ADDIS ABABA UNIVERSITY
ETHIOPIAN INSTITUTE OF WATER RESOURCES

HOSPITAL WASTEWATER TREATMENT THROUGH HORIZONTAL SUBSURFACE FLOW CONSTRUCTED WETLANDS: THE CASE OF HAWASSA UNIVERSITY REFERRAL HOSPITAL

By

Simachew Dires

A Dissertation Submitted to Ethiopian Institute of Water Resources of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.) in Water and Public Health

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This is to certify that the thesis prepared by Simachew Dires, entitled “**Hospital Wastewater Treatment through Horizontal Subsurface Flow Constructed Wetland**” and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy in Water and Public Health complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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ORIGINAL PAPERS

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Acronyms and Abbreviations

AOX	Adsorbable Organic Halogens
APHA	American Public Health Association
APIs	Active Pharmaceutical Ingredients
BOD	Biochemical Oxygen Demand
BOD_5	The Five-Day Biochemical Oxygen Demand
CAS	Conventional Activated Sludge
CFU	Coliform Forming Unit
CLSI	Clinical Laboratory Standards
COD	Chemical Oxygen Demand
CW	Constructed Wetlands
EC_{50}	Half maximal effective concentration
EHNRI	Ethiopian Health and Nutrition Research Institute
FMHACA	Medicine and Health Care Administration and Control
FMOH	Federal Ministry of Health
FWS	Free Water Surface
GIZ	Deutsche Gesellschaft für Internationale Zusammenarbeit
HDPE	High Density Polyethylene Pipe
HPLC	High Pressure Liquid Chromatography
HSSF	Horizontal Subsurface Flow
HWW	Hospital Wastewater
LSD	Least Significant Difference
MBBR	Moving Bed Biofilm Reactor
MBR	Membrane bioreactor
MDR	Multidrug Resistant

MPN	Most Probable Number
MSA	Mannitol Salt Agar
NF	Nano Filtration
PAC	Powdered Activated Carbon
PhACs	Pharmaceutically active compounds
RO	Reverse Osmosis
SBR	Sequencing Batch Reactors
SF	Surface Flow
Sp	Species
SSF	Subsurface Flow
TC	Total Coliform
TKN	Total Kjeldahl Nitrogen
TN	Total Nitrogen
TP	Total Phosphorus
SPE	Solid-phase extraction
SPSS	Statistical Package for the Social Sciences
TSI	Triple sugar iron agar
TSS	Total Suspended Solid
UNWWDR	United Nations World Water Development Report
UV	Ultraviolet
USEPA	United States Environmental Protection Agency
VSSF	Vertical Subsurface Flow
WHO	World Health Organization
WWTP	Wastewater Treatment Plants
XLD	Xylose Lysine Desoxycholate

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Abstract

A huge amount of water consumption in hospitals releases a significant volume of wastewater loaded with complex mixtures of chemical and biological substances. The environmental and public health consequences of the wastewater released in these healthcare setups is immense unless proper wastewater treatment system is in place. Among the treatment systems, constructed wetlands as a reasonable option, have recently received considerable attention to treat a wide variety of wastewater throughout the world. The aim of this study was to examine the potential of subsurface flow constructed wetlands to treat hospital wastewater.

Different composite samples were collected from the inlet and outlet of wetlands, transported and processed for physicochemical, heavy metal and drug analyses as well as enumeration of indicator organisms, bacteriological identification and susceptibility testing were done following the standard procedures. The Fisher's least significant difference (LSD) and Kruskal Wallis H test were used to determine any significant differences in the mean influent and effluent values of parameters, and to compare between planted and unplanted cells in broken brick and gravel bed wetlands during the dry and rainy seasons.

The inlet wastewater of Hawassa referral hospital contained significant amount of organic matters and nutrients. The average concentration of TSS, BOD5, COD, TKN, NH_4^+-N, NO_3^--N and PO_4^{3-}were 535±22 mg/L, 221±31.3 mg/L, 713±86.5 mg/L, 86.3±11.7 mg/L, 0.9±0.2 mg/L, 13.4±4.9 mg/L during the dry season and 496±11.7 mg/L, 185±11.6 mg/L, 673±31.9 mg/L, 98±3 mg/L, 67.5±4.6 mg/L, 1.1±0.2 mg/L, 8±2 mg/L during the rainy season, respectively. The result of the study indicated that the organic matter, ammonium, and phosphate concentration were relatively higher during the dry season while TKN and nitrate were higher during the rainy season. Heavy metal analysis of hospital wastewater demonstrated that the average concentration of cadmium, chromium, nickel, lead, zinc, silver, arsenic, iron, manganese and copper to be 0.078±0.021, 0.1114±0.04, 0.162±0.082, 0.74±0.05, 0.134±0.009, 0.06±0.008, 0.021±0.007, 0.8±0.1, 0.21±0.03, and 0.06±0.008 mg/l, respectively. While the average concentration of sulphamethoxazole and diclofenac were 22.9±2.25 and 1.94+0.06 µg/L, respectively. The concentration of caffeine, salicylic acid, and ethinyl estradiol was below the detection limit.

Bacteriological analysis of samples from the hospital wastewater illustrated that the average bacterial count of total coliforms and fecal coliforms was 1.3 x 10^7 ± 1.2x10^3 and 1.4 x 10^5 ± 5x10^2

MPN/100 ml, respectively throughout the investigation period. The most frequently isolated bacterial species were *Staphylococcus sp* 12 (26.6%), *E. coli* 11 (24.4%), *Klebsiella sp* 9 (20%), *Shigella sp* 5 (11.1%), *Salmonella sp* 4 (8.9%), and others, such as *Pseudomonas sp* and *Citrobacter sp* constitutes 4 (8.9%). Among bacterial isolates, 100% of *Salmonella* species were found to be resistant to ampicillin and 75% to doxycycline, erythromycin, ceftazidime, cefoxitin, and chloramphenicol. About 81.8% of *E.coli* isolates were also found to be resistant to ampicillin and 72.7% to cotrimoxazole and amoxicillin-clavulanic acid. About 80% of ampicillin resistant *Shigella*, as well as 77.8% ampicillin, amoxicillin-clavulanic acid, and ceftazidime-resistant *Klebsiella*, were also frequently found.

The average percent removal efficiency of the constructed wetlands against TSS, BOD_5, COD, TKN, NH_4^+-N, NO_3-N, and PO_4^{-3} were, respectively, 93.2%, 90.4%, 83.7%, 64%, 64.3%, 52.1% and 56.1% in the dry season and 89.7%, 85.8%, 82.9%, 66%, 62.7%, 56.1% and 59.5% in the rainy season. Broken brick bed wetlands gives better removal efficiency of TKN, ammonia, nitrate, and phosphate with average removal rate of 73%, 71.3%, 79.6% and 77.1% in the dry season and 74.7%, 70.7%, 70.9% and 73.6% in the rainy season, respectively. Broken brick beds provide better adsorption of ammonium, nitrate, and phosphate. Typha with broken brick bed was significantly enhanced ($P<0.05$) the treatment performance of constructed wetland systems for the removal of ammonia, nitrate, and phosphate. The seasonal variation couldn't significantly influence the removal of all the pollutants but a better performance of nitrate and phosphate was achieved in a dry season. The use of locally available broken brick as a substrate media can increase the nutrient removal efficiency of constructed wetlands with a cheaper cost when applied in full scale constructed wetlands.

Among the examined wetlands for the removal of heavy metals, those planted wetlands were found to be efficiently removed cadmium, chromium, and nickel with an average removal rate of 100%, 93.7%-100%, 56.8-99.4%, respectively. The average lead removal efficiency of planted and unplanted wetlands was ranged from 75.7% to 100%. Planted broken brick bed wetlands removed 93.4 to 97.8% of lead while the unplanted broken brick bed wetland removed only 40.3%. The average silver and arsenic removal efficiency of wetlands was 79.8% and 83.6%, respectively. Vegetation plays a vital role in the removal of most heavy metals. Typha planted broken brick bed wetland have a better performance in the removal of heavy metals. On the other hand, the average diclofenac removal efficiencies of planted and unplanted reactors were 24%-100% and 8.2%-

34.2%, respectively. The Typha planted broken brick bed wetland removed all the diclofenac from the wastewater. All planted wetlands were significantly removed diclofenac than unplanted gravel bed wetland. There was also a significant difference between Typha planted broken brick bed wetland with other planted and unplanted wetlands of both substrates.

Reductions of antibiotic-resistant bacteria were higher in the vegetated CW of both substrates than the non-vegetated control gravel bed CW throughout the study period. Significantly ($P<0.05$) higher removal (80.8% to 93.2%) of antibiotic-resistant bacteria was recorded in vegetated wetlands. The overall isolated bacterial species in treated wastewater samples were *E.coli* 45(39.5%), *Staphylococcus sp* (35.1%) and *Klebsiella sp* 35 (30.7%). *E.coli*, *Klebsiella*, and *Staphylococcus sp* were the most frequently isolated antibiotic-resistant species in treated wastewater. The frequencies of resistant isolate from treated wastewater to ampicillin were 67/114 (58.8%) followed by cotrimoxazole 45/114 (39.5%), doxycycline 45/114 (39.5%) and chloramphenicol 36/114 (31.6%). Higher removal rate (100%) of *Salmonella* and *Shigella* was achieved in CW1, CW4, and CW5. A maximum removal (93.2%) of resistant bacteria was recorded in CW2 and the least removal rate (42.4%) was observed in non-vegetated gravel bed control wetland (CW8).

The overall pollutant removal efficiency of these constructed wetlands showed how much it is effective to use CWs for the treatment of hospital wastewater. Typha plant with broken brick substrate took part in the removal of heavy metals, nutrients and drug residual. Constructed wetlands with broken brick substrate could help to solve the problem of hospital wastewater treatment and can play significant role in the strategy to reduce water pollution in low-income countries like Ethiopia.

CHAPTER ONE
INTRODUCTION

1.1. Background

Health care industries play a vital role in helping to improve the well-being of all members of society. One of the challenges of these industries is to develop innovative approaches to deliver cost-effective services to patients without compromising the environment or workers health and safety (Friedman et al. 2016). A huge amount of water consumption in hospitals, mainly releases a significant volume of wastewater loaded with complex mixtures of chemical and biological substances such as heavy metals, disinfectants, reagents, detergents, radioactive markers, hormones, pharmaceuticals, endocrine disrupting compounds, anesthetics, cytostatic agents, and AOX (Adsorbable Organic Halogens) and microorganisms (Pauwels and Verstraete 2006; Hawkshead 2008; Kümmerer 2001). The growing application of antimicrobial drugs to treat infectious diseases in these setups can also lead to the release of antibiotic residue in their wastewater (Lien et al. 2016; Tran et al. 2016).

According to Kuchibanda & Mayo (2015) 10 to 25% of the wastes generated by the hospitals are regarded as hazardous wastes, and they can create a variety of health risks. Pharmaceutically active compounds (PhACs) such as analgesics, anti-convulsants, antidepressants, anti-inflammatories, hormones, and antibiotics can enter municipal and natural water systems via hospital effluent. Like many foods and nutritional supplements, they are not always completely absorbed or broken down by the body. In some instances, as much as 50 to 90 percent of an administered drug may be excreted by the body in a biologically active form (Halling-Sørensen et al. 1998).

Hospital effluent could enhance the number of resistant bacteria in the environment by introducing and selection of resistant bacteria as well as inhibiting the growth of susceptible bacteria (Alam et al. 2013). This happens due to those antibiotics, which are mostly released into the environment at low concentration, exert high selective pressure on bacterial communities and, consequently, accelerating their resistance (Larsson 2014). Researchers indicated that wastewater discharged from the hospital contains higher amounts of antibiotic-resistant bacteria, genes, and residual antibiotics than municipal wastewater (Nuñez et al. 2016; Moges et al. 2014).

Thus, the discharge of hospital effluent without treatment might cause a massive health impact through the dissemination of infectious agents and antibiotic-resistant microbes that result in outbreaks of communicable diseases and diarrhea epidemics such as cholera, typhoid fever, dysentery and gastroenteritis (Friedman et al. 2016). Several reports indicated that antibiotic-resistant species are ubiquitously found in various environmental compartments of surface waters, treated wastewater, groundwater, sediments, soils and drinking water (Nain et al. 2015; Vaz-Moreira et al. 2016; Yang et al. 2017; Williams et al. 2016). The major sources of such contamination are due to the release of untreated sewage from municipal services, hospital, industrial, agricultural, and veterinary activities (FAO 2016; Williams et al. 2016).

Among the wastewater treatment systems, constructed wetlands (CWs) as a reasonable option, have recently received considerable attention to treat a wide variety of wastewater throughout the world. This is because of their low construction, operation and maintenance costs, process stability, high nutrient absorption capacity, simplicity, low energy consumption, low excess sludge production, the potential for creating biodiversity, and add aesthetic, ecological, and cultural values. They are used to clean-up not only municipal wastewater but also agricultural effluents, landfill leachates, storm water, polluted river water, urban runoff, food wastes, abattoir effluent, acid mine drainage, industrial effluents, as well as petrochemicals (GIZ 2011; Qasaimeh et al. 2015; USEPA 2000; Vymazal 2011; Skrzypiecbcef and Gajewskaad 2017).

They are complex, well-established, self-contained, integrated and environmentally friendly alternative treatment systems that use natural processes involving water, wetland vegetation, gravel/soils, environment, and their associated microbial assemblages for sewage treatment, pollution control and environmental improvements (Qasaimeh et al. 2015). The main advantages of CWs as the choice of treatment plants in developing countries are due to their high-quality effluent production for multi-purpose reuses as well as their sustainability for self-remediation and self-adaptation to the surrounding conditions and environment.

CWs consist of substrate media that may be planted with different macrophytes for mitigating organic matter, nutrients, trace elements, pharmaceutical contaminants, and pathogenic pollutants from wastewater. Treatment processes in wetland incorporate with complex processes of physical, chemical, and biological mechanisms like filtration, sorption, sedimentation, precipitation, plant uptake, microbial degradation, photolysis, volatilization, and nitrogen transformations (GIZ 2011). They are often highly productive systems that employed more frequently almost in any environment as a way of wastewater treatment facilities for smaller populations (Gikas et al., 2007). Properly designed and constructed artificial wetlands with appropriate plant and substrate media selection and canals arrangement are extremely efficient systems at utilizing and cleaning different pollutants.

Among the treatment wetlands, horizontal subsurface flow (HSSF) are more attractive and widely applied systems due to the view of a decreased risk of nuisance from flies, mosquitoes, animals, and odor, and greater efficiencies in terms of land usage. They are shallow, filled with permeable filter media, and almost completely covered with emerging macrophytes and being managed as water quality improvement systems. Some commonly used macrophytes are cattails (Typha spp.), bulrush (Scirpus spp.), Cyperus papyrus, sugarcane (Saccharum officinarum) and common reeds (Phragmites australis) which are adaptive and water-tolerant rooted in the soil to improve the quality of water by absorbing nutrients with their effective root system (Jethwa and Bajpai 2016; Mateus et al. 2016; Vymazal 2011a).

The materials used as CW filling can directly contribute to the removal of pollutants from the wastewater as a base for the vegetation and a layer of attachment for microorganisms to form a biofilm for degradation as well as acts as a filter and a trap for removing solids and adsorb pollutants depending on the media type (Rana and Laura 2014). In HSSF wetlands, facultative and anaerobic bacteria degrade most organics; the vegetation transfers a limited amount of oxygen to create an aerobic condition in their root zone so as to colonize the area by aerobic bacteria to degrade organics as well. Even if, different constructed wetland designs and plant species were used as effective treatment options for municipal and industrial wastewater, their application in the treatment of wastewater generated from healthcare institutions has not been thoroughly tried (USEPA 2000). Therefore, the current study aims to evaluate the potential of Horizontal subsurface flow constructed wetland treatment systems to remove different pollutants from hospital wastewater.

1.2. Statement of the Problem

The bacteriological and physicochemical composition of Hawassa referral hospital wastewater discharges was not thoroughly investigated. Hospital wastewater in terms of quality is categorized as municipal wastewater or domestic sewage. However, apart from the presence of complex impurities and potentially infectious residues, hospital wastewaters might contain various chemical contaminants that lead to environmental pollution (Emmanuel et al. 2002). As many researchers reported from different countries, hospitals discharge considerable amounts of chemicals and microbial agents in their wastewater such as heavy metals, organic matter, nutrients, multidrug-resistant microbial strains, and pharmaceuticals (Emmanuel et al. 2005; Rezaee et al. 2005).

According to Amouei et al. (2012) and Akin (2016) hospitals released a significant amount of TSS, BOD_5, and COD in their wastewater. They are also key sources of excess amounts of nitrogen and phosphorus compounds into the environment (Wyasu and Okereke 2012). A study conducted by Meo et al. (2014) also showed that the hospitals release Cadmium, Chromium, Lead, Nickel, Zinc, Manganese, and Iron into the wastewater. Previous studies in Ethiopia only focused on the evaluation of the treatment efficiency of the available hospital wastewater treatment plants by considering a few parameters (Beyene and Redaie 2011). The others were also focused on the microbial load and the release of antibiotic-resistant bacteria by this setup (Asfaw et al. 2017; Fekadu et al. 2015). Researches regarding the detailed pollutant loads of the present hospital and other health institutions across Ethiopia are still inadequate. Therefore, studying the characteristics of hospital wastewater is vital to select the type of treatment plant implemented in these particular setups as well as helping to know its short and long term impact on the environment.

The application of broken brick as a subsurface flow wetland media and its capacity to remove nutrients in hospital wastewater to my knowledge was not studied yet. The use of an effective type of substrate in wetlands might increase its potential to remove pollutants. Several researchers use different media type in CWs to remove phosphorous from the wastewater such as basic oxygen furnace slag (BOFS) (Hussain et al. 2015), biochar (Gupta et al. 2016), dolomite (Žibienė et al. 2015), laterite (Mansing and Rout 2013), zeolite, limestone, calcite and other substrates rich in iron, aluminum, and calcium (Yun et al. 2015). Mateus et al. (2016), Abdul and

Ganapathyvenkatasubramanian (2016) and Wang et al. (2012) also investigated the removal of phosphorus by using broken brick media in the vertical flow wetland. Most of these researches applied these media in constructed wetlands to remove phosphorus only from municipal and industrial wastewater, its role in the removal of nitrogenous compounds from complex wastewater types by subsurface flow CWs is still needed the attention of the experts. Therefore, studying the potential of broken brick as a wetland substrate to remove nutrients from the wastewater might motivate the authorities of developing nations to use such type of treatment plants with a cheaper locally available bed material to safeguard their water sources from eutrophication.

Sufficient knowledge about the contribution of plant species for the removal of heavy metals and drug residue in constructed wetlands was lacking. Plants are important components of constructed wetland treatment systems which play a vital role in the removal of different pollutants. A comprehensive review dealing with the potential roles of plants in CWs was discussed by Brix (2013). Vymazal (2011) also published a systematic literature review on plants' role in horizontal subsurface CWs. A variety of research findings also showed the mechanism of heavy metal removal in treatment wetlands through the use of several substrate media and plant species (Matagi et al. 1998; Qasaimeh et al. 2015b; Sheoran and Sheoran 2006). However, these studies have made use of various experimental strategies with different plants, resulting in conflicting findings, with even more variation results when comparisons are made between different types of CWs. Therefore, the current study will show the clear contribution of plant species on the removal of heavy metals and which plant species are more appropriate in the removal of such pollutants from hospital wastewater.

The potential of subsurface flow constructed wetlands for the removal of antibiotic-resistant bacteria from health care wastewater to my knowledge was not studied yet. Antibiotic resistance remained the most important challenge throughout the world (Priyankaa and Nandanb, 2015). Hospital effluent could enhance the number of resistant bacteria in the environment by the introduction and selection of resistant bacteria as well as inhibiting the growth of susceptible bacteria (Alam et al., 2013). Researchers also indicated that wastewater discharged from the hospital contains higher amounts of antibiotic-resistant bacteria, genes, and residual antibiotics than municipal wastewater (Nuñez et al. 2016; Moges et al. 2014). Thus, the discharge of hospital effluent without treatment might cause a massive health impact through the dissemination of infectious agents and antibiotic-resistant microbes that result in outbreaks of communicable diseases and diarrhea epidemics such as cholera, typhoid fever, dysentery and gastroenteritis (Nuñez and Moretton 2007). Even if, different constructed wetland designs and plant species were used as effective treatment options for organic matter and coliforms, there is a lack of information based on the author literature survey, whether these kinds of treatment plants are effective to remove drug-resistant microbial strains from health care institutions. Therefore, the current study can provide baseline information concerning the removal of antibiotic-resistant bacteria to safeguard our environment from the release of resistant strains.

1.3. Significance of the Study

Owing to the current expansion of hospitals and other health institution by the Federal Ministry of Health (FMOH) of Ethiopia, the public health and environmental consequences of the wastewater discharged in these setups will be high unless proper wastewater treatment system is in place. Most of the health institutions constructed in Ethiopia do not have suitable wastewater treatment plants. The existing few treatment systems are not designed by considering the complex nature of healthcare wastes. The ultimate challenge in the water and sanitation sector of our country would be the application of low-cost wastewater treatment plants that would be applied in different institutions with the requirement of less skillful personnel and technical expertise. It is therefore essential that a treatment plant that is economical, efficient and sustainable technology be installed in developing regions.

This dissertation focused on the explanation of the wastewater composition of hospital wastewater, the potential of broken break to remove nutrients from the wastewater, the role of plants to take up heavy metals and the fate and removal of antibiotic resistant bacteria in constructed wetlands with different substrates and plant species. The findings of the dissertation have essential input for the planning of appropriate hospital wastewater treatment plants by the ministry of health, different designers and constructors by considering constructed wetlands as the choice of wastewater treatment plant.

The primary beneficiaries of the study results will be Hawassa referral hospital managements who will be expected to take remedial actions to reduce the potential effect of the pollutants and to protect the receiving water body from these pollutants. The results will also provide important information for health offices, NGOs and another stake holders to design and implement wastewater treatment plants in hospitals. The findings of the study will also serve as a reference material and a guide for future researchers who wish to conduct the same experimental study or any study related to constructed wetlands

1.4. Scope of the Study

The scope of this dissertation is to provide information about horizontal subsurface flow constructed wetlands as a sustainable hospital wastewater treatment option in the case of Hawassa university referral hospital. It includes the construction of pilot scale experimental set-up consisting of 8 cells. Five cells were gravel based and the other three were broken brick based substrates. Among these wetlands six cells were vegetated while two were kept as ‘control’ from both substrates. A continuous flow system was used with a hydraulic residence time of 4 days. Identification of locally available species of plants (macrophytes), which can be used as vegetation for constructed wetlands to polish anaerobically (septic tank) treated effluent. The performance of these macrophytes with respect to the substrates was compared when anaerobically treated effluent was applied. Investigation of the removal efficiencies of constructed wetlands to remove different parameters at different season and the effect of media type selected on the performance of

1.5. Objectives

1.5.1. General Objective

The general objective of this study was to evaluate the performance of horizontal subsurface flow constructed wetlands in treating Hawassa Hospital wastewater using different wetland plants and substrates

1.5.2. Specific Objectives

1. To determine the bacteriological and physicochemical content of Hawassa hospital wastewater;
2. To compare nutrient removal capacity of a broken brick and gravel substrates in subsurface flow constructed wetlands
3. To examine the contribution of wetland plants in the removal of heavy metals and drugs from hospital wastewater;
4. To evaluate the antibiotic-resistant bacteria removal potential of subsurface flow constructed wetlands;

CHAPTER TWO

LITRATURE REVIEW

Throughout the world, hospitals and other healthcare facilities are dedicated to providing innovative and compassionate patient care that meets the quality of life in a cost-effective manner. However, in fulfilling this important mission to care for patients, they induce an impact on the natural environment. We are living in a moment in which the twin crises of public health and the environment are merging; the crosscurrents of disease and ecological deterioration build on one another, becoming increasingly turbulent and damaging forces that are tearing at the very fabric of our societies (Karliner and Guenther 2011).

An increase in water consumption is an inevitable outcome in maintaining the high standard of healthcare service provided and, thereby generated more wastewater. Apart from the presence of the impurities and potentially infectious residues, this wastewater might contain various chemical contents that lead to environmental pollution. On top of this, hospital wastewater also has its biological influences on the environment which might cause cross-infection and outbreaks of diseases.

2. 1. Hospital Wastewater Characteristics

A huge amount of water consumption in hospitals releases a significant volume of wastewater loaded with complex mixtures of chemical and biological substances such as heavy metals, disinfectants, reagents, detergents, radioactive markers, X-ray contrast media, hormones anti-tumor, phenol, chloroform, pharmaceuticals, endocrine disrupting compounds, microorganisms (bacteria, viruses), and biodegradable organic materials (protein, fat, and carbohydrate) (Pauwels and Verstraete 2006; Hawkshead 2008; Kümmerer 2001).

The quantity and type of wastewater released from hospital vary between and within the countries and this variation can be attributed to the size, activity, and nature of hospitals, proportion of in and outpatients, type of institution and specialization, and the prosperity of the country. Hospitals discharge wastewater from medical wards and operating theaters (body fluids and excreta, anatomical waste), laboratories (microbiological cultures, stocks of infectious agents), pharmaceutical and chemical stores; cleaning activities (waste storage rooms), x-ray development facilities, autoclaves, microwave irradiation, chemical disinfection and laundries (Pauwels and Verstraete 2006; Kusuma et al. 2013).

Hospital wastewater in terms of quality is categorized as municipal wastewater or domestic sewage. However, due to the presence of hazardous, toxic, and pathogenic factors, this type of wastewater is considered to be a health and environmental issue. Recent studies indicated that hospitals may represent an incontestable release source of many toxic substances in the aquatic environment (Sun et al. 2006). Many drugs are not completely metabolized after consumption by the patient and are disposed into the wastewater. Disinfectants, in particular, are often highly complex products or mixtures of active substances. Some non-biodegradable materials may pass through the sewage of wastewater treatment plants into surface water or reach underground water after the use of sludge as fertilizer.

The disposal of untreated hospital wastewater which contains antibiotic-resistant bacteria is also a matter of concern since it constitutes a health risk to the population. Furthermore, pathological, radioactive, chemical, infectious, and pharmaceutical wastes, if left untreated could lead to outbreaks of communicable diseases such as diarrhea, cholera, skin diseases, and enteric illnesses (Emmanuel et al. 2002). These activities may lead to a risk directly related to the existence of hazardous substances which could have potential health risks and negative effects on the biological balance of the aquatic ecosystem (Emmanuel et al. 2005). On the one hand, the toxic waste residues passed into the marine ecosystem can cause direct hazards to both the aquatic environment and organisms that reside in it; on the other hand, the impacts can further extend to the land and air arena, in which the terrestrial organisms including human beings and vegetation can be affected by the toxic outcomes (Chan 2005). Thus, hospital wastewater quality and management have become critical issues and taken key attention in national health policies of many countries. These facts emphasize that a pre-treatment is necessary for hospital wastewater to prevent the hazard from being transferred to other water sources through cities' sewerage.

2.1.1. Microbiological Pathogens and Antibiotic-Resistant Microorganisms

During the diagnosis and treatment of diseases, hospitals release a huge amount of harmful infectious agents such as pathogens and microorganisms in their wastewater (Akin 2016). They also act as the storehouse of antibiotic-resistant bacteria due to the widespread use of antibiotics to treat microbial infections (Kümmerer 2001). A study conducted in Mekele hospital, Ethiopia, reported higher total coliform (2.2×10^6 CFU/100 ml) and fecal coliform counts (2.0×10^5 CFU/100 ml) (Asfaw et al. 2017). Likewise, Akin (2016) reported that a higher proportion of Pseudomonas aeruginosa (2×10^4 - 9×10^6 CFU/ml), Acinetobacteria (5×10^4- 6×10^6 CFU/ml) and Coagulase-negative Staphylococcus (7×10^1 - 5×10^2 CFU/ml) were released from the hospital.

Bacterial isolates such as staphylococcus, E.coli, Klebsiella, and Shigella were most frequently detected in hospital wastewater (Fekadu et al. 2015). Researchers in different countries reported that Escherichia coli (E.coli), Staphylococcus sp, Klebsiella sp, Enterobacter sp, Pseudomonas sp, Proteus sp, Shigella sp, Bacillus sp, Citrobacter sp, and Serratia sp were the dominant isolates in hospital wastewater (Moges et al. 2014; Alam et al. 2013; Ashfaq et al. 2013). High counts of bacterial load reflected the potential of the hospital wastewater to pose a significant impact on the health of the community by boosting infectious risks through the spread of infectious diseases caused by bacteria specifically antibiotic-resistant isolates.

In developing countries, the release of wastewater pollutants into the environment without treatment is an extensive practice, thus rendering the contamination of lakes, rivers, groundwater, estuaries, and ponds (Diwan et al. 2010). This practice could make the water bodies a reservoir of multidrug-resistant microorganisms that can contain genes that can be transmissible and exhibit better survival potential for a very long time over a wide range of difficult environmental conditions (Moges et al. 2014). A number of reports indicated that antibiotic-resistant species are ubiquitously found in various environmental compartments of surface waters, treated wastewater, groundwater, sediments, soils, and drinking water (Schwartz et al. 2003; Böckelmann et al. 2009; Vaz-Moreira et al. 2014). The Major sources of such contamination are due to the release of untreated sewage from municipal services, hospital, industrial, agricultural and veterinary activities.

Concerns have been raised about the public health implications of the presence of antibiotic residues in the aquatic environment and their effect on the development of bacterial resistance. The development and proliferation of antibiotic resistance in pathogenic, commensal, and environmental microorganisms are a major public health concern. Conjugative transfer between closely related strains or species of bacteria is an important method for the horizontal transfer of multidrug-resistance genes. The emergence of such resistance is the consequence of a complex interaction of factors. The over-usage of antibiotics in clinics has been believed to be the principal elements involved in the rise of new resistances (Lupo et al., 2012).

The growing application of antimicrobial drugs to treat infectious diseases in hospitals is of great concern to public health as this can lead to the release of antibiotics and the development of multidrug-resistant bacteria (Mao et al. 2015). Hospital effluent could enhance the number of resistant bacteria in the environment by introducing and selection of resistant bacteria as well as inhibiting the growth of susceptible bacteria (Alam et al. 2013). This happens due to the antibiotics, which are mostly released into the environment at low concentration, exert high selective pressure on bacterial communities and, consequently, accelerating their resistance (Mao et al., 2015; Rowe et al. 2017).

Researchers indicated that wastewater discharged from the hospital contains higher amounts of antibiotic-resistant bacteria, genes, and residual antibiotics than municipal wastewater (Nuñez et al. 2016; Moges et al. 2014). A comparative study of hospital and city sewage on drug-resistant coliform bacteria also showed that an average of 26% of coliforms in hospital wastewater had transferable resistance to at least one of the drugs' ampicillin, chloramphenicol, streptomycin, sulfonamide, or tetracycline as compared to an average of 4% in city sewage (Grabow and Prozesky 1973). Similarly, the wastewater of the hospitals carrying more resistant Vibrio cholera as compare to municipal wastewater drains (Ahmad et al. 2012). Likewise, Chitnis et al. (2000) indicated that the percent MDR bacteria for hospital samples ranged widely from 0.58 to 40%, while for residential colony sewage ranged between less than 0.00002 and 0.025%. Yet, the number of MDR bacteria was alarmingly high for the effluent samples from hospitals. The pattern was almost the same for the diverse species (*E.coli, Klebsiella, Enterobacter, Citrobacter,* and *Pseudomonas*) grown from the effluent samples and strongly suggests the prevalence of similar R-plasmids.

The MDR pattern seen in the bacterial isolates from hospital effluent samples included most of the antibiotics used presently for treating human infections. The worst fear apprehended is the transfer of such resistance to bacterial pathogens causing infections in the community. In that case, most of the presently available antibiotics will be futile against the infectious organisms. However, Fekadu et al. (2015) reported that Salmonella sp isolated from Yirgalem and Hawassa referral hospitals effluent were resistant to Ceftriaxone, Tetracycline, Doxycycline, and Gentamycin. Additionally, *S. aureus* was also resistant to Penicillin, Ampicillin, Amoxicillin, and Gentamycin. The origin of such MDR bacterial strains appears to be the hospital environment and the selective pressure responsible for expanding such bacterial populations in hospitals must have been through the use of drugs in humans (Chitnis et al. 2000). Thus, the discharge of hospital effluent without treatment might cause a massive health impact through the dissemination of infectious agents and antibiotic-resistant microbes that result in outbreaks of communicable diseases and diarrhea epidemics such as cholera, typhoid fever, dysentery and gastroenteritis (Nuñez and Moretton 2007).

