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Evaluation of a Horizontal Subsurface Constructed Wetland for the Removal of Antibiotics, Antibiotic Resistant Bacteria and Heavy metals from Hospital Waste water

By

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Declaration

In the undersigned hereby I, Mesenbet Merkeb, declare that the Master Thesis entitled "Evaluation of Horizontal Subsurface Constructed Wetland for the Removal of Antibiotics, Antibiotic Resistant Bacteria and Heavy Metals from Hospital Wastewater" is my Thesis work submitted to Addis Ababa University, Institute of Biotechnology. It has not been presented to any other University for award and all the sources of materials used have been acknowledged.

Name: Mesenbet Merkeb

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Date_____

Approved by _____ (Advisor)

Date _____

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Table of content

List of Tables

List of Figures

List of Appendices

Abbreviations/Acronyms

ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance genes
BOD	Biological Oxygen Demand
CLSI	Clinical and Laboratory Standards Institute
COD	Chemical oxygen demand
CW	Constructed wetland
DO	Dissolved Oxygen
FQ	Fluoroquinolone
FWSCW	Free water surface
HGT	Horizontal gene transfer
HPLC-DAD	High Performance Liquid Chromatography with Diode Array Detector
HRT	Hydraulic Retention Time
MWWTP	Municipal wastewater treatment plant
NCCL	National Committee for Clinical Laboratory Standards
SSF	Subsurface flow
TN	Total Nitrogen
TP	Total Phosphorous

Abstract

Wastewater plays an important role in the dissemination of antibiotic resistant bacteria and source for the release of antibiotics and other pollutants to the environment. Human exposure to antibiotics, heavy metals and antibiotic resistant bacteria are among the major public health concerns. Hence, the aim of this study was to evaluate the efficiency of a horizontal subsurface flow (HSSF) constructed wetland in removing antibiotic-resistant bacteria, heavy metals and antibiotic concentration. Samples were taken from Tikur Anbessa Specialized Referral Hospital periodically for five months in 2018 and fed into a constructed wetland in the College of Natural and Computational Sciences Addis Ababa University. Influent and effluent were collected and analyzed for organic matter, nutrients, heavy metals, antibiotics, antibiotic resistant bacteria and other physiochemical parameters. The result of this study showed that removal of BOD and COD did not exhibit significant (p=0.005) difference between the plants T. latifolia, P. karka and their combination against the unvegetated control. Removal of nutrients (N and P) was more (p=0.05) efficient in Typha latifolia and Phragmite karka than the combined plant. The mean concentration of zinc in raw, influent, typha planted cell, typha and phragmites planted cell, control (non-vegetated) and Phragmites planted treatment were 0.283, 0.353, 0.054, 0.112, 0.219 and 0.048mg/l respectively. P. karka showed good removal efficiency for Nickel (73%) and Zinc (86%). The count of bacteria resistant to ciprofloxacin, gentamicin doxycycline and cefotaxime were decreased in all constructed wetland cells compared to resistant bacteria count in the influent. The mean concentration of ciprofloxacin cefotaxime in the influent were 1.23 ppm and 17 ppm and lower removal efficiency observed in all cell for ciprofloxacin and the highest removal efficiency observed the cell planted with typha latifolia and unvegetated control for cefotaxime. Strong positive correlation (r^2=0.98, P=0.11) was found between the high cefotaxime concentration in the wastewater and high count of resistant bacteria for cefotaxime in the influent water and the water treated with Typha latifolia, while strong negative correlation in unplanted control and Phragmite karka and the combined plants. It was concluded that the wetland process performance optimization schemes need to include antibiotics and heavy metals for maximum removal efficiency in hospital wastewater treatment.

Keywords/ phrases: Cefotaxime, Ciprofloxacin, Macrophyte, Wastewater

1. INTRODUCTION

Hospital wastewater is generated due to different activities in hospitals and contains a great variety of micro-contaminants such as antibiotics, X-ray contrast agents, heavy metals, disinfectants, pharmaceuticals and pathogenic microorganisms (Amouei *et al.*, 2015, Hunachew Beyene and Getachew Redaie, 2011, Sintayehu Fekadu *et al.*, 2015). Antibiotics are widely used as part of human and veterinary medicine practices to control bacterial infections and partly absorbed in human and animals gut with part of them being excreted in faeces and urine. Wastewater undergoing partially proper or improper treatment will facilitate these pollutants to find their way into the environment (Tao *et al*, 2016).

Heavy metal contamination is an emerging concern of environmental and human health due to the harmful effects they may pose on aquatic and terrestrial organisms (Ayangbenro and Babalola, 2017). Among the different heavy metals, cadmium, lead and mercury are the most dangerous and potentially toxic to living organisms (Yamina *et al.*, 2014). Most of the heavy metals are toxic at low concentration with a higher chance of integrating into the food chain where they accumulate and pose damage to living organisms (Ayangbenro and Babalola, 2017). In humans, heavy metals' exposure above threshold levels may result in developmental, immunological and neurological disorders, several types of cancer, kidney damage and endocrine disruption (Mwanyika *et al.*, 2016).

In most cases of the developing world, hospital wastewater is channeled directly to urban sewer systems without pretreatment (Ort *et al.*, 2010). In addition, municipal wastewaters are usually treated together with hospital wastewater using conventional processes in the municipal wastewater treatment plants (MWWTPs). However, municipal systems are not generally designed to remove medical or pharmaceutical wastes (Al Qarni *et al.*, 2016).

Conventional wastewater treatment plants (WWTPs) are developed to remove what is called priority pollutants such as organic matter, inorganic nutrients and suspended solids from effluents. Most of the conventional WWTPs are not efficient in removing pharmaceutical compounds such as antimicrobial agents from wastewater effluents. When in high concentrations, antimicrobial drugs can change the microbial community of the WWTP and disturb the biological wastewater treatment systems (Boto *et al.*, 2016). Hospitals generate

relatively huge amount of wastewater that include antimicrobials among many other micropollutants (Le *et al.*, 2016 and Aali *et al.*, 2014). Therefore, looking for a low cost and efficient alternative technology for wastewater treatment is of importance in developing countries (Wu *et al.*, 2015 and Abd El-Gawad and Aly, 2011).

Since the 1950s, constructed wetlands have been used for retrieval of anthropogenic discharge such as wastewater, urban and rural runoff, sewage treatment and mining activities (Yildırım and Topkaya, 2012).Constructed wetlands can be divided into two major groups according to the flow pattern: free water surface (FWS) and subsurface flow (SSF) (Wang *et al.*,2017). Constructed Wetlands have many unique benefits as a wastewater treatment plant or process, including their nutrient capturing capacity, the ability to self-organize and increase treatment capacity over time, low energy demand, process stability, being rich in biodiversity, and the ability to produce oxygen and consume carbon dioxide, thereby achieving high levels of treatment with minimum maintenance (Shah *et al.*, 2015

In Ethiopia, hospital waste management is often poor and not in the process of development unlike the health care management system. The poor attention given to hospital wastewater management creates a potential risk to public health through the dissemination of pollutants most notably antibiotic resistance to the environment, animals and communities.

Heavy metals, antibiotic resistant bacteria and resistant genes are uniquely abundant in hospital wastewater and eutrophication of lakes, rivers, estuaries, and coastal oceans is mainly due to the presence of excess nutrients and hence, there is considerable requirement to control and remove such nutrients from wastewater. Therefore pretreatment of the wastewater before it joins the main sewer system is required. When pretreatment is malfunctioning or unavailable, these pollutants may persist in the water after the conventional treatment and eventually end up on accumulating in receiving water bodies that are used for irrigation of agricultural fields. The case of Tikur Anbessa Referral Hospital is not different from this scenario. The primary treatment system located in the vicinity of the Hospital is malfunctioning which results the wastewater to directly join the main sewer line heading to Kality treatment plant. Conventional wastewater management practices are not effective in the complete removal of antibiotic resistant bacteria and activated sludge process does not remove most of the heavy metals in efficient manner. Appropriate on-site treatments of hospital effluents prevents antibiotic resistant

pathogens from combination with the resident microorganisms in domestic wastewater treatment plants and also reduce harsh long term problems such as development of antibiotic resistance of pathogens, which are currently worldwide concerns and also to prevent water bodies from eutrophication due to high nutrient and organic substance load from hospital. Thus, this study assesses the removal efficiency of horizontal subsurface flow constructed wetland to remove antibiotics, antibiotic resistant bacteria and heavy metal from Tikur Anbessa specialized hospital waste water.

1.1. Research questions

1. How effective are the Macrophytes *Phragmite karka and Typha latifolia* in the constructed wetland for the removal of heavy metals, antibiotics and antibiotic resistant bacteria from the hospital wastewater?
2. How effective is the combined Macrophytes system in removing nutrient, pathogens, antibiotic resistant bacteria over single species macrophyte planted system of Horizontal Subsurface Flow Constructed wetland?

1.2. Objectives of the study

1.2.1. General objective

The general objective of this study was to evaluate horizontal subsurface flow constructed wetland for removal of antibiotics, antibiotic resistant bacteria and heavy metal from hospital wastewater

1.2.2. Specific objectives

- To assess the removal efficiency of horizontal subsurface flow (HSSF) constructed wetland in removing antibiotic-resistant bacteria, antibiotic residue, heavy metals and nutrients.
- To compare removal efficiency of single macrophyte species planted- based constructed wetland with the combination of the macrophyte species for antibiotics, antibiotic resistant bacteria and heavy metals.
- To determine the relationship between antibiotics concentration and antibiotic-resistant bacteria.

1.3. Hypothesis

- ✓ Horizontal subsurface Constructed wetlands remove hospital wastewater pollutants (antibiotics, antibiotic-resistant bacteria, heavy metals) in addition to nutrients like phosphorus and nitrogen.

