CHARACTERIZATION OF MICROBES AND PERFORMANCE EVALUATION OF INTEGRATED CONSTRUCTED WETLAND

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Islamabad, Pakistan

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Environmental Science

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2023

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This thesis is dedicated to my parents whose continuous support and prayers are always with me whenever and wherever required

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Faria Javed

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LIST OF ABBREVIATIONS

UNESCO	United Nations Educational and Cultural Organization
FILTER	Filter and Irrigated Cropping for Land Treatment and Effluent Reuse
HSSF-CW	Horizontal Subsurface Flow – Constructed Wetland
ICW	Integrated Constructed Wetland
IL	Inlet
ST	Sedimentation Tank
P1	Pond 1
P2	Pond 2
P3	Pond 3
P4	Pond 4
P5	Pond 5
P6	Pond 6
P7	Pond 7
P8	Pond 8
CT	Collection Tank
rpm	Revolution Per Minute
SDGs	Sustainable Development Goals
TDS	Total Dissolved Solids
TKN	Total Kjeldahl Nitrogen
TSS	Total Suspended Solids
NO_3^--N	Nitrate-Nitrogen
NO_2^--N	Nitrite-Nitrogen
PO_4^{3-}-P	Phosphate-Phosphorus
COD	Chemical Oxygen demand
EC	Electrical Conductivity
DO	Dissolved Oxygen
HDPE	High-Density Polyethylene
IESCO	Islamabad Electric Supply Company

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ABSTRACT

The increase in population, rapid industrial growth, and land utilization to fulfill demand for agricultural products have increased water stress. Only less than 10% of wastewater is being treated in developing countries including Pakistan where the groundwater and surface water quality is already low and is continuing to degrade due to unchecked disposal of wastewater. This is resulting in negative impacts on human health as well as the environment. There needs to be a sustainable wastewater treatment method that makes wastewater safe for reuse. One such technology is constructed wetlands that use natural processes and is a sustainable and cost-effective method. The present study aimed at monitoring the performance of an integrated constructed wetland (ICW) located at NUST, H-12 campus, Islamabad. It has a sedimentation tank for pre-treatment, eight ponds planted with different vegetation (*Typha latifolia*, *Pistia stratiotes, and Centella asiatica*), and a FILTER (filtration and irrigated cropping for land treatment and effluent reuse) technology. The objectives of study include divided into the analysis of physicochemical parameters, biological (helminth egg) analysis, microbial (endophyte analysis), and economic valuation of integrated constructed wetland. The water samples for physicochemical analysis were collected from outlet of each sampling point i.e., sedimentation tank, outlet of eight ponds, and collection tank for a period of six months from May to October 2021. All the analysis was conducted using the American Public Health Association (APHA) standard method. The samples for the helminth egg were collected from the same points for one month using a modified USEPA method. The values of temperature, pH, and DO range between 22.6-32.4 ºC, 6.4-7.7, 1.3-6.0 mg/L. The removal efficiency of different parameters was up to 79.01% for Total suspended solids (TSS), 33.13% Total dissolved solids (TDS), 43.12% Total solids (TS), 67.45% Chemical oxygen demand (COD), 73.34%, Electrical conductivity (EC), 15.91% Turbidity, 37.70% Total Kjeldahl nitrogen (TKN), 61.68% Phosphate-phosphorous (PO_4^{3-}-P) and 100% for Helminth eggs. Heavy monsoon rains during sampling month washed helminth eggs away. Eight bacterial strains were isolated from roots, shoots and leaves of *Typha latifolia* from pond 1 of ICW. All isolates were gram-negative and catalase and oxidase positive. The major cost components of ICW were construction cost and human resources for operation and maintenance. Monetary benefits of the studied system were 32.4 million PKR when compared with the cost of purchasing freshwater from CDA.

Chapter 1

INTRODUCTION

1.1. Background

One of the essentials for living is water. According to World Health Organization (WHO), water is an "important source for improving public health" because it considerably improves cleanliness and avoids numerous diseases (Pruss-Ustun & WHO, 2008). The increasing demand for water brought on by urban growth around the world has had a variety of socioeconomic effects that put further stress on already scarce natural resources (Waly *et al.,* 2022). In past 50 years, the world's water consumption has tripled, with maximum use of water in agricultural sector i.e., 80–90% of fresh water supplies that are available (Hussain *et al.*, 2019). Since 2.4 million people have no access to safe water worldwide and over 1.1 billion people experience water scarcity, accessibility to safe water is a privilege for just a select few (Qamar *et al.*, 2022). Based on data from International Monetary Fund (IMF), Pakistan is third among nations with issues with inadequate water and sanitation because 2.1 million Pakistanis lack access to clean water (Zhang *et al.*, 2020). If not addressed immediately, it is expected that Pakistan's water supply and quality issues would be quite problematic. According to Zhang *et al.* (2020) inadequate and unclean water has severely harm many of the nation's essential systems, including agricultural, environmental, and sociological ones. Problems with water sanitation and hygiene have also posed a number of concerns to the public health of Pakistan, as likelihood of contracting waterborne infections is rising rapidly. Pakistan recorded 2.5 million diarrhea-related fatalities in 2017; 40% of country's illnesses and deaths are caused by consuming unclean water (Daud *et al.*, 2017). Numerous pollutants, including feces and microorganisms, metal contaminants, household and industrial waste, antibiotics, and various dangerous drugs, are present in water (Noor *et al.*, 2023). Climate change that affects annual rainfall, inadequate establishment of reservoir structures, and political pressures are some of the causes of Pakistan's current water issue (Mahfooz *et al.*, 2019; Zhang *et al.*, 2020; Daud *et al.*, 2017). These environmental problems are mostly caused by carbon emissions, deforestation, increasing urbanization, and industrialization (Afridi *et al.*, 2019; Anwar *et al.*, 2021; Adewumi 2022). Poor water sanitization cost Pakistan 343.7 billion PKR (1.5 billion USD) in 2019, posing a serious economic burden. Additionally, in 2016–17, with UNICEF's assistance, price of funding services for cleaner water climbed from 48 billion Pakistani Rupees to 72 billion Pakistani Rupees. It might be claimed that providing sanitized water across Pakistan will need funds because even these subpar services were not offered to entire nation. If ongoing trend does not change, a lot of existing issues, such as poverty, disease prevalence, and financial instability, are expected to further worse (Howard, 2021).

As a result, the demand for unconventional water resources linked to sustainable water resource management practices has increased (Dawoud *et al.,* 2020). One of the non-traditional water sources that may help with water scarcity is wastewater reuse. However, due to likelihood that it will be subject to reuse plans, which demand ad hoc wastewater compliance with planned water usage, this option needs careful consideration and design (Najjar *et al.,* 2018). Therefore, it is decided that to mitigate any possible negative effects caused by different use of wastewater applications, efficient wastewater treatment facilities with zero to minimally related hazards are required (Waly *et al.,* 2022).

1.2. Wastewater and its composition

According to Warwick *et al.* (2013), wastewater is a complex matrix with high quantities of particles (total solids 350–1200 mg/L), dissolved and particulate matter (COD 250–1000 mg/L), microbes (up to 109 number/mL), nutrients, heavy metals, and micro-pollutants.

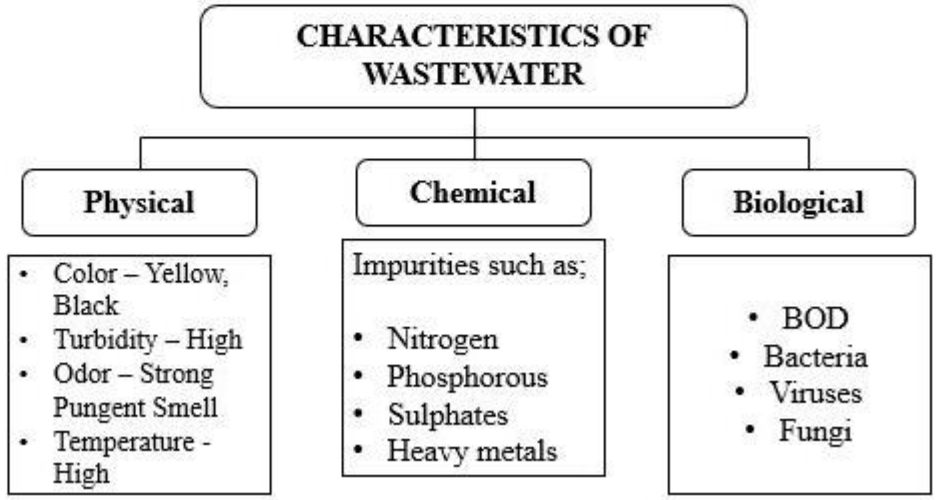


Figure 1.1: Characteristics of wastewater

The following table (Table 1.1) represents the important pollutants in wastewater, sources, and their effects.

Table 1.1: Wastewater pollutants, their sources, and effects

Pollutants	Main representative parameters	Sources				Possible effects of the pollutants
		Wastewater		Storm water		
		Domestic	Industrial	Urban	Agricultural and Pasture	
Suspended solids	Total suspended solids	XXXX	← →	XX	X	• Aesthetic problems • Sludge deposits
Biodegradable organic matter	Biochemical oxygen demand	XXXX	← →	XX	X	• Oxygen consumption • Septic conditions
Nutrients	Nitrogen Phosphorous	XXXX	← →	XX	X	• Excessive algae growth • Pollution of groundwater
Pathogens	Virus, Bacteria, Helminths eggs	XXXX	← →	XX	X	• Water borne diseases
Non-biodegradable organic matter	Pesticides, Some detergents, others	X	← →	X	XX	• Toxicity • Non-biodegradability
Metals	Specific elements (As, Cd, Cr, Cu etc.)	X	← →	X		• Toxicity • Contamination of groundwater
Inorganic dissolved solids	Total dissolved solids Conductivity	XX	← →		X	• Excessive salinity • Toxicity to plants

X: Small, XX: Medium, XXX: High, ← → : Variable, Empty: Usually not important

The implementation of low-energy consuming wastewater treatment methods is sought to aid in accomplishment of Sustainable Development Goal 6, which emphasizes an obligation to ensure access to sanitation (Tortajada, 2020; O'Neill *et al.,* 2022). Constructed wetland (CW) refers to a non-traditional or natural wastewater treatment technology that is inexpensive and simple to use (Hayder *et al.*, 2022; Thao *et al.*, 2022).

1.3. Present study

The study was focused to evaluate performance efficiency (for nutrients and pathogen removal) of an integrated constructed wetland established at the National University of Sciences and Technology (NUST) in 2014 for institutional wastewater treatment. Predominant endophytes were isolated from *Typha latifolia* of pond 1 to identify the endophytes involved in the degradation of contaminants. The cost and benefits including monetary and non-monetary benefits are also analyzed in this study.

1.4. Aims and objectives

1. Performance evaluation of integrated constructed wetland using physicochemical parameters
2. Isolation, morphological and biochemical characterization of bacterial community from roots, shoots and leaves of selected wetland plant
3. Detection and quantification of pathogenic helminth eggs
4. Economic valuation of integrated constructed wetland

CHAPTER 2

LITERATURE REVIEW

2.1. Constructed wetlands

CW is a green technique that treats many types of wastewaters using substrate, water-tolerant macrophytes, and microbes (Patyal *et al.*, 2023). The removal of nutrients, organic materials, suspended particles, pathogens, and metals has demonstrated the effectiveness of CWs (Kumar *et al.*, 2020).

Since the first full-scale plant for the treatment of domestic and industrial wastewater was installed in the 1960s, a great deal of study has been done (Masi *et al.*, 2008; Sheng *et al.*, 2020). To address the difficulties of removing recalcitrant pollutants and achieving the quality of effluent for discharge or reuse, CW enhancement research has increased during the past two decades. Research has been conducted on numerous system elements and aspects as a result of the effort to accelerate CW degradation routes. To increase the effectiveness of removing target contaminants from wastewater, many materials have been researched and employed as substrates in CW (Ballantine & Tanner, 2010; Fu *et al.*, 2020). Similar to this, a great deal of work has been done at the lab and pilot scale on various macrophyte kinds as well as to determine the role of microbes in CW for increasing the effectiveness of treatment (Deng *et al.*, 2021; Li *et al.*, 2010; Datta *et al.*, 2021).

The treatment of wastewater safeguards the general public's health by halting the spread of infectious diseases. The primary microbial pathogens in the final effluents of wastewater are anthropogenically derived bacteria. To prevent microbiological pathogens in wastewater, it is crucial to treat wastewater (Chen *et al.*, 2019).

Additionally, helminths pose a serious risk to one's health and are a significant public health issue, particularly in underdeveloped nations. Globally, helminths currently affect and infect over 2.6 billion people. Helminth eggs spread Helminthiasis (helminthic disease) because it is the infectious stage of their lifecycle. The risk of contracting helminthiasis is correlated to their persistence in the environment. They can remain present in the environment for several years. One egg of helminths is enough to cause the infection and they are highly resistant to different treatment processes. Moreover, they have a high oviposition capacity (Jimenez *et al.*, 2020). Jiménez, 2003 reported that wetlands are proved to be effective in 90-98% removal of fecal coliforms and up to 100% removal of helminth eggs.

The effectiveness of the system can be improved by conducting extensive research on operational considerations like changes in depth, variations in hydraulic loading rate, and different hydraulic retention times, as well as flow pattern changes, different aeration methods, recirculation, and other operational factors (Kolli *et al.,* 2021; Nicolics *et al.*, 2016).

2.2. History and types of constructed wetlands

Early in the 1950s, Käthe Seidel conducted studies at the Max Planck Institute in Germany that explored the potential for wastewater treatment using wetlands (Seidel, 1955). The application of wetland plants for the treatment of many forms of wastewater, particularly phenol wastewater, was then the focus of many experiments conducted by Seidel (Seidel, 1976). However, the first fully constructed wetland was set up with a free water surface in the Netherlands in 1967. Most studies were conducted with either horizontal (HSSF) or vertical subsurface flow-constructed wetlands (VSSF) (DeJong, 1976). Combining different constructed wetlands can increase the treatment impact, particularly for nitrogen. Although vertical flow and horizontal flow systems are two types of constructed wetlands that are typically combined in hybrid systems, any type of constructed wetland might be used in a hybrid system to obtain a more complicated level of treatment efficiency. The wetland type is determined by the flow pattern of wastewater. Surface flow and subsurface flow constructed wetlands are two primary classifications.

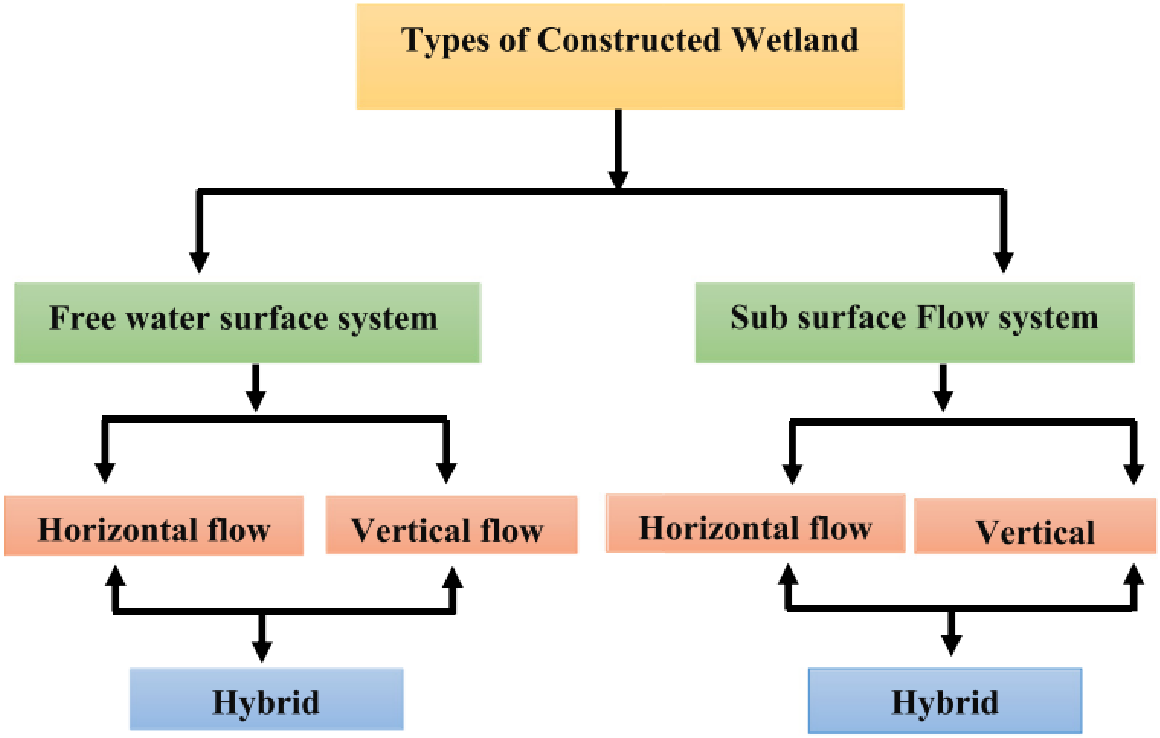


Figure 2.1: Types of constructed wetlands based on flow direction

(Batool and Saleh, 2020)

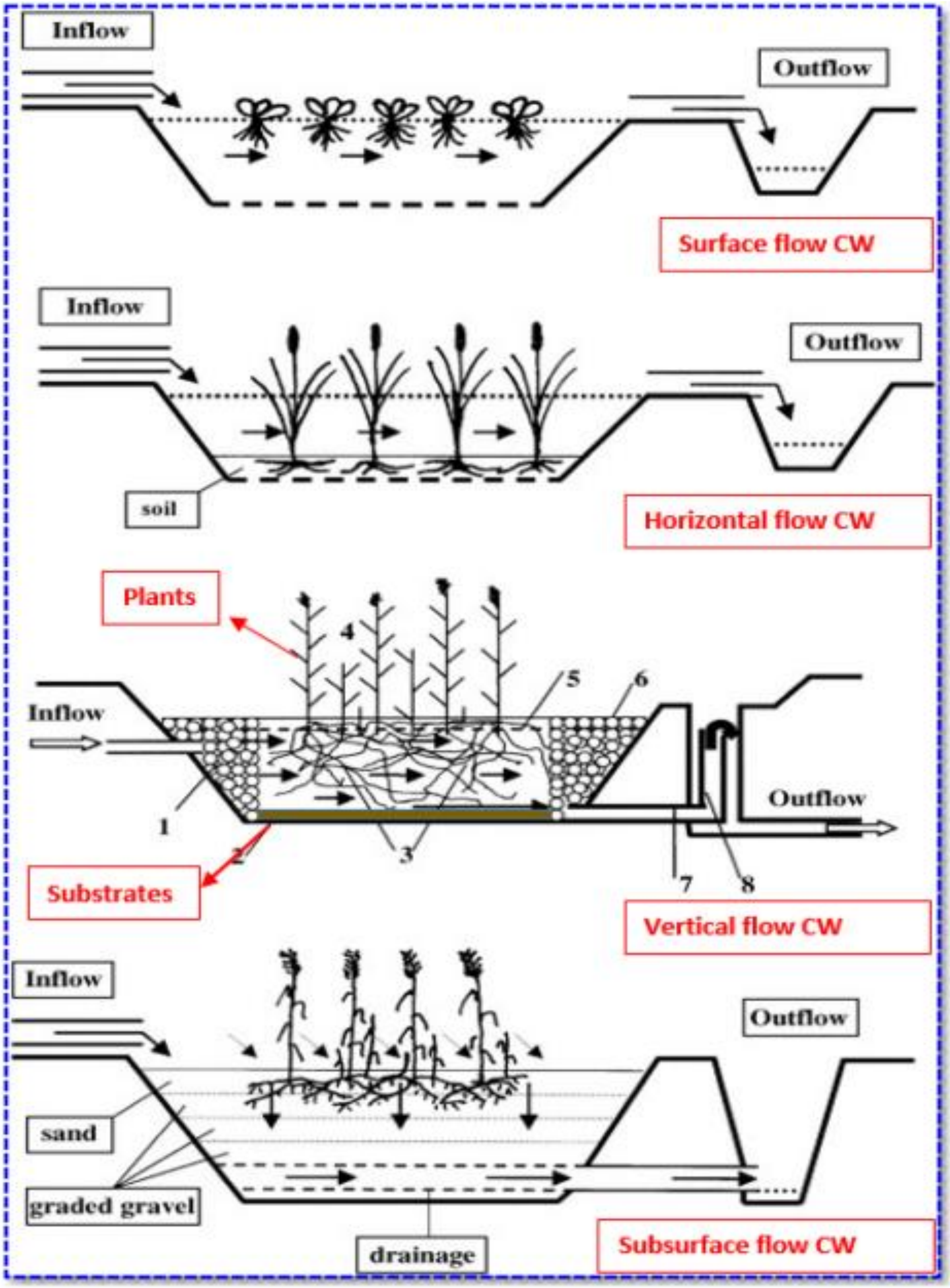


Figure 2.2. Types of constructed wetland (Garcia *et al.,* 2006; Vymazal, 2001; 2010).

2.2.1. Free-water surface system

Free water surface system-constructed wetlands (FWSCW) mimic natural lagoons that are between 0.3 and 0.4 meters deep. Plant stems and leaves are traversed by leachate and wastewater. Additionally, microbes are crucial to the functioning of plant rhizomes. The distribution of vegetation on the free surface of the water might not be uniform or homogeneous.

