant, *Hydrocotyle umbellate* (Manyflower) grow as floating mat along the bank of canals or ponds (Oren and Amit, 2013). Phytoremediation is more than acclimatization and harvesting of selected macrophytes. The site must be designed in a way that prevent flooding or erosion (Tanveer *et al*., 2015). Plants eliminate pollutants when their roots intake nutrients and water from polluted environment. Pollutants are removed to the level at which plant root may be extended. Three main planting practices can be adapted for phytoremediation:

1. When pollutants are with plant root zone plants should be grown on land, usually 3-6 feet.
2. Vegetation type selected for wetland should rely on climate, hydrological condition and geographical location.
3. It is convenient to use diverse species of plants rather than single species. It is difficult to establish some species due to their temperature sensitivity or slow growth. For a specific region native species are better suited (Terrance *et al.,* 2014).

2.5 Microbes and their Role in Phytoremediation

One key limitations in phytoremediation is that due to toxicity of contaminants macrophytes which are resistant to presence of various pollutants usually remain comparatively small as they are accumulating or degrading the product. In previous years attention has been shifted in understanding plant microbe relationship and their role in phytoremediation. Group of bacteria residing inside plant and living harmlessly without causing apparent symptoms of disease are called endophytes (Lee *et al*., 2005). They colonize healthy plant tissue either intracellularly or intercellularly and can be facultative or obligate. With the exclusion of seed endophytes, the main site from where endophytes access their entry into plants is through roots. Their association and interaction with plants are complex. They exhibit complex interactions with their hosts which involves mutualism and antagonism. Plants have limited growth of endophytes and they use different mechanisms that make them gradually adaptive to environment (Anna *et al.,* 2003).

Endophytes have been isolated from many species of plants however their densities varies with highest in roots and decrease through stem to the leaves. Most common genera that have been isolated form plants include *Enterobacteriaceae*, *pseudomonadaceae* and *Burkholderiaceae*. Detailed studies have shown that endophytes microbial community may have the tendency to control insect, nematodes and pathogens. They have also played significant role in adverse conditions by promoting plant growth accelerating seedling emergence and enhance plant growth and development in general, they also enhance plant development, increase resistant to extreme conditions (heat, contamination or drought), reduce disease severity and enhance nutrient uptake. Some endophytes also have the ability to provide Nitrogen to plant (Weyens *et al*., 2009).

In the recent years, research has focused on exploiting the benefits of endophytes to overcome the constraints. Plants in wetland act as source of oxygen to the heterotrophic bacteria present in rhizosphere therefore allowing nitrification and aerobic degradation of contaminants especially organic matter. Plants like other aerobic organisms need oxygen especially from phytotoxins in roots. Around rhizomes and roots aerated micro zones are formed which provide suitable conditions to aerobic microbes in anaerobic environment for biological (Nele *et al.,* 2009). After uptake of pollutants such as organic compounds may be either metabolized or released through evapotranspiration from leaves or stem. Normally end products of metabolism are water, cellular biomass and water. Other Phytoremediation linked advantages allied with endophytic microorganisms are as follows:

1. Efficiency of remediation mechanism can be assessed by quantitative and computable gene expression of microbial contaminant catabolic genes.
2. It is easier to undertake genetic engineering of microbial catabolic pathway rather than pathway of plants.
3. Endophytic bacteria degrade toxic pollutants and convert them into products that have a reduced toxic effect for environment (Zareen, 2011).

To summarize, it is clear that microbe and plant partnerships are very significant for an effective removal and remediation of contaminants. Although plant-endophyte associations remains challenging however with advances in technology and research problems regarding lack of knowledge in this field can be overcome.

2.6 Water Quality Parameters

Water quality may be defined as physical, biological and chemical characteristics and features of water. Water quality and analysis is an important part of environmental parameters. Physical parameters for water quality include turbidity and temperature (Iheanyi *et al.*, 2008). Chemical properties include parameters like dissolved oxygen (DO), chemical oxygen demand (COD), Suspended solids (SS) and pH. Biological pointers of water quality include coliforms and algae. These parameters are not only related to surface water studies of lakes, rivers and oceans but also to industrial processes and groundwater (Munavalli and Mohan, 2003). The following section details out all parameters affecting water quality:

2.6.1 Electric Conductivity

Capability of water to allow flow of current is called as electrical conductivity (EC). It is direct measure of number of ions in water. These ions come from presence of dissolved inorganic solids or salts like, sodium, calcium, phosphates, nitrates, chloride or iron etc. Presence of these solids in water increases its conductivity.

The fewer these conductive ions are less will be conductivity. EC is generally measured in milli or micro siemens per centimeter (mS/cm or µS/cm) (McCleskey and David, 2011).

2.6.2 Dissolved Oxygen

It is defined as the level of non-compound and free oxygen in water. Sources include aeration, surrounding air and as the main byproduct of photosynthesis.

$$\text{Carbon dioxide} + \text{Water} \longrightarrow \text{Oxygen} + \text{Carbon-rich foods}$$

$$CO_2 \quad H_2O \qquad O_2 \quad C_6H_{12}O_6$$

It is one of the most important quality parameter as it effects living organisms in water bodies. Very high or low DO can affect living organisms. Warm water always has low amount of DO as the microorganism overpopulate using more DO. High concentration of fertilizers also results in DO reduction. Normally, DO levels fewer than 3 mg/L are stressful and harmful to many aquatic organisms whereas DO level of 7.0 mg/L or more are preferred to sustain aquatic ecosystem. In general DO is measured in mg/l (Enrique *et al*., 2007).

2.6.3 pH

Presence of hydrogen ions (H^+) and hydroxide ions (OH^-) defines acidity and basicity of water. It is determined on basis of a defined scale ranging from 0-14 where 0 being extremely acidic, 7 being neutral and 14 being highly basic. If pH of water body is too high or low aquatic organisms can die. pH also affects toxicity and solubility of chemicals. The preferred pH range is 6.5-9 however the presence of dissolved substances cause shift in pH (Jianquan and Luhui, 2011).

2.6.4 Temperature

It represents physical character of water expressing how cold or hot it is. It may also be defined as average thermal energy of any body. Temperature of water fluctuates frequently and can be seasonal or even hourly (Wasala *et al*., 2012). It is an important parameter as in addition to its own effect it influences other parameter i.e. conductivity, DO or pH. Factors that influence temperature include color of water, temperature of contaminants released in water and amount of shade received by vegetation (Lishman *et al*., 2012).

2.6.5 Total Solids

It is sum of total suspended solids (TDS) and total dissolved solids (TDS) present in water**.** Total suspended solids are defined as particles that are larger than 2 microns. They are usually the inorganic materials or plant decay that after decomposition enter in water column as TSS. They are important parameter as water clarity depends on them. More are the suspended solids water will be less clear (Anoop *et al*., 2013). Total Dissolved solids include all ion particles that are less than 2 micron. They may include dissolved organic matter and salt ions in wastewater. TDS are measured in mg/l (Maniosa *et al*., 2003).

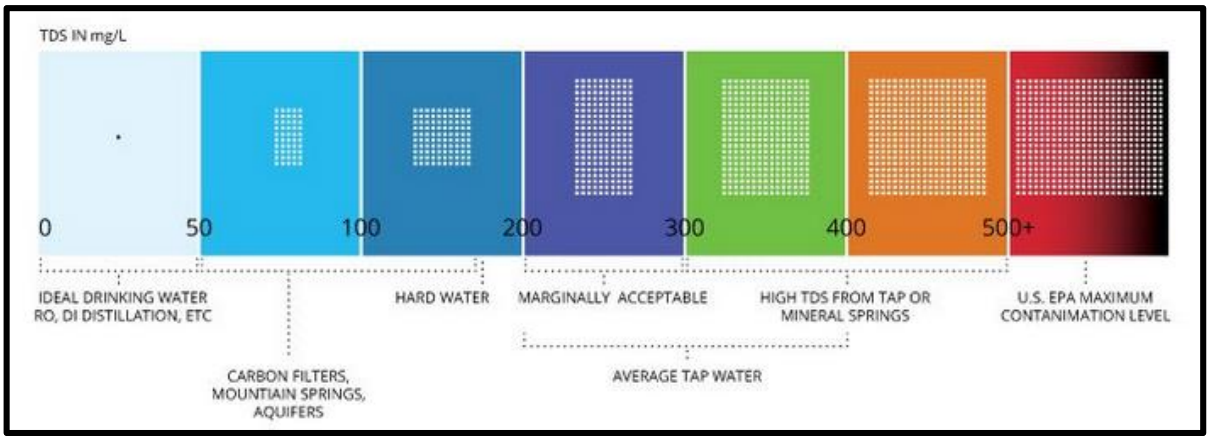


Figure 2.3: EPA recommend an upper limit of 500 mg/L TDS (Atkinson and Brando, 2008)

2.6.6 Chemical Oxygen Demand

The chemical oxygen demand (COD) is a valuable factor and is used in association with biological oxygen demand (BOD) (FuLijun. *et al* 2008). It is used as a measure of the oxygen that is equivalent to content of organic matter of a given sample that is inclined to oxidation through strong chemical oxidant. It is measure of organic matter because it most common content oxidized by DO (Guang and Chen, 2005).

2.6.7 Total Phosphates

For growth of plants and animals phosphorus is an essential nutrient. In water it is present in form of inorganic or organic phosphates. Inorganic phosphate is readily available to plants in general and may be considered as good indicator of problems associated with excessive growth of plants or algae (Claude *et al*., 2012).

Total phosphate/ phosphorus	Effects
0.01-0.03 (mg/L)	Amount of in most uncontaminated lakes
0.025 (mg/L)	Accelerates the eutrophication process in lakes
0.1 (mg/L)	Recommended maximum for rivers and streams

Table 2.2: Phosphate-phosphorus levels and effects (Claude *et al*., 2012)

2.6.8 *Coliforms*

Animal or human waste discharged into waterbodies is source of disease-causing or pathogenic viruses or bacteria such as *Escherichia coli* or fecal *coliform* bacteria. They are considered as indicator of fecal contamination. Other coliforms include species such as *Citrobacter*, *Klebsiella* and *Enterobacter* responsible for number of diseases. They are measured in per 100 ml of water as number of colony forming unit (CFU) (Abbas and Manny, 2003).

Chapter 3

METHODOLOGY

3.1 Study Site

Realizing the need and importance of the wastewater treatment, NUST Institute of Civil Engineering (NICE) was assigned the project entitled “Application of FILTER Technology for Wastewater Treatment - Pilot Study at NUST Islamabad Campus” by the United Nations Educational, Scientific & Cultural Organization (UNESCO). Objective of study was to evaluate performance efficiency of phytoremediation plant at pilot and lab scale unit, comparing the efficiency of all units and to study microbial community structure within selected plants. Installed phytoremediation plant has the capacity to treat water from one sewerage line that is 0.1 Million gallon per day (MGD).

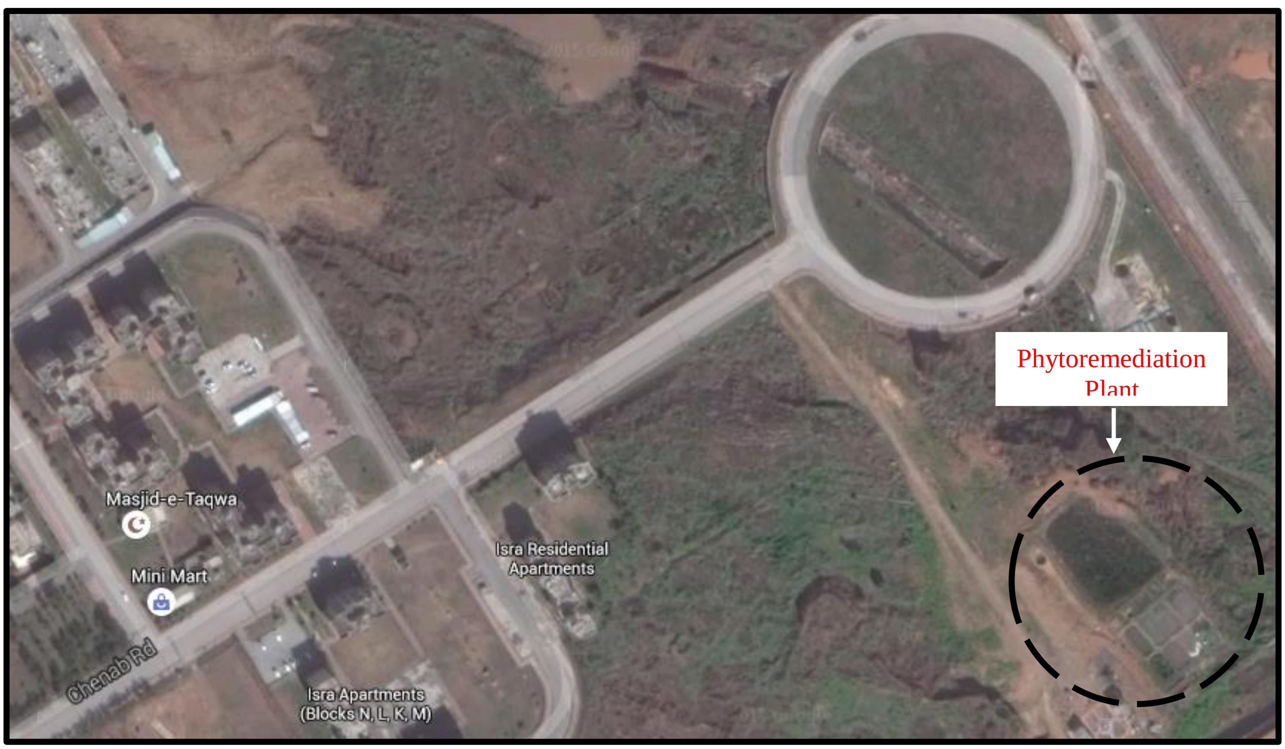


Figure 3.1: Layout of NUST

*Encircled point indicates study site

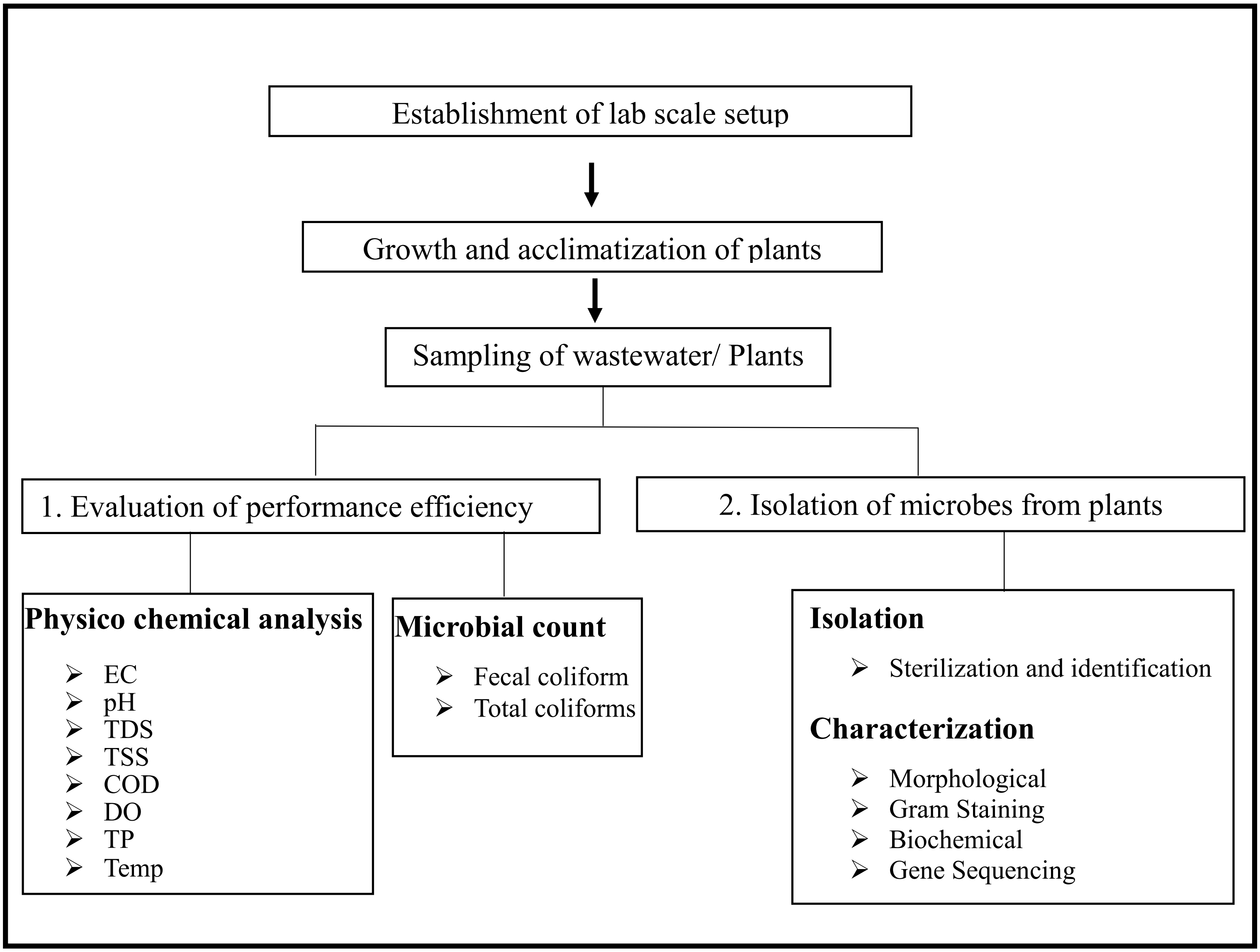


Figure 3.2: Schematic layout of methodology

3.2 Growth and Acclimatization of Plants

Plants selected for evaluating the efficiency of phytoremediation plants and studying the microbial community structure were *Pistia stratiotes* (Water lettuce) and *Centella asiatica* (Pennywort).