2. LITERATURE REVIEW

2.1. The nature of hospital wastewater and its component

Hospital wastewater contains different pollutants like carbonaceous organic compounds represented by Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). A study by Hunachew Beyene and Getachew Redaie (2011) on Hawassa University referral Hospital wastewater showed 632 mg/L and 1388.75 mg/L BOD and COD, respectively. Nitrogen-containing organic and inorganic compounds such as NH_4^+, NH_3 and NO_3 are a number of the pollutants in hospital wastewater (Amouei *et al.*, 2015). Additionally, hospital wastewater contains large amount of Phosphates (Wyasu and Okereke, 2012). In addition to the priority pollutants, hospital wastewater carries pharmaceuticals, intact or partially metabolized (Verlicchi *et al.*, 2010 and Carraro *et al.*, 2016) and heavy metals (Verlicchi *et al.*, 2012).

2.2. Antibiotics and their use

Antibiotics are used widely all over the world to destroy or prevent the growth of bacteria (Chunhui *et al.*, 2016 and Elbossaty, 2017). Antibiotics (antimicrobials at large) are used for preventing and treating animals, different plant infections and also for advancing growth in animal farming (Opris *et al.*, 2013).

Among the different classes of antibiotics, fluroquinolones (FQs), tetracycline, β –lactam and sulfonamide are the main frequently prescribed antibiotics (Oberoi *et al.*, 2019). According to Borghi and Palma (2014), Tetracyclines have low metabolic rate, more than 80% are eliminated mainly as unchanged through the intestinal tract. Rather, fluoroquinolones enter into environment through urine, faeces and hospital wastewater with a large variety of pharmaceuticals (Pena *et al.*, 2010, Seifrtova *et al.*, 2010). Studies on the fate of fluoroquinolones showed their further contamination of agricultural fields and accumulation in crops and vegetables (Seifrtova *et al.*, 2010). Antibiotics occur in hospital wastewater at higher concentrations (in the order of mg/L) than in municipal wastewaters (in the order of ng/L).

According to Diwan *et al.* (2010) among the fluoroquinolones, ciprofloxacin was the most commonly found antibiotic in the hospital wastewater effluent. A study conducted in Africa showed that fluoroquinolones were the most commonly detected drug among five drug group (β-lactam, macrolides, sulfonamides, and tetracyclines) (Faleye *et al.*, 2018). According to Pena *et*

al. (2010) high levels of fluoroquinolone antibiotics (up to 11,000 ng l^{-1} of ciprofloxacin) were found in Coimbra hospital effluents. Ciprofloxacin was present in hospital effluents (13.78µg/l) but lower concentration cefotaxime was detected in hospital effluent (Rodriguez-Mozaz, 2015).

Ciprofloxacin is broad-spectrum antibiotic which belongs to the second generation of fluoroquinolone drug class and effective against gram-positive and gram-negative bacteria (Sharma *et al.*, 2010, Seifrtova *et al.*, 2010) (Table 1). It is used for treatment of many bacterial infections such as bone and joint infections, lower respiratory tract infections, and urinary tract infections (Zuccato *et al.*, 2010, Sharma *et al.*, 2010). Fluoroquinolones and tetracyclines are considerably more stable in the environment than other antibiotics, thus allowing them to persist for longer times, to spread further, and to accumulate to higher concentrations and contaminate water bodies and soils (Hanna *et al.*, 2018).

Aminoglycosides are effective, broad-spectrum antibiotics that act through inhibition of protein synthesis. The aminoglycoside antibiotic, gentamicin is widely used in hospitals for the treatment of serious infections caused by Gram-negative and Gram-positive bacteria. Aminoglycosides are useful primarily in infections involving aerobic, Gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter*, and *Enterobacter* (https://www.drugbank.ca/drugs/DB00798)(accessed date 20/9/2019). Aminoglycosides are used in the treatment of severe infections of the abdomen and urinary tract (https://www.aafp.org/afp/1998/1115/p1811.html,)(accessed date 10/2/20). Cephalosporins are β-lactam antibiotics which are considered the most widely used antibiotics for human and in veterinary with the usage nearly 55 to 70% of the total antibiotic used by humans (Elbalkiny *et al.*, 2019). Cefotaxime, a semisynthetic third-generation cephalosporin exhibits potent activity against many Gram-negative species, including *Escherichia coli, Klebsiella pneumoniae, Haemophilus influenzae* and *Neisseria gonorrhoeae* (Bafeltowska, *et al.*, 2002).

Table 1. List of selected antibiotics, their class, and mode of action and mechanism of resistance

Antibiotic class	Example of an antibiotic	Function	Mode of action	Mechanisms of resistance
Tetracycline	Doxycycline	Broad-spectrum Antibiotics	Inhibits bacterial protein synthesis by binding with the 30S ribosomal subunit	Target modification
Fluoroquinolones	Ciprofloxacin	Broad-spectrum Antibiotics	Inhibition of DNA replication, inhibits the activity of DNA gyrase,	Mutations in topoisomerase II or IV
Cephalosporin	Cefotaxime	Broad-spectrum Antibiotics	Inhibit enzymatic reactions needed for stable bacterial wall synthesis by binding to PBPs(penicillin binding protein)	Extended spectrum Betalactamase production
Aminoglycosides	Gentamicin	Broad-spectrum antibiotics	Inhibition of protein synthesis by binding to 30S ribosomes	Enzymatic modification
Beta Lactam	Ampicillin	Broad-spectrum Antibiotics	Inhibition of the peptidoglycan layer during cell wall synthesis	Production of Beta lactamase enzyme
Penicillin Combinations	Amoxacilin/ Clavulanic acid	Broad-spectrum Antibiotics	Inhibition of the peptidoglycan layer of cell wall and blockage of Beta lactamase by clavulanic acid	Beta lactamases and ESBLs

Source: Correia *et al.*, 2017, (Davies and Davies, 2010), Gad *et al.*, 2011, (Jayaraman, 2009), Sharma *et al.*, 2010)

2.3. Epidemiology of antibiotic resistant bacteria and mechanism

Antibiotic resistance is defined as the ability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would inhibit or kill the organism (Hardley 1927 *cited in*: Jacoby, 2017). Intrinsic antimicrobial resistance is the one that occurs naturally, and is one of the many traits that bacterial subpopulations possess, enabling them to out-compete and out-survive their microbial neighbors and overcome host strategies aimed against them (Jacoby,

2017). This phenomenon is nearly as old as the discovery of antimicrobials themselves (Table 2). Antibiotic resistance occurs when bacteria change or modify in some way that decrease or eliminate the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections (Priyankaa and Nandanb, 2014).

Furthermore, as a consequence of sequential, cumulative acquisition of resistance traits against diverse antibiotics, more bacterial pathogens with multiple-drug resistance are being reported worldwide. As a result, many bacterial organisms, including major human and animal pathogens like Mycobacterium and Salmonella species, have become resistant to antibiotics which were previously quite efficacious. Excessive use of antibiotics is the leading cause to antibiotic resistance in bacteria and also development of resistance to an antibiotic depends on how much is being used and how often bacteria are exposed to the drug (Priyankaa and Nandanb, 2014).

Horizontal gene transfer (HGT), a transfer of resistance genes from one bacterium to another, is one of the most important mechanisms leading to the distribution of antibiotic resistance in the environment (Barancheshme and Munir, 2018). There are four different mechanisms of HGT: conjugation, transformation, and transduction and via gene transfer agents (Giedraitiene *et al.*, 2011).

Table 2. Timetable of antibiotic discovery and resistance

Antibiotic	Discovered/ Reported	Clinical Use	Resistance Identified	Organism
Sulfonamide	1935	1936	1939	*Streptococcus pneumonia*
Penicillin G	1928, purified in 1940	1941	1942 1965	*Staphylococcus aureus* *Streptococcus pneumoniae*
Methicillin	1960	1960	1961	*Staphylococcus aureus*
Oxyimino Beta lactams	1978	1981	1983	*Klebsiella pneumoniae*
Streptomycin	1944	1946	1946	*Escherichia coli*
Tetracycline	1948	1952	1959	*Shigella dysenteriae*
Erythromycin	1952	1955	1957	*Staphylococcus aureus*
Vancomycin	1956	1958	1987	*Entrococcus. Faecium*
Gentamicin	1963	1967	1970	*Klebsiella pneumoniae* *Pseudomonas aeruginosa*

Source: Jacoby, 2017

2.3.1. Multidrug resistant bacteria

Multidrug resistant (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories (Basak *et al.*, 2016).The rate of development of Multidrug Resistant (MDR) and Extensively Drug Resistant (XDR) bacteria is alarming (Faleye *et al.*, 2018). The MDR phenotype often associates a decrease in porin synthesis with an increased activity of efflux pumps to restrict the intracellular concentration of various antibiotics, including β-lactams, tetracyclines, chloramphenicol, and quinolones(Chollet *et al.*,2004). In clinical isolates which exhibit high resistance to broad-spectrum antibiotics, MDR is the result of enzymatic responses, mutations in the antibiotic target, and modifications in envelope permeability, including porin alteration and induction of drug efflux (Charrel *et al.*, 1996).

2.3.2. Extensively drug resistant bacteria

Extensively drug resistant (XDR) was defined as nonsusceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories (Basak *et al.*, 2016). The risk of global dissemination of these XDR pathogens has become a recognized global threat and the dramatic increase in prevalence of infections caused by multidrug-resistant(MDR) and extremely drug resistant (XDR) pathogens belonging to the *Enterobacteriaceae* group poses a great concern(Navon-Venezia, *et al.*,2017)

2.3.3. Extended spectrum b-lactamases

ESBL is an enzyme that is produced by bacteria to become resistant to extended-spectrum penicillin, cephalosporins, and monobactams except for cephamycins and carbapenems (Dejenie Shiferaw Teklu, Abebe Aseffa Negeri, Melese Hailu Legese, Tesfaye Legesse Bedada, Hiwot Ketema Woldemariam and Kassu Desta Tullu. β-lactamase inhibitors such as clavulanic acid inhibit the resistance mechanism (Dowling *et al.*, 2017). β-lactamases hydrolyze nearly all β-lactams that have ester and amide bond, e.g., penicillins, cephalosporins, monobactams, and carbapenems (Kapoor *et al.*, 2017). The Extended Spectrum β-Lactamases (ESBLs) are typically plasmid-mediated enzymes that hydrolyze the penicillins, the third generation cephalosporins and aztreonam (Kaur *et al.*, 2013).