2.2.2. Subsurface flow system

Wetland depth is between 0.3 and 0.9 meters, and water flow is in touch with the roots and substrates. This system offers a setting for the growth of biofilm and the elimination of contaminants. Bulrush and common reed are frequently employed in subsurface flow systems.

2.2.3. Horizontal flow system

Water moves horizontally through the granular bed at a flow rate of 0.05–0.1 cubic meters and a depth of 0.3–0.9 meters (García, 2011). A horizontal flow system varies in effectiveness and has a good circulation system of pipes. For filtration, coarse gravels are poured into the input and output. In horizontal sub-surface flow (HSF), *P. australis* is frequently planted.

2.2.4. Vertical flow system

Instead of being constantly inundated, water moves vertically downwards across a bed of substrates. With the same organic loading rate, the vertical flow system's efficacy for treatment is higher than that of a horizontal flow system. Additionally, as underground pipes are 0.05–0.01 placed in soil, they are susceptible to clogging.

2.2.5. Hybrid systems

For the removal of nitrogen and nitrates, hybrid systems combine horizontal (HSSF) and vertical subsurface flow (VSSF) systems. Using various wetland vertical subsurface flow types with free water surface and other combinations, similar and distinct combinations are also feasible.

2.3. Vegetation in constructed wetlands

Typical activities performed by vegetation include chemical transformation and removal of toxins. According to Kadlec and Wallace (2008), sustainable plant growth supports the wetland's effective operation. Plant growth patterns are grouped according to the water's surface.

1) Submerged aquatic plants

2) Emergent woody plants

3) Floating vegetation

4) Emergent soft tissue plant

5) Floating mats

Emergent soft tissue plants, which have a rhizome network and deep roots, predominate among macrophytes. Examples are bulrush (*Scirpus*), cattail (*T. latifolia*), and common reed (*P. australis*).

2.4. Design and operation of constructed wetlands

To obtain good water quality during the treatment process, constructed wetlands are probably designed to closely resemble natural wetlands in every area of their structure (Vymazal, 2001; Hammer, 2020). Many different systems and designs have been adopted to fulfill individual needs of wastewater treatment, sites are frequently available, and a wide range of native plant species can be chosen. The planning step is crucial and significant in the design of constructed wetlands. Additionally, each of the chosen sites is distinct, and each will have a different engineered wetland system (Kadlec and Wallace, 2008; Davis, 1995; Hammer, 2020). Four elements are needed to create a constructed wetland: a liner, substrate, (plants) vegetation, and an underdrain system. The liner stops water leaks and prevents groundwater and the environment from being contaminated by wastewater.

2.5. Economics of constructed wetlands

Whether or not constructed wetlands are a more cost-effective treatment option than other existing or traditional treatment methods will determine how they are used. To find if the establishment of a constructed wetland will generate an additional economic value advantage, each activity must be analyzed separately (Zhang *et al.*, 2009).

Some essential elements that must be considered in an economic analysis include (Vymazal, 2010; Wallace and Knight, 2006):

1. Costs associated with construction.
2. Nutrients value lost during wetland treatment.
3. Cost of equipment and labor required to apply wastewater to the land.
4. Cost of the land used to establish the wetland.
5. Cost of crops lost because the CW took land out of production and,
6. Cost of operation and maintenance

2.5.1. Benefits of constructed wetlands

Wildlife is usually attracted by constructed wetlands. Numerous birds, animals, amphibians, reptiles, and different kinds of insects make the wetlands their habitat. Constructed wetlands are recognized to have additional advantages other than improving water quality, such as aesthetic landscape enhancement, improved biodiversity, recreational opportunities, and hunting opportunities (Koskiaho, 2009). The design team can add extra components to the wetland structure that doesn't interfere with the main objective of wastewater treatment. Aesthetic appeal, value for education, opportunities for recreation, and ecological value are just a few of these advantages.

CHAPTER 3

METHODOLOGY

3.1. Study site

The study site was integrated constructed wetland which is located at the National University of Science and Technology (NUST), Sector H-12, Islamabad, Pakistan at the following global coordinates.

Latitude: 33°38'31.1"N Longitude: 73°00'13.7"E

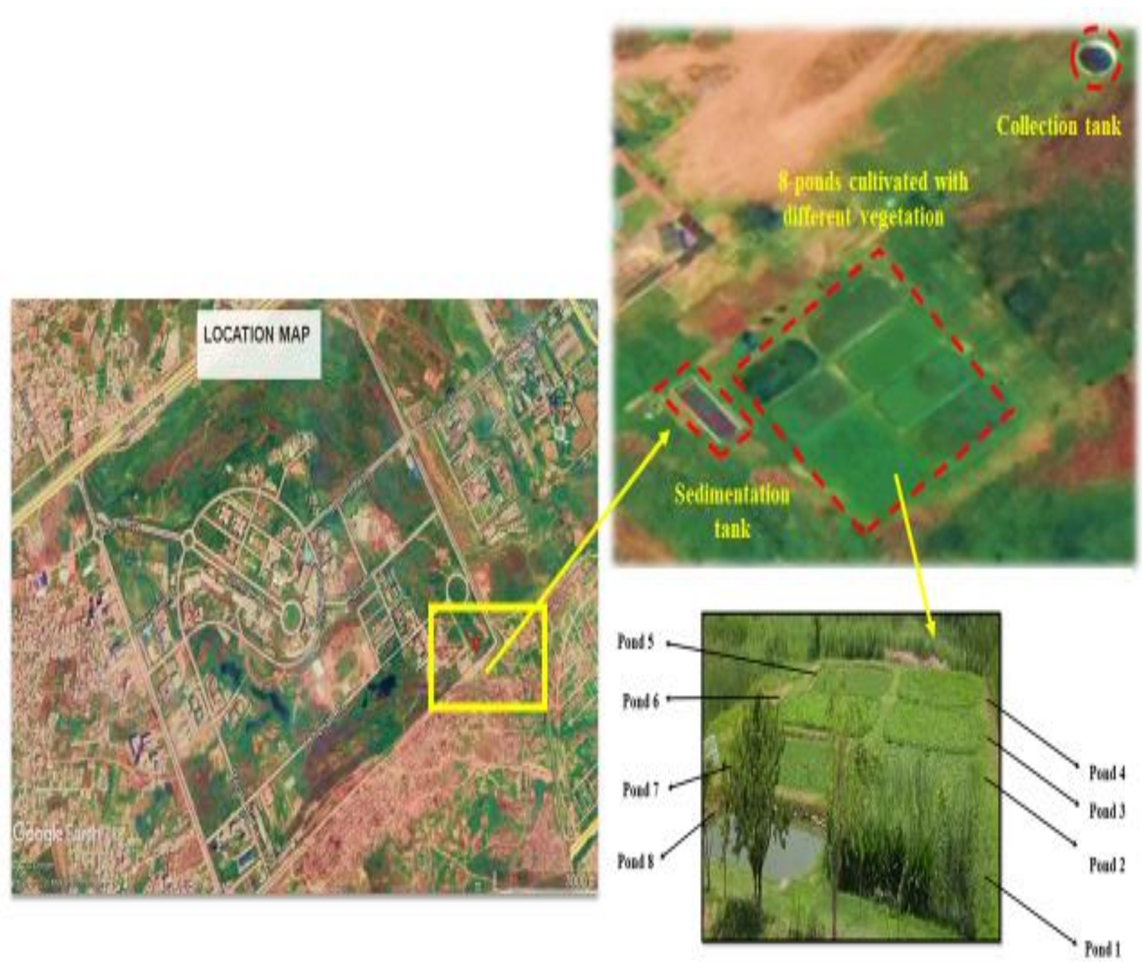


Figure 3.1: Study site

The United Nations Educational and Cultural Organization (UNESCO) funded the integrated constructed wetland. It was inaugurated on 13th November 2014 by the Minister of Science and Technology. In 2022 total population of NUST is around 6000 and it covers an area of 707 acres. The total number of students residing in hostels is approximately 3456. The approximate number of residences, flats, and houses for faculty and staff is 238. The total volume of wastewater generated at NUST by different schools, institutes, hostels, and residential areas is about 200,000 US gallons/day. Of which 60-65% of wastewater enters an integrated constructed wetland. CWs installed at NUST may treat around 0.1 million gallons of water per day. The flow into ICW is maintained at 70000 US gallons per day at inlet. The layout of the wetland system consists of a sedimentation tank, 8 ponds planted with different species of plants, and FILTER technology that further treats wastewater from 8th pond and water is then stored in collection tank. Detailed characteristics are discussed in table 3.2. About 70000 US gallons of water per day are being treated and used for horticulture purposes in NUST. The current number of trees in NUST is about 18000. The salient features of the project are shown in Table 3.1.

Table 3.1: Salient features of integrated constructed wetland

Location	**Northern Corner of NUST H-12 Campus, Islamabad**
Latitude and Longitude	33.6417767 and 73.003592
Treatment Capacity	75,000 Gallons/Day
Total Area of ICW	33000ft^2 (0.76 Acre)
Size of Ponds	120 ft. x 100 ft. (Each-pond 20 ft. x 50 ft.)
Size of FILTER	120 ft. x 80 ft.
Cost of UNESCO Sponsored Project	65,000 USD $

Table 3.2: Specifications of integrated constructed wetland

Descriptions		Total Capacity (ft^3)	Substrates	HRT (hours)	Plantations
Sedimentation tank		2520	---	----	Empty
HSSF-CW	Pond 1	5500	Dry Stone (Gravel) Pitching on slopes & top	6.87	Broadleaf cattail
	Pond 2	7700		10.30	Penny wort
	Pond 3			9.16	Penny wort
	Pond 4			11.44	Penny wort
	Pond 5		Dry Stone (Gravel) Pitching on top	14.88	Water lettuce
	Pond 6			10.07	Penny wort
	Pond 7			9.16	Water lettuce
	Pond 8			5.61	Empty
FILTER Technology		---	Soil, Sand and Gravel	---	Narrowleaf cattail

3.2. Materials and Methods

3.2.1. Sampling

A total of 12 sampling visits were conducted throughout six months for physicochemical analysis, from May to October 2021. Properly washed and autoclaved bottles (for 15 minutes at 120 ^{O}C and oven dried at 105 ^{O}C for 120 minutes) were used to collect samples. 11 samples per visit were collected from outlet of each pond as shown in Figure 3.2.

For microbial analysis, plant sample (i.e., *Typha latifolia*) from pond 1 was selected to gain insights into the bacteria that contribute to plant adaptation in the contaminated environment. Aspond 1 is the first pond of integrated constructed wetland after sedimentation tank and gets the maximum pollutant load.

For helminth egg analysis 11 samples from outlet of each pond were collected.

The collected samples were instantly transported to Environmental Microbiology laboratory of IESE (Institute of Environmental Sciences and Engineering) for further physicochemical and biological analysis.

3.2.2. Physicochemical parameters

The physicochemical parameters of collected water samples were analyzed. For this study selected parameters which includes pH, Temperature, Electrical Conductivity (EC), Turbidity, Total Dissolved Solids (TDS), Total Suspended Solids (TSS), Total Solids (TS), Chemical Oxygen Demand (COD), Dissolved Oxygen (DO), Nitrate-Nitrogen (NO_3^--N), Nitrite-Nitrogen (NO_2^--N), Total Kjeldahl Nitrogen (TKN), and Phosphate-Phosphorous (PO_4^{3-}-P). The parameters' characteristics, instruments, and methods used for analysis are described in Table 3.3. All analysis was completed according to the APHA standard methods for the examination of water and wastewater (APHA, 2017).

Table 3.3: Physicochemical analysis parameters

Parameters	Units	Instrument and methods	Reference
pH	-	HACH 156 pH meter	APHA, 2017
Temperature	°C	Laboratory method-HACH	
Turbidity	NTU	Turbidity meter (HACH 2100N)	
Electric Conductance	μS/cm	Potentiometric method-conductivity meter	
Dissolved Oxygen	mg/L	Crison Oxi 45 DO meter	
COD		COD digester	
TSS, TDS		Gravimetric dried method	
TKN		Kjeldahl apparatus	
NO_3^--N		UV-spectrophotometer, colorimetric method	
NO_2^--N			
PO_4^{3-}-P			

3.2.3. Plant sampling, isolation, and identification of bacteria

The leaves, stem, and root tissues of *Typha latifolia* were collected from pond 1 of integrated constructed wetland. Plastic bags were used to store the plant samples. Samples were shifted to the IESE microbiology lab and processed immediately. Plant samples were cut into small pieces. Initially, the small pieces of roots, shoots, and leaves were washed with tap water. Followed by surface disinfection for 5 minutes in 5% sodium hypochlorite solution. Later the samples were washed with sterile distilled water three times. For sterility test, 50 microliters (μL) of the final rinse from previous step was spread on (N/A) nutrient agar medium. Small pieces were further crushed in pestle and mortar under sterile conditions and kept in 4–5 ml distilled water for 30 minutes. Then 50 μL from this was collected via pipette and spread onto a nutrient agar plate. All samples were kept for incubation including controls at 26–28 °C for 2 weeks. And samples were daily observed for growth (Tashi-Oshnoei *et al.*, 2017; Maulani *et al.*, 2019). The colonies based on their morphology were selected. They were then purified on the same nutrient agar media by using streak plate method. These isolates were used for physiological and biochemical analysis. The biochemical tests used include catalase and oxidase test, and gram reaction (Schaad *et al.,* 2001).

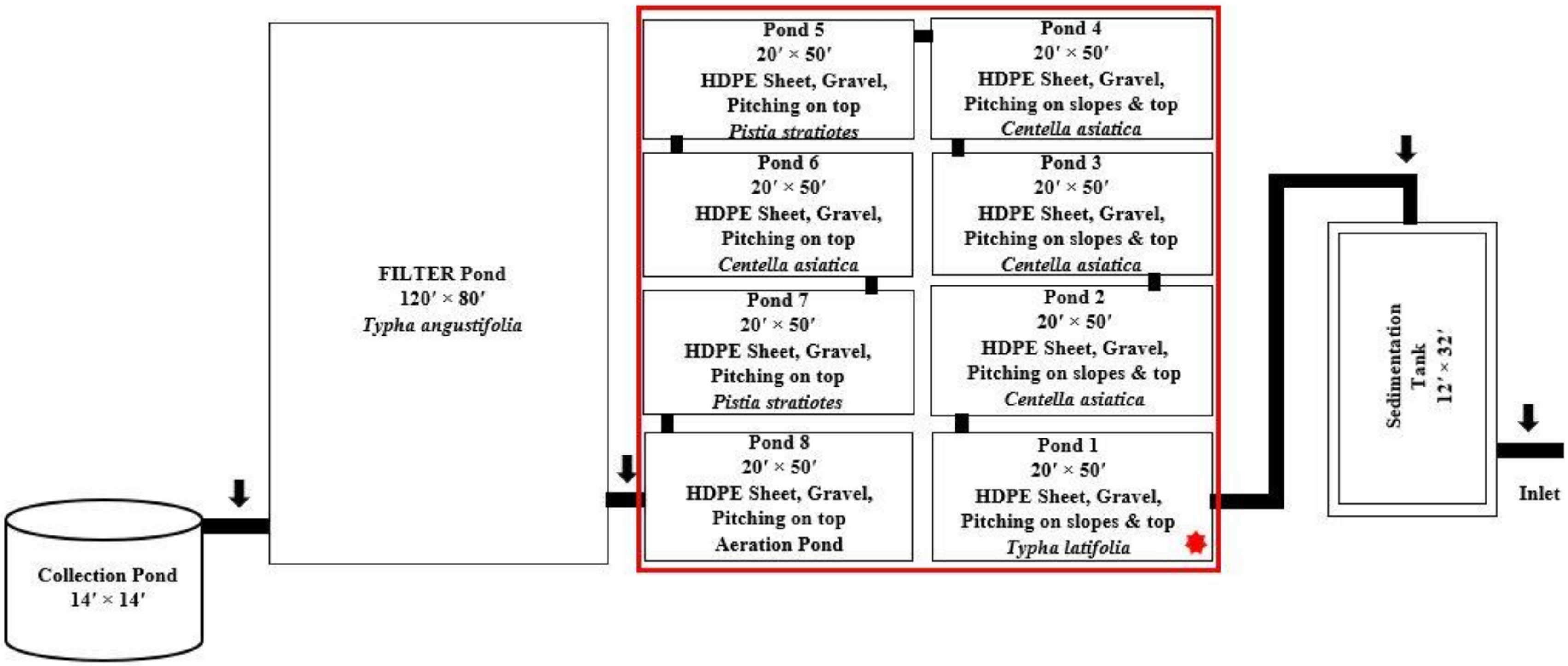


Figure 3.2. Sampling layout

Sampling Points (Physicochemical, helminths) ⬇ ☐ (Microbial) ✸

HDPE: High-density polyethylene **FILTER:** Filtration and irrigated cropping for land treatment and effluent reuse

3.2.4. Morphological characterization

Bergey's Manual of Determinative Bacteriology (Parte, 2012) was used to analyze bacterial colonies morphologically. Table 3.4 describes the commonly observed morphological features along with their description (Tortora *et al.,* 2004).

Table 3.4: Morphological characteristics

Morphological characteristics	Description
Size	small, large, medium
Shape	filamentous, punctiform
Color	white, off-white, yellow, orange, pink, green
Elevation	convex, umbonate, raised, pulvinated, flat
Margin	curled, entire, lobate, undulate
Texture	dry, smooth, wrinkled
Opacity	opaque, transparent, translucent

3.2.5. Biochemical characterization

3.2.5.1. Gram staining

Gram staining is a specific technique for differentiation among gram-positive and gram-negative bacteria based on alteration in their cell wall structure. This technique also assures us that colony is fully purified. In 1884 Danish Physician Hans Christian Gram developed a procedure for Gram staining. Gram-positive bacteria have a thick layer of peptidoglycan around the cell wall and can retain crystal violet stain which causes a purple appearance on cell wall while a thin layer of peptidoglycan on the cell wall of gram-negative bacteria is unable to retain crystal violet strain and appears pink in microscope after staining. The procedure was followed as described by (Fawole & Oso, 2004).

3.2.5.2. Catalase and oxidase tests for purified microbes

Catalase and oxidase tests for purified microbes were performed by following the procedure described by (Cheesbrough, 2005).

3.2.6. Helminths egg analysis

For helminth egg analysis first step was sample collection. After stirring/shaking the sample well the sample is passed through the sieve of 150-180 µm (already wet sieves (soaked in water) followed by passing through a smaller sieve of 20 µm via funnel (plastic). As larger particles will stay on upper sieve and remaining will pass through a smaller funnel (plastic) and get trapped on the sieve of 20 µm ensurig that all helminth eggs of the sample are captured on the sieve. Sieves were then washed, including the container that originally contained the sample, with tap water at least three times. The remaining sample is then transferred into a falcon tube of 50 mL by washing the sieve with tap water and making sample volume 50 mL as the sample size on sieve was usually less than 50 mL for less turbid samples (treated wastewater), and for more turbid samples (wastewater not treated), sometimes it is necessary to transfer samples into two or more 50 mL falcon tubes. The sample is then centrifuged once at 3000 rpm for 10 minutes (or 660 g/ 10 minutes). The supernatant is then removed with pipette, and remaining sample (up to 1.5-2 mL) was then observed under the microscope in the Sedgewick rafter chamber by using a dropper or a plastic pasteur pipette. For turbid samples, like wastewater (not treated) reagent ($ZnSO_4$ 1.3 density solution) was added. Sample was then centrifuged at 3000 rpm for 10 minutes (or 660 g/ 10 minutes). The supernatant from tubes was collected one by one and passed through the sieve of 20 µm. The sieve was washed, first with distilled water, and after with tap water, and with the help of the funnel was shifted to a 50 mL falcon tube. The sample is then again centrifuged at 3000 rpm for 10 minutes (660 g/ 10 minutes). The supernatant is removed and observed the remaining sample (up to 1.5-2 mL) under the microscope in Sedgewick rafter chamber by using a dropper or a plastic pasteur pipette. For more turbid samples 20 ml of a biphasic solution of 1 normal sulfuric acid and 70 % alcohol (70-30 ratio), and 10 ml of ethyl acetate was added and mixed and making sample volume to 50 mL. Sample was then centrifuged at 3000 rpm for 10 minutes (660 g/ 10 minutes). Small particles, which may be difficult to read under a microscope, get trapped in the ethyl acetate layer. The layer and supernatant were carefully removed until the particles containing the helminth eggs remain in the falcon tube, with the help of funnel. The pellet was then passed through the sieve of 20 µm, sieve and the tube were rinsed with tap water and making the sample volume to 50 mL in a falcon tube. Centrifuged again at 3000 rpm for 10 minutes

(660 g/ 10 minutes), supernatant is removed. The sample was collected with a dropper or a plastic pasteur pipette and transferred to a Sedgewick rafter chamber and observed under the microscope. The sample was then left to settle in the chamber on a flat surface for 5 minutes before its examination. This way, all the eggs settled to the bottom. Sedgewick rafter chamber was kept under the microscope and all the samples were examined at 10x objective (Ayres *et al.*, 1996; Mifsut 2015; Jimenez *et al.*, 2020). Helminth eggs were calculated by using the following formula.

$$\mathrm{HO/L} = \frac{\mathrm{No.HO}}{\mathrm{1L}}$$

$\mathrm{HO/L}$ = number of eggs per liter of sample

HO: number of helminth ova counted in the sample

1L: volume of sample analyzed

3.2.7. Economic valuation of integrated constructed wetland

3.2.7.1 Basic cost components of constructed wetland

The basic cost components of constructed wetland system include capital cost, operational maintenance, and reuse cost. Categories for the capital cost of constructed wetland include:

1. Land acquisition
2. Site survey, planning, and design
3. Excavation including leveling and shifting of soil
4. Construction of sedimentation tank and interconnection manholes
5. HDPE lining of constructed wetland and detention ponds
6. Substrate of constructed wetland
7. Installation of interconnecting pipes
8. Introduction of aquatic plants
9. Startup

The categories for operation and maintenance of constructed wetland treatment facility include:

1. Human resources for facility operation and maintenance.

2. Consumables, supplies, capital equipment, and utilities

3. Water quality analysis

4. Repair and maintenance

3.2.7.2. Basic benefits components of constructed wetland

To analyze the benefits of constructed wetland treatment facility developed at NUST, the benefits were assessed in four broad categories.

