Microbial Characterization and Water Quality Assessment of Integrated Constructed Wetland

By

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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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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]*

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ABSTRACT

Inadequate sanitation system lead towards surface water contamination. In order to maintain surface water quality and decrease environmental risk, there was a need for wastewater treatment system. Objective of the current study is to monitor the efficacy of multi-stage integrated constructed wetland consisting of sedimentation tank, eight ponds of HSSF-CW and FILTER technology. Specific hydrophytes such as (Typha latifola, Pistia stratiotes, Centella asiatica) were used in treatment system. Samples were collected from ten sites including sedimentation tank (inflow), ponds and collection tank (outflow) to measure organic removal efficiency at each site. Selected physicochemical and microbiological parameters were analyzed according to standard method of examination (APHA) to demonstrate the sustained and stable removal of organics through wetland system, that includes pH, Temperature, Turbidity, Electrical Conductivity (EC), Dissolved Oxygen (DO), Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Total Kjeldahl Nitrogen (TKN), Nitrate, Nitrite, Phosphate, Total coliforms and Helminths quantification was carried out by using Ballinger method. Significant spatial variations were observed with higher organics removal efficiency and final effluent proved to comply with the NEQS regulations. An excellent treatment efficiency was exhibited by the water quality parameters such as COD 69%, TSS 74%, BOD 29%, Nitrite 48%, Nitrate 59%, TKN 47%, Phosphate 33%, Total Coliform 83% and Helminth eggs 100%. The high removal rates were achieved at higher temperature as well as weather parameters (Global Horizontal Irradiance and Rainfall) showed a significant positive and negative correlation with the removal efficiencies. Predominant species isolated and identified from wastewater of integrated constructed wetland belongs to the phyla Proteobacterium and Firmicute. While, predominant bacterial species isolated and identified from surface and sediment samples of different ponds includes *Pseudomonas* sp. and *Bacillus* sp. integrated constructed wetland has proved as an appropriate technology for treating domestic wastewater, land limitation is a major issue that need to be resolved. However, minor energy requirements and low principal cost are the supreme advantage for the decision makers to take into consideration.

1. Introduction

1.1 Background

Water is the most vulnerable resource existed on earth because only 1% water from all sources is easily accessible for human beings. With the increasing demand, stress on water resource is also increasing. Inadequate provision of safe and clean water has become one of the most prevalent problems which is expected to rise in the coming years. 844 million people lacked even a basic drinking water service and 263 million people spent over 30 minutes per round trip to collect water from an improved source (constituting a limited drinking water service) (WHO/UNICEF, 2017). To meet the rising water demands people are overexploiting the natural resources that are resulting in various environmental consequences like ecosystem deterioration (Zhang et al., 2015b). Impaired water conditions due to anthropogenic pollution will enhance pollution driven water scarcity. Polluted water loses its ability to support affiliated biotic communities as well as does not remain portable for human use.

Majority of the water pollutants are being carried by rivers into the longer water bodies, ultimately making them impure and posing risks to the human health (Song *et al*., 2019). It is well understood that in this situation the cost for rectifying is high, so the only way is to provide at least some degree of treated water and economically sound and sustainable sanitation solutions (Johnstone, 2013). Water pollution has resulted in many problems all over the world which include drinking water supply, sanitation supplies and survival of the biotic species. Direct water pollution refers to the release of pollutants from refineries, factories, sewage treatment plants, directly into the urban water provisions while indirect pollution refers to the addition of contaminants in the drinking water supply from ground/soil water system and from the atmosphere through rain water. Some major pollutants found in water include organic matter, metals, xenobiotic, nutrients and acidic gases such as Sulphur dioxide. Discharge of pollutants from domestic and industrial sources has detrimental effects on the aquatic ecosystem as this can result in deposition of large amount of nutrients, organic matter and pollutants leading to eutrophication, oxygen deficiency in the aquatic ecosystem and deposition of pollutants in the receiving water bodies (Wakelin *et al*., 2008).

Water conditions are getting worse in developing countries like Pakistan, which is suffering from lack of proper surface wastewater treatment systems in the rural and peri-urban areas (Corcoran, 2010). According to WHO/UNICEF, Pakistan is one of those country in which 76-90% population used an improved basic service from 30 minutes' round trip to collect water and 50-75% population used basic sanitation service while remaining still lack a basic sanitation service.

On the day of 25 September 2015, United Nation's Member State, adopted Sustainable Development Agenda 2030, which is comprise of 17 goals which also includes clean water and sanitation, good health and wellbeing, resource consumption and production.

Pakistan government has its own national Sustainable Development targets directed by the worldwide ambitions, and also considering national conditions. Pakistan also adopt National Sustainable Development Goals (SDGs) framework (MPDR, 2018), to significantly reduce the release of chemicals and all other solid, liquid or gaseous wastes to atmosphere, water and soil in order to curtail their dangerous impacts on human health and environment by 2020.

Pakistan Environmental Protection Act clarifies the need and necessity of the treatment of waste water before disposing into water bodies. Municipal waste includes refuse, garbage, sewage like liquid or semi-solid wastes and waste from slaughter house, sludge and human excreta. "Prohibition of certain discharges or emissions" regulates the disposal of waste water. According to National Environmental Quality Standards no person is allowed to discharge or emit any effluent, waste or atmospheric pollutant in concentration higher than National Environmental Quality Standards. Limited number of wastewater treatment plants are currently working under Water and Sanitation Agency (WASA) and need particular input to improve their capacities.

1.2 Wastewater and its composition

1.1.1 Sources of wastewater

Mostly wastewater characterize into four types such as, domestic wastewater, agricultural water, industrial wastewater and storm water (Crini and Lichtfouse, 2019). Domestic wastewater is the combination of all discharges including human excreta together with gray water, consist of laundry, washing, cleaning, food waste and water from kitchen and households, institutions, and commercial buildings (Boutin and Eme, 2016).

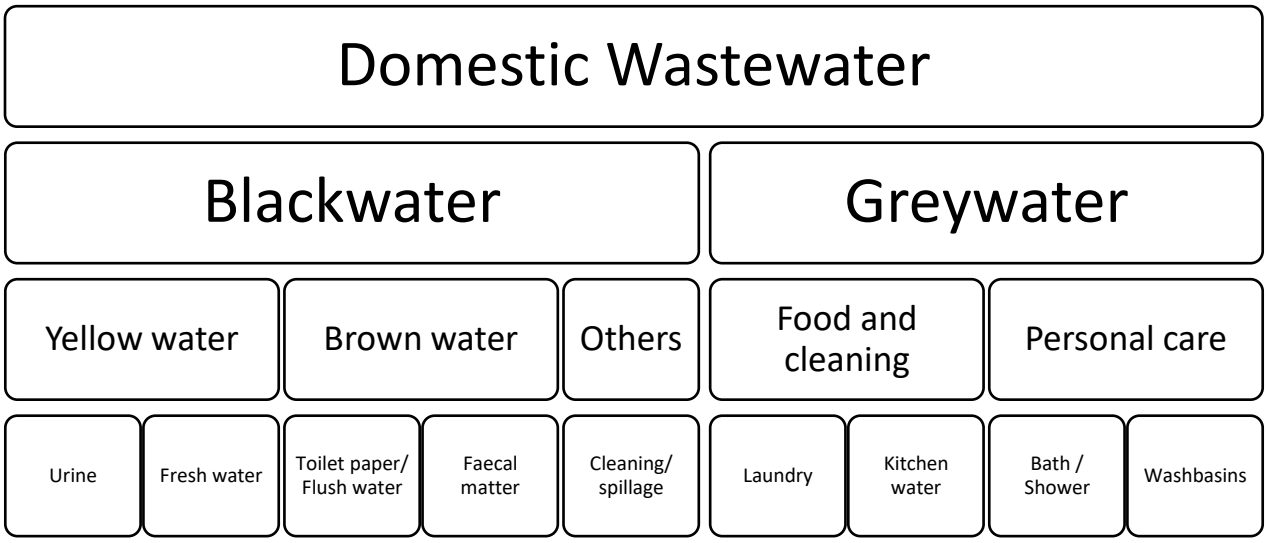

Figure 1-1.Source of domestic wastewater

Industrial wastewater is generated during manufacturing and processing plants. Unlike developed countries, in Pakistan, due to absence of proper wastewater management and treatment facilities, large proportion of waste is continually discharge into rivers, nearby canals or waterways. In Pakistan, municipal sewage network serves also serve as storm water drain which eventually increase the volume of sewage for disposal.

1.1.2 Domestic wastewater composition

Wastewater composition may vary in different communities, while main constituents of municipal wastewater remains same. Two proportions including wet mass and dry mass further includes various compounds. Organic and inorganic matter (dissolved minerals), Nutrients (Nitrogen, Calcium, Phosphorus, Potassium) and Pathogens are present in domestic wastewater. Brief summary of domestic wastewater elements, parameters, and possible impacts are explained in Table 1.

Table 1-1. Domestic wastewater composition

Proportion	Pollutants			Percentage in Domestic wastewater	Parameter	Impacts***
Mass Wet (75%)** 99%* Mass Dry 25%** 1%*	Organics and nutrients	**Biodegradable organics**	Proteins, carbohydrates, fats, etc.	70% of solids, from which 48% protein, 15% carbohydrates and 7% fats	BOD, COD	- depletion of dissolved oxygen - unsuitable environment - fish mortality - humus build-up
		Stable organics	Phenols, pesticides chlorinated hydrocarbon	Mainly depend on community due to their less use	GC, HPLC	- persist in the environment - toxic to environment - may make wastewater unsuitable for irrigation
	Inorganics	**Suspended solids**	Volatile compounds, colloidal impurities, Salts, grit etc.	30% of solid part of wastewater	TSS	development of sludge deposits - plugging of irrigation equipment and systems such as sprinklers
		Dissolved solids			TDS	- cause salinity and associated adverse impacts - phytotoxicity - affect permeability and soil structure
		Heavy metals			ASS	
	Pathogens****	**Viruses**	Adenoviruses Hepatitis A gastrointestinal, viruses	Mainly depend on community	Depend upon nature of pathogen	Cause communicable diseases
		Bacteria	*Escherichia coli* *Salmonella typhi* *Shigella* sp.			
		Helminth Eggs	*Taenia saginata* *Ascaris lumbricoides* Schistosoma spp.			

*(WWAP, 2017), **(Mara, 2003), ***(Hussain *et al*., 2002), ****(Chin, 2006)

1.1.3 Wastewater strength and flow

Domestic wastewater production and its strength depends upon various factors

- Water consumption
- Stormwater and Graywater intrusion

Domestic wastewater flow differs during 24 hours because of varying amount of water usage at different times. Amount of wastewater production from specific area also depends upon the size of the community. Areas where graywater and stormwater ultimately dumped into sewage system also effect wastewater strength. Flow rate of domestic wastewater is normally measured from domestic water consumption and number of populations connected to the sewerage system as shown in 1.1 eq.

$$Q_{ww} = 10–3kqP \text{ …….1.1}$$

Q_{ww} = wastewater flow m3/day

q = Water consumption, l/person day

P = Population connected

k = Return factor (0.8–0.9)

1.1.4 Pollution due to domestic wastewater

In developing countries 60% of population is connected to wastewater collection (sewerage) systems. In this system wastewater is removed by direct runoff or percolation into the nearby watercourses and aquifers, often causing water pollution and only less than 1% is being treated before its reuse or disposal into surface water bodies (WWAP, 2017; Corcoran, 2010; Zhang *et al*., 2015)

1.1.5 Water scarcity in Pakistan

Limited availability of water is not only problem but its deterioration due to wastewater intrusion also limit its uses. Discharge of untreated wastewater into nearby surface water-bodies cause contamination and damage to environment and human health (Wu *et al.,* 2018).

1.1.6 Wastewater treatment technologies

For the protection of environment and restoration of water various wastewater treatment technologies are used that consist of combined physical, mechanical, chemical and biological processes for solids removal including organic matter, nutrients, soluble contaminants like metals, organics, pathogens, etc. Multiple methods are involved in various wastewater treatment technologies to improve removal of contaminants and various recovery processes as shown in Fig 2.

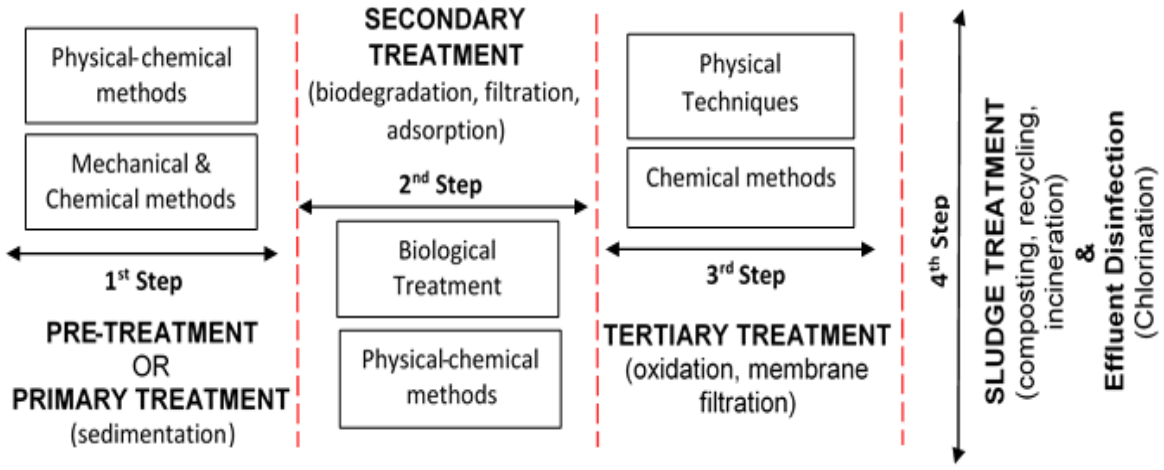

Figure 1-2 Wastewater treatment teachnologies

Method selection to treat wastewater thus depends upon area, weather conditions and wastewater characteristics (composition, flow, loading rate, etc.) Although biological processes are encouraged due to their diversity in removal of contaminants and pathogens.

Table 1-2 Advantages and Disadvantages of Treatment systenms

TREATMENT METHODS	TYPES	ADVANTAGE	DISADVANTAGE
Physical methods	Sedimentation, screening, grit removal	Remove all unpleasant and disturbing material before secondary treatment	------
Mechanical methods	Adsorption, Filtration		
Chemical methods	Coagulation/flocculation, Ion exchange, Chemical precipitation	Simple technologically, efficient in high pollutant loads removal	Chemical consumption, cost, sludge production, handling and disposal
Biological methods	Wetlands, & water stabilization ponds	simple, economically beneficial, acknowledged by public, high removal of biodegradable organics, and emergent contaminants	Slow process, large land area required
Bio-mechanical methods	Bioreactors	High removal efficiency	High operational and maintenance cost Require specialized labor for proper function of system

(Crini and Lichtfouse, 2019)

1.3 Present study

The study was focused to evaluate the treatment efficiency (with respect to nutrients and pathogen removal) of integrated constructed wetland established at National University of Sciences and Technology (NUST) in 2014 for institutional wastewater treatment. Predominant microbial species were isolated from surface and sediments of various ponds to identify predominant microbiota involved in degradation.