Antibiotic resistance remained the most important challenge throughout the world (Priyankaa and Nandanb, 2015). It became an emergent healthcare crisis due to the fact that the currently existing antimicrobial drugs couldn't effectively work against the disease agent and the incidence of multidrug-resistant genes in both developed and developing countries. Its impact in developing countries is extremely serious where the infectious disease burden is high and access to effective diagnostic and treatment options in health institutions with enough manpower is a fundamental problem. In these countries, overuse, misuse, and damping of antibiotics is a common practice (Mustapha et al. 2016). It is difficult to evaluate and substitute antibiotics to which resistance is developed by newly fabricated ones within a short time. These make the treatment option limited, increasing costs of treatment, an extra length of stays in the hospital, therapeutic failures, and death (Sipahi 2008).

Conventional wastewater treatment plants (WWTP) play a crucial role in the reduction and removal of many pollutants including organic matters, pathogenic microorganisms, and even antibiotics. However, such treatment processes serve as collection points for resistant organisms and antibiotics from various sources as well as enhance the proportion of bacteria resistant to antibiotics by creating a suitable environment for the horizontal gene transfer via conjugation, transduction, and transformation (Priyankaa and Nandanb, 2015; Szczepanowski et al. 2009). Thus, WWTP have to be considered as a hot-spot site for the dissemination of antibiotics, antibiotic-resistant bacteria and genes which can survive long, harsh routs and final disinfection processes into surface and drinking waters (Mao et al. 2015; Rowe et al. 2017).

2.1.2. Organic Matter

BOD_5 and COD parameters are widely used to indicate the biodegradability potential of the organic matter content of wastewater. Most of the pollutants released from the hospital and other health care institutes are biodegradable except for some recalcitrant hazardous chemicals and organic matter. A study conducted by Aliahmad and Dehbashi (2013) in the hospital of Iran showed that the average influent concentration of COD was 688 mg/l. In a similar country studied by Rezaee et al. (2005) indicated that the concentration of COD and BOD_5 was 450 mg/L and 270 mg/L, respectively. Likewise, the COD concentration of raw hospital wastewater varied from 115.2 mg/l to 617.5 mg/l and BOD_5 of 40 mg/l to 188.1 mg/l with COD/BOD_5 ratio equal to 2.83 mg/l (Mesdaghinia et al. 2009). In another study, the maximum and the minimum concentration of BOD and COD regard to its pollution strength are in the ranges of 280 to 380 mg/L and 480 to 690 mg/L, respectively (Mahmoudkhani et al. 2012).

Similarly, the mean value of TSS, BOD, and COD in influent wastewater of all studied hospitals was 296, 400, and 616 mg/L (Amouei et al. 2012). In most of the hospitals, the BOD_5 and COD concentrations of wastewater are almost similar to the domestic wastewater values. On the other hand, Emmanuel et al. (2002) reported a high organic matter concentration of raw hospital wastewater with BOD_5 and COD values of 603 mg/L and 1223 mg/L, respectively. Similarly, the minimum, maximum and mean concentrations of BOD_5 (161, 648, and 372 mg/L) and COD (379, 1187, and 687 mg/L) were also reported for the investigated hospitals (Amouei et al. 2015). Some hospital clinical laboratory wastewater also account a higher amount of chemically oxidizable organic matter (COD) (934.2 mg/L) than that of BOD (75.3 mg/L) and it was seen that COD/BOD ratio could reach to a range of 10 to 12 (Akin 2016). Therefore, the organic matter in the hospital wastewater had higher biodegradability in comparison with domestic wastewater. The high biodegradability of organic matters is very desirable from the viewpoint of wastewater treatment and promotes the efficiency of wastewater treatment plants.

2.1.3. Nutrients

Nutrients are considered to be the primary sources of water pollution and limiting factors for algal blooms in lakes and ponds. Nitrogen and phosphorous are two main nutrients found in the municipal wastewater, storm-water runoff from urban and agricultural lands, and wastewater from various types of industrial processes and hospitals. Environmental and health problems associated with an excessive amount of certain forms of nitrogen and phosphorus in the environment have been established already. For example, high concentrations of nitrate in drinking water supplies can cause methemoglobinemia, or “blue baby” syndrome, in infants. Unionized ammonia (NH_3), found in some wastewater effluent is potentially toxic to many aquatic and marine organisms.

Moreover, eutrophication of surface water is frequently linked with elevated nitrogen and phosphorus concentrations, especially in coastal and estuary environments. Phosphorus is present in the wastewater in the form of orthophosphate and organic phosphorous while nitrogen exists in many forms such as inorganic and organic forms in the environment and transformations among different forms may occur rapidly and frequently. Municipal and industrial wastewater may have significant amounts of both organic, and inorganic forms of N. Inorganic nitrogen, i.e. nitrate, nitrite and ammonium, may also be present at high concentrations in agricultural and urban runoff.

Hospitals can release excess amounts of nitrogen and phosphorus into the environment. According to Wyasu and Okereke (2012), the minimum and maximum concentrations of nitrate and phosphate were 23.83 ±2.69 to 30.33±1.50 mg/l and 7.86 ±1.16 to 31.00± 1.48 mg/l in Ahmadu Bello University Teaching Hospital of Nigeria. Similarly, the average PO_4^{-3} and NO_3^- concentrations of Al-Sadr Teaching Hospital in Iraq were 4.3 and 18.6 mg/l, respectively (Al-enazi 2016). Another study conducted in Iran hospital indicated that the averaged influent concentrations of NH_3-N and PO_4-P were 11.8 and 3 mg/l, respectively (Aliahmad and Dehbashi 2013).

Rezaee et al. (2005) indicated that the concentration of ammonium nitrogen from raw wastewater was 18 mg/l, while the concentration of total phosphorous from hospital wastewater was 8.8 mg/l (Emmanuel et al. 2002). Amouei et al. (2015) also described that the average TKN and TP values of hospital wastewater were reached to 289 ± 132 mg/L and 15 ± 5.5 mg/L, respectively. The study done by Alrhmoun et al. (2014) also showed that the concentration of TN from raw hospital wastewater was 91 mg/l. These results indicated that hospital wastewater needs proper nutrient removal technology before release to the aquatic environment.

2.1.4. Heavy Metals

Metals and its compounds are of great concern, even at trace levels, primarily due to their potential toxicity to all aspects of the environment. The risks of their bioaccumulation in the food chains pose one of the major environmental and health problems of our modern society. Trace quantities of many metals are found in sewage from various sources. Among them, arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) are classified as potentially toxic elements, which mean that depending on the concentration and time of exposure, they can pose problems of acute or chronic human health effects, carcinogenicity, phytotoxicity, and bioaccumulation (Mudhoo and Kumar 2013).

Hospitals are one of the major sources of heavy metals in the environment. This is influenced by the volume, the number, and intensity of services provided by hospitals. They were mainly released during the radiology, X-ray, pharmacy, laboratory, and dental amalgam services of the hospital. According to Torke (1994), the heavy metals of concern from hospital wastewater are Cadmium, Chromium, Copper, Lead, Mercury, Nickel, Selenium, Silver, Barium, and Zinc. These contaminants are especially problematic to water quality control program due to the need of new, extremely stringent discharge limits to water bodies (Kusuma et al. 2013).

Hospitals are also recognized as the main source of mercury discharge to the wastewater system. The sources of mercury in hospital wastewater include potable water supply, medical equipment breakage (thermometers, blood pressure cuffs, and the manometers), laboratory chemicals, medicinal wastes, amalgam from dental clinics, human amalgam and dietary waste retained in wastewater sumps and traps. Cleaning products used in hospitals may contain trace mercury levels from the caustic soda used in the production of soap or from chemicals that may contain traces of mercury as an impurity. The study reported by Kaur (2014) showed that the quantity of mercury released from the hospital ranged between 0.100 mg/L to 0.150 mg/L. This higher concentration is found in wastewater samples collected from the laboratory, radiology department, and operation theatre.

A study conducted by Meo et al. (2014) showed that the total levels of Cadmium, Chromium, Lead, Nickel, Zinc, Manganese, and Iron in raw hospital wastewater were ranged from 0.032-0.676 mg/l, 0.042-0.107 mg/l, 0.012-0.229 mg/l, 0.593-0.643 mg/l, 0.077-0.174 mg/l, 0.027-0.057 mg/l, and 0.339-0.447 mg/l, respectively. Another study of hospital laboratory wastewater showed that the level of copper was higher in concentration varied between 0.44 and 0.85 mg/L, whereas the average Al, Cr, Cd, and Zn values were measured as 0.06, 0.07, 0.02,and 0.07 mg/L, respectively and Mn, Co, Ni, and Zn values were <0.01 (Akin 2016). Likewise, in Sri Lanka the maximum concentration of Cr (VI), Mn, and Pb were reported as 0.23, 0.52 and 0.90 mg/l (Kumarathilaka et al. 2015). Babaahmadi et al. (2017) also indicated that the concentration of Pb, Cr, Cd, Ni, Hg, Fe, and Cu were 0.53+0.08, 0.9 7+ 0.20, 0.0357+0.008, 0.867 +0.09, 0.0027+ 0.00, 1.317+ 0.51, and 0.437+ 0.10, respectively.

The average lead, chromium, iron, manganese, and zinc concentrations of Wolaita Sodo Teaching Referral Hospital were also 0.01 mg/l, 0.03 mg/l, 0.795 mg/l, 0.034 mg/l, and 0.06 mg/l, respectively (Moga et al. 2017). Urban et al. (2015) indicated the concentration of Arsenic, Cadmium, Lead, Mercury, and Chromium were ranged from 0.003 ± 0.00082 to 0.14 ± 0.0082 mg/l. These results indicated that hospital wastewater releases a significant amount of heavy metals that can adversely affect the receiving environment. Therefore, there should be optimal treatment technology for hospital wastewater to remove heavy metals and continuous monitoring and evaluation of the effluent quality before discharging into the environment is of paramount importance.

2.1.5. Pharmaceuticals

The word "pharmaceutical" refers to a chemical prepared or dispensed in pharmacies and which treats or prevents or alleviates the symptoms of disease or physiologic function. Pharmaceuticals are the drugs including the diagnostics (e.g., x-ray contrast media, including certain radiological), nutritional and dietary supplements (ephedra is an example), illicit drugs, cosmetic or lifestyle drugs not essential for medical purposes, and the broad range of personal care product ingredients (Daughton 2007). They range in prevalence from Naproxen, which is a prescription drug, to caffeine, which is found in coffee, tea, and chocolate among other goods (Shaver 2011). Besides the active substances, formulation adjuvant, and in some instances, pigments and dyes are also drug components. Most pharmaceuticals are chemically stable and slightly lipid-soluble. They can also be divided into acidic, basic, or neutral compounds (Daughton 2007). A large number of these pharmaceuticals are polar and neither volatile nor biodegradable (Mohamad 2010).

The main routes for human pharmaceuticals to reach the environment are through use with patients in hospitals, medical centers or the community, and disposal of unused or outdated drugs. In addition to this; the ubiquitous use of pharmaceuticals in veterinary medical practices, aquaculture, and agricultural products has led to the continual release of a wide array of pharmaceutical chemicals. These pharmaceuticals enter the environment through many routes, including human or animal excreta, wastewater effluent, treated sewage sludge, industrial waste, medical waste from health-care and veterinary facilities, landfill leachate, and biosolids (Kümmerer 2009; Ternes 1998).

Among different localities hospitals are suspected, or implied, to be a major and highly variable source of pharmaceuticals that substantially contributes to the total wastewater load. Their contribution to wastewater treatment plants ranged from less than 10% for substances used in the communities (diclofenac and atenolol), to well in excess of 50% for antibiotics and x-ray contrast media (Helwig et al. 2013). This is depending on the size of the facility, its service spectrum, the size of the community served in the facility, and the season of the year (Coutu et al. 2013).

A study conducted in Pakistan also showed that the highest antibiotic concentration was observed in hospital wastewater (HWW) ranged from 7.31 to 39.13 μg/L (Ahmad et al. 2012). In another study, antibiotics were released from hospitals dominated by the beta-lactam, quinolone, and sulphonamide groups (Watkinson et al., 2009). An estimated 89, 1, and 25 ng/L/day of fluoroquinolones, metronidazole and sulfamethoxazole respectively, might be getting released into the environment per 100 hospital beds (Diwan et al. 2013). Similarly, thirteen of the eighteen measured cardiovascular active pharmaceutical ingredients (APIs) positively confirmed and quantified from 12 hospitals (Nagarnaik et al. 2010).

Pharmaceuticals are ubiquitously found in the environment. They have been detected in wastewater, river water, groundwater and even in drinking water (Kümmerer 2001). After administration at health care facilities (hospitals and long-term-care homes), incompletely metabolized pharmaceuticals are excreted by patients into wastewater. Unused medications are also sometimes disposed of in drains. The drugs enter the aquatic environment and eventually reach drinking water if they are not biodegraded or eliminated during sewage treatment (Kümmerer 2001; Riaz 2010). Their occurrence in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry.

Recent studies have shown that residues of pharmaceuticals can be detected in concentrations greater than 1 μg/L in influents and effluents of municipal wastewater treatment plants. Such residues can also be detected in wastewater-affected rivers and groundwater in concentrations above 0.1 μg/L-0.5 μg/L (Kanda et al. 2003). About 28 pharmaceutical compounds were detectable in sewage treatment plant effluents, surface water, and sediment. The therapeutic classes included antibiotics, analgesics and anti-inflammatories, lipid regulators, beta-blockers, anti-convulsant, and steroid hormones (Hernando et al. 2006). In some investigations, more than 80 compounds, pharmaceuticals, and several drug metabolites have been detected in the aquatic environment (Kolpin and Meyer 2002).

The non-steroidal anti-inflammatory drug diclofenac has frequently been detected in almost all environmental surveys at concentrations ranging from 0.001 μg/L to 30 μg/L (Mohamad 2010). Watkinson et al. (2009) also indicated that antibiotics were identified quite frequently in the low ng/L range up to 2µg/L in the surface waters of six investigated rivers including freshwater, estuaries, and marine samples. Similarly, In California Caffeine, trimethoprim, sulfamethoxazole, gemfibrozil, fluoxetine, ibuprofen, carbamazepine, xylene, nonylphenol, and nonylphenol ethoxylates were detected at one or more monitoring site of the river (Schaefer and Johnson 2009). Benotti et al. (2009) also detected a diverse group of pharmaceuticals, potential endocrine-disrupting compounds (EDCs), and other unregulated organic contaminants from the source water, finished drinking water, and distribution system (tap) water from 19 U.S. water utilities. Similarly in China, about 17 pharmaceuticals were detected in 89% of samples of tap water, with most detectable concentrations (92%) less than 50 ng/L.

Caffeine occurring in 88% of samples at a median concentration of 24.4 ng/L, but exceeding 400 ng/L (maximum, 564 ng/L) in a few samples (Leung et al. 2013). Carbamazepine, sulfamethoxazole, ibuprofen, bisphenol, caffeine, and a significant concentration (102–104 ng/L) of a number of Endocrine disrupting substances were also detected from 13 groundwater (Lapworth et al. 2012). Soils that were variously irrigated with wastewater were also accumulating different types of pharmaceuticals. Total concentrations of ciprofloxacin, sulfamethoxazole, and carbamazepine increased with irrigation duration reaching 95% of their upper limit of 1.4 mg/kg (ciprofloxacin), 4.3 mg/kg (sulfamethoxazole), and 5.4 mg/kg (carbamazepine) in soils irrigated for 19–28 years (Dalkmann et al. 2012).

The pharmaceutical products have become a major problem that contributes negatively to the environment. The identified problems associated with their presence include the fact that these compounds are biologically active, some of them are toxic in nature, and a number of compounds have the potential to foster and maintain drug-resistant microorganisms. Antibiotics and disinfectants are supposed to disturb the wastewater treatment process and the microbial ecology in surface waters (Kümmerer 2001). The anti-tumor agents such as cyclophosphamide and ifosfamide were not biodegraded by municipal sewage treatment plants and can persist in the aquatic environment and to enter drinking water via surface water. Such pharmaceuticals are connected to the input of genotoxic mutagenic, teratogenic, fetotoxic, and carcinogenic effects in the aquatic environment (Kümmerer 2001, 2009).

Drinking water with low levels of cytotoxic drugs could also cause damage to the most vulnerable members of our society, the unborn, babies, and children (O ’keefe 2011). A similar link is also established between the ingestion of these compounds through drinking water and human health

(Rahman et al. 2009). There is a limited understanding of chronic exposure but significant research findings demonstrate reproductive disorders in males along with elevated incidences of various forms of cancer(Emmanuel et al. 2002; Sumpter 2007). They have adverse effects on ecosystem health, notably in the fish population; the observed effects in fish include intersex changes, impaired reproductive potential, and tissue accumulation of active substances (Wennmalm, 2011). A similar study indicated that Ethinyl estradiol, is adversely affecting fish through its "feminization" of males. Diclofenac has also caused the deaths of millions of vultures in Southeast Asia through its use in veterinary medicine (Sumpter 2007).

Another study also showed that 29 of the 53 total analyzed hospital wastewater samples were toxic to P.subcapitata (between 18 and 55 % inhibition), whereas only 8 samples were toxic to C. Vulgaris (between 21 and 50 % inhibition). Of the two tests used, P.subcapitata was the most sensible, resulting in the most suitable species to be used in hospital wastewater monitoring (Magdaleno et al. 2012). A 24-h EC_{50} on Daphnia Magna demonstrated the high toxicity effect of hospital wastewater (Emmanuel et al. 2004). This high toxicity is due probably to the presence of organohalogen compounds as a result of the use of the hypochlorite sodium and some iodized substances in the disinfection of hospital effluents (Emmanuel et al. 2002). Detailed characterization of pollutants released from our country's hospitals wastewater were not thoroughly investigated.

2.2. Hospital Wastewater Treatment Technologies

Hospital wastewater contains several pollutants that are resistant to biological degradation. Some emergent pollutants released from hospitals showed resistance toward conventional biological treatment processes. These complex matrixes showed resistance toward conventional activated sludge biological treatment process (Kajitvichyanukul and Suntronvipart, 2006). Therefore, traditional wastewater treatment (activated sludge) does not eliminate most of the so-called "emerging pollutants" (Polar, 2007). Emmanuel (2002) also points out that these emerging pollutants leave most wastewater treatment plants (WWTP) without any degradation. By leaving the WWTP, these chemical compounds can provoke the pollution of the natural environment by entailing a biological imbalance.

Several researchers use different technologies to remove pollutants from hospital wastewater. Conventional and other advanced wastewater treatment systems may play a key role in the treatment of hospital wastewater because of their high removal capacity of BOD, COD, nutrients, and bacteria. Activated sludge treatment and oxidation ditch were less effective in removing bacteria and parasites from hospital effluent. Exceeded levels of total coliforms and fecal coliforms were found in 5.6% and 20.8% of samples in 8.3% and 41.7% of treated hospital wastewater, respectively. Pathogenic bacteria like vibrio and Salmonella sp. were also found in 3.1% of samples and in 12.5% of treated hospital wastewater (Danchaivijitr et al. 2005). Another study also showed that enterococci were decreased below the detection limit in the MBR and indicator organisms such as fecal coliforms were decreased by 1.4 log units in the CAS system compared to a 3.6 log removal in the MBR (Pauwels and Verstraete 2006).

The activated sludge process with extended aeration and sequencing batch reactors (SBR) was used in Iran to treat hospitals' wastewater. The results showed that the BOD, COD, and TSS removal efficiency of extended aeration system was 91%, 90.8%, and 95.7%, respectively, while these values for the SBR system were found to be 91.7%, 91.9%, and 95.3%, respectively (Heidari et al. 2016). A similar study carried out by Amouei et al. (2012) showed that the BOD, COD, TSS, and total coliform (TC) removal efficiency of extended aeration method was 74.3%, 79.6%, 76.5%, and 99.7%, respectively. Hashemzadeh et al. (2017) also indicated that the BOD, COD, TSS, and TC removal of Extended Aeration Biological Systems was 85.21%, 82.46%, 86 %, and 90.15%. It was also illustrated that the removal efficiency of 88.5% and 92.5% for turbidity and COD were achieved by Conventional activated sludge (CAS), respectively (Karami et al. 2018).

In another study, an integrated anaerobic-aerobic fixed-film reactor with arranged media, fed with hospital wastewater, achieved COD removal efficiencies of 95.1%. It was found out that most of the COD were removed through aerobic oxidation (85%), while the anaerobic removal was only 15% (Rezaee et al. 2005). Similarly, the average removal efficiency of moving bed biofilm reactor (MBBR) with polyethylene media as a biofilm support carrier was 79.5%, 74.5%, 78%, and 79% for BOD_5, COD, TSS, and nitrogen, respectively (Jasem et al. 2018).

MBR treatment was highly efficient for hospital wastewater treatment in terms of the removal of solids and nutrients and can be a suitable pre-treatment solution for further trace pollutant removal (Beier et al. 2012). The percentage removal efficiency of reverse osmosis (RO) and ultrafiltration (UF) membrane for COD and BOD were also found to be more than 99% for RO and more than 97% for UF. The TSS and TDS were found to be removed almost 100% for both units. The removal of TN, NH_4, and NO_3 was 85%, 95%, and 95% respectively for UF and it was 90%, 98%, and 96% respectively for RO. The performance of RO found to be much better than UF (Jadhao and Dawande 2012).

MBR also removed NH_3-N, NO_2-N, NO_3-N, and PO_4-P by 82.6, 82.1, 82.1, and 60.2%, respectively (Kootenaei et al. 2013). A membrane bioreactor (MBR) equipped with nano-membranes (NF-MBR) under a pilot-scale test has also shown that high COD and NH_3-N removal efficiencies of 92 ± 4% and_88 ± 2, respectively and PO_4-P removal efficiency of 68% (Aliahmad and Dehbashi 2013). Another study by Sani and Dareini (2014) also showed that the removal rate of TN and BOD_5 parameters for vetiver plant-based CWs were 88.46% and 88.54%, respectively, and 75.03% and 82.52% for the reed bed CWs.

Some conventional and high-grade treatment plants have effectively removed micropollutants from hospital wastewater. A study done in India showed that the activated sludge treatment systems effectively remove genotoxicity and cytotoxicity substances from hospital wastewater (Sharma et al. 2014). Similarly, extended aeration activated sludge (EAAS) system effectively remove tetracycline (TC) from the same wastewater (Neisi et al. 2017). A batch fluidized bed bioreactor under sterile and non-sterile conditions also removed PhACs and EDCs with the overall load elimination of 83.2% and 53.3% in their respective treatments (Cruz-Morató et al. 2014). Similarly, Aerated Fixed Film Biofilter with Ozonation (AF_2B/O_3) reactor removes BOD_5, fecal coliforms, phenol, and Pb pollutants by 97.92 %, 99.23 %, 100%, and 100%, respectively. Thus, it can be concluded that AF_2B/O_3 process has a large capability of pollutant removal in hospital wastewater (Kusuma et al. 2014).

On the other hand, a pilot-scale hospital wastewater treatment plant consisting of five post-treatment technologies including ozone (O_3), O_3/H_2O_2, powdered activated carbon (PAC), and low-pressure UV light with and without TiO_2 was tested to eliminate 56 micropollutants. The results showed that ozonation, PAC, and UV eliminate 90%, 86%, and 33% of micro pollutants, respectively (Kovalova et al. 2013). A full-scale MBR with further treatment technologies of NF (nanofiltration) as well as an RO (reverse osmosis) was evaluated to remove pharmaceutical residues from hospital wastewater. The results showed that RO is highly efficient to remove pharmaceuticals than NF (Beier et al. 2010). Alrhmoun et al. (2014) also showed that MBR achieved very good organic removal efficiencies and high removal efficiencies for more than ten different pharmaceuticals compounds. Researches on the treatment of hospital wastewater by constructed wetland systems and their performance to remove various pollutants and antibiotic-resistant bacteria and other forms of pathogens especially from hospital origin however are limited.

2.3. Constructed Wetlands

“Constructed wetland is a shallow, manmade, complex, engineered, marsh-like area which is designed, constructed and operated to treat wastewater by attempting to optimize the physical, chemical, and biological process of natural ecosystem.” It can be effective, reliable, simple, and relatively inexpensive as compared to conventional systems (GIZ, 2011). Constructed wetlands as a reasonable option, have recently received considerable attention to treat a wide variety of wastewater throughout the world.

They are preferred as the wastewater treatment option because of low construction, operation and maintenance costs, process stability, high nutrient absorption capacity, simplicity, low energy consumption, low excess sludge production, the potential for creating biodiversity, and add aesthetic, ecological, and cultural values. If properly built, maintained and operated, constructed wetlands can effectively remove many contaminants including nitrogen (N), phosphorus (P), mineral oils, pathogens, trace contaminants (such as pesticides, heavy metals, radionuclides), and emerging pollutants (such as brominated flame retardants, estrogenic compounds). They can be used for primary, secondary and tertiary clean-up of not only municipal wastewater but also agricultural effluents, landfill leachates, storm water, polluted river water, urban runoff, food wastes, abattoir effluent, acid mine drainage, industrial effluents, as well as petrochemicals (GIZ 2011; Qasaimeh et al. 2015; USEPA 2000; Vymazal 2011).

Constructed wetlands are complex, well-established, self-contained, integrated, and environmentally friendly alternative treatment systems that use natural processes involving water, wetland vegetation, gravel/soils, animals, environment, and their associated microbial assemblages for sewage treatment, pollution control and environmental improvements (Qasaimeh et al. 2015). Constructed wetlands are now widely accepted as a viable, cost-effective, ‘natural’ wastewater treatment technology, particularly appropriate for small communities, towns, industries, and agriculture (Zhang et al. 2010).

2.3.1. Types of Constructed Wetlands

Constructed wetlands are characterized as simulated wastewater treatment plants containing low (typically under 1 m significant) channels that were inserted with aquatic plants treated through various methods. CWs have basic classification based on the type of macrophytic growth; further classification is usually based on the water flow regime. CWs can be designed in a variety of hydrologic modes. However, nowadays the two main types of CWs are categorized as surface flow (SF) and subsurface flow (SSF) CWs (Vymazal 2007a). SSF CWs are further subdivided into the horizontal flow (HSSF) and vertical flow (VSSF) systems depending on the direction of water flow through the porous soil (usually sand or gravel) (Kadlec and Wallace 2009).

Subsurface flow constructed wetlands can be filled with different media types (such as crushed rock, small stones, gravel, sand, or soil) and which can be planted with aquatic plants. The pollutant removal performance of these systems depends on many factors including influent wastewater quality, hydraulic and pollutant loading, climate, and the physical characteristics of the system. SF CWs are vegetated systems with open water surface and typically have water depths of less than 0.4 m. In SSF CWs, no free water is visible because the water flows through a porous medium planted with emergent water plants (helophytes) (Jethwa and Bajpai 2016). The main advantage of a SSF system over a free water surface (FWS) wetland system is the isolation of the wastewater from vectors, animals, and humans (USEPA 2000). Constructed wetlands may be planted with a mixture of submerged, emergent, and in the case of FWS, floating vegetation.

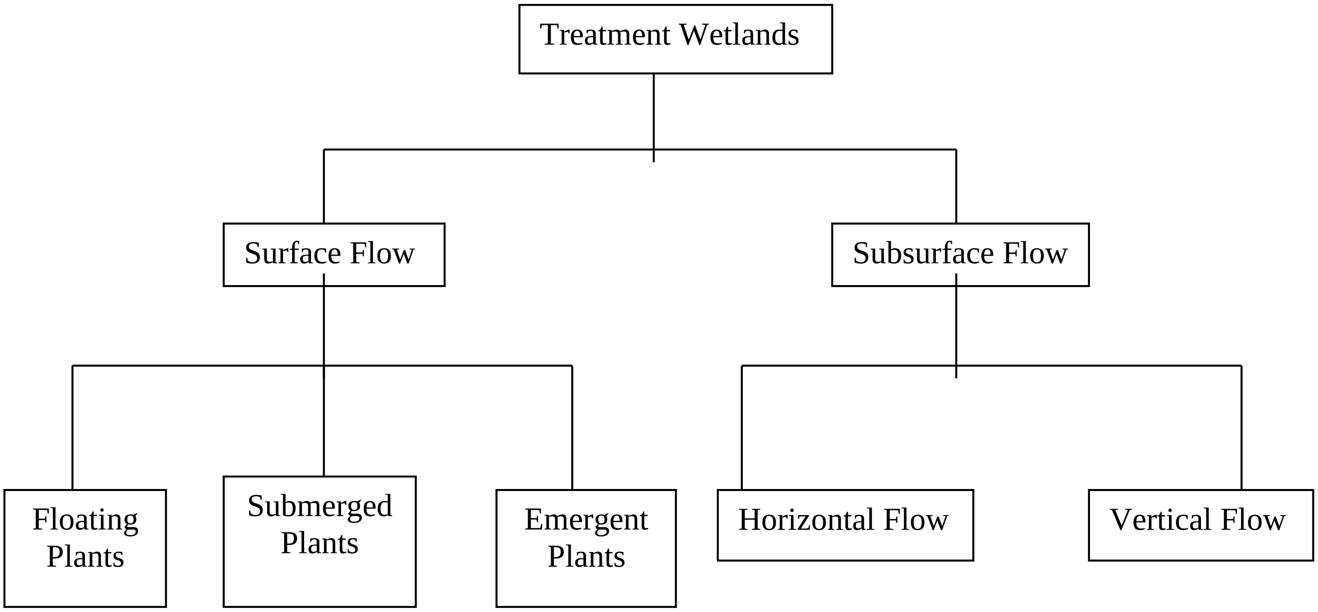

Figure 1. Classification of constructed wetlands, source: Almuktar et al. (2018)

2.3.1.1. Free Water Surface Systems (FWS)

Free water surface (FWS) flow constructed wetlands nearly resemble natural wetlands in appearance and function, with a combination of open-water areas, emergent vegetation, standing water on the surface usually 0.5 to 1 ft deep, and other typical wetland features. These systems basically consist of basins or channels, lined to prevent seepage containing soil or another growth medium to support emergent vegetation. The wastewater flows at a shallow depth above the soil surface that is exposed to the atmosphere on a year round basis (Abdel-Sabour 2014). These systems generally are cheaper to install (no gravel media), simple hydraulics, fewer clogging problems, and maybe more suitable for most communities (USEPA 2000). Preliminary treatment must be provided prior to entry into the system to reduce the influent solids.

Water found near the bottom of the wetland is in an anoxic state which inhibits nitrification (Vymazal 2007b). Near the surface, there is aerobic water condition which is aided by emergent plants. Oxygen is available at the water surface and on the living plant surfaces, root, and rhizome surfaces so aerobic reactions are possible within the system (Bendoricchio et al. 2000). Mosquito control can be an issue with this system due to pond type conditions and areas of open water. These conditions also attract a variety of other wildlife including fish, amphibians, reptiles, and birds. Besides municipal wastewater, FWS CWs with emergent vegetation has been used to treat various types of wastewater. They are the most commonly used for advanced treatment of effluent from secondary or tertiary treatment processes. SF wetland systems offer low construction cost, but they generally have a lower contaminant removal efficiency compared with SSF systems (Vymazal 2010).

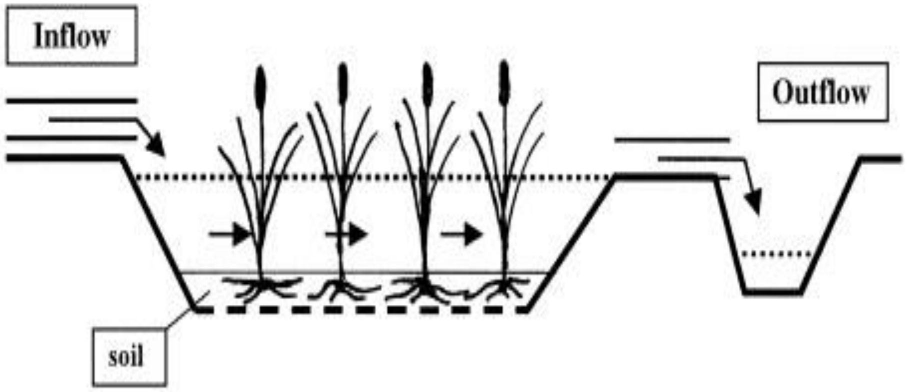

Figure 2. Free water surface CW (Vymazal 2007a)

2.3.1.2. Horizontal Subsurface Flow Wetlands (HSSFW)

HSSF wetlands consist of a bed that may be planted with wetland vegetation in which the water level is completely below the porous media, i.e. gravel, sand or soil. Wastewater is intended to flow horizontally and stay beneath the media surface and flow around the rhizomes of the plants (Kadlec and Wallace 2009). The startup cost HSSF wetland is higher than free water surface wetlands due to the higher amount of media required. These systems can easy applied in cold weather areas and where mosquito control is a priority. DO levels in these wetlands are generally too low to provide adequate nitrification for a large scale operation (Vymazal 2009). Because of this, denitrification is the dominant removal mechanism of nitrogen from the system.