1. Improvement in surface water quality

2. Availability of treated water

3. Carbon sequestration

4. Landscape enhancement

5. Educational and recreational activities

3.2.7.3. Data collection

The data for cost and benefits analysis of constructed wetland treatment facility was collected primarily from interviews of the principal investigator (Dr. Hamza Farooq Gabriel) of the project, and plant operators. The financial valuation of treated water was based on hedonic price analysis (Hussain *et. al*, 2001; Robertson, 2011). The cost related to the integrated constructed wetland was calculated using data provided by the Works Directorate (Ex. PMO). The values of per unit electricity cost of pumping through an electric motor were used by Islamabad electric supply company (IESCO) and the calculation of purchasing freshwater was made through the data available on the capital development authority (CDA) website.

3.3. Statistical analysis

3.3.1 Descriptive statistics

The mean value for each month for physicochemical parameters was calculated and the standard deviation was applied using Microsoft Excel.

3.3.2 Pearson’s Correlation Matrix

Significant and non-significant effects among physicochemical and weather parameters were noted with the level of significance at $p<0.05$ using Microsoft Excel.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Temporal variation of physicochemical parameters

The removal efficiency of organic pollutants was measured from the effluent of each pond of the integrated constructed wetland. Organic pollutants removal involves plant uptake, aerobic, anaerobic, and rhizosphere digestion (Wu *et al.*, 2018). In the present study, there is aerobic digestion in the upper 2 feet of the HSSF-CW design. Aerobic digestion is because of factors such as atmospheric diffusion, convection through wind, and plant roots within the rhizosphere. Whereas anaerobic digestion from 3-7 feet on the benthic surface and degradation through plant mechanisms i.e., phytodegradation, phytoextraction, and rhizo-filtration.

4.1.1. Temperature and pH

In the present investigation, temperature of the integrated constructed wetland ranged between 22.6-32.4 ºC. For efficient pollutant degradation through plant and microbial activity, moderate temperature is important (Naseer *et al.*, 2021). The pH of sampling points ranged from 6.4-7.7. pH effluent values ranged within wastewater discharge and international agriculture reuse standards.

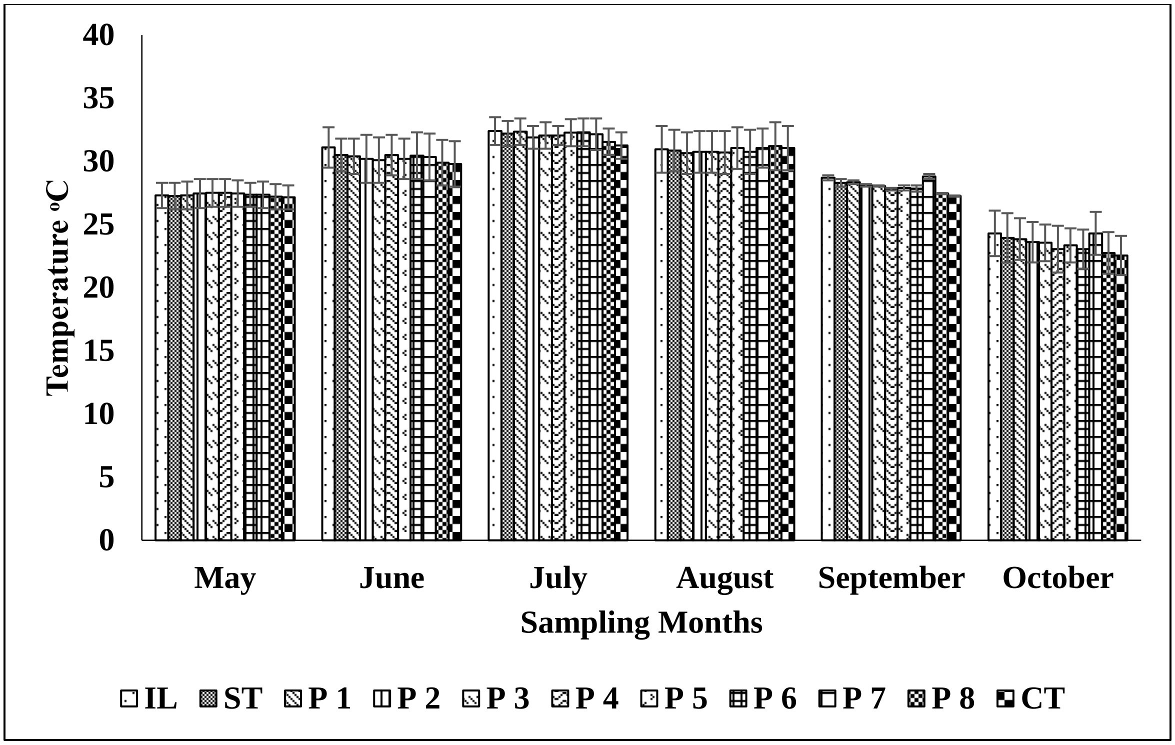


Figure 4.1: Temporal variation of temperature in ICW

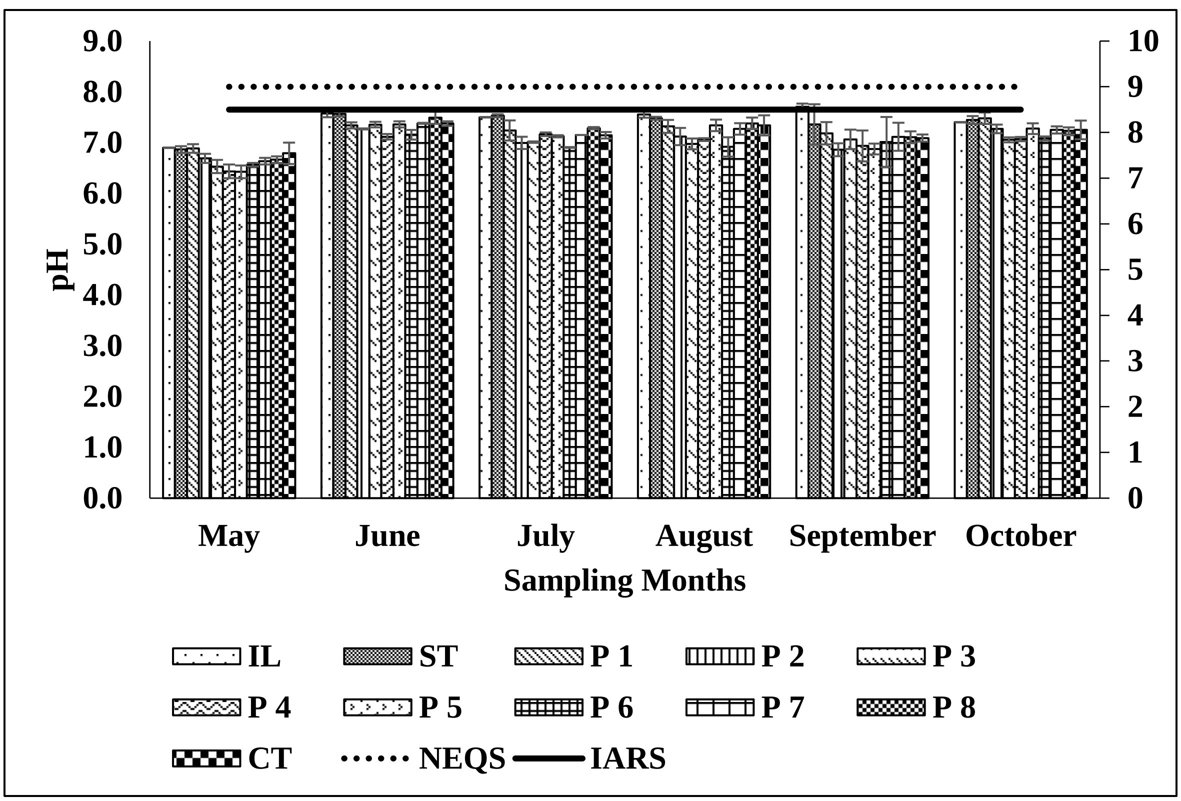


Figure 4.2: Temporal variation of pH in ICW

4.1.2. Electrical conductivity (EC)

EC was highest in pond 3 in June which may be due to low volume of water in the dry summer season. As reported by *Ávila et al.,* 2021 and Bakhshoodeh *et al.,* 2017 this may be due to different factors such as high evapotranspiration, bed granules dissolution, and mineralization of organic content in wetland. Amiri *et al.*, 2022 also observed a similar trend. High EC values may be due to the decaying activity of organic matter, and plants that result in mineralization. Also due to the high evapotranspiration and bed granules dissolution (Amiri *et al.*, 2022). The highest removal efficiency of EC was recorded in September which is 22.3%. However, effluent values of EC were below international agriculture reuse standards.

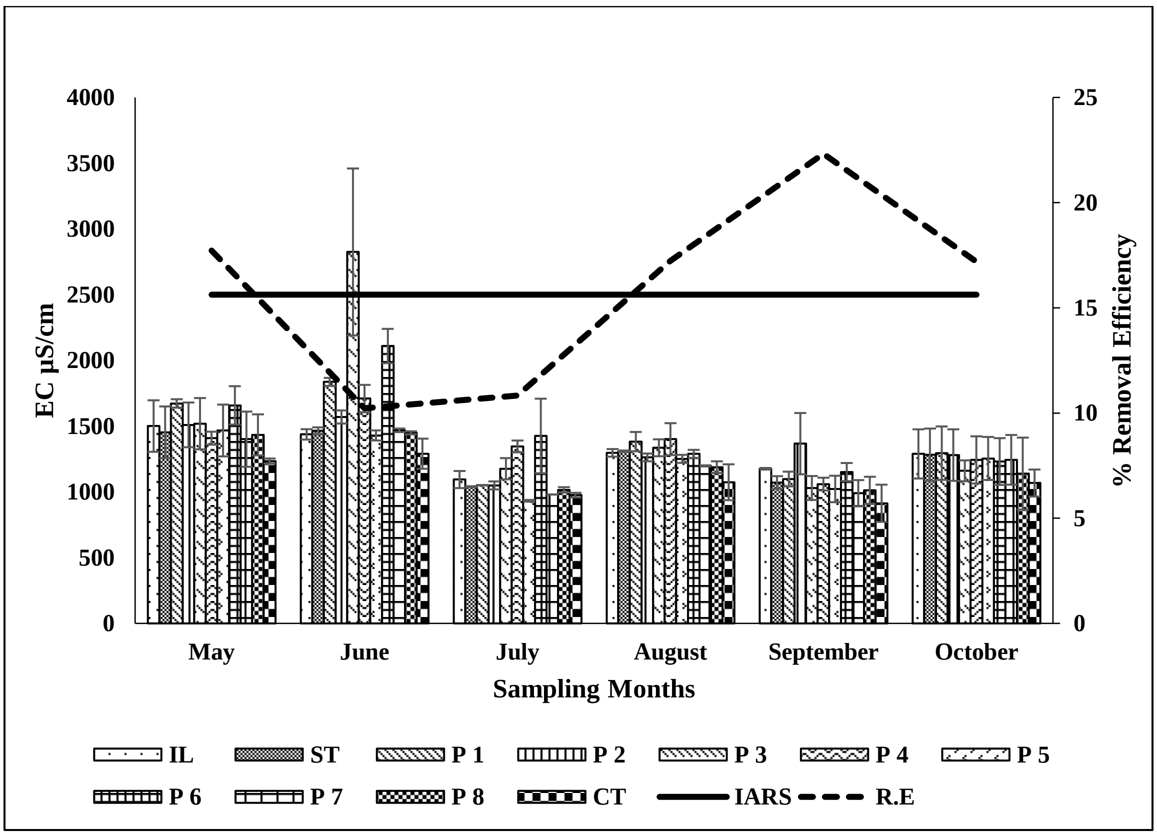


Figure 4.3: Temporal variation of EC in ICW

4.1.3. Turbidity

Turbidity was high in June and maximum removal efficiency was also reported in same month which is 88%. High turbidity was may be due to the suspension of small particles attached to roots and other plant debris in vegetated ponds while minimum values were observed in pond 8 and the collection tank throughout the study period. High turbidity was maybe due to degradation of organic matter in the wetland. This might be due to significant relation between the activity of microorganisms and turbidity which leads to an increase in particles in constructed wetland (Sani *et al.*, 2013).

Sanchez *et al.*, 2018 observed a difference between raw and treated wastewater, which showed that physical removal mechanisms of integrated constructed wetland were efficient. Moreover, color change from turbid to clear has also proved that total suspended and total dissolved solids have also been removed.

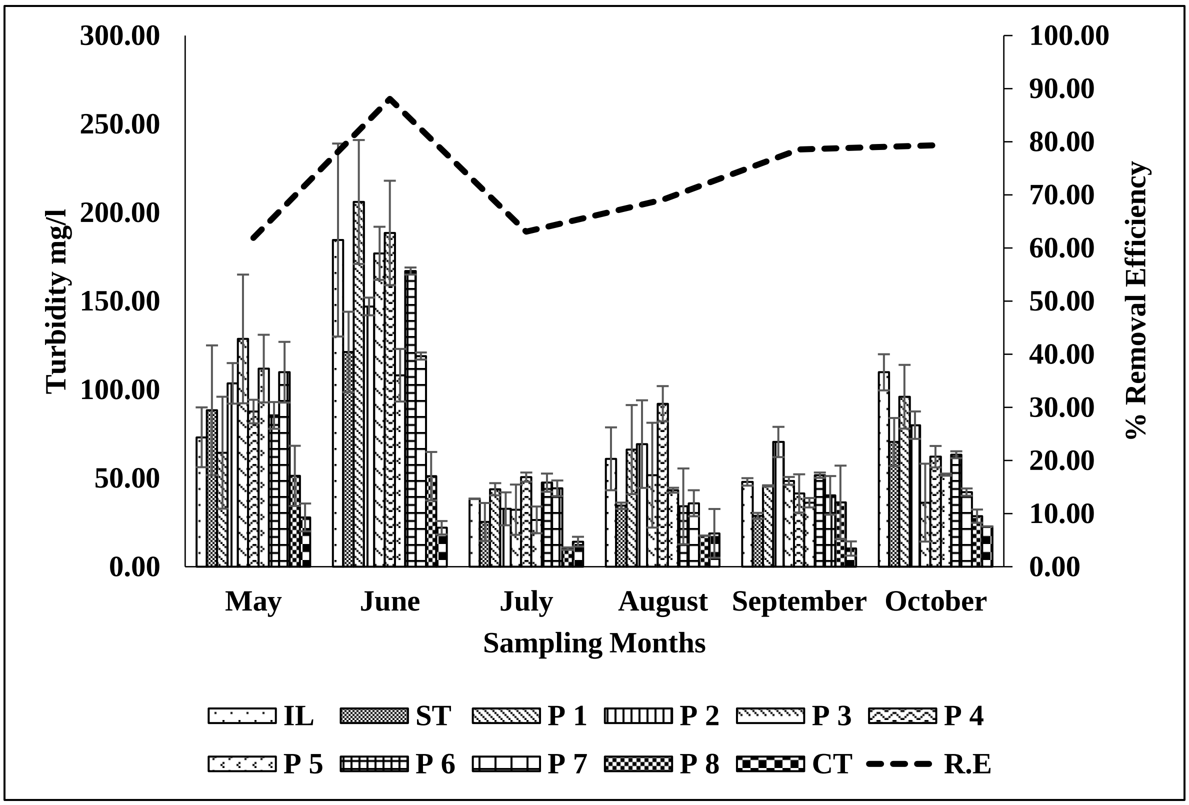


Figure 4.4: Temporal variation of turbidity in ICW

4.1.4. Total suspended solids, total dissolved solids, and total solids (TSS, TDS, and TS)

TSS is a measure of wastewater clarity and includes all mineral and organic suspended particles (Johal *et al.*, 2014). In addition, a high TSS concentration may obstruct light from entering the water, reduce amount of dissolved oxygen present, and impede growth of aquatic life. Maximum removal efficiency of TSS was observed in September which is

86.6% and maximum values were observed in June specifically in Pond 3. This may be because TSS content might also be influenced by particles of organic matter from decaying substances. Small organic particles may separate from decaying algae, plants, and animals and enter into the water column as suspended solids (Belghyti *et al.,* 2009). Whereas, as reported by Afzal *et al.* (2019) TSS concentration has significantly reduced in constructed wetlands due to presence of macrophytes. The direct contact of the roots of macrophytes with microorganisms may have resulted in transformation of dissolved particles. In a wetland, TSS is removed through filtration by root structure, settling down, and absorption (Sathe and Munavalli, 2019). Minimum TSS values were observed in final effluent. Overall, effluent values were within IARS and NEQs.

Similarly, for TDS and TS minimum values were observed in final effluent. Overall, effluent values of TDS were within IARS and NEQs. As shown by Sanchez *et al.,* 2018 there was a statistically significant difference between color of raw wastewater and treated water of eight sampling points on average confirming efficient removal of dissolved particles from treatment facility.