Plant species were collected from a local nursery. Plants were grown in hydroponic culture for three weeks. For fast and uniform growth plant species were supported by a layer of clay and uniformly sized cut thermocole sheet. During acclimatization phase system was supplied with reservoir water. Uniformly sized and healthy plant species were obtained after completion of acclimatization phase

3.3 Establishment of Pilot and Lab scale setup

Efficiency of Phytoremediation plant was evaluated in three main phases:

3.3.1 Pilot Scale Unit

A constructed wetland for the treatment of wastewater was established at NUST H-12. Treated water could be used for horticulture purpose. Wastewater from residential colony, offices and student hostels was directed toward sedimentation tank from where it is directed towards eight ponds connected in series for treatment. Quality of treated water was further enhanced by filter irrigated with *Typha latifola*. Tile drainage system was used for collection of water from filter. Sludge (solid waste) was separated at sedimentation and was dried for use as a fertilizer. Each pond was coated with plastic sheet to avoid infiltration. Ponds were connected in series and bed preparation consist of organic soil and gravel.

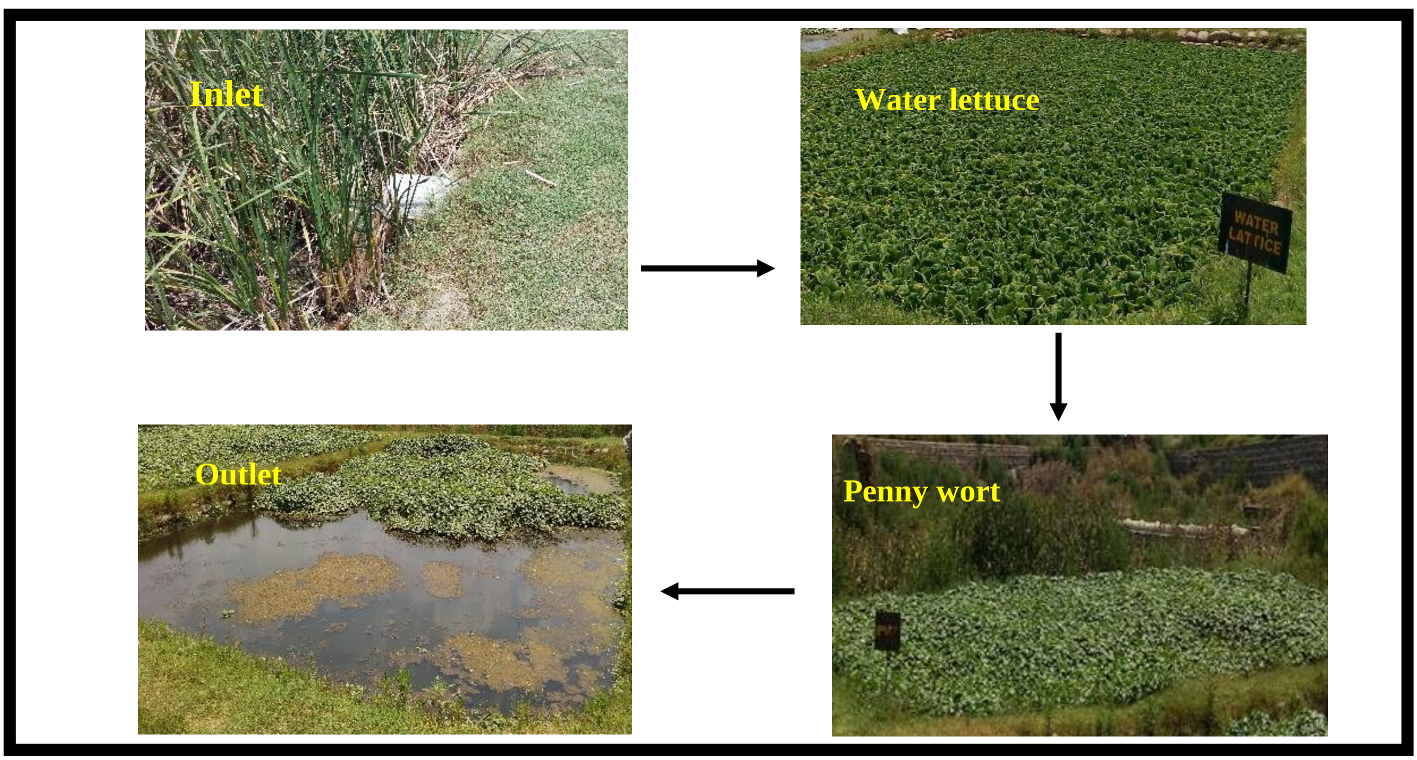


Figure 3.3: Schematic layout of pilot scale constructed wetland

1st pond in constructed wetland was inlet for *Pistia stratiotes* and was cultivated with *Typha latifola* (Bulrush) which provided support through gravel and soil. 2nd pond (Inlet for *Centella asiatica*) contained *Pistia stratiotes* (water lettuce) which do not require additional support for its growth. 3rd pond (Oulet for *Pistia stratiotes*) and 4th pond consisted of *Centella asiatica* (Pennywort) which were further sustained by thermocole sheet to support roots and organic soil. 4th pond was oulet for system.

3.3.2 Lab Scale Unit

A lab scale unit was established to better analyze performance of wetland. It was further categorized in three subunits

1. **Wetland Replica**

   Purpose of this subunit was to analyze different working conditions and treatment aspects. Each pond was facilitated with the same plant as that in pilot scale unit. System was provided with wastewater from sedimentation tank at pilot scale unit.

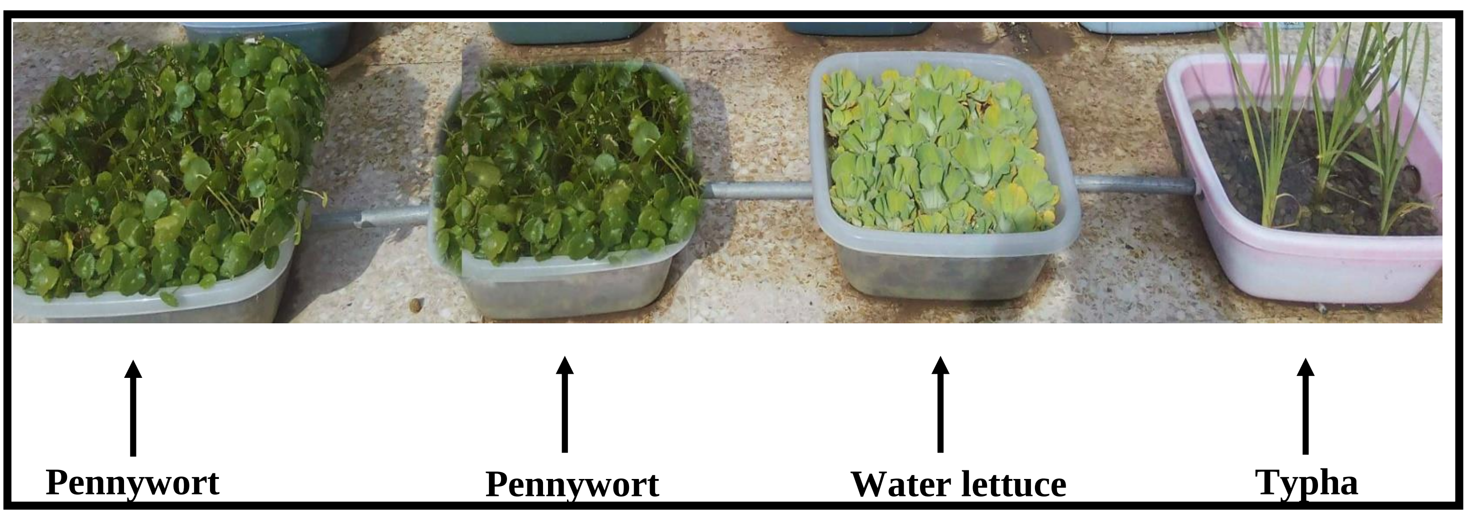


Figure 3.4: Design of Lab scale wetland replica

As a replica of pilot scale unit four plastic tubs were lined with same supporting materials as that in pilot scale unit. Tubs were connected with each other through pipes each 1foot (ft.) in size. This unit was assembled in a manner that plants were exposed to maximum sunlight. Plants were grown in same order as for pilot scale unit.

2. Parallel Scale Unit

This subunit was designed to measure the uptake efficiency of pennywort and water lettuce individually. Plastic tubs of same size and dimension as that of wetland replica were used. However the tubs were not connected in series. Each tub was provided with wastewater via plastic pipes connected to sedimentation unit and water was released from each tub individually through pipes connected at other end after certain retention time.

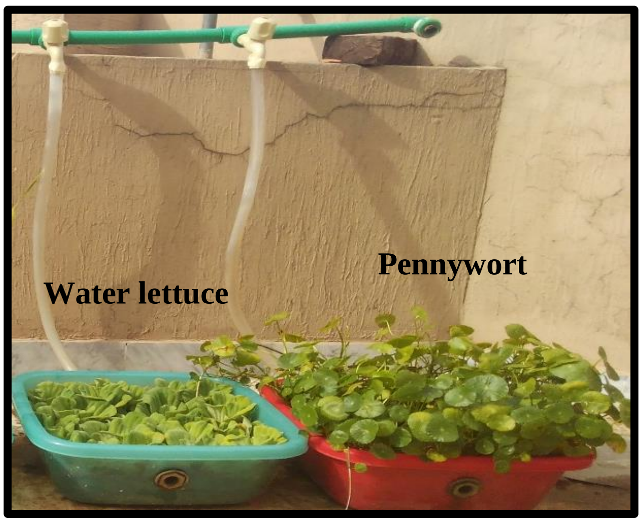


Figure 3.5: Parallel scale unit

3. Control Unit

For this subunit the treatment setup was established without introduction of plants in plastic tubs. Purpose was to confirm either the treatment process is exclusively due to phytoremediation or there is contribution of other physical processes.

3.4 Sampling

3.4.1 Glassware Preparation

250 mL Schott (glass) and leak proof sterile bottles were used for sample collection. All bottles used for sampling were washed properly with detergent and further rinsed with distilled water. Sample water used for collection of bacterial samples were further autoclaved for 15 minutes at 121ºC and 15 pounds per square inch (psi). They were oven dried for one hour at 105°C. Sample bottles were tightly wrapped and capped.

3.4.2 Sample Collection

For physicochemical analysis of wastewater samples were collected on weekly basis from inlet, and outlet of each selected plant. For parallel system sample was collected from each tub whereas for control unit sample were collected from inlet and outlet. For bacterial analysis for each of the experimental unit sample were collected from inlet and outlet. Samples were immediately transferred to microbiology lab at Institute of Environmental Sciences and Engineering (IESE) for further analysis. Throughout study samples were preserved at 4ºC. All the procedure for collection transport and storage of samples were carried according to Standard Methods for Examination of Water and Wastewater (APHA, 2012) to avoid any change in its physical and chemical characteristics.

3.5 Water Quality Analysis

3.5.1 Physicochemical Analysis

Physicochemical analysis included DO, EC, pH, Temperature, TP, COD, TSS and TDS. Table 3.1 characterizes methods and instruments used for analysis of these parameters.

Parameters	Units	Equipment Used	Method of Analysis
pH	---	pH Meter	Potentiometric Method
Temperature	^{0}C	HACH Session 1	Laboratory Method
Conductivity	µS/cm	Conductivity Meter	Potentiometric Method
Total Dissolved Solids	mg/L	Conductivity Meter	Potentiometric Method
Total Suspended Solids	mg/L	Analytical Mass Balance	Gravimetric Dried Method
Chemical Oxygen Demand	mg/L	Through Titration	The Closed Reflux Method
Total Phosphorus	mg/L	HACH DR 2010	Spectrophotometer
Dissolved oxygen	mg/L	Crison Oxi 45	DO meter

Table 3.1: Methods and Instruments for Physicochemical Parameters (APHA, 2012)

1. On Site Analysis

Dissolved oxygen, temperature and pH were measured on site using DO meter (Crison Oxi 45), HACH session 1 and HACH 156 respectively. All the analysis were executed according to the Standard Methods for the Examination of Water and Wastewater (APHA, 2012).

2. Laboratory Analysis

EC, TDS, TSS, COD and TP were analyzed in laboratory within 3 hours of sample collection. Electric conductivity and TDS were measured using portable conductivity meter. Total suspended solids were measured through gravimetric dried method. Total phosphates through spectrophotometer using HACH DR 2010 and using Molybdovanadate as reagent. Chemical oxygen demand was analyzed using close reflux method through titration. Sample for titration was prepared according to Standard Method (APHA, 2012) and was titrated against ferrous ammonium sulfate 0.1 N.

3.5.2 Microbial Analysis

Microbial analysis of collected wastewater samples was performed weekly. Table 3.2 indicates microbial parameters that were analyzed along with technique and media used.

Parameters	Technique Used	Media Used	Measured Units
Total *Coliforms*	Membrane Filtration (MF)	Eosin Methylene Blue Agar	CFU/100 ml
Fecal *Coliforms*	Membrane Filtration (MF)	Eosin Methylene Blue Agar	CFU/100ml

Table 3.2: Bacterial parameters and techniques used

Agar plates were autoclaved at 121ºC for 15 minutes and oven dried. Eosin methylene blue (EMB) agar was used as it is selective agar for coliforms. It was prepared as 2.8 gram per 100 ml of water in volumetric flask erlenmeyer flask. After media preparation flask was sealed tight with aluminIum flask and was autoclaved. Molten and liquefied agar was poured in pre autoclaved petri plates. After pouring plates were incubated for 24 hours at 37ºC for sterility test.

1. Dilution Preparation

For preparation of dilution erlenmeyer flask was filled with 100 ml of distilled water each and sealed with aluminum foil. Flask were autoclaved and preserved at 4 ºC for further use.

2. Membrane Filtration

Before analysis section surface was disinfected with ethanol. Serial dilution technique was used. Through 10ml disposable pipette sample was transferred in the first flask. 10 ml from 1st flask was then transferred in the next one and so on. After Transfer of sample till the 10th flask filter assembly was assembled and was fitted with membrane filter with a rate pore size that retained coliforms selectively. Sample from each flask was allowed to pass through membrane filter. For each flask different filter was used and each filter was then placed in prepared EMB agar plates. Plates were incubated at 37 ºC for 24 hours. Plates were removed from incubator and colonies were counted in colony counter.