Subsurface flow wetlands are generally applied to serve individual septic tank systems or small communities. The choice of treatment media (gravel shape and size, for example) and selection of vegetation is essential to boost the treatment performance. The materials used as CW filling can directly contribute to the removal of pollutants from the wastewater as a base for the vegetation and a layer of attachment for microorganisms to form a biofilm for degradation as well as acts as a filter and a trap for removing solids and adsorb pollutants depending on the media type (Rana and Laura 2014). In HSSF wetlands, facultative and anaerobic bacteria degrade most organics; the vegetation transfers a limited amount of oxygen to create an aerobic condition in their root zone to colonize the area by aerobic bacteria to degrade organics as well (Abdel-Sabour 2014).

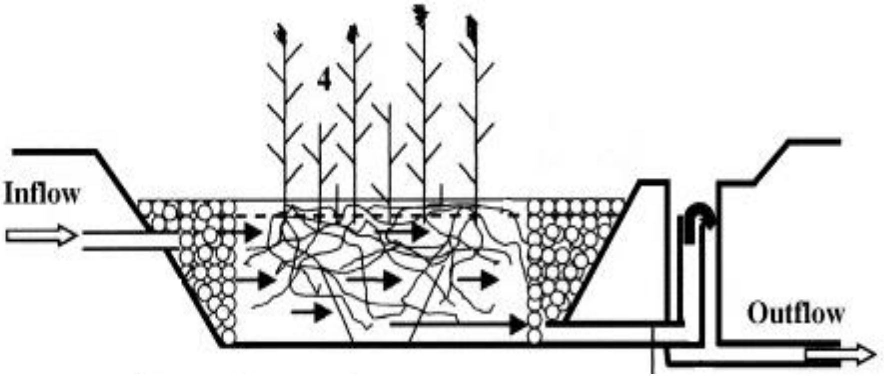

Figure 3. Horizotal subsurface flow CW (Vymazal 2007a)

2.3.1.3. Vertical Subsurface Flow Wetland

Vertical subsurface flow constructed wetlands (VF CWs) basically filter beds that are planted with aquatic plants. Wastewater flows from the top and gradually penetrates down through the filter media and collected at the bottom of the bed by drainage pipes (USEPA 2000). VSSF CWs consist of sand or gravel bed typically planted with wetland vegetation and water is equally distributed across the wetland surface. An essential factor is that the bed has to completely drain free and it allows air to refill the bed. This process leads the CWs to have good oxygen transfer and hence the ability to nitrify. As HSSF wetlands have a limited capacity to oxidize NH_4^+ due to limited oxygen transfer, VSSF CWs were developed to provide higher levels of O_2 transfer, thus producing a nitrified effluent.

The biggest difference between a VF and HF wetland is not the direction of the flow, but the aerobic conditions. The amount of oxygen is far greater in a VF bed. Its design is rather complicated and needs more knowledge and materials. This process, therefore, provides alternating oxidizing/reducing conditions in the soil promoting alternating nitrification and denitrification reactions, effectively removal of BOD_5, COD, and pathogen and P adsorption. It also has an advantage in sizing because it requires a considerably smaller area than the HF system. As compared to HSSF CWs, VSSF CWs require less land (Brix & Arias 2005). Such CWs can clean contaminated waters with very high concentrations of contaminants even during cold winters (Brix et al. 2002). The ability of VSSF wetlands to oxidize NH_{4+} has resulted in their use in applications with higher NH_4^+ than municipal or domestic wastewater. Landfill leachates and food processing wastewaters can have NH_4^+ levels in the hundreds of milligrams per liter, and the key to reduction is the ability to nitrify. Thriving VSSF wetlands, therefore, have formed part of the treatment process for those wastes (Kadlec and Wallace 2009).

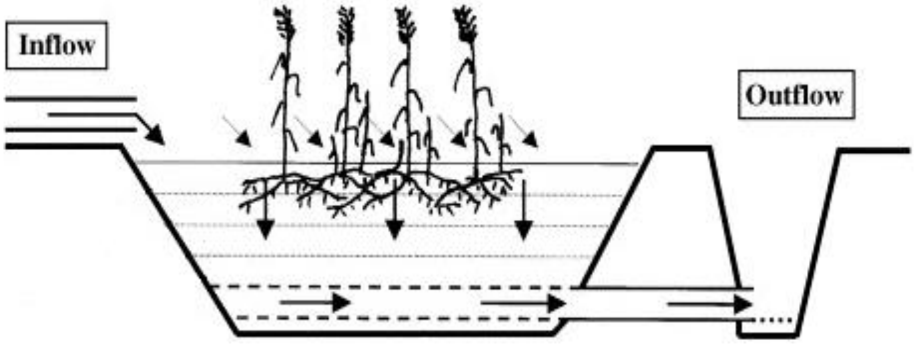

Figure 4. Vertical subsurface flow CW (Vymazal 2007a)

2.3.2. Pollutants Removal Mechanism of HSSF CWs

Constructed wetlands are highly complex systems that separate and transform contaminants by physical, chemical, and biological mechanisms like filtration, absorption, adsorption, ion exchange, sedimentation, precipitation, plant uptake, microbial degradation, photolysis, volatilization and nitrogen transformations (GIZ 2011) that may occur under aerobic, anoxic, or anaerobic conditions. Several researchers have intensively reviewed the removal mechanisms of different pollutants in horizontal subsurface flow constructed wetlands. The predominant mechanisms and their sequence of reactions are dependent on the external input parameters to the system, the internal interactions, and the characteristics of the wetland. The external input parameters most often of concern include the wastewater quality and quantity and the system hydrological cycle.

2.3.2.1. Suspended Solids Removal Mechanism

Removal of suspended solids by SSF is greatly attributed by physical processes like mechanical filtration and microbial breakdown of an organic portion of suspended solids (Gupta et al. 2016). Additionally, flocculation and settling of colloidal by sedimentation, straining and entrapment, and adsorption onto a substrate and plant media play a significant role in their reduction (USEPA 2000). These systems are capable of achieving higher efficiency of suspended solids' removal from the water column because of the relatively low velocity and high surface area in the SSF media. HSSF wetlands are typically better at TSS removal than BOD removal, because TSS removal is a completely physical mechanism. Suspended matter in water may contain a number of contaminants, such as nutrients, heavy metals, and organic compounds. In cases where particulate matter considered as the bulk of contaminant load, the physical settling of suspended solids can result the efficient removal of the contaminants from the wastewater streams. Particles that are larger than pore size may be trapped within the filter media (Abdel-Sabour 2014).

2.3.2.2. Organic Matter Removal Mechanism

BOD is the commonly used parameter for available biological carbon, which is a measure of the rate of O_2 consumption by micro-organisms utilizing the available organic carbon in the water or soil. Wetlands contain a vast number of organic carbon utilizing micro-organisms adapted to the aerobic surface waters and anaerobic soils (Abdel-Sabour 2014). Bacterial degradation may play a key role in the removal of BOD and COD in HSSF CWs to change into CO_2, H_2O, and NH_3 by micro-organisms attached to plant and sedimentation surface (Abdul and Ganapathyvenkatasubramanian 2016). The organic matter is dominantly degraded by facultative and anaerobic heterotrophic microorganisms in the wetland reactors due to minimal oxygen concentration in the bed (USEPA 2000). Furthermore, filtration, adsorption, sedimentation/interception, and oxidation are also responsible for their reduction in wetlands (Skoczko et al. 2017; Lee et al. 2004).

Oxygen sources to the SSF wetlands would be limited to some small amount of surface aeration and plant-mediated transport. The presence of plant root structure in planted wetlands would provide additional surface for biofilm attachment. Macrophytes may also contribute some oxygen to the granular bed that makes aerobic metabolism occur in the beds. However, reviewed literature indicated that the rates of oxygen transport by macrophytes are very thin restricted to the soil layer adjacent to the roots (Vymazal 2011; Vymazal and Kröpfelová 2009). Thus, the predominant metabolic pathways are most likely anaerobic. The anaerobic pathways for the removal of BOD from the system would be methanogenesis, sulfate reduction, or denitrification, all yielding gaseous end products. The organic matter from the influent to the system will most likely be degraded by a biofilm of microorganisms attached to the media, plant roots, and plant litter accumulated at the bed surface or within the interstices of the media.

2.3.2.3. Nitrogen Removal Mechanism

Nitrogen is a major component of municipal wastewater and wastewater from various types of health care institutions. Environmental and health problems associated with excessive amounts of certain forms of nitrogen have been well documented (Braun 2007; Suddick et al. 2012). High concentrations of nitrate in drinking water can cause methemoglobinemia or "blue baby" syndrome, in infants (Braun 2007). Wetlands are generally well suitable for nitrogen removal (Vymazal 2007b). Several researchers have intensively reviewed the removal mechanisms of nitrogen and phosphorus in subsurface flow constructed wetlands (Shi et al. 2018; Vymazal 2007b). The removal of nitrogen and phosphorus varies greatly depending on the loading rate of the wastewater, type of substrate, and the type and composition of the wastewater. The nitrification processes in SSF wetlands are negligible due to insufficient oxygen supply in the filtration beds.

Any nitrification occurring may be found in the root zone that is adjacent to rhizomes or near the bed surface, where some surface oxygen transfer might occur (Vymazal 2007b). Therefore, nitrogen removal in SSF wetlands mostly depends on denitrification, assimilation, volatilization, nitrogen fixation, and plant uptake through microbial activity (Shi et al. 2018). Based on the filtration materials used adsorption and burial also play a significant role to retain nitrogen in the beds of constructed wetlands (Shi et al. 2018); however, such removal is not considered to be a long-term sink due to the adsorbed nitrogen is released easily when water chemistry conditions change. While, organic nitrogen from dead plant material can accumulate in the soil as peat, a long term storage mechanism (Abdel-Sabour 2014). Harvesting of plant biomass is another removal mechanism since nitrogen is an essential plant nutrient and stored as an organic form in wetland vegetation (Wu et al. 2013).

2.3.2.4. Phosphorus Removal Mechanism

Phosphorus is present in the water in the form of orthophosphate and organic phosphorus. Phosphorus like nitrogen is a major plant nutrient. Its addition to the environment often contributes to eutrophication of lakes and coastal waters as well as downgrading of the quality of water bodies and impacts on industries such as shellfish growing and tourism (Akunna et al. 2017). The removal of phosphorus in CWs occurs through various processes such as adsorption on the media surface, complexation, bacterial assimilation, precipitation, accretion, chemical transformations, retention in sediments, and plant uptake (Vymazal 2007; Avsar et al. 2007). Adsorption has been considered to be the most important mechanism for phosphorus removal based on substrate media type (Bama et al. 2013).

Adsorption of phosphorus occurs due to reactions with iron, calcium, and magnesium present in sediments. Precipitation reactions can occur when the wastewater comes into contact with available aluminum, iron, calcium, and other clay minerals in the substrate (Abdel-Sabour 2014). Plant uptake may be another essential process and this is dependent on their growth rates, the sediment characteristics, the oxygen transfer capability of the plants into the root-zone, plant density per unit area, the concentration of nutrients in the plant tissue, the ultimate potential for biomass accumulation, plant harvesting and climate (Reddy et al. 1995). Phosphorus is removed from the water and is either adsorbed to the metals ions or taken up by plants or fixed in the clay minerals.

The adsorbents that have been tested by different scholars for the removal of phosphorous as constructed wetland substrate include broken brick (Wang et al. 2012; Mateus et al. 2016), basic oxygen furnace slag (BOFS) (Hussain et al. 2015), biochar (Gupta et al. 2016), dolomite (Žibienė et al. 2015), laterite (Mansing and Rout 2013), zeolite, limestone, calcite and other substrates rich in iron, aluminum, and calcium (Yun et al. 2015). Broken brick is considered to be a universal absorbent because of its application in the removal of fluoride from groundwater in many countries (Kumar et al. 2017; Panchore et al. 2016). It is also well known to remove phosphorus as it has a greater surface area to provide better adsorption (Mateus et al. 2016). Additionally, Wang et al. (2012) stated that broken brick was appropriate for the enrichment of microorganisms and the growth of plants as a filter medium in CWs. The contents and chemical forms of broken brick could also be the principal factors for phosphorus removal by precipitation process (Wang et al. 2012).

2.3.2.5. Trace Metal Removal Mechanism

The major metal removal mechanisms in wetlands include cation exchange and chelation with wetland soils and sediments; binding with humic materials; precipitation as insoluble salts of sulfides, carbonates, and oxyhydroxides; and uptake by plants, algae, and bacteria. Metals may be incorporated into the wetland biomass. For macrophytes, metals are taken up through the root system and distributed through the plant (Wang et al. 2014). The extent of uptake is dependent on the metal species and plant type. A number of metals, including Cd, Cu, Ni, Pb, and Zn, forms nearly insoluble compounds with sulfides under anaerobic conditions in wetland soils (Abdel-Sabour 2014). These bound metals are often not bio-available and removed from the system. Studies have shown that metals like cadmium, chromium, copper, lead, mercury, nickel, and zinc can be sequestered by wetland soils and biota or both (Chen et al. 2009; Dufresne et al. 2015).

2.3.2.6. Removal of Toxic Organic Compounds

Organic compounds include pesticides, fertilizers, process chemicals, pharmaceuticals, and other multitude degradation-resistant plus toxic natural and human-made organic compounds that may be present in wastewater. The possible mechanisms for removal of this organics in wetlands are volatilization, photochemical degradation, sedimentation/interception, biodegradation, adsorption, and uptake (Ávila et al. 2015). The substrate soils may absorb organic compounds via chemisorption or physical adsorption (USEPA 2000). Microbes are capable of degrading most classes of organic pollutants, but the rate of degradation varies considerably, depending on the chemical and structural properties of the organic compound and the chemical and physical environment in the soil.

2.3.2.7. Fecal Coliforms and Pathogens Removal Mechanisms

Wetland systems have excellent pathogen removal capability. The removal of indicator, pathogenic and antibiotic-resistant bacteria in wetlands is mostly caused by filtration, sedimentation, adsorption, aggregation, natural die-off, the influence of toxins from plants and other microorganisms, unfavorable water chemistry, biofilm interaction, exposure to biocides and oxidative damage (Bôto et al. 2016; Karimi et al. 2014; USEPA, 2000). Competition with the consortium of organisms surrounding them and predation also plays a great role in the destruction of many bacteria (USEPA 2000). As intestinal organisms, most pathogenic bacteria normally require a rich substrate and high temperature to compete favorably. Most will not survive in this competition. They will also be destroyed by UV irradiation if they are near the open water surface.

Their removal appears to be correlated with suspended solid removal and hydraulic residence times (Karimi et al. 2014; USEPA, 2000; Gonzalez et al. 2009). Li et al. (2015) indicated that longer retention periods in wetlands increased bacterial deposition rates in sand columns. The concentration of fecal bacteria, protozoa, helminthes, and viruses are removed by disinfection mechanisms through extended retention in the media, exposure to unfavorable environmental conditions, and grazing by free-living protozoa. Macrophytes play an essential role for fecal pathogen removal by the supply of oxygen to the roots for the grazing predators like protozoan, nematodes, zooplankton, and lytic bacteria and viruses. Macrophytes also affect fecal pathogens by excreting toxic antimicrobial substances from their roots.

2.3.3. Plant Species Used in Constructed Wetlands

The larger aquatic plants growing in natural wetlands are commonly called macrophytes, which include aquatic vascular plants, aquatic mosses, and some larger algae that have tissues and are easily visible (Brix 2013). A wide variety of macrophytes can be used in constructed wetlands designed for wastewater treatment. The suitable species that are selected to use in wastewater treatment wetlands depend on local conditions, the water depth, the design of wetland (surface or subsurface flow), characteristics of the wastewater, quality and quantity of the wastewater loads, medium type, and plant management, such as harvesting regime.

The plants used in HF CWs designed for wastewater treatment must be ecologically friendly, i.e. it shouldn’t have significant weed or disease risk or danger to the ecological or genetic integrity of surrounding natural ecosystems; should tolerate local climatic conditions, pests, and diseases; should be resistant to high organic and nutrient loading; easy to propagate, and rapid spread and growth; good pollutant removal capacity, either through direct assimilation and storage or indirectly by enhancement of microbial transformations, such as nitrification (via root-zone oxygen release) and denitrification (via the production of carbon substrates); Have rich below ground organs (i.e. roots and rhizomes) in order to provide the substrate for attached bacteria and oxygenation (even very limited) of areas adjacent to roots and rhizomes and have high above-ground biomass for winter insulation in cold and temperate regions and for nutrient removal via harvesting (Almuktar et al. 2018).

Most frequently, it is recommended to use plant species that are locally available in nearby natural wetlands and have confirmed endurance and purification capacity. The mechanisms by which plant species enhance the treatment efficiency of CWs are well known. However, types of plants grown whether they are native or exotic, their level of tolerance to pollutant load, oxygen supply to their roots, type of microbial growth on the root surface, affect the performance (Jethwa and Bajpai 2016).

The macrophytes growing in wetlands may be classified in the following major groups according to their life form:

(1) **Emergent aquatic macrophytes**: These are dominantly found in wetlands and marshes, growing within a water table range from 50 cm below the soil surface to a water depth of 150 cm or more. They produce aerial stems and leaves and extensive root and rhizome system. The plants are morphologically adapted to growing in a waterlogged or submersed substrate by virtue of large internal air spaces for transportation of oxygen to roots and rhizomes. This life form comprises species like Common Reed (Phragmites australis), cattail (*Typha latifolia, T.orientalis* or *T.domengensis*), Bulrushes (Scirpus sp.), *Cyprus papyrus* and *Zizania aquatica* (Wild Rice). These emergent plants play a fundamental role in the removal and retention of nutrients in a constructed wetland (Prasad and Jasutkar 2016). These plants are stable towards the climatic changes and the type of medium in which it is grown.

(2) **Floating-leaved aquatic macrophytes**: The freely floating species are highly diverse in form and habit, ranging from large plants with floating leaves and well-developed submerged roots, to minute surface-floating plants with few or no roots (Jethwa and Bajpai 2016). The floating plants are the species which are free floating on the water surface and used in the aquatic plant treatment system. A wide range of aquatic plants have been used to assist in the breakdown of wastewater. The Water Hyacinth (Eichhornia crassipes), and Duckweed (Lemna) are some floating aquatic plants frequently used to reduce concentrations of BOD, TSS, total phosphorus, and total nitrogen (Arias et al. 2016; Gupta et al. 2012; Ingole and Bhole 2002).

(3) **Submerged aquatic macrophytes:** These have their photosynthetic tissue entirely submerged but usually the flowers exposed to the atmosphere. Two types of submerged aquatics are typically

recognized: the elodeid type (e.g. Elodea, Myriophyllum, Ceratophyllum), and the isoetid (rosette) type (e.g. Isoetes, Littorella, Lobelia).

2.3.4. The Role of Wetland Plants in Constructed Wetlands

The role of plants in the performance of constructed wetlands treating domestic sewage is a matter of considerable debate among researchers because of the multiple angles that surround this analysis: physical influence of the roots in the hydraulic conductivity of the units, possibility of oxygen transfer through the root zone, the release of exudates, contaminant adsorption in the biofilm zone around the roots, nutrient uptake from the plants, and other aspects. The degree of influence is dependent on several factors, such as plant species, influent composition, the grain size distribution of the filter media, applied hydraulic and mass loading rates, and geometric configuration of the wetland units (da Costa et al. 2015). The significant roles of macrophytes in constructed treatment wetlands are (Prasad and Jasutkar 2016):

2.3.4.1. Physical Effects

The presence of vegetation in wetlands is mainly distributing and reducing the flow velocities of the water in the bed (Shelef et al. 2013). This creates better conditions for sedimentation of suspended solids, decreased re-suspension of settled material, prevention of clogging, improves hydraulic conductivity of the substrate or media, and increases the contact time between the water and the plant surface areas (Jethwa and Bajpai 2016). The movements of plants due to wind and the growth of roots within the filter medium also help to decompose organic matter and prevent clogging. Another important effect of the plants is facilitating physical filtration of wastewater, making shade to prevent algal growth, and provides a perfect insulation from radiation in the spring and frost in the winter (Prasad and Jasutkar 2016).

2.3.4.2. Surface Area for Attached Microbial Growth

The roots and stems of macrophytes that are submerged in the wetland soil provide a vast surface area for attached growth of microbial biofilms. These biofilms are responsible for biodegradation of organic matter taken place in constructed wetland systems (Jethwa and Bajpai 2016). The plant tissues are colonized by dense communities of photosynthetic algae as well as by bacteria and protozoa (Shelef et al. 2013). Thus, biofilms are present on both the above and below-ground tissue of the macrophytes. Since microorganisms are considered to be key drivers in the treatment process, any factor that changes their composition has a significant impact on the biodegradation efficiency of the whole CW (Prasad and Jasutkar 2016).

2.3.4.3. Nutrient Uptake

Wetland plants require many macro and micronutrients for growth and reproduction. Nitrogen (N) and Phosphorus (P) are key nutrients in the life cycle of wetland plants and the rooted macrophytes utilizing N, P, and other nutrients for growth and simultaneously reduce their concentrations that would be considered pollutants in CWs (Jethwa and Bajpai 2016). Plants can also accumulate phytotoxic elements, such as heavy metals, in vascular or granular compartments (Shelef et al. 2013). Wetland plants can be bound considerable amounts of nutrients in their biomass and can remove nutrients if the biomass is harvested (30 to 150 kg P/ha/year and 200 to 2500 kg N/ha/year). Plants play a vital role in the assimilation of nutrients, but as the plants senesce, some nutrients are released back into the water. Parts of the nutrients are retained in the undecomposed fraction of the plant litter and accumulate in the soils. Wetland plants are able to tolerate high concentrations of nutrients and in some cases even to accumulate more nutrients than are needed for growth when supplemental nutrients are available (luxury uptake). However, the concentrations of nutrients that can be removed by harvesting are generally negligible compared to the loading into the constructed wetlands with the wastewater (Prasad and Jasutkar 2016).

2.3.4.4. Root Release of Gas

Another key effect of aquatic macrophytes in CWs is that, they translocate oxygen from the shoots into the rhizosphere and that this release influences the biogeochemical cycles in the sediments. Hollow vessels of the plant tissues are able to transport oxygen from the leaves to the root zone and to the surrounding soil (Jethwa and Bajpai 2016). The oxygen leakage at the root-tips has been shown to have a significant impact on important mechanisms of wastewater treatment in CWs, including increases soil redox potential and aerobic niches, improves aerobic decomposition of organic matter, supports heavy-metal sedimentation, enhances microbial density and activity, oxidized and detoxify potentially harmful substances, and increases nitrification (Prasad and Jasutkar 2016). However, some researchers argued that root-derived aerobic dynamics is very limited in horizontal CWs and its role is minor in periodically loaded vertical CWs with short hydraulic-retention times (Shelef et al. 2013; USEPA 2000).

2.3.4.5. Other Functions

Plants have additional values by providing habitat for wildlife and making wastewater treatment systems aesthetically pleasing (Jethwa and Bajpai 2016). Additionally, eliminate pathogens, insects, and offensive odors as well as being a source of carbohydrates for microbes, use as halophyte (salt-tolerant plant) for salt phytoremediation, and the use of plants as bioindicators (Shelef et al. 2013).

2.3.5. Types of Wetland Plants

2.3.5.1. Bulrushes Sp.

Bulrush is a perennial, emergent, grass-like wetland plant often found growing in large colonies. They may have a triangular shaped stem, often hollow, with long, slender green leaves that appear to be a continuation of the stem similar to those of grasses. Bulrush can grow 5' to 10' above the water with a cluster of small reddish-brown flowers and seeds at the tip of the stem. Bulrushes (Scirpus sp.) are ubiquitous plants that grow in a diverse range of inland and coastal waters, brackish and salt marshes and wetlands as well as capable of growing well in water that is 5 cm (2 in) to 3 m (10 ft) deep. Desirable temperatures are 16-27°C (61-81°F) and growing in a pH of 4-9.

Bulrush is a wetland plant that plays a significant role in the removal of different pollutants from different wastewater sources. It has also a higher potential for uptaking nitrogenous and phosphorus compounds. A report by Gersberg et al. (1986) showed that a reduction of ammonia concentration from 24.7 mg/l to 1.4 mg/l by bulrush bed as compared to a mean value of 22.1 mg/l for the unvegetated (control) bed. For the vegetated beds, the mean effluent ammonia values were significantly below that of the unvegetated bed and for the inflow. The bulrushes proved to be superior at removing ammonia, both with mean effluent levels considerably below that for the cattail bed. In a similar study, bulrush removes a higher amount of ammonia (85%) from wastewater (Richardson et al. 2000). The high ammonia-N (and total N) removal efficiencies shown by the bulrush bed is attributed to the ability of these plants to transport oxygen down to the roots, thereby establishing an oxidized rhizosphere (Bezbaruah and Zhang 2005; Gersberg et al. 1986).

Similarly, BOD removal efficiencies were highest in the bulrush bed than cattle and reeds, both with mean effluent BOD levels (5.3) significantly below that for the unvegetated bed (36.4 mg/l) and equal to or better than secondary treatment quality (30 mg I-') (Gersberg et al. 1986). Likewise, TSS efficiency was generally very high from 86.8 – 96.9%, and effluent concentrations were from 2.55 – 8.31 mg/l. The removal efficiency of bulrush was higher than the vetiver with 96.9% (Tan and Vu 2011). These results demonstrated that higher aquatic plants could indeed play a significant role in secondary and advanced (N removal) wastewater treatment by wetland systems, a role that is completely associated with their pollutant uptake capacity.

2.3.5.2. Typha Sp.

Typha sp. (*Cattails*) is erect rhizomatous perennial plants with joint less stems. The plants are up to 3 m tall with an extensive branching horizontal rhizome system. Leaves are flat or slightly rounded on the back, in their basal parts spongy. It is a plant of the warm temperate zone through to the tropics, where it is found at elevations up to 1,500 meters. Typha sp. grows best in areas where annual daytime temperatures are within the range 16-28°C but can tolerate 14-30°c. It prefers a mean annual rainfall in the range 400-1,400 mm, but tolerates 250-1,600 mm and prefers a pH in the range 6.5-7.5, tolerating 6-8. Plants can be very invasive, spreading freely at the roots when in a suitable site, and if rhizomes planted at approximately 1 m (3.3 ft) intervals, it can produce a dense stand within three months.

Cattail species are ubiquitous in distribution, hardy, capable of thriving under diverse environmental conditions, and easy to propagate and thus represent an ideal plant species for constructed wetlands. They are commonly found inhabiting shallow bays, irrigation ditches, lakes, ponds, rivers, and both brackish and freshwater marshes. It is the most frequently used in constructed wetlands with free water surface and HF CWs for various types of wastewater around the world, and they share common characteristics of high biomass productivity. Typha sp. is tolerant to high organic matter and nutrient concentrations, as it has been used in experiments/applications in which COD, TKN and TP concentrations were up to 4000, 360, and 71 mg/L, respectively.

The efficiency of Typha sp. planted setups in the open environment was fairly good for parameters like COD and BOD_5 which was showing reduction up to 82.5% for COD and 82.6% for BOD_5 and phosphates were removed up to 80.58% (Aziz et al. 2015). Similarly, Arivoli and Mohanraj (2013) showed that the removal efficiency of cattail planted at 36 hours HRT was found to be

84.66% for TDS, 92.90% for Turbidity, 80.53% for COD, 75.49% for BOD_5, 83.51% for PO_4^{-3}, 88.48 % for NO_3.

They are extremely efficient in removing nutrients from the water and are thus very effective filters. According to Gersberg et al. (1986), significantly higher ammonia-N removals were observed in the cattail bed. This could be due to the vital role of aquatic plants in translocating oxygen from the upper parts of the plants to the ends of the roots, which facilitates the nitrification of ammonia into nitrate. However, some authors have pointed out plant uptake as the main responsible factor for ammonia-N removal (Grismer and Shepherd 2011; Prasad et al. 2016). They are known to be tolerant of heavy metals, can colonize industrially degraded habitats, and capable of accumulating heavy metal ions preferentially from wastewater than from sediments. The accumulation of metals in plant organs attained the highest values in roots, rhizomes, and old leaves (Zingelwa and Wooldridge 2009).

Rhizofiltration was found to be the best mechanism to explain *Typha domingensis* phytoremediation capability (Hegazy et al. 2011; Mojiri 2012). Cattail has already been tested and found suitable for the removal of several organic compounds from wastewater, being commonly used in CWs. Typha sp. can tolerance higher pharmaceutical compounds concentration in the wastewater, with no visible signs of toxicity for the tested concentrations. Kucerak (2014) reported that they have higher removal efficiencies for all pharmaceutical compounds tested when compared to the unplanted control beds. They have already been tested and found suitable for the removal of several organic compounds from wastewater, being commonly used in CWs (Dordio et al. 2010)

2.3.5.3. Cyperus Papyrus

Cyperus papyrus widely referred to as papyrus, is one of the most prolific emergent macrophytes in African subtropical and tropical wetlands, often as an ornamental species, to other warm parts of the world and growing in both lentic and lotic environments. It can form dense and extensive wetland stands and grows either rooted in shallow water or large, free-floating clumps. The roots are tough, extending 1 m or more in a suitable substrate. The most conspicuous feature of the plant is the bright green, smooth, rounded culms (flowering stems) which are up to 40 mm thick at the base and maybe up to 5 m tall in ideal conditions. Each is topped by a dense cluster of thin, bright green, shiny stalks, which resemble a feather duster when young.

The stalks elongate later and bend gracefully downward under their own weight so that the cluster becomes almost spherical in shape. Colors fade and sheaths split ventrally with age. *C.papyru*s plants take about 6-9 months to mature with a highly reliable natural regrowth and replenishment on a site after harvesting. *C. papyrus* requires an annual rainfall of 10-420 cm, yearly temperatures of 20-30^{o}C, and a pH of 6.0-8.5 and it can tolerate a few degrees of frost. Botanical studies have shown that stands of papyrus are capable of accumulating large amounts of nutrients and have high standing biomass. This plant has a relatively high potential of producing biomass from solar energy with dry weight biomass generation rates of up to 6.00 kg m^{-2} years^{-1} and a significant nutrient uptake rates of up to 7.10 kg ha^{-1}days^{-1} and 0.24 kg ha^{-1}days^{-1} of, respectively, nitrogen and phosphorus (Lema et al. 2014). Nevertheless, the configuration of the system had a significant influence on the reduction of parameters like NO_3-N, NH_4-N, and PO_4^{-3}-P which had higher reduction in the HSSF than in the HSF (Theophile et al. 2011).

Papyrus plant reactor shown to remove BOD values to 93.17%, TSS parameter 96.49% and NH3-N values can be reduced 99.13% (Erina and Wiyono 2012). Similarly, Cyperus papyrus-planted treatment beds had markedly higher phosphorus removal efficiency with higher total biomass and nutrient levels (leaf N and root P) in plant tissues (Kassa and Mengistou 2014). As far as heavy metals are concerned, Cyperus papyrus had a greater removal of Cu and Fe and the pesticide endosulfan and permethrin (Lema et al. 2014). The ability of *C. papyru*s to use nutrients from the wastewater and the incorporation of heavy metals and organics into its phytomass added to its easy management by regular harvesting, makes it one of the most suitable plants to be used in wastewater phytoremediation in tropical areas (Mburu et al. 2015). The wetland with papyrus spp. provides ecological and socio-economic services related to the harvesting of aerial biomass, wastewater treatment, hydrological functions, and climate modification.

2.3.5.4. Sugarcane (Saccharum Officinarum)

Sugarcane is a large perennial grass of up to 250-300 cm tall and 2-5 cm wide. The culms are usually in small clumps, greenish, yellowish, or dark purple. The leaves are broadly linear, 80-150 x 4-6 cm with the leaf sheaths covered with irritant hairs. The inflorescence is a sizeable silky panicle, 15-40 cm tall (Sinclair et al. 2004). Sugarcane is cultivated for the stems, from which sugar is extracted. The leftover from sugar extraction, the molasses, is used as a feed, as a fertilizer or for the production of yeast, etc. Alcohol can also be obtained from molasses (Khan et al. 2017). The fibrous residue, the bagasse, is used as fuel for energy generation in the sugarcane factory. It can also be used to make fiberboard, plastic, paper, and furfural. Juice impurities with lime added are useful to maintain soil moisture (Mateus et al. 2016). The plant is considered to be moderately tolerant of saline soil conditions and relatively tolerant of acid soils. Prefers a pH in the range 5-8, but can tolerate 4.5-9 (Khan et al. 2017).

Sugarcane is a significant source of income and development. It is the primary raw material for the manufacture of sugar, ethanol, and spirits. It is also used as a forage plant in its fresh form. The high rate of biomass accumulation is due to intense photosynthetic activity throughout the growing season and high leaf area index (LAI) of the plant (Sinclair et al. 2004). Sugarcane, usually grown in tropical and subtropical climates, has higher productivity and lower production costs and can replace the plants commonly used in CWs. The use of CWs to simultaneously treat wastewater and produce biomass for energy purposes can constitute an eco-efficient alternative for energy crop production and improvement of CW sustainability (Mateus et al. 2016).