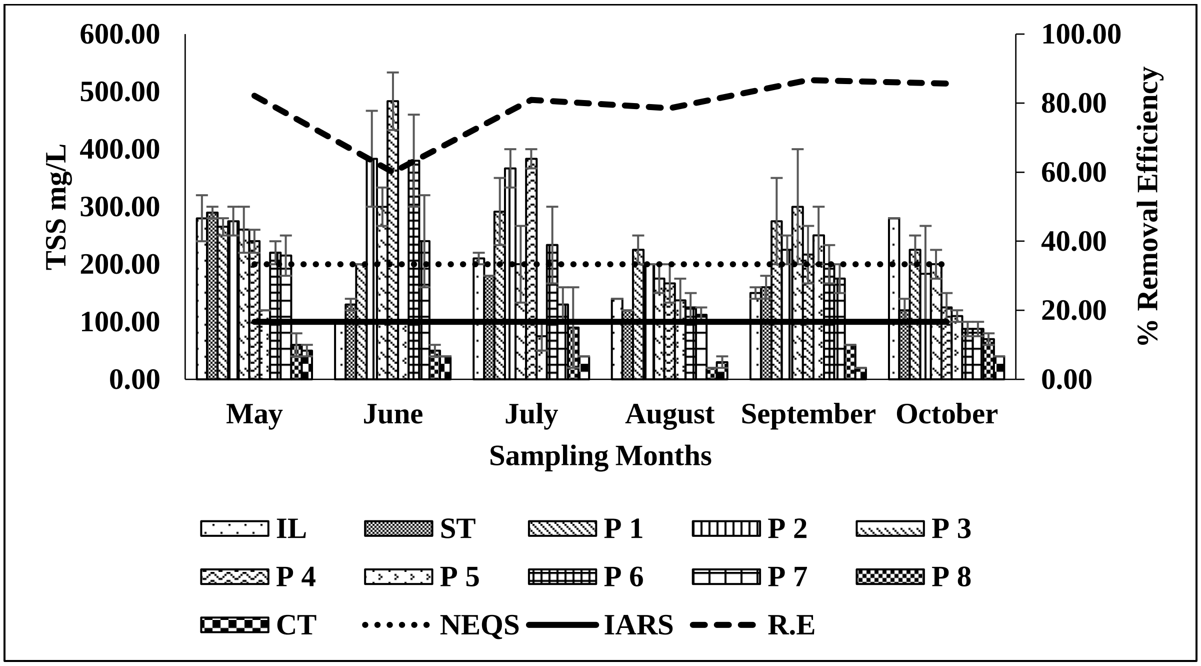


Figure 4.5: Temporal variation of TSS in ICW

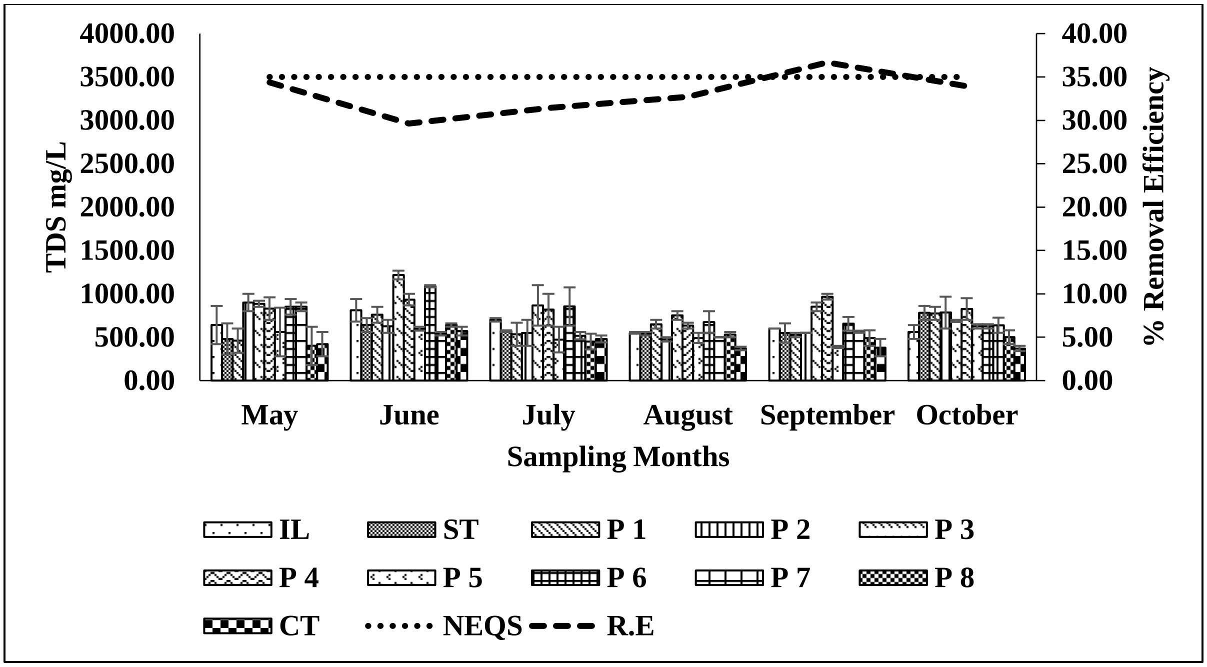


Figure 4.6: Temporal variation of TDS in ICW

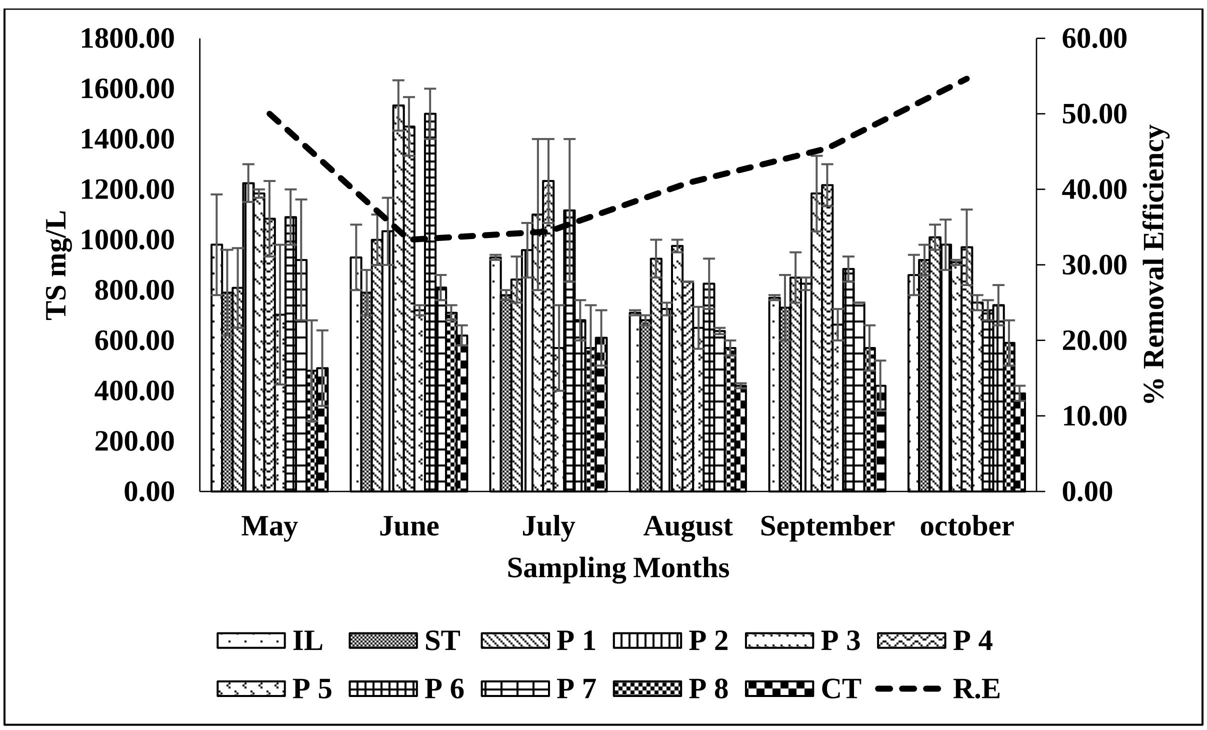


Figure 4.7: Temporal variation of TS in ICW

4.1.5. Chemical oxygen demand and dissolved oxygen (COD and DO)

The COD values ranged between 150.40 in influent and 31.68 mg/L in effluent. The lowest values were recorded in the collection tank for all months. Ho *et al.* (2020) also observed a similar trend in removal performance. This might be caused by material's rapid degradation through aerobic and anaerobic processes, as well as filtering.

A crucial element in regulating physical, chemical, and biological processes is dissolved oxygen. Like in the present study Gaballah *et al.* (2020) also recorded higher effluent values of DO in all seasons.

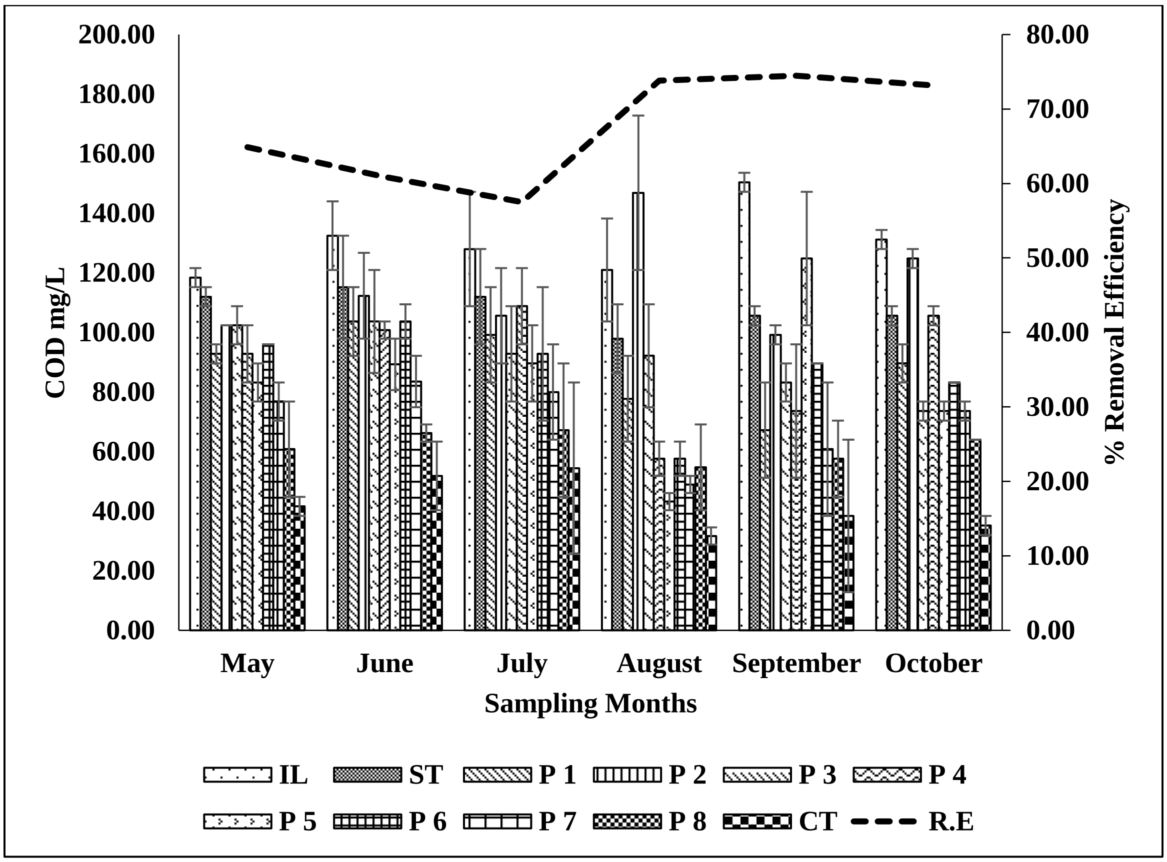


Figure 4.8: Temporal variation of COD in ICW

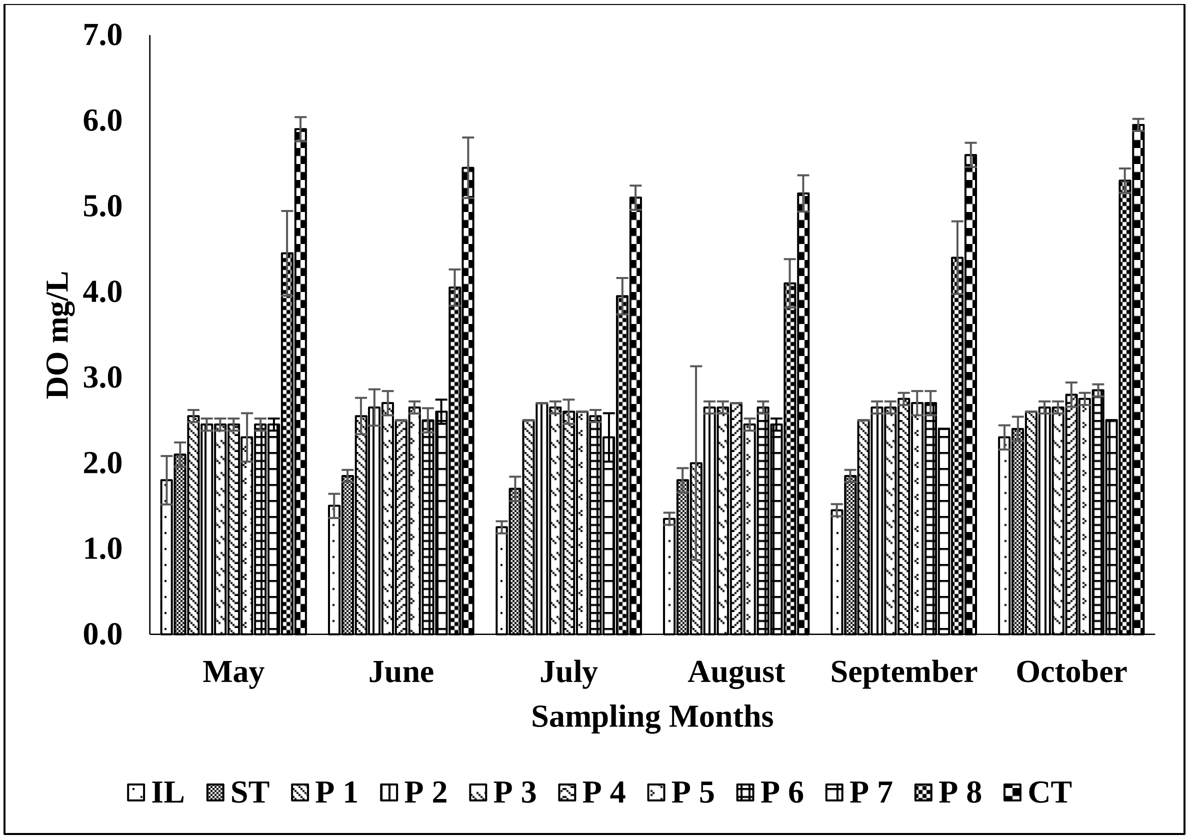


Figure 4.9: Temporal variation of DO in ICW

4.1.6. Nitrate-nitrogen and nitrite-nitrogen (NO_3^--N and NO_2^--N)

NO_3-N and NO_2-N have shown a different trend where the concentration increased, in the effluent. High nitrate in effluent may be due to the oxidation of nitrogen by the nitrification process which may be due to increased DO concentrations at P8, and CT. High DO concentrations resulted in nitrification process i.e., conversion of ammonium into nitrate and nitrite (EPA, 2000). However, effluent values were below EPA standards i.e., 10 mg/L for nitrate and 1 mg/L for nitrite.

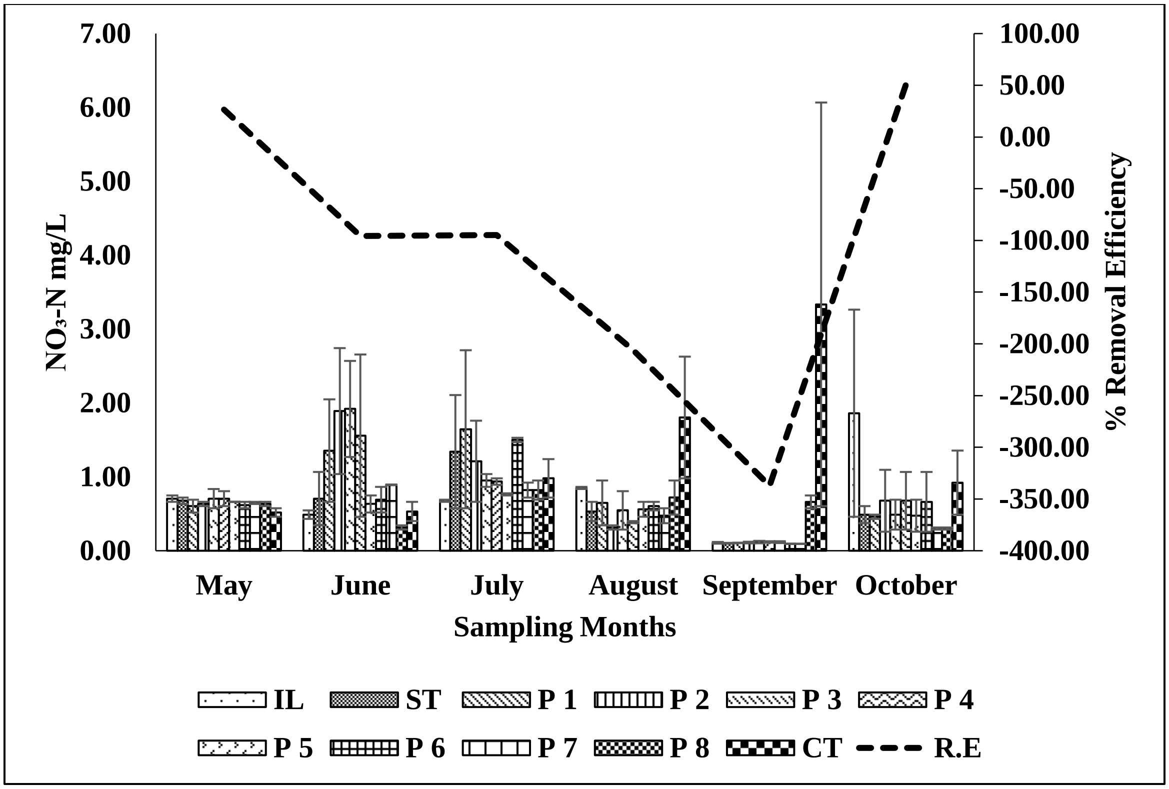


Figure 4.10: Temporal variation of NO_3^--N in ICW

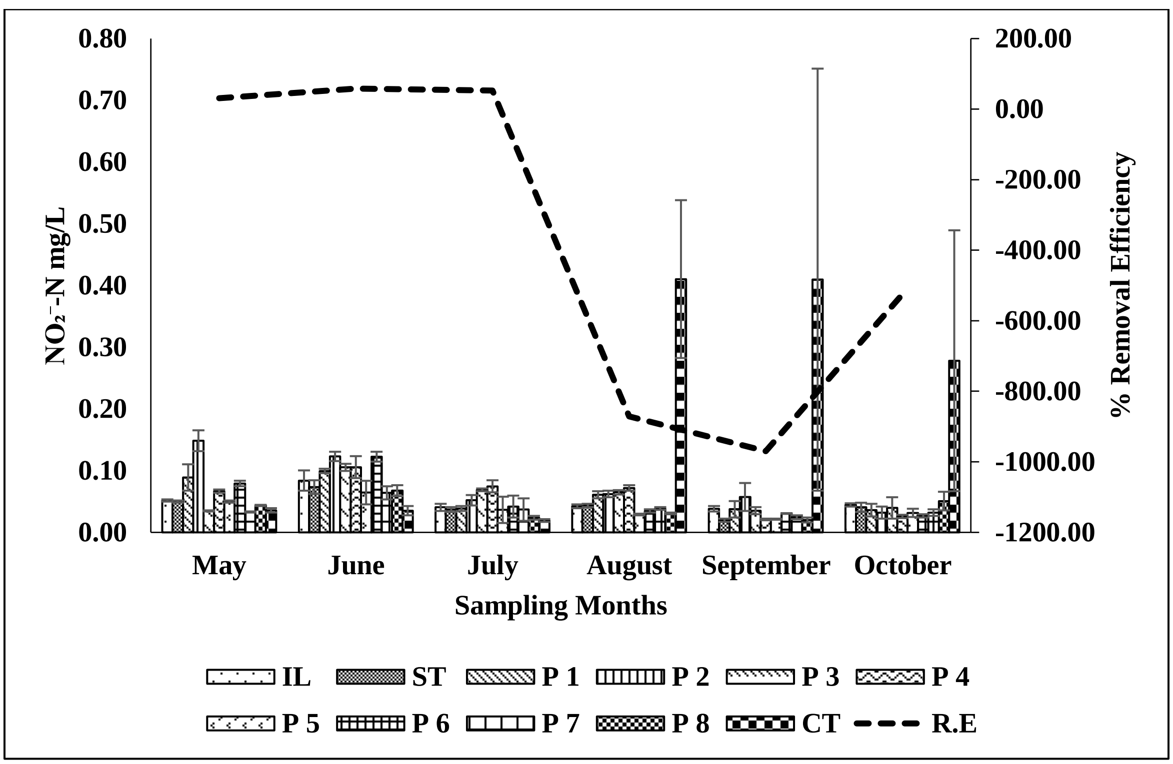


Figure 4.11: Temporal variation of NO_2^--N in ICW

4.1.7. Total kjeldahl nitrogen (TKN)

TKN is a combination of organic nitrogen and ammonia nitrogen. The maximum TKN values were recorded in May while minimum values were recorded in August with a maximum removal efficiency of 45.6%. Hua *et al.* (2017) reported that the reduction of the organic and ammonia nitrogen values may result in the high removal performance of TKN. As these are the important contributing factors to high TKN effluent values.

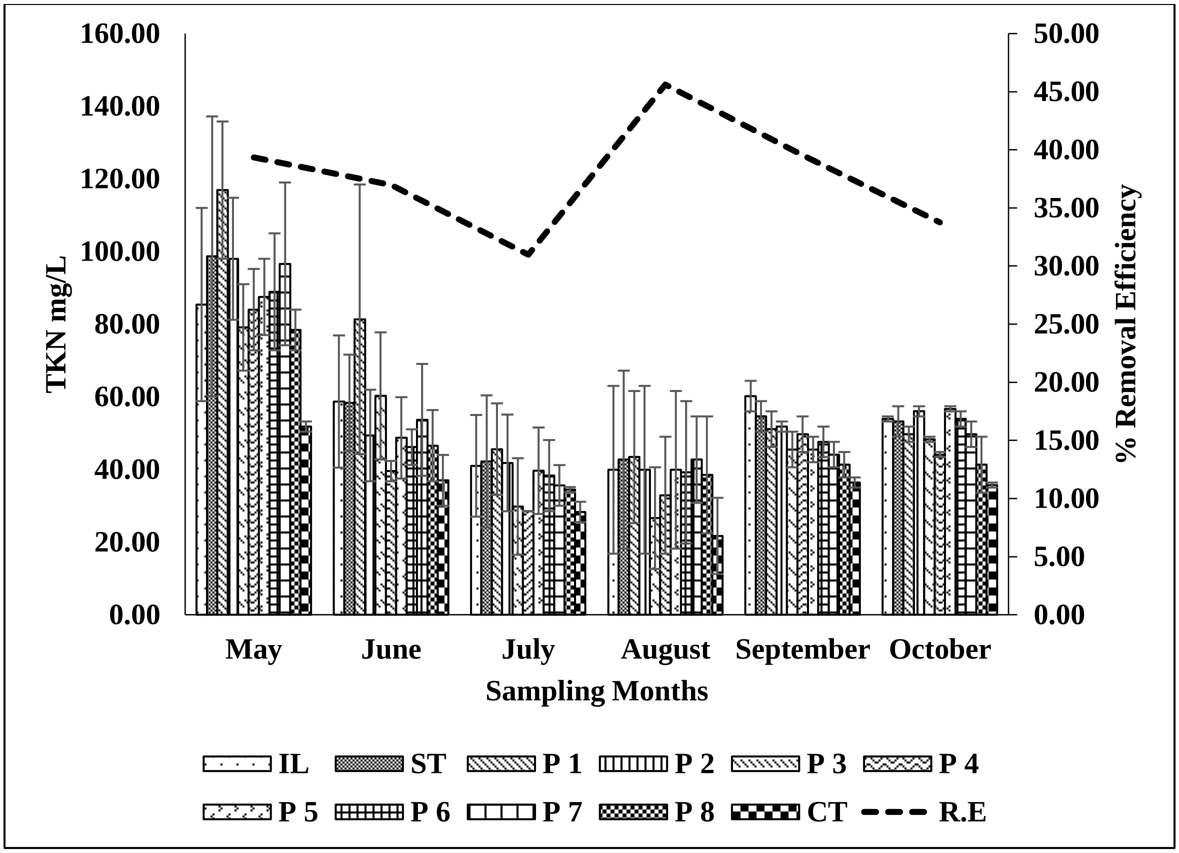


Figure 4.12: Temporal variation of TKN in ICW

4.1.8. Phosphate-phosphorous (PO_4^{3-} -P)

Phosphate phosphorous values ranged between 9.74 in the inlet to 2.66 in the effluent. Low effluent values of PO_4^{3-} -P were recorded in all months, especially in August, September, and October. The low concentrations PO_4^{3-} -P at effluent were may be due to the process of adsorption by substrate material, plants, and microbe uptake mechanism in constructed wetlands (Mujtaba *et al.,* 2017).