3.6 Isolation of Bacteria

Bacteria were isolated from roots and leaves of *Pistia stratiotes* (water lettuce) and *Centella asiatica* (Pennywort). Process of sample collection disinfection and isolation were performed as follows:

3.6.1 Sample Collection

Roots and leaves of selected plants were excised by sterile scalpel and immediately retained in disinfected sample bags. Sample was transferred to laboratory for disinfection within 1 hour.

3.6.2 Surface Disinfection

Surface disinfection was performed for each plant segment separately. The collected roots and leaves were rinsed with running tap water and then rinsed with distilled water so that any superficial injury visible could be excluded. Roots and leaves were then dried in absorbent and soaked in 70% ethanol for 30 seconds. After that plant sample were immersed in 30% sodium hypochlorite (NaClO) for 4 minutes and finally rinsed with sterile distilled water (SDW) thrice. In order to confirm the disinfection protocol was accurate, aliquots from last rinse of SDW were plated in agar plates and incubated at 37 ºC for 24 hours. Absence of microbial growth verified the accuracy of disinfection.

3.6.3 Isolation

Roots and leaves were aseptically incised with sterile knife and transferred carefully on the prepared agar plates so as to allow release of microorganisms and plates were incubated. After

24 hours plates were examined for growth of bacterial colonies. Single colonies were carefully selected and further streaked on agar plates until pure colonies were achieved (Hui *et al*., 2014).

3.7 Bacterial Identification

Bacterial isolates may be identified through conventional methods relying on their morphological and biochemical characteristics.

3.7.1 Colony Morphology

For identification of unknown isolates it is important to observe a single colony. After purification of isolates following morphological characters were observed.

Morphological features	**Description**
Form	Circular, small, large filamentous, punctiform
Color	White, off-white, yellow, green
Elevation	Convex, umbonate, raised, pulvinate, raised
Margin	Lobate, Curled, undulate, entire
Surface texture	Dry, smooth, wrinkled
Opacity	Transparent, translucent

Table 3.3: Morphological features for bacteria

3.7.2 Gram Staining

Bacterial smear was prepared by thoroughly mixing culture with drop of distilled water on clean slide. Slide was left for air dry and was heat fixed. After heat fixation crystal violet (Primary stain) was applied on smear for one minute. Slide was swamped with iodine solution for 1 minute and then with distilled water. Iodine was used as mordant, which means it increases affinity for crystal violet making it difficult to remove from cell. After that slide was flooded with decolorizing agent for 30 seconds. Since gram-positive bacteria consist of thick layer of peptidoglycan which is a polymer (Alfred, 2011). The cross linkage helps gram positive bacteria to retain crystal violet which on other hand washes off from gram negative bacteria when flooded with ethanol (Christopher and Kimberly, 2003). Slide was then treated with safranin for 40 seconds which is counter stain and air dried. A drop of oil immersion was applied on slide cover slip and it was observed under light microscope at 100X.

3.7.3 Motility Test

Cell motility was examined through hanging drop technique. On cover slip a drop of distilled water was carefully placed. Smear was made by introducing bacterial culture and gently mixing it with water. Cover slip was overturned carefully so that smear drop hanged in cavity slide. A drop of oil immersion was subjected and slide was observed under 100X of resolution through light microscope.

3.7.4 MacConkey Agar Test

MacConkey agar is a specialized and selective growth medium for gram-negative bacteria. After preparation of agar plates bacterial isolated were streaked. Agar plates were incubated at 37ºC for 24 hour, isolates that transformed in pink color were lactose fermenters.

3.7.5 EMB Agar Test

Gram-negative enteric bacilli may be differentiated by Eosin Methylene Blue (EMB) agar, Agar plates for EMB media were prepared. Plates were streaked with bacterial isolates and after incubation period were analyzed. Bacterial colonies which turned blue indicates that they are lactose fermenters (James, 2011).

3.8 Biochemical Characterization

As bacterial colonies show wide difference in their morphologies their different species can be identified. Since these tests are not extremely specific i.e. gram staining only differentiate between gram negative and positive, it is not practical to rely on them. In order to assist in more defined and authentic identification biochemical tests are used as they may distinguish between closely related organisms.

3.8.1 Catalase Test

Catalase enzyme are produced by aerobic bacteria or mostly facultative anaerobes. This reaction solely decomposes Hydrogen peroxide (H_2O_2) into water and oxygen (Roberto, 1993).

$$2H_2O_2 + \text{Catalase} \longrightarrow 2H_2O + O_2$$

Bacteria was streaked on an agar plate and incubated for 24 hours at 37 ºC. With help of sterile inoculating loop carefully a single colony of bacteria was collected and was applied on a clean microscopic slide. 3% H_2O_2 solution was prepared carefully and one drop was applied on slide. Instant bubble production indicated test is catalase positive while no bubble production represented that test is catalase negative (Reiner, 2012).

3.8.2 Oxidase Test

Bacteria having aerobic respiration normally have cytochrome c oxidase and cytochrome c. For this test N, N, N', N'-tetramethyl-p-phenylenediamine I (TMPD) s used which comprises an artificial electron acceptor. Reliant on redox state this electron acceptor transforms into some colored product i.e. purple or dark blue (Roberto, 1993). Filter paper was cut in strips and was autoclaved. TMPD solution was prepared as per instruction. Filter paper strips were immersed in the TMPD solution and were allowed to dry in air. With inoculating loop bacterial culture were picked and rubbed gently on paper strips. Blue color indicated oxidase positive while no change indicated oxidase negative (Reiner, 2012).

3.9 Molecular Characterization

3.9.1 16S rRNA Gene Sequencing

Molecular characterization techniques are important tools for microbial analysis from multiple sources. These techniques are fast and effective for exploration of bacterial diversity in different environments. Genetic diversity help in identification of individual organisms generating information from some unique section of their RNA or DNA giving absolute information on its diversity.16s ribosomal RNA is an element of the 30s submit derived from prokaryotic ribosomes. An attractive potential use of 16s rRNA gene sequencing is to provide species as well as genus identification of bacterial isolates that do not fall in any renowned biochemical profiles for strains processing low acceptable identification (Malik *et al*., 2008). 16S rRNA gene sequences is by far used most commonly as a genetic marker to study microbial taxonomy and phylogeny for various reasons such as: it is present in all bacterial life forms usually as operons or multigene family, the function of this genetic marker has not changed overtime, signifying that random changes in sequences are a more precise time measurement (evolution) and the 16S rRNA gene is sufficiently large for informatics studies (Sharron, 2007).

Bacteria isolated from roots and leaves of *Pistia stratiotes* (water lettuce) and *Centella asiatica* (Pennywort) were further preserved for DNA sequencing. Bacterial isolates were wiped gently with distilled water with help of bent glass rod and collected in eppendorf tubes. Tubes were centrifuged to separate supernatant from bacterial culture and supernatant was removed. 1 ml of 50% glycerol and 3 ml of 30% nutrient broth was introduced in eppendrof tubes and preserved at -20ºC. For further 16S rRNA sequencing the preserved isolates were sent to Genome Analysis Department Macrogen Inc. Korea.

3.9.2 Phylogenetic Analysis

Phylogenetics is the study that helps in understanding ties between ancestors and descendants. Phylogenetic tree is a branched diagram that deduce evolutionary relationship. This diagram depicts a projected image that indicates link of genetic relations among organisms (Brinkman, 2001). Phylogenic tree may be rooted having same ancestor or unrooted having unknown ancestors Gene sequence may be utilized to construct the phylogeny of selected bacteria. These studies specify that bacteria at first diverged from the eukaryotic/archaeal lineage. For phylogenetic studies of molecular sequences there are four basic steps: (1) assortment of an appropriate molecule or molecules also known as phylogenetic marker (2) achievement of selected molecular sequences (3) multiple sequence alignment (MSA) (4) construction and evaluation of phylogenetic tree. Phylogenetic studies are capable of serving multiple purposes such as comparative studies for two or more sequences, analyzing genetic families which may include functional prediction and for valuation of evolutionary linkages between different species (Olena and Marco, 2008).

Once microbes were identified through morphological and biochemical characterization sequences were processed through BLAST nucleotide search from databases of National Center for Biotechnology Information (NCBI). After proper detection of mismatch CLUSTALW was used for sequence alignment. MEGA4 was used for construction of phylogenic tree. It demonstrates the phylogenetic connection and linkage of identified bacterial strains with strain selected from GeneBank.

Chapter 4

RESULTS AND DISCUSSION

4.1 Wastewater Sources and Composition

The sources and composition of wastewater was examined. It was observed that concentration of selected parameters in wastewater varied on daily and seasonal basis. Major sources of phosphates in wastewater included; soaps and detergents used in laundry and kitchen, personal care products since phosphates are an important ingredient of commercial cleaning products and discharge of garden fertilizer due to soil erosion from lawns or due to animal waste. Researcher observed domestic sources of phosphates and their contribution in wastewater. Results indicated that natural dishwasher detergents contributed 9%, personal care products 1%, domestic laundry 6% and food waste 1% discharge of phosphorus in form of phosphates in water (Comber *et al.*, 2013). As COD is chracterizied by presence of organic mtater its major contributors were plant waste or dead plants such as bushes or leaves, decomposed organic material, fruits and vegetables or other natural food and fertilizer runoff. Results for analysis of primary pollutants in domestic water showed that animal and plant waste and green manure are responsible for a high COD (Tjandraatmadja *et al.*, 2009).

Water conductivity was caused by; dssolved inorganic solids such as sulphates, chlorides, calcium, magnesium, iron etc., urban runoff from paved surfaces and nutrients. Researchers used EC as water quality indicator and observed total dissolved solids were responsible for high EC and may be used to measure changing runoff sources (Dan *et al*., 2008). TSS are formed when organic particles from process of decomposition break into tiny organic particles and enter into water column. Very small amount of inorganic particulates also form TSS. According to a study organic matter was responsible for 70% contribution of TSS and had direct influence on aquatic biota

(Bilotta, 2008). Coliforms are present in intestine of animals and are introduced in water environment from feces. Fecal *coliforms* are pathogenic type of *coliform* and are responsible for adverse human health. Fecal *coliforms* and their sources in an urban watershed were observed. Research indicated that humans and wild animals were major sources of fecal coliforms with wild animals as most dominant source (John *et al*., 2002).

4.2 Evaluating Performance Efficiency of *Pistia stratiotes* and *Centella asiatica* at Pilot Scale

Pilot scale system is a series unit that means ponds are connected with one another. *Pistia stratiotes* was planted in 2nd pond whereas *Centella* asiatica was planted in 3rd pond. Inlet and outlet concentration of designed parameters were measured for both plants. Figure 4.1 depicts temperature profile of pilot scale unit.

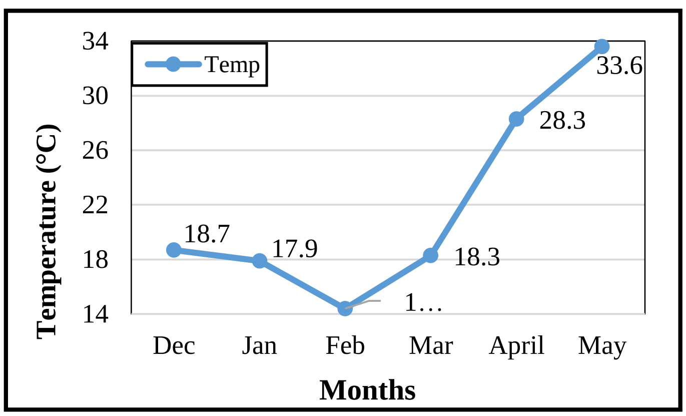


Figure 4.1: Temperature profile of pilot scale unit

For all physicochemical parameters evaluated temperature played a significant role. In all cases removal efficiency of phytoremediation plant in month of winter was slow as compared to summers due to limited plant growth and death of already grown plants. Highest rainfall was recorded for month of March thereby resulting in wastewater dilution. As a result average profile for all parameters varied significantly in March. Researchers studied effect of temperature on pennywort and vetiver grass and stated that higher temperature favors their growth and multiplication (Girija *et al*., 2011)

Temperature and TSS

High value of suspended solids and least removal was observed during winter season in months of December, January and February due to (a) Death of plants and growth retardation (b) An increase in organic matter overall contributes an increase in suspended solids (El Refaie and Ghada, 2010). Significant decrease in influent suspended solids was observed in March due to heavy rainfall which led to dilution of pollutants. A decrease of inlet effluent was observed during summer as vegetation growth was maximum in months of May and June. Figure 4.2 (a) shows relation between temperature and TSS. At temperature of 14.4 ºC minimum plant efficiency was observed and at 33.6 ºC minimum influent TSS was observed and plants performed efficiently.

Temperature and DO

DO was high in winters with peak value of influent by 5.4 mg/L in case *Pistia stratiotes* and 5.6 mg/L in case of *Centella asiatica* in March due to heavy rainfall that led to influent dilution the rain continuously interacts with oxygen in atmosphere. High value of DO in winters was related to the fact that cold water have the tendency to hold more dissolved oxygen as warm water becomes easily saturated (Vassilis *et al*., 2003). However at temperature as low as 14 ºC DO level was as high as 10.2 mg/L (EPA, 2012) but in current study following factors were probably responsible for DO fluctuation (a) At low temperature wastewater was loaded with decaying organic matter and bacteria consume more oxygen for decomposition through chemical oxidation (b) High suspended solids also block sunlight at surface, light reaching via water is reduced and hence process of photosynthesis is reduced. Figure 4.2 (b) represents relation between DO and temperature.

Conductivity and Temperature

Electrical conductivity of water increases invariably as temperature increase. Warm water has less viscosity and more electric moment and dissolved solids are in concentrated value it allows electric current to flow freely (Todd *et al.*, 2005; David, 2004) therefore at high temperature of 33.6 ºC in May influent conductivity reached a value of 1134 µs/cm. Figure 4.2 (c) represents relation between conductivity and temperature**.** Variations in temperature resulted in marked conductivity changes. For winters at lowest temperature of 14.6 ºC conductivity recorded was 789 µs/cm. In addition introduction of rainfall also lowered conductivity because rainfall has less EC and minerals are diluted due to addition of water. As a result In March although temperature was 18.3ºC but conductivity recorded was 715 µs/cm.

COD and Temperature

Highest COD values were recorded in winters with peak influent value for *Pistia stratiotes* as 539 mg/L and for *Centella asiatica* as 456 mg/L in February when temperature was 14 ºC. High COD resulted due to increase in organic content. Another factor played a key role that at low temperature plant growth was retarted which led to low aeration and high chemical oxygen demand of bacteria for decomposition. During summers organic matter was significantly decreased and temperature was suitable for plant growth. In May COD value recorded for *Pistia stratiotes* was 167 mg/L and for *Centella asiatica* was 117 mg/L. Figure 4.2 (d) shows relation between temperature and COD.

pH and Temperature

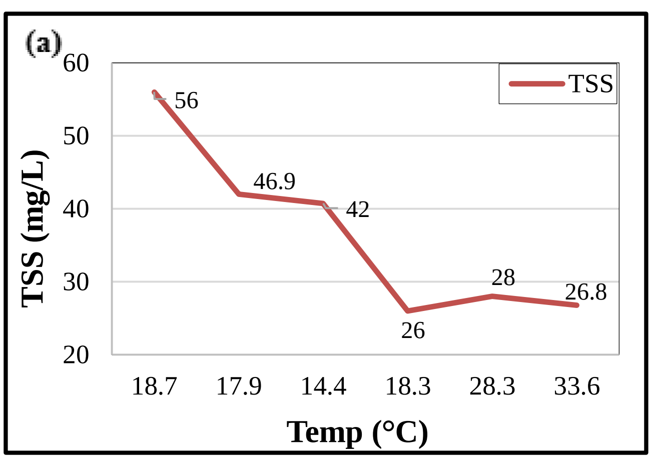
Temperature has a very slight effect on pH. Normally based on environmental condition pH value differ. At high temperature water molecules have small tendency to split into its elements, hydrogen and oxygen. As temperature continue to increase it leads to more release of hydrogen ion hence shifting pH towards slightly acidic. At low temperature breakdown of water molecules is less (Clark, 2002). Highest pH value was observed in February with influent pH of 7.4 while lowest pH value was observed in May with influent pH value of 7.19. Figure 4.2 (e) indicates relation between pH and temperature.