1.4 Aims and objectives

I. Performance evaluation of constructed wetland through physiochemical parameters and its removal efficiency
II. Isolation and identification of dominant microbial communities within constructed wetland
III. Phylogenetic analysis of microbes through 16s RNA gene sequencing
IV. Identification and quantification of helminths eggs by using HEAD (Helminths Egg Automatic Detector)

Literature review

2.1 Constructed wetlands

Constructed wetland is most convenient, cost-efficient, ecologically delicate, or reliable technology for treatment of wastewater coming from human dwellings that always been a primary target to treat domestic and municipal wastewater. Numerous processes including physical, chemical, and biological, are involved in wetland treatment system. These processes are universal in nature and can function within other treatment systems. First, primary or pretreatment, to remove potential solids through sedimentation, screening and grit removal from raw or inadequately treated wastewaters that might cause nuisance within wetland. Secondary treatment involves the removal of organic matter through microbial degradation that can also be enhanced by mutualistic relation of microbes and plants. (Wallace, 2009)

2.2 History of wetland

The first effort proposed for wastewater treatment through Constructed wetlands was implemented by Käthe Seidel after that from 1960s to 1970s continuous experiments were carried out and further used for wastewater treatment. At the start of experimental phase, constructed wetlands were largely used for treating municipal wastewater. But now its applications have been extended to the treatment of industrial and agricultural effluents, landfill leachate, mine drainage, polluted lakes and rivers,11 urban runoff and it is implemented in various weather conditions around the worlds such as tropical, arid and semiarid regions, hot and humid climate (Zhang et al., 2010)

Constructed wetland is an attractive substitute for wastewater treatment in developing countries, where thousands of Constructed wetlands had been functional as wastewater treatment technologies (He et al., 2015)

2.3 Types of treatment wetlands

constructed wetlands are artificial systems that are designed to accelerate specific features of wetland systems. Treatment wetlands are constructed on large area with a variation of layout design, flow patterns, plantation, substrate and hydraulic retention time. Different types of wetlands (natural or constructed) are shown in Figure.

Free Water Surface Wetlands (FWS):

Area with free-flowing water like natural marshes or lagoons.

Horizontal subsurface flow constructed wetlands (HSSF-CW):

Typically constructed with a substrate of gravel or sand also planted with vegetation and water flows horizontally from the inlet to the outlet.

Vertical subsurface flow constructed wetlands (VSSF-CW):

Water move across the surface of soil, sand or gravel bed planted with wetland vegetation.

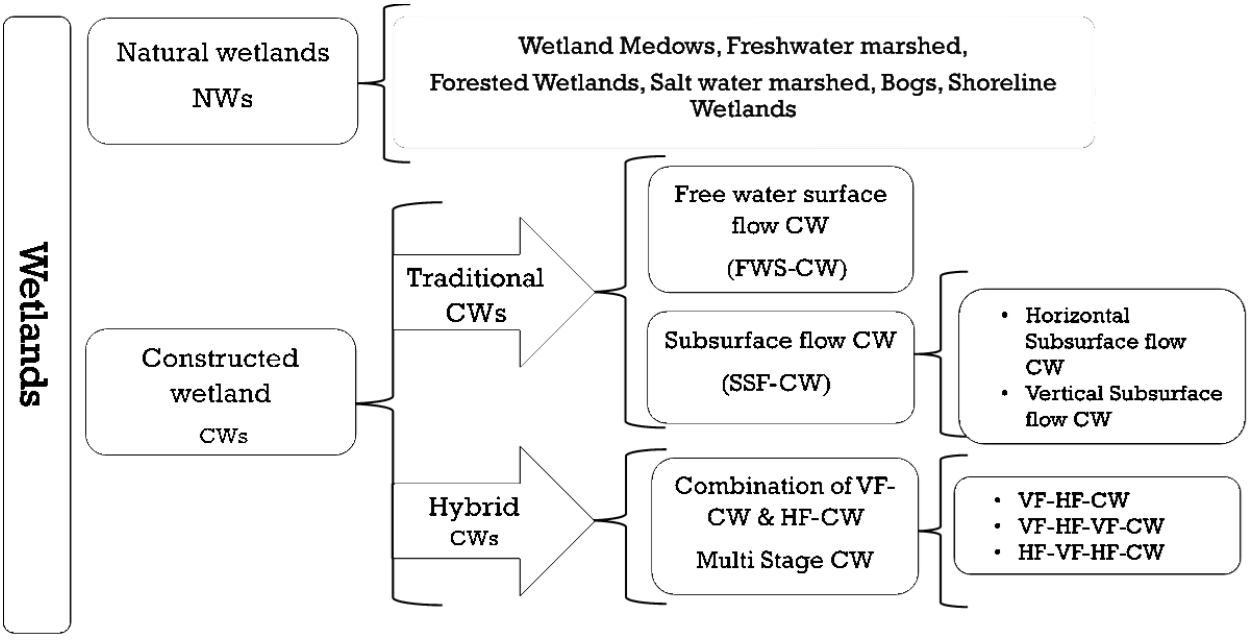

Figure 0-1: Types of treatment wetlands

Operation and design

The criteria for constructed wetland operation and design include vegetation selection, site/ area selection, type of wastewater, selection of substrate, width, length and depth of wetlands, hydraulic retention time (HRT), hydraulic loading rate (HLR), operation and maintenance procedures (Haris *et al*., 2018 ; Kadlec., 2012) are crucial to create a feasible CW system and attain the sustainable performance.

2.4 Plant selection criteria

Macrophytes containing unique properties to treat wastewater could play a deliberate role in CWs, and are considered to be the vital component of the design and operation of Constructed wetland treatments. However, only a few plant species have been largely used in this treatment system (Andleeb and Hashmi, 2018; Vymazal, 2013a; Wu et al., 2014). For the selection of macrophytes factors which are mainly considered include: Tolerance of hyper-eutrophic and waterlogged-anoxic conditions.

One of the previous studies shows that multi-stage constructed wetland for the treatment of secondary effluents from urban land shows 63% COD removal efficiency in autumn which decrease to 30% in winters. Maximum COD removal of 68% was observed in summer due to increased plant growth (Wu *et al*., 2018).

Another study shows that constructed wetlands associated with disinfection systems for the treatment of urban wastewater reported that HSSF-CW shows 80% removal of TSS and 60% for BOD and COD with 2.3 days HRT. Lack of significant correlation existed between pollutants removal efficiency and temperature in Mediterranean region (Russo *et al*., 2019).

Comparative lab-scale study for rural wastewater treatment through single stage vertical flow constructed wetland (VFCW) and a hybrid system reported 80% COD removal from both hybrid and single stage CW that contradicts with other existed studies While TKN removal was 70% in hybrid-CW and 57% in single stage CW (Kraiem *et al*., 2019).

Helminth eggs treatment by centralized and decentralized treatment plants reported 91% removal efficiency of helminths eggs by centralized biological Treatment plant at 12h HRT. 41% and 48% removal was recorded by decentralized aerobic and anaerobic treatment system (Amoah *et al*., 2018).

activated sludge and natural lagoons for helminth egg treatment in Morocco Activated sludge treatment show 100% Helminth egg removal efficiency while natural lagoon treatment shows 94% removal (Dennis *et al*., 2017).

Methodology

3.1 Study site

Study was conducted on Biological wastewater treatment plant (constructed wetland) located at NUST, treat Domestic wastewater from schools, institutes, hostels and residential areas. Constructed wetland project was funded by United Nation Educational and Cultural Organization (UNESCO) and inaugurated by Minster of Science and Technology on 13th November 2014. At present its maintenance is under NUST research and development fund. Total population of NUST is around 6000 and it covers an area of 707 acres. The total volume of wastewater generated by NUST is about 200,000 US gallons per day and the flow into the treatment facility is maintained at 75000 US gallons per day at the inlet of CWs. Layout of treatment is represented in Table 3. Over the wetland operating season from October to March, 30-year climate normal vary from 7 °C in January to 35.5 °C in October with an average of 21.2 °C.

The layout of wetland system consists of sedimentation tank, 8 ponds cultivated with different species of plants, FILTER technology that polish treated water from 8th pond and eventually stored in collection pond. Detailed characteristics are discussed in table 3. CWs installed at NUST may treat around 0.1 Million gallons of water per day. About 18850 US gallons of wastewater is first pretreated in the sedimentation tank daily after that it is loaded in eight ponds and further filtered through FILTER technology 10850 US gallons are stored in collection tank. The salient features of the project are as below:

Table 0-1: Structure specifications of integrated constructed wetland

Location:	Northern Corner of NUST H-12 Campus, Islamabad
Latitude and Longitude	33.6417767 and 73.0035925
Treatment Capacity:	75,000 Gallons/Day
Size of Constructed Wetland:	120 ft. x 100 ft. (8-ponds, each of 22 ft. x 50 ft.)
Emergent/floating/ submergent vegetation:	Cattail, Water Hyacinth, Duckweed, etc.
Size of FILTER:	120 ft. x 170 ft
Total Area of CW-FILTER	33000ft^2 (0.76 Acre)
Cost of UNESCO Sponsored Project:	USD $ 65,000
Operation & Maintenance (O&M) Cost	Rs. 36,000/- p.m. (Salaries of two Malis)

Table 0-2: Specifications of integrated constructed wetland

Ponds	**Seasonal characteristics**		**Description**	**Dimensions** (L, W, D)	**Total capacity (US-G)**	**HRT (Hours)**
Sedimentation tank	Primary settling of sludge and sediments		Sludge recovered to be used as fertilizer (in compost for digestion further use in fertilizer)	35'×12'×6'	18850	3-4
Pond 1	Planted with *Typha latifolia* during whole year		Large persistent grasses native to tropical and temperate areas (Vymazal, 2011). Approx 15 plants per m2 are cultivated	50'×22'×7'	41142	6.87
Pond 2	*Planted with Pistia stratiotes*	Empty in winter	Light greenish-yellow shell like plant, long unbranched roots and is frost sensitive (Pott & Pott, 2002). Approx 10 plants per m2 are cultivated	50'×22'×7'	57600	10.30
Pond 3	*Planted with Centella asiatica*		Considered effective for pollutant removal in summer however the removal potential can drop to even 50% in winters (Li. *et al.*, 2018). Approx 20 plants per m2 are cultivated	50'×22'×7'	57600	9.16
Pond 4	*Planted with Centella asiatica*		Approx 20 plants per m2 are cultivated	50'×22'×7'	57600	11.44
Pond 5	*Planted with Pistia stratiotes*	Empty in winter	Only aquatic and sediment microbial community and natural settling are the removal mechanisms present	50'×22'×7'	57600	14.48
Pond 6	*Planted with Pistia stratiotes*	Empty in winter	Only aquatic and sediment microbial community and natural settling are the removal mechanisms present	50'×22×7'	57600	10.07
Pond 7	*Planted with Pistia stratiotes*	Empty in winter	Approx. 10 plants per m2 are cultivated	50'×22'×7'	57600	9.16
Pond 8	Aeration/ Stablization pond		Aerators were installed to boost up oxygen level in the system	50'×22'×7'	57600	5.61
FILTER	Cad Tale is used as filter plants		Approx. 10 plants per m2 are cultivated	120×170×5'	57600	——
Storage tank	Final treated water ready to be used for horticultural purposes					

3.2 Sampling

A total of 12 sampling visits were conducted throughout six months, from October 2018 to March 2019. Properly washed and autoclaved bottles (for 15 minutes at 120ºC and oven dry at 105ºC for 120 minutes) were used to collect sample. 10 samples per visit were collected from outlet of each pond as shown in Figure 3.

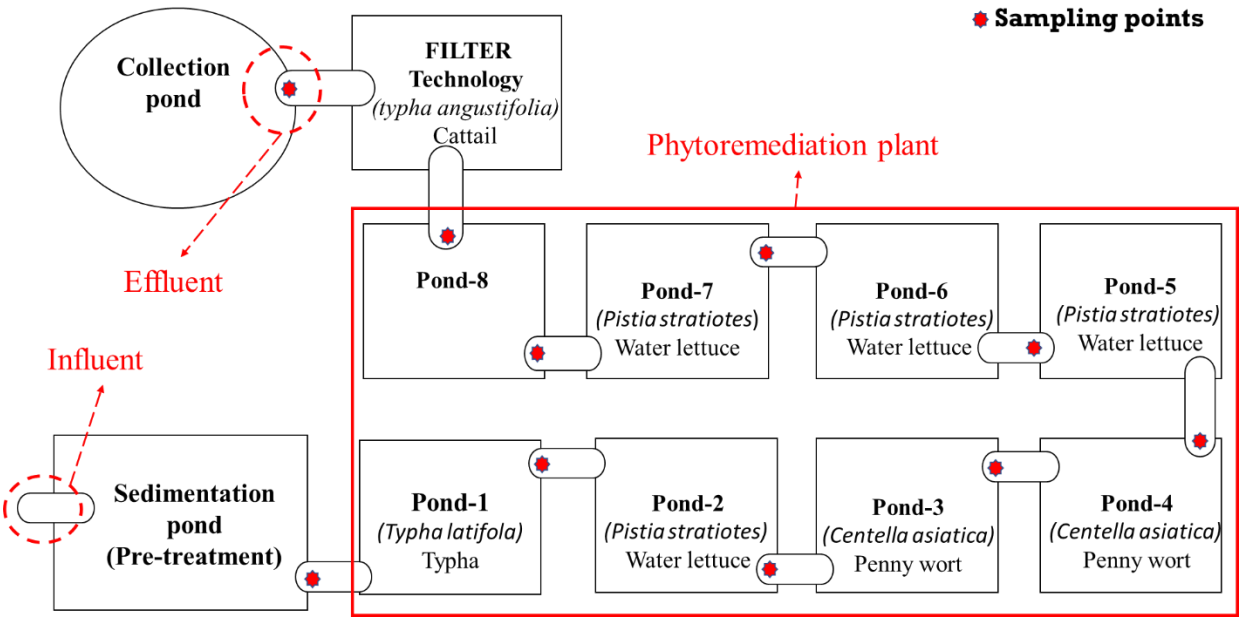

Figure 0-1:Schematic layout of Integrated Constructed Wetland

The collected samples were instantly transported to Environmental microbiology laboratory of IESE (Institute of Environmental Sciences and Engineering) for further physico-chemical and biological analysis. All sampling and analysis procedure were complete under the standard method for examination of water and wastewater (APHA, 2017)

3.3 Analysis of water quality parameters

1.4.1 Physicochemical parameters

Physicochemical parameters of collected water samples were analyzed. In this study selected Parameters include pH, Temperature, Turbidity, Electrical Conductivity (EC), Total Dissolved Solids (TDS), Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Total Kjeldahl Nitrogen (TKN), Nitrate, Nitrite and Phosphate. Characteristics of the parameters along with their instruments and method used for analysis are described in Table 3.2. All analysis was completed according to the APHA standard methods for water and wastewater (APHA, 2017)**.**

1.4.2 Microbiological parameters

Total coliform (TC) through membrane filtration technique is used to detect indicator organisms for determining the coliform removal efficiency according to the standard protocol (APHA, 2017) and the measuring unit was CFU/100mL.

1.4.3 EMB agar plate preparation

Glass/disposable petri plates were used, glass plate was autoclaved as per protocol described in (APHA, 2017). EMB (Eosin Methylene Blue) agar was prepared in w/v concentration 28g/1000mL of distilled water in volumetric flask covered with aluminum foil and autoclaved prior to use. After autoclaving media was cool down to 45˚C in water bath and glass Petri plates were oven dried for 60-120 minutes as required. Molten liquid media at 45˚C was poured in sterilized petri plates in sterile environment of laminar flow hood. Plates were placed within laminar flow hood solidify for 10-15 minutes under UV-light for proper solidification of media and then placed in incubator for 24 hours to confirm sterility.

1.4.4 Membrane filtration

Grab samples were placed near the filtration assembly and were unsealed. Serial dilution of each sample was performed in laminar flow hood and serially diluted sample was allowed to pass through filter paper (0.45 µm size) fitted in filtration assembly. Each filter paper having coliform bacteria retained in it was placed onto EMB agar media plates without any gape produced by air between media and filter that inhibits microbial growth and leads to false results. These plates were incubated (not inverted) for 24 hours at 37°C. After 24 hours' colonies were calculated, using colony counter.