2.3.4. Pan-drug resistance

Pan-drug resistant (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories (Basak *et al.*, 2016). Pan-drug resistance is not an exceptional phenotype in *E. aerogenes*, since resistant strains to all antibiotics, including colistin *pmrA* substitution, were isolated and described to be associated with colistin resistance (Davin-Regli and Pages.J-M, 2015)

2.4. Health problem of micro pollutants and antibiotic resistant

The release resistant of antibiotic bacteria to the environment create a potential public health impact in different ways. When the resistant bacteria are carrying a transmissible gene, they transfer resistant genes to other community of bacteria and also act as a vector or reservoir of resistant genes (Sintayehu Fekadu et al., 2015)The greatest increase in antibiotics use was recorded in Low and Middle Income Countries (LMIC). As long as Ethiopia is one of the LMICs, antibiotic resistance is a major challenge (Alemayehu Reta et al., 2019).

Pharmaceutical wastes are introduced into the environment all the way through hospitals, veterinary clinics, pharmaceutical industry effluents and household effluents. Among these pharmaceutical wastes antibiotics poses to be of great threat (Mondal and Sinha, 2014). Antibiotics are the main medicines in the hospitals and also large amounts of antibiotics can be released or emission in to hospital wastewater due to excretion of used antibiotics and also disposal of unused compounds (Lien *et al.*, 2016).

Antibiotic residues within the environment can exhibit negative influence on aquatic and terrestrial organisms, resulting in a series of potential ecological hazards, such as the development of antibiotic resistance gene and antibiotic resistant bacteria (Chunhui *et al.*, 2016 and Lien *et al.*, 2016). Bacterial antibiotic resistance may be a serious public health issues and also continuous exposure to antibiotics within the environment has led to the development of antibiotic resistance in microorganisms (Kim *et al.*, 2018).

Pharmaceuticals generally dissolve easily in aqueous media and don't usually evaporate at normal temperatures or pressure; they make their way into the soil and aquatic environments via sewage, treated sewage sludge and irrigation with reclaimed waters (Shraim *et al.*, 2017). Infections caused by antibiotic-resistant bacteria are often difficult, or sometimes impossible to cure, leading to death (Kim *et al.*, 2018). The impact of antibiotic resistance like increased treatment costs, disease spread and duration of illness, posing a serious challenge to the future of chemotherapy (Feleke Moges *et al.*, 2014 and Crouch *et al.*, 2015). Conventional wastewater treatment technologies don't sufficiently remove pharmaceuticals and their metabolites and degradation by-products from wastewater, and thus allow them to reach surface, marine, ground, and drinking waters (Shraim *et al.*, 2017).

2.5. Sources of antibiotic pollution in the environment

The potential source of antibiotics within the environment include the effluents released from hospitals, sewage, livestock farm, aquaculture farm, pharmaceutical industries, households, agricultural and urban runoffs (Oberoi *et al.*, 2019). Additionally, the improper disposals of such unused or expired antibiotics contribute immensely to environmental pollution (Mondal and Sinha, 2014). Healthcare centers and hospitals are the foremost important facilities with reference to antibiotic consumption and subsequent excretion since only partially metabolized

(up to 90%) are being excreted in its original, active form in urine and faeces and if not properly treated, the wastewater may be a potential source of antibiotic resistant bacteria (ARB) and ARGs (Chunhui *et al.*, 2016, Barancheshme and Munir, 2018).

2.6. Heavy metals in hospital wastewater and its environmental impact

Heavy metals are categorized under micro-pollutant group within the hospital wastewater that cause risk to environment and human health because they're not biologically degradable and transformable pollutants (Akin, 2016). A number of the heavy metals like zinc, nickel, cobalt and chromium have biological importance in trace amount but their high concentrations (above threshold level) and long-term exposures produce harmful effects on various organisms (Oves and Hussain, 2016). Other heavy metals like cadmium, mercury and lead haven't any biological role, and really toxic at very low concentrations (Rahman *et al.*, 2016).

Vegetables, particularly leafy vegetables, accumulate or store higher amounts of heavy metals because of they absorb or taken up these metals in their leaves. When heavy metals are transferred into food chains and accumulate in vital organs, like liver, kidneys, and bones, there's an immediate threat to human health which will end in numerous serious health disorders (Sardar *et al.*, 2013 and Hu *et al.*, 2017).

Hospitals are one among the main sources of heavy metals within the environment and also recognized as a source of mercury discharge to the wastewater system. Mercury is used in thermometers, blood pressure cuffs, batteries and a lot of drugs (Kaur *et al.*, 2014).

A study by Ji *et al.* (2012) in China, the correlations between metal content and ARGs were stronger and metal contaminations (heavy metals released from waste) may act as a serious factor that contributes to ARG abundances. Laffite *et al.* (2016) reported that ARGs (except for *bla*TEM) have a direct correlation with *E. coli* and metals (Cd, Cr, Cu, Hg, and Zn)

2.7. Treatment of hospital wastewater

The composition of hospital wastewater is different from municipal wastewater due to the high level of pharmaceuticals and bacterial pathogens in addition to the priority pollutants (Carbon, Nitrogen and Phosphorous) it contains (Amouei *et al.*, 2015). Pharmaceuticals enter the water systems through various sources like hospital effluents, human sewage, and discharge from industrial pharmaceutical plants (Zuccato *et al.*, 2010). Hospital wastewater, if disposed with

insufficient treatment, results in great damage to the environment and groundwater resources. General treatment can't be used for each hospital wastewater due to its variable composition and thus specific treatment is required for specific type of wastewater (saleem, 2007). To reduce the buildup of those pollutants from environment, different types of treatment processes are employed to make sure the water released has reduced level of pharmaceuticals (Mondal and Sinha, 2014).

2.8. Constructed wetland Treatment Systems

Constructed wetland (CWs) is a land-based wastewater treatment system that contains floating or emergent rooted wetland vegetation (Ozengin and Elmaci, 2016). These wetlands are constructed to simulate the natural processes to treat different sort of wastewater by reducing pollutant level to a dischargeable limit (Chen *et al.*, 2016 and Wu *et al.*, 2015).

Constructed wetlands can help or facilitate the removal or elimination of pharmaceuticals through natural processes involving plants, microorganisms, solid matrix components and sunlight (Li *et al.*, 2014). Growth media (gravel) is that the most ordinarily used media in CW and provides not only physical support for plant growth but also additional sites for biofilm growth and adsorption of nutrients promote the sedimentation and filtration of pollutants (Abdelhakeem *et al.*, 2016).

The root zone, also referred to as the rhizosphere, is that the active reaction zone of constructed wetlands where physico-chemical and biological processes happen or takes places as a result of the interaction of plants, microorganisms, soil and pollutants(Fig 1). Direct uptake, accumulation and translocation of micro contaminants by plants are as important mechanisms for phytoremediation technology (Zhang *et al.*, 2014). Different process like biodegradation, substrate adsorption and plant uptake, all play a particular role in decreasing or reducing the loadings of nutrients, antibiotics, and ARGs in the constructed wetlands (Barancheshme and Munir, 2018).

Compared to conventional treatment, constructed wetland utilization provide a low-cost alternative which make them suitable for wastewater treatment because they depend upon natural process, less costly to construct, operate, and maintain (Mburu *et al.*, 2015 and Zhang *et al.*, 2012).

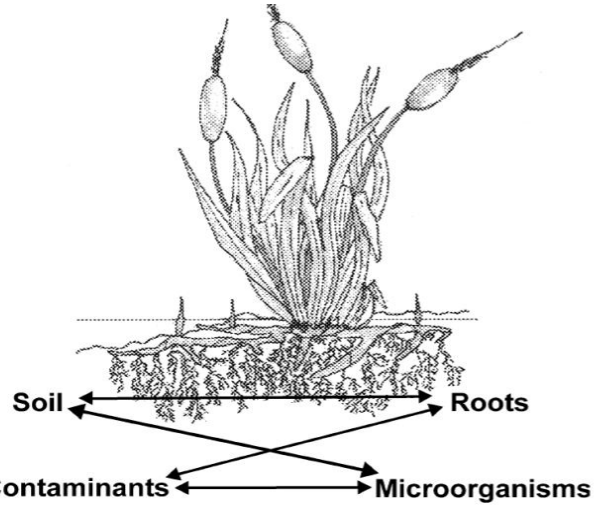

Figure 1. Possible interactions in the rhizosphere of plants in wetlands for wastewater treatment

(Source: Stottmeister *et al.*, 2003)

2.8.1. Types of Constructed Wetland Treatment Systems

There are two types of constructed wetlands based on differences in the wastewater flow pattern or settings, namely: surface flow (SF) and subsurface flow (SSF) wetlands (Prihatini *et al.*, 2015). Consistent with the direction of water movement within the system, subsurface flow constructed wetlands could also be classified into vertical (SSFCW) and horizontal (HSSF) (Almuktar *et al.*, 2018).

2.8.1.1. Free water surface (FWS) flow constructed wetland

Free water surface flow constructed wetlands are infrequently or rarely used for wastewater treatment due to the higher chance of human exposure to pathogens (Almuktar *et al.*, 2018). As compared to FWS CWs, SSF CWs are very effective in removal of organics, suspended solids, microbial pollution and heavy metals (Wu *et al.*, 2015). In free water surface constructed wetlands, the wastewater is above the substrate and therefore the near surface layer is aerobic while the deeper waters and substrate are usually anaerobic (Choudhary *et al.*, 2011).

According to Yidong *et al.* (2017), the removal efficiencies of constructed wetland for the removal of sulfonamides, quinolones, macroldes, teteracyclin and β-lactams were 83.7%, 29.2%, 70.1%, 15.9%, and 51.1%, respectively. Liu *et al.* (2019) reported that the removal efficiency of Quinolone antibiotics (QNs) and Tetracycline antibiotics (TCs) by CWs was the highest with removal of efficiency of greater than 70%-90%.

Surface flow constructed wetland with floating macrophytes was efficient within the removal of four antibiotics (ofloxacin, leucomycin, sulfamonomethoxine, and trimethoprim) (Chen *et al.*, 2014). Berglund *et al.* (2014) have reported that surface-flow CWs are highly competitive alternatives for removing antibiotics and also better performance was observed for removal of antibiotics particularly norofloxacin and Azithromycin (NOR and AZI).