Temperature and TP

Amount of phosphates in winter was considerably low. In summer consumption of cleaning compounds, waste from kitchen, laundries and personal care was far much high as compared to winters. This led to high discharge of phosphates in wastewater. Maximum influent phosphate recorded for *Pistia stratiote* and *lla asiatica* were 34 and 31 mg/L in May respectively while minimum influent phosphate recorded for *Pistia stratiote* and *Centella asiatica* were 11.5 and 11 mg/L respectively. Figure 4.2 (f) represents relation between TP and temperature.

Temperature and *Coliforms*

Growth rate of bacteria is high at high temperature as compared to low temperature (Sakyi and Patrick, 2012). Maximum growth of total and fecal *coliforms* at inlet was observed for month of May as $27x10^5$ and $15x10^5$ respectively. Whereas lowest growth was observed for February as $11x10^5$ and $3x10^5$ respectively. Figure 4.1 (a) to (f) represent effect of temperature on selected parameters.



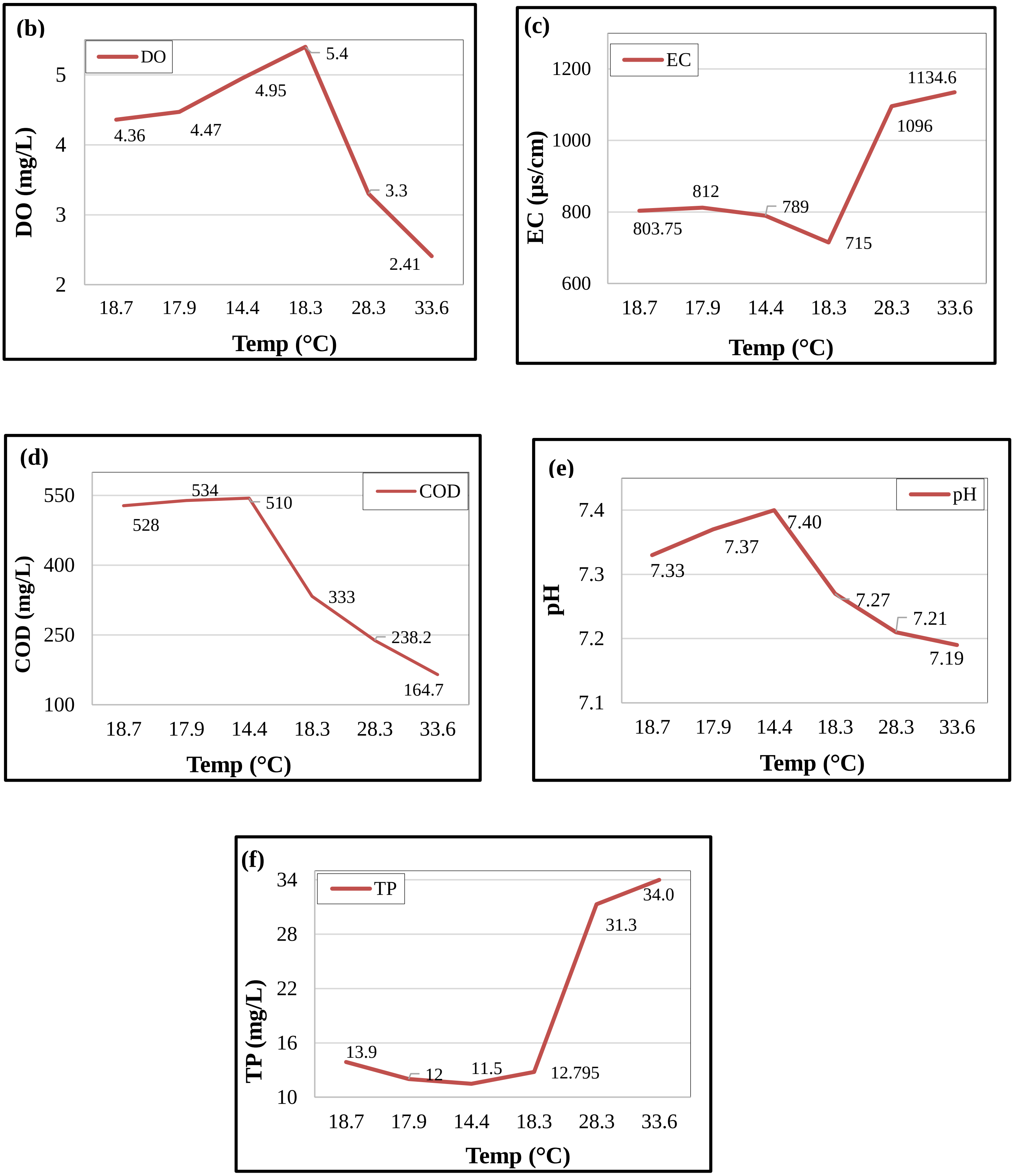
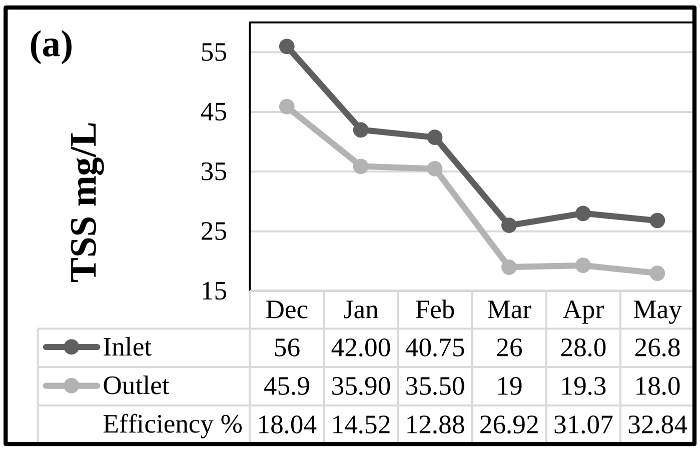


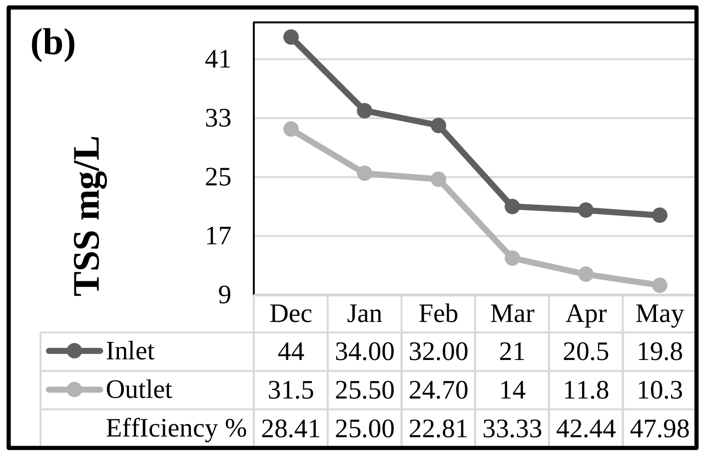
Figure 4.2 (a) to (f): Relation between temperature and selected parameters

4.2.1 Total Suspended Solids

In cases of both plants lowest removal efficiency was achieved in month of February with removal efficiency of 12.8 % for *Pistia stratiotes* and 22.7% for *Centella asiatica.* Maximum removal efficiency was achieved in month of May as 32.8 % for *Pistia stratiotes* and 47.9 % for *Centella asiatica.* Figure 4.3 (a) and (b) represents average monthly profile of TSS at pilot scale for *Pistia stratiotes* and *Centella asiatica.*



	Dec	Jan	Feb	Mar	Apr	May
Inlet	56	42.00	40.75	26	28.0	26.8
Outlet	45.9	35.90	35.50	19	19.3	18.0
Efficiency %	18.04	14.52	12.88	26.92	31.07	32.84



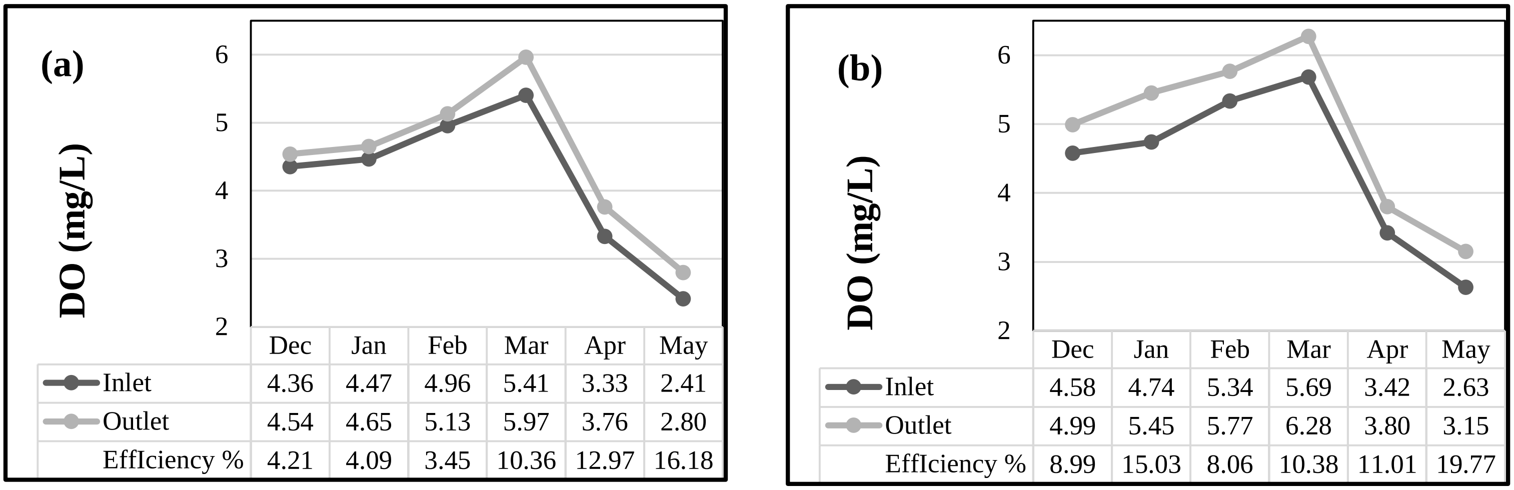
	Dec	Jan	Feb	Mar	Apr	May
Inlet	44	34.00	32.00	21	20.5	19.8
Outlet	31.5	25.50	24.70	14	11.8	10.3
EffIciency %	28.41	25.00	22.81	33.33	42.44	47.98

Figure 4.3: Average TSS removal by (a) *Pistia stratiotes* (b) *Centella asiatica* at pilot scale

Studies were performed by Esi and coworkers to record removal efficiency of water lettuce in stabilization ponds. COD removal reached 59 %, total phosphorus was removed by 9 % and total dissolved solids (TDS) were removed by 70 % (Esi *et al*., 2004).

4.2.2 Dissolved Oxygen

DO was measured immediately after collection of sample. For DO lowest efficiency of plants was achieved in month of February. Maximum efficiency was achieved in month of May as 16.1% for *Pistia* stratiotes and 19.7 % for *Centella asiatica.* Figure 4.4 (a) and (b) represents average monthly profile of DO at pilot scale unit for *Pistia stratiotes* and *Centella asiatica.*



(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	4.36	4.47	4.96	5.41	3.33	2.41
Outlet	4.54	4.65	5.13	5.97	3.76	2.80
EffIciency %	4.21	4.09	3.45	10.36	12.97	16.18

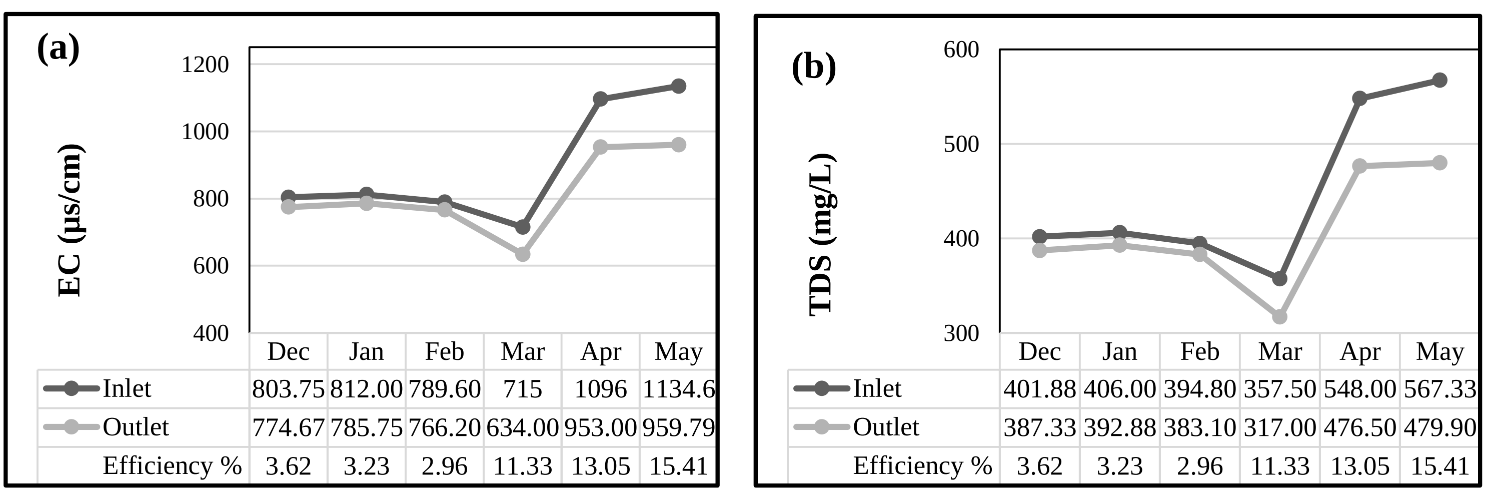
(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	4.58	4.74	5.34	5.69	3.42	2.63
Outlet	4.99	5.45	5.77	6.28	3.80	3.15
EffIciency %	8.99	15.03	8.06	10.38	11.01	19.77

Figure 4.4: Average increase in DO by (a) *Pistia stratiotes* (b) *Centella asiatica* at pilot scale

4.2.3 Conductivity and Total Dissolved Solids

Conductivity and TDS are directly related to one another as EC is a measure of dissolved solids in water. For both EC and TDS lowest removal efficiencies were recorded for February at 14°C as 2.96 % for *Pistia stratiotes* and 3.46% for *Centella asiatica.* Figure 4.5 (a) and (b) gives average decrease in EC and TDS by *Pistia stratiotes* and Figure 4.6 (a) and (b) gives average decrease in EC and TDS by *Centella asiatica*.