1.4.5 Helminths egg detection and identification

Helminth eggs detection was carried out by using modified Ballinger method given by USEPA. Sample was collected while sample volume depends upon the recovered sediments (Mes, 2003) e.g., 1 litter for untreated or partially treated and 10 litter for treated wastewater sample were placed into beakers for sedimentation up to 3 hours to collect settled eggs. Almost 90 % of the supernatant removed by using vacuum.

Collected sediments were centrifuged after transferring to several 50ml tubes for 20 min at 1000g. supernatant was discarded and sediments collected from all the tubes was subjected to centrifugation in a single tube for 20 min at 1000g. Again, discard supernatant. Add 5 volumes of 30% ZnSO4 solution, use vortex for proper mixing of sediments so that all the eggs float into ZnSO4 solution. Centrifuged at, same described above, supernatant was recovered and further washed by Acetoacetic buffer solution and Ethyl acetate the pellet was suspended in it. Volume of the pellet was recorded. The mixture was vortex again before transferring to Sedgewick Rafter chamber slide. The slide was then viewed under a microscope for the enumeration of helminths eggs at 10X and 40X magnification (Ayres *et al*., 1996 ; Dennis *et al*., 2017). Number of eggs found in one slide can be counted by equation 3.1.

Equation- N = AX/PV……..3.1

Where,

N = number of eggs/L

A = mean of counts from the 3 slides,

X = final product volume (mL),

V = sample volume (L)

P = volume used in Sedgewick Rafter chamber slide (0.5 mL)

Total 320 high quality images of helminth eggs were captured using optical microscope (Carl Zeiss Axio-Lab A1) and HD-color camera (2560 x 1920-pixel) with operating system (IDS-UI-1480LE-C-HQ USB2). Captured images include different stages of egg development (larval and non-larval eggs and morphological variations) Helminths egg identification was carried out using HEAD software (Maya *et al*., 2016).

3.4 Isolation of bacteria

Surface water samples and sediment samples from benthic region were collected from sedimentation tank from each 8 ponds planted with Pistia stratiotes, Centella asiatica, Typha latifolia and collection Tank. Process of sample collection, storage and isolation was performed according to the standard method. Collected samples using sterile sampling bottles were transferred to laboratory. Surface disinfection was performed through 70% ethanol to maintain sterility. Serial dilution of each sample was carried out and appropriately 0.2 ml portion of serially diluted samples were spread onto nutrient agar petri plates by using Spread Plate Technique, and incubated for 24 hours at 37°C. After incubation, different bacterial dominating colonies were picked after observing them on colony counter. Further, these colonies were streaked on nutrient agar plates through streak plate technique for their pure cultures. In this process, the whir loop was first sterilized by holding the loop in the flame of spirit lamp until the loop appeared as red hot. Then the loop was allowed to cool down by holding. Total of 12 strains were obtained and were designated as KN1-KN12.

3.5 Isolated bacterial strains identification

Bacterial strains were identified and further characterized by morphological, biochemical and molecular analysis. The details of which are mentioned below:

1.6.1 Morphological characterization

Examination of the structure and form of bacterial colonies is named as colony morphology and is often used as a first step in bacterial characterization. For identification of unknown isolates, it is important to observe a single colony. After purification of isolates following morphological characters were observed. Bergey's Manual of Determinative Bacteriology (Parte, 2012) was used to analyze the bacterial colonies morphologically. Table 3.3 describes the commonly observed morphological features along with their description (Tortora *et al*., 2004).

Morphological chacteristics	**Description**
Size	small, large, filamentous, punctiform
Color	white, off white, yellow, orange, pink, green
Elevation	convex, umbonate, raised, pulvinated, flat
Margin	curled, entire, lobate, undulate
Surface texture	dry, smooth, wrinkled
Opacity	opaque, transparent, translucent

Figure 0-2: Morphological chactaristics

1.6.2 Biochemical characterization

1.6.2.1 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 the colony is fully purred. In 1884 Danish Physician Hans Christian Gram developed a procedure for Gram staining. Gram positive bacteria have thick layer of peptidoglycan around cell wall and able to retain crystal violet strain which cause purple appearance of cell wall while thin layer of peptidoglycan on cell wall of gram-negative bacteria is unable to retain crystal violet strain and appears pink in microscope after staining. Procedure was followed as described by (Fawole and Oso, 2004)**.**

1.6.2.2 EMB agar test

This test was performed by following the procedure as described by (APHA, 2017)**.**

1.6.2.3 Catalase test for purified microbes

Catalase test on for purified microbes was performed by following the procedure as described by (Cheesbrough, 2006)**.**

1.6.2.4 Oxidase test for purified microbes

Oxidase test on purified microbes was carried out by following the procedure as described by (Cheesbrough, 2006)**.**

1.6.2.5 Molecular characterization

16s RNA sequence analysis was used for molecular identification of bacterial strains isolated from surface and sediments of each pond. The method followed was mainly the culture dependent method.

1.6.2.6 Primer sequences

PCR primers and sequences used in this study are mentioned in Table The sequences were compared with the NCBI (National Center for Biotechnological Information) gene bank database by using BLAST function (http://www.ncbi.nlm.nih.gov).

1.6.2.7 DNA extraction

Genomic bacterial DNA was extracted by using Invitrogen Pure Link Genomic DNA Mini Kit by following manufacturers instruction (Cat no K1820-01, USA).

Table 0-3: Oligoprimes used in PCR

Primers	Sequence F & R	Target genes	Reference
518F	CCAGCAGCCGCGGTAATACG	16S rRNA	Waheed *et al.*, 2013
800R	TACCAGGGTATCTAATCC	16S rRNA	

1.6.2.8 Agarose gel electrophoresis:

Agarose gel electrophoresis was carried out to visualize the extracted DNA. 1% (w/v) agarose gel was prepared by adding 0.6 grams of agarose gel in 60 ml of 1X TBE buffer. Ethidium bromide solution (50µg/ml) was added as a staining agent. Electrophoresis was performed at 100 volts for 30 minutes. After that gel was observed by placing it under UV trans-illuminator.

1.6.2.9 PCR amplification

PCR was performed to amplify the extracted DNA. The reaction mixture was prepared (25 µl) having composition mentioned in Table **5.**

Table 0-4: Recipe of PCR reaction mixture

Reagents	**Volume (µl)**
Taq PCR master mix	25
DNA template	1
Primer F(10µM)	2
Primer R(10µM)	2
Nuclease free water (doubled distilled H_20)	20
Total volume	50

For the 16SrRNA gene detection , the PCR program includes 5 min at 95°C for template denaturation, and 40 cycles for template amplification consisting of three steps: 95°C for 1 min for DNA denaturation into single strand, 61°C for 1 min for primer to anneal to their complementary sequences on either side of the target sequence, 72°C for 1 min for extension of complementary DNA strand from each primer and final elongation at 72°C for 10 min for Taq-DNA polymerase to synthesize any unexpended strand left.

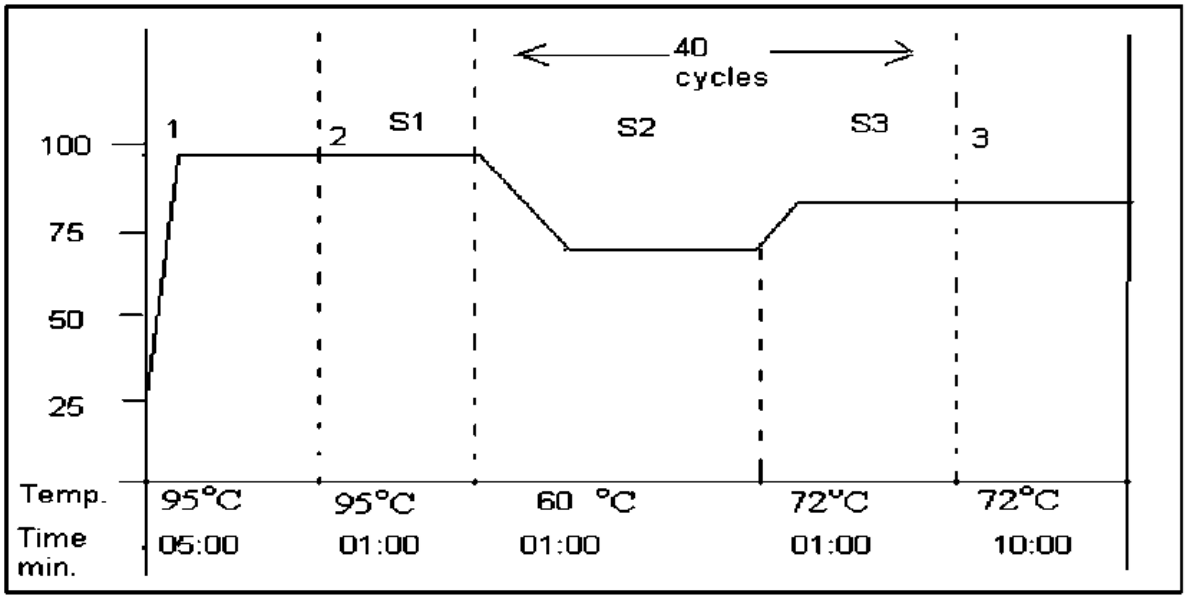

Figure 0-3 : PCR program for 16SrRNA gene amplification

1.6.2.10 16S rRNA sequencing

PCR products were kept in ice box and the preserved isolates were sent to Genome analysis department Macrogen, Seoul, South Korea for 16S rRNA sequencing.

1.6.2.11 Phylogenetic analysis

Phylogenetic analysis through phylogenetic tree show the evolutionary relationships among the various biological entities based on the differences in their genetic characteristics (Tamura et al., 2013). Once the sequences were obtained, they were trimmed through Bio-edit software and junk data was removed. Once the noise was removed and the sequences were properly trimmed, they were analyzed through BLAST tool of National Center of Biotechnological Information (NCBI). After proper detection of the obtained species, accession numbers were obtained from NCBI gene bank library. FASTA sequences were run in MEGA 7 software to obtain the phylogenetic tree which showed linkages between the isolated strains and those at GENEBANK of NCBI.

3.6 Statistical analysis

1.7.1 Descriptive statistics

Mean value for each month with standard error was calculated and standard deviation applied.

1.7.2 Correlation

Significant and non-significant effects among physicochemical and biological parameters were noted with the level of significance at $p<0.05$ and at $p<0.01$using SPSS.

1.7.3 MANOVA (Multivariate Analysis of Variance)

The MANOVA is an extension of Two-Way-ANOVA, it was used to assess significant differences in two or more dependent variables by a Categorical independent variable(s).

Results and Discussion

1. Physicochemical and biological parameters were analyzed to determine organic and pathogen removal pattern of Integrated constructed wetland system.
2. Statistics was applied on physicochemical and biological parameters to analyze the significant and nonsignificant impacts of spatial and temporal variation on performance efficiency of Integrated constructed wetland system.
3. Monthly percentage removal efficiency of both HSSF-CW and FILTER-Technology were examined.
4. Identification of helminths egg found in wastewater with HEAD (Helminths Egg Detector) and specie abundance with respect to temperature and seasons.
5. Microbial isolation and characterization of microbes from surface and sediments of each pond of Integrated constructed wetland.
6. Weather variations including Rainfall (mm) and GHI (W/m²) during sampling period is given in Figure *0-1*.

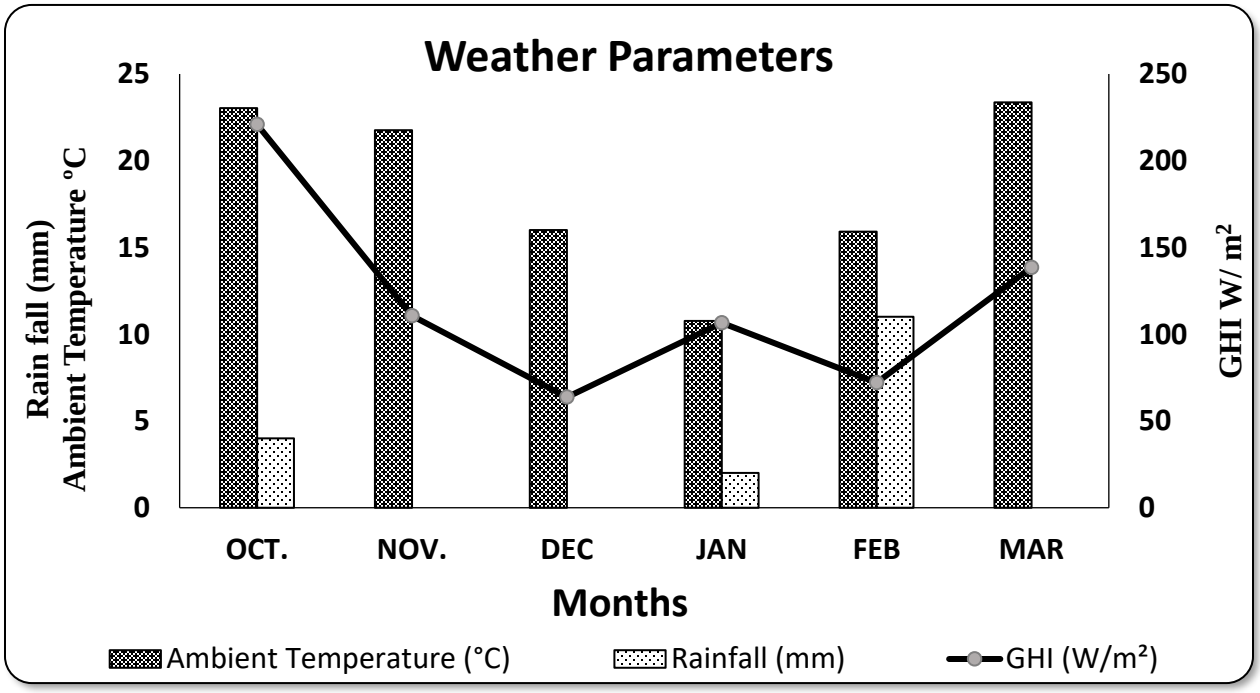

Figure 0-1: Weather variations during sampling period

4.1 Physicochemical and biological parameters

Removal efficiency of organic pollutants was measured from effluent of each pond of HSS-CW and finally treated water from FILTER technology. Organic pollutants removal involves plant uptake, aerobic, anaerobic and rhizosphere digestion (Wu *et al*., 2018). In present study, design of integrated constructed wetland contributes aerobic digestion in upper 2 feet of HFSSF-CW through atmospheric diffusion, convection through wind and plant roots with in rhizosphere., anaerobic digestion from 3-7 feet on benthic surface and plant involved mechanisms such as phytodegradation, phytoextraction and rhizo-filtration. Data showed stable and sustained removal of organics with significant improvement in wastewater quality. Each parameter pH, Temp., EC, TDS, TSS, COD, BOD, Helminth eggs comply with agriculture reuse standards (Table *0-1*: National Environmental Quality Standards & Agriculture Reuse Standards) except for total coliform (TC). Although system showed TC removal from log 9 to 7 (Waller and Bruland, 2016) showed that wetlands are efficient for organic removal and still need tertiary treatment (disinfection) for TC removal.