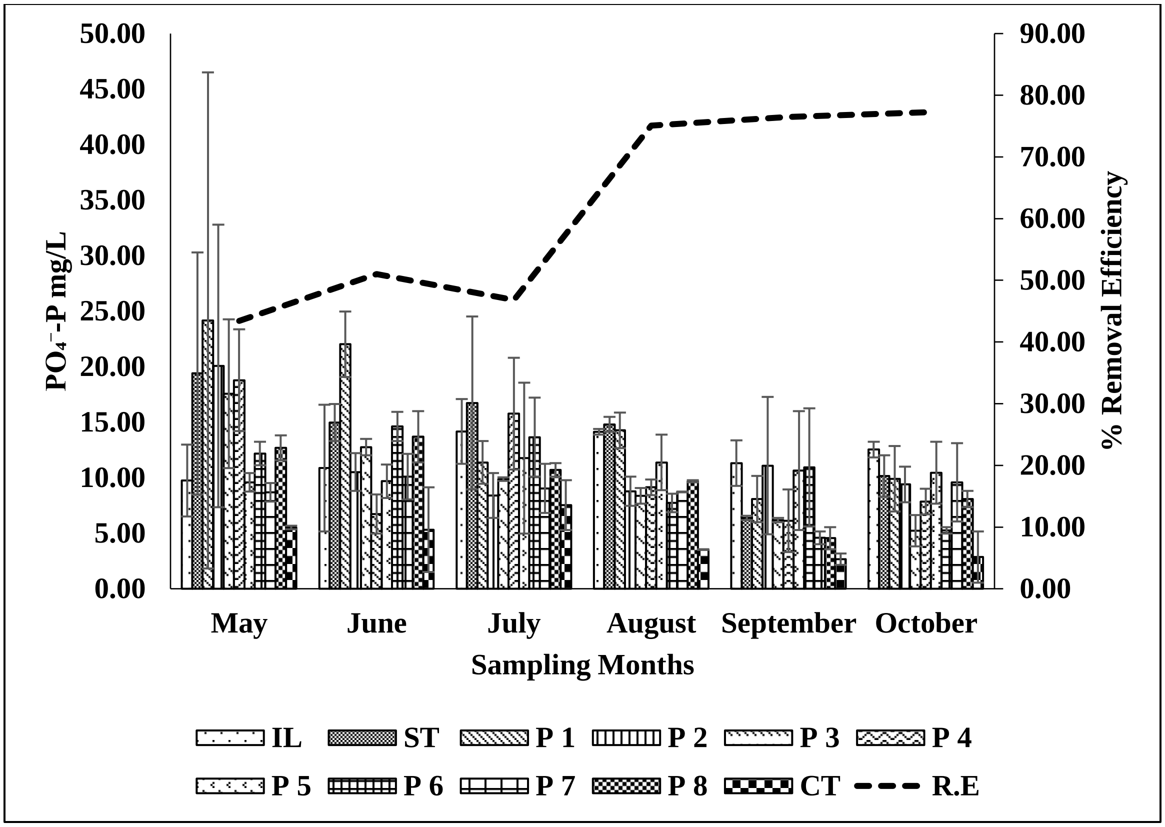


Figure 4.13: Temporal variation of PO_4^{3-} -P in ICW

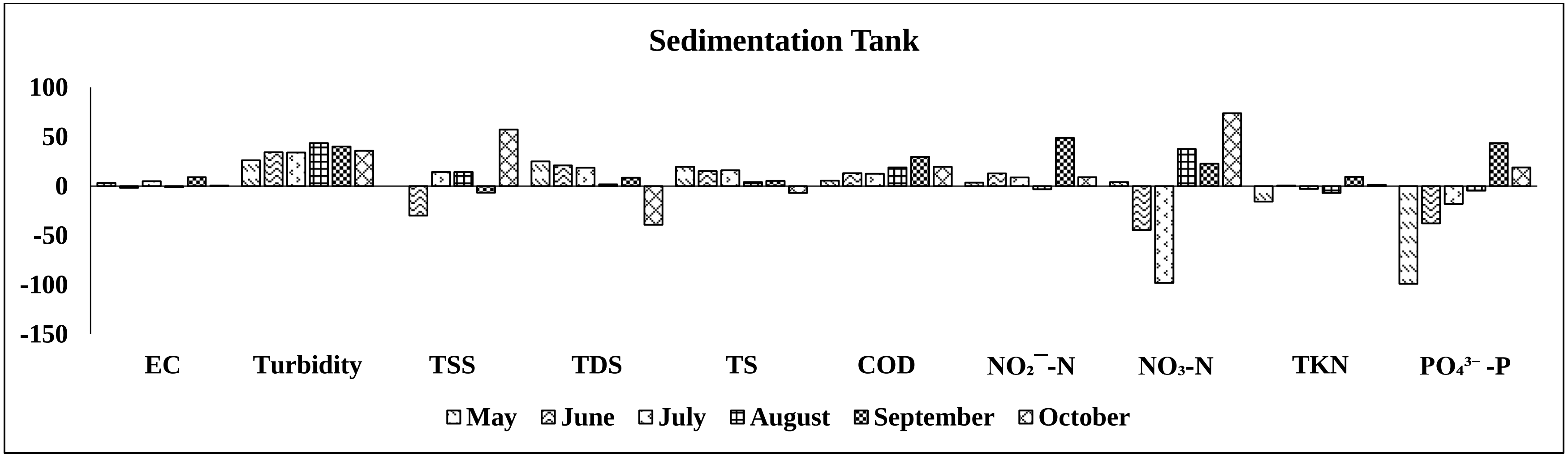
Data showed stable and sustained removal of organics with significant improvement in wastewater quality. Each parameter pH, Temp., EC, TDS, TSS, and COD comply with national environmental quality standards & international agriculture reuse standards (Table 4.1).

Table 4.1: Wastewater discharge and international agriculture reuse standards

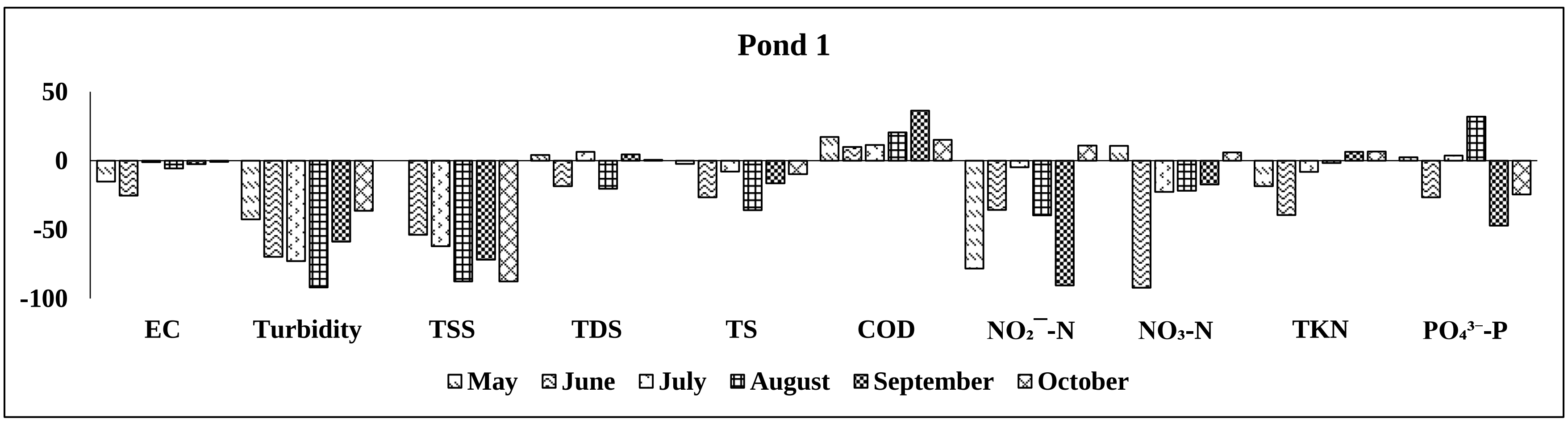
Parameters	Units	Current effluent values Avg. (Min-Max)	Revised Standards for wastewater discharge			International Agricultural reuse standards (Non-fodder crops)	Remarks
			Inland waters	Sewage	Sea	----	✓
Temperature	^{o}C	28.17 (22.55-31.25)	≥3	≥3	≥3	6.5-8.5	✓
pH	-	7.2 (6.8-7.4)	6 - 9	6 - 9	6 - 9	<150	✓
COD	mg/L	42.19 (31.68-54.40)	150	400	400	<100	✓
TSS	mg/L	36.67 (50-20)	200	400	200	---	✓
TDS	mg/L	431.67 (370-570)	3500	3500	3500	>2500 unacceptable	✓
EC	µS/cm	1092.58 (913.5-1290.5)	---	---	---	<1	✓
Helminths	eggs/L	---	---	---	---		✓
References	---	---	NEQS, 2000			US-EPA, 2006	---

4.2. Percentage removal efficiencies of ICW

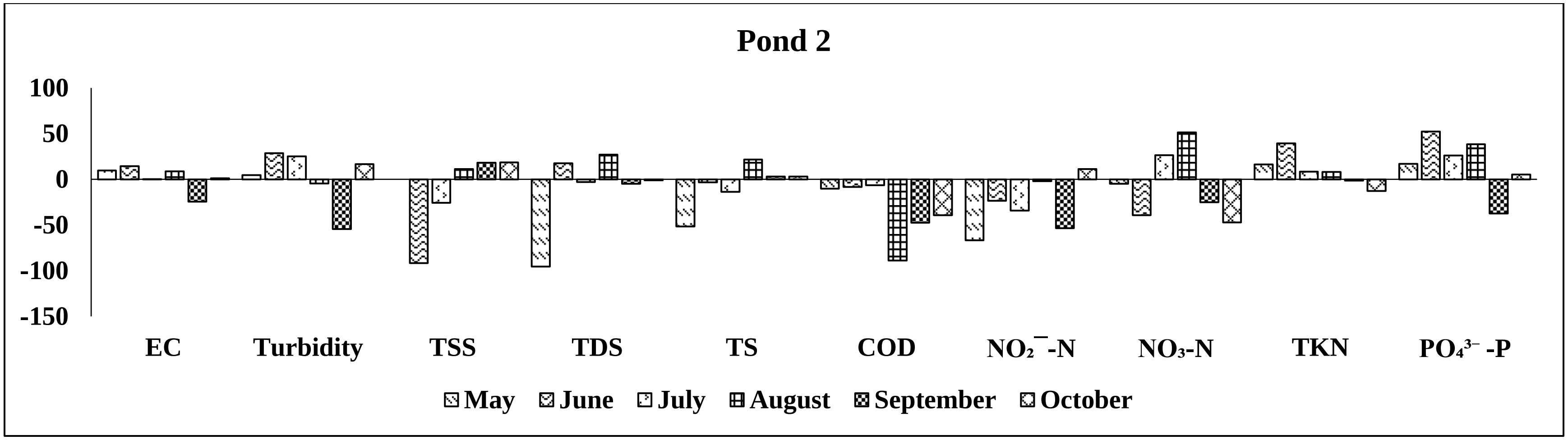
Sedimentation tank showed maximum removal efficiency for nitrate-nitrogen in October which is 73.76% and 57.14% for TSS. Maximum removal efficiencies for pond 1 were found to be for COD in September which is 36.36% and for phosphate-phosphorous in August which is 31.99%. Pond 2 observed maximum removal efficiency for nitrate-nitrogen in August which is 51.34% and 52.26% for phosphate-phosphorous in June. Pond 3 showed maximum removal efficiency for nitrite-nitrogen in May which is 76.55%. Maximum removal efficiency for pond 4 was observed in June which is 47.24%. Pond 5 showed maximum removal efficiency for TSS in June and July which varied between 79.31% and 80.43%. Maximum removal efficiency for nitrate-nitrogen was achieved in September for pond 6 which is 63.56%. Similarly, maximum removal efficiency of 58.06% for pond 7 was observed in September. Pond 8 exhibited maximum removal efficiencies of 57.02%, 76.94%, and 51.05% for turbidity in June, July, and August, and for TSS 79.17%, 30.77%, 82.22%, and 65.71% in June, August, and September respectively. Whereas, 64.72% for nitrate-nitrogen in June. Maximum removal efficiencies of FILTER technology were observed for turbidity in June 56.99%, and September 71.69%, and for TSS in July 55.56% and September 66.67%. Similarly, for phosphate-phosphorous in May 56.57%, June 61.12%, August 65.58%, and October 64.68%. For nitrate-nitrogen and nitrite-nitrogen FILTER technology showed very high negative values for August, September, and October. Furthermore, percentage removal efficiencies of individual units of ICW are shown below in the following figures.


Sedimentation Tank
100
50
0
-50
-100
-150
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

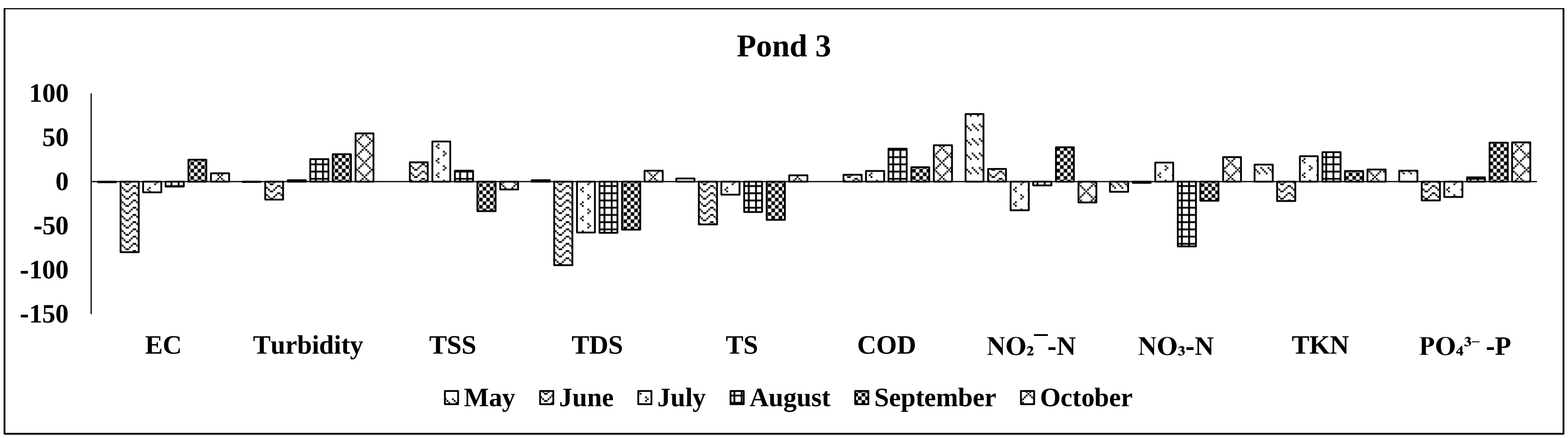
(a)


Pond 1
50
0
-50
-100
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻-P
May
June
July
August
September
October

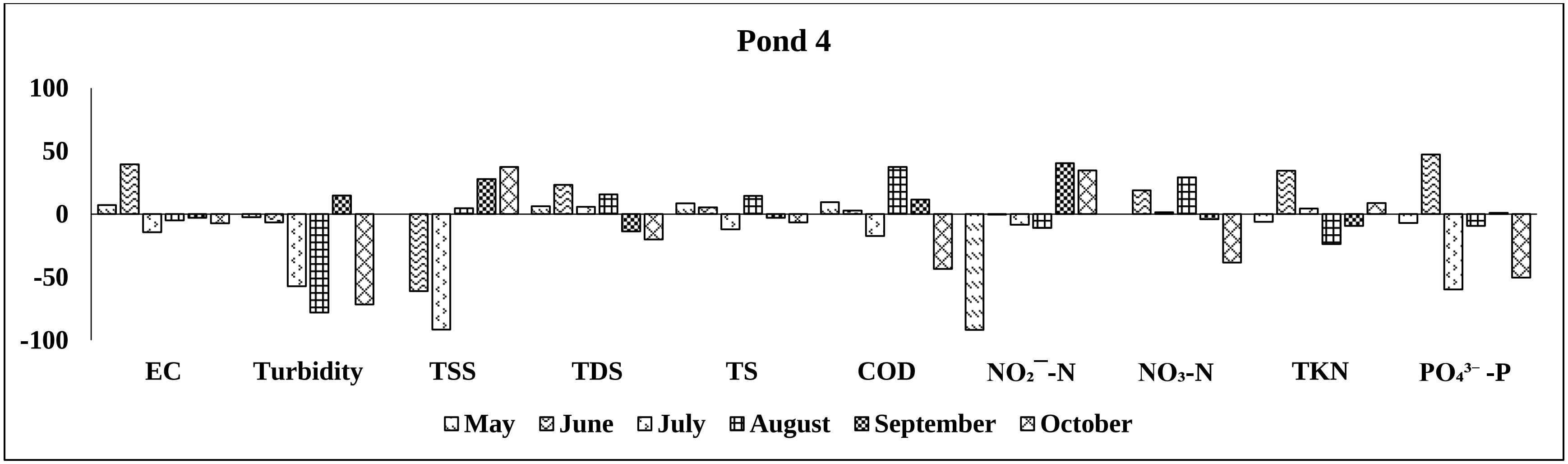
(b)


Pond 2
100
50
0
-50
-100
-150
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

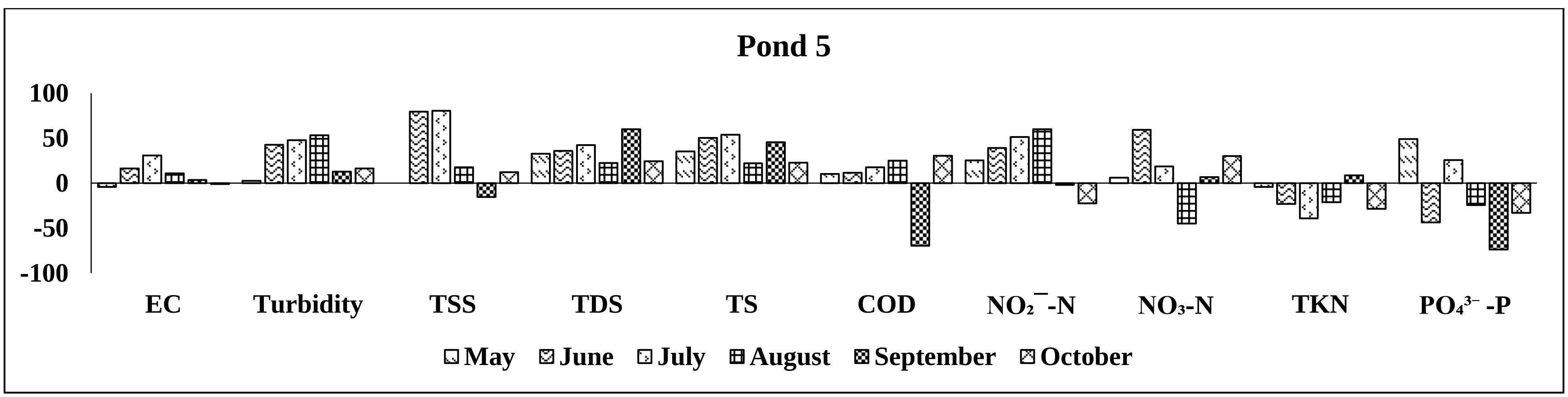
(c)


Pond 3
100
50
0
-50
-100
-150
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