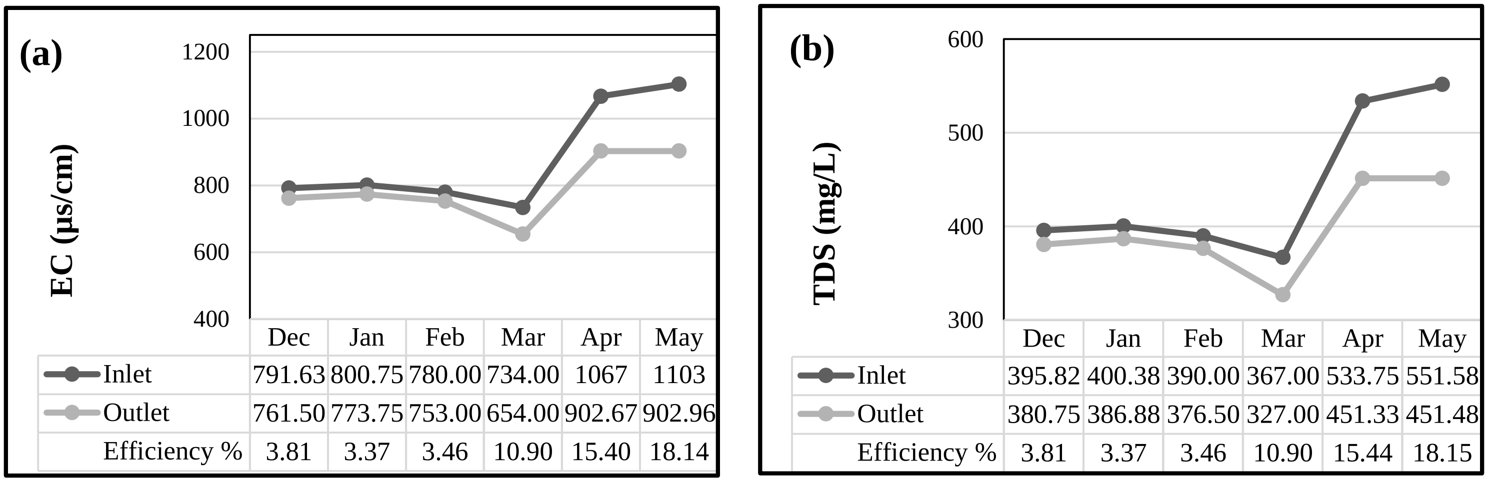
(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	803.75	812.00	789.60	715	1096	1134.6
Outlet	774.67	785.75	766.20	634.00	953.00	959.79
Efficiency %	3.62	3.23	2.96	11.33	13.05	15.41

(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	401.88	406.00	394.80	357.50	548.00	567.33
Outlet	387.33	392.88	383.10	317.00	476.50	479.90
Efficiency %	3.62	3.23	2.96	11.33	13.05	15.41

Figure 4.5: Average removal by *Pistia stratiotes* (a) EC (b) TDS at pilot scale



(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	791.63	800.75	780.00	734.00	1067	1103
Outlet	761.50	773.75	753.00	654.00	902.67	902.96
Efficiency %	3.81	3.37	3.46	10.90	15.40	18.14

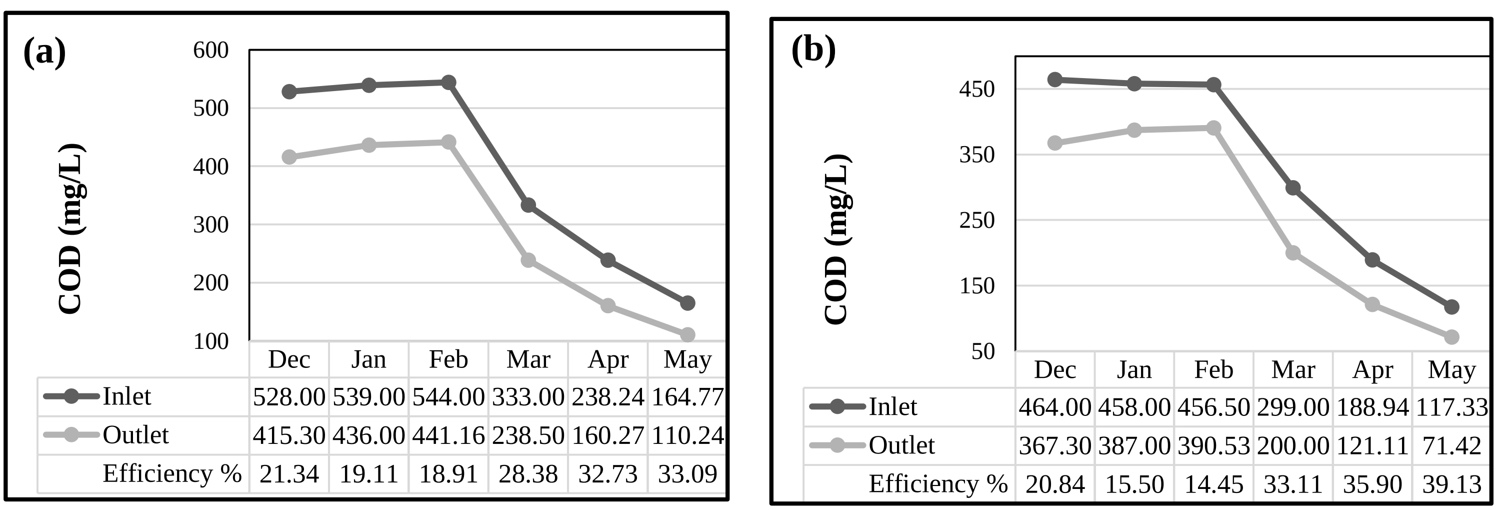
(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	395.82	400.38	390.00	367.00	533.75	551.58
Outlet	380.75	386.88	376.50	327.00	451.33	451.48
Efficiency %	3.81	3.37	3.46	10.90	15.44	18.15

Figure 4.6: Average removal by *Centella asiatica* (a) EC (b) TDS at pilot scale

4.2.4 Chemical Oxygen Demand

COD values for *Pistia stratiotes and Centella asiatica w*ere measured. Lowest removal efficiencies recorded were for month of February as 18.5 % for *Pistia stratiotes* and 14 % for *Centella asiatica.* Maximum efficiency for *Pistia stratiotes* was recorded as 33.3 % and for *Centella asiatica* as 39.1 %. Figure 4.7 (a) and (b) indicates average decrease in COD by *Pistia stratiotes* and *Centella asiatica* respectively.



(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	528.00	539.00	544.00	333.00	238.24	164.77
Outlet	415.30	436.00	441.16	238.50	160.27	110.24
Efficiency %	21.34	19.11	18.91	28.38	32.73	33.09

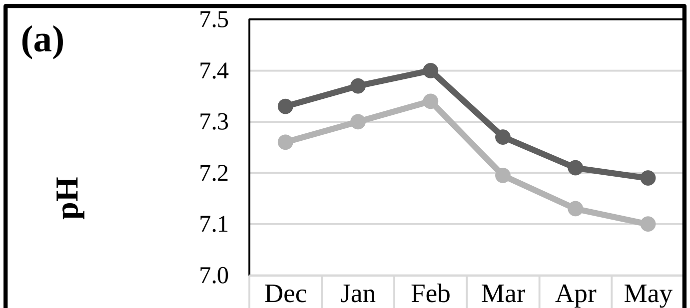
(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	464.00	458.00	456.50	299.00	188.94	117.33
Outlet	367.30	387.00	390.53	200.00	121.11	71.42
Efficiency %	20.84	15.50	14.45	33.11	35.90	39.13

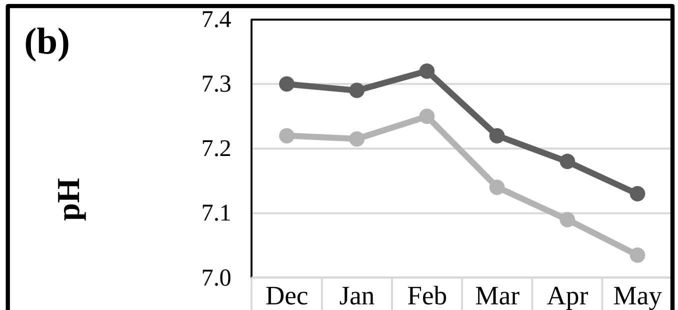
Figure 4.7: Average decrease in COD (a) *Pistia stratiotes (*b) *Centella asiatica* at pilot scale

4.2.5 pH of Wastewater

pH of wastewater was neither found significantly high neither significantly low at both temperature extremes. Highest efficiencies for pH shift were recorded as 1.25 and 1.33 % by *Pistia stratiotes* and *Centella asiatica* respectively for month of May. Figure 4.8 (a) and (b) indicates average shift in pH by *Pistia stratiotes* and *Centella asiatica* respectively.



	Dec	Jan	Feb	Mar	Apr	May
Inlet	7.33	7.37	7.40	7.27	7.21	7.19
Outlet	7.26	7.30	7.34	7.195	7.13	7.1
Efficiency %	0.95	0.95	0.81	1.03	1.11	1.25

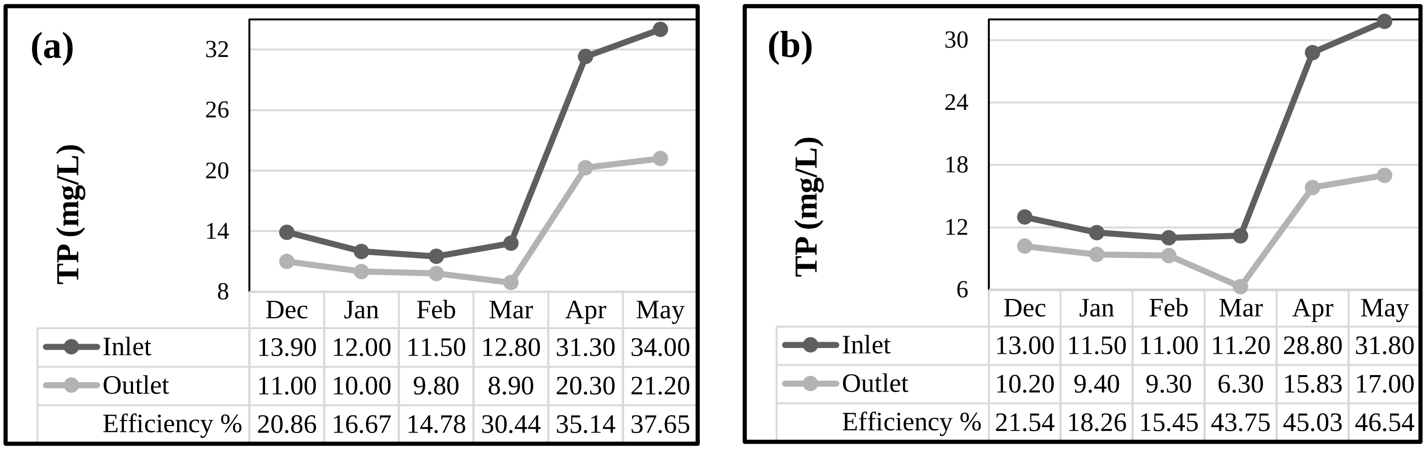


	Dec	Jan	Feb	Mar	Apr	May
Inlet	7.30	7.29	7.32	7.22	7.18	7.13
Outlet	7.22	7.22	7.25	7.14	7.09	7.04
Efficiency %	1.10	1.03	0.96	1.11	1.25	1.33

Figure 4.8: Average shift in pH by (a) *Pistia stratiotes* (b) *Centella asiatica* at pilot scale

4.2.6 Total Phosphates

Total phosphates were measured at inlet and outlet. Lowest efficiencies were recorded in February as 14.7 and 15.4 % while highest efficiencies for phosphate removal were 37.6 and 46.5 % for *Pistia stratiotes* and *Centella asiatica* respectively. Figure 4.9 (a) and (b) indicates average shift in pH by *Pistia stratiotes* and *Centella asiatica* respectively. According to research efficiency of three macrophytes i.e, Pennywort, duckweed and water lettuce was evaluated in series unit. In case of pennywort average reduction in effluent value were 46.38 % for COD, 18.76 % for Phosphorus and 40.34 % for Nitrogen. For duckweed the reduction efficiency was 26.37 % for COD, 15.25 % for Phosphorus and 17.59 % for Nitrogen respectively while for water lettuce removal level was 28.59 % for COD, 10.69 % for Phosphorus and 14.45 % for Nitrogen (Mumtaz and Hashim, 2014).



(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	13.90	12.00	11.50	12.80	31.30	34.00
Outlet	11.00	10.00	9.80	8.90	20.30	21.20
Efficiency %	20.86	16.67	14.78	30.44	35.14	37.65

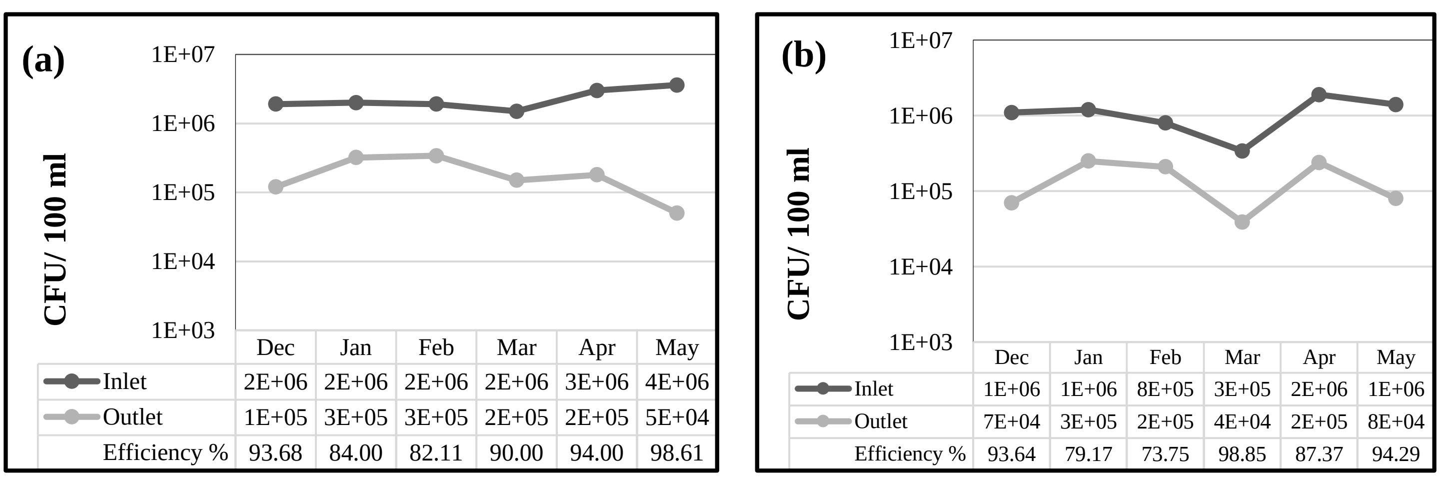
(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	13.00	11.50	11.00	11.20	28.80	31.80
Outlet	10.20	9.40	9.30	6.30	15.83	17.00
Efficiency %	21.54	18.26	15.45	43.75	45.03	46.54

Figure 4.9: Average removal of TP by (a) *Pistia stratiotes* (b) *Centella asiatica* at pilot scale

4.2.7 *Coliforms*

Samples were collected from inlet of *Pistia stratiotes* and outlet of *Centella asiatica***.** For total *coliforms* lowest efficiency achieved was in month of February as 84.5 % while for fecal *coliforms* lowest efficiency was 86 % for February. Highest efficiencies for total and fecal *coliforms* were achieved in month of May as 99.2 and 99.85 % respectively. Figure 4.10 (a) and (b) indicates average removal of total *coliforms* and fecal *coliforms* respectively. Esi and coworkers observed a reduction of 78% in fecal *coliforms* for water lettuce planted in series unit of stabilization (Esi *et al.*, 2004).



(a)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	2E+06	2E+06	2E+06	2E+06	3E+06	4E+06
Outlet	1E+05	3E+05	3E+05	2E+05	2E+05	5E+04
Efficiency %	93.68	84.00	82.11	90.00	94.00	98.61

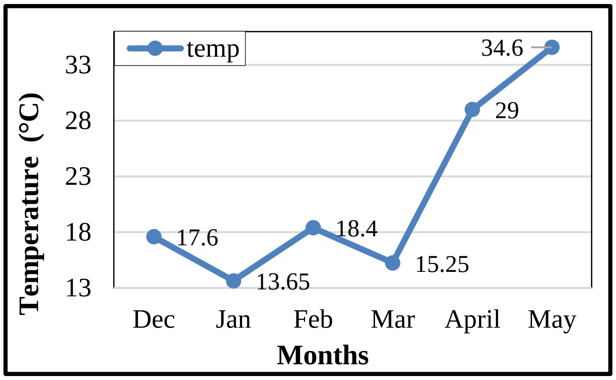
(b)

	Dec	Jan	Feb	Mar	Apr	May
Inlet	1E+06	1E+06	8E+05	3E+05	2E+06	1E+06
Outlet	7E+04	3E+05	2E+05	4E+04	2E+05	8E+04
Efficiency %	93.64	79.17	73.75	98.85	87.37	94.29

Figure 4.10: Average removal of (a) Total *coliforms* (b) Fecal *coliforms* at pilot scale

4.3 Evaluating Performance Efficiency of *Pistia stratiotes* and *Centella asiatica* at Wetland Replica

Lab scale replica of wetland unit is also a series unit. *Pistia stratiotes* was planted in 2nd pond whereas *Centella* asiatica was planted in 3rd and 4th pond. Inlet and outlet concentration of designed parameters were measured. Figure 4.11 depicts temperature profile of study time for lab scale unit.