Parameter	Existing Standards for wastewater discharge	Revised Standards for wastewater discharge			International Agricultural reuse standards
		Inland waters	Sewage	Sea	
Temperature	40°C	≥3°C	≥3°C	≥3°C	----
pH	6 - 10	6 - 9	6 - 9	6 - 9	6.5-8.5
BOD_5	80	80	250	80	<30 mg/L
COD	150	150	400	400	<150 mg/L
TSS	150	200	400	200	<100mg/L
EC	---	---	---	---	>2500 µS /ml unacceptable
TDS	3500	3500	3500	3500	---
TC					<1000/100 CFU/mL
Helminthes	---	---	---	---	<1 egg/ L
Reference	NEQS, 1995	NEQS, 2000			US-EPA, 2006

Table 0-1: National Environmental Quality Standards & Agriculture Reuse Standards

Physicochemical and biological parameters showed spatial and temporal variations within treatment system due to variation in weather conditions (Andleeb and Hashmi, 2018) density and diversity of plants(Vymazal, 2013a), pollutant concentration from source (Maine et al., 2017).

These variations were further configured by Multivariate analysis MANOVA, an extension of two-way ANOVA. Minimum value of Wilks' Lambda showed significant difference existed within groups. Effects Between-Subjects is explained by ANOVA and further variations are described using Post hoc (Tuckey) test. Spatial and Temporal variation across each pond is further described by post hoc (Tuckey) test. As explained by effects Between-Subjects (ANOVA) pH, DO, Turbidity, TSS, BOD, COD, Phosphate, TKN, TC, Helminths eggs showed significant while Temperature, EC, TDS, Nitrate and Nitrite showed non-significant spatial variations. pH, DO, and nitrite showed non-significant variation while Temperature, EC, TDS, Turbidity, TSS, BOD, COD, Nitrate, TKN, Phosphate, TC, and Helminths eggs significantly varied across time.

1.7.4 pH

pH is the measure of Hydrogen ion or simply acidity or alkalinity of water. pH of domestic wastewater water mainly varied from 5.6-11 due to acidic or basic nature of the organic or inorganic pollutants.

pH was maximum in P1 and ST while shows decreasing trend and got minimum values in P3, P6 and P4 and increased in pond 7, 8, CP (effluent) and showed significant spatial variations (p=0.0), Table 3. Reduced pH values may be due to degradation (L. yu Zhang et al., 2010), nitrification (Saeed and Sun, 2012) and other processes involved in reduction of alkalinity.

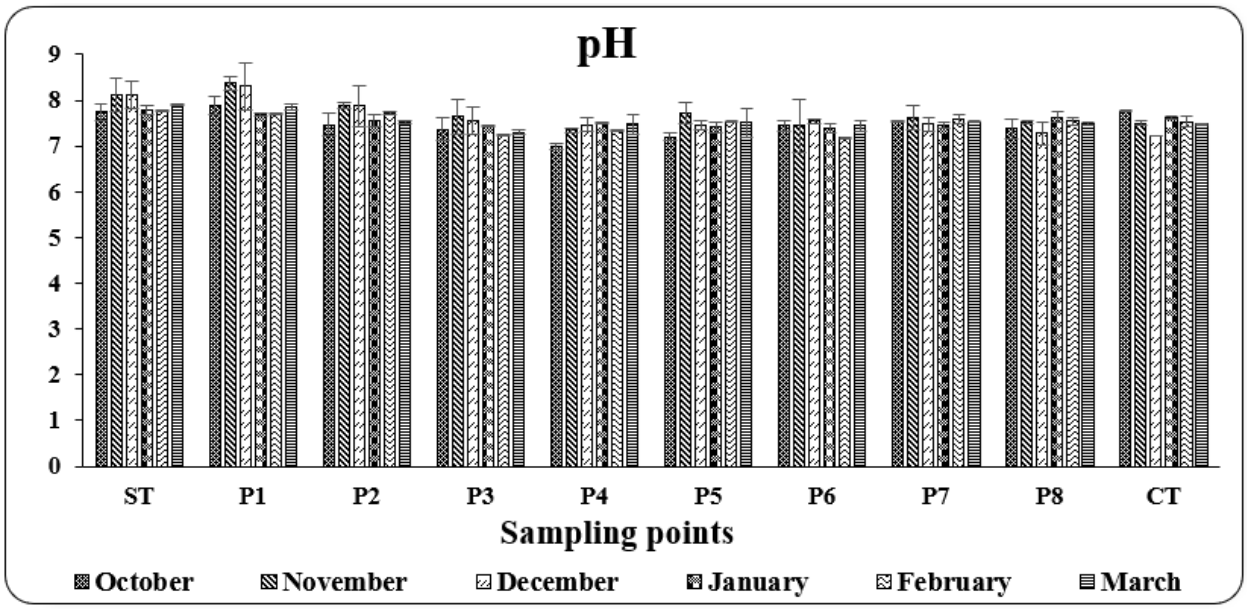

Figure 0-2: Spatial and temporal variation of pH

pH range between 7-7.9 with in the whole treatment system that is ideal for vast diversity of microbes and also hampered microbial degradation processes (Paredes et al., 2007; Vymazal, 2013b). Similar varied pH values in HSS-CW was also experienced by (Saeed et al., 2019). Significant difference in pH values was not detected in monthly variation this may be because of similar effluent concentrations in each month, although influent values varied a little. pH shows weak correlation with BOD (0.467) COD (0.269) TKN (0.364) TC (0.484) and helminths egg (0.431).

1.7.5 Temperature

Moderate temperature is effective for microbial degradation and plant mechanism activity (Feher et al., 2017; Osland et al., 2018; Wu et al., 2014) whereas degradation rate slows down as temperature decreases (Faulwetter et al., 2009; Meng et al., 2014). So, temperature of October, November and March was better for microbial degradation while it decreases in DEC, JAN and FEB shows low degradation of organic matter. Temperature shows strong negative correlation with EC (-0.602) and TDS (-0.588) while positive correlation with Helminths egg (0.429).

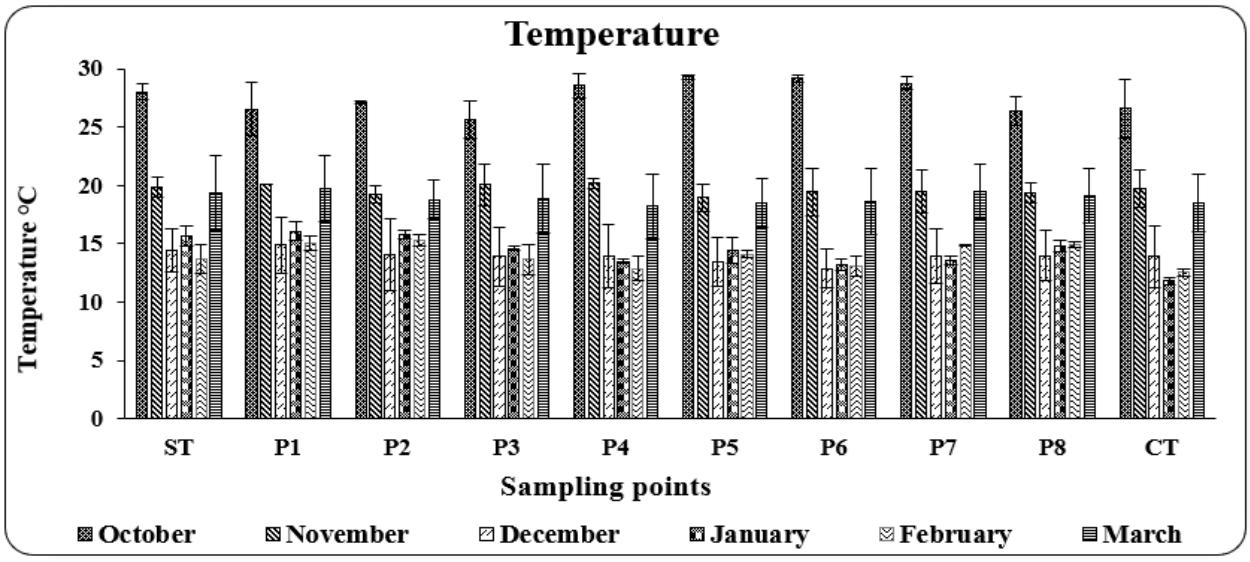

Figure 0-3: Spatial and temporal variation of Temperature

DO

DO increased over the treatment system and max. value was recorded in effluent (CT). Rather than monthly variation DO significantly varied within different ponds.

Plant roots, and diffusion through air increase dissolved oxygen that enhance microbial mediated degradation even in depth(Meng et al., 2014; Ye and Li, 2009). High value of oxygen also shows improved quality water because high turbidity also effects dissolved oxygen in water. High value of DO in DEC was due to Lower temperature that enhance DO in water(Shen et al., 2019). DO is negatively corelated with TC and helminths egg that may be because of low turbidity values. Because TC and helminths egg attached with sediments that further cause decrease in oxygen.

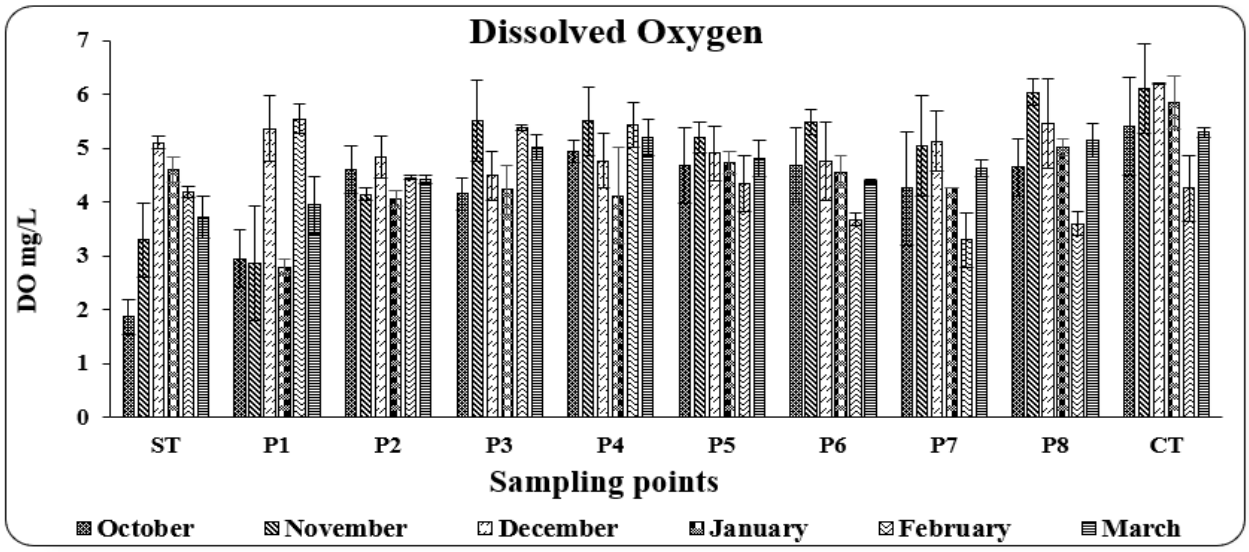

Figure 0-4: Spatial and temporal variation of DO

1.7.6 EC and TDS

EC and TDS values showed non-significant spatial variation while temporal variations were significantly higher in lower temperatures like Dec and Jan while it decreases in Feb due to high(massive) rain fall. Oct was having minimum EC due to low influent values, plant uptake. Removal efficiency of HSSF-CW and FILTER-Technology was 4% and 6% respectively. Non-significant removal efficiency of 10% was observed in overall treatment. High water consumption and frequent pumping at source are main factors that cause dilution effect on EC values (Biagi., et al. 2019).

Similar monthly variation of EC and TDS was observed in wastewater stream by Dietler et al., 2019. Temperature and plant (diversity and density) effect overall evapotranspiration rate that may cause variation in EC values in different ponds (Sandoval., et al. 2019). Increased EC values in planted ponds was reported by (Coleman et al., 2001) which could be due to high evapotranspiration rates**.** EC and TDS are highly corelated with each other (0.989), while negatively corelated with temperature (-0.602, -0.588) and weak positive correlation existed with TSS (0.365, 0.362), BOD (0.471, 0.468), COD (0.424, 0.421), Nitrate (0.453, 0.469), Phosphate (0.459, 0.464) and TKN (0.383, 0.377) respectively.

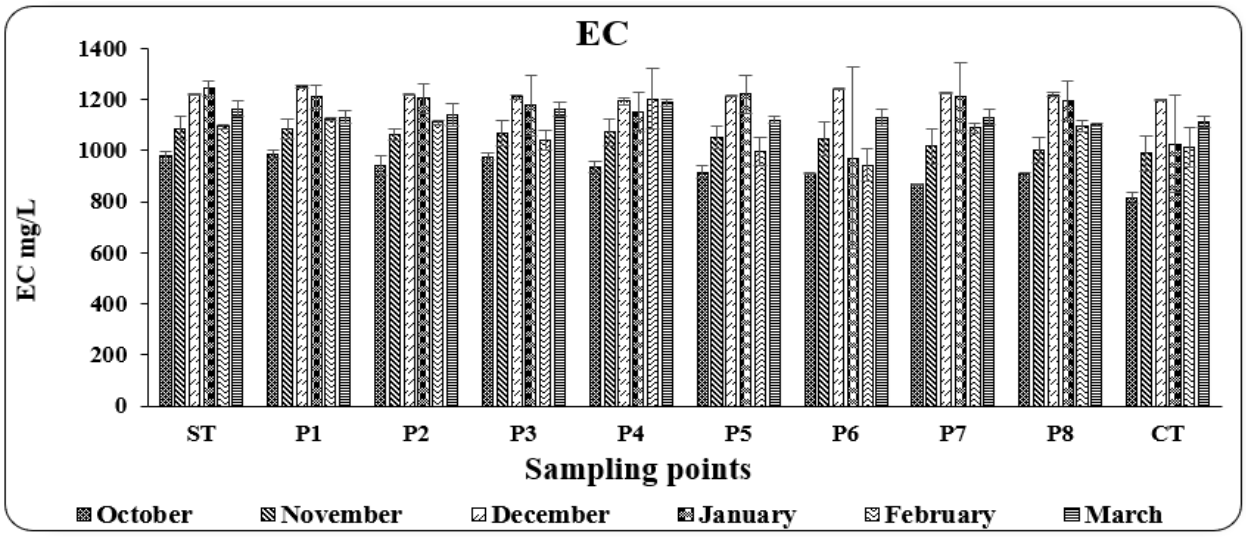

Figure 0-5:Spatial and temporal variation of EC

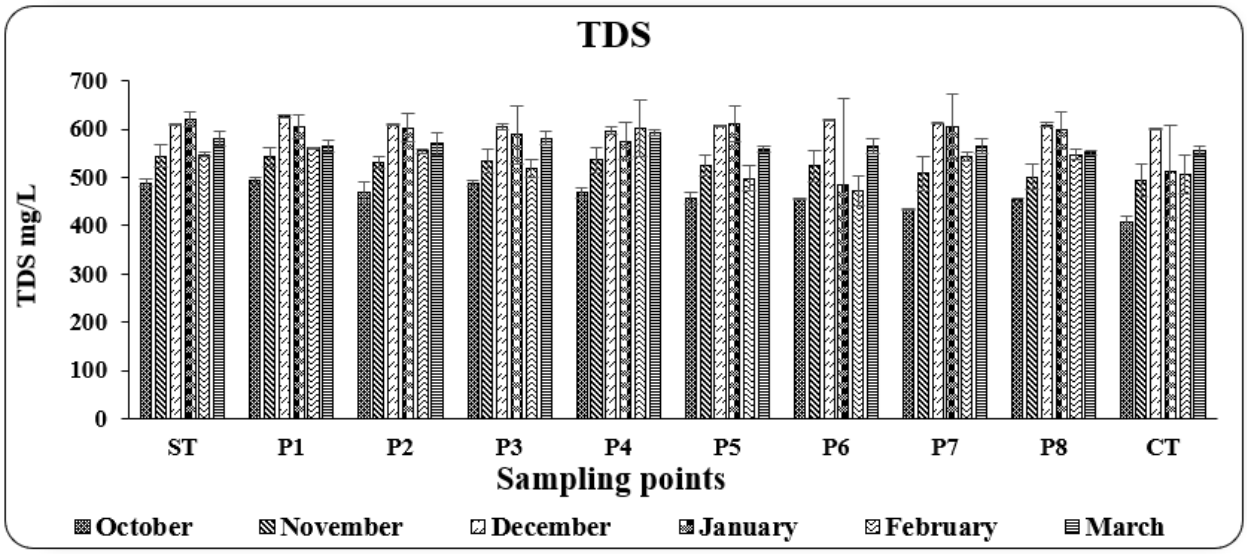

Figure 0-6:Spatial and temporal variation of TDS

1.7.7 Turbidity and TSS

Significant spatial &temporal variation of Turbidity and TSS was existed. Turbidity and TSS values were consistently increase within initial ponds (P4>P5>P1>P3>P2), (P1>P2>P4>P3>P5) respectively. It is mainly due to resuspension of small particles attached on roots and other plant debris. while minimum values were detected in pond 8 and collection pond. Turbidity and TSS removal efficiency of HSSF-CW was 47% and 39% respectively. While FILTER-Technology shows 34% removal efficiency for Turbidity and 69% for TSS.