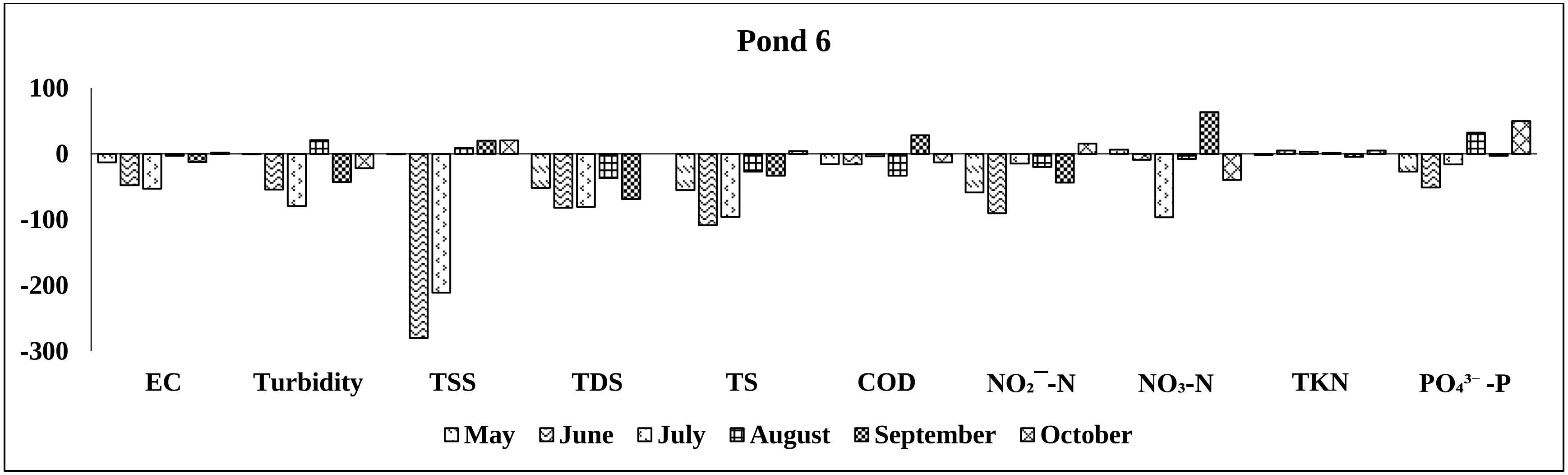
(d)


Pond 4
100
50
0
-50
-100
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

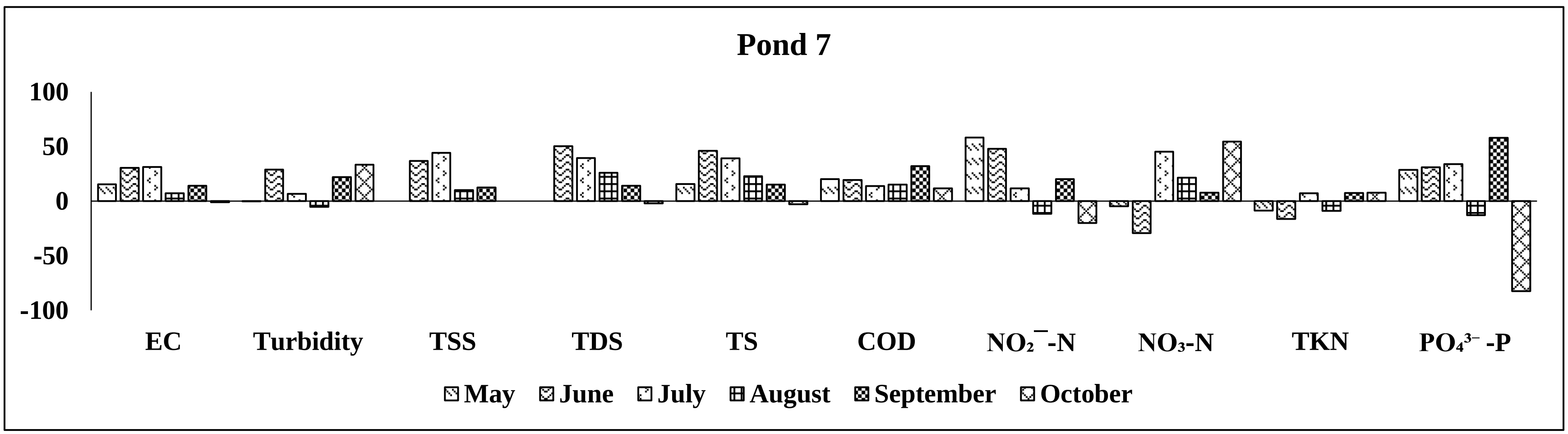
(e)


Pond 5
100
50
0
-50
-100
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

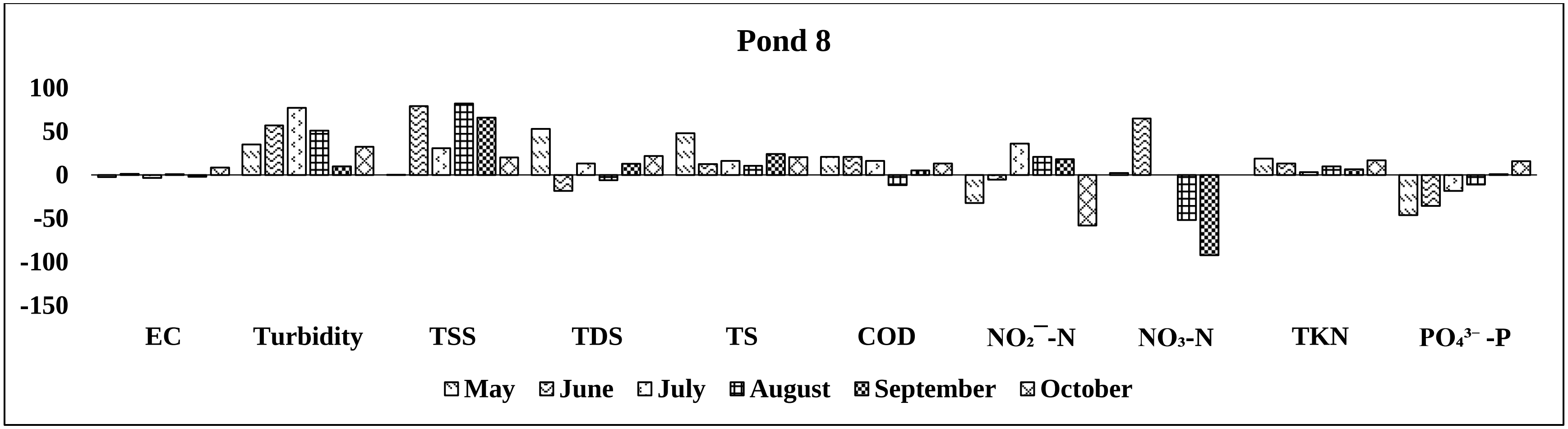
(f)


Pond 6
100
0
-100
-200
-300
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

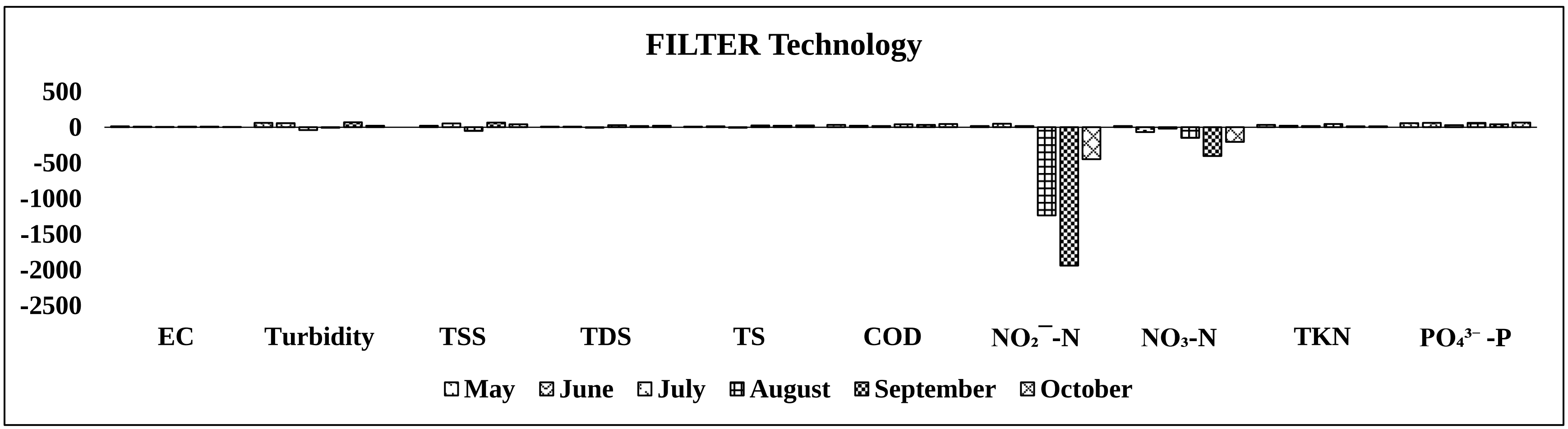
(g)


Pond 7
100
50
0
-50
-100
EC
Turbidity
TSS
TDS
TS
COD
NO₂⁻-N
NO₃-N
TKN
PO₄³⁻ -P
May
June
July
August
September
October

(h)



(i)



(j)

Figures 4.14 (a-j): Monthly variations in percentage removal efficiencies of ICW for physicochemical parameters

4.3. Pearson's correlation matrix

The following table shows the parameters that have shown a strong correlation with their p-values. The correlation was significant when p-values were less than 0.05.

Table 4.2: Parameters and their p-values

Parameters	**p-values**
EC → Turbidity	0.002
EC → TDS	0.047
TDS → Turbidity	0.011
TS → Turbidity	0.039
TS → TDS	0.005
TS → TSS	0.031
COD → TSS	0.043
COD → TS	0.020
GHI → PO_4^{-}-P	0.038
GHI → TKN	0.006
Wind Speed → PO_4^{3-}-P	0.001
Wind Speed → GHI	0.010
Temperature → 1/DO	0.003

Table 4.3: Pearson's correlation matrix among physicochemical and weather parameters

	Temperature	pH	EC	Turbidity	TDS	TSS	TS	COD	DO	NO_2^--N	NO_3^--N	PO_4^--P	TKN	GHI	Wind Speed
Temperature	1.00														
pH	0.21	1.00													
EC	0.05	-0.05	1.00												
Turbidity	-0.03	0.06	*0.97	1.00											
TDS	-0.09	0.12	*0.82	*0.91	1.00										
TSS	0.35	-0.30	0.45	0.51	0.63	1.00									
TS	0.14	0.00	0.74	*0.83	*0.94	*0.85	1.00								
COD	0.08	0.03	0.36	0.51	0.79	*0.82	*0.88	1.00							
DO	*-0.96	0.04	-0.06	0.06	0.19	-0.35	-0.06	0.05	1.00						
NO_2^--N	0.14	0.31	0.65	0.63	0.30	-0.07	0.19	-0.25	-0.15	1.00					
NO_3^--N	0.57	0.38	0.33	0.33	0.50	0.44	0.56	0.62	-0.39	-0.11	1.00				
PO_4^--P	0.34	-0.54	0.61	0.46	0.43	0.61	0.54	0.38	-0.45	-0.01	0.49	1.00			
TKN	-0.39	-0.84	0.51	0.45	0.38	0.44	0.39	0.22	0.19	0.02	-0.26	0.61	1.00		
GHI	-0.07	-0.83	0.58	0.46	0.36	0.53	0.43	0.22	-0.13	0.05	-0.03	*0.84	*0.93	1.00	
Wind Speed	0.23	-0.71	0.54	0.42	0.40	0.68	0.54	0.41	-0.37	-0.09	0.33	*0.97	0.75	*0.92	1.00

*Correlation is significant at 0.05 level

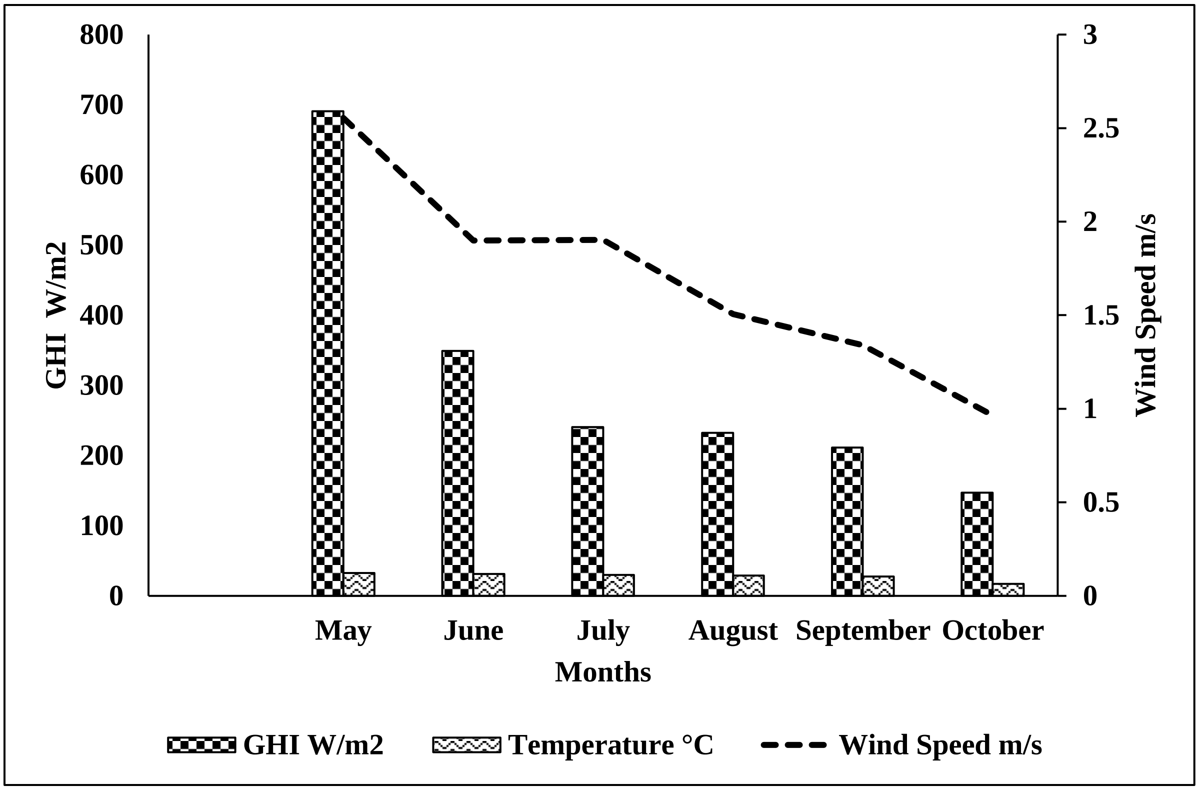


Figure 4.15: Seasonal variations of meteorological parameters

Source: USPCASE (May - October 2021)

Above figure shows average temperature, wind speed, and GHI values during the study period. Wind speed GHI showed a decreasing trend throughout the months. Whereas there were slight variations in temperature in May, June, July, and August which has decreased further in September and October.

Table 4.4: Correlation between meteorological and physicochemical parameters

Meteorological Parameters	Water Quality Parameters	
Temperature	DO **(Strong – Negative)**	pH, EC, Turbidity, TDS, TSS, TS, COD, DO, NO_3^--N, NO_2^--N, PO_4^{3-}-P, TKN, GHI, Wind Speed **(Non-significant)**
GHI	PO_4^{3-}-P, TKN, Wind Speed **(Strong – Positive)** pH, **(Strong – Negative)**	EC, Turbidity, TDS, TSS, TS, COD, DO, NO_3^--N, NO_2^--N **(Non-significant)**
Wind Speed	PO_4^{3-}-P, GHI **(Strong – Positive)**	pH, EC, Turbidity, TDS, TSS, TS, COD, DO, NO_3^--N, NO_2^--N, TKN **(Non-significant)**

Temperature showed a strong negative correlation with DO. While it had a non-significant correlation with other parameters. Andleeb and Hashmi (2018) also reported an inverse relation between DO and temperature showing high DO values i.e., 5.2 mg/L at low temperatures i.e., 18.25 ºC. GHI showed a strong positive correlation with phosphate phosphorous, TKN, and wind speed, a strong negative correlation with the pH, and non-significant with other parameters. As reported by Baldovi *et al.* (2021) high total phosphorous removal was achieved due to increased solar radiation that resulted in the high growth rate of plants present in wetland. An increase in GHI resulted in improved photosynthetic activity by plants which in turn resulted in enhanced uptake of nutrients and lesser release of decaying organic matter, hence, increase in GHI resulted in a decrease in value of pH for phytoremediation system (Herrera, 2013). Wind speed showed a strong positive correlation with GHI (Wooten, 2011) and phosphate-phosphorous while non-significant with other parameters. Chao *et al.* (2017) demonstrated that both strong and weak wind conditions have resulted in increase of total phosphorous in the water column.

4.4. Morphological identification and characterization of plant endophytes

Based on morphology a total of eight bacterial strains were isolated from leaves, shoots, and roots of *Typha latifolia*. Three bacteria were isolated from leaves, two from shoots, and three from roots. Morphological characters studied were shape, color, size, elevation, margin, surface texture, and opacity. Colony morphology is used to illustrate bacterial properties (Enos-Berlage and McCarter, 2000). Previously endophytes from roots and shoots of *Pistia stratiotes* and *Centella asiatica* were isolated from the same wetland based on physicochemical parameter's removal efficiency of ponds. The results showed that *Centella asiatica* was more efficient than *Pistia stratiotes* (Abeerah *et al.,* 2013). In the present investigation, an attempt has also been made to isolate bacteria from leaves, shoots, and roots of *Typha latifolia*. The results of present study have demonstrated the contribution of *Typha latifolia* in overall treatment efficiency. Morphological characteristics of bacterial strains isolated from leaves, shoots, and roots of plants are presented in below figures.