A feasibility study conducted by Mateus et al. (2016) demonstrated that Saccharum officinarum (sugarcane) can be used as a vegetation and mineral wastes for filling in constructed wetlands (CWs) designed for the removal of nutrients from wastewater. Based on the results, sugarcane biomass production was 107 ton/ha for the brick fragments filled CW and 67 ton/ha for the fragmented limestone filled CW. As well as, sugarcane planted CWs show better nutrient removal efficiencies than the unplanted with an average efficiency of 77% for TP and 60% for TN. Additionally, they concluded that the use of sugarcane as CW vegetation is a viable alternative to produce a bioethanol raw-material without the use of arable land and irrigation water, while it maintains the wastewater treatment capabilities of CWs. The contribution of sugarcane on the removal of organic matter and microbial organisms as well as its uptake potential of heavy metals was not thoroughly tried.

Sugar cane bagasse ash, used as a wetland substrate, can also act as an effective adsorbent for the removal of dyes from aqueous solution (Kanawade et al. 2010). Similarly, a vertical flow (VF) constructed wetlands that were filled with organic sugarcane bagasse and brickbats as the main media planted with reed plant, Typha latifolia, and one controlled system was studied. The results showed that the bagasse reactor removed nitrogen by75% while the brick reactor removed both COD and phosphates by 70% and 75% respectively (Abdul and Ganapathyvenkatasubramanian 2016). Lab-scale constructed wetlands that were filled with organic sugarcane bagasse and Sylhet sand for the treatment of textile wastewater showed that sugarcane bagasse facilitated denitrification in the VF wetlands and provided efficient color removal under predominantly anaerobic condition (Saeed and Sun 2013).

2.4. Determination of Different parameters

Several studies showed that the level of TSS in the wastewater samples is determined by using the gravimetric method (Aniyikaiye et al. 2019 L; Sarafraz et al. 2007; Amouei et al. 2015; Amouei et al. 2012). After filtering the wastewater through a weighed glass fiber filter, it will be dried in the oven at a temperature of 105± 2°C. Then the filter paper will be removed from the oven and allowed to cool to room temperature and weighted to constant weight. Chapman (1996) also described that the TSS determination is based on gravimetric measurement after following the appropriate procedures, i.e. filtration, evaporation, drying, and ignition. Total suspended solids (TSS) can also be calculated as the difference between TS and TDS (Ojo and Adeniyi 2012)

There are two principal methods for the determination of BOD by analyzing initial and final dissolved oxygen. The older, titration method (Winkler method) involves the chemical fixation of the oxygen in a water sample collected in an air-tight bottle (Aniyikaiye et al. 2019). The other alternative method is the use of membrane-electrode or oxygen probe. This method is quick and can be used in situ or for continuous monitoring, although a high degree of accuracy may be difficult to maintain (Chapman 1996).

There are also two methods for COD determination. The first method is the open reflux method which is suitable for a wide range of wastes where a large volume of samples are required. This can be carried out by the addition of mercuric sulphate and sulphuric acid into an aliquot of wastewater sample in a reflux flask. On cooling, the obtained solution is then reacted with a known concentration of potassium dichromate and a known volume of sulphuric acid. The solution is refluxed for 2 h and cooled. The obtained solution is diluted to twice its volume, cooled to room temperature, and excess $K_2Cr_2O_7$ in it determined by titrating with ferrous ammonium sulphate (FAS) using ferroin indicator (Aniyikaiye et al. 2019). The second method of COD analysis is closed reflux methods, in which small quantities of metallic salt reagents are required and small quantities of hazardous waste is produced. In the closed reflux method, ampoules and culture tubes with premeasured reagents are used and then samples are placed in the tube, and COD is determined. The other COD measurements are made using low-and high-range ampoules (HACH Chemical) with a spectrophotometer (HACH, DR5000) (Hur et al. 2010).

Ammonium (NH_4^+), and nitrate (NO_3^-) ions can be determined by spectrophotometer methods (Ojo and Adeniyi 2012). Nitrate in wastewater can also be determined by using a UV spectrometric method. Some simple field determinations, of limited accuracy, can be made using colorimetric comparator methods available as kits (Chapman 1996). The simplest ammonia ions measuring instruments suitable for waters with little or no pollution are colorimetric methods using Nessler's reagent or the phenate method. For high concentrations of ammonia found in wastewater, a distillation and titration method is more appropriate. Total ammonia nitrogen and organic nitrogen are also determined by the Kjeldahl method which gives total ammonia nitrogen plus total organic nitrogen (Kjeldahl N). Photochemical methods can also be used in place of the Kjeldahl method. These methods oxidize all organic nitrogen (as well as ammonia) to nitrates and nitrites. If samples are filtered total dissolved nitrogen is determined instead of total organic nitrogen (Chapman 1996).

The phosphate level in the water can be determined by UV spectrophotometric method. Phosphate ions are determined by developing color reagent and measuring the color intensity in a spectrophotometer (Lüderitz and Gerlach 2002). Soluble reactive phosphorus samples can be filtered and analyzed by standard automated colorimetric methods (Leader et al. 2003). Chapman (1996) also stated that the determination of phosphate involves conversion to orthophosphate which is then measured colorimetrically.

Metals can quantitatively be estimated by using flame atomic absorption spectrometry (FAAS) (Sukender et al. 2012; Amouei et al. 2015). Moreover, they can also be performed according to the HACH DR5000 UV-Vis Spectrophotometer (Ali and Shakrani 2014). The low concentrations of metals in natural waters necessitate determination by instrumental methods. Photometric methods, sometimes in combination with extraction, are the oldest and most inexpensive techniques. However, as these have high detection limits, they can only be used for the analysis of comparatively polluted waters. According to Chen et al. (2009) and Mwanyika et al. (2016) atomic emission spectroscopy methods were used to determine numerous metal elements simultaneously. Si et al. (2014) also analyzed heavy metals concentrations from different samples by using inductively coupled plasma-atomic emission spectroscopy. Chapman (1996) and Ali and Shakrani (2014) also stated that inductively coupled plasma-optical emission spectrometry (ICP-OES) is becoming popular, especially in the industrially developed countries, due to its high productivity and wide range of quantifiable determinations.

Analytical determinations of pharmaceuticals were performed by a RP-HPLC (Varian 9012) with fluorescence detection (Fram and Belitz 2011; Caracciolo et al. 2012). Chen et al. (2006) and Diwan et al. (2010) also stated that liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine pharmaceutical from the wastewater samples. Gas chromatography-mass spectrometry (GC-MS) was also be used to analyze acidic and neutral pharmaceuticals (Lee et al. 2013; Gros et al. 2010). Diclofenac was analyzed either by GC/MS or HPLC. The sensitivity of GC/MS and HPLC is in the low or sub µg/l range and only with a pre-concentration by the factor of 100 – 1000 environmental concentrations of PhACs can be measured (Jiskra et al. 2007).

The total coliforms and fecal coliforms of microbial samples can be determined by using the multiple tubes fermentation technique (Amouei et al. 2015; Rezaee et al. 2005). According to Beyene and Redaie (2011) and Fekadu et al. (2015), the Membrane Filtration Plate Count method was used to analyze total coliforms and fecal coliforms from water and wastewater samples. A standard Kirby-Bauer disk diffusion was the commonly used method to determine the antimicrobial susceptibility test of bacterial isolates (Asfaw et al. 2017; Fekadu et al. 2015; Moges et al. 2014).

In this study, the constructed wetlands were design based on the manual of GIZ (2011) and USEPA (2000). The macrophytes were selected based on the availability of long root size to rich belowground in order to provide a substrate for attached bacteria and oxygenation, accessibility in the nearby natural wetlands (local area) and their potential to remove pollutants as stated somewhere (Jethwa and Bajpai 2016; Mateus et al. 2016; Vymazal 2011). The broken brick was selected based on Mateus et al. (2016), Abdul and Ganapathyvenkatasubramanian (2016), and Wang et al. (2012). The analysis of TSS level in wastewater samples was determined based on the methods used by Aniyikaiye et al. (2019) and Amouei et al. (2015). The analysis of the five days biodegradable organic matter (BOD_5), COD, TKN, Ammonium (NH_4^+), Nitrate (NO_3^-), and Phosphate (PO_4^{3-}) ions were also determined based on the methods stated by Chapman (1996). The study focused on the physicochemical and bacteriological characterization of hospital wastewater and the nutrient removal capacity of the broken brick substrate in the subsurface flow constructed wetlands.

In the present study, inductively coupled plasma-optical emission spectrometry (ICP-OES) was used based on the methods recommended by Chapman (1996) and Ali and Shakrani (2014) in order to determine the potential of plant species for the removal of heavy metals in constructed wetlands. (Amouei et al. 2015; Rezaee et al. 2005). The multiple tubes fermentation technique was used to determine the total coliforms and fecal coliforms according to Amouei et al. (2015) and (Rezaee et al. (2005). The Kirby-Bauer disk diffusion method was also used to determine the antimicrobial susceptibility test of bacterial isolates (Asfaw et al. 2017; Fekadu et al. 2015; Moges et al. 2014) so as to evaluate the potential of constructed wetlands for the removal of antibiotic-resistant bacteria.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Description of Study Area

The experiments were carried out from December 2016 to December 2017 in the compound of Hawassa University Referral Hospital, southern Ethiopia, located 7.06 latitude and 38.48 longitudes with an elevation of 1697 meters above sea level. Its climate is tropical with an average annual rainfall of 945 mm. It has a long wet season from March to October and a short dry season from November to February. The mean minimum and maximum temperatures during the study period were 13°C and 27°C, respectively. The hospital has been working as a teaching institute and a medical referral center. At the time of data collection, the hospital had around 300 beds in 6 wards and the number of patients visited per day was ranged from 200 to 350.

The examined wastewater was generated from different wards of the hospital mainly from inpatient wards, outpatient services, delivery rooms, operation theaters, pathological services, dental and therapeutic services, X-ray room, different laboratory units, hospital laundry, kitchen, and toilets. The wastewater generated in the hospital have been reached to 143,285 liters per day (Beyene and Redaie 2011) and pass through a septic tank pretreatment before it inters to the waste stabilization pond for final treatment. The hospital wastewater discharge is then released into the vicinity of Lake Hawassa, which is an ecologically sensitive area. The wastewater samples for characterization were taken from the manhole of the sewer line that has been transported from the septic tank pretreatment to the waste stabilization pond for final treatment.

The waste stabilization pond employed in the hospital comprises of two facultative ponds and two maturation and a fish ponds for fish farming. The total hydraulic residence of the wastewater on the pond was 29.18 days. The main purposes of the waste stabilization ponds were not only to treat the wastewater but also to rear fish in the treated water and lastly to reuse the treated wastewater for compound gardening and fire hydrant for the hospital. However, different researchers claimed that the existing waste stabilization pond is unsatisfactory to give the essential treatment level of pollutants and there is a fear that the lake might be seriously damaged (Beyene and Redaie 2011; Sewhunegn, 2012).

3.2. Experimental Setup

Eight horizontal subsurface flow pilot-scale wetlands were constructed in the compound of Hawassa University Teaching Referral Hospital to determine their effectiveness in treating hospital wastewater after passes through septic tank pretreatment. Each bed was constructed with cement blocks of size 4 m×1.2 m having a depth of 0.6 m (Figure 5). The inside wall of the structures was plastered with cement and lined with a 3.00 mm high-density polyethylene (HDPE) sheet to avoid the percolation of wastewater into the groundwater and to protect the wastewater from being eroded out of the system. The HDPE geomembrane sheet was bought from one of the known plastic materials importer and distributer center of Hawassa City. It is made from high-grade polyethylene by triple layer coextrusion & film-blowing technology and consists of 97.5% high-density polyethylene and 2.5% anti-aging materials. The bottom slope of each cell was 0.5% to ease the movement of water from the inlet to the outlet.

This study investigated the efficiency of two growing media, namely, gravel and broken brick to remove different pollutants. Burnt Bricks were purchased from the local building material shops and grounded into a uniform particle size of 20-25 mm. Based on the laboratory test reports obtained from Hawassa University soil department; the brick used for wetland media have the following chemical composition: Silica (SiO_2) (56%), Alumina (Al_2O_3) (24%), Iron oxide (Fe_2O_3) (10%), Lime (CaO) (5%), and Magnesium Oxide (MnO) (5%). The crushed gravel of 40-50 mm, 20-25 mm, and 5-10 mm diameter was also purchased from the nearby market. Before placing in the bed, all substrates were cleaned and thoroughly washed with piped water, to remove the dirt materials and fine-sized particles to reduce clogging. Coarse gravel of 40 to 50 mm in diameter was arranged at the inlet and outlet zones of the wetlands to prevent clogging and facilitate wastewater distribution. The main treatment zone of five CWs was filled with gravel and three CW with a broken brick substrate having a particle size of 20-25 mm at a depth of 45 cm (Figure 5 to 8) (GIZ 2011; USEPA 2000). The upper top layer (15 cm) of all wetlands was filled with a gravel of 5-10 mm to provide better rooting of plants (Figure 7). The depth of the substrate for each wetland was 60cm with a water depth of 55cm. The top surface of the media was leveled for easier planting and root maintenance. The water table was kept 5 cm below the surface. There was an additional 10 cm freeboard for water accumulation. The outlet structures were adjustable to enable the regulation of the water level in the substrate. Each cell has an inlet connected to a valve for controlling the inflow.

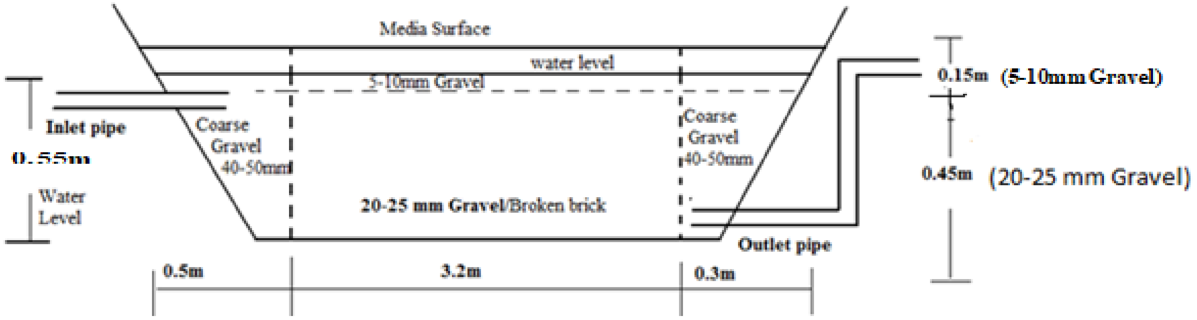


Figure 5. The cross-sectional dimension of constructed wetlands

3.3. Plant Material and Experimental Start-up

Young shoots of wetland plants were collected from the swampy area of the shore of Lake Hawassa and Tikur Wuha River. Shoots were washed with piped water and transplanted at a density of 12shoots/m^2 then irrigated with piped water for one month during which they grew and adapted the new environment to have good biological activity. Four of the gravel bed wetlands were planted with emergent macrophytes including cattails (*Typha domingensis*), *Cyperus Papyrus* (*Papyrus*), Dark Green Bulrush (*Scirpus Atrovirens*) and Sugarcane (*Saccharum Officinarum*) which are known to be suitable for usage in constructed wetlands (Jethwa and Bajpai 2016; Mateus et al. 2016; Vymazal 2011a) and two broken brick bed wetlands were planted with *Typha domingensis* and *Cyperus Papyrus*. The remaining two from both media left unplanted to act as the control (Table 1 and Figure 6).

The experiment of this study consists of a simultaneous comparison of wetlands with different plants and substrate media. Part of the primary treated wastewater was pumped into a temporary collection tank before entering the experimental wetland to achieve a constant flow rate during the study period using HDP pipes (3/4 inch in diameter). Both planted and unplanted wetlands were operated under continuous flow conditions with an equal hydraulic flow rate of 240 l/day. The bed capacity measured from the porosity of the gravel and the broken brick filters (with a porosity of 40%) was 960 liters. The hydraulic retention time (HRT) of 4 days was then calculated from the equation

HRT= bed capacity/inflow rate---------------------------------------(1)

Table 1. Dimension and feature of constructed wetlands (CWs)

Parameter	CW1	**CW2**	**CW3 (Control)**	**CW4**	**CW 5**	**CW6**	**CW7**	**CW8 (Control)**
Type of bed	Broken Brick	Broken Brick	Broken Brick	Gravel	Gravel	Gravel	Gravel	Gravel
Type of wetland plant	Papyrus	Typha	No plant	Sugar cane	Typha	Papyrus	Bulrush	No plant
Gravel Depth (cm)	60cm	60cm	60cm	60cm	60cm	60cm	60cm	60cm
Water Depth (cm)	55cm	55cm	55cm	55cm	55cm	55cm	55cm	55cm

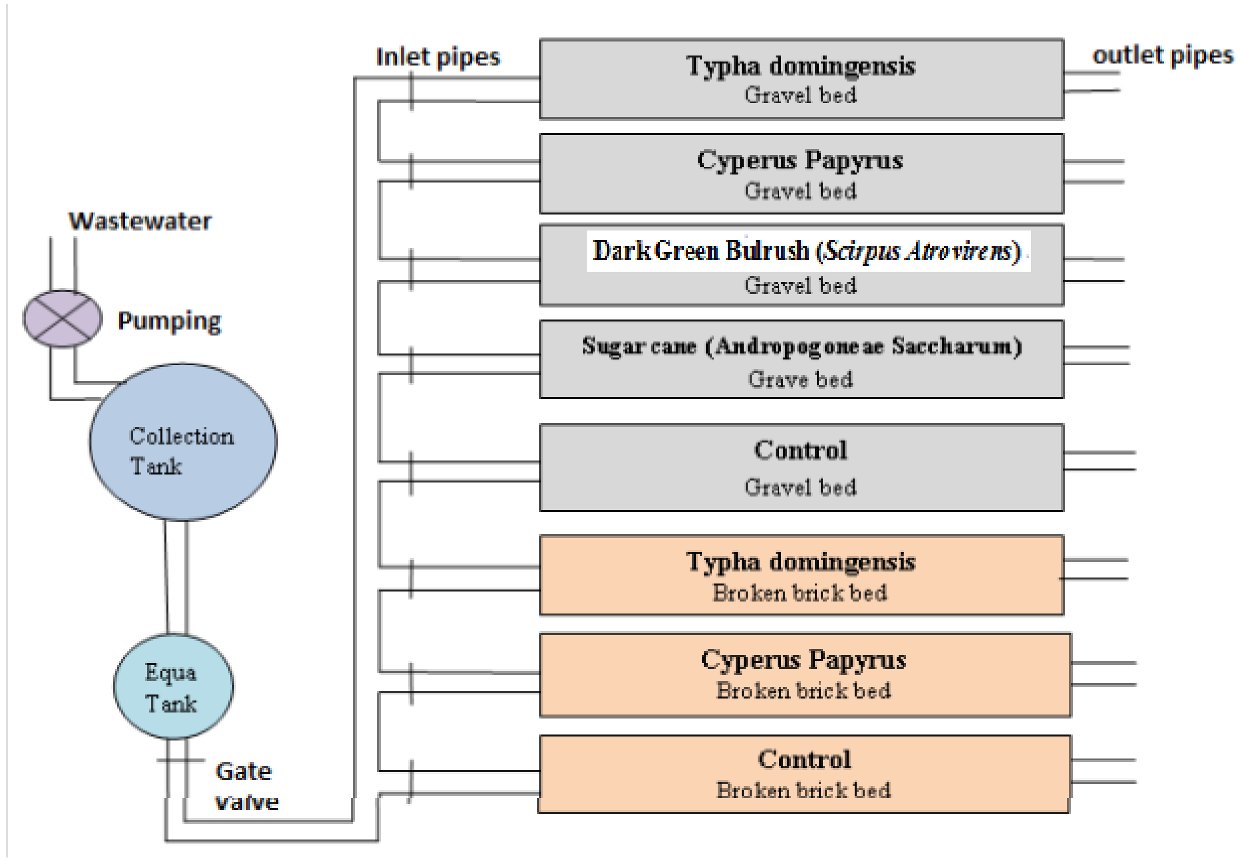


Figure 6. Schematic diagram of the pilot scale subsurface flow constructed wetlands

Figure 7. Photographic presentation of the Pilot scale subsurface flow constructed wetlands

Figure 8. Photographic presentation of the Pilot scale subsurface flow constructed wetlands

Figure 9. Substrate media used in the experiment.

3.4. Sampling and Sample Collection

Wastewater treatment performance of organic matter and nutrients were monitored over six sampling periods, three times in a dry season (November to December 2017) and three times in a rainy season (June to August) on a monthly base. A total of six composite samples from inflow wastewater and 48 composite samples from outflow wastewater were collected simultaneously. The wastewater samplings were undertaken three times a day with three hours interval in the morning and afternoon (9:00 AM, 12:00 AM, and 3:00 PM) in 250 ml cleaned and sterile screw-capped containers and transported on ice (≈4°C) and stored in refrigerator at 4^oC. Then all the samples pooled aseptically to 500 ml sized cleaned and sterilized containers and analyzed within 24 hours and kept refrigerated (4^oC) during transportation to Leather Industry Development Institute Research Laboratory and Addis Ababa University Environmental Science laboratory.

For heavy metals and drug analysis, a total of 3 composite samples from inflow and each outlet of the wetlands were collected in 500ml cleaned high-density polythene containers based on APHA (1998) standard methods. The samples for heavy metals were collected three times a day with three hours interval in the morning and afternoon (9:00 AM, 12:00 AM and 3:00 PM) and acidified to 0.05 M with nitric acid then transported on ice (≈4°C) to Leather Industry Development Institute Research Laboratory. Similarly, 500 ml composite samples were collected and transported on ice to Ethiopian Quality and Standards Authority for drug analysis.

For bacteriological analysis, a total of 8 composite inflow wastewater samples were collected from the outlet of a temporary collection tank and a total of 32 composite samples were collected from the outlets of wetlands (four times from each) at a monthly interval for bacteriological analysis, identification and susceptibility testing. Samples were collected three times a day with a two-hour interval (10:00 AM, 12:00 AM, and 2:00 PM) in 250 ml cleaned and sterile screw-capped containers and transported on ice (≈4°C) to the microbiology laboratory of Hawassa University and stored in the refrigerator at 4^oC. After the last sample collection (2:00 PM), all the samples were pooled aseptically to 750 ml sized cleaned and sterilized containers in the laboratory and analyzed immediately according to APHA (1998).

3.5. Sample Analysis

A total of 25 parameters were analyzed from inlet hospital wastewater and outlets of treatment wetlands. The physicochemical parameters such as pH, T°, BOD_5, COD, TN, ammonium, nitrate, phosphate and TSS, heavy metals (arsenic, cadmium, chromium, nickel, lead, zinc, silver, iron, manganese, copper and aluminum), anti-inflammatory drug (diclofenac), antibiotics (sulfamethoxazole), indicator organisms (total Coliform, fecal coliforms) and drug-resistant strain were analyzed.

3.5.1. Physicochemical Parameters Analysis

All parameters were analyzed based on APHA (1998) standard methods: Total Suspended Solids (TSS; gravimetric method through filtration at 2 µm filter paper and drying at 103-105°C), Biological Oxygen Demand (BOD_5; Winkler titration method was used to measure initial and final DO after dilution), Chemical Oxygen Demand (COD; open reflux method), Total Kjeldahl Nitrogen (TKN; Kjeldahl digestion), Ammonia nitrogen (NH4-N; colorimetric methods using Nessler's reagent), Nitrate nitrogen and phosphate (P; microwave digestion, colorimetry with vanadomolybdophosphoric acid). The samples for water temperature and pH were analyzed in the field with portable digital pH/Temperature meter.

3.5.2. Heavy Metals Analysis

Metals concentration in the wastewater samples was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES PerkinElmer model 7300 DV, USA) using argon and nitrogen gases.

Sample preparation

For the determination of heavy metals, a 100-mL aliquot from a well-mixed, acid-preserved sample was transferred to a 250 mL beaker then 2.0 mL (1+1) nitric acid and 1.0 mL of (1+1) hydrochloric acid was added. Subsequently, the beaker was placed on a hot plate (85°C) for solvent evaporation. The volume of the sample aliquot was reduced to about 20 mL by gentle heating at 85°C without boiling. Then the lip of the beaker was covered with a watch glass to reduce additional evaporation and gently refluxed for 30 minutes and cooled. The sample solution was quantitatively transferred to a 50-mL volumetric flask and made up to the mark and allowed to settle overnight. Each sample was prepared into three replicates. Two water blanks were run with each batch of samples. The instrument working wavelengths were set as suggested by the APHA (1998).

3.5.3. Drug Analysis

All used solvents were HPLC-grade. Acetonitrile, methanol, phosphoric acid, hexane, diethyl ether and ascorbic acid were obtained from Zaf Pharmaceuticals Drug and Medical Supplies Importer and Wholesale. GracePureTM C18 solid-phase extraction (SPE) cartridges (500 mg, 6 mL) and the 0.45-μm glass fiber filters (vol., 47 mm) (Whatman) were purchased from Afro-German Chemicals Est Plc. Antibiotic working standards were obtained from Ethiopian Food, Medicines, and Health Care Administration and Control Authority (FMHACA).

A stock solution of the pharmaceutical mixture was prepared by dissolving the right quantities of the powdered standards in methanol and stored at 4^{o}C in a darkened place. The mass concentrations of each pharmaceutical drug in this mixture are 100 mg/L. The standard work solution (50 mg/L) is obtained by the dilution of the stock standard solution with methanol and its stability was one month.

Sample preparation

Prior to extraction, the wastewater was filtered through Whatman filter paper to eliminate suspended matter and then filtered twice through a 0.45 mm membrane filter to remove particulate matter. Samples were stored at 4^{o}c. Before analysis, the pH was adjusted to 4.0 with a hydrochloric acid solution (0.1 mol/L) 21625, 24049

SPE extraction and HPLC-DAD analysis

Before applying the spiked water samples, the C-18 cartridges mini-column was conditioned with 5 mL of n-Hexane: Diethyl Ether (1:1), 3ml of methanol, and 5ml of 0.01M Ascorbic Acid buffer. A sample volume of 100 mL was applied to the cartridge and the flow was kept at no greater than 4 ml/min.

The sorbent was never allowed to dry during either the conditioning period or sample loading procedures. After the vacuum drying period of the sorbent, the loaded cartridges were eluted with methanol (eight volumes of sorbent bed). Following the elution, the cartridge was dried by using a vacuum pump for 10min and the dried residues were dissolved in 2500µl of methanol to obtain a 100-fold preconcentration. The extraction efficiencies of sorbents were determined by HPLC.

The mobile phase used in the chromatographic separation consists of acetonitrile, methanol, and 0.05 mol/L phosphoric Acid (48:12:40) solvents. The flow rate started with 1.5 ml/min, which was maintained for 6 min, followed by a 19-min linear gradient to 0.8 ml/min and a 5-min linear gradient back to 1 ml/min. This flow rate gradient provides good separation of all pharmaceuticals investigated and avoids the peaks overlap. The investigated pharmaceuticals are eluted within 20 min. The separation was monitored at an absorbance wavelength of 280 nm with a column temperature of 30^{O}C. The injection volume was 5µl. In the first set of experiments, the extraction efficiency for the selected five pharmaceuticals was tested on 5 different 500-mg sorbents. In the second set of experiments, the extraction efficiency of experiments was repeated with 200 mg of sorbent bed.

3.5.4. Bacteriological Analysis

Bacteriological Determination

In a standard multiple-tube technique, samples were subjected to presumptive, confirmed, and completed tests to estimate total and fecal coliform groups of bacteria as described in Standard Methods for the Examination of Water and Wastewater (APHA 1998). To determine the total coliform and fecal coliform count, 1 mL of aliquot sample was transferred aseptically to serial 10-fold dilutions prepared by physiological saline in a series of 5 tubes of each sample containing Durham tube and MacConkey broth. Tubes were gently shaken and incubated for 48 hours at 37ºC for total coliforms and 48 hours at 44.5ºC for fecal thermotolerant coliforms. Production of gas and lactose fermentation was observed as positive reactions. In the confirmed test, all tubes showing positive results were gently shaken and inoculated into an eosin methylene blue agar plate and incubated for 24 hours at 37ºC for total coliforms and 24 hours at 44.5ºC for fecal coliforms. Positive colonies were then subjected to lactose fermentation broth for the completed test. Those showing lactose fermentation and gas production were recorded as positive and used for most probable number analysis.

For the *Staphylococcus* count, 0.1 mL of the aliquot was streaked on mannitol salt agar (MSA) and incubated at 37°C from 24 to 48 hours. Colonies showing a yellow zone of fermentation were used for Gram staining. Gram-positive cocci colonies identified were counted using a digital colony counter as *staphylococci* and described as CFU/mL (Fekadu et al. 2015).

For the *Salmonella* and *Shigella* count, 0.1 mL of the aliquot was streaked on Xylose Lysine Desoxycholate Agar (XLD) and incubated at 37^{O}C for 24 hours. Those typically produce clear to light pink/red colonies with 2-4 mm distinct black centers were considered as *Salmonella* colonies and colonies 1-2 mm showing red color were considered as *Shigella* colonies. The isolated colonies were subjected to the biochemical test for further screening (WHO 2010).

Identification of Bacterial Isolates and Sensitivity Test

Biochemical Test: All samples were streaked on MacConkey agar, Mannitol salt agar, and Xylose Lysine Desoxycholate Agar (XLD) to obtain pure colonies of gram-negative coliforms, *staphylococcus*, and *salmonella* and *shigaella*, respectively. Further identification of gram-negative isolates was done using gram staining and biochemical test with dihydrolase L-lysine, Urea hydrolysis test, Oxidation-fermentation of glucose, H_2S production, Indole formation, Motility test, Oxidase test, Triple sugar iron agar (TSI) and Citrate test (Hemraj et al. 2013). Gram-positive isolates were also identified by Gram staining, catalase, and coagulase tests.

Antimicrobial sensitivity test: A standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profile of bacterial isolates as recommended by the Clinical and Laboratory Standards Institute (CLSI 2007). Bacterial isolates were inoculated into a tube containing 5ml sterile nutrient broth and grown to a 0.5 McFarland turbidity standard within 4hrs. Using a sterile cotton swab, a bacterial lawn was created onto Mueller Hinton agar plates and Mannitol salt agar plates. The following commonly prescribed drugs were tested on bacterial isolates: amikacin (30µg), ceftriaxone (30µg), chloramphenicol (30µg), erythromycin (15 µg), cefoxitin (30 µg), ciprofloxacin (5µg), cotrimoxazole (25µg), gentamicin (10µg), doxycycline (30µg), ceftazidime (30µg), amoxicillin-clavulanic acid (30µg), ampicillin (10µg), naldlic Acid (30µg). After incubation for 18 hours at 35°c, the zone size was measured in millimeter using a manual caliper to classify as susceptible and resistant (intermediate considered as resistant in this study) based on the diameters of inhibition zones (CLSI 2007). Resistance to three or more antimicrobial drugs was taken as multiple antibiotic resistant (MAR).

3.6. Quality Control

The sampling and sample handling were performed based on the procedures stated. Calibration of all devices was performed according to manufacturers' instructions with standard solutions appropriate to the type of instrument and the linear range established for the analytical method.

All measurements were made on independent replicates the number of which is given in the method descriptions. Blank samples were included for all wastewater sample measurements. All standard solutions were analyzed as samples before and after the sample run during ICP determination. Wastewater sample collection, handling, transportation, and bacteriological analysis were carried out based on the standard operating procedures. Prior to the actual work, the quality of all media, reagents, stains, and antibiotic disks were checked following the manufacturer's instructions. All the media were incubated overnight without inoculums to ensure sterility. Besides, reference strains of *Escherichia coli* (25922) and *Staphylococcus* were obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) and employed every time to check the performance of biochemical and disk diffusion tests.

3.7. Statistical Analyses

Statistical analysis for organic matter, nutrients, heavy metals, and drugs were performed using SPSS 24.0 version software package for Windows. Fisher's Least Significant Difference (LSD) test was used as post hoc to determine any significant differences in the mean influent and effluent values of the different parameters and pollutant removal efficiencies of the wetlands with different plant species combinations, with $p < 0.05$ defined as a significant difference. A comparison between wastewater analysis results for planted and unplanted cells in broken brick and gravel beds during the dry and rainy season was performed to evaluate the effect of seasons and the plant/media combination on the pollutants' removal.

Means, percentages, and standard deviation of bacteriological results were computed using Microsoft Excel 2007. Kruskal-Wallis H-tests were run in SPSS v.24.0 to detect any significant differences in the mean concentration of coliforms and antibiotic-resistant bacteria in the inlet hospital wastewater and outlets of wetlands. A comparison between mean values of planted and unplanted cells in broken brick and gravel bed wetlands was also performed to evaluate the plant and bed type effect on the removal efficiency. All tests were considered statistically significant at P values of < 0.05. The removal efficiency of the pollutant was calculated by the percentage of reduction in concentration for each pollutant as follows:

Removal efficiency = $(C_{inf} - C_{eff}/C_{inf}) \times 100$ %------------------------------(2)

Where: C_{inf} and C_{eff} are the influent and effluent concentrations in mg/L.