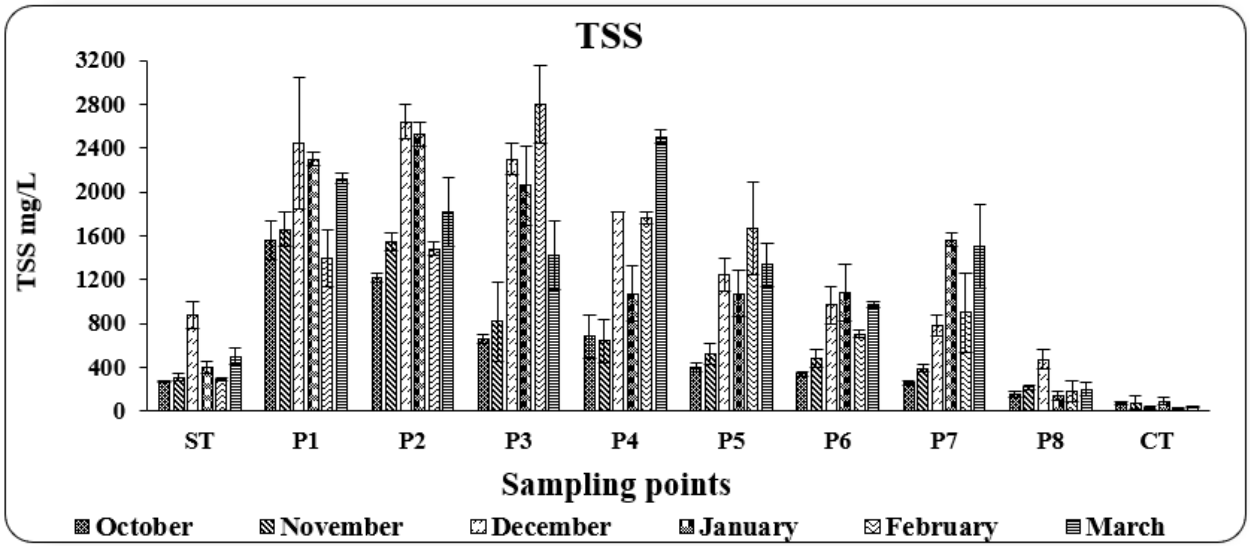

Figure 0-7:Spatial and temporal variation of TSS

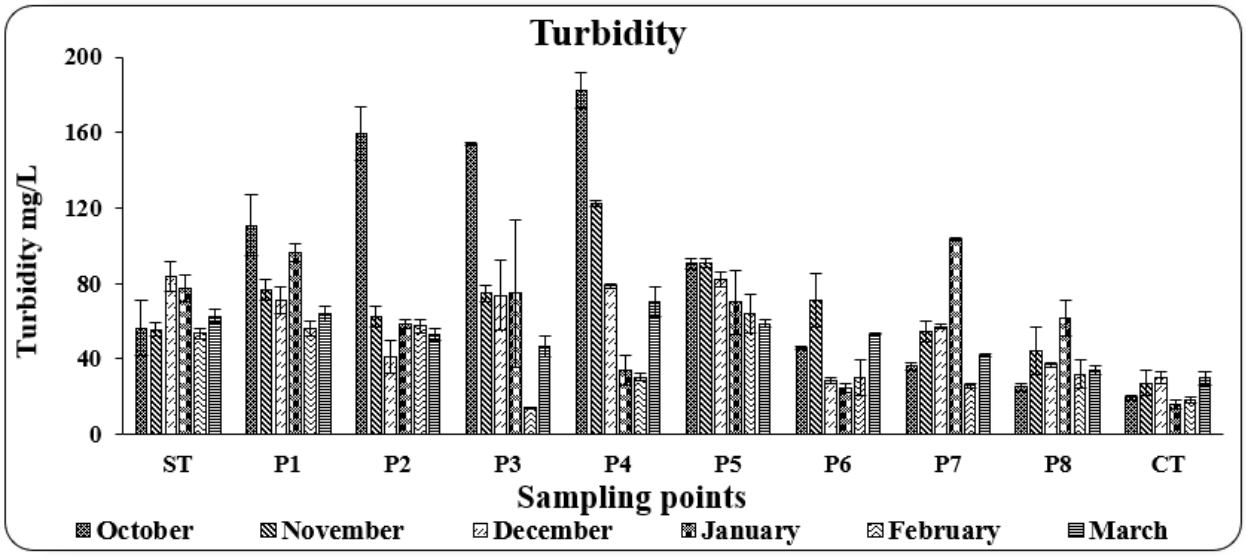

Figure 0-8:Spatial and temporal variation of Turbidity

Overall removal efficiency of Turbidity and TSS is 63% and 84% respectively. Turbidity removal efficiency of both treatment system is almost similar. While FILTER-Technology shows higher removal rate than HSSF-CW. TSS and Turbidity are mostly considered as strongly related parameters and linear relationship among these parameters is explained by previous research(Hannouche et al., 2011). In actual this concept is not applicable to every environmental condition. In present study TSS and Turbidity values varied even at same point at different time, that may be due to higher concentration of large suspended particles or dissolved organic matter (Harvey and Mannino, 2001). Turbidity could make good estimation of TSS concentration in water sample, however TSS cannot directly measured from turbidity. Recent recaches showed variations in TSS and Turbidity values which is mainly due to sensitivity of sensor technology, particle size, surface texture, shape, colour, density and scattering efficiency. In natural environment suspended solids, their size and density are continuously changing with other environmental factors. For example, compounds like dyes increase turbidity while not consider in estimation of TSS (Chapalain et al., 2019; Druine et al., 2018). Significant monthly variations of Turbidity (p-0.0) and TSS (p-0.0) were also existed. High TSS in January and December was due to decaying organic matter (from water lettuce in pond 2, 5, 6, and 7), and minimum TSS values were detected in October. Maximum Turbidity values were detected in October which is totally opposite to TSS, these values increased may be due to high dissolved organic content as explained earlier.

TSS and Turbidity removal was mainly due to sedimentation and Filtration by plant roots which is totally independent process and temperature has no effect on its removal efficiency (Russo et al., 2019 ; Toscano., et al. 2015 ; Wallace, 2009) . Turbidity is weakly corelated with COD, TKN, TC and Helminths eggs. However, TSS is weakly corelated with BOD and total coliform.

1.7.8 COD

Sustained and stable removal of COD was confirmed from the final effluent of integrated Constructed Wetland. However, average concentrations of COD in each Pond change significantly with different removal efficiency in each month. Furthermore, significant variations were observed in ST, P1, P2 and P3 with time that effect overall treatment efficiency of each pond of HSSF-CW.

These variations were due to wide range in influent values and microbial degradation, decaying of plant debris, suspended and dissolved organic matter. While all other ponds show continuous decrease in COD values except pond 7 in JAN and FEB.

Higher COD values were recorded in December while in October and February, minimum COD values were recorded. Average COD concentration gradually decreased along the treatment system, from 185.28 mg/L at the inlet to 40.82 mg/L in the outlet, representing removal efficiency of 78%. While removal efficiency of HSSF-CW and FILTER-Technology is 57% and 45% respectively.

Various study showed that multi stage constructed wetlands are more efficient than single-stage constructed wetlands in COD removal (Owuor and Corresponding, 2017; Sgroi et al., 2018). While contradictory statements exist like Dong & Sun (2007) at field scale and Kraiem and his coworkers (2019) at lab scale showed equal results for COD removal from single-stage and multi-stage constructed wetlands. COD is strongly corelated with BOD, phosphate, TKN, and TC while weakly correlation exist with EC, TDS and turbidity.

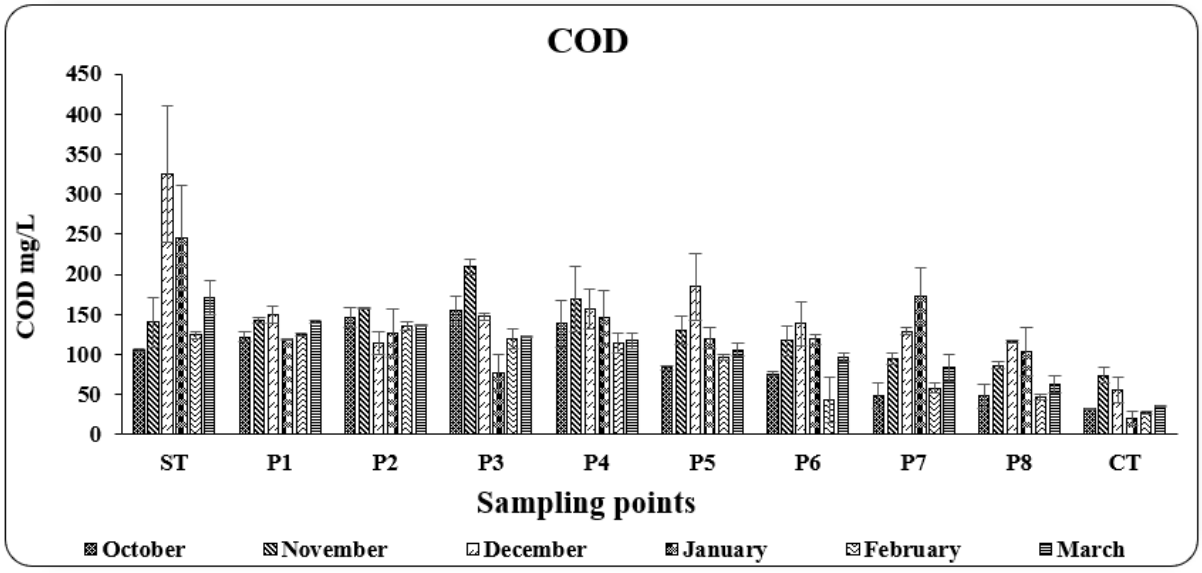

Figure 0-9:Spatial and temporal variation of COD

1.7.9 BOD

BOD removal was consistent in each pond and minimum values were recorded in Collection pond. While in December and January higher BOD values shows increased number of microbes in influent or because of higher number of coliforms in wastewater. Lower degradation rate, minimization of filtration and sedimentation due to absence of plants.

High DO values in winter as in December could be the reason of enhanced microbial growth even at lower temperature. While October shows maximum removal efficiency. And lower February values was due to high(massive) rainfall.

Total removal efficiency of treatment system was 66% while HSSF-CW shows 56% and FILTER-Technology shows 24% removal efficiency. BOD shows significant correlation with TC (r=0.742), TKN (r=0.668), COD (r=0.681), pH (r=0.467), EC (r=0.471), TDS (r=0.468), TSS (r=0.422) at ($p < 0.01$)

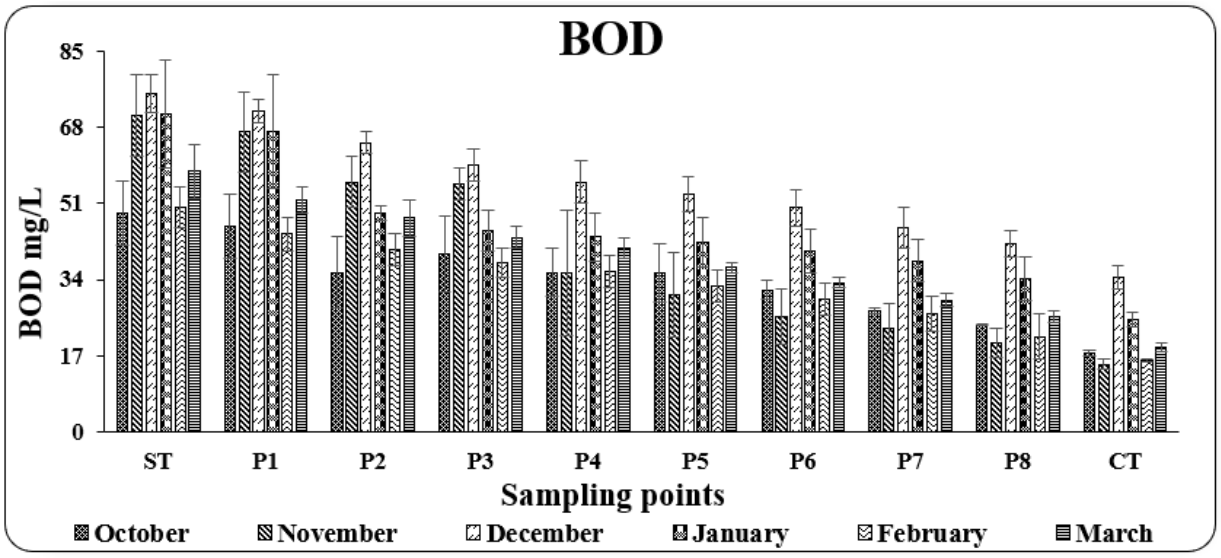

Figure 0-10: Spatial and temporal variation of BOD

1.7.10 TKN

TKN, nitrate and nitrate play major role in removal of nitrogen from constructed wetland. TKN is a combination of organic nitrogen and ammonia-nitrogen that varied across different ponds and minimum values was recorded in final effluent (CT). Influent values varied with time because of variation in wastewater composition. High organic nitrogen in wastewater influent cause increase in TKN values in first few ponds may be because of its conversion into ammonium ions (He., et al, 2018). While consistent decrease in values of pond P3, P4, P5, P6, P7, P8 respectively and lowest value was detected in final effluent of collection pond. Removal efficiency of TKN varied in HSSF-CW and FILTER-Technology due to different substrate, existence of aerobic and anaerobic conditions. TKN removal efficiency of HSSF-CW and FILTER-Technology was 44% and 23% while removal efficiency of treatment system was 56%.

Varied values of **Nitrate and Nitrite** was mainly due to microbial oxidation of NH4-N through nitrification (Saeed and Sun, 2012). Nitrate removal efficiency in HSSF-CW and FILTER-Technology was 10% and 73% respectively while whole treatment efficiency was 37%.

Nitrate production in HSSF-CW was enhanced by facultative microbes present in HSSF-CW. (Vymazal, 2007) report nitrate formation from ammonia through nitrification in HSSF-CW. Nitrite removal efficiency was 39% in HSSF-CW because of its continuous conversion into NO3-N and 23% in FILTER-Technology while overall removal efficiency reported was 47%.