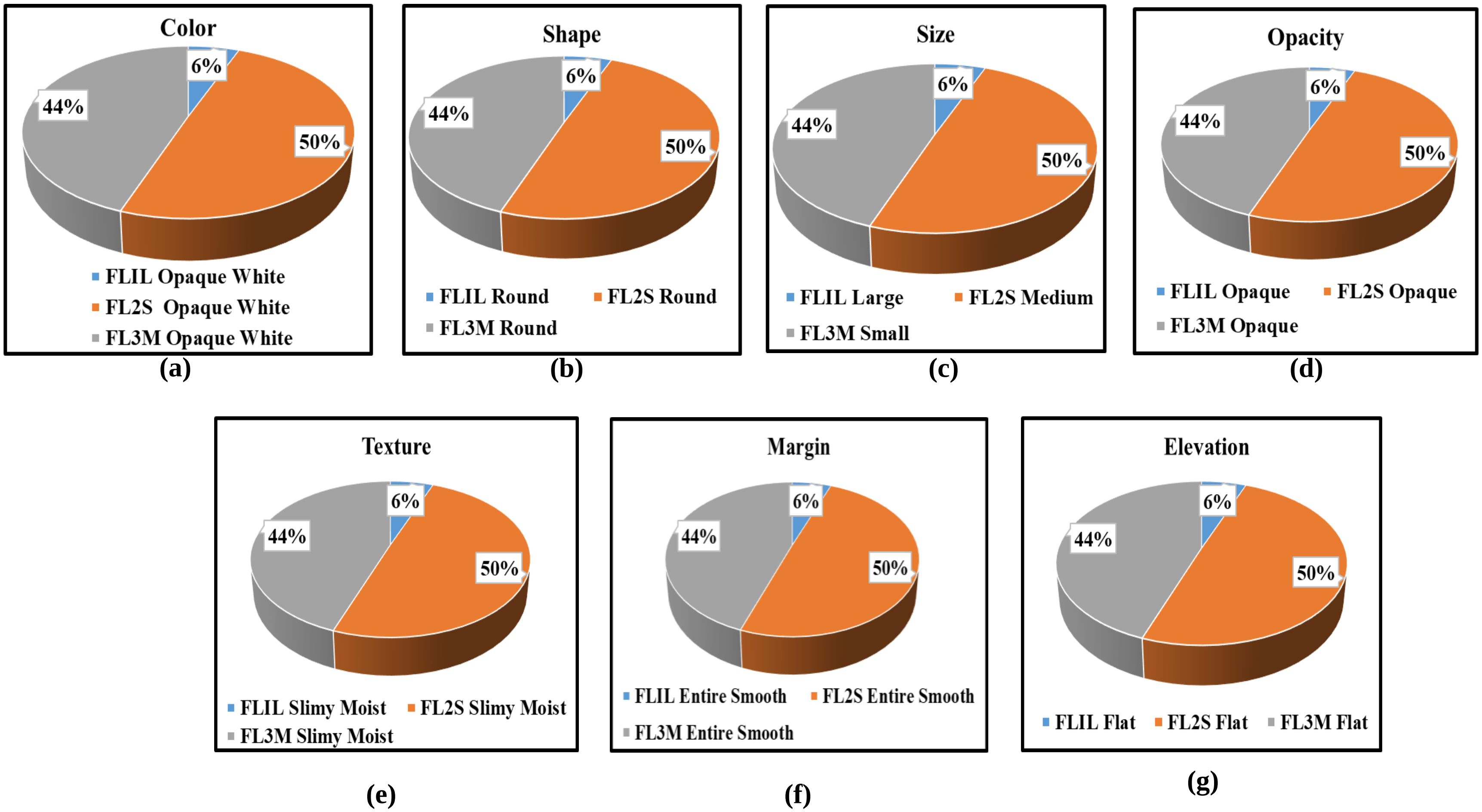


Figure 4.16 (a-g): Morphological characterization of leaves

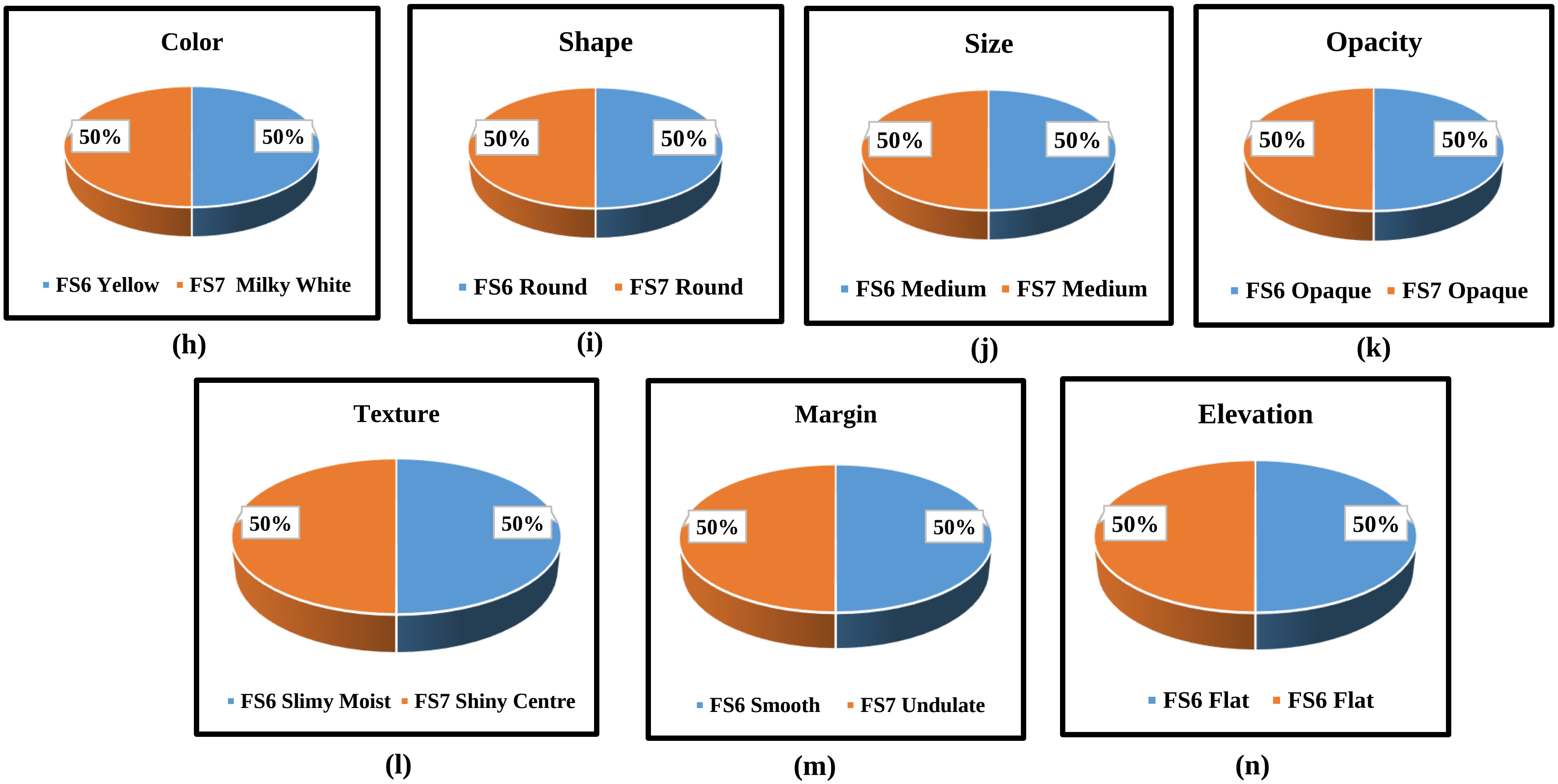


Figure 4.17 (h-n): Morphological characterization of shoots

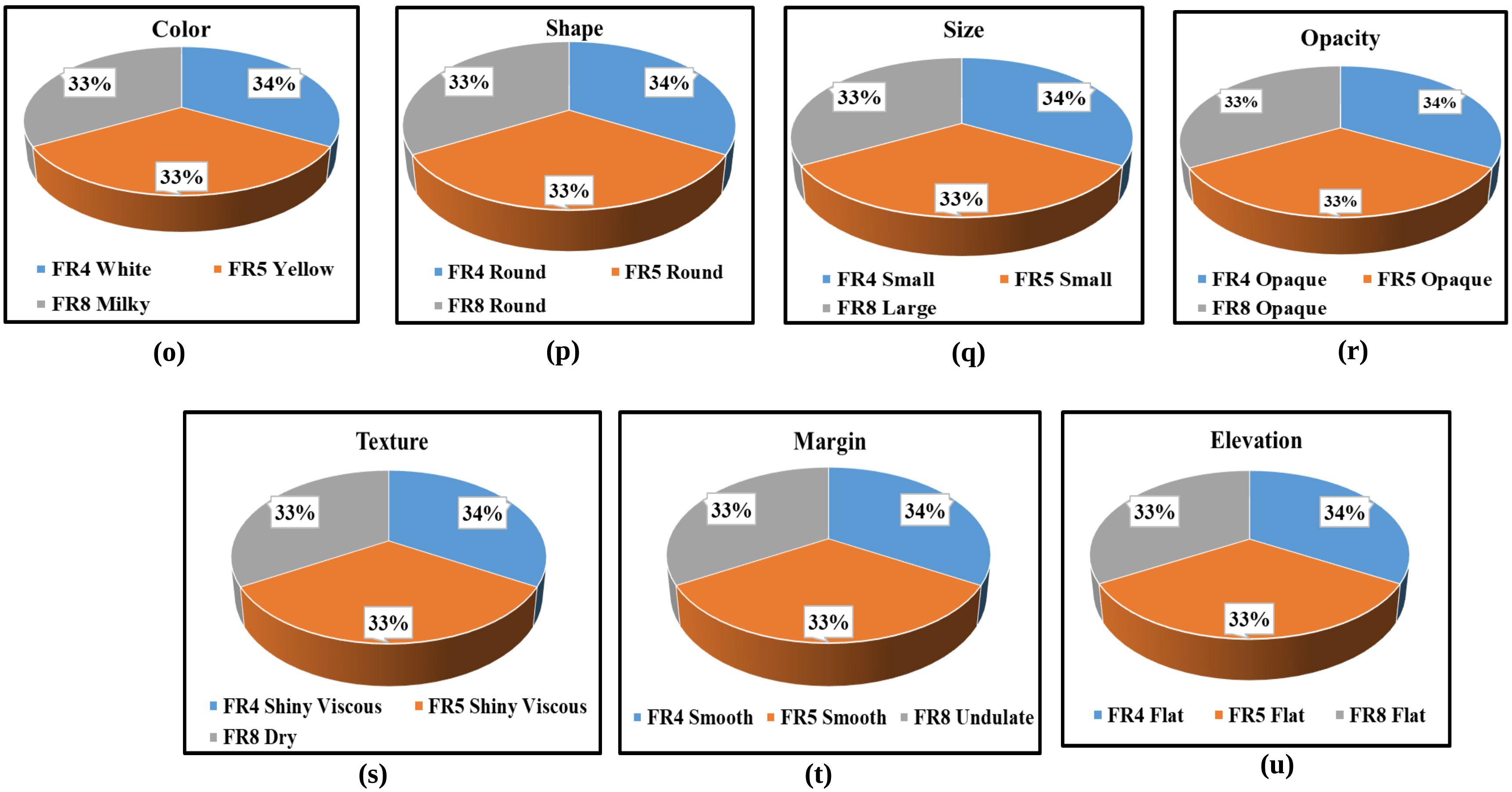
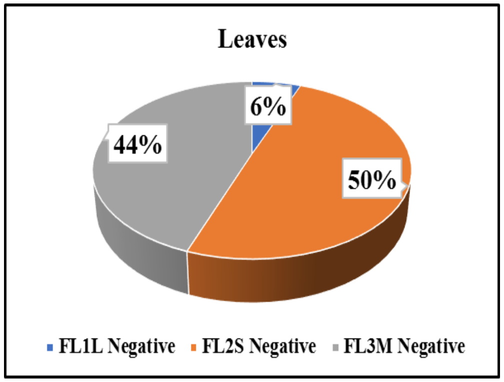


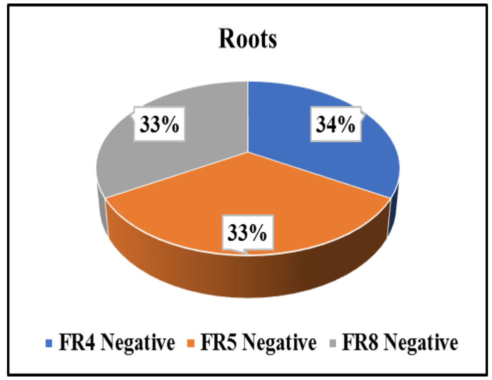
Figure 4.18 (o-u): Morphological characterization of roots

4.5. Biochemical characterization of isolates

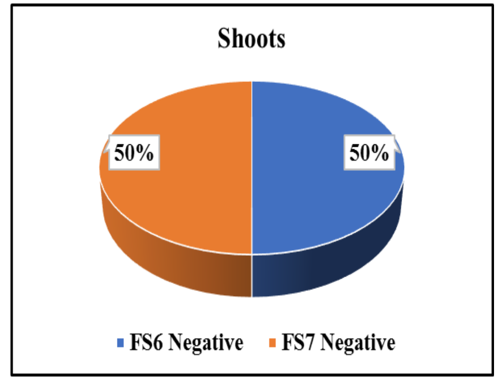
Biochemical characterization of isolates showed that all bacteria were gram-negative and catalase and oxidase positive. Biochemical characterization of isolated bacterial strains in terms of gram reaction, catalase test, and oxidase test is presented in figures 4.19 (a-c), 4.20 (d-f), and 4.21 (g-i) respectively.



(a)

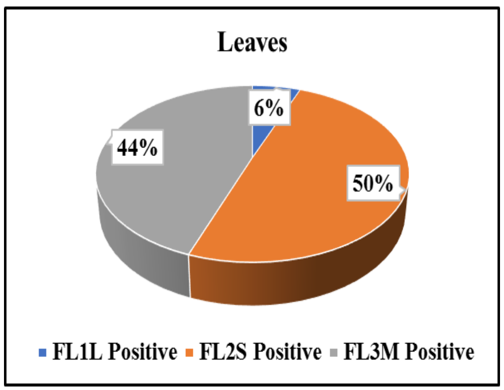


(b)

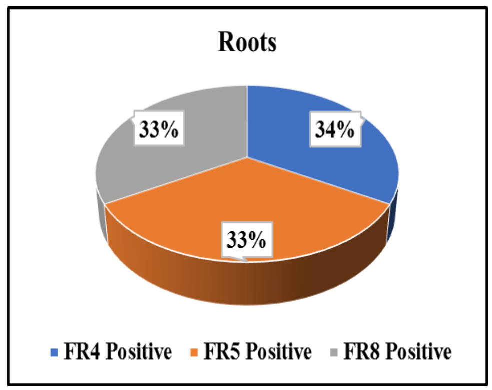


(c)

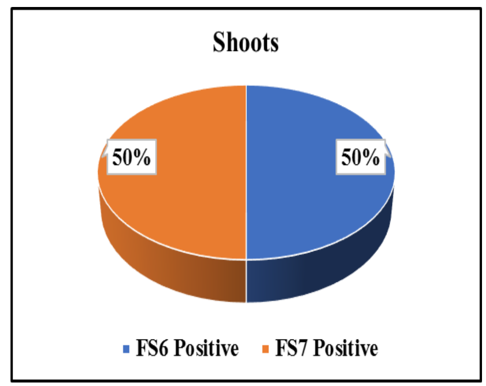
Figure 4.19 (a-c): Gram reaction



(d)

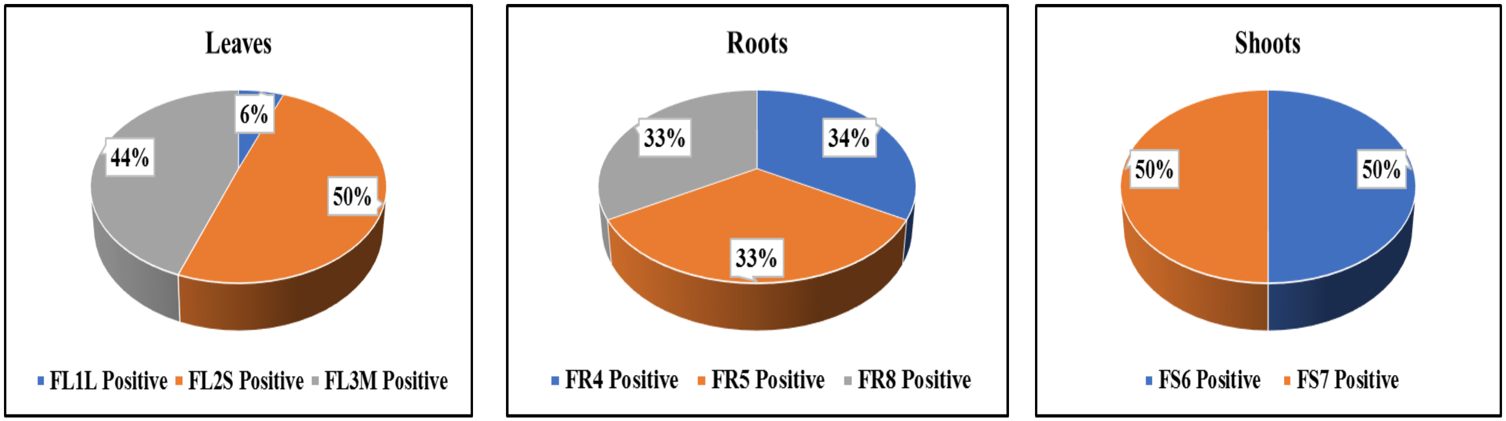


(e)



(f)

Figure 4.20 (d-f): Catalase test

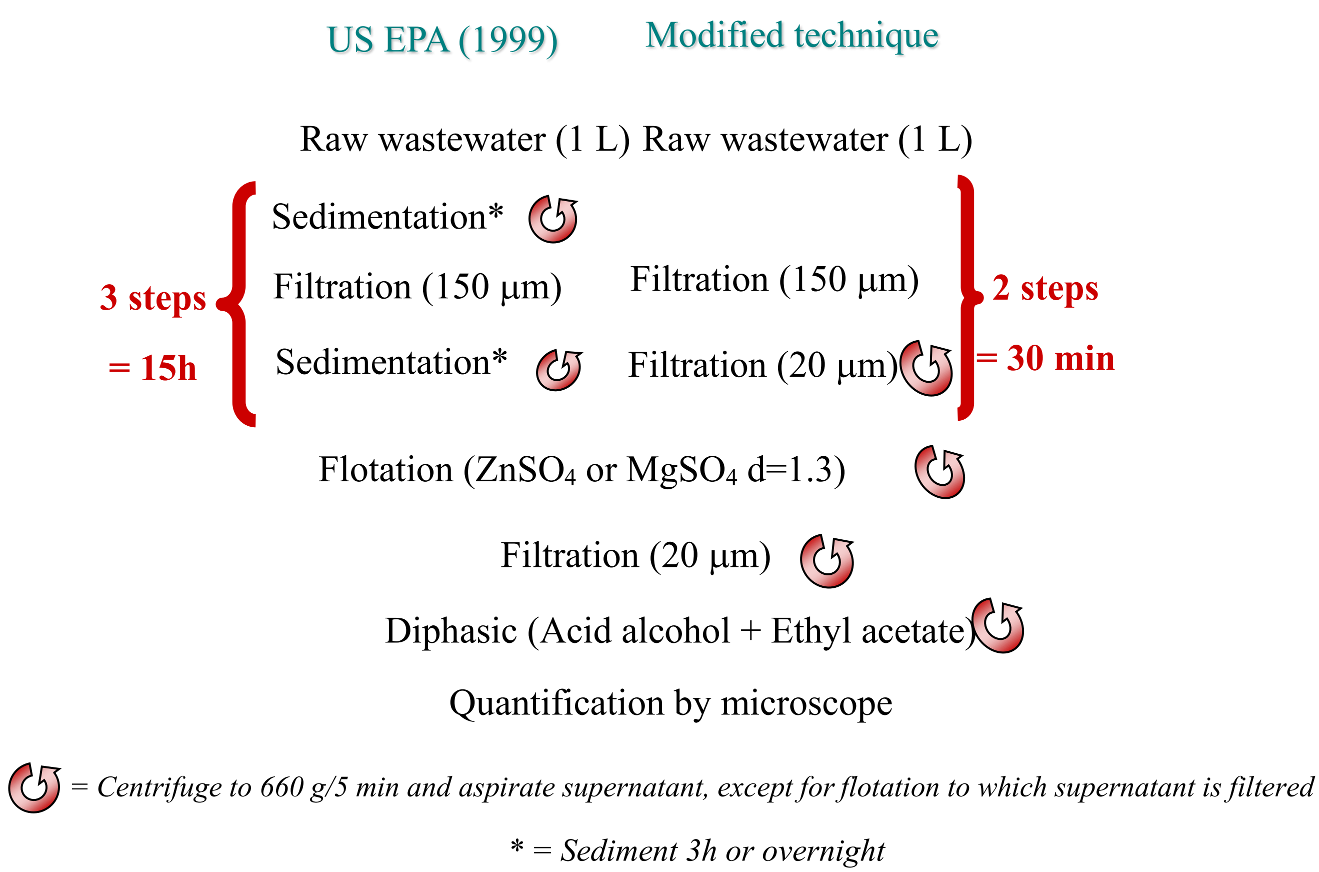


(g) **(h)** **(i)**

Figure 4.21 (g-i): Oxidase test

4.6. Helminth egg analysis

For helminth egg analysis modified USEPA Technique was used. The validation of technique was performed during a one-month analysis of wastewater samples collected from ICW. Helminth eggs were observed under Carl Zeiss Axio-lab A1 microscope at 10x objective lens and identified. Species other than helminth eggs were also identified manually. In comparison to the USEPA (1999) technique, the modified method reduces analysis time to less than two hours in terms of effective analysis time.



During analysis, only one helminth egg was identified i.e., *Fertile ascaris*. The probable reason for only one egg identified was maybe the heavy Monsoon rains during the sampling period that washed the helminth eggs away. Outwater *et al.* (2019) also found strong evidence of the effect of the season because of precipitation and possibly water temperature. Heavy monsoon rains washed all the helminth eggs and larvae and there was no helminth egg found at the season's end. Since no helminth egg was identified in the collection tank it means the treated wastewater showed compliance with the international agriculture reuse standards (IARS). Following are the other species that were observed in samples apart from helminth eggs using Carl Zeiss Axio-lab A1 microscope at 10x objective lens.

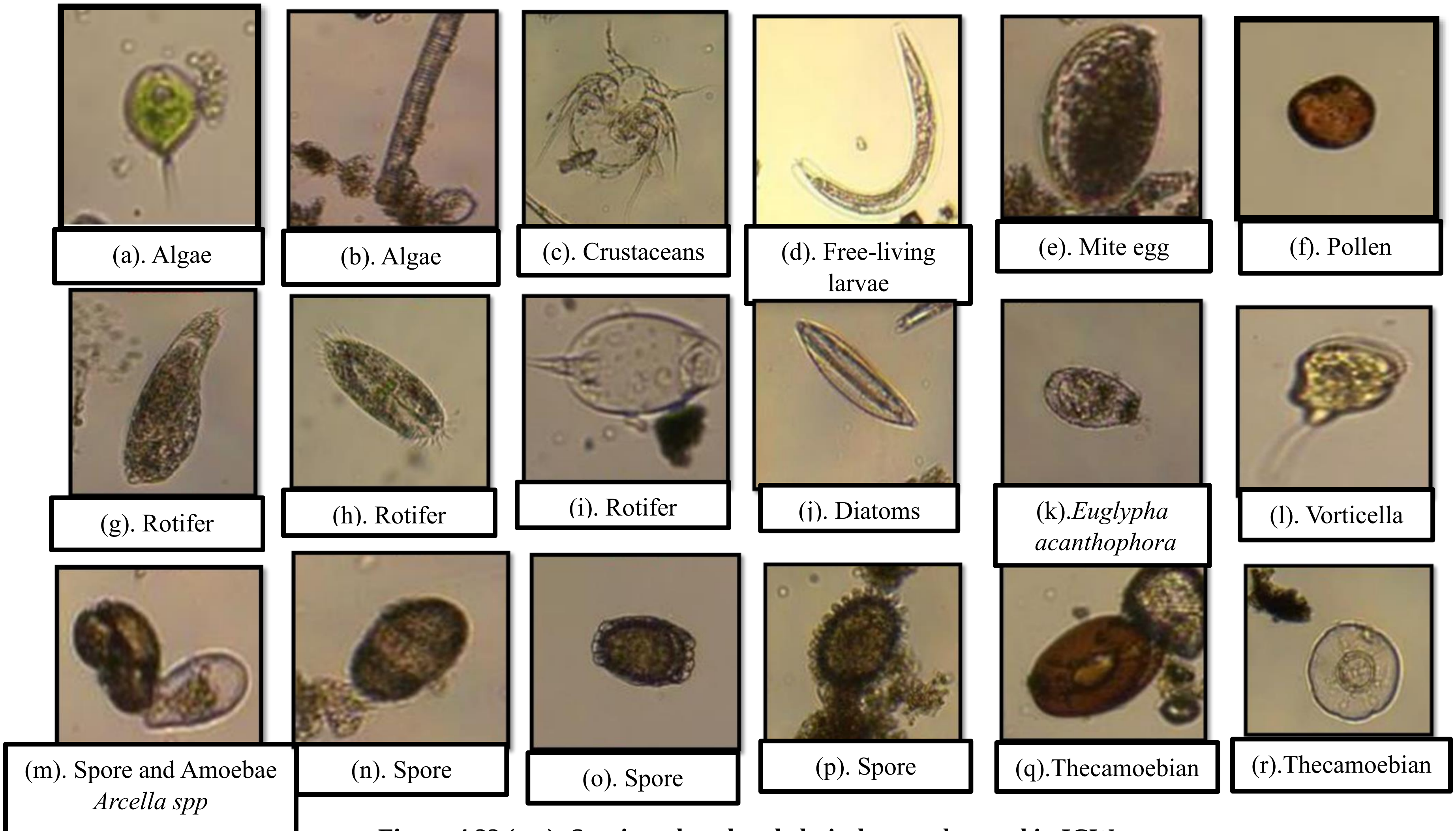


Figure 4.22 (a-r): Species other than helminth eggs observed in ICW

These are indicator of wastewater treatment and according to Akpor *et al.* (2014) microbes such as fungi, protozoans, crustaceans, and bacteria play an important part in converting organic waste into less toxic and more stable substances in natural treatment systems i.e., constructed wetlands and engineered treatment plants.