4.11: Temperature profile of lab scale unit

4.3.1 Total Suspended Solids

In case of both plants lowest removal efficiency was achieved in month of February when temperature was lowest 14.4 ºC with efficiency of 20 % for *Pistia stratiotes* and 22.7 % for *Centella asiatica.* Maximum removal efficiency was achieved in month of May 44 % for *Pistia* stratiotes and 53 % for *Centella asiatica*. Fernanda and coworkers treated municipal wastewater using pennywort and calculated a decrease of 45 % in TSS concentration for month of May (Fernanda *et al.*, 2012)

4.3.2 Dissolved Oxygen

DO was measured immediately after collection of sample. For DO lowest efficiency of plants was achieved for February with efficiency of 6.1 % for *Pistia stratiotes* and 8.59 % for *Centella asiatica.* Maximum efficiency was achieved for May 17.9 % for *Pistia* stratiotes and 18.3 % for *Centella asiatica.* Sudden increase of DO in month of March was due to heavy rainfall.

4.3.3 Conductivity and Total Dissolved Solids

Conductivity and TDS were measured for labs scale unit. Lowest efficiency values of EC and TDS for *Pistia stratiotes* were recorded in February as 3.08 % in both cases. In case of *Centella asiatica* lowest efficiencies for EC and TDS were 3.68 % for February. Maximum efficiencies were achieved in month of May for both plants with EC and TDS values for *Pistia stratiotes* 16.70 % and for *Centella asiatica* as 20.7 %. Pennywort was evaluated for treatment of TDS from municipal water and concentration decrease of 60 % was recorded (Fernanda *et al.*, 2012).

4.3.4 Chemical Oxygen Demand

COD values for *Pistia stratiotes* and *Centella asiatica w*ere recorded. Lowest removal efficiencies measured were for month of February as 15.5 % for *Pistia stratiotes* and 18.9 % for *Centella asiatica.* Maximum efficiency for *Pistia stratiotes* was recorded as 35 % and for *Centella asiatica* as 39 %.

4.3.5 pH of Wastewater

Lowest pH shift were recorded as 0.83 and 0.82 % by *Pistia stratiotes* and *Centella asiatica* respectively for month of February. Highest efficiencies for pH shift were recorded as 1.21 % and 1.34 % by *Pistia stratiotes* and *Centella asiatica* and respectively for month of May.

4.3.6 Total Phosphates

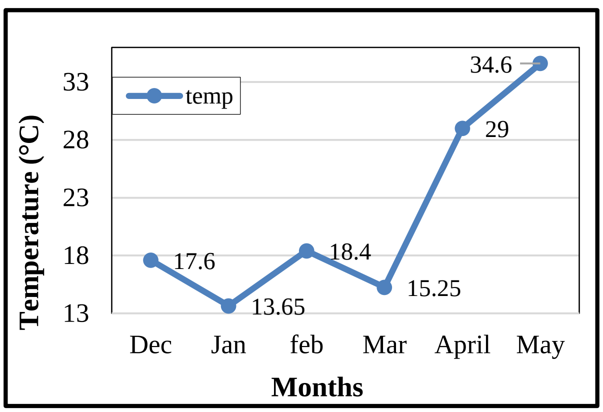
Total phosphates were analyzed. Lowest efficiencies were recorded in February as 14.8 and 16.38 % for *Pistia stratiotes* and *Centella asiatica* respectively while highest efficiencies for phosphate removal were 36.4 and 46.2 % for *Pistia stratiotes* and respectively. A reduction in total phosphates by 24 and COD by 30% was recorded for winter season using water lettuce in an engineered wetland system during course of winter efficiency was increased by 60% for total phosphates and 55% for COD respectively (Zhanga *et al*., 2010).

4.3.7 *Coliforms*

Samples were collected from inlet of *Pistia stratiotes* and outlet of *Centella asiatica* as both plant ponds are connected adjacently in series. For total *coliforms* lowest efficiency achieved was in month of February as 85.4 % and for fecal *coliforms* lowest efficiency was 86.1 % in February. Highest efficiencies for total and fecal *coliforms* were achieved in month of May as 98.3 and 99.2 % respectively.

4.4 Evaluating Performance Efficiency of *Centella asiatica* and *Pistia stratiotes* at Parallel Scale

This unit was designed to check the removal efficiency of individual plant. As inlet for both plants was same but was connected seperately to each plant. As plant growth was lowest and plant decay was highest in winters removal effeciency was considerably low than in summers. Figure 4.12 depicts temperature profile of lab scale unit.



4.12: Temperature profile of parallel unit

4.4.1 Total Suspended Solids

For parallel unit lowest removal efficiency was achieved in month of with efficiency of 27 % for *Pistia stratiotes* and 37 % for *Centella asiatica*. Maximum removal efficiency was achieved in month of May 62 % for *Pistia* stratiotes and 83 % for *Centella asiatica*. Figure 4.13 (a) shows average removal in TSS by *Pistia stratiotes* and *Centella asiatica* in parallel unit.

4.4.2 Dissolved Oxygen

DO was measured instantly after collection of sample because it varies with time. Figure 4.13 (b) indicates average increase in dissolved oxygen by *Pistia stratiotes* and *Centella asiatica* respectively. Maximum efficiency was achieved in month of May 53 % for *Pistia* stratiotes and 64 % for *Centella asiatica.* Wastewater was treated using water lettuce and pennywort in parallel units. For water lettuce and pennywort dissolved oxygen (DO) was increased from 0.75 to 6.02 and from 0 to 0.45 respectively whereas TDS was removed by 70 and 55.93 % respectively (Piyush *et al*., 2012).

4.4.3 Conductivity and Total Dissolved Solids

Conductivity and TDS were measured for parallel scale unit. Lowest efficiency values of EC and TDS for *Pistia stratiotes* were in February and were 9.97 % in both cases. In case of *Centella asiatica* lowest efficiencies for EC and TDS were 13.22% for February. Maximum efficiencies were achieved in May for both plants with EC and TDS values for *Pistia stratiotes* as 37.64 % and for *Centella asiatica* 49.71 %. Figure 4.13 (c) gives average decrease in EC and Figure 4.13 (d) gives average decrease in TDS by *Pistia stratiotes* and *Centella asiatica*.

4.4.4 Chemical Oxygen Demand

Lowest removal efficiencies of COD measured were for month of February as 45.17 % for *Pistia stratiotes* and 58.7 % for *Centella asiatica.* Figure 4.13 (e) indicates average decrease in COD by *Pistia stratiotes* and *Centella asiatica.* COD removal was recorded as 65% for pennywort and 48% for water lettuce during course of summer (Piyush *et al*., 2012).

4.4.5 pH of Wastewater

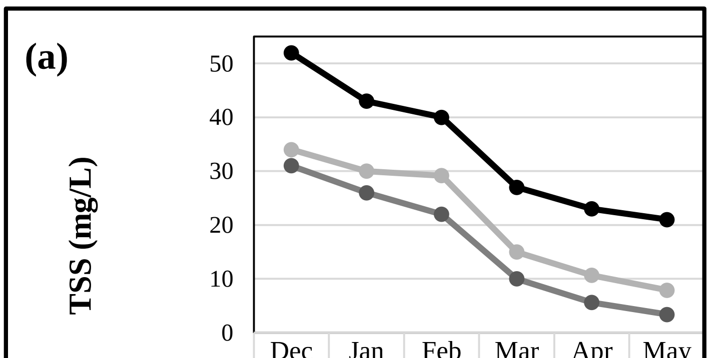
Lowest efficiencies for pH shift were recorded as 0.94 and 1.21 % by *Pistia stratiotes* and *Centella asiatica* respectively for month of February. Highest efficiencies for pH shift were recorded as 2.21 % and 3.04 % by *Pistia stratiotes* and *Centella asiatica* respectively for month of May. Figure 4.13 (f) indicates average shift in pH by *Pistia stratiotes* and *Centella asiatica.*

4.4.6 Total Phosphates

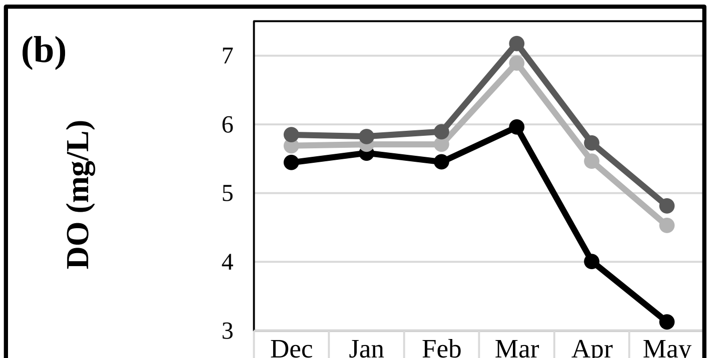
Lowest efficiencies were recorded in February as 24.81 and 39.84% for *Pistia stratiotes* and *Centella asiatica* arespectively while highest efficiencies for phosphate removal were 64.28 and 81.71% for *Pistia stratiotes* and *Centella asiatica* respectively. Figure 4.13 (g) indicates average shift in Total phosphates by *Pistia stratiotes* and *Centella asiatica* respectively.

4.4.7 *Coliforms*

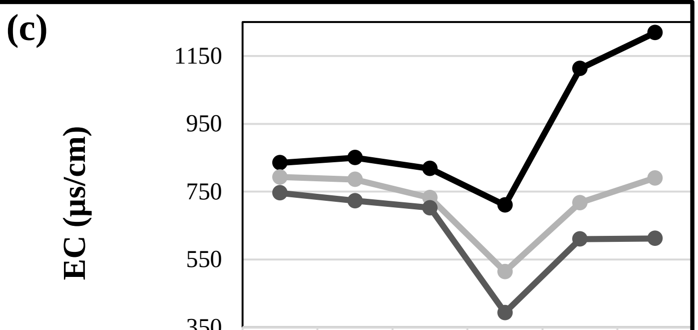
Samples were taken from inlet and outlet of *Pistia stratiotes* and *Centella asiatica* individually as plant ponds are separately connected to inlet. For total *coliforms* lowest efficiency achieved by *Pistia stratiotes* and *Centella asiatica* was in February as 88.8 and 90 % respectively. For fecal *coliforms* lowest efficiency was 88 and 92 % in February by *Pistia stratiotes* and *Centella asiatica* respectively. Figure 4.13 (h) and (i) indicates average removal of total *coliforms* and fecal *coliforms* by *Pistia stratiotes* and *Centella asiatica* respectively.



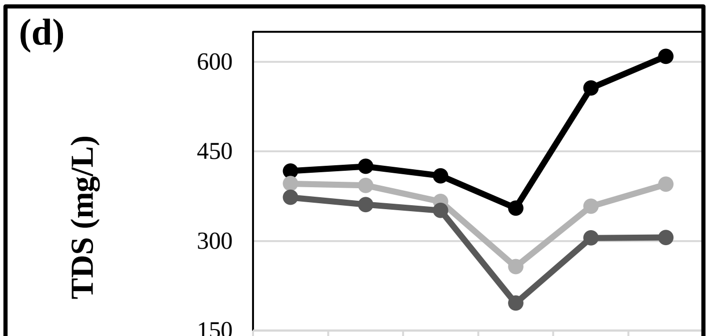
	Dec	Jan	Feb	Mar	Apr	May
Inlet	52.00	43.00	40.00	27.00	23.00	21.00
Water lettuce	34.00	30.00	29.17	15.00	10.67	7.90
Efficiency %	34.62	30.23	27.08	44.44	53.62	62.38
Penny wort	31.00	26.00	22.00	10.00	5.60	3.40
Efficiency %	40.38	39.53	45.00	62.96	75.65	83.81



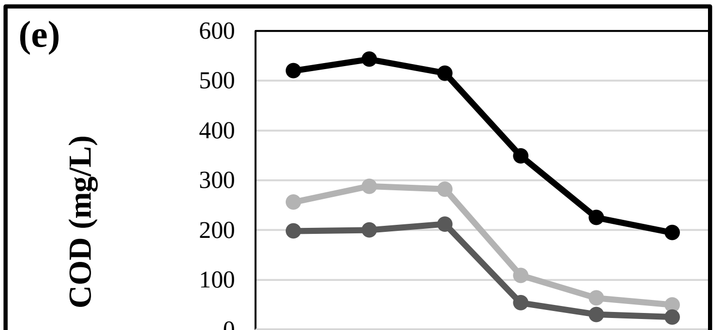
	Dec	Jan	Feb	Mar	Apr	May
Inlet	4.95	5.08	4.95	5.46	3.51	2.63
Water lettuce	5.19	5.21	5.21	6.39	4.96	4.03
Efficiency %	4.92	2.51	5.18	17.09	41.54	53.24
Penny wort	5.35	5.33	5.39	6.68	5.23	4.32
Efficiency %	8.15	4.78	8.89	22.28	49.10	64.13



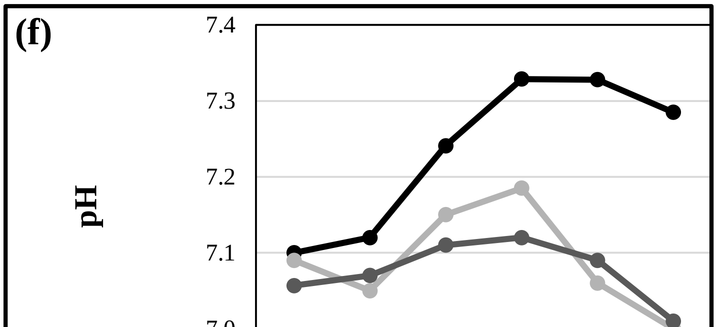
	Dec	Jan	Feb	Mar	Apr	May
Inlet	835	850	818	710	1113	1219
Water lettuce	793	786	732	514	717	790
Efficiency %	5.03	7.53	10.51	27.61	35.58	35.19
Penny wort	746	723	702	393	610	612
Efficiency %	10.66	14.94	14.18	44.65	45.19	49.79



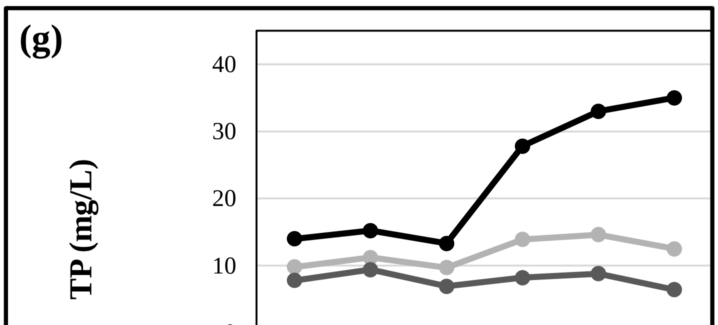
	Dec	Jan	Feb	Mar	Apr	May
Inlet	417	425	409	355	556	609
Water lettuce	396	393	366	257	358	395
Efficiency %	5.04	7.53	10.51	27.61	35.61	35.14
Penny wort	373	361	351	196	305	306
Efficiency %	10.55	15.06	14.18	44.79	45.14	49.75