2.8.1.2. Subsurface flow constructed wetland

In subsurface flow CWs, it's generally accepted that the most sources of oxygen are either diffusion and/or translocation of oxygen from the plant shoots into the rhizosphere. The improved oxidizing conditions in the planted beds may stimulate microbial growth and increase the range of the microbial community related with the rhizosphere. Macrophytes have great potential of directly taking up and assimilating certain pharmaceuticals (Zhang *et al.*, 2015). The water level is designed to remain below the top of the substrate (Choudhary *et al.*, 2011). Subsurface flow (SSF) constructed wetland, during which the wastewater flows horizontally or vertically through the highly permeable substrate of the bed which is planted with vegetation (Thalla *et al.*, 2019). The wastewater treatment depends on different processes like biological, chemical and physical processes that are happening during a natural environment (Ozengin and Elmaci, 2016). During the passage of the wastewater will come into contact with a system of aerobic (occurs around root), anaerobic and anoxic zones (Vymazal, 2014).

Horizontal subsurface flow constructed wetlands (HSSFCWs) have been the most frequently employed aquatic plant-based systems as secondary treatment for removal of pharmaceutical compounds (Zhang *et al.*,2014). Some pharmaceuticals are best reduced under aerobic conditions (ibuprofen) however removal of others is favoured by anaerobic conditions (clofibric acid, diclofenac) (Verlicchi *et al.*, 2010).

Hijosa-Valsero *et al.* (2011) reported that Erythromycin could only be removed by a *Phragmites australis*-horizontal SSF system and also doxycycline removal efficiency was higher in SSF systems. Chen *et al.* (2014) reported that sulfamonomethoxine was eliminated by subsurface flow constructed wetland (SSFCW) with removal of 36% and also contributed 73 and 62% in the removal of ofloxacin and trimethoprim, respectively.

High hydraulic retention times promote biodegradation and photodegradation reactions involved within the removal of emerging contaminants (Verlicchi *et al.*, 2010). The presence of plants was beneficial to the removal of pollutants, and therefore the subsurface flow constructed wetland have higher pollutant removal compared to the surface flow constructed wetlands, mainly for antibiotics (Chen *et al.*, 2016).

There are two types of subsurface flow constructed wetland types: the horizontal (HSSF) and vertical (VSSF). In the horizontal subsurface-horizontal flow constructed wetlands (HSSFCWs), the wastewater is fed into the wetland at the inlet and flow slowly horizontally through porous medium (gravel) under the surface of the bed which is planted with vegetation (Fig 2) (Li *et al.*, 2014). In the vertical subsurface flow constructed wetland, the wastewater flows within the wetland is directed downwards through the porous medium in the root zone. The water will be finally channeled through a pipe to flow out of the wetland (Fig. 2). Organic-matter reduction in HSSF-CW is primarily achieved by microorganisms attached to the substrate media and to plant roots through aerobic, anoxic, and anaerobic processes (Mburu *et al.*, 2013).

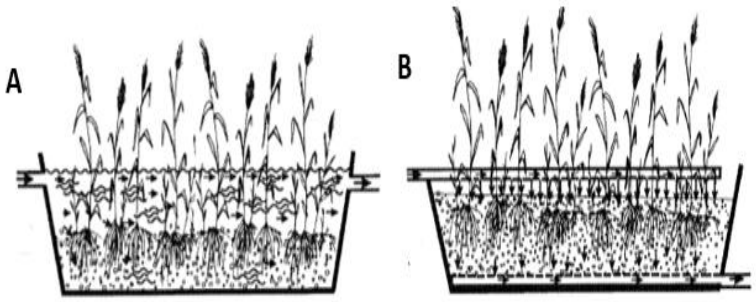

Figure 2. Subsurface Flow Constructed Wetland types (A) Horizontal subsurface flow CW and (B) Vertical subsurface flow CW

(Source: Stottmeister *et al.*, 2003)

2.9. Macrophytes: Typha latifolia and phragmite karka

Aquatic plants are natural absorbers of heavy metals and other nutrients (Kumari and Tripathi, 2015). Srivastava *et al.,* 2014 reported that Species of genera *Phragmites*, mainly *australis, karka* and *communis,* are well recognized for their ability to mitigate environmental pollution. The genus *Phragmites* is a well-known wetland plant commonly named as "reed bed" and its efficiency in the removal of TP in addition to the common organic pollutants is reported as the second most efficient wetland plant as studied by various researchers (Akratos and Tsihrintzis, 2007; Vyamzal, 2001; Ghosh and Gopal, 2010).

T. latifolia and *P. karka* have long been known among the most important wetland plants and the high efficiency of these plants in the removal of ammonia nitrogen both in natural wetland and constructed wetland settings were reported by (Vymazal 2017, Ciria *et al.,* 2005).*Typha latifolia and Phragmites* are well known hyper accumulator emergent plants and capable to accumulate metals copper, cadmium, chromium, nickel and lead up to 0.1% and iron and zinc up to 1% of the plant dry weight (Kumari and Tripathi, 2015).

Saxena (2015) reported that Total phosphorus (TP) and SRP (soluble reactive phosphorus) reduction in *Phragmite karka* planted constructed wetland system was 74.4% and 54.4 %(Saxena, 2015).Golda (2014) Reported that the percentage pollutant removal of Typha species was found to be 85.4% for BOD,78% for COD , 57%for TS and also horizontal subsurface flow constructed using *P.karka* with percentage removal of total solid (70.7%),coliform bacteria(98.7%), COD(77.8%), TKN(8.9%), BOD(65.7%), Nitrate nitrogen(62%) and Ammonium nitrogen(53.35 %).

2.10. Determination of pharmaceuticals in hospital wastewater

A study conducted in Qatar showed that eight (penicillin, amoxicillin, gentamicin, ciprofloxacin, tetracycline, erythromycin, metronidazole and clavulanic acid) selected antibiotics tested by using solid-phase extraction (SPE) method followed by Liquid Chromatography Mass Spectrometry (LC–MS), among the detected antibiotics: metronidazole and ciprofloxacin were the most abundant type in the Hospital wastewater (HWW) at concentrations of 5.46 µg/L and 1.99 µg/L respectively(Al-Maadheed *et al.,* 2019).

According to Wang *et al.* (2018) among fourteen antibiotics, the concentrations of quinolones, tetracyclines, and cephalexin were particularly higher than those of macrolides and sulfonamides

in all three hospitals in central China using solid-phase extraction and Utra Performance Liquid Chromatography Mass Spectrophotometry/Mass Spectrometry analysis.

Previous studies have also found high concentration of ciprofloxacin (236.6 µg/l) from fluoroquinolone class and hospital associated water analyzed using solid phase extraction followed by liquid chromatography/tandem mass spectrometry technique (Diwan *et al.*, 2010).

A study conducted in Thailand reported that Ciprofloxacin was found at greatest concentration because of its larger number of being prescribed than others (ceftriaxone, norfloxacin and tetracycline) and the detection method was high-performance liquid chromatography / tandem mass spectrometry (HPLC-MS/MS) (Jaidumrong *et al.*,2016).

2.11. Efficiency of wastewater treatment systems for the removal of pathogens

Horizontal subsurface flows CWs have better capacity than free water surface flow CWs for the removal of *E. coli* and FC (Wu *et al.,* 2016). Removal efficiencies for four different indicator organisms (total coliforms, faecal coliforms, *faecal streptococci* and *E. coli*) ranging from 65% to 99%, where the highest removal rates were observed in HFCW (Adrados *et al.*, 2018).

Most of the studies investigated pathogen removal in horizontal subsurface flow CW and free water surface flow CW that have macrophytes possess a great potential to reduce a significant number of fecal bacteria (Wu *et al.* 2016). SSF are found to remove a significantly higher number of bacterial pathogens from domestic wastewater and also the highest removal efficiency of helminths (*Ascaris sp.*) and protozoan cyst (*Giardia sp.*) found in SSF wetland (Shingare *et al.*, 2019)

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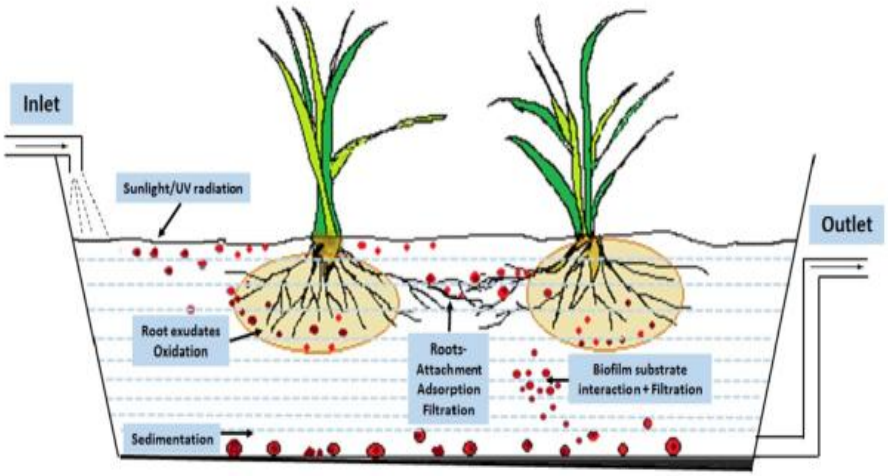

Figure 3. Mechanisms involved in pathogen removal in horizontal subsurface Constructed wetland**.** Source**: (Shingare et *al.*, 2019)**

3. MATERIALS AND METHODS

3.1. Sample site description and wastewater sampling

Tikur Anbessa Specialized Hospital is a University hospital of the College of Health Sciences, Addis Ababa University. It is the largest specialized hospital in Ethiopia, with over 700 beds, and serves as a training center for medical students, dentists, nurses, midwives, pharmacists, medical laboratory technologists, radiology technologists, and residents who specialize in various specialty areas. The study area is located College of Health Sciences, Addis Ababa University, Addis Ababa (8°58′ 50.1708″ N 38°45′ 27.9396″). The main source of wastewater released from hospital including students' dormitory, toilets, laboratories and cafeterias flow directly to the main sewage line that joins Kality wastewater treatment plant and also the hospital does not have functional onsite treatment plants. The wastewater sources generated from the hospital including students' dormitory, toilets, laboratories and cafeterias flow directly to the main sewage line that joins Kality wastewater treatment plant and also the hospital does not have functional onsite treatment plants.