4.7. Economic valuation of integrated constructed wetland

In Pakistan wastewater treatment through constructed wetlands is a relatively new technology. Although globally constructed wetland treatment technology has been used for several decades and its acceptance and recognition has increased considerably. This technology is slightly gaining in Pakistan. In Pakistan, conventional treatment technologies have been used that require high capital and operation cost. The major cost associated with these technologies is the high use of electricity. Keeping in view the economic situation and shortage of electricity, wetland treatment technology is a more reliable and sustainable technology for wastewater treatment. This economic valuation of an integrated constructed wetland may help and encourage the management and policymakers in decision-making and help in the replication of this technology throughout the country.

4.7.1. Basic Cost components of Constructed Wetland

The basic cost components of constructed wetland system are capital cost, operational and maintenance cost.

4.7.1.1 Components of Capital Cost

4.7.1.1.1. Land acquisition

The major cost applied while establishing constructed wetlands is land acquisition as this treatment technology is mostly constructed near residential areas, with high land costs. However, land for ICW construction, which is approximately half an acre of land, was provided by NUST. So, in this case no cost was applied for land acquisition.

4.7.1.1.2. Site survey, planning, and design

The design of constructed wetland treatment facility is highly site-specific. An evaluation of terrain, depth of the groundwater, kind of soil, and gravity flow of wastewater into and out of the treatment facility are all included in site survey. Wastewater is pumped into or out of the system if gravity flow is not possible. If pumping is necessary, it is advised that pumping wastewater at input rather than outlet be given priority. This prevents flooding of constructed wetland treatment plant in case the pumping system fails. Because wastewater had been diverted from main sewerage line, gravity flow was possible for the system under study. The site was designed by the principal investigator and other team members with the consultation of private consultants. The private consultant charged Rs. 80000 for the ICW project in 2014.

4.7.1.1.3. Construction

The site was designed, and then construction work started. The construction of wetland basins is typically the initial stage. Excavations for eight wetland ponds and a sedimentation tank were made, and the excess soil was then leveled and shifted. According to data collection interviews, the cost of pond excavation was around Rs. 6–8/cft. The surface of the pond and t berms were leveled following the excavation. The extra soil was moved to appropriate location. The cost of about Rs. 3–4/cft is incurred when soil is leveled and moved. After the ponds had been leveled, a 150-micron high-density polyethylene sheet was used to line the basins. For eight basins, 16500 square feet of HDPE lining were used. At time of construction, market price for HDPE liners was Rs. 230/kg, with each kg containing roughly 100 ft^2 of 150-micron HDPE. The plastic lining is available in widths of 12, 16, and 20 feet. To address this problem, skilled laborers joined together HDPE using a variety of materials.

Building the sedimentation tank was the next step in constructing wetland treatment facility. For walls and internal baffles of sedimentation tank, primarily brickwork was used. Concrete with ratio of 1:2:4 was used to build sedimentation tank's base. To make maintenance and cleaning easy, top surface of sedimentation tank was left uncovered.

RCC pipes with a 1 ft. diameter were used to connect all ponds and sedimentation tank. The substrate was added to wetland following the designed parameters. As a substrate for constructed wetland, boulders from Korang River were collected. Aeration equipment was installed in last pond that holds treated water for further use. The project's overall cost included the cost of aeration system. A rotational valve was used to finally connect the wastewater from inlet to the system.

4.7.1.1.4. Plantation

One of the essential elements of a constructed wetland treatment system is aquatic plants. Aquatic macrophyte planting costs covered collecting and installation in specific ponds. The aquatic macrophytes used in this treatment plant were emergent and floating.

Upon site completion, a rotating valve-controlled wastewater input, which was linked, and site was subsequently filled with wastewater.

4.7.1.1.5. Break-even point

The break-even point for ICW occurred after 6 months upon completion of constructed wetland project. If 1 rupee to produce 1 gallon is assumed. Then for 70000 gallons per day, it will be 70000 rupees which in six months will be Rs. 12.775 million which is approximately equal to 13 million PKR capital cost for the ICW.

4.7.1.2. Operation, maintenance, and reuse cost components

Although constructed wetland treatment systems require less maintenance than traditional treatment methods, it is still a crucial element in a project's success. Maintenance of ICW treatment facility includes ensuring smooth inflow and outflow of wastewater, harvesting mature aquatic plants, removing sludge, cleaning entrapped sediments in sedimentation tank, and maintaining walkways that connect the ponds. The major operation and maintenance cost components include the following categories.

4.7.1.2.1. Human resource for plant operation and maintenance

To ensure the regular operation and maintenance of the constructed wetland treatment facility, two workers were hired. At the start of project in 2014, these plant operators were paid Rs. 12,000 per month each. Their pay gradually increased to Rs 19,400/- per month each in 2017. Therefore, at beginning of project, total cost of human resources is estimated to be 0.28 M/annum and 0.46 M/annum in the year 2017. In the year 2020, the salaries of workers were PKR. 0.49 M/annum and in the year 2022 the salaries were PKR 0.72 M/annum. The salaries have increased over the years. Unrestricted wastewater flow within the treatment facility, regular cleaning of scum, large floating materials, and sediments from sedimentation tanks, hoeing and weeding of unwanted grass, trimming of grass on treatment facility berms, harvesting of mature aquatic plants and moving to designated locations, operation of aeration facility in treated water reservoir in last pond, and cutting of emergent vegetation, are the main tasks for smooth operation.

4.7.1.2.2. Consumables, supplies, capital equipment, and utilities

Consumables, supplies, capital equipment, and utilities needed to conduct maintenance operations are all included in the cost of maintenance. Only provision of overall, boots and gloves are necessary for maintenance of treatment facility. The brush cutter for cutting grass. Except for pumping water from the collection tank to transport it for horticultural purposes, no energy or other utilities were required for ICW project because wastewater enters and exits the system using gravity force.

4.7.1.2.3. Water quality analysis

The constructed wetland treatment system works like a natural system. Therefore, it is imperative to continuously assess the effectiveness of the treatment. This includes routinely sampling water and analyzing it to make sure treated water remains within acceptable limits of water quality. In the laboratories of Institute of Environmental Science and Engineering, water quality is regularly examined. The cost applied in this category is on the resources used in IESE laboratories to conduct the analysis.

4.7.1.2.4. Repair and maintenance

The stability of berms, walkways between ponds, and masonry structures are all part of integrated constructed wetland treatment facility repair and maintenance. Fountains, solar plates, batteries, damaged PVC membrane sheets, hardwood planks, gate valves, motor pumps, etc. all need repair and maintenance. A total of Rs. 0.8 M/annum cost is applied to the repair and maintenance.

4.7.1.2.5. Reuse cost

If 0.15 Rupees per gallon for 70,000/GPD is assumed the cost of distribution of water inside NUST will be 3.833 M/annum. The cost is associated with i.e., the Diesel used in tanker, electric bill for the motor, driver, and 1 staff member salary for water handling.

4.7.1.3. Overall capital and O&M cost of the treatment facility

The integrated constructed wetland treatment facility developed at NUST includes a sedimentation tank, wetland, detention ponds, and (FILTER Technology) tile drainage filtration system. The cost incurred on the construction of the ICW treatment facility was 13 million PKR in 2014. The overall cost for operation and maintenance of treatment facility is 0.8 M/annum and the reuse cost is 3.833 M/annum.

4.7.1.4. Cost comparison with other methods

Through the concept of hedonic price analysis, a cost comparison of ICW with other methods generating the same amount of water has been estimated. Hedonic price analysis suggests that a monetary value equal to cost incurred on an alternative source for the generation of the same amount of treated water may be assigned to treated water (Hussain *et. al,* 2001; Robertson, 2011).

A comparison was made between operation, maintenance, and reuse costs of ICW, pumping of groundwater through an electric motor, and purchasing freshwater through CDA. As described earlier different cost components of ICW are presented here.

4.7.1.4.1. Integrated constructed wetland (ICW)

The cost related to the salaries of workers is 0.72 M/annum. The repair and maintenance cost is 0.8 M per annum. The reuse cost is 3.88 M/annum. The total cost related to the integrated constructed wetland is 5.43 M/annum.

4.7.1.4.2. Pumping through the electric motor

If a 7.5 kW electric motor that generates 40 m^3 of water per hour is considered it will take 6.6 hours to generate 70000 US gallons per day.

Electrical consumption = 49.5 KWhr/day

Considering Rs. 32/KWhr per day cost = Rs. 1584/day

Per year cost of an electric motor of 7.5 KWhr capacity = 0.42 M/annum

Considering same cost as ICW for salaries of workers, repair and maintenance,

Total cost = 2.02 M/annum.

If the total cost of ICW and pumping through the electric motor is compared constructed wetland has more cost than groundwater pumping. But through groundwater pumping groundwater source is depleted whereas integrated constructed wetland treats the wastewater and converts it into a reusable form.

4.7.1.4.3. Purchasing freshwater through CDA

Following calculations show the total cost for purchasing water from CDA.

Per tanker cost from CDA = Rs. 5500

Capacity of 1 Tanker = 10,000 Liters

10,000 Liters = 2640 US gallons

Water capacity required = 70,000 US gallons per day

For 70,000 USG tanker required = $\frac{70000}{2640}$ = 26.51 tanker/day

Total cost per day = 26.51 tanker cost/day = 26.51 x 5500 = Rs. 145,805/day

Total Cost = 37.9M/annum

Considering total costs of these three methods pumping through an electric motor has lowest cost whereas purchasing freshwater through CDA has highest cost. But considering non-monetary benefits and sustainability of the approach wastewater treatment through ICW is a more reliable and sustainable method. Comparison of ICW with purchasing freshwater from CDA indicate monetary benefits of ICW will be 32.4 M/annum. The following table shows the comparison of costs between the three treatment methods.

4.7.1.5. Benefits of constructed wetland treatment facility

Although water treatment is primary goal of developing constructed wetland treatment facilities, this natural system also offers many secondary advantages. The Wetland Reserve Programme report for natural wetlands assessment method was used to analyze marketable and non-marketable benefits of constructed wetlands (Kormos, 1995). The benefits that can be given a monetary value are included in the marketable benefits, and vice versa. The marketable or monetary benefits include.

4.7.1.5.1. Availability of treated water

The constructed wetland treatment facility has a daily capacity of 75000 US gallons. To use the treated water for horticulture within NUST, tankers collect it from reservoir. 5-7 tankers with a capacity of 10,000 US gallons are collected on average from this site. This indicates that irrigation and horticulture purposes utilize 50,000 to 70,000 gallons of treated water per day. The average cost for a freshwater tanker in the federal capital area is Rs. 145,805/day (CDA) which is 37.9M/annum. Subtracting this value from the total cost of integrated constructed wetland i.e., 5.43 M/annum. The monetary benefit will be 32.4 M/annum.

The nonmarketable benefits of constructed wetland treatment include the benefits that may not be assigned a monetary value. The nonmarketable benefits include:

4.7.1.5.2. Improvement in water quality

The provision of reusable water for agricultural purposes and improvement in surface water quality are two main benefits of constructed wetlands. It is evident from the results of the physicochemical and biological analysis that the water quality has been improved and is showing compliance with national and international regulations.

4.7.1.5.3. Enhancement in landscape

The wetland treatment facility is frequently built on abandoned or deteriorated land. The establishment of natural treatment methods improves landscape in those locations where damaged land is recovered and restored to aesthetical beautiful area. After collection, the university's effluent is directed through a single sewage pipe, which eventually empties into the allocated channel or waterway. This sewage line was not far from the area designated for construction of treatment plant. This area of institution was deserted at time of construction. The addition of green vegetation and water ponds by construction of treatment facility improved the landscape.

4.7.1.5.4. Carbon sequestration

Constructed wetlands have potential to sequestrate carbon. Each wetland provides a potential sink area for atmospheric carbon.

4.7.1.5.5. Improvement in aquatic habitat

The aquatic life benefits from improved surface water quality even if wastewater treated by this system is not used specifically for fish or other related uses.

4.7.1.5.6. Educational and recreational services

The constructed wetland treatment system developed at NUST is used for both educational and recreational purposes. This site is visited by several visitors on annual basis.

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Integrated constructed wetlands are efficient and reliable systems for the removal of contaminants from wastewater and thereby reusing it for agriculture.

Conclusions of research drawn are mentioned below:

1. Physicochemical parameters removal efficiency was up to 79.01%, 67.45%, 73.34%, 37.70%, and 61.68% for TSS, COD, Turbidity, TKN, and Phosphate-phosphorous respectively. Removal efficiency of constructed wetlands varies with changes in seasons.
2. Eight bacterial strains were isolated from the roots, shoots & leaves of *Typha latifolia.* All of them were gram-negative and catalase and oxidase positive.
3. Strong relation found between the rainfall and helminth egg removal. Monsoon rains washed helminth eggs away, 1 egg was found in pond 5 and none were found in the effluent.
4. Wastewater management through constructed wetland treatment facilities is a cost-effective and environment-friendly solution. Monetary benefit of ICW was 32.47 M/annum when compared with cost of purchasing freshwater from CDA.

5.2. Recommendations

1. Usage of alternative plants to increase the performance efficiency of integrated constructed wetland.
2. The isolated endophytes from *Typha latifolia* may be used to gain further insights into the bacteria that contribute to plant adaptation in contaminated environment.

3. Regular monitoring of helminths eggs through reliable techniques such as modified USEPA technique is important to ensure compliance with global and international regulations.
4. Cost effectiveness of constructed wetland treatment may be further enhanced through efficient hydraulic design as it will result in optimum land utilization.

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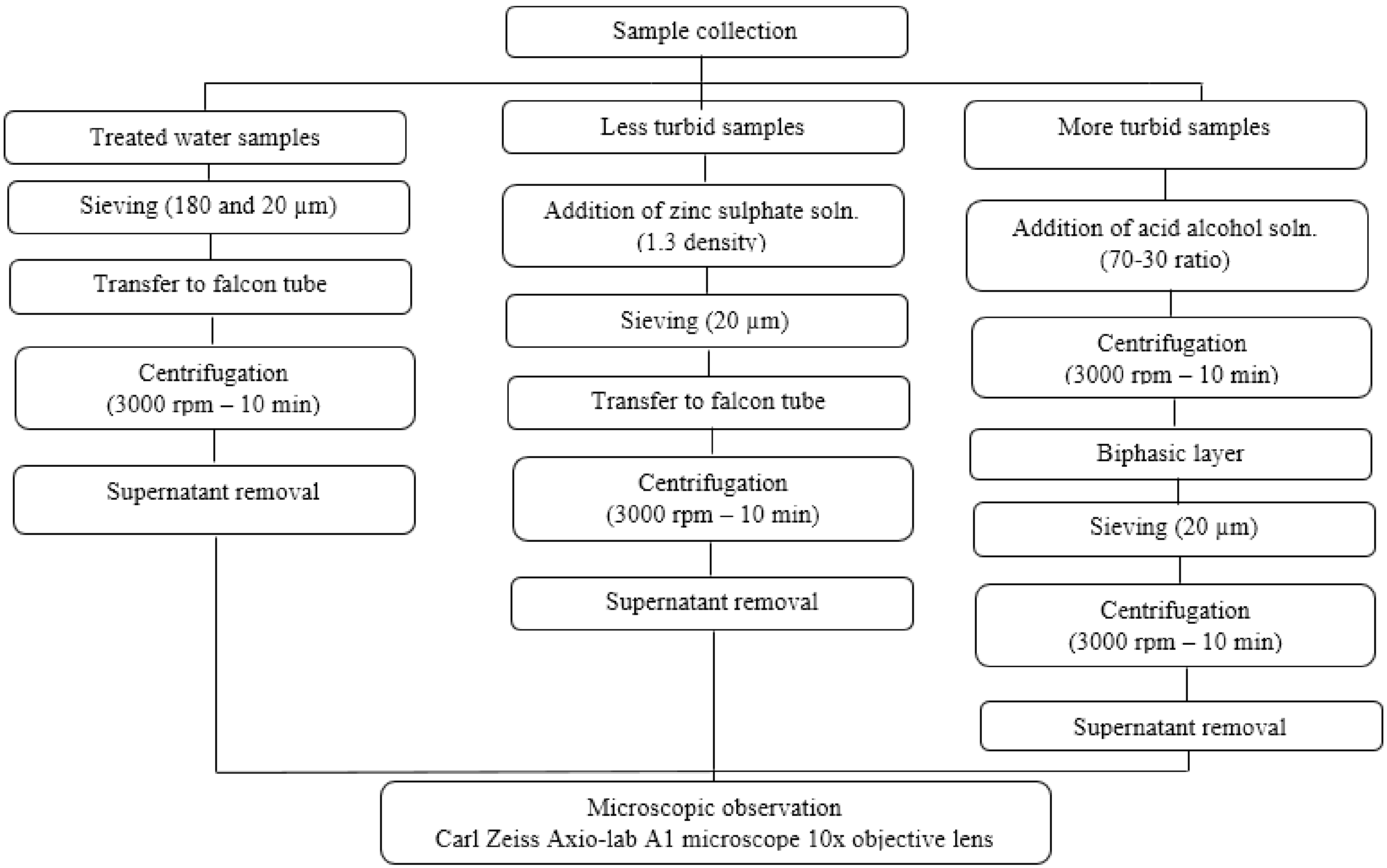

Figure 4.23. Flowchart for helminth egg analysis

Table 4.5: Cost comparison between ICW, pumping through the electric motor, and purchasing freshwater through CDA

Integrated Constructed Wetland	**Pumping through Electric Motor**	**Purchasing Freshwater through CDA**
• PKR. 0.72 M/annum (Salaries of Workers) • PKR. 0.8 M/annum (Repair & Maintenance) • PKR. 3.833 M/annum (Reuse Cost) • Total Cost = 5.43 M/annum	• 7.5 kW electric motor @ 40 m^3/hr • 70000 US gallons/day in 6.6 hours. • Electrical consumption 49.5 KWhr/day • Rs. 1584/day @ Rs. 32/KWhr (IESCO). • Rs. 0.42 M/annum • PKR. 0.72 M/annum (Salaries of Workers) • PKR. 0.8 M/annum (Repair & Maintenance) • Total Cost = 2.02 M/annum	• Rs. 5500 per tanker (CDA) • 1 Tanker = 10,000 Liters • 10,000 Liters = 2640 US gallons • Water capacity required = 70,000 US gallons per day • For 70,000 USG, $\frac{70000}{2640}$ = 26.51 tanker/day • 26.51 tanker cost/day = 26.51 x 5500 = Rs. 145,805/day • Total Cost = 37.9M/annum

Table 4.7: Cost comparison of ICW from 2014-2022

Operation, Maintenance & Reuse Cost			
(Year, 2014)	**(Year, 2017)**	**(Year, 2020)**	**(Year, 2022)**
• PKR. 0.28 M/annum (Salaries of Workers) • PKR. 0.35 M/annum (Repair & Maintenance) • PKR.1.53 M/annum (Reuse Cost) • Total Cost = 2.17 M/annum	• PKR. 0.46 M/annum (Salaries of Workers) • PKR. 0.56 M/annum (Repair & Maintenance) • PKR. 2.47 M/annum (Reuse Cost) • Total Cost 3.51 M/annum	• PKR. 0.49 M/annum (Salaries of Workers) • PKR. 0.60 M/annum (Repair & Maintenance) • PKR. 2.61 M/annum (Reuse Cost) • Total Cost = 3.70 M/annum	• PKR. 0.72 M/annum (Salaries of Workers) • PKR. 0.88 M/annum (Repair & Maintenance) • PKR. 3.83 M/annum (Reuse Cost) • Total Cost = 5.43 M/annum