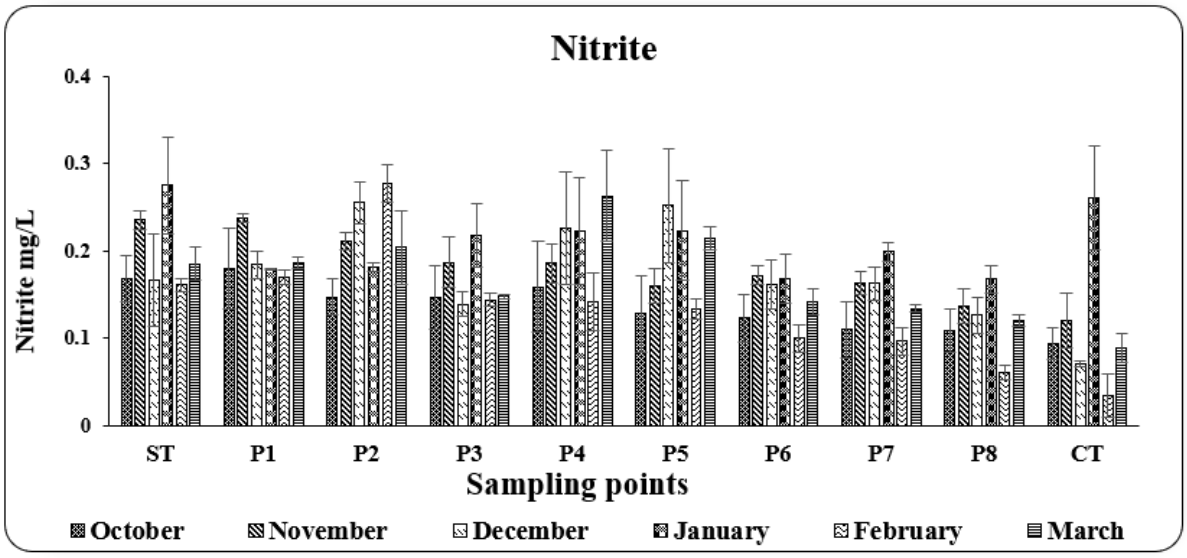

Figure 0-11:Spatial and temporal variation of Nitrite

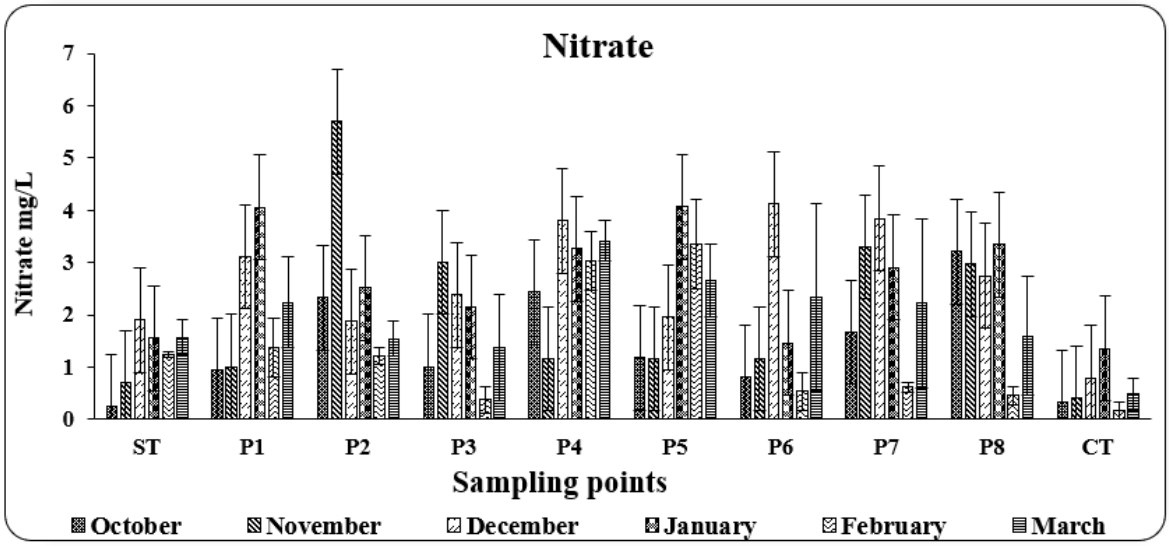

Figure 0-12:Spatial and temporal variation of Nitrate

Denitrification is carried out by facultative microbes and can be a significant for nitrogen loss pathway in wetlands, especially if the dominant species of N in the effluent is the oxidized NO3(Day et al., 2004; Vymazal, 2007). This may be the reason that nitrate removal efficiency was high in FILTER-Technology. Several studies also showed the effect of plant on removal efficiency of nitrogenous compounds. So, changes could be because of plant species or absence or presence of plants.

Nitrate is weakly corelated with EC (0.453) and TDS (0.469) while TKN corelates with BOD (0.668), COD (0.582) and TC (0.545) and weak correlation also exists with Turbidity (0.402).

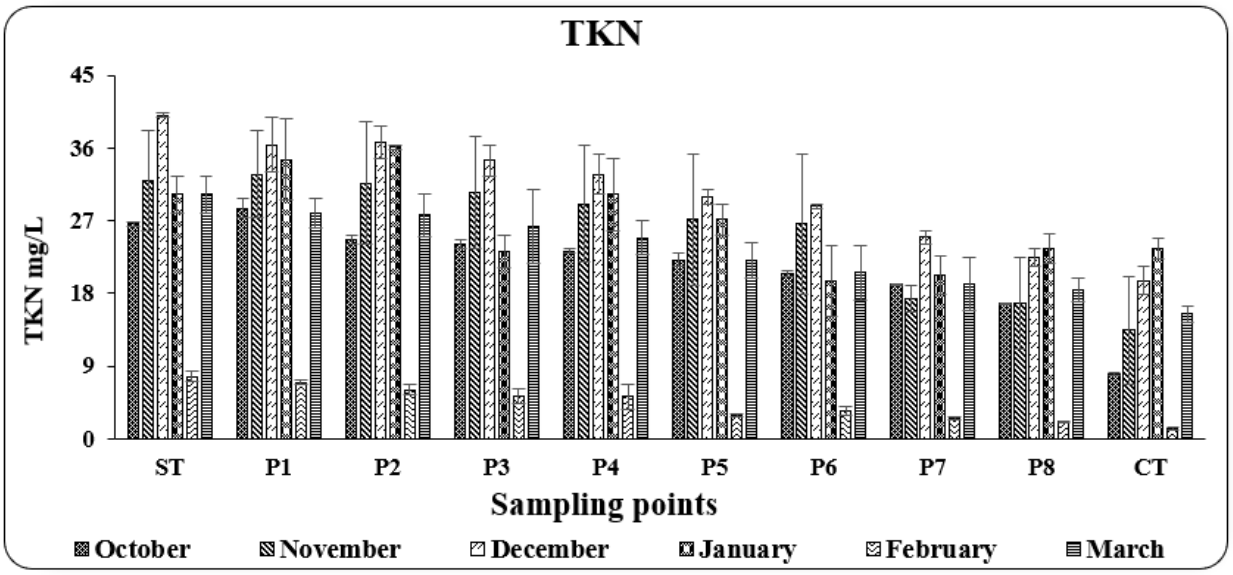

Figure 0-13: Spatial and temporal variation of TKN

1.7.11 Phosphate

Phosphate removal efficiency mainly due to adsorption and very minute amount remove from plant uptake and microbial degradation. Phosphate removal shows consistent decrease in each sampling point and minimum value recorded at collection tank. December shows high influent values while treatment efficiency was consistent and there was not significant difference was detected in effluent values in each month. Minimum influent values were detected in October and February(rainfall). Total phosphate removal efficiency of treatment system was 57% while HSSF-CW shows 27% and FILTER-Technology shows 37% removal efficiency. Phosphate shows significant correlation with TC (r=0.490), BOD (r=0.506), COD (r=0.589), EC (r=0.459), TDS (r=0.464), at ($p < 0.01$)

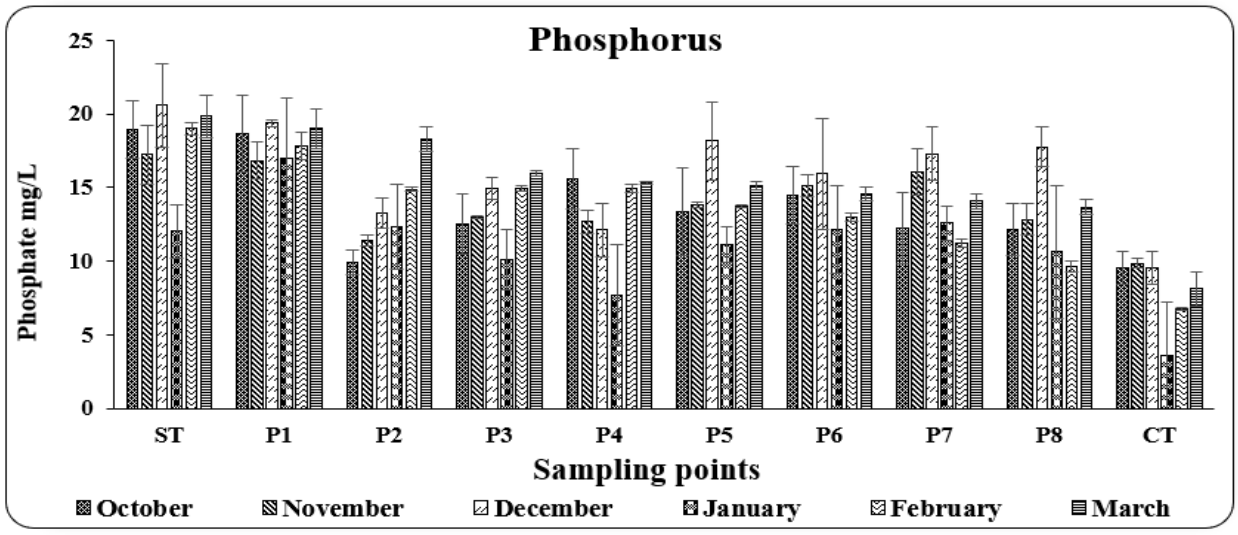

Figure 0-14:Spatial and temporal variation of Phosphate

1.7.12 Total coliform

Coliforms are dangerous microorganisms and predict contamination with human excreta. Due to diseases caused by coliforms it is necessary to remove them from wastewater. Wetlands are efficient in removing microbes from wastewater. TC removal shows consistent decrease from Sedimentation tank to collection tank.

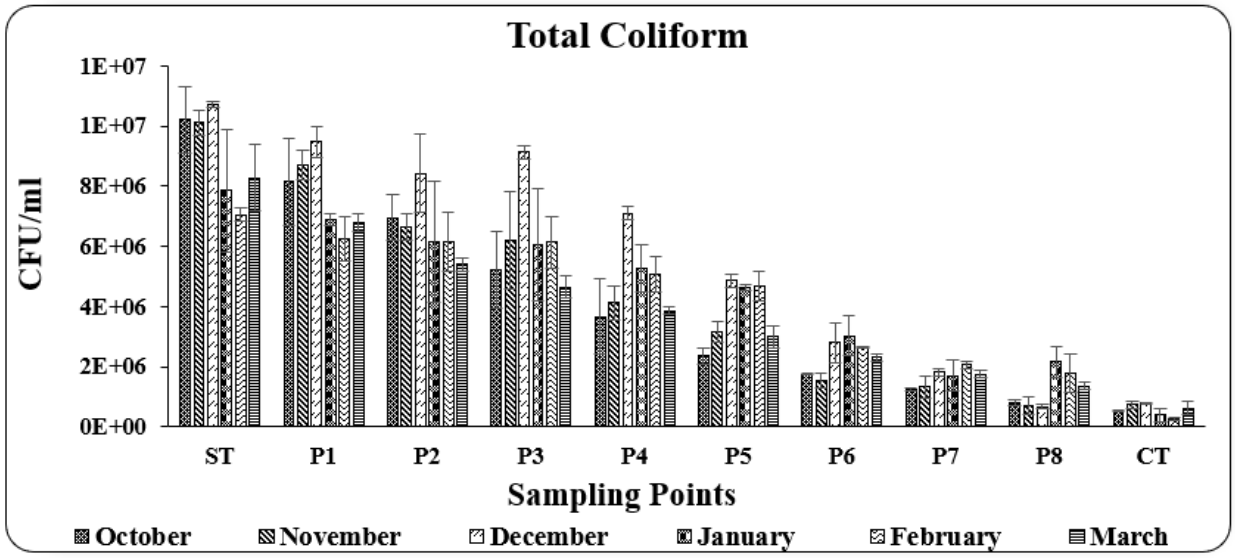

Figure 0-15:Spatial and temporal variation of TC

Number of TC was higher in Dec, Jan & Nov while Feb, Oct & Mar got minimum values this is mainly due to variation in influent values. Higher removal efficiency of TC by integrated

constructed wetland is 94% while HSSF-CW shows 85% removal and FILTER-Technology shows 42%. TC still need disinfection (Tertiary treatment) because effluent from integrated constructed wetland did not comply with agriculture reuse standards. TC shows significant correlation with BOD (r=0.742), COD (r=0.591), Phosphate (r=0.490), TKN (r=0.545), Helminths eggs (r=0.598), pH (r=0.484) at ($p < 0.01$).

1.7.13 Helminths eggs

Helminths egg showed constant decreasing trend in each pond. Mainly Helminths egg removal was due to sedimentation and stabilization on bed of ponds. Higher number of helminths eggs was recorded in Nov, Oct, Dec and Mar while minimum eggs were found in Feb and Jan. Integrated constructed wetland is efficient technology in removing helminths eggs. Higher values of helminths egg showed presence of helminths related diseases within society that could be only due to consumption of contaminated of half cooked food. Drop in influent values may be affected by their survival rate at lower temperature or may be their reproductive cycle stops at lower temperature.

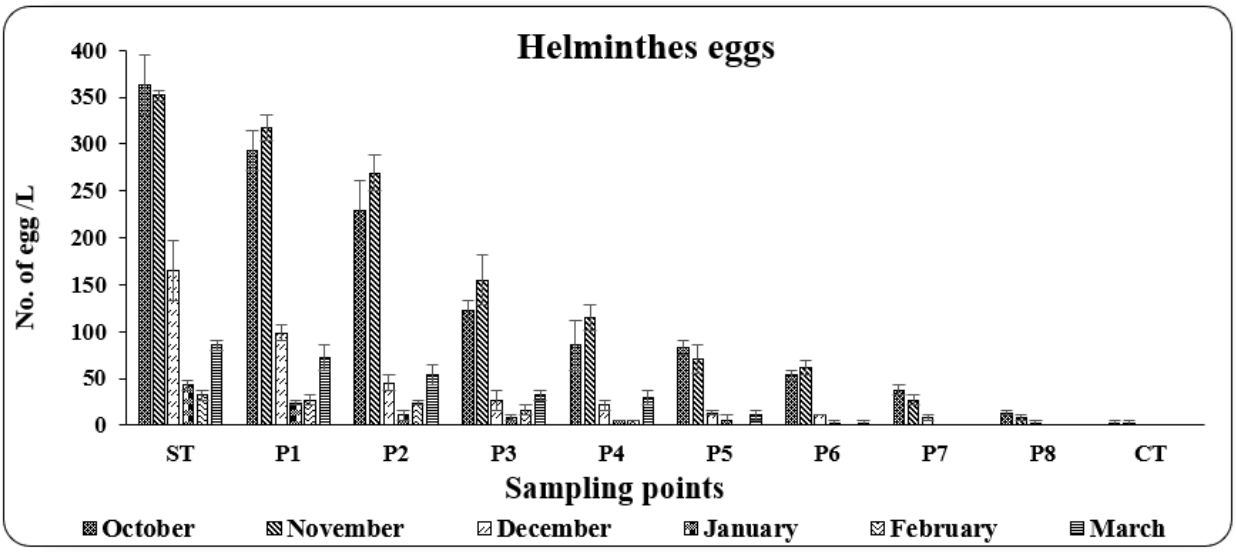

Figure 0-16:Spatial and temporal variation of Helminths eggs

Helminths egg removal efficiency of HSSF-CW and FILTER-Technology was 98% and 41% while removal efficiency of treatment system was 99% helninthes (egg laying season, temperature, specie variation). Helminths egg shows significant correlation with pH (r=0.431), Temperature (r=0.429), TKN (r=0.442), TC (r=0.548) at ($p < 0.01$)

4.2 Helminths egg identification

Quantification of helminths eggs in water is very necessary for identification of infection level in environment. As presence of helminths parasites is mainly linked with inadequate sanitation, health facilities and also poverty. (Mahvi *et al*., 2006). Microscopic observation of effluent water indicated a variety of helminths parasites in inlet water samples and treated water. Recommended limit of Agriculture reuse standards for helminth eggs is <1 egg/Liter. These eggs are mainly common in domestic wastewater (Grego *et al*., 2018). Most predominant helminths species of Phylum Nematode, Trematode and Cestode were identified.