CHAPTER FOUR

RESULTS

4.1. Wastewater Composition of Hawassa Referral Hospital

4.1.1. Physicochemical Characteristics

Analysis of physicochemical parameters showed that the wastewater released from the hospital was slightly acidic to neutral. The average pH of the hospital wastewater was ranged from 6.7 in the rainy season to 6.9 in the dry season. The investigation of temperature demonstrated that a little seasonal variation was recorded with a minimum value of 21.5^{0}C during the rainy season and a maximum of 24.6^{0}C during the dry season (March 2011 to Feb 2013) with a mean value of 23^{0}C. As presented in Table 2, the pretreated wastewater of the hospital was loaded with a significant amount of organic matter and nutrients. The result of the study indicated that the organic matter, ammonium, and phosphate concentration were higher during the dry season while TKN and nitrate were higher during the rainy season. There was a very low concentration of nitrate in the wastewater throughout the study period.

Table 2. Physicochemical concentration (± standard deviation) of pretreated hospital wastewater

Parameter	**Dry season**	**Rainy season**
pH	6.9±0.1	6.7±0.2
Temp (^{O}C)	24.6±0.4	21.5±0.5
TSS (mg/L)	535±22	496±11.7
BOD_5 (mg/L)	221±31.3	185±11.6
COD (mg/L)	713±86.5	673±31.9
TKN (mg/L)	86.3±11.7	98±3
NH_4^+N (mg/L)	73.4±0.6	67.5±4.6
NO_3^--N (mg/L)	0.9±0.2	1.1±0.2
PO_4^{3-}(mg/L)	13.4±4.9	8±2

The hospital wastewater sample exhibited a high concentration of cadmium, chromium, lead, and silver metals while all other metals detected were below the WHO standard. The average concentration of cadmium, chromium, lead and silver, were 0.078±0.021, 0.1114±0.04, 0.74±0.05, and 0.06±0.008 mg/l, respectively.The concentration of Sulphametoxazol and Diclofenac were 22.9±2.25 and 1.94+0.06 ug/L, respectively while the concentration of Caffeine, Salicylic Acid, and Ethinyl Estradiol was below the detection limit (Table 3).

Table 3. Heavy metals and pharmaceuticals concentration (± standard deviation) hospital wastewater

Parameter	**Concentration**	**WHO Standard for wastewater reuse and discharge (2006)**
Aluminum (mg/L)	0.08±0.001	5.0
Cadmium (mg/L)	0.078±0.021	0.01
Chromium (mg/L)	0.1114±0.04	0.02
Nickel (mg/L)	0.162±0.082	0.2
Lead (mg/L)	0.74±0.05	0.2
Zinc (mg/L)	0.134±0.009	2
Silver (mg/L)	0.06±0.008	0.01
Arsenic (mg/L)	0.021±0.007	0.05
Iron (Fe^{2+}) (mg/L)	0.8±0.1	5.0
Manganese (mg/L)	0.21±0.03	0.2
Copper (mg/L)	0.06±0.008	0.2
Caffeine (µg/L)	*NOD**	
Sulphametoxazol (µg/L)	22.9+2.25	
Salicylic Acid (µg/L)	*NOD**	
Ethinyl Estradiol (µg/L)	*NOD**	
Diclofenac (µg/L)	1.94+0.06	

*Note detected

4.1.2. Bacteriological Characteristics

Bacteriological analysis of the pretreated hospital wastewater was explained in Table 4. The Results showed that the average bacterial count of total coliforms and fecal coliforms was $1.3 \times 10^7 \pm 1.2x10^3$ and $1.4 \times 10^5 \pm 5x10^2$ MPN/100 ml, respectively, throughout the investigation period. Among the total samples, 45 bacterial isolates were detected. The most frequently isolated bacteria from the pretreated hospital wastewater samples were *Staphylococcus sp* 12(26.6%), *E. coli* 11(24.4%) and *Klebsiella sp* 9(20%) (Table 4).

Table 4. The mean (± standard deviation) number of bacterial isolates of hospital wastewater and the outlet of wetlands by biochemical test.

Bacterial Isolates	**Average No of Isolates (%)**
Escherichia coli	10.8±3 (24%)*
Shigella sp	4.8±1 (10.7%)
Pseudomonas sp	2 (4.4%)
Staphylococcus sp	11.8±3.5 (26.2%)
Klebsiella sp	9±2.1 (20%)
Salmonella sp	4.3±2.1 (9.6%)
Citrobacter sp	2.3±0.5 (5.1%)
Total	45±12.2 (100%)

* Percent in the bracket represents the specific isolated species over the total isolates of the hospital wastewater times 100

Among bacterial isolates from the pretreated hospital wastewater, 100% of *Salmonella* isolates were found to be resistant to ampicillin, and 75% to doxycycline, erythromycin, ceftazidime, cefoxitin, and chloramphenicol. About 81.8% of *E.coli* isolates were also found to be resistant to ampicillin and 72.7% to cotrimoxazole and amoxicillin-clavulanic acid. About 80% of ampicillin-resistant *Shigella*, as well as 77.8% ampicillin, amoxicillin-clavulanic acid, and ceftazidime-resistant *Klebsiella*, were also frequently found (Figure 10).

From the total bacterial isolates of the pretreated hospital wastewater, most of them 38 (84.4%) were resistant to ampicillin followed by cotrimoxazole 32 (71%), amoxicillin-clavulanic acid 29 (64.4%), chloramphenicol 27 (60%), ceftriaxone 25 (55.6%), and doxycycline 25 (55.6%). However, relatively medium resistance was observed among bacterial isolates to cefoxitin 11 (24.4%), ceftazidime 12 (26.7%) and erythromycin 15 (35.5%). All bacterial isolates were susceptible to amikacin (Figure 10).

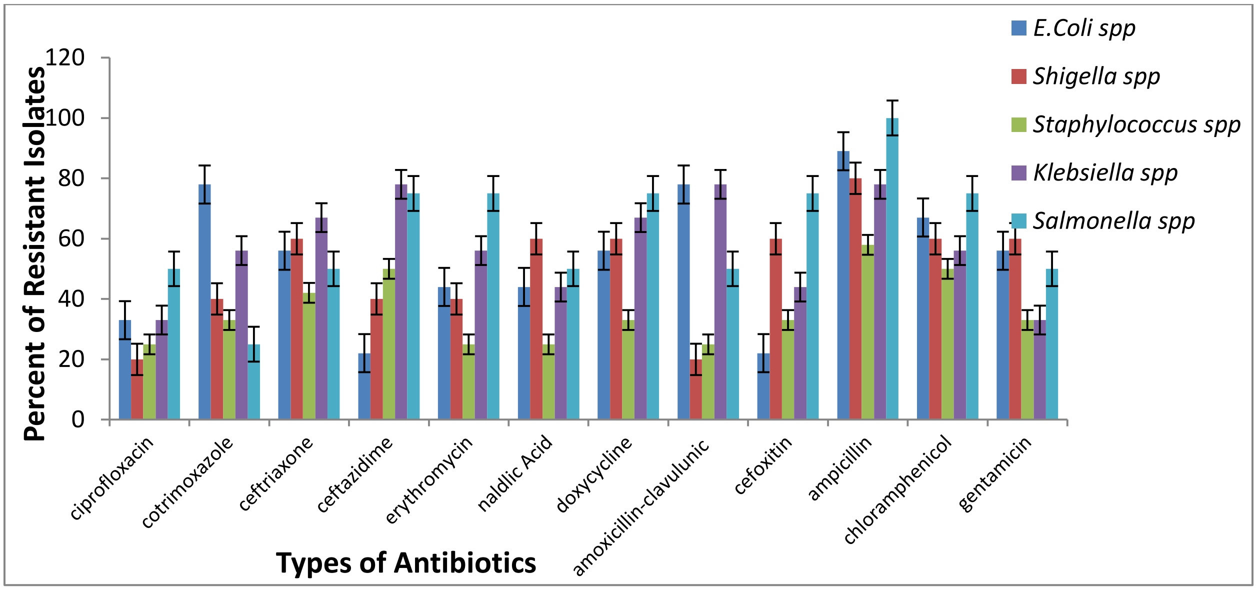


Figure 10. The average resistance percentage of bacterial isolates from inlet hospital wastewater.

4.2. Use of Broken Brick to Enhance the Removal of organic matter and Nutrients in CWs

4.2.1. Suspended Solids Removal

The average concentration and removal percentage of TSS are shown in Table 5 and Figure 11. Influent suspended solids concentration was ranged from 335±22 mg/l in the dry season to 306±11.7 mg/l in the rainy season. Mean outflow concentration was ranged from 9-32.5 mg/l in planted to 12.5-31 mg/l in unplanted wetlands during the dry season and 14.2-42.3 mg/l in planted to 21.2-47 mg/l in unplanted wetlands during the rainy season.

The average suspended solid percent removal efficiency in this study was 93.2% in the dry season and 89.7% in the rainy season. Gravel based beds containing different plants managed to reach the removal values of 90.7%-97.3% in the dry season and 84.6-95.3% in the rainy season. Wetlands containing broken brick as substrate produced an effluent with an average concentration of less than 34.5 mg/L and a percentage removal between 90.3% and 96.3% in the dry season and 88.7% and 93.1% in the rainy season. There is no statistical difference between media types in the removal of TSS.

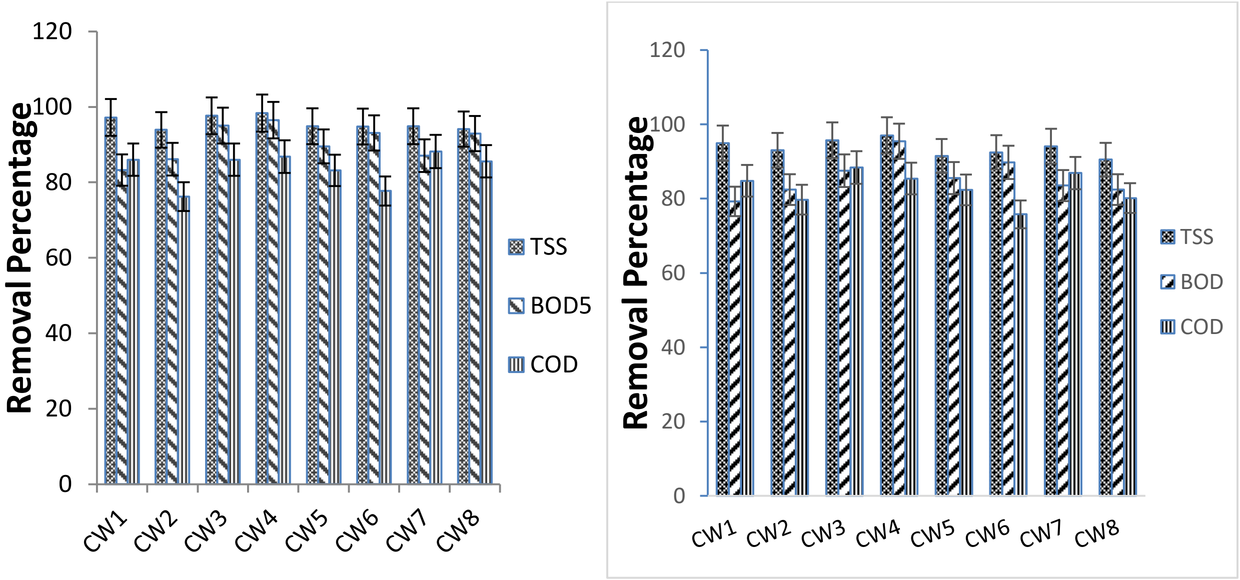
The best performance was attained by the sugar cane planted gravel bed wetland with an almost constant removal rate above 97% and an average effluent concentration of less than 10 mg/l. The unplanted broken brick bed was reached to removal values of 96.3%. The Papyrus planted broken brick bed wetland produced an effluent with an average concentration of 15 mg/l and a percent removal of 95.5% in the dry season. However, there was no significant difference in the removal of suspended solids among the eight wetlands in the dry and rainy seasons. Moreover, there was no significant difference observed between the performance of the planted and unplanted wetlands in both substrates. The similarity in TSS treatment between planted and unplanted systems in this study persisted in all seasons of the year, with the broken brick and gravel bed wetlands providing the most consistent performance even during the rainy season.

Table 5. Organic matter and nutrient concentration of the inlet and outlet wastewater of wetlands during the dry and rainy seasons

Parameter	**Season**	**HWW**	**CW1**	**CW2**	**CW3**	**CW4**	**CW5**	**CW6**	**CW7**	**CW8**
pH	Dry season	6.9±0.1	7.3±0.2	7.2	7.3±0.1	7.2±0.1	7.2±0.2	7.2	7.2±0.1	7.4±0.2
	Rainy season	6.7±0.2	7.1	7±0.4	7.3±1	7.2±1	7.2±2	7.3	7.1±2	7.3±1
Temp(^{O}C)	Dry season	25.2±0.4	24.8±0.6	25±0.2	25.2±0.1	25.1±0.5	24.4±0.9	24.7±0.6	24.2±0.8	25±0.5
	Rainy season	24.6±6	22.8±0.2	22±0.4	23±0.2	22.8±0.7	22.4±1	22.8±0.4	22.1±0.4	23.1±0.2
TSS (mg/L)	Dry season	535±22	15±10	32.5±19.5	12.5±11.5	9±7	27.5±17.5	28±2	27.5±13.5	31±17
	Rainy season	496±11.7	25±2.2	34.5±4.1	21.2±1.1	14.5±0.8	42.3±2.8	37.4±7.1	29.3±3.4	47±8.9
BOD_5 (mg/L)	Dry season	221±31.3	37± 1.45	30.6 ±5.7	10.9± 1.3	7.8± 0.6	23.1±5.6	15.3±7.6	28.6±10	15.9±5.2
	Rainy season	185±11.6	38.4±4.6	32.5±3.1	23.1±5.6	8.4±5.1	26.7±3.6	18.9±7.2	30.4±1.2	32.4±5.1
COD (mg/L)	Dry season	713±86.5	99.9±4.4	169.8 ±6.7	99.9±11.4	94±16	120±4.3	159±0.9	84.5±35.5	103±23
	Rainy season	673±31.9	102.2±6.5	136.5±12.4	78±9.6	98.2±1.8	118.7±15.4	163±7.4	88±9.3	133.6±21.8
TKN (mg/L)	Dry season	86.3±11.7	29.8±9.3	28.3±13.8	35.9±3.7	28.3±8.8	31.1±11.4	31.1±10.2	42.1±3	45.9±18.5
	Rainy season	98±3	25.4±3.2	29.6±8.4	41.7±7.6	33.2±4.7	35.5±5.5	32.4±6.2	41.9+7.7	50.1±2.1
NH_4^+-N (mg/L)	Dry season	73.4±0.6	20.9±3.5	16.6±4.1	25.8±1.2	24.7±4.4	21.6±6	11.7±3.2	29.5±6.5	30.9±0.5
	Rainy season	67.5±4.6	16.9±1.1	16.7±4.3	23.4±2.3	26.3±2	20.4±3	*27.5+6.1*	28.5±1.5	30.1±0.9
NO_3^-–N (mg/L)	Dry season	0.9±0.2	0.3±0.1	0.5±0.1	0.6±0.2	0.2±0.1	0.6±0.2	0.5±0.1	0.4±0.2	0.7±0.2
	Rainy season	1.1±0.2	0.5±0.3	0.4±0.1	0.6±0.1	0.5±0.2	0.6±0.1	0.6±0.2	0.5±0.1	0.7±0.2
PO_4^{-3}(mg/L)	Dry season	13.4±4.9	1.5±0.3	2±0.7	4±0.5	4.1±0.6	1.8±0.3	4.1±1	2.8±0.3	5.8±0.5
	Rainy season	8±2	2.5±1	3.2±1.2	3.8±0.8	4.6±0.4	2.1±0.2	4.8±1	3.1±1.3	5.6±0.5

4.2.2. BOD_5 and COD Removal

Organic matter concentration in influent and effluents was presented in Table 5. Influent BOD_5 and COD concentration was 221±31.3 mg/l and 713±36.5 in dry season and 185±11.6 mg/l and 673±31.9 mg/l in rainy season, respectively. The average BOD_5 percent removal efficiency in this study was 90.4% in the dry season and 85.8% in the rainy season. Gravel based beds containing different plants managed to reach the removal values of 87%-96.5% in the dry season and 83.6-95.5% in the rainy season. There was also higher removal percentage of BOD_5 in unplanted broken brick bed wetland (ranged from 95% in the dry season to 87.5% in the rainy season) than in planted broken brick wetlands (ranged from 84.7% in dry season to 80.8% in rainy season). The best performance was attained by the sugar cane planted gravel bed wetland with an almost constant removal rate above 96.5% and an average effluent concentration of less than 10 mg/L. Unplanted broken brick bed removal was 95%. The average COD removal efficiency of both planted and unplanted wetlands ranged from 76.2% to 88.1% in the dry season and 75.8% to 88.4% in the rainy season. However, with respect to BOD5 and COD, the least significant difference (LSD) test analysis showed that the removal percentages for these parameters were not significantly different in the planted and unplanted cells. Likewise, the vegetated cells in both substrates did not differ significantly ($P > 0.05$).

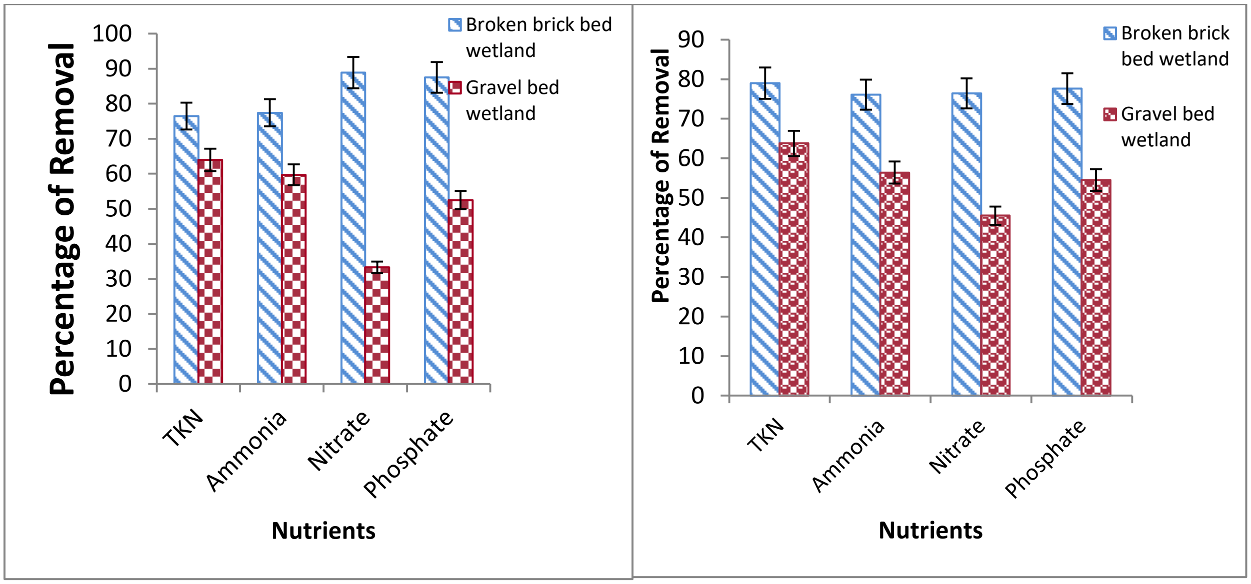


(a) dry season (b) rainy season

Figure 11. Organic matter removal efficiency of constructed wetlands

4.2.3. Nutrient removal

Table 5, Fig 12, and Figure 13 present the influent and effluent concentrations of TKN. The average concentration of TKN in the influent wastewater was 86.3±11.7 mg/l in the dry season and 98±3 mg/l in the rainy season. The level of TKN in inflow was significantly higher ($p<0.05$) than in the outflow of wetlands in both seasons. The overall average TKN removal efficiency of broken brick bed wetlands was 73% in the dry season and 74.9% in the rainy season while gravel bed wetlands achieved 58.6% in the dry season and 60.6% in the rainy season. Relatively lower removal of TKN was recorded in unplanted gravel bed wetland in both seasons. In the dry season, broken brick bed planted wetlands exhibited a higher removal percentage of TKN (75.6%) than unplanted broken brick wetlands (67.7%). Similarly, planted gravel bed wetlands achieved 61.6% removal than unplanted gravel bed wetlands (46.8%) in the same season. Both planted and unplanted broken brick bed cells in the rainy season were significantly higher ($P<0.05$) removal efficiency than unplanted gravel-bed wetland. Moreover, Typha plant in broken brick bed wetland (CW2) was significantly higher ($p<0.05$) removal efficiency than bulrush plant in gravel bed (CW7) wetland in the rainy season. A maximum of 76.5% to 79% of TKN removal was achieved by Typha species.



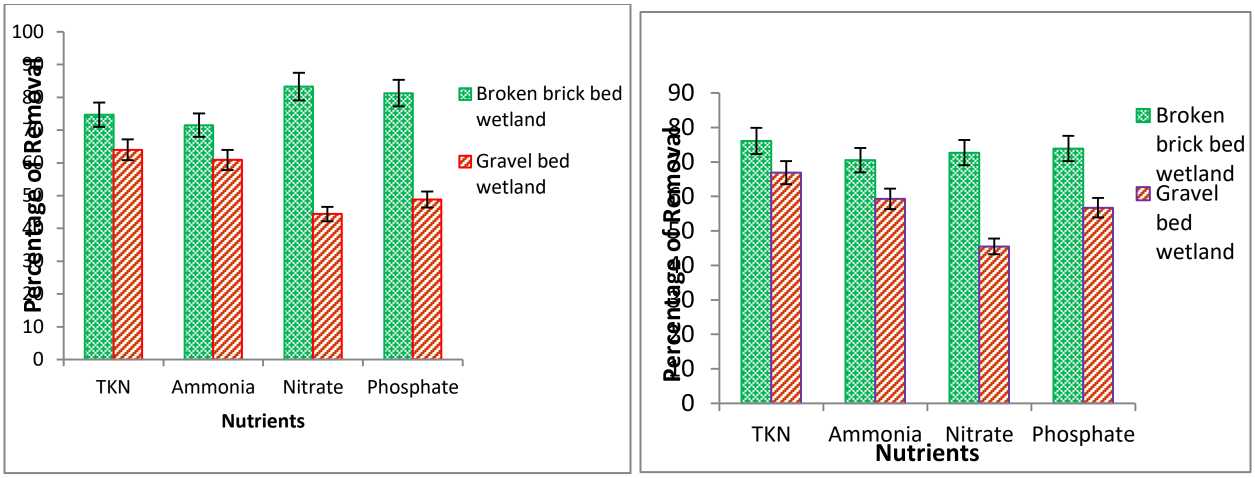
(a) Dry season

(b) Rainy season

Figure 12. Comparison of nutrient removal percentage of broken brick and gravel bed Typha planted wetlands

As expected the NH_4^+-N concentration generally decreased through the subsurface flow constructed wetland system. The concentration of NH_4^+-N in the effluent after the constructed wetland treatment was varied from 16.6±4.1 to 30.9±0.5 mg/l in the dry season and 16.1±4.3 to 30.1±0.9 mg/l in the rainy season. The concentration of NH_4-N in influent was significantly higher ($p<0.05$) than in the effluent in both seasons. Media type influences the removal of NH4-N in this study. It was revealed that the wetlands with broken brick were more efficient as compared to the wetland with gravels with an average removal rate of 71.3% in the dry season and 70.7% in the rainy season, while gavel bed wetlands achieved 60.1% in the dry season and 58% in the rainy season. Typha in the broken brick bed wetland was significantly improved ($P<0.05$) the treatment performance of the constructed wetland systems for NH4$^+$-N compared to Typha in gravel-bed wetland (Figure 12 and 14). Likewise, C.papyrus in broken brick had higher removal performance than C.papyrus in gravel-bed (Figure 12). In this study, seasonal variations couldn't influence the removal efficiency of the CWs.

The level of NO_3^--N in hospital wastewater was very low with an average concentration of 0.9±0.2 mg/l in the dry season and 1.1±0.5 mg/l in the rainy season with an average effluent concentration of 0.43 mg/l in the dry season and 0.48 mg/l in the rainy season. Its concentration was significantly ($P<0.05$) higher in inflow wastewater than the outflow of broken brick bed wetlands in the dry season and in all wetlands in the rainy season. However, there was no significant improvement in all gravel bed wetlands during the dry season and in unplanted gravel bed wetland in the rainy season. There was a higher removal percentage of NO_3-N in planted broken brick cells (ranged from 86.1% in the dry season to 74.5% in the rainy season) than in unplanted broken brick cell (ranged from 64.9% in the dry season to 63.6% in the rainy season). Typha plant in broken brick bed wetlands (CW2) was significantly ($P<0.05$) higher removal percentage of nitrate than Typha plant in gravel-bed wetland (CW5) and in other gravel bed wetlands (CW7 and CW8) in the dry season (Figure 11). Similarly, the C.papyrus plant (CW1) in the dry season and Typha plant (CW2) in the rainy season also removed significantly ($P<0.05$) higher NO_3-N than unplanted control gravel bed wetland (CW8). Likewise, C.papyrus in broken brick had higher removal performance than C.papyrus in gravel-bed (Figure 12). The higher reduction of nitrate-nitrogen in planted wetlands was observed during the study period in wetlands.



(a) Dry season (b) Rainy season

Figure 13. Comparison of nutrient removal percentage of broken brick and gravel bed C.Papyrus planted wetlands

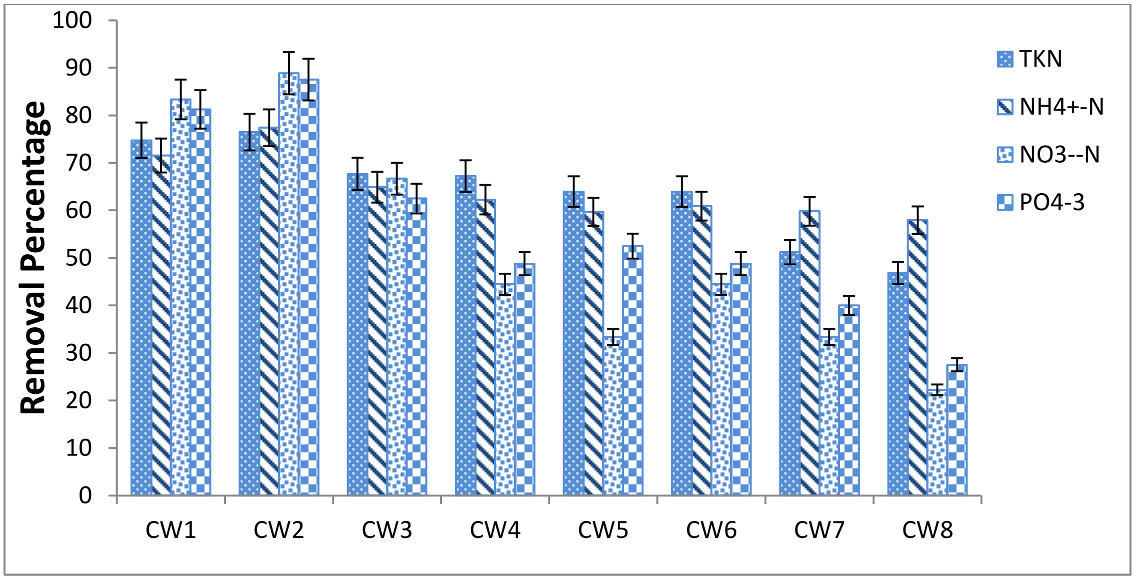


Figure 14. Nutrient Removal Efficiency of CWs during the dry Season

The average PO_4^{3-} concentration of the inflowing wastewater was 8 ± 1.4 mg/l in the dry season and 13.4 ± 4.9 mg/l in the rainy season. Data of influent PO_4^{3-} concentration and its removal for each wetland is summarized in Table 5 and Figure 13 &14. Its level in the outflow wetlands was significantly ($P < 0.05$) reduced than in inflow wastewater except for unplanted grave bed wetland in both seasons. The planted wetlands with broken brick media were significantly ($P<0.05$) higher removal efficiency of PO_4^{3-} as compared to the wetlands with gravel beds with an average removal rate of 84.4% in the dry season. Typha plant in broken brick bed wetland was significantly ($P<0.05$) removed PO_4^{3-} than unplanted gravel bed wetland with an average removal rate of 77.6% to 87.7% in a rainy and dry season, respectively. In the dry season, the unplanted broken brick wetland was also significantly ($p<0.05$) higher removal efficiency of PO_4^{3-} than unplanted gravel bed wetland. Typha plant in the broken brick bed wetland was significantly improved ($P<0.05$) the treatment performance of the constructed wetland systems for PO_4^{3-}compared to Typha in gravel-bed wetland (Figure 11). Likewise, C.papyrus in broken brick had higher removal performance than C.papyrus in gravel-bed (Figure 12).

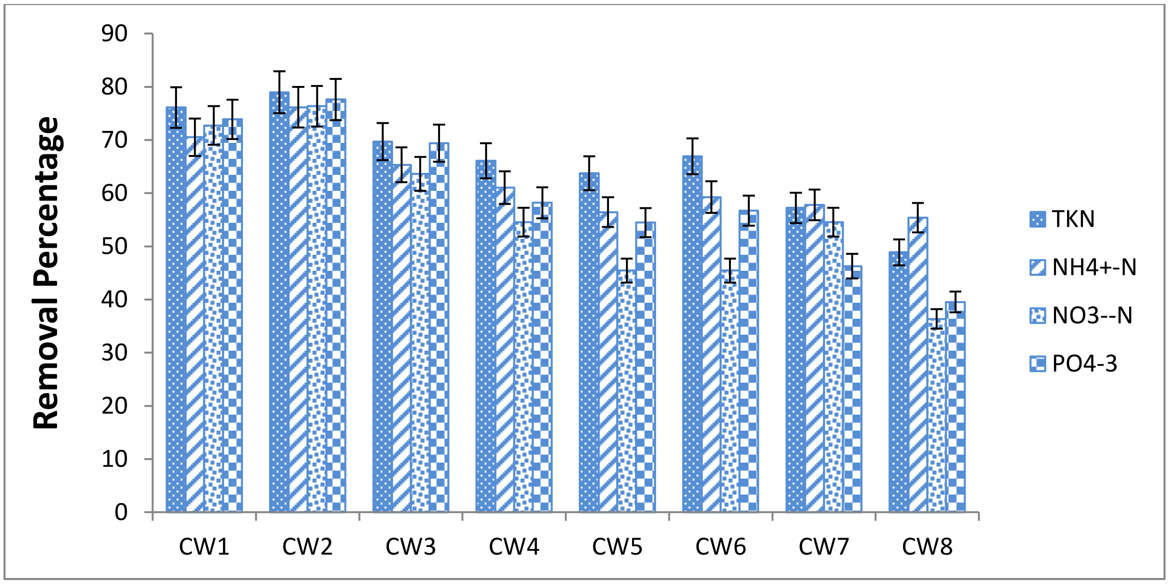


Figure 15. The nutrient removal efficiency of wetlands during Rainy Season

4.3. The Contribution of Wetland Plants in the Removal of Heavy Metals and Diclofenac from Wastewater

4.2.1. Heavy Metals Removal

The average hospital wastewater concentration and removal percentage of heavy metals were shown in Table 6 and Figure 16. The mean concentration of cadmium found in the input hospital wastewater was 0.078 mg/l in a range from 0.057-0.099 mg/l. Cd concentrations in the out-let wastewater samples were significantly lower ($P < 0.01$) than the inlet of the CWs, indicating that the CW has effectively removed the Cd from the wastewater. A reduction in the level of cadmium was evidenced in each of the eight wetlands of vegetated and non-vegetated with an average removal efficiency of 60.3-67.9% in unplanted wetlands and 100% in planted wetlands. The planted wetlands with broken brick media were significantly ($P<0.05$) higher removal efficiency of Cd as compared to the unplanted broken brick and gravel bed wetlands.

The influent chromium concentration of hospital wastewater was 0.1114±0.04 mg/l. The vegetated wetlands had higher removal of chromium with a removal efficiency ranged from 93.7%-100% while the non-vegetated wetlands achieved a lower removal of chromium ranged from 32.8%-45.2%. It was found that Cyprus Papyrus and Typha species of broken brick bed wetlands achieved a higher percent removal of chromium than other species from gravel bed wetlands.

The average level of Nickel in influent hospital wastewater was 0.162±0.082 mg/l with average effluent concentration ranged from 0.001-0.087 mg/l. Higher percent removal of nickel was recorded in broken brick planted wetlands than unplanted control wetlands. The removal efficiency of unplanted wetlands was ranged from 46.3% to 60.5% while the percent removal of planted gravel and broken brick bed wetlands was varied from 56.8-99.4%. The mean concentration of lead at the inflow and outflow were ranged from 0.69-0.79 mg/l and 0-0.18 mg/l, respectively. The average lead removal efficiency of planted and unplanted wetlands was ranged from 75.7%-100%.