Wastewater samples were collected periodically four times for five months from February to June 2018 and used for the different methods described below. In all the sampling times, the wastewater samples were brought from Tikur Anbessa Specialized Hospital aseptically using clean jerrycans with the capacity of 20 Liter. The jerrycans were emptied to the storage tanker with capacity of 1000L to feed the wetland. Physico-chemical analysis of the raw wastewater was analyzed onsite, while nutrient analyses were conducted in JIJE Analytical Testing services Laboratory, Addis Ababa (Fig. 4).

Figure 4. Onsite pH, temperature and conductivity analysis of the raw wastewater at Tikur Anbessa Specialized Referral Hospital at the time of sample collection (Feb-June 2018)

3.2. Wetland design and operational procedures

The constructed wetland is located in the premises of the College of Natural and Computational Sciences and consists of four cells planted with *Typha latifolia, Phragmite karka,* combined (*T.latifolia* and *P.karka*) and unplanted control. The wetland was constructed as a horizontal subsurface flow constructed wetland by the Center for Environmental Sciences, AAU, with the dimensions of 2 m × 0.68 m × 0.45 m of length, width and height, respectively (Fig 5). Total amount of wastewater added to the collection tanker was 440L. Raw wastewater from the collection tank was periodically released through the gate valve to the equalizer tank which then flows into each cell at an optimized flow rate of 0.72 L/hr that meets the hydraulic retention time (HRT) of four days. During construction, a slope of 1% at the bottom of each bed was maintained to ease the circulation of water from the inlet to the outlet.

The wastewater was distributed into four cells with the hydraulic retention time (HRT, verage residence time during the water remains within the wetland system) of four days. The raw wastewater, the influent (wastewater that enters the cells) and effluent (water that comes out from the cells after the retention time) was taken for various analyses described in the sections below.

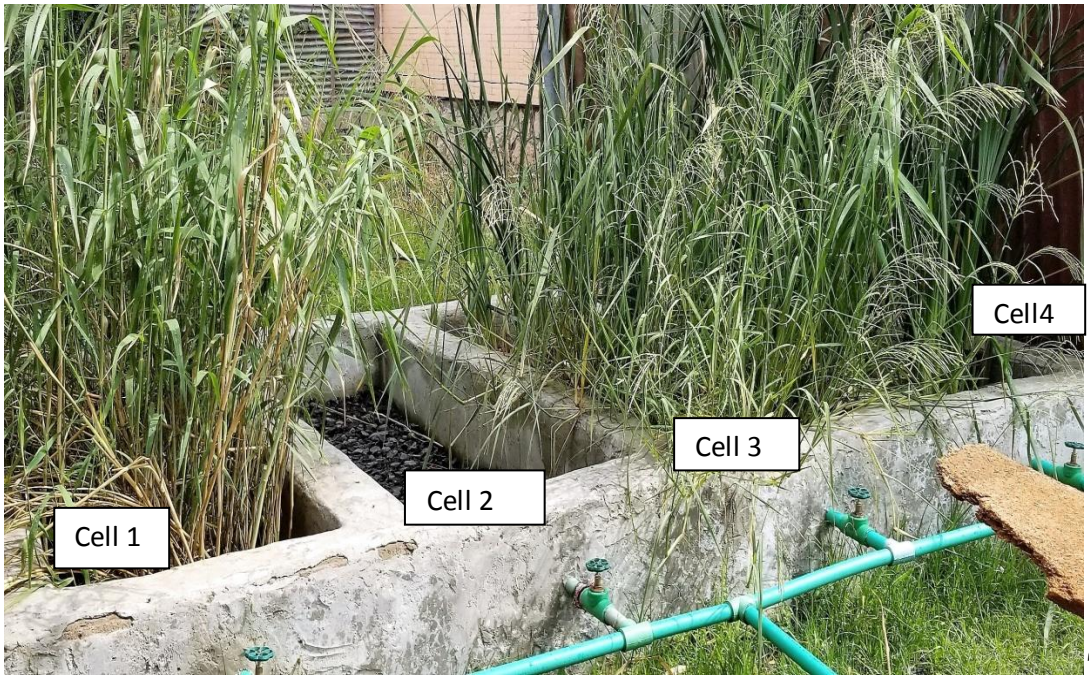

Figure 5.Horizontal subsurface flow constructed wetland

*cell1, 2, 3, 4 stands for cell with *P.karka*, control, combined and cell with *Typha latifolia*

3.3. Physicochemical analysis

The physical parameters like pH, dissolved oxygen (DO), electric conductivity and temperature were measured onsite using Avante multi-meter probe (Avante multi parameter meter 900b and India). The chemical parameters were measured as follows: NH_3 ,Nesseler method(HACH, 1999), briefly, sample was diluted 1:100 with deionized water and three drops of mineral stabilizer (potassium sodium tartrate,sodium citrate and demineralized water) was added to the sample and blank (Deionized water). The mixing cylinder was inverted several times to mix using stopper on the mixing cylinder. Three drops of polyvinyl alcohol water were added to each cylinder and mixed it well. One ml of Nesseler reagent (Sodium hydroxide, Sodium iodide, Demineralized water and Mercuric iodide) was added to each mixing cylinder and mixed it well. After 1 minute of incubation the sample was measured spectrophotometrically (HACH, DR/2010) at 425 nm.

Total nitrogen (TN), Total phosphate (TP) and Orthophosphate ($PO4^{3-)}$ analyses were carried out at JIJE Analytical Testing services Laboratory, Addis Ababa. Total nitrogen (TN) was measured by using Semi-Micro-Kjeldhal method as in protocol # APHA 4500 B (APHA, 1998). Both total phosphate (TP) and Orthophosphate (Po_4^{3-}) were analyzed using vanadomolybdophosphoric acid based on protocol # APHA 4500 C (APHA, 1998).

COD was measured by using Manganese III Digestion Method (HACH, DR/ 2000 and DR/3000 instrument) according to HACH instructions and BOD analyses were carried out using titration method in the Department of Environmental Science research laboratory, AAU.

TDS was measured onsite using TDS instrument (HACH, 1999). All the analyses were made in triplicate for each parameter. The removal efficiency of the system was calculated based on the formula:

$$(C_{influent} - C_{effluent} / C_{influent}) * 100$$

Where: - $C_{influent}$ is concentration of the pollutant in the influent wastewater and $C_{effluent}$ is concentration of the pollutant in the effluent water.

3.4. Heavy Metal Analysis

The analyses of the following heavy metals namely**:** Lead (Pb), Zinc (Zn), Cadmium (Cd), Cobalt (Co), Nickel (Ni), Chromium (Cr) and Mercury (Hg) were conducted at JIJE Analytical Testing Service Laboratory, Addis Ababa. The above-mentioned heavy metals were analyzed using Air-Acetylene flame method as in protocol #APHA 3111C (APHA, 1998).

3.5. Antibiotic Sensitivity Testing

The following antibiotics were used for the sensitivity testing**:** Ciprofloxacin (4 µg/ml), Gentamicin (16 µg/ml), Cefotaxime (64 µg/ml) and Doxycycline (16 µg/ml). The choice of these antibiotics was based on availability for the tests and frequency of application (commonly used) in the hospital. Antibiotic susceptibility test was performed based on standard reference values according to National Committee for Clinical Laboratory Standards, now Clinical and Laboratory Standards Institute (CLSI) described in (NCCLS, 2015).

The wastewater samples (influent and effluent) were serially diluted and plated them on to nutrient agar in duplicate containing the selected antibiotics: Ciprofloxacin, Gentamicin, Cefotaxime and Doxycycline with their maximum value of the minimum inhibitory concentrations as described in Boto *et al.* (2016).

Stock and working solution preparation for the following antibiotics (cefotaxime, gentamicin ciprofloxacin and doxycycline) respectively, 0.05 g cefotaxime was diluted in 1ml of sterilized distilled water. From the stock solution of cefotaxime 0.13 ml (130 µL) was diluted in 9.87 ml of distilled water. A 40 µL Gentamicin was diluted in 100 ml of distilled water. 0.004g of ciprofloxacin was diluted in 1000 ml of distilled water and also 0.003 g of doxycycline was diluted in 100 ml of sterilized distilled water. From each dilution of antibiotics, 100 µL volumes of antibiotics were spread plated in duplicate and after antibiotics were spread on nutrient agar plate, 100 µL volume of sample was spread on to antibiotic containing plate and parallelly, 100 µL volume of sample was spread on Nutrient Agar plates without antibiotics all of which in duplicate and incubated for 24 hours at 37°C. The plates with antibiotics were used to determine the number of antibiotic resistant bacteria and the plates without antibiotic were used to determine the total number of heterotrophic resistant bacteria.

The percentage removal was calculated by the ratio between CFU/ml in treated and in initial wastewater:

$$\% \text{ Removal} = \left(1 - \frac{(\text{CFU/mL}) \text{ in treated waste water}}{(\text{CFU/mL}) \text{ in initial waste water}} \times 100\right)$$

For each antibiotic, the percentage proportion of antibiotic resistance was calculated for each sample:

$$\%\text{proportion} = \frac{(\text{CFU/mL}) \text{medium with antibiotics}}{(\text{CFU/ml}) \text{medium with out antibiotic}} \times 100$$

3.6. HPLC- based determination of selected antibiotics

Quantitative determination of the level of ciprofloxacin and cefotaxime in the raw wastewater, influent and effluents from each constructed wetland cell was done using High Performance Liquid Chromatography (HPLC) at the Department of Chemistry. The samples collected from each cell using sterilized bottles were kept at -20^{o}c until subject to analysis. Ciprofloxacin and cefotaxime were selected based on the frequency of application in the hospital and suspected environmental impact of different antibiotics.