1.8.1 Nematode

Various species of Nematodes were identified according to their size and appearance. Identified species includes *Physaloptera* sp., Trichostrrongylus sp., Physocephalus sp., Ascaris lumbricoide, Trichuris trichiura and Capillaria sp. various nematode species were identified at lower temperature that confirms its resistance to toward external conditions which allow it to remain viable for longer (Chaoua *et al.*, 2018).

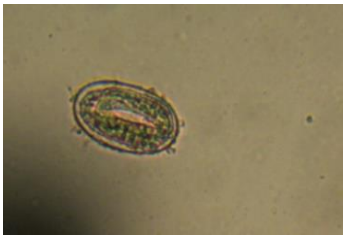

Figure 0-20:Capillaria sp.

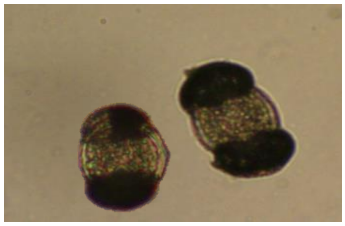

Figure 0-22:Trichuris trichiura

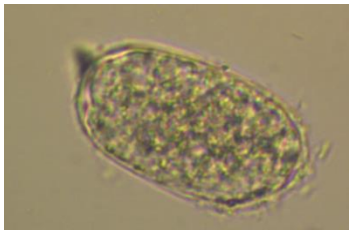

Figure 0-21: Physaloptera sp.

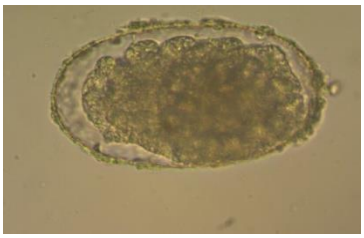

Figure 0-19: Trichostrongylus sp

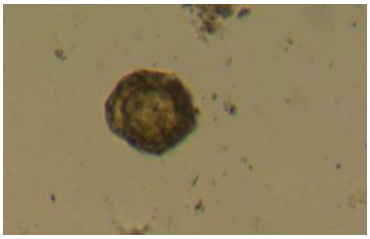

Figure 0-17: Ascaris lumbricoide

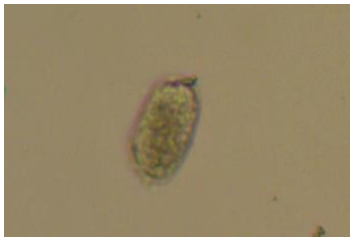

Figure 0-18:Physocephalus sp.

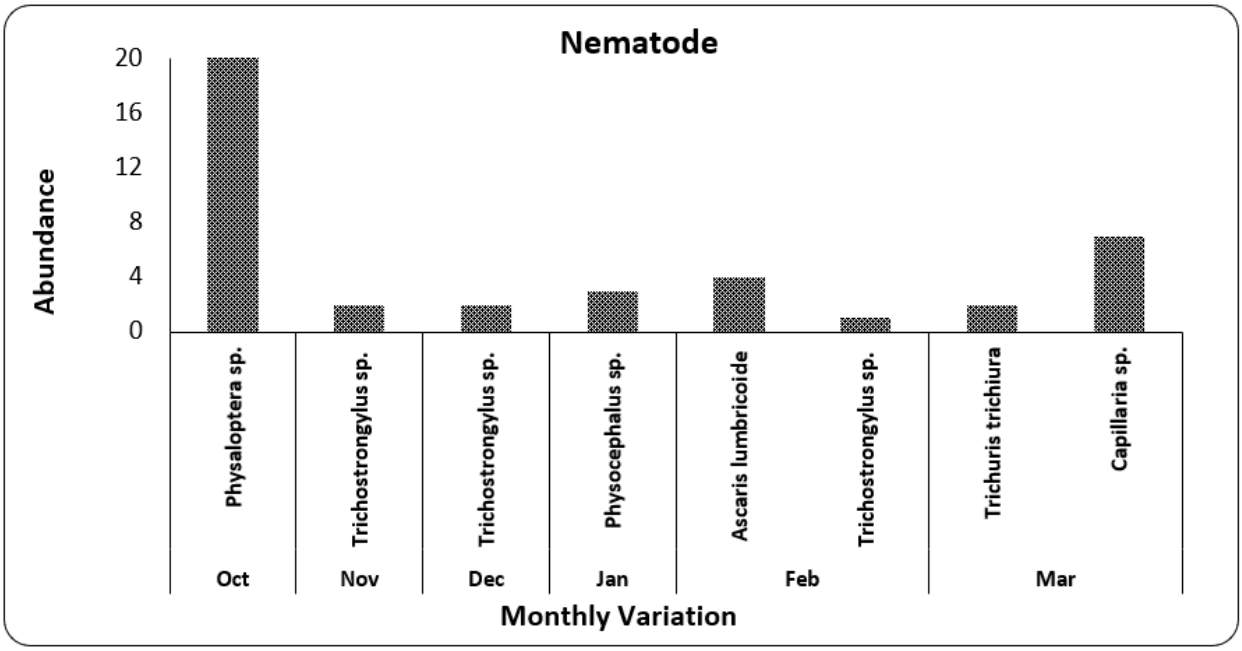

Figure 0-23:Nematode abundance during each month

1.8.2 Trematode

Various species of Trematode were identified according to their size and appearance. Identified species include *Paragonimus*, *Clonorchis sinensis* and *Nanophyetus salmincola*.

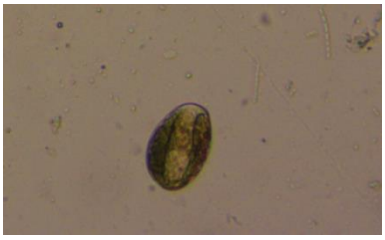

Figure 0-25: Nanophyetus salmincola

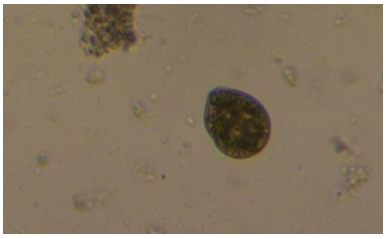

Figure 0-26:Clonorchis sinenis

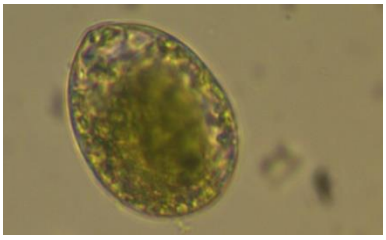

Figure 0-24:Paragonimus westermani

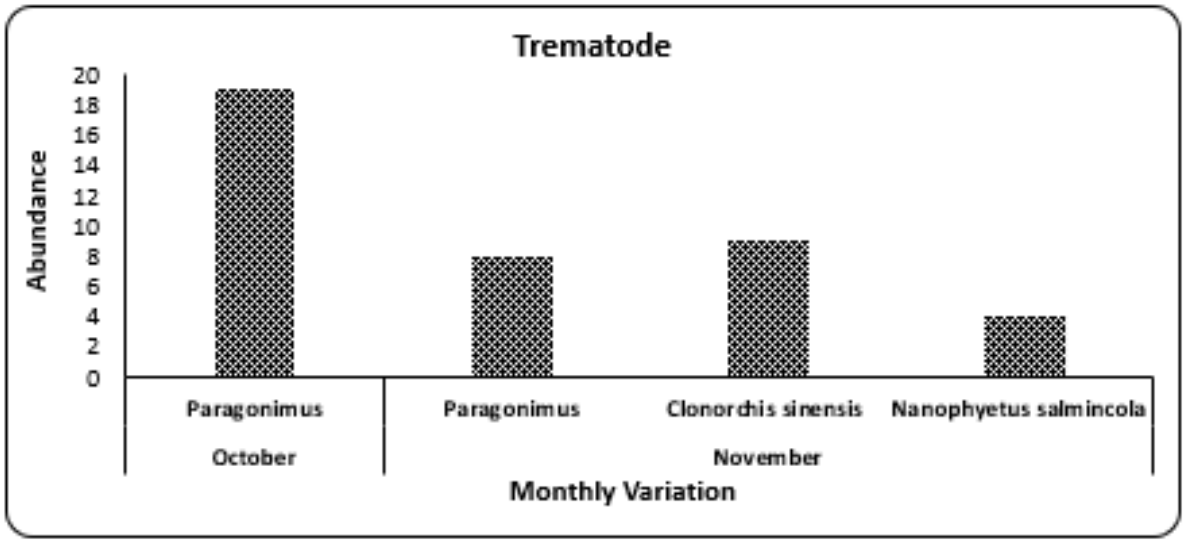

Figure 0-27:Trematode specie abundance during each month

1.8.3 Cestode

Only one species of Cestodes was identified in the month of March that show low prevalence and contamination of Cestodes in environment.

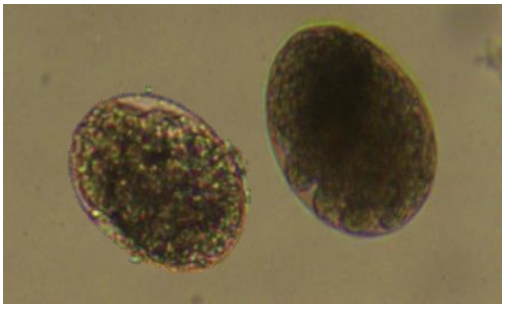

Figure 0-28:Oesophagostomum sp.

4.3 Microbial characterization from surface and sediments of integrated constructed wetland

Isolated strains KN 1 to KN 20 belong to the surface and sediments of 8-Ponds The detail of identification of bacterial species is mentioned below.

1.9.1 Morphological characterization of isolates

1.9.1.1 Colony morphology

Colony morphology of isolated strains (KN1-KN20) is given in Table-5. Colony morphology was studied in terms of form, color, elevation, margin, surface texture and opacity. Maximum percentage of strains had circular shape, white color, raised elevation, smooth texture and were opaque. Colony morphology is used to illustrate bacterial properties. Bacteria that form smooth colonies were capable of making more biofilms polysaccharides (Enos-Berlage & McCarter, 2000).

Table 0-2:Colony morphology of bacterial strains isolated from surface and sediments of integrated constructed wetland system

Colony Morphology							
Strain	**Source**	**Form**	**Color**	**Elevation**	**Margin**	**Surface texture**	**Opacity**
KN1	ST	Circular	Crystal clear	Convex	Entire	Smooth	Opaque
KN2	P6	Irregular	Yellow	Raised	Filiform	Rough	Opaque
KN3	ST	Circular	yellow	Raised	Entire	Smooth	Opaque
KN4	P2	Circular	Light yellow	Raised	Entire	Smooth	Opaque
KN5	P2	Circular	White	Convex	Entire	Smooth	Opaque
KN6	P3	Circular	Yellow	Raised	Entire	Smooth	Opaque
KN7	P4	Circular	Light yellow	Crateriform	Endulated	Smooth	Opaque
KN8	P5	Irregular	Off-white	Raised	Filiform	Rough	Opaque
KN9	P7	Circular	Light yellow	Convex	Entire	Smooth	Opaque
KN10	P8	Circular	White	Raised	Entire	Smooth	Opaque
KN11	CT	Circular	Circular	Light yellow	Raised	Entire	Opaque
KN12	CT	Circular	White	Raised	Entire	Smooth	Crystal
KN13	ST	Circular	Yellow	Raised	Entire	Smooth	Crystal
KN14	P1	Circular	Off-white	Convex	Entire	Smooth	Opaque
KN15	P2	Circular	White	Convex	Entire	Smooth	Opaque
KN16	P3	Circular	White	Raised	Entire	Smooth	Opaque
KN17	P6	Circular	Crystal clear	Raised	Entire	Smooth	Opaque
KN18	P6	Circular	White	Raised	Entire	Smooth	Opaque
KN19	P5	Circular	White	Raised	Entire	Smooth	Opaque
KN20	P4	Irregular	White	Raised	Lobate	Rough	Opaque

1.9.1.2 Cell morphology and Biochemical characterization of isolates

Cell morphology and biochemical characterization of isolated bacterial strains in terms of gram reaction, shape, Oxidase test and Catalase test is mentioned in detail in Table-6. Most of the isolated strains were identified as gram Positive and maximum percentage of bacteria had Cocci shape. Results were compared with previous identified species and only 11 anonymous species were further analyzed.

Table 0-3:Cell morphology and biochemical characterization of bacterial strains isolated from surface and sediments of integrated constructed wetland system

Biochemical characterization					
Strain	**Gram Reaction**	**Shape**	**Oxidase**	**Catalase**	**Similarity with Identified Strains**
KN1	Positive	Bacillus	Negative	Negative	---
KN2	Positive	Cocci	Positive	Negative	---
KN3	Negative	Cocci	Positive	Positive	---
KN4	Negative	Cocci	Positive	Negative	---
KN5	Positive	Cocci	Negative	Negative	---
KN6	Positive	Cocci	Positive	Negative	---
KN7	Positive	Cocci	Negative	Negative	---
KN8	Positive	Rode	Positive	Negative	---
KN9	Positive	Bacillus	Negative	Negative	---
KN10	Positive	Cocci	Positive	Positive	---
KN11	Positive	Cocci	Positive	Negative	---
KN12	Positive	Cocci	Positive	Negative	---
KN13	Negative	Bacillus	Positive	Negative	KB14
KN14	Negative	Cocci	Positive	Negative	KB6
KN15	Positive	Cocci	Negative	Negative	KN5
KN16	Negative	Bacillus	Positive	Negative	KN10
KN17	Positive	Rode	Negative	Negative	KN1
KN18	Positive	Cocci	Positive	Positive	KN10
KN19	Positive	Rode	Negative	Negative	KN5
KN20	Positive	Cocci	Positive	Positive	NB14

1.9.2 Molecular characterization

Strains characterized by the side of genus and specie level by using PCR amplification method and 16S-rRNA sequencing process.

1.9.2.1 DNA extraction and PCR amplification

DNA of the isolated and purified strains was taken out by using DNA Extraction Kit by NORGEN-BIOTEK-CORP. 1% agarose gel was used to examine the amplified DNA segments for genus identification.

785 F-primers and 907 R-primers were used for amplification process. Stained with loading dye and was observed under UV transilluminator. Figure-7 is the gel picture of amplified genes of isolated strains.