The Zn concentration of the wetlands outflow was 0.003-0.1. The removal efficiency of planted broken brick bed wetlands were 93.4-97.8% while the unplanted broken brick bed wetland was 40.3%. Likewise, Typha planted gravel bed wetlands were also removed about 92.7% of Zn than unplanted gravel bed wetlands (25.4%). The highest percent removal (97.7%) was recorded in Typha based broken brick wetland and the least removal (25.4%) of Zn was recorded in unplanted gravel bed wetland. The planted gravel bed wetlands in this study were significantly removed Zn than unplanted gravel bed wetlands. Papyrus planted broken brick bed wetland was also significantly reduced Zn than papyrus planted gravel bed wetland (Figure 16). There was no significant difference between Typha plant broken brick and Typha plant gravel bed wetlands but the highest percent removal (97.7%) was recorded in Typha based broken brick wetland (Figure 17).

The influent concentrations of silver and arsenic in the hospital wastewater were 0.06±0.008 and 0.021±0.007 mg/l, respectively, but dropped in the effluent after passing through the wetlands. The average silver and arsenic removal efficiency of wetlands was 79.8% and 83.6%, respectively.

The highest percent (100%) removal of silver was seen in Typha based broken brick bed wetland. On the other hand, Typha in broken brick and gravel bed wetlands achieved the highest removal (100%) of arsenic than the other wetlands.

Table 6. Heavy metals concentration of inlet wastewater and the outlet of each wetland

Parameter	**HWW**	**CW1**	**CW2**	**CW3**	**CW4**	**CW5**	**CW6**	**CW7**	**CW8**
Cd (mg/l)	0.078±0.021	0	0	0.025±0.001	0	0	0	0	0.031±0.001
Cr (mg/l)	0.1114±0.04	0.001±0.0002	0	0.061±0.03	0.006±0.002	0.004±0.001	0.0062±0.002	0.007±0.003	0.075±0.008
Ni (mg/l)	0.162±0.082	0.002±0.001	0.001±0.0002	0.064±0.02	0.03±0.009	0.004±0.002	0.07±0.003	0.05±0.02	0.087±0.008
Pb (mg/l)	0.74±0.05	0.0023±0.001	0	0.11±0.06	0.005±0.001	0.0013±0.0008	0.041±0.002	0.067±0.002	0.18±0.05
Zn (mg/l)	0.134±0.009	0.0089±.003	0.003±0.001	0.08±0.006	0.06±0.002	0.01±0.006	0.03±0.01	0.08±0.007	0.1±0.006
Ag (mg/l)	0.06±0.008	0.006±0.002	0	0.017±0.005	0.01±0.005	0.02±0.007	0.02±0.01	0.01±0.007	0.014±0.006
As (mg/l)	0.021±0.007	0.0046±0.002	0	0.009±0.004	0.0023±0.001	0	0.001±0.0004	0.003±0.001	0.0076±0.001

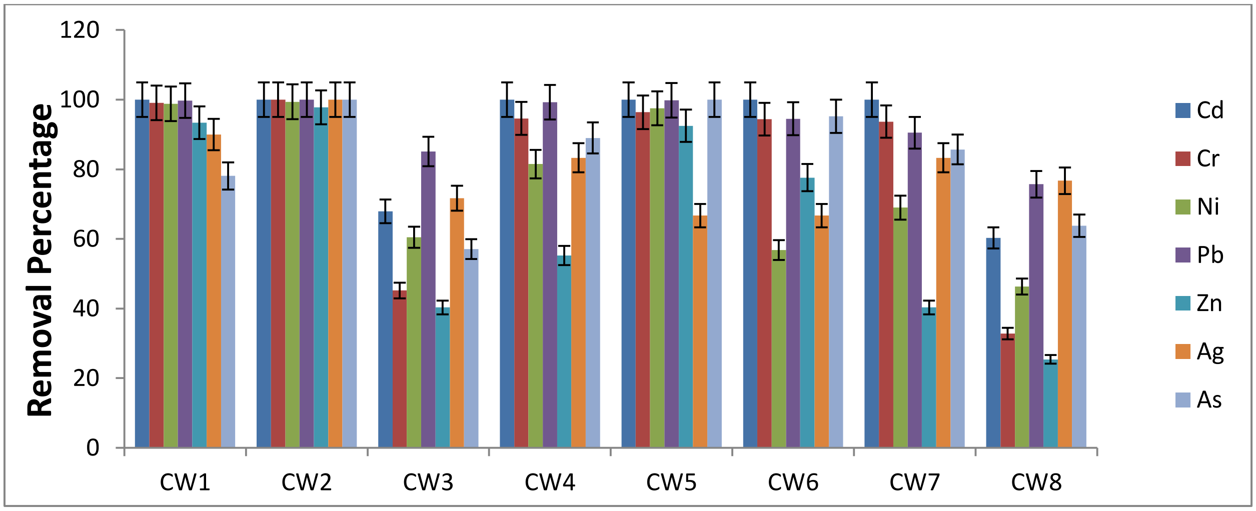


Figure 16. Heavy metals removal efficiency of wetlands

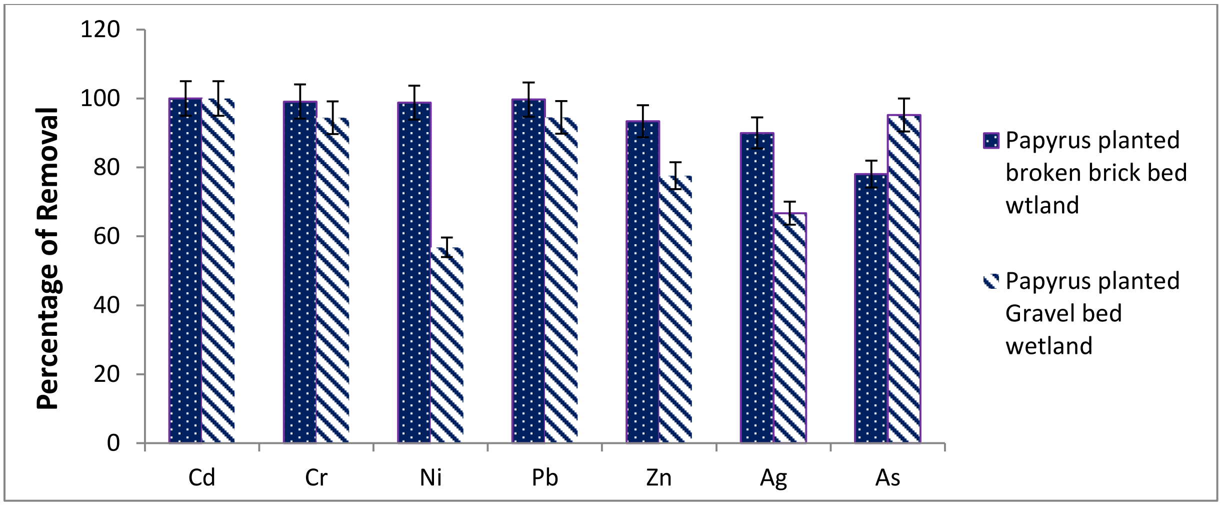


Figure 17. Comparison of Heavy metals removal percentage of broken brick and gravel bed C.Papyrus planted wetlands

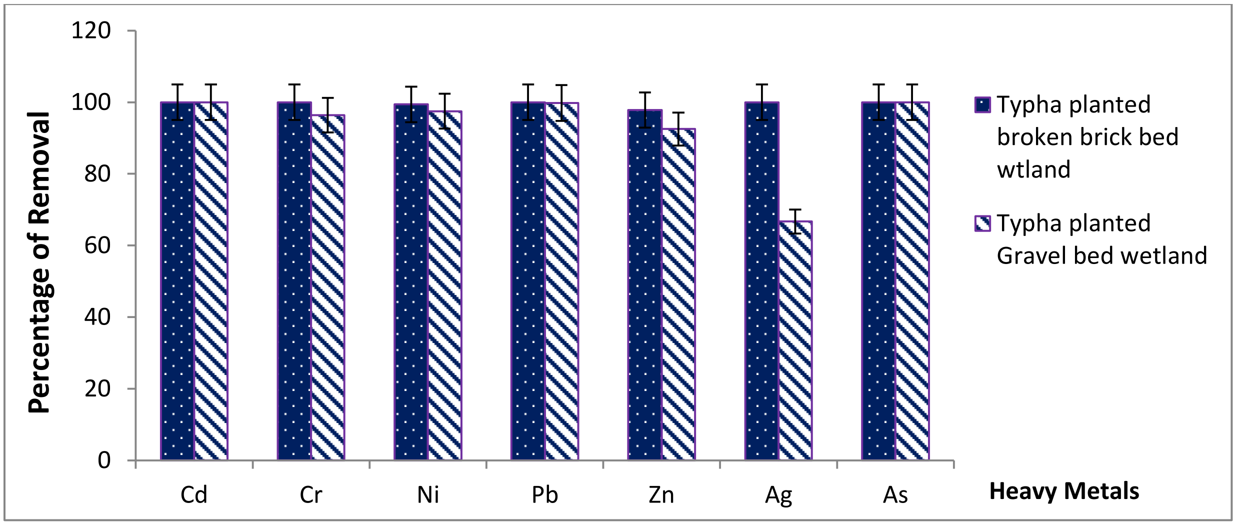


Figure 18: Comparison of heavy metals removal percentage of broken brick and gravel bed Typha planted wetlands

4.2.3. Drug Residue Removal

The average Diclofenac concentration of inflow hospital wastewater was 1.94±0.06 μg/L. Its concentration in the outlet of planted wetlands was ranged from 0-1.5 μg/L while in the unplanted wetlands reached a concentration of 1.33-1.9 μg/L. The average Diclofenac removal efficiencies of planted and unplanted reactors were 24%-100% and 8.2%-34.2%, respectively. The Typha planted broken brick bed wetland removed all the diclofenac from the wastewater (Figure 18). There was a significant difference in the removal of diclofenac between Typha planted broken brick bed wetland with other planted and unplanted wetlands of both the gravel and broken brick substrates. There was also a significant difference between Typha planted gravel bed wetland and planted and unplanted wetlands of both substrates (except Papyrus planted broken brick bed wetland). However, there was no significant difference between Papyrus planted broken brick bed wetland and unplanted broken brick bed wetland. All planted wetlands were significantly removed diclofenac than unplanted gravel bed wetland. There was also significantly higher removal efficiency in Typha and Papyrus planted broken brick bed wetlands than that of Typha and Papyrus planted gravel bed wetlands (Figure19). The load of ibuprofen and trimethoprim in this study were below the detection limit.

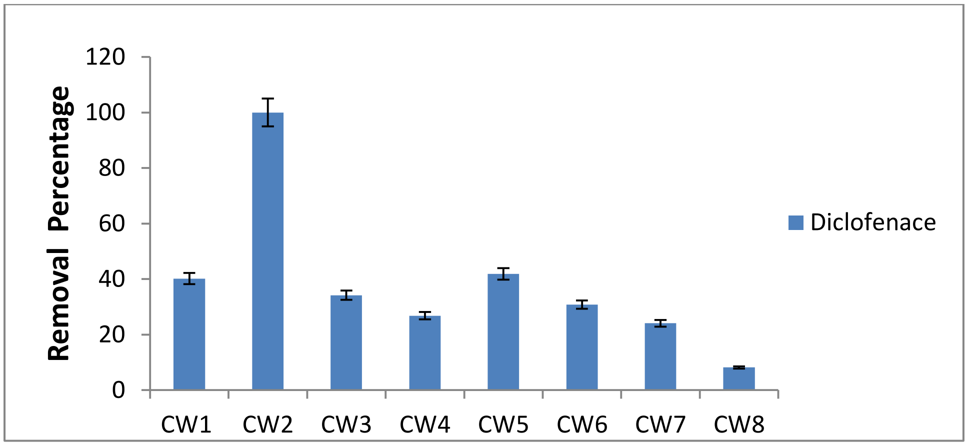


Figure 19. The average diclofenac removal efficiency of wetlands

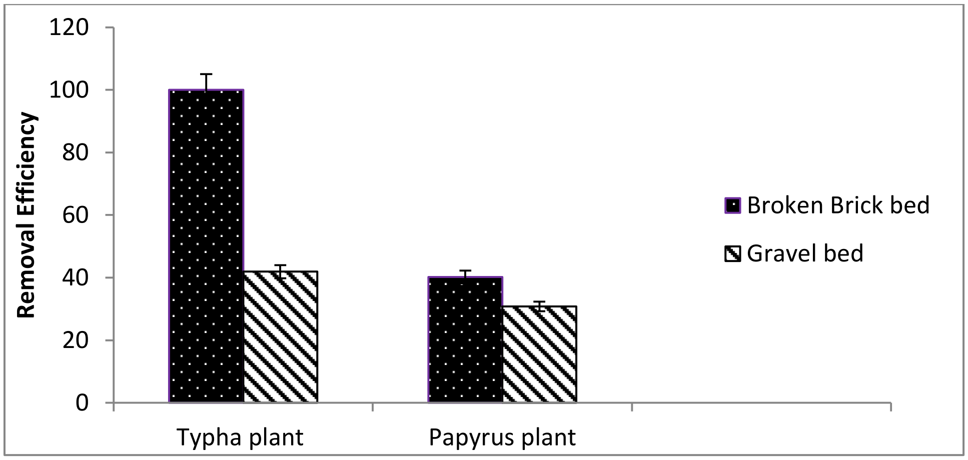


Figure 20. Comparison of diclofenac removal percentage of broken brick and gravel bed Typha and Papyrus planted wetlands

4.2.6. Coliform and Antibiotic-Resistant Bacterial Removal potential of CWs

The average number of total and fecal coliforms detected in hospital wastewater reached up to 1.3 x 10^7 ± 1.2 x 10^3 and 1.4 x 10^5 ± 5 x 10^2 MPN/100 ml that attained 7.1 log10 and 5.1 log10 removals in the outlet of both vegetated and non-vegetated wetlands, respectively. The final effluent released an average value of 1 x 10^3 - 7.9 x 10^3 and 1.1 x 10^2 - 4.5 x 10^3 MPN/100ml, respectively. Higher removal (100%) of *Salmonella* and *Shigella* was achieved in Papyrus planted broken brick bed (CW1), gravel bed sugar cane (CW4), and Typha planted wetlands (CW5). While lower removal was recorded in unplanted broken brick (CW3) and gravel bed (CW8) wetlands, respectively (Table 7).

Table 7. The Xmean (± standard deviation) bacterial load of hospital wastewater and outlets of CWs

Indicator Organism	**Hospital WW**	**CW1**	**CW2**	**CW3**	**CW4**	**CW5**	**CW6**	**CW7**	**CW8**
Total coliforms (MPN/100mL)	1.3 x 10^7 ± 1.2x10^3	1.2 x 10^3± 1x10^2	1 x 10^3 ± 70	7.9 x10^3± 1.5x10^2	1.6 x10^3 ± 96	1.4 x10^3± 80	2.3 x10^3± 1.2x10^2	1.3 x10^3± 73	5.1 x 10^3 ± 7.1x10^2
Fecal Coliforms (MPN/100mL)	1.4 x 10^5 ± 5x10^2	1.3 x 10^2± 25	1.1 x10^2± 17	2.3 x10^2± 42	1.5 x10^2± 24	2.1 x10^2± 17	1.6x10^2± 35	3.3 x10^2± 50	4.5 x 10^2± 70
Staphylococcus (CFU/mL)	3.2 x 10^3 ± 55	10±3	12±5	30±4	20±7	15±6	8±2	25±8	50±12
Salmonella (CFU/mL)	4±1	0	1	1±1	0	0	1	0	2±1
Shigella (CFU/mL)	5±2	0	0	2±1	0	0	0	1	1±1

Among the total samples, 159 bacterial isolates were detected, and 45(28.3%) were from inlet hospital wastewater and 114(71.7%) were from the outlets of eight CWs. The most frequently isolated bacteria from inlet hospital wastewater samples were found to be *Staphylococcus sp* 12(26.6%) followed by *E.coli* 11(24.4%), *Klebsiella sp* 9(20%), *Shigella sp* 5(11.1%), and *Salmonella sp* 4(8.9%). Similarly, *E.coli* 34(29.8%), *Staphylococcus sp* 28(24.6%), *Klebsiella sp* 26(22.8%), and *Pseudomonas sp* 9(7.9%) were frequently detected in the overall outlets of wetland samples (Table 8).

Table 8. The mean (± standard deviation) number of bacterial isolates from inlet hospital wastewater and the outlet of wetlands

Isolates	**Hospital WW**	**CW1**	**CW2**	**CW3**	**CW4**	**CW5**	**CW6**	**CW7**	**CW8**	**Total**
Escherichia coli	10.8±3 (24%)*	4.8±1.3 (43.2%)	5.8±2.5 (31.2%)	4.3±2.1 (20.8%)	2.8±1 (36.8%)	2(28.2%)	3.8±1.7 (41.8%)	3.3±1.9 (25.2%)	6.8±3 (25.2%)	44.4±16.5 (27.9%)
Shigella sp	4.8±1 (10.7%)	0(0%)	0(0%)	2.3±1.3 (11.1%)	0(0%)	0(0%)	0(0%)	1(7.6%)	1.3± 0.5 (4.8%)	9.4±3.6 (5.9%)
Pseudomonas sp	2(4.4%)	1(9%)	1(5.4%)	1(4.8%)	0(0%)	1(14.1%)	1(11%)	2(15.3%)	1.8±1 (6.7%)	10.8±1 (6.8%)
Staphylococcus sp	11.8±3.5 (26.2%)	3.3±1.9 (29.7%)	5.8±1 (31.2%)	3.8±1 (18.4%)	0(0%)	2.3±1.3 (32.4%)	0(0%)	4.8±1.3 (36.6%)	7.5±2.1 (27.8%)	39.3±12.1 (24.7%)
Klebsiella sp	9±2.1 (20%)	2± 0.8 (18%)	3.5±1.9 (18.8%)	6.3±2.6 (30.4%)	2.8±1 (36.8%)	0.8±1 (11.3%)	3.3±2.1 (36.3%)	2±0.8 (15.3%)	5.3±2.8 (19.6%)	35±15.1 (22%)
Salmonella sp	4.3±2.1 (9.6%)	0(0%)	1.5±0.5 (5.3%)	1(4.8%)	0(0%)	0(0%)	1(11%)	0(0%)	2±0.8 (7.4%)	9.8±3.4 (6.2%)
Citrobacter sp	2.3±0.5 (5.1%)	0(0%)	1(5.4%)	2±0.8 (9.7%)	2(26.3 %)	1(14.1%)	0(0%)	0(0%)	2.3±1.3 (8.5%)	10.6±2.6 (6.7%)
Total	45±12.2 (100%)	11.1±4 (100%)	18.6±5.9 (100%)	20.7±7.8 (100%)	7.6±2.3 (100%)	7.1±2.3 (100%)	9.1±3.8 (100%)	13.1±4 (100%)	27±11.5 (100%)	159.3±53.8 (100%)

* Percent in the bracket represents the specific isolated species over the total isolates of respective wetland and inlet hospital wastewater

E.coli, *Klebsiella*, and *Staphylococcus sp* were the most frequently isolated antibiotic-resistant species in treated wastewater. Among the isolated bacteria in constructed wetlands, 71.4% of *E.coli* were resistant to ampicillin in unplanted (control) gravel bed wetland as well as 50% of ampicillin-resistant *E.coli* were isolated in unplanted (control) broken brick bed, Typha planted gravel bed, and Papyrus planted gravel bed wetlands. Similarly, 50% of isolates found in both control unplanted wetlands were ampicillin, cotrimoxazole, and doxycycline resistant *Staphylococcus sp*. Higher ampicillin (66.7%) resistant *Klebsiella sp* was found in the control unplanted broken brick wetland. Likewise, a higher proportion (60%) of ampicillin, cefoxitin, and doxycycline resistant *Klebsiella sp* was found in control gravel bed wetland.

The frequencies of resistant isolates from treated wastewater to ampicillin were 67/114 (58.8%) followed by cotrimoxazole 45/114 (39.5%), doxycycline 45/114 (39.5%) and chloramphenicol 36/114 (31.6%). Relatively lower resistance among bacterial isolates was observed to gentamicin 21/114 (18.4%), ciprofloxacin 20/114 (15.5%), and amikacin 7/114 (6.7%) (Figure 20). From the total resistant isolates of treated wastewater, a higher proportion of ampicillin-resistant *Pseudomonas* 8/11 (72.7%), *Salmonella* 3/5 (60%), *Klebsiella* 16/26 (61.5%), *E.coli* 20/34 (58%) and *Shigella* 2/4 (50%) were observed. Similarly, 50% of *Shigella* isolates were found to be resistant to cotrimoxazole, chloramphenicol, and doxycycline.

Among isolates from hospital wastewater, the overall prevalence of multi-drug resistance (MDR) (resistant to three or more antibiotics) was found to be 30/45 (66.7%). *Escherichia coli* and *Klebsiella* isolates were subsequently characterized as 77.8% MDR followed by *Shigella* (75%) and *Salmonella* (60%). Similarly, the overall MDR prevalence in treated wastewater was 39/114 (34.2%). Of these, *E.coli* and *Klebsiella* account 47% and 46.1%, respectively.

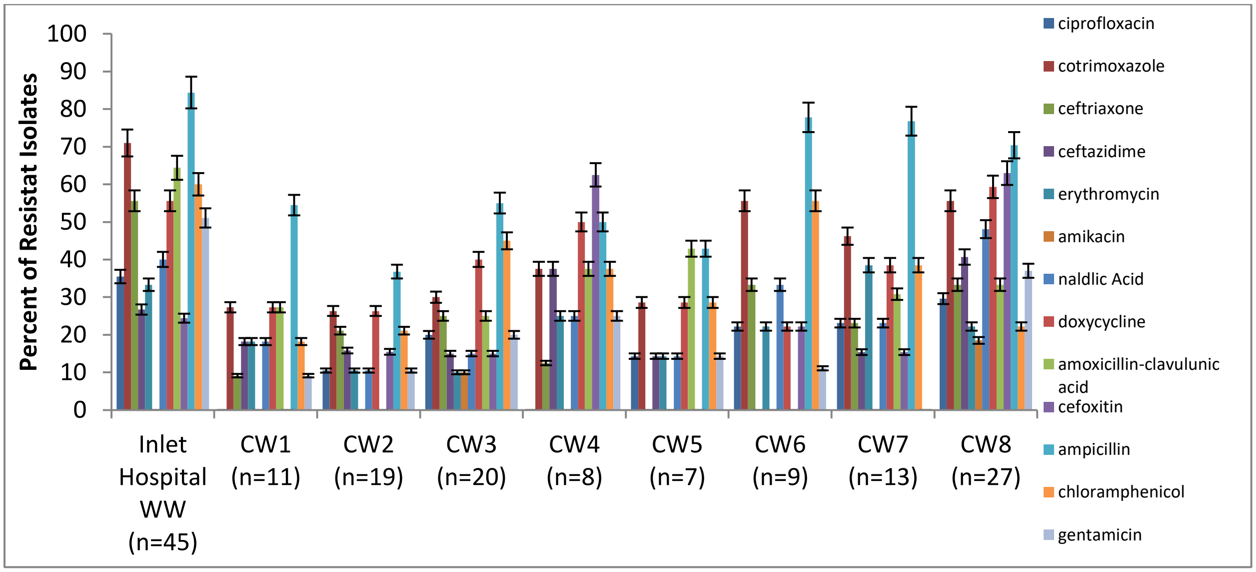


Figure 21. The overall antibiotic resistance pattern of bacterial isolates from the inlet hospital wastewater and the outlets of CWs

The frequency of antibiotic resistance in bacterial isolates to the most tested drugs was consistently increased in the hospital wastewater than outlets of the wetlands. The overall removal of antibiotic-resistant bacteria in the constructed wetlands was highly significant ($P<0.05$) but there was no significant reduction in non-vegetated gravel bed CW. A maximum removal (93.2%) of resistant bacteria was recorded in Typha planted broken brick bed wetland and the least removal (42.4%) was observed in non-vegetated gravel bed control wetland (Figures 21). Reductions of antibiotic-resistant bacteria were significantly higher (P<0.05) in the vegetated wetland in both substrates than in the non-vegetated control gravel bed CW throughout the study period. On the contrary, there was no significant difference in the removal rates between vegetated wetlands and the non-vegetated broken brick bed wetland. Additionally, Wetlands vegetated with different plants were proven to be continuously efficient in the removal of antibiotic-resistant bacteria, with no significant differences between them. Control broken brick bed wetland was significantly (P<0.05) reduce antibiotic-resistant bacteria than control gravel bed wetland.

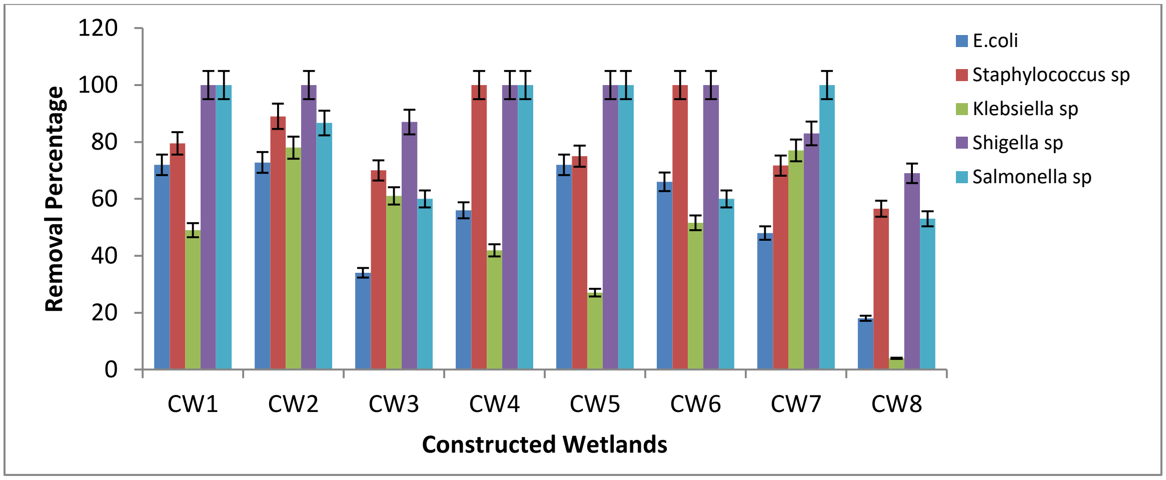


Figure 22. The average reduction rate of antibiotic-resistant isolates in CWs

CHAPTER FIVE

DISCUSSION

5.1. Wastewater Composition of Hawassa Referral Hospital

Hospital wastewater composition refers to the actual amounts of physical, chemical, and biological constituents present in the wastewater. During the diagnosis and treatment of diseases, hospitals release a considerable amount of harmful chemicals, organic matter, and infectious agents such as pathogens and microorganisms in their wastewater (Akin 2016). Based on the results obtained, the average pH values observed in the current hospital wastewater were 6.7 in the rainy season and 6.9 in the dry season. A similar study reported by Amouei et al. (2015) indicated that the pH of Babol University hospital of Iran to be ranged from 6.9-8.3. Likewise, the pH of Shiraz hospital in Iran reaches to 7.19 (Amirhossein and Bahareh 2016). Sarafraz et al. (2007) also reported that the average pH of 7 hospitals in Hormozgan province was 7.42. These results agreed well with the current studies and it seems to be slightly acidic to neutral condition. The mentioned range was also comparable to pH of domestic wastewater and might be suitable for the biological processes from the viewpoint of wastewater treatment. According to the standards of USEPA, the acceptable level of pH for agricultural reuse of wastewater is 6 to 9 (Kramer and Post 2005), considering our study findings it is clear that the pH of studied hospital wastewater was compatible with these standards.

The other common parameter used in defining the strength of wastewater is TSS. The TSS content of the pretreated wastewater of the hospital was ranged from 513-557 mg/L and 484.3-507.7 mg/L with the average concentration being 535 mg/l and 496 mg/L in the dry and the rainy seasons, respectively. A similar study also showed that raw hospital wastewater contains a TSS content of 539 mg/L (Berto et al. 2009). Beyene and Redaie (2011) also found an average TSS of 469.00±1.41 mg/L concentration of the current hospital wastewater. These results are closer to the obtained results of our study. Contrarily, different levels of TSS in hospital wastewater have been observed in various studies of Iran: 231.25 mg/L (Sarafraz et al. 2007), 289 ± 132 mg/L (Amouei et al. 2015), 296 mg/L (Amouei et al. 2012), 312 mg/L (Amirhossein and Bahareh 2016) and 72 and 383 mg/L (Mesdaghinia et al. 2009). Another study in Sri Lankan also reported a TSS content of raw hospital wastewater to be 314 mg/L (Kumarathilaka et al. 2015). Likewise, Emmanuel et al. (2004) reported a range of 155-298 mg/L in south France hospital. Also, TSS value varied between

98-162 mg/L ranges in the hospital laboratory wastewater of Turkey (Akin 2016). Such discrepancy in the concentration of TSS between different hospitals may be due to the number of patients served, the capacity of the hospital, solid waste management system, and the functionality of all the sewerage systems of the hospital. The release of such excessive levels of TSS into water bodies can have significant deleterious impacts on the physical, chemical, and biological properties of the water body. Particularly, phytoplankton, macrophytes, aquatic invertebrates, and fishes are highly affected by excessive release of TSS. It's effete on macrophytes and algae, primarily through affecting the amount of light penetrating through the water column (Bilotta and Brazier 2008).

The average concentration of BOD_5 in this hospital in terms of wastewater strength was reported to be 221±31.3 mg/L and 185±11.6 mg/L in the dry season and rainy season, respectively. Sarafraz et al. (2007) reported that the mean value of BOD_5 in 7 hospitals of Hormozgan province in raw wastewater was 242.25 mg/L. Another study reported by Amirhossein and Bahareh (2016) showed a BOD_5 of 272.98 mg/L in raw wastewater of Medical Sciences University of Shiraz hospitals. Similarly, In Malaga city of Indonesia, the concentration of BOD_5 in raw wastewater of 3 hospitals was in the range of 86.9-238.98 mg/L (Prayitno et al. 2013). The observed findings of the above studies are in accordance with the present study in biodegradable organic waste loads. However, other studies reported the higher values of BOD_5 in different hospital wastewater: 372 ± 173 mg/L (Amouei et al. 2015), 400 mg/L (Amouei et al. 2012), 228-768 mg/L (Mesdaghinia et al. 2009), and 6-1950 mg/L (Kumarathilaka et al. 2015).

As shown in Table 5 the average COD in this hospital wastewater was 713±86.5 mg/L in the dry season and 673±31.9 mg/L in the rainy season. Kootenaei and Rad (2013) also reported that the influent COD of Babol Clinic Hospital of Iran varies in a range of 635-744 mg/L in the period of the experiment. Different researchers also reported a similar concentration of COD ranged from 517.89-813 mg/L (Amouei et al. 2015; Sarafraz et al. 2007; Amouei et al. 2012; Mahvi et al. 2009; Amirhossein and Bahareh 2016). The result of the present research was agreed well with the above findings. However, some researchers reported a higher concentration of COD in hospital wastewater than that of the present study: 934.2 mg/L (Akin 2016), 130-1183 mg/L (Kumarathilaka et al. 2015), 362 to 1492 mg/L (Emmanuel et al. 2004), and 435-1362 mg/L (Mesdaghinia et al. 2009).

Such discrepancy in the concentration of BOD_5 and COD between different hospitals may be due to the size of a hospital, the bed density, number of inpatients and outpatients, the number and the type of wards, the number and types of services. Such a higher concentration of organic matter can affect the treatment efficiency of wastewater treatment plants as well as if they are released to water bodies without treatment, can negatively impact people and ecosystems. As a result, the accumulation of organic pollutants in rivers stimulates microbial growth, leading to oxygen depletion that disappears fish and aquatic invertebrates as well as disturb the entire river ecosystem (Wen et al. 2017). The degradability of the organic matter based on the BOD_5/COD ratio in the present study was in the range of 0.28-0.3 which is agreed well with the research done by Mesdaghinia et al. (2009) and Sarafraz et al. (2007). These results indicated that organic matter in the hospital wastewater had lower biodegradability. This resistance to biodegradation has its own impact from the viewpoint of wastewater treatment and to promote the efficiency of wastewater treatment plants.