Extraction of the target analytes from the samples was done following the salting out assisted liquid-liquid extraction method described by Teshome Gezahegn and colleagues (2018). Initially, each water sample was filtered through 0.45 µm membrane filter to remove any impurities and acidified with hydrochloric acid to pH 3.0. Ten milliliters of sample were added into 50 ml falcon tube and then 5 ml of acetonitrile (CH_3CN) and 4 g of $MgSO_4$ were added respectively. Then the sample, all added solvents and salt were shaken for 6 minutes after which they were centrifuged at 4000 rpm for 5 minutes. The organic layer of the sample was withdrawn with micropipette into a clean test tube and then dried using rotavapor. Finally, the dried sample was reconstituted by 500 µl methanol and transferred into loading vial for chromatographic analysis.

3.6.1. Preparation of analytical standards

a. Stock solution preparation

Stock standard solution was prepared by weighing ciprofloxacin (10 mg) and cefotaxime (1 mg) dissolving it in methanol (10 ml and 5 ml respectively). This solution was used to make up all working solutions by diluting portion of stock with mobile phase. From stock solution of ciprofloxacin and cefotaxime, 2000 µl stock solution of ciprofloxacin and 1000 µl stock solution cefotaxime were used to prepare dilution series of 0.3125 ppm, 0.625 ppm, 1.25 ppm, 2.5 ppm, 5

ppm, 10 ppm, 20 ppm and 40 ppm. The readings of these dilutions were used to draw the standard curve and check for the accuracy of the measurement.

b. Instrumentation and sample run

The chromatographic analysis was performed using Agilent 1200 series HPLC, equipped with quaternary pump, Vacuum Degasser and autosampler (Agilent Technlogies, Germany). Chromatographic separation was performed on C18 analytical column (ZORBAX Eclipse XDB, 150 mm × 4.6 mm id, particle size 5 m, Agilent Technologies) and the column temperature was 35°C. Data acquisition and processing were performed with LC Chemstation software (Agilent Technologies).

The mobile phase composition was methanol and water (75:25 v/v) which was pumped at a flow rate of 2.2 ml/min. The detection wavelength of ciprofloxacin and cefotaxime was 278 and 254 nm, respectively with a band width of 4 nm and the injection volume was 20 µl. peak area was used as response of HPLC with run time of 15 minutes.

3.7. Statistical analysis

Statistical analyses were performed using the software SPSS Version 20. The data was analyzed through analysis of variance (ANOVA). Pearson correlation analyses were conducted to study correlations between antibiotic residue concentration and antibiotic resistant bacteria.

4. RESULTS

4.1. Characterization of Tikur Anbessa Specialized Hospital wastewater

Table 3. Characteristics Tikur Anbessa Hospital wastewater in the study time

Parameter	Unit	Mean ± SD
Temperature	^{o}C	20±4.2
PH		7.3±0.25
Conductivity	µS	1260±140.6
DO	mg/l	5.19±5.17
BOD	mg/l	243±95
COD	mg/l	1312±255
TP	mg/l	6.4±4.4
SRP	mg/l	5.01±3.41
TN	mg/l	61.98±39.4
NH3-N	mg/l	2.32±3.18
TDS	mg/l	242.66±205.73
Hg	µg/l	1.29±1.48
Pb	mg/l	BDL*
Cr	mg/l	BDL*
Cd	mg/l	BDL*
Zn	mg/l	0.28±0.38
Ni	mg/l	0.078±0.088
Co	mg/l	BDL*
Ciprofloxacin	Ppm	1.3
Cefotaxime	Ppm	18

*BDL refers to below the method dictation limit

During the study period, the sampled raw wastewater of Tikur Anbessa Specialized Hospital had temperature ranging from 18.2 to 25°C with an average temperature of 20.16°C (Table.3). The raw wastewater also had pH that ranged between 7.05 - 7.34 with an average PH of 7.3. Dissolved oxygen (DO) in the hospital wastewater ranged from 0.11 to 10.06 mg/l with an average concentration of 5.91 mg/l (Table.3). The average concentration of BOD and COD of the wastewater from Tikur Anbessa Hospital were 243 mg/l and 1312 mg/l (Table.3).

The total nitrogen of the raw hospital wastewater ranged between 16 - 88.85 mg/l with an average concentration of 62 mg/l (Table.3). Ammonia in the raw wastewater ranged between 0.43 – 6 mg/l with average of 2.3 mg/l. Total suspended solid of the raw hospital wastewater ranged between 480 and 1115mg/l with an average value of 243 mg/l (Table.3). Conductivity of the hospital wastewater ranged between 1156 to 1420 µS with an average of 1260 µS (Table.3). The average concentrations of total phosphorus and soluble reactive phosphorus (SRP) were 6.62 and 5 mg/l respectively (Table.3).

Among the tested heavy metals, Mercury, Zinc and Nickel were detected with average concentrations of 1.29 µg/l, 0.28 and 0.078 mg/ respectively (Table.3). Lead, cadmium and cobalt were measured below the method detection limit.

The concentration of ciprofloxacin in the raw wastewater was 1.3 ppm, while that of cefotaxime was 18 ppm (Table 3).

4.2. Removal of organic matter and nutrients in the constructed wetland

4.2.1. BOD and COD

The influent wastewater from the equalization tank had a mean BOD of 161.69 mg/L. After treating using the different plants and combination, the mean BOD of the effluent of the wetland cell planted with *T. latifolia* was 97.13 mg/L, resulting in a 40% efficiency of *T. latifolia* in BOD removal. The cell planted with *P. karka* had effluent with BOD of 66.77 mg/L, making its efficiency 59% in BOD removal (Table 4). The wastewater treated with the combination of both plants had BOD of 73 mg/L with the efficiency of 55% in BOD removal. The unplanted control had BOD of 73.07 mg/L, with 55 % BOD removal efficiency.

The COD of the influent wastewater in the equalization tank was 681 mg/L. After being treated by *T. latifolia*, COD decreased to 179 mg/L with the removal efficiency of 74 %, while the treated water (effluent) from *P. karka* had COD of 146.3 mg/L, making its removal efficiency to 79 %. The wastewater treated by the combination of two plants had COD of 147 mg/L with removal efficiency of 78%, whereas the unplanted control-based treatment brought a COD of 97.23 mg/L, with removal efficiency of 86 % (Table 4). Comparative analysis showed that there is no significant (P=0.05) variation between the influent and treated effluents with regard to the concentrations of organic carbon in the form of BOD and COD

Table 4.concentration of organic matter and efficiency of the wetland

Plant type	Mean concentrations* (mg/l) and removal efficiencies (%)	
	BOD (RE %)	COD (RE %)
Influent	161.69[a]	681.67[b]
T.latifolia	97.00[a] (40 %)	179.00[b] (74 %)
P.karka	66.67[a] (59 %)	146.33[b] (79%)
Combined	73.00[a] (55 %)	147.00[b] (78%)
Control	73.00[a] (55%)	97.00[b] (86%)

*Within each column, the mean values with different letters are significantly different at $p < 0.05$

4.2.2. Removal of phosphorous

Total phosphorous (TP) of the influent was 5.61 mg/L and upon treatment with *T. latifolia*, the mean concentration was reduced to 0.69 mg/L resulting in 88% removal (Table 5). Treatment with *P. karka* reduced the total phosphorus to 0.28 mg/L, resulting in 95% removal. Treatment using the combined plants reduced the TP to 1.43, mg/L while the unplanted control had 3.43 mg/L TP. The removal efficiency of the combined plants treatment was 74% and the unplanted control had 39%. Comparative analysis showed that the concentration of TP in the influent was significantly (P=0.05) the highest of all except the unplanted control effluent (Appendix 1).

Soluble reactive phosphorous (SRP) concentration of the influent wastewater was 4.01 mg/L, which decreased to 0.33 mg/L and 0.02 mg/L upon treatment with *T. latifolia* (92%) and *P. karka* (99%), respectively. Treatment with the combined plants decreased the SRP to 0.83 mg/L with a removal efficiency of 79%. The effluent from the unplanted control had SRP of 2.86 mg/L with a removal efficiency of 29% (Table 5). The SRP of the effluent treated with *P. karka* had significantly the lowest concentration of all, including the influent (Appendix1).

Table 5.concentration of total phosphorous and soluble reactive phosphorous

Plant type	Mean concentrations* (mg/l) and removal efficiencies (%)	
	TP (RE %)	SRP (RE %)
Influent	6.00[c]	4.00[e]
T.latifolia	0.67[d] (68 %)	0.33[e] (92%)
P.karka	0.00[d] (95%)	0.00[f] (99%)
Combined	1.33[d] (74 %)	1.00[e] (79%)
Control	3.33[c] (39%)	2.67[e] (29%)

*Within each column, the mean values with different letters are significantly different at $p < 0.05$

4.2.3. Removal of Nitrogen

The mean concentration of total nitrogen (TN) in the influent wastewater was 48.76 mg/L. The effluent treated by *T. latifolia* and *P. karka* had mean TN concentrations of 3.33 and 5.72 mg/L, with removal of 98 and 88% respectively. The mean TN concentration of water treated using the combined plants was 6.93 mg/L while the water from untreated control had a mean TN of 24.03 mg/L (Table 6). The combined and control had removal efficiency of 86 and 51% respectively. Comparative analysis using ANOVA showed that concentration of TN in the effluent treated with *T. latifolia* was significantly the lowest of all the other effluents including TN of the influent wastewater (Appendix 1).

Regarding ammonia (NH_3), the mean concentration of the influent was 22 mg/L and effluent water treated by *T. latifolia* and *P. karka* had NH_3 concentrations of 0.33 mg/L and 0.66 mg/L respectively. The mean of NH_3 concentration of water treated using the combined plants were 1.33 mg/L and water from the untreated control had a mean concentration of 5.33 mg/L (Table 6). The efficiency of the treatment system for the removal of NH_3 planted with *T. latifolia* and *P. karka* was 98% and 97% respectively. The combined and the control had removal efficiency of 94 and 76% respectively (Table 6). Comparative analysis using ANOVA showed that ammonia

concentration in the influent was significantly higher than all except the unplanted control effluent (Appendix1).