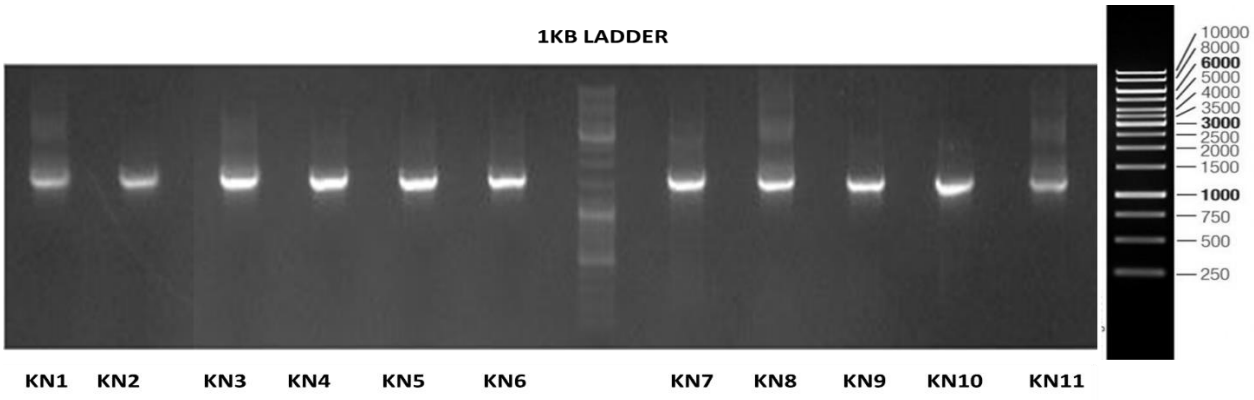

Table 0-4: PCR amplification with refference to 1KB ladder

1.9.2.2 16S rRNA sequencing

PCR products were sent to genome analysis department, Macrogen. Sequences that were obtained were trimmed through Bio edit software and were identified through BLAST tool of NCBI. After getting the accession number (Table 8) phylogenetic tree (Figure 7) was constructed which demonstrate the relatedness and linkages of different bacterial strains identified.

Strain ID	Source	Organism	Accession Number
KN1	ST	*Pseudomonas alcaliphila*	MN192139
KN7	P4	*Pseudomonas mendocina*	MN192140
KN4	P2	*Bacillus paranthracis*	MN192141
KN8	P5	*Bacillus haynesii*	MN192142
KN10	P8	*Bacillus stratosphericus*	MN192143
KN11	CT	*Bacillus zhangzhouensis*	MN192144
KN6	P3	*Glutamicibacter* sp.	MN192145
KN5	P2	*Acinetobacter vivianii*	MN192146
KN2	P6	*Staphylococcus gallinarum*	MN192147
KN9	P7	Bacillus sp.	MN207310

Table 0-5: Source and scientific name of identified species along with the accession number

Figure 0-29: Phylogenetic tree demonstrating relatedness and linkage to different bacterial strains

Categorization of microbial communities within CW for domestic wastewater degradation described that these systems are reliant on microbial compositions for optimum wastewater treatment. Dominant bacterial species isolated from Phytoremediation system belong to the Phylum Proteobacteria and Firmicutes (Ibekwe *et al.,* 2003; Baptista *et al.,* 2003; Nicomrat *et al.,* 2006)

This is perfectly in line with the study conducted by Calheiros and his coworkers in 2009 have worked on the identification of bacterial communities from wetlands and the results revealed γ-Proteobacteria being the most dominant phyla responsible for removal of phenols and organic compounds from wastewater. Previous studies have reported that aerobic autotrophic ammonia oxidizing bacteria, denitrifying bacteria and methanogens belong to the phyla proteobacteria and have an impressive role in pollutant removal from wetlands (Gorra *et al.,* 2007; Tietz *et al.,* 2007). Calheiros and his colleagues in 2009 have worked on the bacterial community dynamics of HSFCW and have identified *Firmicutes*, *Actinobacteria*, *α*, *β,* and *γ Proteobacteria* being dominant ones.

5. Conclusions and Recommendations

5.1 Conclusions

Integrated constructed wetland systems are efficient and reliable for elimination of pollutants from domestic wastewater and reusing it for agriculture.

Conclusions of overall research includes:

1. Overall Physicochemical parameters removal efficiency was up to 74% for TSS, 70.2% COD, 95% Turbidity, 79% BOD, 68% Nitrite, 71% Nitrate, 69% TKN, 47% Phosphate, 83% Total Coliform and 100% for Helminth eggs with hydraulic Retention time of 3 days.
2. Predominant phyla of Integrated Constructed Wetland system were proteobacteria (Pseudomonas sp.) and Firmicutes (Bacillus sp.)
3. The overall treatment performance of horizontal subsurface flow constructed wetland was higher than FILTER-Technology but HSSF-CW was unable to treat TSS, Turbidity and Nitrate which further treated by Filter technology.
4. Wastewater management through constructed wetland treatment facilities is a cost effective and environment friendly solution.
5. Helminths eggs were higher in the month of October, November and march shows decreased prevalence with decrease in temperature. Nematodes prevalence was higher than trematodes and cestodes

5.2 Recommendations

1. It is recommended to detect the effect of treated wastewater after irrigation on soil structure and its microbiota.
2. Usage of Alternative plants to increase the performance efficiency of Integrated Constructed Wetland
3. Further research is recommended for the better removal of the TC so that it can comply with agriculture reuse standards
4. Plant biomass must treat or properly dump to decrease spread of contaminants, uptake by plants in environment.

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IV-Interaction	MANOVA	Effects Between-Subjects (ANOVA)	Post hoc (Tuckey)
Sampling Points	F (135,373) =7.605, P<0.05, nP^2=0.7, SIG	pH: F (9,60)=7.76, p=0.0, np2=0.54, SIG Temp: F (9,60)=0.45, p=0.9, np2=0.06, NOT-SIG DO: F (9,60)=2.42, p=0.02, np2=0.27, SIG EC: F (9,60)=1.7, p=0.1, np2=0.23, NOT-SIG TDS: F (9,60)=1.7, p=0.1, np2=0.23, NOT-SIG Turbidity: F (9,60)=19.6, p=0.0, np2=0.7, SIG TSS: F (9,60)=56.4, p=0.0, np2=0.9, SIG BOD: F(9,60)=26.6, p=0.0, np2=0.8, SIG COD: F(9,60)=20.6, p=0.0, np2=0.8, SIG Nitrate: F(9,60)=1.6, p=0.1, np2=0.2, NOT-SIG Nitrite: F(9,60)=1.6, p=0.1, np2=0.2, NOT-SIG Phosphate: F(9,60)=12.6, p=0.0, np2=0.6, SIG TKN: F(9,60)=22.4, p=0.0, np2=0.7, SIG TC: F(9,60)=94.5, p=0.0, np2=0.9, SIG Helminth eggs: F(9,60)=181.5, p=0.0, np2=0.9, SIG	pH: P1>ST>P2>P7>CT>P8>P5>P3>P6>P4 Temp: CT>P6>P3>P4>P8>P5>P7>P2>ST>P1 DO: CT>P6>P8>P5>P4>P2>P7>P3>P1>ST EC: ST>P1>P4>P2>P3>P7>P5>P8>P6>CT TDS: ST>P1>P4>P2>P3>P7>P5>P8>P6>CT Turbidity: P4>P5>P1>P3>P2>ST>P7>P6>P8>CT TSS: P1>P2>P4>P3>P5>P7>P6>ST>P8>CT BOD: ST>P1>P2>P3>P4>P5>P6>P7>P8>CT COD: ST>P3>P4>P2>P1>P5>P7>P6>P8>CT Nitrate: P2>P7>P8>P4>P1>P5>P3>P6>ST>CT Nitrite: P4>P2>P3>P8>ST>P1>P5>P6>P7>CT Phosphate: ST>P1>P5>P6>P7>P2>P3>P4>P8>CT TKN: P2>ST>P1>P3>P4>P5>P6>P7>P8>CT TC: ST>P1>P2>P3>P4>P5>P6>P7>P8>CT Helminth eggs: ST>P1>P2>P3>P4>P5>P6>P7>P8>CT
Sampling Frequency	F (75,224) =20.19, P<0.05, nP^2=0.9, SIG	pH: F(5,60)=2.1, p=0.06, np2=0.1, NOT-SIG Temp: F(5,60)=101.4, p=0.0, np2=0.8, SIG DO: F(5,60)=2.1, p=0.07, np2=0.1, NOT-SIG EC: F(5,60)=29.7, p=0.0, np2=0.7, SIG TDS: F(5,60)=28.6, p=0.0, np2=0.7, SIG Turbidity: F(5,60)=20.8, p=0.0, np2=0.6, SIG TSS: F (5,60)=35.3, p=0.0, np2=0.7, SIG BOD: F (5,60)=21.3, p=0.0, np2=0.6, SIG COD: F (5,60)=12.7, p=0.0, np2=0.5, SIG Nitrate: F(5,60)=4.8, p=0.01, np2=0.2, SIG Nitrite: F(5,60)=0.85, p=0.5, np2=0.06, NOT-SIG Phosphate: F(5,60)=2.4, p=0.04, np2=0.1, SIG TKN: F(5,60)=101.0, p=0.0, np2=0.8, SIG TC: F(5,60)=6.8, p=0.0, np2=0.3, SIG Helminth eggs: F(5,60)=285.3, p=0.0, np2=0.9, SIG	pH: Nov>Dec>Mar>Jan>Feb>Oct Temp: Oct>Nov>Mar>Jan>Feb>Dec DO: Dec>Nov>Jan>Oct>Mar>Feb EC: Dec>Jan>Mar>Feb>Nov>Oct TDS: Dec>Jan>Mar>Feb>Nov>Oct Turbidity: Oct>Jan>Nov>Dec>Mar>Feb TSS:Jan>Dec>Mar>Feb>Nov>Oct BOD: Dec>Jan>Nov>Mar>Feb>Oct COD: Dec>Nov>Jan>Mar>Oct>Feb Nitrate:Jan>Dec>Mar>Nov>Feb>Oct Nitrite: Jan>Dec>Feb>Oct>Nov>Mar Phosphate: Dec>Mar>Jan>Nov>Oct>Feb TKN: Dec>Nov>Jan>Oct>Mar>Feb TC: Dec>Jan>Nov>Feb>Oct>Mar Helminth eggs: Nov>Oct>Dec>Mar>Feb>Jan

Sampling Points * Sampling Frequency	F (675,724) =1.76, P<0.05, nP^2=0.6, SIG	pH: F(45,60)=0.7, p=0.8, np2=0.3, NOT-SIG Temp: F(45,60)=0.3, p=1, np2=0.1, NOT-SIG DO: F(45,60)=1.1, p=0.3, np2=0.4, NOT-SIG EC: F(45,60)=0.4, p=0.9, np2=0.2, NOT-SIG TDS: F(45,60)=0.4, p=0.9, np2=0.2, NOT-SIG Turbidity: F(45,60)=29.6, p=0.0, np2=0.7, SIG TSS: F(45,60)=4.79, p=0.0, np2=0.7, SIG BOD: F(45,60)=0.6, p=0.9, np2=0.3, NOT-SIG COD: F(45,60)=2.39, p=0.0, np2=0.64, NOT-SIG Nitrate: F(45,60)=0.91, p=0.6, np2=0.4, NOT-SIG Nitrite: F(45,60)=1.52, p=0.06, np2=0.53, NOT-SIG Phosphate: F(45,60)=0.84, p=0.71, np2=0.38, NOT-SIG TKN: F(45,60)=0.79, p=0.7, np2=0.3, NOT-SIG TC: F(45,60)=1.4, p=0.08, np2=0.5, NOT-SIG Helminth eggs: F(45,60)=25.0, p=0.0, np2=0.9, NOT-SIG	Oct (Turbidity): ST<P1<P2>P3<P4>P5>P6>P7>P8>CT Nov (Turbidity):ST<P1>P2<P3<P4>P5>P6>P7>P8>CT Dec (Turbidity):ST>P1>P2<P3<P4<P5>P6<P7>P8>CT Jan (Turbidity): ST<P1>P2<P3>P4<P5>P6<P7>P8>CT Feb (Turbidity):ST<P1<P2>P3<P4<P5>P6>P7<P8>CT Mar (Turbidity):ST<P1>P2>P3<P4>P5>P6>P7>P8>CT Oct (TSS): ST<P1>P2<P3<P4>P5>P6>P7>P8>CT Nov (TSS): ST<P1>P2>P3>P4>P5>P6>P7>P8>CT Dec (TSS): ST<P1<P2>P3>P4>P5>P6>P7>P8>CT Jan (TSS): ST<P1<P2>P3<P4<P5>P6<P7>P8>CT Feb (TSS): ST<P1<P2<P3<P4<P5>P6<P7>P8>CT Mar (TSS): ST<P1>P2>P3<P4>P5>P6<P7>P8>CT Oct (COD): ST<P1<P2<P3>P4>P5>P6>P7>P8>CT Nov (COD): ST<P1<P2>P3>P4>P5>P6>P7>P8>CT Dec (COD): ST>P1>P2>P3<P4<P5>P6>P7>P8>CT Jan (COD): ST>P1<P2>P3<P4<P5>P6<P7>P8>CT Feb (COD): ST=P1<P2<P3>P4>P5>P6<P7>P8>CT Mar (COD): ST>P1>P2>P3>P4>P5>P6>P7>P8>CT Oct (Helminth egg): ST>P1>P2>P3>P4>P5>P6>P7>P8>CT Nov (Helminth egg): ST>P1>P2>P3>P4>P5>P6>P7>P8>CT Dec (Helminth egg): ST>P1>P2>P3>P4>P5>P6>P7>P8>CT Jan (Helminth egg): ST>P1>P2>P3>P4>P5>P6>P7=P8=CT Feb (Helminth egg): ST>P1>P2>P3>P4>P5>P6=P7=P8=CT Mar (Helminth egg): ST>P1>P2>P3>P4>P5>P6>P7=P8=CT

Table 0-1: Multi Variate Analysis of Variance

Parameter	pH	TEMP.	DO	EC	TDS	Turbidity	TSS	BOD	COD	Nitrate	Nitrite	Phosphate	TKN	TC	H-eggs	Rain fall	GHI
pH	1																
TEMP.	-.110	1															
DO	-.144	-.067	1														
EC	.178	-.602**	.018	1													
TDS	.184*	-.588**	.021	.989**	1												
Turbidity	-.044	.302**	-.034	.026	.031	1											
TSS	.121	-.320**	-.117	.365**	.362**	.203*	1										
BOD	.467**	-.266**	-.097	.471**	.468**	.273**	.422**	1									
COD	.269**	-.148	-.051	.424**	.421**	.423**	.257**	.681**	1								
Nitrate	.059	-.323**	-.003	.453**	.469**	.089	.258**	.115	.199*	1							
Nitrite	.070	-.111	-.044	.183*	.184*	.156	.172	.087	.084	.180*	1						
Phosphate	.285**	-.050	-.167	.459**	.464**	.260**	.185*	.506**	.589**	.240**	-.031	1					
TKN	.364**	.068	.059	.383**	.377**	.402**	.317**	.668**	.582**	.128	.162	.387**	1				
TC	.484**	-.036	-.343**	.297**	.302**	.364**	.449**	.742**	.591**	.032	.186*	.490**	.545**	1			
Helminth	.431**	.429**	-.345**	-.194*	-.190*	.373**	-.040	.373**	.320**	-.200*	.023	.203*	.442**	.598**	1		
Rainfall	-.18	.21*	-.24**	-.47**	-.46**	-.02	-.13	-.34**	-.32**	-.30	-.02	-.17	-.63**	-.06	-.02	1.00	
GHI	-.12	.89**	-.07	-.57**	-.57**	.32**	-.24	-.29	-.20	-.30	-.09	-.08	.06	-.10	.34	.25	1.00

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 0-2: Corelation