In this study, the average concentration of TKN and NH_4^+-N in hospital wastewater were 86.3±11.7 mg/L and 73.4±0.6 mg/L in the dry season and 98±3 mg/L, 67.5±4.6 mg/L in the rainy season, respectively. A similar study conducted by Amouei et al. (2015) described that the average TKN values of hospital wastewater could reach 289 ± 132 mg/L while another study showed the mean contents of TKN was as low as 15 mg/L (Amouei et al. 2015). There was a relatively higher concentration of ammonium nitrogen in the present hospital wastewater than other studies done by different researchers. Rezaee et al. (2005) indicated that the concentration of ammonium nitrogen in Iran hospital was 18 mg/L. Another study in Germany hospital also showed the concentration of NH4-N was 13 mg/L (Beier et al. 2012). A study conducted in Iran hospital also indicated that the averaged influent concentrations of NH_3-N was 11.8 mg/L (Kootenaei and Rad 2013). This discrepancy might be due to the breakdown of organic nitrogen into ammonium in the septic tank pretreatment of the wastewater in this study. Similarly, the treatment process and the flow of wastewater in this hospital were in a closed way from the starting point up to the final treatment system. This might concentrate on the amount of ammonium in the wastewater.

The NO_3^-N concentration of the present hospital wastewater was 0.9±0.2 mg/L in the dry season and 1.1±0.2 mg/L in the rainy season. Irianto et al. (2013) indicated that the nitrate concentration of the Sanglah General Hospital Centre in Indonesia was 1.62 mg/L. Also, the nitrate concentration of 7 hospitals wastewater in Iran was 0.1-16.1 mg/L (Mahvi et al. 2009). Other researchers reported relatively a higher concentration of nitrate in hospital wastewater than the above studies which was in the range of 9.06-30.33 mg/L (Kootenaei and Rad 2013; Al-enazi 2016; Wyasu and Okereke 2012). The level of nitrate-nitrogen in the input wastewater was negligible in the present hospital. This might be as a result of anaerobic pretreatment of the nitrogenous compounds. As it was assessed during data collection, the wastewater from different wards was collected in the sewer system into septic tank pretreatment and transported to the final treatment system through sewers without exposure to external air.

The other most important nutrient frequently considered as a limiting agent in aquatic productivity is phosphorus. The concentration of PO_4^{3-} in the raw wastewater composition of this hospital was found to be 13.4±4.9 mg/L in the dry season and 8±2 mg/L in the rainy season. Wyasu and Okereke (2012) explained that the concentration of PO_4^{3-} in the Nigerian hospitals was ranged from 7.86 ±1.16 to 31.00± 1.48 mg/L. Akin (2016) also found out that the average PO_4^{3-} level of hospital wastewaters in Turkey was 15-30 mg/L. Another study obtained the concentration of PO_4^{3-} in 7 hospitals of Kerman province (southwest Iran) ranged from 6-51.5 mg/L (Mahvi et al. 2009). However, several studies confirmed that there was a relatively lower concentration of PO_4^{3-} (2.31-6.5 mg/L) than the present hospital (Prayitno et al. 2013; Al-enazi 2016; Aliahmad and Dehbashi 2013; Kootenaei and Rad 2013). This discrepancy on PO_4^{3-}concentration between different hospitals may be as a result of patient flow, availability of separate laundry wastewater management system, and the complexity of services given.

As it is discussed above, the concentration of nutrients in the present hospital was relatively higher. If it is not properly treated, its impact on the receiving environment may be enormous. As reported by many researchers, nutrient pollution has impacted many streams, rivers, lakes, bays, and coastal waters, resulting in serious environmental and human health issues, and impacting the economy (Kremser & Schnug 2002; Munn et al. 2019; Zhu et al. 2010). Excess amounts of nutrients can cause over-stimulation of growth of aquatic plants and algae. Excessive growth of these organisms, in turn, can clog water intakes, use up dissolved oxygen as they decompose, and block light to deeper waters. Some other major adverse effects include hypoxia of fresh and coastal waters, ocean acidification, long-term harm to human health, and increased emissions of greenhouse gases (Munn et al. 2019; Zhu et al. 2010).

Different metal concentrations in the raw hospital wastewater were measured for the last study period. Among the measured heavy metals in this study, Cd, Cr, Pb, and Ag had the highest concentration beyond the limit of discharge to the environment set by Kramer and Post (2001) and WHO (2006) with the average value of 0.078+0.021, 0.1114+0.04, 0.74+0.05, 0.06+0.008 mg/L, respectively. A similar study conducted in Turkey hospital showed that the average values of Cr and Cd were measured as 0.07 and 0.02 mg/L in a six-month period (Akin 2016). Kumarathilaka et al. (2015) also found out that the maximum concentration of Cr (VI) and Pb in Sri Lanka hospital was reported as 0.23 and 0.90 mg/L. Another study conducted by Meo et al. (2014) also showed the total levels of Cadmium, Chromium, and Lead in raw hospital wastewater were ranged from 0.032-0.676 mg/L, 0.042-0.107 mg/L, and 0.012-0.229 mg/L, respectively. Likewise, Babaahmadi et al. (2017) indicated that the concentration of Pb, Cr and Cd were 0.53+0.08, 0.9 7+ 0.20, and 0.0357+0.008 mg/L, respectively. However, there were relatively lower average lead and chromium concentrations in Wolaita Sodo Teaching Referral hospital, with average values of 0.01 mg/L and 0.03 mg/L, respectively (Moga et al. 2017). These results indicated that hospital wastewater releases a significant amount of toxic heavy metals when compared to the WHO standard that can adversely affect the receiving environment.

According to Tchounwou et al. (2012) due to their higher degree of toxicity, cadmium, chromium, and lead ranked among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants to plants, animals, and human beings that are known to induce multiple organ damage, even at lower levels of exposure. They are also considered as human carcinogens. They are non-biodegradable and persistent in nature, therefore get accumulated in soils, plants, and other animals. Uptake of heavy metals by plants and its subsequent accumulation along the food chain is also a potential threat to animal and human health. Heavy metals can also affect the key microbial processes and decrease the number and activity of soil microorganisms (Jiwan & Ajah 2011). Their impact on aquatic organisms is also higher. Due to the movement of these pollutants from various sources, they give rise to coincidental mixtures in the ecosystem. Thus posing an immense threat to aquatic fauna especially to fishes which constitutes one of the major sources of protein-rich food for mankind (Rajeswari & Sailaja 2014; Tchounwou et al. 2012).

Currently, pharmaceuticals are found in every compartment of the environment (Kümmerer 2001). Hospital is one of the major sources of these pollutants. The wastewater treatment plants are poorly designed to treat these types of compounds efficiently. Diclofenac, a widely used analgesic nonsteroidal anti-inflammatory pharmaceutical drug, was detected in all influent hospital wastewater samples and were present at the concentrations of 1.94±0.06 μg/L. It has also been detected in hospital wastewater of Switzerland at concentrations of 0.858±0.186 μg/L (Kovalova et al. 2013), in Slovenia at 1.1-3.2 μg/L (Klančar et al. 2016), in South Africa at 2.38 ± 4.46 μg/L (Kanama et al. 2018) and in the Netherlands at concentrations up to 2-4 μg/L (Langenhoff et al. 2013). Alawi and Alahmad (2012) also reported high concentrations of Diclofenac (3-7 μg/L) in the influent of Jordan hospital. The present study is in line with some of the above-published hospital wastewater studies, and confirms differences in the loading patterns for specific hospitals maybe depend on the administration and consumption rate, disposal of unused or expired drugs and the number of patients served. According to Bonnefille et al. (2018), if marine organisms exposed to 1 µg/L Diclofenac concentration, it will have a negative effect on reproduction (e.g. sperm motility, fertilization success) and osmoregulation as well as can cause oxidative stress and alteration of immune functions.

Hospitals release a huge amount of harmful infectious agents such as pathogens and microorganisms in their wastewater (Akin 2016). They also act as the storehouse of antibiotic-resistant bacteria due to the widespread use of antibiotics to treat microbial infections (Kümmerer 2001). In the present study, it was found that hospital wastewater released an average concentration of l.3x10^7 MPN/100 total coliforms and 1.4×10^5 MPN/100ml fecal coliforms. A similar study conducted in Mekele, Ethiopia, reported a comparable concentration of total coliform (2.2×10^6 CFU/100ml) and fecal coliform counts (2.0×10^5 CFU/100ml) (Asfaw et al. 2017). Likewise, Akin (2016) reported that a higher proportion of *Pseudomonas aeruginosa* (2×10^4-9×10^6 CFU/ml), *Acinetobacteria* (5×10^4-6×10^6 CFU/ml) and *Coagulase-negative Staphylococcus* (7×10^1-5×10^2 CFU/mL) was released. The number of coliforms released from hospitals mostly depends on the number of patients served, hospital capacity, and the type of cases managed.

In the present study, bacterial isolates such as *staphylococcus*, *E.coli*, *Klebsiella*, and *Shigella* were most frequently detected in hospital wastewater. The same result was found in Hawassa and Yirgalem referral hospitals studied in 2010/11 (Fekadu et al. 2015). Researchers in different countries reported that *E.coli*, *Staphylococcus sp, Klebsiella sp, Enterobacter sp, Pseudomonas sp, Proteus sp, Shigella sp, Bacillus sp, Citrobacter sp, and Serratia sp* were the dominant isolates in hospital wastewater (Moges et al. 2014; Alam et al. 2013; Ashfaq et al. 2013). High counts of bacterial load reflected the potential of the hospital wastewater to pose a significant impact on the health of the community by boosting infectious risks through the spread of infectious diseases caused by bacteria specifically antibiotic-resistant isolates.

Among bacterial isolates from the present hospital wastewater, 100% of Salmonella isolates were found to be resistant to ampicillin, and a higher proportion of ampicillin resistant *E.coli*, *Shigella*, and *Klebsiella* were also observed. In similar studies, ampicillin-resistant *E.coli* (Galvin et al. 2010), *Klebsiella, Shigella,* and *Citrobacter sp* (Alam et al. 2013) were detected in every sample of hospital effluent. Moges, et al. (2014) also reported that all the isolates of *S. aureus*, *E.coli*, *Citrobacter sp,* and *Enterobacter sp* were found to be 100% resistant to ampicillin. In general, most of the bacterial isolates in this study were highly resistant to cotrimoxazole, amoxicillin-clavulanic acid, chloramphenicol, ceftriaxone, and doxycycline. A similar study in Mekele showed simultaneous resistance to penicillin, tetracycline, doxycycline, cotrimoxazole, amoxicillin-clavulanic acid, and ceftriaxone (Asfaw et al. 2017). Likewise, the study reported by Alam et al., (2013) showed concurrent resistance to the same drugs in extended-spectrum beta-lactamases (ESBL) producing enteric bacteria.

In the present study, fairly higher percent (66.7%) of multi-drug resistant isolates were detected in hospital wastewater which was similar to the results reported elsewhere (Exner et al. 2017; WHO 2014). Compared to the present study, an increased proportion (76.2%-81.5%) of multi-drug resistant bacteria among hospital wastewater isolates was reported (Asfaw et al. 2017; Moges et al. 2014). The widespread distribution of such MDR strains in hospital wastewater is essentially due to higher lactamase(s) production of enteric bacteria against β-lactams antibiotics (Alam et al. 2013; Egbule 2016), the horizontal transfer and exchange of resistant mobile genetic elements (Egbule 2016) and proliferation of resistant bacteria due to selective pressures imposed by antimicrobial residue (Jamali et al. 2015).

5.2. Use of Broken Brick to Enhance the Removal of organic matter and Nutrients in CWs

Wastewater temperature is one of the essential factors among the various physicochemical parameters in any aquatic ecosystem, since it influences the growth, activity, and distribution of microorganisms, other flora, and fauna. The removal rate of total nitrogen is also highly influenced by ambient temperature since the ammoniation, nitrification. and denitrification process are related to temperature in wetlands (Shi et al. 2018). In addition to this, plant photosynthesis and transpiration are also strictly associated with temperature to provide oxygen to the wetland through plant roots (Lee et al. 2009). In the present study, a little seasonal variation was recorded in the outflow of wetlands with a minimum value of 22.4^0C during the rainy season and a maximum of 25.6^0C during the dry season (March 2011 to Feb 2013) with a mean value of 24^0C which is suitable to nitrification and denitrification (Shi et al. 2018).

pH is also the other important factor that determines nutrient removal rate through nitrification/denitrification, volatilization, and ammonification in the wetland treatment system (Lee et al. 2009). It also determines the adsorbent surface charge, ionization degree, and speciation of the adsorbent in the aqueous media. Overall, suitable pH for bacteria growth and activity in biological waste water treatment processes ranges between 6.5 to 8.5 (Emmanuel et al. 2002). The average pH of influent hospital wastewater was 6.7 in the rainy season and 6.9 in the dry season which is favorable for wetland biological activity. After the wastewater passing through the wetland beds, pH increased to 7 and 7.4 which were observed to be near neutral that is conducive for aquatic life and microbial activity. All horizontal flow constructed wetland units showed lower pH variations throughout the study period. These results were within the USEPA's acceptable level of 6 to 9 for agricultural reuse of wastewater (Kramer and Post 2005).

The average suspended solid percent removal efficiency in this study was 93.2% in the dry season and 89.7% in the rainy season. The wetlands containing broken brick as substrate remove TSS between a range of 90.3% -96.3% in the dry season and 88.7% -93.1% in the rainy season. The effluent of most wetlands in this study achieved the minimum USEPA standard of 30 mg/L SS to use the wastewater for agricultural purposes (Kramer and Post 2005). These high suspended solid removal rates by horizontal subsurface flow constructed wetlands were also reported in Spain (Andreo-Martínez et al. 2016) and in Korea (Gupta et al. 2016). Lee et al. (2004) also obtained

96–99% removal of TSS by horizontal subsurface flow wetland in Taiwan. However, there was no significant difference in the removal percentage of suspended solids among the eight wetlands in the dry and the rainy seasons, this result agrees well with the results of Prost-Boucle *et al.* (2015) and Wang *et al.* (2017).

Moreover, there was no visible difference observed between the performance of the planted and unplanted wetlands in both substrates. Similar recent studies also confirmed that plants have usually no effect on the removal of suspended solids (Martin et al. 2012; Gupta *et al.* 2016). The similarity in TSS treatment between planted and unplanted systems in this study persisted in all seasons of the year, with the broken brick and gravel bed wetlands providing the most consistent performance even during the rainy season, this result agrees well with the results of Elfanssi et al. (2018). This confirms that TSS removal is greatly attributed to physical processes like mechanical filtration and microbial breakdown of the organic portion of suspended solids (Gupta et al. 2016). Additionally, flocculation and settling of colloidal by sedimentation, straining and physical capture, and adsorption onto substrate play a great role in their reduction (USEPA 2000).

The average BOD_5 percent removal efficiency in this study was 90.4% in the dry season and 85.8% in the rainy season. A similar result by Gikas et al. (2007) showed that the mean BOD_5 removals were 89% and 93.5% for temperatures below and above 15ºC, respectively. Even if there is no statistical difference, the gravel bed wetlands remove BOD_5 more than the broken brick bed wetlands, that achieved the USEPA standard of 30 mg/l BOD (Kramer and Post 2005). This might be due to the higher compaction potential of the gravel-based beds to facilitate the rate of filtration of organic matter in the system. The average COD removal efficiency of both planted and unplanted wetlands ranged from 76.2% to 88.1% in the dry season and 75.8% to 88.4% in the rainy season that is consistent with other researchers' findings (Martin et al. 2012). Zhang et al. (2011) also reported that the removal efficiencies of CWs were 92.6% (planted) and 89.6% (unplanted) for COD.

However, there was no statistically significant difference in the removal percentage of BOD5 and COD among planted and unplanted cells in both media types throughout the study period, comparable findings also observed by Qing et al. (2011). Similarly, Albalawneh et al. (2016) reported that plants haven't a significant effect on the reduction of COD. Likewise, the planted cells in both substrates did not differ significantly ($P > 0.05$), this result agrees well with the results of Mairi et al. (2012). In this study, the influence of temperature was not so vital for the removal of BOD_5 and COD, comparable findings also observed by Prost-Boucle et al. (2015) and Wang et al. (2017).

The above results indicated that role plants in BOD_5 and COD removal was minimal since their existence is not directly proportional to the BOD and COD levels reduction. Thereby, bacterial degradation may play a key role in the removal of BOD and COD in HSSF CWs (Abdul and Ganapathyvenkatasubramanian 2016). The organic matter in wastewater was dominantly degraded by facultative and anaerobic heterotrophic microorganisms in the wetland reactors due to minimal oxygen concentration in the bed (USEPA 2000). Furthermore, filtration, adsorption, sedimentation, and oxidation are also responsible for their reduction in wetlands (Skoczko et al. 2017; Lee et al. 2004).

The nitrogen removal efficiency was only modest in subsurface flow constructed wetlands. The results of this study showed that broken brick bed wetlands provide better removal of nutrients than gravel beds. In the dry season, broken brick bed planted wetlands exhibited a higher removal percentage of TKN (75.6%) than unplanted broken brick bed wetlands (67.7%). Lima et al. (2018) also reported that the vegetated CW with clay bricks presented the best performance for TKN removal with maximum removal efficiencies of 68%. Both planted and unplanted broken brick bed cells in the rainy season were significantly higher ($P<0.05$) removal efficiency than unplanted gravel bed wetland. A maximum of 76.5% to 79% of TKN removal was achieved by Typha species which is in agreement with other reported literature (Sun et al. 2009; Basker et al. 2014). This result indicated that the removal of nitrogen in constructed wetlands is mainly due to the plant uptake in the planted wetlands as compared to denitrification that occurs in the unplanted wetlands under anoxic conditions (Vymazal 2007b).

As expected, the NH_4^+-N concentration generally decreased through the subsurface flow constructed wetland system. Media type influences the removal of NH_4^+-N in this study. It was revealed that the wetlands with broken brick were more efficient as compared to the wetland with gravels with an average removal rate of 71.3% in the dry season and 70.7% in the rainy season. Additionally, Typha planted broken brick bed wetland was significantly improved ($P<0.05$) the treatment performance of the constructed wetland systems for NH_4^+-N compared to Typha plated gravel bed wetland. Abdul and Ganapathyvenkatasubramanian (2016) also showed that an effective removal performance was achieved by using brickbats as a CW media. Similarly, Lima et al. (2018) reported that the vegetated CW with clay bricks presented the best performance for total ammonia nitrogen removal. This might be due to the higher potential of broken brick for the enrichment of microorganisms and the growth of plants as well as its adsorption capability of pollutants (Wang et al. 2012). The higher elimination rate can also be explained by the higher cation exchange capacity of broken brick (Yang et al. 2016).

In this study, seasonal variations couldn't influence the removal efficiency of the CWs. However, the study done by Andreo-Martínez, et al. (2016) reported that ammonia removal was influenced by the level of temperature in the reactor. High temperature facilitates the blockage of nitrogen by plants growing in the wetland as nitrates and ammonia and the activity of bacteria responsible for nitrogen removal became better.

The removal of NH_4^+-N was significantly improved in planted systems than in unplanted systems ($p < 0.05$) which is in agreement with somewhere else (Caselles-Osorio et al. 2017; Qing et al. 2011). Mancilla et al. (2013) also explained that plants have a positive influence in the removal of ammonia by direct assimilation or uptake of ammonia, and indirectly due to the supply of oxygen by plant roots for the nitrification process. This indicated that the removal of NH_4^+-N is mainly due to the plant uptake. The nitrification process was usually considered to be limited under low oxygen concentration conditions, resulting in a high ammonium removal rate by denitrification. According to Keffala and Ghrabi (2005), the oxygen released from roots in anaerobic condition was too low to enhance ammonia oxidation. In general, this study indicated that plant uptake and sediment storage were the key factors limiting NH_4^+-N removal (Wu et al. 2013).

In this study, there was a higher removal of NO_3^--N in planted broken brick cells than the unplanted broken brick cells. Several experimental studies also confirmed that unplanted treatment wetlands had lower nitrogen removal than planted treatment wetlands (Kassa and Mengistou 2014; Tadesse and Seyoum 2015). Martin et al. (2012) also reported that CWs with vegetation showed better removal efficiency of nutrients than the non-vegetated CWs in both seasons. The higher reduction of nitrate-nitrogen in planted wetlands was observed during the study period, this might be because of the taken up of the existing lower concentration of nitrate in influent by plants and incorporated into the biomass (Rana and Laura 2014). This is confirmed by Lima et al. (2018) that direct uptake of the nitrogen by plant species was varied from 4 to 74%. Additionally, the progressive increase in the plant density, shoot length and stem diameter was positively correlated with the nutrient removal efficiency of the treatment beds. These macrophytes had the ability to accumulate high biomass and remove nutrients and therefore have high potential in biological nutrient removal processes (Kassa and Mengistou 2014).

The core removal mechanisms of nitrogen in CWs include nitrification/denitrification, volatilization, ammonification, plant uptake, and matrix adsorption (Vymazal 2007). However, the nitrification process in horizontal subsurface flow wetland is usually considered to be limited due to the release of lower oxygen concentration by plant roots and the available small concentration is mostly consumed by competitive microorganisms to degrade organic matter (Gupta et al. 2016). Furthermore, volatilization couldn't be the mechanism of removal in this study since the pH of the wastewater was nearer to neutral. According to US-EPA (2002), a maximum nitrogen removal by volatilization occurs predominately at pH values of 9.0 and above. Denitrification seems to be the dominant mechanism of removal in this study due to the fact that the anoxic and/or anaerobic condition as well as the pH and temperature of wastewater is conducive for denitrification (Shi et al. 2018). Additionally, adsorption of nitrogen ions by brick media may play a part in its removal. The microorganisms attached to different parts of plants and media were also the contributors to the degradation of organic nitrogen and its removal from the wastewater (Sirianuntapiboon and Jitvimolnimit 2007).

The minimum, mean and maximum PO_4^{3-} concentrations of the outflow wastewater were 1 mg/l, 3.5 mg/l, and 5.8 mg/l in the dry season and 2 mg/l, 5.2 mg/l, and 8.1 mg/l in the rainy season, respectively. The wetland outlet samples during the dry season were below the permissible limit (5 mg/L) set for wastewater reuse and discharge (WHO 2006a) except the unplanted grave bed wetlands. However, during the rainy season only broken brick bed planted and unplanted wetlands were achieved the minimum permissible limit. Its level in the outflow of wetlands was significantly ($P < 0.05$) reduced than in inflow wastewater except for unplanted grave bed wetland in both seasons. The planted wetlands with broken brick media were significantly ($P<0.05$) higher removal efficiency of PO_4^{3-} as compared to the wetland with gravel beds with an average removal rate of 84.4% in the dry season. Lima et al. (2018) also reported that both planted and unplanted clay brick bed wetlands efficiently removed phosphorus with mean removal efficiencies of 82% and 87%, respectively.

Typha planted broken brick bed wetland was significantly ($P<0.05$) remove PO_4^{3-} than unplanted gravel bed wetland with an average removal rate of 77.6% to 87.7% in the rainy and the dry seasons, respectively. A similar study reported by Sun et al. (2009) showed that phosphorus removal rate reached 88.9% by cattail (Typha). Kassa and Mengistou (2014) also reported that there were statistically significant differences ($p<0.05$) in the removal of PO_4^{3-} between planted and control treatment bed and also among planted treatment beds. They also added that the planted treatment beds have better efficiency in the removal of PO_4^{3-}compared to the unplanted treatment beds. Likewise, higher performance was also reported by Mateus et al. (2016), Abdul and Ganapathyvenkatasubramanian (2016), and Wang et al. (2012) that vertical flow wetland with broken brick media achieved 80% to 90% phosphorus removal. The result indicates that plants and broken brick media must work together for the better removal of phosphate, suggesting a synergistic mechanism.

In the dry season, the unplanted broken brick wetland was also significantly ($p<0.05$) higher removal efficiency of PO_4^{3-} than unplanted grave bed wetland. Clay bricks could significantly enhance phosphorus removal capacity in CWs (Lima et al. 2018). It is also well known that the broken brick substrate had better phosphorus removal abilities as they have a greater surface area to provide better adsorption (Mateus et al. 2016). Lima et al. (2018) reported that no desorption or increase of phosphorus in effluent samples after 296 days of operation of wetlands, this indicating that the clay brick was not yet saturated and phosphorus probably presents a strong binding to the media. Additionally, Wang et al. (2012) stated that broken brick plays a vital role for the enrichment of microorganisms and the growth of plants as a filter medium in CWs. The contents and chemical forms of broken brick could also be the principal factors for phosphorus removal by precipitation process (Wang et al. 2012). Phosphorus might precipitate in the form of calcium phosphate, and this could be caused by high calcium content in the brick media in this study, as explained by other literature (Albalawneh et al. 2016).

There was no significant difference between planted and unplanted gravel bed wetlands in the removal of phosphate in the dry season. Similarly, in the rainy season, planted and unplanted broken brick and gravel bed wetlands haven’t a significant difference in PO_4^{3-} removal. This supports the fact that the plants have limited ability for the uptake of $PO_4^{3-}$and that adsorption or precipitation by bed media contributes to phosphorus removal (Mateus et al. 2016). On the other hand, phosphorus removal was dependent on water temperature. In this study, better removal of phosphate was recorded in planted broken brick wetlands during the dry season with an average removal rate of 81.3% to 87.5% than in the rainy season with an average removal rate of 73.9% to 77.9%. Similarly, Villalobos et al. (2013) stated that seasonal variations strongly affected the phosphorous removal in HSSF. This might be related to the increase in plant growth and microbial activity in the warm season (Elfanssi et al. 2018).

5.3. The Contribution of Wetland Plants in the Removal of Heavy Metals and Diclofenac from Wastewater

The release of heavy metals into the environment arisen from a wide range of natural and anthropogenic sources (Qasaimeh et al. 2015). Unlike organic pollutants, heavy metals cannot be degraded through biological processes. However, different research findings showed the mechanism of heavy metals removal in treatment wetlands through the use of several substrate media and plant species (Matagi et al. 1998; Qasaimeh et al. 2015; Sheoran and Sheoran 2006). Matagi et al. (1998) discuss the potential for heavy metal removal mechanisms by wetlands through sedimentation, absorption, co-precipitation, cation and anion exchange, complexation, microbiological activity, and plant uptake.

Many metals such as Co, Cu, Fe, Mn, Mo, Ni, and Zn are essential micronutrients for plants because they are involved in numerous metabolic processes as constituents of enzymes and other proteins. The rhizosphere of the aquatic plants provide substrate and supporting media for the growth of microorganisms, which help in the immobilization of heavy metals and uptake by plants (Guittonny-philippe et al. 2014). The removal of metals can also be achieved via sorption with the use of effective sorption media in CWs. These adsorption processes are due to an electrostatic bond between the metal and the charged surfaces in a substrate. Substrate surface charge is highly dependent on pH, with a greater negative charge at higher pH (Justin et al. 2008). Another mechanism of metal cations removal from CWs is by precipitation reactions which form new solid phases, usually in association with a corresponding anion already present in the solution.

The Cd removal performance of all planted CW was 100% in view of the fact that Cd was not detected in the outflow of any planted cell. Also, Sarafraz et al. (2009) reported that the removal rate of Cd was found to be almost 100%. Similarly, a vertical flow CW with *C.alternifolius was* effectively removed (100%) Cd and remained stable over an operational period (Cheng et al. 2002). Likewise, the Cd removal efficiencies of the CW in Pakistan were 91.9% (Khan et al. 2009). Justin et al. (2008) also explained that through the use of test substrates like sand, peat and ceramic waste particles in the model-scaled CW achieved 97.8%-98.0% removal of Cd. The average removal percent of the unplanted broken brick and gravel bed in these wetlands was 67.9% and

60.3%, respectively. Interestingly, the Cd removal efficiency was higher when compared with other heavy metals and this could be related to high mobilization of Cd and further uptake of plants.

Planted CWs were significantly ($P<0.05$) higher removal efficiency for Cd than unplanted wetlands. This value agreed well with the findings of the previous studies (Kumari and Tripathi 2015). George and Gabriel (2017) also revealed that significantly higher removal of heavy metals was recorded by planted than the unplanted CWs. There is evidence that wetland plants such as Typh, papyrus, sugarcane and bulrush plants can accumulate heavy metals in their tissues (Mojiri 2012). Moussa (2015) and Mojiri (2012) proposed that high accumulation of metals by plants, particularly in the root tissues might have resulted from complexation of the metals with sulfhydryl groups.

According to Khan et al. (2009) and Moussa (2015) plant uptake and accumulation of Cd varies from one species to another and high concentrations were found in root tissues as compared to aerial tissues. Gill et al. (2014) also revealed that the Cd was measured in all the root samples. However, Moussa (2015) and Mojiri (2012) oppositely reported that the concentration of Cd in the root is relatively lower than other heavy metals. Rhizofiltration was found to be the best mechanism to explain Typha domingensis phytoremediation capability (Mojiri 2012). On the other hand, Khan et al. (2009) reported that the bottom substrate retained a large amount of Cd through adsorption. Mohammed and Babatunde (2017) explained that up to 91% of Cd can be removed through the adsorption process.

Chromium level in the influent wastewater was significantly ($P < 0.05$) higher than in effluents except for unplanted control gravel bed wetland, which indicates the CW has effectively removed Cr from the wastewater. Its concentration in the outlet of wetland samples ranged from 0 to 0.075 mg/L which was lower than the permissible limit (0.1 mg/L) set for wastewater reuse and discharge (WHO 2006). The Cr removal efficiency of vegetated wetlands was ranged from 93.7%-100%. Similarly, in the planted unit maximum Cr removal efficiencies of 100% were recorded at HRT's of 1 day in Greece (Sultana et al. 2015). Also, Sultana et al. (2013) revealed that the removal of Cr reached 100% in planted wetlands. Other studies in different countries also showed effective removal of Cr by planted wetlands such as 82.21 ± 10.9% (Choesin et al. 2016), 87-90% (Gikas et al. 2013), 81.02 and 99.42% (Githuku et al. 2018), 97–99.6% (Mant et al. 2005) and 99.3% (Tadesse and Seyoum 2015).

In this study, the vegetated wetlands were significantly ($P<0.05$) removed chromium than non-vegetated wetlands. This result agrees well with the results of Sultana et al. (2015), Choesin et al. (2016), and Sultana et al. (2013). The least significant difference statistical analysis showed no significant variations between the planted CW, inferring that the main pollutant removal mechanism is plant accumulation which is agreed with the results of Sultana et al. (2013). Mant et al. (2005) reported that 97–98% of all the chromium remained below ground is mostly taken up by plants. Tadesse and Seyoum (2015) also reported that based on mass balance calculation 23-48.68% of the Cr is either up taken by plants or adsorbed on to the root surface.

The plant roots accumulated the highest amount of heavy metals, followed by the leaves, and then the stem (Mustapha et al. 2018; Mant et al. 2005). According to Tadesse and Seyoum (2015) and Mustapha et al. (2018), Cr is mainly retained in the roots of plants that could be only partially transported to the leaves of the plants. Plant analysis by Tadesse and Seyoum (2015) showed that most of the chromium taken up at the plants remained in the root (up to 83%). The percent removal of the Cr by plants depend on retention time, increased removal with an increase in retention time (Githuku et al. 2018). The non-vegetated wetlands achieved a lower removal of chromium ranged from 32.8%-45.2%, which is consistent with the findings of Sultana et al. (2013).

In this study, it was found that Cyprus Papyrus and Typha species of broken brick bed wetlands achieved a higher percent removal of Cr than other species from gravel bed wetlands. Similar studies also showed that the Typha species had the best Cr removal performance (Tadesse and Seyoum 2015; Githuku et al. 2018; Mustapha et al. 2018). These plants have significantly greater chromium concentration in their roots than any other plants (Tadesse and Seyoum 2015). The reason of Cyprus Papyrus and Typha species have high concentrations of Cr in its roots may be that the plants have fast-growing and spongy root which enables the plant to absorb more Cr as compared to other plants and probably due to a better oxygen transfer as they had a larger leaf surface area (Maine et al. 2009). The adsorption of Cr by the broken brick substrate may also play a great role for the higher removal of Cr by Cyprus Papyrus and Typha species. Tadesse and Seyoum (2015) explained that clay soil can have a chemical reaction with Cr species and a cation exchange capacity of clay soils is responsible for the adsorption of chromium. This report also supported by Mohammed and Babatunde (2017) that more than 91% of Cr can be removed by adsorption in the wetland substrates.

The average Pb removal efficiency of planted broken brick bed wetlands was ranged from 99.7%-100%. Similarly, the planted gravel bed wetlands removed 90.5-99.8% of Pb from the wastewater. Also, Sarafraz et al. (2009) reported that the removal rates of Pb in the wetlands were found to be almost 100%. The level of Pb in all outlet wastewaters was below the permissible limit (0.2 mg/L) set for wastewater reuse and discharge (WHO 2006). In this study, significantly ($p < 0.05$) higher removal efficiency of Pb was registered in vegetated wetlands (CW1, 2, 4, and 5) than non-vegetated control gravel bed wetland (CW8). These results were similar to the study done by Gikas et al. (2013). Typha plant from both substrates in the present study was removed a higher amount of Pb which is consistent with the results of Gikas et al. (2013) and (Githuku et al. 2018). Anning et al. (2013) and Hegazy et al. (2011) explained that these native aquatic plant species Typha accumulates high concentrations of Pb in their roots and there were significant positive relationships between the concentrations of Pb in the Typha roots (Hegazy et al. 2011). Bonanno and Giudice (2010) also explained that the belowground organs (root) were the primary areas of Pb accumulation.