Table 6.Mean concentration of TN and removal efficiency

Plant type	Mean concentrations* (mg/l) and removal efficiencies (%)	
	TN (RE %)	NH_3 (RE %)
Influent	48.66^{g}	22.00^{i}
T.latifolia	3.33^{h} (94%)	0.33^{j} (98%)
P.karka	6.00^{g} (88%)	0.67^{j} (97%)
Combined	7.00^{g} (86%)	1.33^{j} (94%)
Control	24.00g (51%)	5.33i (76%)

*Within each column, the mean values with different letters are significantly different at $p < 0.05$

4.3. Heavy metal concentration in Tikur Anbessa Hospital wastewater and its removal

Zinc, Nickel and Mercury were detected in the raw wastewater with samples among the range of metals screened including Lead, cadmium, chromium and cobalt (Table 7). The average concentration of Zinc in the raw wastewater was 0.28 mg/l while Nickel had an average concentration of 0.08 mg/L in the raw wastewater. There was no significant (P=0.05) variation between the influent and treated effluents with regard to the concentrations of Zinc and Nickel.

Table 7. Mean concentrations and percentage efficiency of the constructed wetland for the removal of Zinc and Nickel from the hospital wastewater

Constructed Wetland Cell	Mean concentration (RE %)	
	Zinc	**Nickel**
Influent	0.33	0.09
T. latifolia	0.05 (84%)	0.04 (59%)
P. karka	0.045 (86%)	0.02* (73%)
Combined	0.11 (65%)	0.16 (-78%)
Control	0.22 (34%)	0.05 (41%)

*The concentration of Nickel in the treatment cell using *P. karka* in some of the triplicate samples was below the detection limit.

The efficiency of *T. latifolia*, *P. karka* and the combined plants in removing zinc was 84, 86 and 65 percent, respectively while the unplanted control had 34% removal efficiency. The efficiency of *T. latifolia* and *P. karka* in removing nickel was 59 and 73% respectively. The combined cell showed an increased concentration of nickel compared to the influent and hence had the lowest efficiency (below zero), while the unplanted control had 41% removal efficiency (Table 7).

4.4. Removal of heterotrophic bacteria in treated effluents using the different plants

The total heterotrophic bacteria in the influent hospital wastewater ranged between 2.59E+05 and 1.83E+05 CFU/ml, with mean of 2.12E+05 CFU/ml (Table 8). The heterotrophic bacteria after treatment with the different treatment cells ranged between 6.98E+04 and 4.35E+04 CFU/ml. Maximum removal efficiency was obtained with *T. latifolia*; with 80% removal efficiency in the effluent (Table 8). The second highest removal of heterotrophs was obtained by treatment with *P. karka* (72%) removal of the heterotrophic bacteria. The CW cell with mixed plants and the unplanted control had 67 and 68% of heterotrophic bacteria removal efficiency, respectively. The mean count of heterotrophic bacteria in the effluent from the four cells was

significantly (p=0.05) lower than the influent hospital wastewater. Despite this, the heterotrophic bacterial count in the effluent from each cell did not have significant difference between each other.

Table 8.Mean colony count and efficiency of the constructed wetland for the removal of total heterotrophic bacteria in Tikur Anbessa Hospital wastewater

Plants	Mean of influent (CFU/ml)	Mean of effluent (CFU/ml)	Removal efficiency (%)
T. latifolia	2.12E+05	4.35E+04	80
P. karka		5.86E+04	72
Combined		6.98E+04	67
Control		6.84E+04	68

4.5. Removal of antibiotic resistant bacteria in the treated effluents using the different plants

In the influent wastewater from the equalization tank, 87% of the cultured bacteria were capable of growing in 4 µg/ml ciprofloxacin (Table 9). In the presence of 16 µg/ml gentamicin, 66% of the cultured bacteria grew and in the presence of 16 µg/ml doxycycline, 84% of the cultured bacteria grew. In the presence of 64 µg/ml cefotaxime, 83% of the cultured bacteria grew (Table 10). In the cell with *T. latifolia*, 99% bacteria grew in the presence of same concentration of gentamicin, while only 20% cultivated heterotrophs grew in the same concentration of cefotaxime. The cell with *P. karka* had more than 100% heterotrophs growth in the presence of ciprofloxacin followed by 72% of bacteria that grew in the presence of gentamicin. Similar to *T. latifolia*, the cell with the combined plants had more than 100% heterotrophic bacteria capable of growing in the presence of ciprofloxacin, while 89% in the presence of gentamicin. In the control cell, 71% of the heterotrophs grew in ciprofloxacin and 70% in gentamicin (Table 9).

In medium containing 4 µg/ml ciprofloxacin, the CFU/ml of the influent that grew in the presence of ciprofloxacin was significantly the highest of all the effluents from the different treatment cells. Similarly, the CFU/ml of the influent wastewater grown on 64 µg/ml cefotaxime and 16 µg/ml gentamicin was significantly higher than all the effluents (Table 9). The CFU/ml of the influent grown with 16 µg/ml doxycycline was significantly higher than the effluent

treated with *P. karka*, which is followed by *T. latifolia*, the combined plants and the unplanted control (Table 9).

Table 9.Mean and percent proportion of resistant bacteria with the total heterotrophic bacterial count in the influent and effluents in paired plating with and without the listed antibiotic

Plant type	Log-transformed mean ± SE CFU/ml of bacteria with and without the antibiotics and percent proportion (%) of bacteria grown in the presence of the antibiotics											
	Ciprofloxacin (4µg/ml)			**Cefotaxime** (64 µg/ml)			**Doxycycline (**16 µg/ml)			**Gentamicin (**16 µg/ml)		
	Without	**With**	**%**	**Without**	**With**	**%**	**Without**	**With**	**%**	**Without**	**With**	**%**
Influent	5.28±0.07 a	5.21±0.49 a	87	5.25±0.03 a	5.17±0.02 a	83	5.29±0.04 a	5.21±0.04 a	84	5.40±0.03 a	5.21±0.06 a	66
T. latifolia	4.69±0.09 b	4.51±0.89 b	63	4.40±0.16 b	3.71±0.15 b	20	4.55±0.19 b	4.10±0.28 bc	41	4.48±0.13 b	4.42±0.21 b	99
P. karka	4.57±0.15 b	4.64±0.10 b	102	4.72±0.15 b	4.13±0.07 b	18	4.61±0.23 b	4.48±0.15 b	46	4.67±0.14 b	4.45±0.24 b	72
Combined	4.65±0.15 b	4.64±0.10 b	138	4.57±0.21 b	3.92±0.27 b	33	4.63±0.20 b	4.17±0.27 c	46	4.59±0.24 b	4.48±0.25 b	89
Control	4.53±0.23 b	4.45±0.17 b	71	4.75±0.15 b	3.68±0.17 b	9	4.76±0.15 b	3.85±0.12 c	11	4.72±0.15 b	4.39±0.24 b	70

* within each column, value with different letters are significantly different ($P < 0.05$)

** Percentage proportion of antibiotic resistant heterotrophic bacteria with respect to the total bacteria grown without antibiotics

4.6. Removal of antibiotic residues in the treated effluents using the two plants

The mean concentration of ciprofloxacin in the influent was 1.23 ppm which decreased to 1.13 and 1.16 ppm upon treatment with the combined plants and *P. karka*, respectively (Table 10). The concentration of ciprofloxacin in the outlet of the wetland planted with *T. latifolia* and the unplanted control had 1.2 ppm. In all the plant types, the removal efficiency of ciprofloxacin was below 10% (Table 10).

The other analyzed antibiotic residue, cefotaxime, was found to be present in the influent with an average concentration of 17 ppm which decreased by more than 50% to 6.66 ppm and 6.33 ppm upon treatment with *T. latifolia* and the unplanted control, respectively. The concentration of cefotaxime in the outlet of the wetland planted with *P. karka* and combined plant had 12.33 and 11.3 ppm, respectively. The highest performance was observed with *T. latifolia* (61%) and control (62%) relative to the other treatment cells (Table 10).

Table 10. Removal of ciprofloxacin and cefotaxime residues from the hospital wastewater using the plants T. latifolia, P. karka and their combination using HSSFCW

Mean (SD)	**Drug (ppm)**		**Removal efficiency (%)**	
	Ciprofloxacin	**Cefotaxime**	**Ciprofloxacin**	**Cefotaxime**
Influent	1.23±0.05	17±7.5		
T. latifolia	1.2±0.1	6.66±2.3	3	61
P. karka	1.16±0.05	12.33±4.1	5	28
Combined	1.13±0.05	11.33±1.1	8	33
Control	1.2±0.0	6.33±2.5	2	62

4.7. Relationship between the load of antibiotic residues and antibiotic resistant bacteria

Upon examining the presence of cross-sectional relationship between antibiotic concentration and resistant bacteria in the wastewater, ciprofloxacin and ciprofloxacin resistant bacteria in the influent had strong positive correlation (r^2=0.58, P=0.61). Strong negative correlation (r^2= -0.99, P=0.076) with the effluent treated with *P. karka* while the wetland planted with *T. latifolia* had weak positive correlation (r^2=0.31, P=0.798) in all the cases with ciprofloxacin, the relationships were statistically insignificant (Table 11).

Regarding cefotaxime and cefotaxime resistant bacteria, strong positive (r^2=0.98, P=0.11) correlation was exhibited in the influent and the cell with *T. latifolia*, the latter being statistically significant (Table 11). Cefotaxime and cefotaxime resistant bacteria in the outlet of the wetland planted with *P. karka* and the unplanted control had strong negative (r^2=-0.977,-0.746, P=0.138, 0.464) correlation which is statistically insignificant. Strong negative correlation was observed between cefotaxime and cefotaxime resistant bacteria after treated with combined plant and statistically significant (p=0.01).