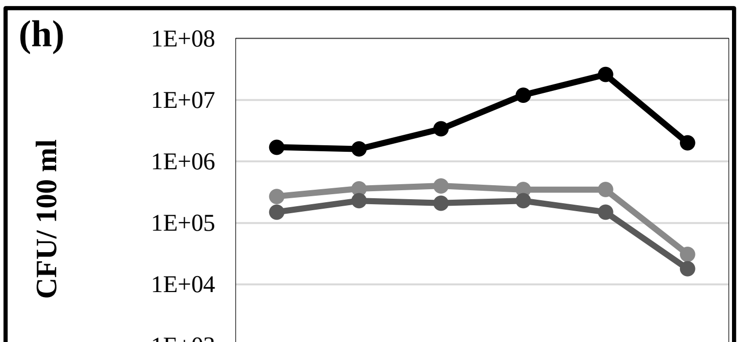
	Dec	Jan	Feb	Mar	Apr	May
Inlet	520	543	515	349	225	195
Water lettuce	256	288	282	109	63.50	49.67
Efficiency %	50.77	46.96	45.24	68.77	71.78	74.53
Penny wort	198	200	212	53.96	30.50	25.33
Efficiency %	61.92	63.17	58.83	84.54	86.44	87.01



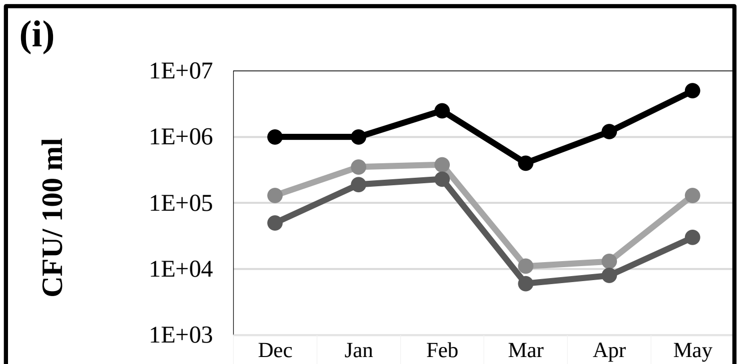
	Dec	Jan	Feb	Mar	Apr	May
Inlet	7.10	7.12	7.24	7.33	7.33	7.29
Water lettuce	7.09	7.05	7.15	7.19	7.06	7.00
Efficiency %	0.14	0.98	1.26	1.96	3.66	3.91
Penny wort	7.06	7.07	7.11	7.12	7.09	7.01
Efficiency %	0.61	0.70	1.81	2.85	3.25	3.77



	Dec	Jan	Feb	Mar	Apr	May
Inlet	14.00	15.20	13.30	27.80	33.00	35.00
Water lettuce	9.80	11.20	9.70	13.90	14.60	12.50
Efficiency %	30.00	26.32	27.07	49.99	55.76	64.29
Penny wort	7.80	9.4	6.90	8.2	8.80	6.40
Efficiency %	44.29	38.16	48.12	70.50	73.33	81.71



	Dec	Jan	Feb	Mar	Apr	May
Inlet	2E+06	2E+06	3E+06	1E+07	3E+07	2E+06
water lettuce	3E+05	4E+05	4E+05	4E+05	4E+05	3E+04
Efficiency %	84.12	77.50	88.24	97.08	98.65	98.45
Penny wort	2E+05	2E+05	2E+05	2E+05	2E+05	2E+04
Efficiency %	91.18	85.63	93.82	98.08	99.42	99.10



	Dec	Jan	Feb	Mar	Apr	May
Inlet	1E+06	1E+06	3E+06	4E+05	1E+06	5E+06
water lettuce	1E+05	4E+05	4E+05	1E+04	1E+04	1E+05
Efficiency %	87.00	65.00	84.80	97.25	98.92	97.40
Penny wort	5E+04	2E+05	2E+05	6E+03	8E+03	3E+04
Efficiency %	95.00	81.00	90.80	98.50	99.33	99.40

Figure 4.13: Removal efficiency of of *Pistia stratiotes* and *Centella asiatica* for (a) TSS (b) DO (c) EC (d) TDS (e) COD (f) TP (g) pH (h) Total *coliforms* (i) Fecal *coliforms*

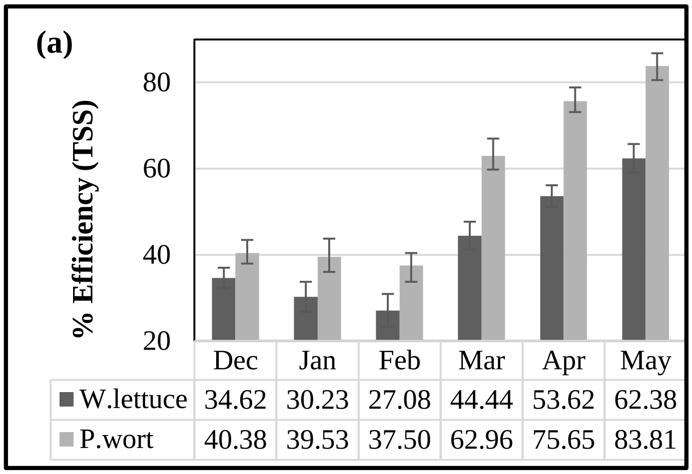
4.5 Comparative Removal Efficiency of *Pistia stratiotes* and *Centella asiatica* analysis Of Pilot Scale Unit, Lab Scale Replica and Parallel Unit

Removal efficiency of *Pistia stratiotes* and *Centella asiatica* was evaluated. This study helped in better understanding of macrophytes and how their performance differ in series unit in which each ponds were linked to one another and in parallel unit in which removal was based on merely uptake of individual plant.

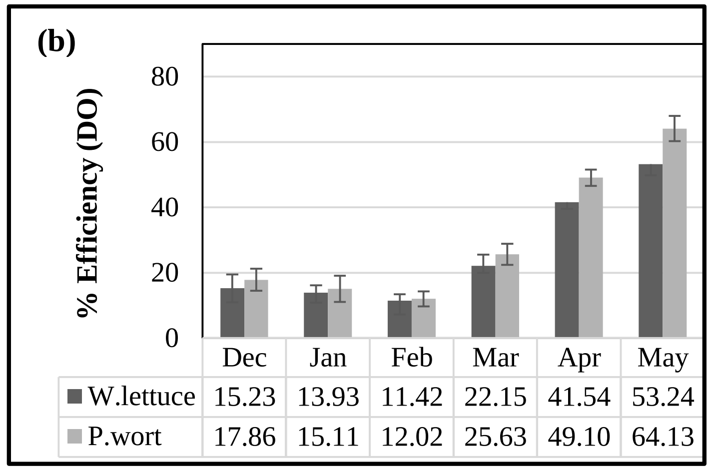
In both plants efficiency of series unit was lower as compared to parallel unit. It can be directed to factor that in case of parallel unit the the retention time for waste water was more as compared to series unit in which water has to flow with a fast rate from one pond to another leading to less contact of water with plants More retention time and comparatively less flow rate of water in case of parallel unit provided more contact of plants and their endphytes with wastewater which resulted in high removal efficiency.

4.6 Comparative Removal Efficiency of *Pistia stratiotes* and *Centella asiatica*

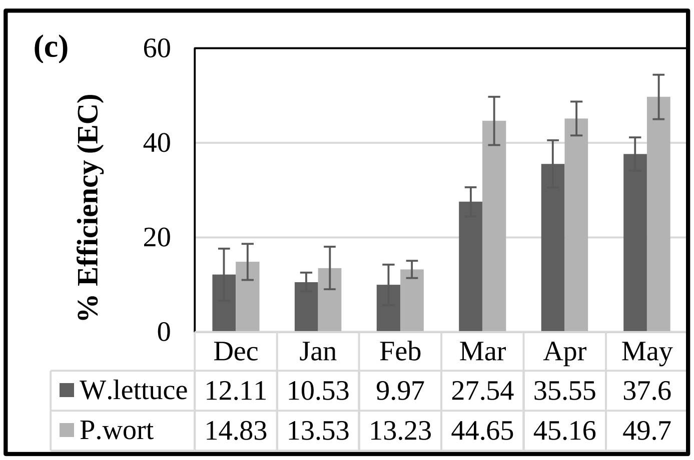
In all three cases; Pilot scale unit, Lab scale replica unit and parallel unit efficiency of *Pistia stratiotes* and *tica* was examined and it was observed that in all cases *Centella asiatica* was more efficient than *Pistia stratiotes.* Figure 4.14 (a) to (i) represents the comparative efficiencies of *Pistia stratiotes* and *Centella asiatica* for parallel unit.



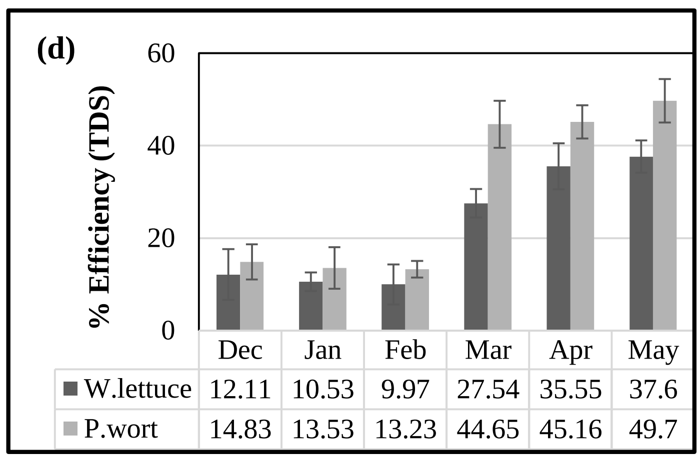
	Dec	Jan	Feb	Mar	Apr	May
W.lettuce	34.62	30.23	27.08	44.44	53.62	62.38
P.wort	40.38	39.53	37.50	62.96	75.65	83.81



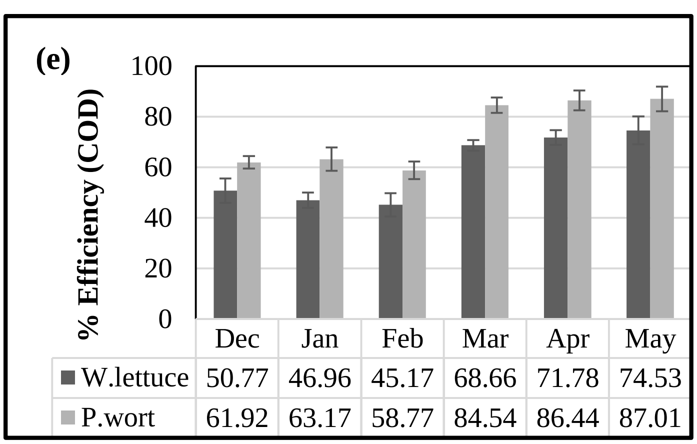
	Dec	Jan	Feb	Mar	Apr	May
W.lettuce	15.23	13.93	11.42	22.15	41.54	53.24
P.wort	17.86	15.11	12.02	25.63	49.10	64.13



	Dec	Jan	Feb	Mar	Apr	May
W.lettuce	12.11	10.53	9.97	27.54	35.55	37.6
P.wort	14.83	13.53	13.23	44.65	45.16	49.7



	Dec	Jan	Feb	Mar	Apr	May
W.lettuce	12.11	10.53	9.97	27.54	35.55	37.6
P.wort	14.83	13.53	13.23	44.65	45.16	49.7



	Dec	Jan	Feb	Mar	Apr	May
W.lettuce	50.77	46.96	45.17	68.66	71.78	74.53
P.wort	61.92	63.17	58.77	84.54	86.44	87.01

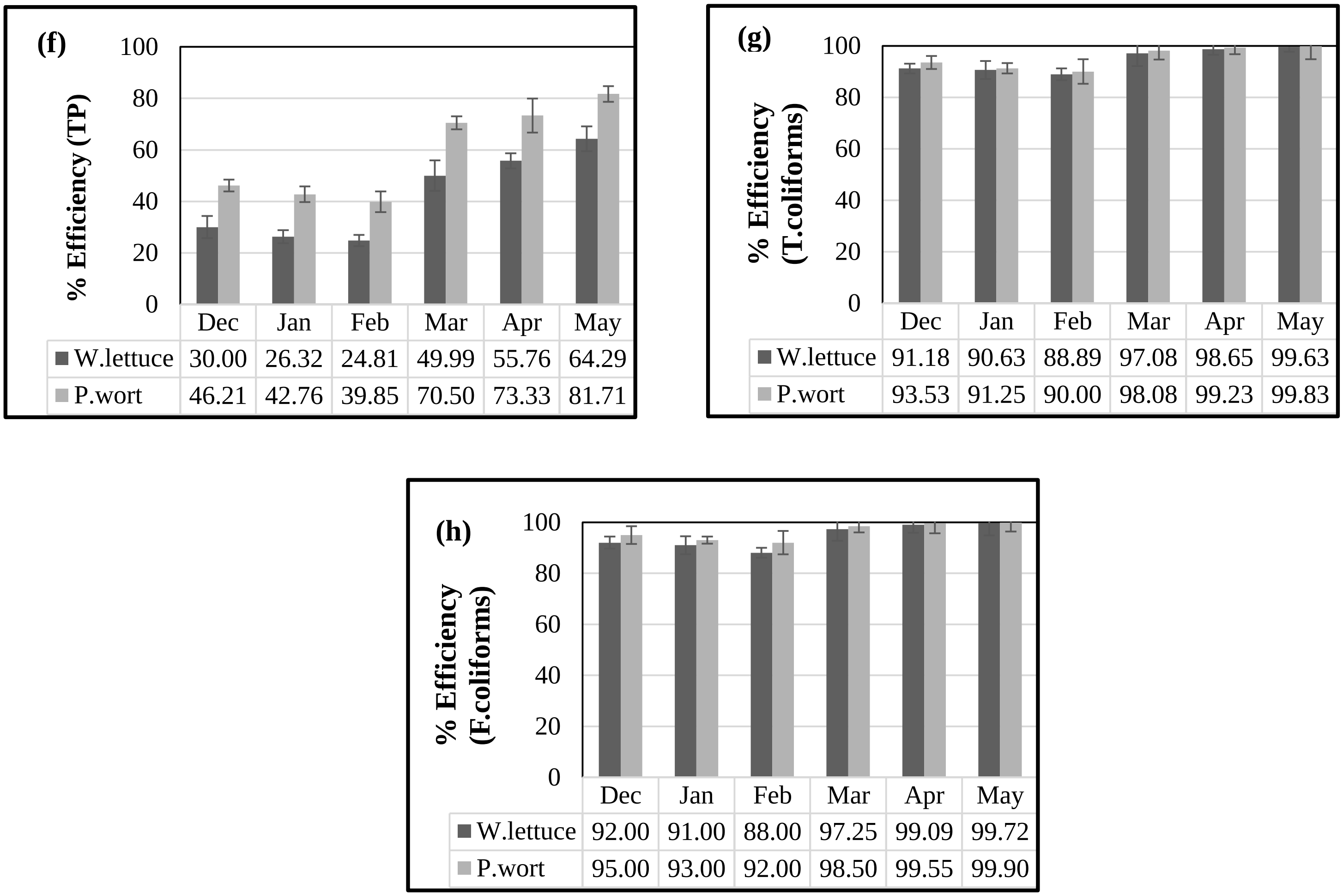


Figure 4.14: Comparative efficiency of *Pistia stratiotes* and *Centella asiatica* for (a) TSS (b) DO (c) EC (d) TDS (e) COD (f) TP (g) pH (h) *T.coliforms* (i) *F.coliforms*.

Although both plants are excellent phytoremediation agent as compared to aquatic macrophytes still there are some limitations.

1. One of the most important factor that makes *Centella asiatica* an efficient macrophyte than *Pistia stratiotes* is that former is more temperature resistant (Oren and Amit, 2013). While *Centella asiatica* is slightly more tolerant to temperature *Pistia stratiotes* is hard non winter plant, and at 15 ºC having a minimum growth. Also it requires more solar radiation than *Centella asiatica* and showed slower growth rate in case of heavy rainfall.

2. For an efficient phytoremediation agent, optimal growth of plant is a vital parameter. Environmental factors such as solar radiation influence plant growth. Growth rate of *Centella asiatica* is high and it spreads like a carpet over surface of water covering maximum area as compared to *Pistia stratiotes* even in case of moderate or high solar radiation. Growth of *Centella asiatica* leaves are in more vertical direction which provide more surface area for sunlight absorption. Growth rate of *Centella asiatica* is reported to be 5-10 days while that for *Pistia stratiotes* is 25 days (Gupta, 2012).
3. Deep root system of *Centella asiatica* provides maximum contact of endophytes with wastewater (Vymazal and Lenka, 2008) which results in high nutrient uptake capacity and efficient nutrient removal as compared to *Pistia stratiotes.*

4.7 Removal Efficiency of Control Unit

Control unit (without any macrophytes) was designed specifically to study either removal is based on exclusively phytoremediation or there are other physical processes involve. For this water was allowed to pass from inlet and through control unit to outlet and samples were taken from inlet and outlet of control unit.