The average silver removal efficiency of the wetlands was 79.8%. Odinga et al. (2013) also indicated that 75.9% of silver was removed from wastewater by adsorption and absorption into the filtration matrix and the leaves, shoots, and rhizomes of the wetland plants. The highest percent (100%) removal of silver was seen in Typha based broken brick bed wetland. Typha planted wetlands in both substrates were significantly ($P<0.05$) removed silver than unplanted gravel and broken brick bed wetlands. Similarly, Bao et al. (2019) reported that CWs with plants were more effective in removing silver nanoparticles than the unplanted CWs. Likewise, Eid et al. (2012) also showed that there is a higher removal of Ag by *Typha domingensis.* Plant biomass, root activity, peroxidase activity of leaves, and biofilm biomass were significantly altered silver concentration ($P < 0.05$) (Bao et al. 2019). The bioaccumulation of silver in plant biomass was decreased according to the order of rhizome > root > leaf (Eid et al. 2012).

Based on the least significant difference (LSD) analysis, the concentrations of diclofenac in the outlet wastewater samples were significantly lower than the inflowing wastewater. The concentration of diclofenac in the outlet of planted wetlands was ranged from 0-1.5 μg/L while in the unplanted wetlands reached a concentration of 1.33-1.9 μg/L. The average diclofenac removal efficiencies of planted and unplanted reactors were 24%-100% and 8.2%-34.2%, respectively. Vymazal et al. (2017) reported that diclofenac removal of the HSSF CWs in rural areas of the

Czech Republic was only 41%. Similarly, Zhang et al. (2012) confirmed that the diclofenac removal of HSSF CWs were 44%-55% in planted and 24%-32% in unplanted wetlands operated under batch and continuous flow systems. Li et al. (2014) also reported low removal efficiencies between 20% and 50% in constructed wetlands. Also, the free water flow system with plants obtained a maximum of 69.3% diclofenac removal efficiency (Zhai et al. 2016).

All planted wetlands were significantly removed diclofenac than unplanted gravel bed wetland which was agreed with other works of literature (Qing et al. 2011; Zhang et al. 2012). It has been reported that the presence of plants in constructed wetlands played a positive role in the removal of some diclofenac (Hijosa-Valsero et al. 2010; Li et al. 2014; Hijosa-Valsero et al. 2011). Zhang et al. (2012) also indicated that the presence of plants exerts a stimulatory effect on pharmaceutical removal for diclofenac in batch and continuous mode. There was a significant (P<0.05) difference between Typha planted broken brick bed wetland and all other planted and unplanted wetlands of both substrates. There was also a significant difference between Typha planted gravel bed wetland and planted and unplanted wetlands of both substrates except papyrus planted broken brick bed wetland. The Typha planted broken brick bed wetland removed all (100%) the diclofenac from the wastewater. A similar study reported by Susi et al. (2017) described that the wetlands planted with *Typha* and *Cyperus were removed* 98% of diclofenac from wastewaters having passed through the biodigester. For this higher removal, plants of the species *Typha* and *Cyperus* used in the wetland were generated excellent results. Vertical subsurface flow constructed wetland (VFCW) was also highly removed diclofenac from wastewater with the removal efficiency between 70 and 90% (Matamoros et al. 2007).

The results of this research clearly indicated that plant existence favored diclofenac removal in CWs. Hijosa-Valsero et al. (2010) also reported that plant uptake played a significant role in the pharmaceutical removal process (Hijosa-Valsero et al. 2010). Herklotz et al. (2010) and Reinhold et al. (2010) demonstrated that active plant uptake can, directly and indirectly, affect the fate of emerging organic pollutants in wetland systems. According to Zhai et al. (2016), the mass balance study of the experimental system showed that the estimated plant uptake and in plant conversion of diclofenac contributed about 21.4% of the total diclofenac removal in the mesocosm while the remaining 78.6% diclofenac was eliminated through the biotic and abiotic conversion of diclofenac in the water phase. Based on their analysis the maximum bioaccumulation factor of diclofenac was calculated in roots (21.04) followed by root surface (20.49), stems (4.19), and leaves (0.16), respectively.

Additionally, the broken brick as a substrate plays a significant role in the removal of diclofenac than the gravel substrate. Accordingly, there was significantly higher removal efficiency in Typha and Papyrus planted broken brick bed wetlands than that of Typha and Papyrus planted gravel bed wetlands. Qing et al. (2011) reported that the sorption of diclofenac onto organic matter is an important removal mechanism due to their hydrophobic structure, which could be ascribed to specific structural characteristics. Wetlands can promote the removal of these pharmaceutical compounds through a number of mechanisms including photolysis, plant uptake, microbial degradation, and sorption to the soil (White et al. 2006**.** Microorganisms usually play the main role in the processes of degradation, transformation and mineralization of pharmaceuticals (Hijosa-Valsero et al. 2011). Their degradation by microorganisms in constructed wetlands may also be influenced by the substrate, vegetation, oxygen, and redox potential, temperature, pH, nutrient availability, and presence of toxic substances (Li et al. 2014). The results of this study and other studies indicated that diclofenac became a recalcitrant compound in wetland experiments and other wastewater treatment plants (Cherik et al. 2015). However, its removal could be enhanced under anaerobic conditions. White et al. (2006) demonstrated that anaerobic biofilms yielded a 30–40% reduction for diclofenac.

5.4. Coliform and Antibiotic-Resistant Bacterial Removal potential of CWs

In the present study, treatment of hospital wastewater particularly bacterial removal using constructed wetlands was meticulously studied. Both the vegetated and non-vegetated wetlands had been effectively removed total coliforms, fecal coliforms, and *Staphylococcus sp* with an average reduction rate of 99.98%, 99.84%, and 99.3%, respectively. While *Shigella* and *Salmonella sp* removal was reached to 50-100% which is comparable to the findings of Song et al. (2008). Likewise, the other study was shown that an integrated wetland system removed up to 99.97% of coliforms (Sehar et al. 2013). In general, reports by multiple authors employing a number of different constructed wetland designs indicated the overall removal was in the range of 98-99.9% for total coliforms and 93-99% for fecal coliforms (Karimi et al. 2014; Bôto et al. 2016; Mustafa 2013).

The concentration of antibiotic-resistant bacteria in the inlet hospital wastewater was consistently higher than the outlets of wetlands to the most tested drugs. A significant amount of antibiotic-resistant bacteria was removed in this constructed wetlands ($H(8)=29.197$, $P=0.000$, $\eta^2=0.25$). But there was no significant reduction in non-vegetated gravel bed CW ($H(1)=0.718$, $P=0.397$, $\eta^2=0.029$). A similar study by Hien et al. (2017) showed that the outflow of the vegetated wetland has a significant removal ($P<0.05$) than the inflow. Absolute abundances of antibiotic-resistant bacteria were reduced from the influent to the effluent by 80.8% to 93.2% in planted and 42.4% to 74% in unplanted wetlands. Another study done by Bôto et al. (2016) showed that above 95% removal of antibiotic-resistant bacteria was obtained after three weeks of adaptation in CWs. Targeted antibiotic-resistance genes were also significantly removed by parallel horizontal subsurface flow mesocosm cells (Nõlvak et al. 2013) and integrated CW system with removal rates of above 99 % (Chen et al. 2015). These results showed that constructed wetlands could be a promising technology for the removal of fecal coliform and antibiotic-resistant bacteria.

There has been some debate on the importance of plants in coliforms and antibiotic-resistant enteric bacteria removal by constructed wetland treatment systems. Some investigations have shown that wetland system with vegetation has a higher efficiency of the total, fecal and antibiotic-resistant bacteria removal than without vegetation, and the contribution of plants for bacteria disinfection has been emphasized by some researchers (Lekeufack et al. 2017; Martin et al. 2012). The results of the present study agreed with the above findings that significantly higher removal of antibiotic-resistant bacteria was recorded in vegetated broken brick and gravel bed wetlands than the non-vegetated gravel bed wetlands (H(1)=0.718, P=0.397, η^2=0.029). Bôto et al. (2016) indicated that aquatic plants significantly increased the die-off of *E.coli* and other bacteria in wetland treatment systems. The presence of plants enhances aerobic or facultative microbial density and activity particularly on root surface due to root oxygen release. Microbes were also present as an attached biofilm on sand and roots to degrade and reduce enteric bacteria (Gagnon et al. 2007).

The other debate on which several experiments reported was that the role of plants in constructed wetlands for the reduction of coliforms and pathogenic bacteria were minimal (Kipasika et al. 2016; Sidrach-Cardona and Bécares 2013). The results of the present study also agreed well with the above researchers that, there was no significant difference in the rate of removal of indicator bacteria, *Staphylococcus, Salmonella,* and *Shigella sp* between the vegetated and the non-vegetated wetlands. These findings were also similar to those reported by Theophile et al. (2011) that no significant difference in the die-off of fecal, total coliforms, and *Staphylococcus sp* observed in wetlands with different types of plants grown in broken brick and gravel bed cells. Higher removal of antibiotic-resistant bacteria was observed in unplanted broken brick beds (77.5%) than unplanted gravel bed (46.8%) wetlands in this study (H(1)=6.205, P=0.013, η^2=0.25). Akadar (2014) stated that reduction of indicator and antibiotic-resistant bacteria sometimes depends on the filtration capacity of wetland media. Carvalho et al. (2016) also reported that filtration is effective at removing bacteria including *E.coli* and protozoa in wetlands. The other mechanisms of removal are predation, sedimentation, and natural die-off due to competition with the consortium of organisms and toxins from plants and other microorganisms (Bôto et al. 2016; Karimi et al. 2014).

Even if promising disinfection of indicator and resistant bacteria was recorded in wetland treatment systems, an increased proportion of antibiotic-resistant bacteria were discharged into treated effluent samples. Ampicillin resistant *E.coli, Klebsiella,* and *Staphylococcus* were frequently detected. Some (34.2%) of the isolates were recorded to be multi-drug resistant in treated wastewater. This was supported by the study conducted in North Carolina which showed that tetracycline, erythromycin, and ampicillin-resistant and higher MDR (46.3%) isolates were released from CWs (Ibekwe et al. 2016). Similarly, lab-scale parallel horizontal subsurface flow mesocosms and full-scale Grand Marais treatment wetland discharged significant amounts of antibiotic-resistance genes into the environment (Nõlvak et al. 2013; Anderson et al. 2013).

Studies conducted in South Africa (Adefisoye et al. 2016), Iran (Karimi et al. 2016), China (Mao et al. 2015), Portugal (Silva et al. 2006) and Brazil (Santoro et al. 2015) showed that activated sludge treatment system discharged excessive amount (30%-70%) of antibiotic-resistant bacteria, genes as well as MDR bacteria in final effluents. Similarly, in Ethiopia, waste stabilization ponds applied to treat hospital wastewater also released a higher percentage of resistant bacteria (Asfaw et al. 2017; Fekadu et al. 2015; Moges et al. 2014). Increasing the capability of the wastewater treatment process to remove antibiotic-resistant bacteria is pivotal, but some treatment facilities in different countries proved that there is a direct release of a higher rate of ARB than untreated wastewater to the environment (Lood et al. 2017; Mao et al. 2015; Asfaw et al. 2017). This is because of the favorable growth conditions provided by WWTPs for horizontal transfer of genetic material by means of conjugation. Moreover, they provide a favorable environment for further exposure to the antibiotics collected from different sources (Mao et al. 2015). Thus, WWTPs sometimes have to be considered as a hot-spot site for the collection, proliferation, and dissemination of ARB and genes into the environment.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The results of these findings indicated that the hospital releases excessive amounts of organic matter, TKN, ammonium, and phosphate into the environment. There was no significant difference in the release of pollutants during the dry and rainy seasons. Hospitals are also the hot spot sources of heavy metal, drug, and antibiotic-resistant bacteria. The major toxic metals were above the detection limit set by the WHO. It was indicated that most of the bacterial isolates in the hospital wastewater were highly resistant to the commonly prescribed antibiotics and a considerable number of bacterial isolates were multidrug-resistant. Ampicillin and cotrimoxazole resistant bacterial isolates were dominantly found in the wastewater.

The application of constructed wetlands for hospital wastewater treatment was shown to be appropriate and able to remove significant amounts of the pollutants of concern. HSSF CWs had excellent removal potential of BOD5, COD, TSS, phosphate, and nitrogenous compounds from hospital wastewater. There was no statistical difference between planted and unplanted wetlands in both gravel and broken brick beds for the removal of BOD5, COD, and TSS. However, broken brick bed wetlands provide better removal of TKN, ammonia, nitrate, and phosphate from wastewater than gravel bed wetlands and it provides better adsorption sites for ammonium, nitrate, and phosphate. Typha with the broken brick bed was significantly improved ($P<0.05$) the treatment performance of the constructed wetlands for the removal of ammonium, nitrate, and phosphate. The seasonal variation couldn’t significantly influence the removal of all the parameters. However, better performance of nitrate and phosphate removal was achieved in the dry season.

The HSSF CWs were effectively removed heavy metals from the wastewater. Most of the studied heavy metals in the outlets of the wetlands were below the detection limit set by WHO. Heavy metals removal efficiencies were enhanced by the presence of aquatic macrophytes. The vegetated wetlands were significantly ($P<0.05$) removed heavy metals than non-vegetated wetlands. Typha planted broken brick bed wetland, in particular, had higher removal efficiency than the other plants. Plant uptake was the main mechanism of removal than adsorption by wetland media. There was a lower removal efficiency of diclofenac in most of the wetlands. However, Typha planted broken brick bed wetland effectively removed diclofenac than the other wetlands. All planted wetlands were also significantly removed diclofenac than the unplanted gravel bed wetland.

Both the vegetated and non-vegetated wetlands had been effectively removed total coliforms, fecal coliforms, and *Staphylococcus sp.* A significant number of antibiotic-resistant bacteria were removed in vegetated broken brick and gravel bed wetlands than the non-vegetated gravel bed wetlands. This indicates the positive use of plants in antibiotic-resistant bacteria removal from wastewater. However, there was no significant difference in the rate of removal of indicator bacteria, *Staphylococcus, Salmonella,* and *Shigella sp* between the vegetated and the non-vegetated wetlands. In general, horizontal subsurface flow wetlands could help to solve the problem of cost-effective disposal of hospital wastewater, being a suitable treatment for reducing indicator, pathogenic and antibiotic-resistant bacteria which is comparable to activated sludge treatment system.

Most CW systems around the world were designed and constructed with gravel or other substrates to treat raw or pre-treated sewage and domestic wastewater. However, this study demonstrates the importance of broken brick as a substrate bed in wetlands particularly for the removal of emergent pollutants like nutrients, heavy metals, diclofenac, and antibiotic-resistant bacteria from complex types of wastewater like hospital origins. Compared to previously studied wetland systems, the present study is relatively high in its removal efficiency. In addition, the wastewater nutrients could be mainly removed by broken brick media rather than by emergent macrophytes. According to this research, the removal of nutrients in broken brick bed wetlands can be maintained throughout all seasons of the year. From the investigated plant species, the Typha plant greatly removes different pollutants from the wastewater. The combined effect (broken brick bed with Typha plant) will be the future possible solution for the treatment of hospital wastewater, predominantly, for developing nations to whom advanced wastewater treatment plants are inaccessible due to the shortage of budget.

6.2. Recommendations

Hospital wastewater is very complex in its characteristics since it contains different pollutants. CWs are promising technologies for the removal of different pollutants contained in hospital wastewater. Therefore, it is strongly recommended that the treatment of hospital wastewater with CWs be promoted in a strategy to reduce water pollution in low-income countries like Ethiopia. Testing of the effects of pharmaceuticals, disinfectants and other chemicals on wetland microbial communities in terms of their distribution and activity is also recommended.

This research was focused on broken brick which has been found in the local area with described characteristics, the innovative substrate tested provide benefits for a wide variety of settings. In particular, they can be applied everywhere with similar setup for high standards of effluent quality required in terms of nutrient removal. Even though broken brick is effective to remove nutrients in the pilot plants, it is costly to implement at large scale. So, further research is needed on another clay soils which have similar mineral composition to decrease the cost of the media when applied in full scale constructed wetlands. Moreover, long-term studies were also required to realize its exhaustion as a result of adsorption of nutrients and how to regenerate when exhaustion occurred.

One of the main issues concerning heavy metals removal using CWs is the post-treatment management of plant biomass that contains high heavy metals concentrations. For effective removal of pollutants, harvesting of the plants should be carried out at the end of each vegetation cycle. After harvesting, the contaminated plants should either be used as substrate material for biogasification or chopped for further extraction of heavy metals for recycling purpose.

Previous studies have examined the role of plant species and porous media on the removal of antibiotic-resistant bacteria and diclofenac by CWs, to further improve their performance and better control of these pollutants in constructed wetlands (CWs), more controlled studies involving effects of important factors such as hydraulic loading rate and residence time are necessary.

These pilot scale works were undertaken in actual environmental conditions in the field but to further improve CWs performance on hospital wastewater treatment, specific design and operation parameters should be examined, including step feeding with different HRT in order to understanding about the acceptable hydraulic loadings to maximizes the use of constructed wetlands. In general, it requires more research in order to fully understand how this research can be applied to larger scale units for hospital wastewater treatment systems.

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Annexe 1. Lab Procedures to assess pollutants

1. Total Suspended Solids by Gravimetric Determination

Total suspended solids are a measure of the undissolved solid matter in water that remains on the surface of a filter after all the water has been evaporated. A known volume of a well-mixed sample is filtered through a standard glass-fiber filter, collecting the solid residue on the surface of the filter. The filter and residue is evaporated to a constant weight condition in an oven maintained at a temperature of 103-105°C. The mass of the dried residue is determined and used to calculate the concentration of total suspended solids in the sample. This method is applicable for measurement of total suspended solids in all natural waters, in raw, process and treated agricultural, municipal and industrial wastewaters. This method is not considered applicable to wastewater slurries behaving as a Newtonian fluid, non-Newtonian fluids or treated drinking water.

Apparatus

a. Glass-fiber filter, with a 47 mm diameter, nominal pore size ≤ 2.0 µm and ≥ 1.0 µm, and no binders.

b. Graduated cylinder, Class A

c. Wide-bore pipet, Class B

d. Forceps capable of lifting and holding a filter without tearing or puncturing it.

e. Filter pans, aluminum or other inert material, to hold filters.

f. Convection oven operated at 103-105°C for drying samples to a constant weight condition.

g. Muffle furnace operated at 550 ± 50°C. SOP AMBL-105-DPage 3 of 5

h. Desiccator containing a desiccant that responds (color change) to moisture or a hygrometer that measures moisture.

i. Analytical balance capable of weighing to the nearest 0.1 mg or less.

j. Magnetic stirrer and stir bar (optional).

k. Blender or homogenizer (optional)

l. Beaker, low-form Class B or Class A having a volume sufficient enough to fully contain the sample and prevent sample loss from spillage or splattering when mixing.

m. Filtration funnel assembly for a 47 mm size diameter filter.

n. Vacuum suction flask, 1000 mL capacity.

Procedure

A known volume of a well-mixed sample is filtered through a standard glass-fiber filter, collecting the solid residue on the surface of the filter. The filter and residue is evaporated to a constant weight condition in an oven maintained at a temperature of 103-105°C. The mass of the dried residue is determined and used to calculate the concentration of total suspended solids in the sample.

Calculation and Reporting

a. Calculate the concentration of total suspended solids

$$\text{Total Suspended Solids, as mg TSS/L} \quad \frac{(A - F) \times 1{,}000}{S}$$

Where: A = final 103°C weight of the dried residue + the tared filter, mg,
F = tared filter weight, mg, and
S = mL of sample volume.

b. Report as “Total Suspended Solids (TSS) = ____ mg/L” or as “____ mg/L TSS”

c. Identify any sample that yields a residue mass < 2.5 mg or > 200 mg and report the results as an “estimate” because the mass has exceeded the criteria of this analysis.

2. The Winkler Method - Measuring Biological oxygen Demand

The Winkler Method is a technique used to measure dissolved oxygen in freshwater systems. The Winkler Method uses titration to determine dissolved oxygen in the water sample. BOD measurement requires taking two samples at each site. One is tested immediately for dissolved oxygen, and the second is incubated in the dark at 20 C for 5 days and then tested for the amount of dissolved oxygen remaining. The difference in oxygen levels between the first test and the second test, in milligrams per liter (mg/L), is the amount of BOD. This represents the amount of oxygen consumed by microorganisms to break down the organic matter present in the sample bottle during the incubation period. Because of the 5-day incubation, the tests should be conducted in a laboratory.

Reagent List:

- 2ml Manganese sulfate
- 2ml alkali-iodide-azide

- 2ml concentrated sulfuric acid
- 2ml starch solution
- Sodium thiosulfate

Procedure:

1. Carefully fill a 300-mL glass Biological Oxygen Demand (BOD) stoppered bottle brim-full with sample water.
2. Immediately add 2mL of manganese sulfate to the collection bottle by inserting the calibrated pipette just below the surface of the liquid. (If the reagent is added above the sample surface, you will introduce oxygen into the sample.) Squeeze the pipette slowly so no bubbles are introduced via the pipette.
3. Add 2 mL of alkali-iodide-azide reagent in the same manner.
4. Stopper the bottle with care to be sure no air is introduced. Mix the sample by inverting several times. Check for air bubbles; discard the sample and start over if any are seen. If oxygen is present, a brownish-orange cloud of precipitate or floc will appear. When this floc has settle to the bottom, mix the sample by turning it upside down several times and let it settle again.
5. Add 2 mL of concentrated sulfuric acid via a pipette held just above the surface of the sample. Carefully stopper and invert several times to dissolve the floc. At this point, the sample is "fixed" and can be stored for up to 8 hours if kept in a cool, dark place. As an added precaution, squirt distilled water along the stopper, and cap the bottle with aluminum foil and a rubber band during the storage period.
6. In a glass flask, titrate 201 mL of the sample with sodium thiosulfate to a pale straw color. Titrate by slowly dropping titrant solution from a calibrated pipette into the flask and continually stirring or swirling the sample water.
7. Add 2 mL of starch solution so a blue color forms.
8. Continue slowly titrating until the sample turns clear. As this experiment reaches the endpoint, it will take only one drop of the titrant to eliminate the blue color. Be especially careful that each drop is fully mixed into the sample before adding the next. It is sometimes helpful to hold the flask up to a white sheet of paper to check for absence of the blue color.

9. The concentration of dissolved oxygen in the sample is equivalent to the number of milliliters of titrant used. Each mL of sodium thiosulfate added in steps 6 and 8 equals 1 mg/L dissolved oxygen

The procedures for collecting samples for BOD testing consist of the same steps described for sampling for dissolved oxygen (see above), with one important difference. At each site a second sample is collected in a BOD bottle and delivered to the lab for DO testing after the 5-day incubation period. Follow the same steps used for measuring dissolved oxygen with these additional considerations:

- Make sure you have two BOD bottles for each site you will sample. The bottles should be black to prevent photosynthesis. You can wrap a clear bottle with black electrician's tape if you do not have a bottle with black or brown glass.
- Label the second bottle (the one to be incubated) clearly so that it will not be mistaken for the first bottle.
- Be sure to record the information for the second bottle on the field data sheet.

The first bottle should be analyzed just prior to storing the second sample bottle in the dark for 5 days at 20 C. After this time, the second bottle is tested for dissolved oxygen using the same method that was used for the first bottle. The BOD is expressed in milligrams per liter of DO using the following equation:

DO (mg/L) of first bottle - DO (mg/L) of second bottle
= BOD (mg/L)

3. Open Reflux Method- Measuring Chemical Oxygen Demand.

The chemical oxygen demand (COD) determines the amount of oxygen required for chemical oxidation of organic matter using a strong chemical oxidant, such as, potassium dichromate under reflux conditions.

REAGENTS:

- Standard Potassium dichromate ($K_2Cr_2O_7$) digestion solution, 0.01667M
- Sulfuric acid reagent:
- Ferroin Indicator solution

- Standard ferrous ammonium sulfate titrant (FAS), approximately 0.10M:

PROCEDURE:

1. Wash culture tubes and caps with 20% H_2SO_4 before using to prevent contamination.
2. Place sample (2.5 mL) in culture tube and Add K2Cr2O7 digestion solution (1.5 mL).
3. Carefully run sulphuric acid reagent (3.5 mL) down inside of vessel so an acid layer is formed under the sample-digestion solution layer and tightly cap tubes or seal ampules, and invert each several times to mix completely.
4. Place tubes in block digester preheated to 150°C and reflux for 2 h behind a protective shield.
5. Cool to room temperature and place vessels in test tube rack. Some mercuric sulfate may precipitate out but this will not affect the analysis.
6. Add 1 to 2 drops of Ferroin indicator and stir rapidly on magnetic stirrer while titrating with standardized 0.10 M FAS.
7. The end point is a sharp color change from blue-green to reddish brown, although the blue green may reappear within minutes.
8. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample.
9. COD is given by

$$\text{COD (mg O2 /L)} = [(A-B) \times M \times 8000) / (V \text{ sample})$$

Where: A = volume of FAS used for blank (mL)

B = volume of FAS used for sample (mL)

M = molarity of FAS

8000 = milli equivalent weight of oxygen (8) ×1000 mL/L.

4. Kjeldahl method- Measuring Total Kjeldahl Nitrogen (TKN)

Total Kjeldahl nitrogen is the sum of free-ammonia and organic nitrogen compounds which are converted to ammonium sulfate $(NH_4^2)\ SO_4$, under the conditions of digestion described.

This method covers the determination of total Kjeldahl nitrogen in drinking, ground, and surface waters, domestic and industrial wastes. The procedure converts nitrogen components of biological origin such as amino acids, proteins and peptides to ammonia, but may not convert the nitrogenous compounds of some industrial wastes such as amines, nitro compounds, hydrazones, oximes, semicarbazones and some refractory tertiary amines.

Reagents and Standards

- Reagent water: Ammonia free distilled or deionized water, free of the analyte of interest.
- Mercuric sulfate:
- Digestion solution: (Sulfuric acid-mercuric sulfate-potassium sulfate solution):
- Sulfuric Acid solution (4%):
- Stock Sodium Hydroxide (20%):
- Stock Sodium Potassium Tartrate solution (20%):
- Stock Buffer solution:
- Working Buffer solution:
- Sodium Salicylate/Sodium Nitroprusside solution:
- Sodium Hypochlorite solution:
- Ammonium chloride, stock solution:

Procedure

1. Pipet 25.0 mL of sample, standard or blank in the digestor tube.
2. Add 5 mL of digestion solution (Section 7.3) and mix with a vortex mixer (See Note 1).
3. Add four to eight Teflon boiling chips (Section 7.12). CAUTION: An excess of Teflon chips may cause the sample to boil over.
4. Place tubes in block digestor preheated to 160°C and maintain temperature for one hour.
5. Reset temperature to 380°C and continue to heat for one and one half hour. (380°C Must Be Maintained For 30 Minutes)
6. Remove digestion tubes, cool and dilute to 25 mL with reagent water.
7. Excluding the salicylate line, place all reagent lines in their respective containers, connect the sample probe to the sampler and start the pump.
8. Flush the sampler wash receptacle with about 25 mL of 4% sulfuric acid (Section 7.4) (See Note 2).
9. When reagents have been pumping for at least five minutes, place the salicylate line in its respective container and allow the system to equilibrate. If a precipitate forms after the addition of salicylate, the pH is too low. Immediately stop the proportioning pump and flush

the coils with water using a syringe. Before restarting the system, check the concentration of the sulfuric acid solutions and/or the working buffer solution.

10. To prevent precipitation of sodium salicylate in the waste tray, which can clog the tray outlet, keep the nitrogen flowcell pump tube and the nitrogen Colorimeter "To Waste" tube separate from all other lines or keep tap water flowing in the waste tray. 351.2-10
11. After a stable baseline has been obtained, start the sampler and perform analysis.

Analysis and Calculations

1. Prepare a calibration curve by plotting instrument response against standard concentration. Compute sample concentration by comparing sample response with the standard curve. Multiply answer by appropriate dilution factor.
2. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalyzed.
3. Report results in mg N/L.

5. Colorimetric Method For Nitrate Measurement

Nitrate is an essential plant nutrient; it constitutes a risk to human health because it plays a part in the formation of methemoglobin and nitrosamines. Negligible amounts of nitrite occur in plants and drinking water. However, under unfavorable conditions nitrite may enter the food chain via microbial reduction of nitrate thus endangering human health.

This method is applicable to the analysis of drinking, surface and salinewaters, domestic and industrial wastes. Applicable range of concentrations is 0.1 to 2 mg NO3-N/liter.

Apparatus

1. Spectrophotometer or filter photometer suitable for measuring absorbance at410 nm.
2. Sufficient number of 40-50 mL glass sample tubes for reagent blanks, standardsand samples.
3. Neoprene coated wire racks to hold sample tubes.
4. Water bath suitable for use at 100°C. This bath should contain a stirringmechanism so that all tubes are at the same temperature and should be ofsufficient capacity to accept the required number of tubes without significantdrop in temperature when the tubes are immersed.
5. Water bath suitable for use at 10-15°C.

Reagents

- Distilled water free of nitrite and nitrate is to be used in preparation of all reagents and standards.

- Sodium chloride solution (30%).
- Brucine-sulfanilic acid reagent:
- Potassium nitrate stock solution
- Potassium nitrate standard solution
- Acetic acid
- Sodium hydroxide (lN):

Procedure

1. Adjust the pH of the samples to approximately 7 with acetic acid (6.7) orsodium hydroxide (6.8). If necessary, filter to remove turbidity.
2. Set up the required number of sample tubes in the rack to handle reagentblank, standards and samples. Space tubes evenly throughout the rack to allow for even flow of bath water between the tubes. This should assist in achievinguniform heating of all tubes.
3. If it is necessary to correct for color or dissolved organic matter which willcause color on heating, a set of duplicate samples must be run to which allreagents except the brucine-sulfanilic acid have been added.
4. Pipette 10.0 mL of standards and samples or an aliquot of the samples dilutedto 10.0 mL - into the sample tubes.
5. If the samples are saline, add 2 mL of the 30% sodium chloride solution (6.2)to the reagent blank, standards and samples. For fresh water samples, sodiumchloride solution may be omitted. Mix contents of tubes by swirling and placerack in cold water bath (0 - 10°C).
6. Pipette 10.0 mL of sulfuric acid solution (6.3) into each tube and mix byswirling. Allow tubes to come to thermal equilibrium in the cold bath. Be surethat temperatures have equilibrated in all tubes before continuing.
7. Add 0.5 mL brucine-sulfanilic acid reagent (6.4) to each tube (except theinterference control tubes, 7.3) and carefully mix by swirling, then place therack of tubes in the 100°C water bath for exactly 25 minutes.
8. Remove rack of tubes from the hot water bath and immerse in the cold waterbath and allow to reach thermal equilibrium (20-25°C).
9. Read absorbance against the reagent blank at 410 nm usinga1cmorlongercell.

Calculation

- Obtain a standard curve by plotting the absorbance of standards run by theabove procedure against mg NO3-N/L. (The color reaction does not alwaysfollow Beer's law).
- Subtract the absorbance of the sample without the brucine-sulfanilic reagentfrom the absorbance of the sample containing brucine-sulfanilic acid anddetermine mg NO3-N/L. Multiply by an appropriate dilution factor if less than10 mL of sample is taken.

6. Phosphate Determination by (Vanadomolybdo phosphoric Acid colorimetric Method)

Phosphorous occurs in waters and wastewaters almost solely as phosphates. The forms of phosphates arise from a variety of sources. Some small amounts of orthophosphate are added to some water supplies during treatment.

Reagents:

1) PH. PH Indicator
2) Conc. HCl 1:1 _conc. 1 HCl: 1dH_2O Or conc. 1 H_2SO_4:1 dH_2O
3) Activated carbon, used only when water is colored –
4) Vanadate-molybdate reagent 1-Solution
5) Standard phosphate solution:

Procedure:

1- Adjust PH: -If pH of sample >10, add 1 drop pp Indicator to 50ml sample -discharge (remove) the red color with 1+1 HCl before diluting to 100 ml by dH2O If pH<10 proceed:

2- Remove samples color (if it is colored only)

-Shake about 50ml sample with 200 mg activated carbon for 5 minutes

-Filter through filter paper to remove carbon, color 3

a) Place about 50 ml sample and blank in 50 ml volumetric flask the same thing with standards (1,2,3,4,5ppm)

b) Add 10 ml vanadate-molybdate reagent dilute to the mark (50ml) by dH2O After 10 minutes or more:

c) For samples, Blank, standards prepared, read at λ =400nm- Plot a calibration cure "straight line" Calculation: From the calibration curve, find the concentration of the unknown sample.

Annex 2. Photographic presentation of the Pilot scale subsurface flow constructed wetlands