Readings at both sample points were compared to examine removal of contaminants. It was observed that without introduction of phytoremediation no removal efficiency was achieved in some cases value for control outlet was the same as that taken from inlet. In other cases values for control outlet was negligibly less than control inlet and cannot be considered as a removal pattern. Overall study of control unit helped in clarifying the fact that without introduction of plants in wetland removal efficiency cannot be achieved.

4.8 Identification of isolated strains from roots and leaves of *Pistia stratiotes* and *Centella asiatica*

From roots of *Pistia stratiotes* and *Centella asiatica* 10 different strains were obtained AHR1-AHR10 while from leaves eight different species were obtained as AHL1-AHL8.

4.8.1 Morphological Characterization

Isolated bacterial starins were studied for their form, color, opacity, elevation, margin and surface. Table 4.1 (a) and (b) represent colony and cell morphology of bacterial isolates from roots of *Pistia stratiotes* and *Centella asiatica* respectively. Table 4.2 (a) and (b) represent colony and cell morphology of bacterial isolates from leaves of *Pistia stratiotes* and *Centella asiatica* respectively.

Strains	Form	Color	Elevation	Margin	Surface	Opacity
AHR1	Punctiform	Off white	Flat	Entire	Dry	Opaque
AHR2	Irregular	White	Raised	Lobate	Smooth	Translucent
AHR3	Circular	Off white	Raised	Lobate	Smooth	Opaque
AHR4	Circular	Yellow	Convex	Entire	Smooth	Translucent
AHR5	Punctiform	Off white	Flat	Entire	Dry	Opaque
AHR6	Punctiform	Off white	Flat	Entire	Dry	Opaque
AHR7	Punctiform	Off white	Flat	Entire	Dry	Opaque
AHR8	Punctiform	Off white	Flat	Entire	Dry	Opaque
AHR9	Irregular	White	Raised	Lobate	Smooth	Translucent
AHR10	Punctiform	Off white	Flat	Entire	Dry	Opaque

Table 4.1 (a):Colony morphology of bacterial isolates from roots of *Pistia stratiotes* and *Centella asiatica*

Strains	Gram Reaction	Shape	Arrangement	Motility
AHR1	Positive	Bacilli	Single/Pairs	+
AHR2	Positive	Bacilli	Pairs/Groups	+
AHR3	Positive	Bacilli	Pairs/Groups	++
AHR4	Positive	Bacilli	Chained/Groups	+
AHR5	Positive	Bacilli	Single/Pairs	+
AHR6	Positive	Bacilli	Single/Pairs	+
AHR7	Positive	Bacilli	Single/Pairs	+
AHR8	Positive	Bacilli	Single/Pairs	+
AHR9	Positive	Bacilli	Pairs/Groups	+
AHR10	Positive	Bacilli	Single/Pairs	+

+ Fast ++ Very fast

Table 4.1 (b):Cell morphology of bacterial isolates from roots of *Pistia stratiotes* and *Centella asiatica*

All isolates were gram positive when observed under microscope and were all bacilli. AHR3 showed very fast motility and rest of isolates showed fast motility as studied under microscope at 100X. AHR1, AHR5-AHR8 and AHR10 appeared in single and pair form, AHR2 and AHR3 appeared in pairs and groups while AHR4 in chained and group form.

Strains	Form	Color	Elevation	Margin	Surface	Opacity
AHL1	Punctiform	Off white	Flat	Entire	Smooth	Opaque
AHL2	Circular	Off white	Convex	Undulate	Dry	Opaque
AHL3	Circular	Off white	Raised	Entire	Smooth	Opaque
AHL4	Circular	Off white	Flat	Lobate	Smooth	Opaque
AHL5	Circular	Off white	Raised	Entire	Smooth	Opaque
AHL6	Punctiform	Off white	Flat	Entire	Dry	Translucent

AHL7	Circular	Yellow	Flat	Entire	Smooth	Opaque
AHL8	Circular	Off white	Raised	Entire	Smooth	Opaque

Table 4.2 (a):Colony morphology of bacterial isolates from leaves of *Pistia stratiotes* and *Centella asiatica*

Strains	Gram Reaction	Shape	Arrangement	Motility
AHL1	Positive	Bacilli	Single/Pairs	+
AHL2	Negative	Bacilli	Pairs	+
AHL3	Positive	Bacilli	Single/pairs	+
AHL4	Positive	Bacilli	Pairs/Groups	+
AHL5	Positive	Bacilli	Single/Chained	+
AHL6	Positive	Bacilli	Single/Chained	+
AHL7	Positive	Bacilli	Single/Chained	+
AHL8	Positive	Bacilli	Single/Pairs	+

+ Fast ++ Very fast

Table 4.2 (b):Cell morphology of bacterial isolates from leaves of *Pistia stratiotes* and *Centella asiatica*

Only AHL2 appeared gram negative when studied under microscope. All strain showed fast motility when studied under microscope at 100X. AHL1, AHL3 and AHL8 showed single and paired arrangements. AHL2 showed pair arrangement, AHL5-AHL7 showed single/chained arrangements and AHL3 showed pair/group arrangements

4.8.2 Biochemical Characterization

After morphological characterization strains were studied for biochemical characters. Table 4.3 (a) represent biochemical characterization of bacterial isolates from roots of *Pistia stratiotes* and *Centella asiatica* respectively. Table 4.3 (b) represent biochemical characterization of bacterial isolates from leaves of *Pistia stratiotes* and *Centella asiatica* respectively.

Strains	**Oxidase**	**Catalase**	**MacConkey agar**	**EMB agar**
AHR1	Positive	Positive	Negative	Negative
AHR2	Positive	Positive	Negative	Negative
AHR3	Positive	Positive	Negative	Negative
AHR4	Positive	Positive	Negative	Negative
AHR5	Positive	Positive	Negative	Negative
AHR6	Positive	Positive	Negative	Negative
AHR7	Positive	Positive	Negative	Negative
AHR8	Positive	Positive	Negative	Negative
AHR9	Positive	Positive	Negative	Negative
AHR10	Positive	Positive	Negative	Negative

Table 4.3 (a):Biochemical characterization of bacterial isolates from roots of *Pistia stratiotes* and *Centella asiatica*

Strains	**Oxidase**	**Catalase**	**MacConkey agar**	**EMB agar**
AHL1	Negative	Positive	Negative	Negative
AHL2	Negative	Positive	Positive	Positive
AHL3	Negative	Positive	Negative	Negative
AHL4	Positive	Positive	Negative	Negative
AHL5	Positive	Positive	Negative	Negative
AHL6	Positive	Positive	Negative	Negative

AHL7	Positive	Positive	Negative	Negative
AHL8	Negative	Positive	Negative	Negative

Table 4.3 (b):Biochemical characterization of bacterial isolates from leaves of *Pistia stratiotes* and *Centella asiatica*

In case of strains from both roots and leaves only AHL1 showed positive results on MacConkay agar and EMB agar all other strains showed negative response. AHL1-AHL3 and AHL8 showed negative results for oxidase test all other isolated strains showed positive reaction to oxidase and catalase test.

4.8.3 Selection of Strains for Gene Sequencing

After detailed analysis of isolated strains through morphological and biochemical characterization strains showing similarity in all of the tests performed were not considered for sequencing .Specific strains (AHR1-AHR3) (AHL1-AHL7) were selected for further Full length 16S rRNA gene sequencing. It was performed at Genome Analysis Department Macrogen Inc. Korea.

The strains were screened and noise was removed manually. Strains were identified through BLAST search (Althushul, 1997) available at National Center for Biotechnology Information (NCBI) databases revealing up to 99% similarity to different bacterial species. Schloss in 2004 market the limit of 97% for identification of species (Schloss, 2004).

Complete sequence alignment was performed through CLUSTALW. A phylogenetic tree, assembled through MEGA 4 program demonstrates the phylogenetic relatedness and linkage among identified strains, shown in Figure 4.15

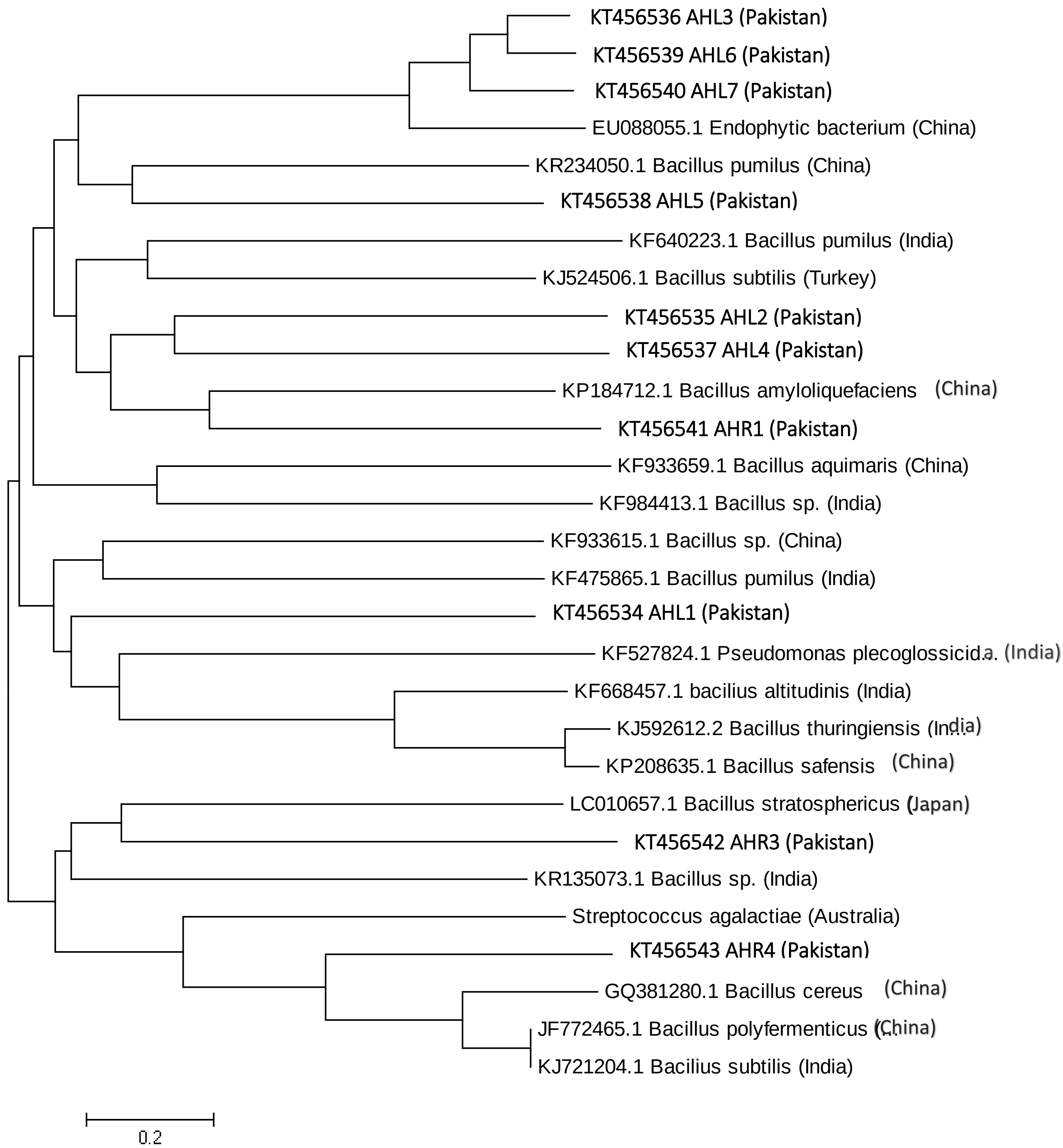


Fig 4.15:Phylogenetic tree demonstrating the relatedness and linkage of bacterial strains. Genus identified were *Pseudomonas* and *Bacillus*

Strains AHL1-AHL7 were identified as *Bacillus thuringiensis, Pseudomonas plecoglossicida, Bacillus pumilus, Bacillus aquimaris, Bacillus safensis, Bacillus amyloliquefaciens* and *Bacillus anthracis* respectively. Strains AHR1-AHR4 were identified as *Bacillus altitudinis, Bacills cereus and Bacillus subtilis* respectively. 16S rRNA sequences of identified species were submitted to GenBank under accession numbers KT456534, KT456535, KT456536, KT456537, KT456538, KT456539, KT456540, KT456541, KT456542, and KT456543. The data is now available online at http://www.ncbi.nlm.nih.gov/.

Weyens and coworkers evaluated function of endophytes isolated from pennywort used for phytoremediation and studied degradation pathways of organic compounds and toxic materials. Plant-microbe partnership assisted in degradation and conversion of harmful substances (Weyens *et al*., 2009). Vymazal and Brix concluded microorganisms in rhizosphere to be base of phytoremediation. They elaborated the importance of the rhizosphere for forming enhanced conditions for numerous microorganisms utilized in CWs (Oren and Amit, 2013). A study led by Huiping and coworkers observed Microbial variation between roots and shoots of water lettuce and isolated *Bacillus pumilus* and *Bacillus cereus* from roots while *Bacillus sp* from shoots of selected plants (Huiping *et al*., 2009). Zareen reported *Entero-bacteriaceae, Pseudomonaceae* and *Burkholderiaceae* as among the most notable genera of endophytes in plants (Zareen, 2011). Erick isolated endophytes of *Centella asiatic* and studied their impact on plant pathogen control. Dominant isolated species were *Bacillus pumilus, Bacillus subtilis, Acinetobacter sp, Microbacterium sp, Pseudomonas putida and Cohnella luojiensis* (Erick *et al*., 2012). Shehzadi and coworkers isolated 41 strains from roots and shoots of *Pistia stratiotes*, *Eichhornia crassipes* and *Typha domingensis*. Isolated species of endophytic bacteria belonged to mainly genera *Bacillus* (39%), *Microbacterium* (12%) and *Halomonas* (12%) (Shehzadi *et al*., 2015)

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Basic aim of study was to evaluate efficiency of constructed wetland both in series and parallel unit. This study helped in determining compatibility of water lettuce and pennywort with respect to seasonal variation. Isolation of endophytes assisted in identification of microbial species involved in phytoremediation. Following conclusion were drawn from current study;

1. Plants exhibited high removal efficiency during course of summers as compared to winters due to rapid and maximum plant growth. Heavy rainfall during month of March resulted in wastewater dilution and in turn low influent values.
2. Efficiency of series unit was observed to be less than parallel due to (a) low retention time of wastewater (b) Less contact of water with plants.
3. *Centella asiatica* showed comparatively good removal efficiency than *Pistia stratiotes* due to: its temperature resistivity, fast growth and deep root system. For *Pistia stratiotes* and *Centella asiatica* removal efficiencies were recorded as, for: TSS (62.3 and 83 %), DO (53.7 and 64 %), COD (74 and 87 %), TP (64 and 81 %), Fecal *coliforms* (99.7 and 99.9 %) and Total *coliforms* (99.6 and 99.9 %).
4. A total of 10 species of endophytes were isolated from roots and leaves of *Pistia stratiotes* and *asiatica.* 16S rRNA gene sequencing was performed and isolated strains were identified as *Bacillus altitudinis, Bacills cereus, Bacillus thuringiensis, Pseudomonas plecoglossicida, Bacillus subtilis, Bacillus pumilus, Bacillus aquimaris, Bacillus safensis, Bacillus amyloliquefaciens* and *Bacillus anthracis.*

5.2 Recommendations

1. Detailed study of microbial diversity within wastewater.
2. Degradation pathways of contaminants and enzymes responsible may be explored for better understanding of degradation process.
3. Effect of total sunshine hours and rain pattern may be studied.

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