Table 11. Correlation between concentration of antibiotic residue and antibiotic resistant bacteria

Plant type	Ciprofloxacin Vs ciprofloxacin Resistant Bacteria		Cefotaxime Vs cefotaxime resistant bacteria	
	Pearson's Correlation	P-value	Pearson's Correlation	P-value
Influent	0.58	0.61	0.98	0.11
T.latifolia	0.31	0.798	0.998*	0.044
P.karka	-0.993	0.076	-0.977	0.138
Combined	-0.521	0.651	-1.000**	0.000
Control	-	-	-0.746	0.464

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

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

5. DISCUSSION

The organic matter of the wastewater in the current study (243 mg/l BOD and 1312 mg/L COD) was lower than that of a previous study conducted in the same hospital wastewater, which was 288.8 mg/l BOD and 1764.8 mg/l COD (Mahder Mekonnen, 2017). Such fluctuation of organic matter in hospital wastewater depends on factors including number of patients, number of students and residents in the hospital at the particular time of the sampling. Despite this, the BOD and COD of the wastewater were above the permissible limit of the US EPA standard which is 30 mg/l. A study conducted in Hawassa referral hospital, the average concentration of BOD and COD in influent (632±31.11 and 1388.75±206.83 mg/L) higher than the result obtained from this study (161.69 mg/L and 681 mg/L). In another study of hospital wastewater in Iran, it was reported that the BOD ranged between 161 – 648 mg/l and COD ranged between 379 - 1187 mg/l, which was much higher than the present study (Amouei *et al.*, 2015). According to Mesdaghinia *et al.*, (2009), the average concentrations of BOD_5 and COD (768 and 1362 mg/L) were the highest compared to this study.

In the current study, the wetland cells with *T. latifolia* and *P. karka* showed higher removal efficiency for BOD and COD (59 and 86%, respectively) but this difference was not statistically significant ($p > 0.05$), which may be due to lower difference between these wetland cells planted with different plant species and unplanted cell.

Among the different plant types and their combinations, the highest organic matter removal was observed in the *P. karka* planted cell for BOD (59% RE) and the unplanted control cell for COD (97% RE). The highest removal of COD in the cell with no plantation is unusual and is in contrary to the fact that plant roots in wetlands facilitate organic matter removal through providing surface area for microorganisms, increasing uptake of nutrients and oxygen transfer to their roots (Kadlack, *et al.*, 2000). The reason for the highest COD removal in the unvegetated wetland cell requires further investigation.

When comparing the performance of *T. latifolia, P. karka* and their combination with respect to BOD and COD removal, no significant difference was found between the treatment types and with the unvegetated control. It was expected that the wetland cell with the combined plants would show higher performance in the removal of organic matter as reviewed by (Vymazal

2011). The way of planting macrophyte in wetland, early and maturity stage of the macrophyte might make difference in their performance.

The wastewater from Tikur Anbessa referral Hospital had total Nitrogen content of 62 mg/L and ammonia 2.3 mg/L. Compared to the report by Hunachew Beyene and Getachew Redae (2011) where total nitrogen and ammonia of Hawassa Hospital wastewater was 98 mg/L and 0.2 mg/L, respectively, the Nitrogen load of Tikur Anbessa Hospital wastewater was lower despite its being a referral hospital. This can be explained by factors that affect the movement of patients the referral hospital from various parts of the country. One of the observed factors at the sampling time was the civil unrest that occurred in many parts of Oromiya region in 2018, which might be an obstacle for patients to come to the referral hospital for treatments.

The constructed wetland planted with *T. latifolia* showed the highest Nitrogen removal (94%), indicating *T. latifolia* as an efficient plant for the removal of total Nitrogen. Similarly, this plant showed the highest removal of NH_3, where 98% removal was observed in constructed wetland cell planted with *T. latifolia* followed by *P. karka* (97%). *T. latifolia* and *P. karka* have long been known among the most important wetland plants and the high efficiency of these plants in the removal of ammonia and nitrogen both in natural wetland and constructed wetland settings were reported earlier (Vymazal 2017, Ciria *et al.*, 2005).

Total phosphorus and soluble reactive phosphorus in the current study was also much lower than that of Hawassa referral Hospital, with 37 mg/L of TP and 22 mg/L of SRP (Hunachew Beyene and Getachew Redae, 2011). This is accounted for the number of patients who came to the hospital for service at the time of the sampling months. The constructed wetland cell planted with *P. karka* showed 95% removal for TP and 99% removal for SRP. The genus *Phragmites* is a well-known wetland plant commonly named as "reed bed" and its efficiency in the removal of TP in addition to the common organic pollutants is reported as the second most efficient wetland plant as studied by various researchers (Akratos and Tsihrintzis, 2007; Ghosh and Gopal, 2010; Vyamzal, 2001).

The concentration of Zinc and Nickel in the influent was below the permissible limit set by WHO, which are 3 and 0.2 mg/l, respectively. Even though the concentrations of Zinc and Nickel in effluents from all the cells (including the unvegetated control) were below the permissible limit as set by the WHO standards, *P. karka* and *T. latifolia* was found to be the

most efficient plant for Zinc removal with 86% and 84% efficiency. For Nickel reduction, *P. karka* was found to be the most efficient with 73% removal efficiency. For Nickel removal, the combined cell showed negative performance with increasing concentration of Nickel in the effluent. Based on the current study, it is difficult to explain the reason for such an increase of Nickel in the effluent from the combined plant cell but it is worth considering measurement repeats for Nickel. Nevertheless, regarding these two metals, the obtained effluent concentration values were below the standard limit values, indicating the effectiveness of the constructed wetland in fulfilling the regulatory limit values to discharge the effluent.

The mean concentration of total heterotrophic bacteria in the influent was 2.12×10^5 CFU/ml which is lower compared to a study conducted in Mekelle hospital raw wastewater, which was 1.6×10^6 CFU/ml (Tsegahun Asfaw *et al.*, 2017) and Hawassa referral hospital wastewater which was 7.4×10^7 CFU/ml (Hunachew Beyene and Getachew Redae, 2011). Tikur Anbessa wastewater hetroplate count was also lower than the concentration of total cultivable heterotrophic bacteria in raw hospital wastewater from three tertiary hospitals in central China, which ranges from 2.03×10^9 CFU/L to 3.31×10^9 CFU/L (Wang *et al.*, 2010). According to Aali *et al.*, (2014), the mean concentration of heterotrophic bacteria in hospital wastewater was 6.05×10^7. After treatment with different plants and no vegetated cell, the concentration of total heterotrophic bacteria was reduced 10^5 to 10^4.As presented in table 9,*T.latifolia* showed highest performance for removal of total heterotrophic bacteria and the mean difference between inlet and outlet statistically significant $P<0.05$ but there no difference among the cells ($p>0.05$).

In this study, the mean concentrations of ciprofloxacin in the inlet and outlet were higher than a study conducted by Le *et al.* (2016) in Singapore, the concentration ranges of detected ciprofloxacin from hospital wastewater were 8.74–76.44 and 1.72–4.34 µg/l. Recent study by Rodrigues-Silva et *al.* (2019) showed that the concentration ranges of ciprofloxacin were 1.3-33.9 ng mL^{-1} in hospital raw sewage and also found in treated wastewater sample (0.5-5.6 ng mL^{-1}). According to Wang *et al.* (2010), the concentration of ciprofloxacin residue was 1334.10 ±180.02 ng/L, still much lower than the current study. In the current study, the difference in removal of ciprofloxacin between planted cells and unplanted control had less than 10%. This might be due to the short retention time and longer retention might improve the removal

efficiency, leading to further study in hydraulic retention time in the framework of antibiotics removal in constructed wetland system.

The mean concentration of cefotaxime in the inlet and outlet of constructed wetland planted and unplanted cells had higher levels. A study conducted in Pakistan, cefotaxime was not detected in hospital wastewater which may possibly be due to degradation of the drug by complex matrix effects, sunlight and also adsorption on soil. Wang *et al.*, (2011) have also reported that concentration of cefotaxime in the influent and effluent of WWTP ranged from none detected to 0.82 µg/L and none detected to 0.13µg/L respectively. Higher removal efficiency was observed in the unplanted control and the cell planted with *T. latifolia* than the rest of the cells. For the unplanted control, a number of factors including sun light degradation may contribute for degradation of the compound since no coverage of vegetation and the water is prone to photoreaction that the vegetated wetland cells.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusion

The following conclusion drawn based on the result obtained during this study, results revealed that a large occurrence of resistant bacteria and antibiotics, such as ciprofloxacin and cefotaxime in hospital wastewater. Better removal /reduction were found in HSSFCW for cefotaxime compared to ciprofloxacin. Reduction of nutrient, organic matter, heavy metal, antibiotics and antibiotic resistant bacteria were observed in the influent and effluent of wastewater using HSSFCW.

Single macrophyte species planted based constructed perform better removal /reduction compared to combination of the macrophyte species for nutrient , organic matter, antibiotics, antibiotic resistance and heavy metals. There was strong positive correlation between ciprofloxacin and ciprofloxacin resistant bacteria in the influent. Strong positive relationship was found between cefotaxime and cefotaxime resistant bacteria in the influent, effluents from cell planted with *T.latifolia* and mixed plant.

6.2. Recommendations

- ✓ Hospital waste water contains antibiotics, heavy metal and antibiotic resistant bacteria so onsite hospital treatment is required before the hospital wastewater joined to sewage system.
- ✓ Further research is required to evaluate the pollution level of antibiotics and ARGs released from hospital and also the association between ARG, antibiotics and antibiotic resistant bacteria.
- ✓ Further research is recommended for the removal of pharmaceuticals and resistant bacteria using high retention time in the constructed wetland.

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Appendex

Appendix 1.ANNOVA result for organic matter, nutrient and heavy metals

Multiple Comparisons

LSD

Dependent Variable	(I) PLANT	(J) PLANT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TP	Influent	Typha	5.33333*	1.75119	.012	1.4314	9.2352
		PHRAG	6.00000*	1.75119	.006	2.0981	9.9019
		COMBINED	4.66667*	1.75119	.024	.7648	8.5686
		CONTROL	2.66667	1.75119	.159	-1.2352	6.5686
	Typha	influent	-5.33333*	1.75119	.012	-9.2352	-1.4314
		PHRAG	.66667	1.75119	.711	-3.2352	4.5686
		COMBINED	-.66667	1.75119	.711	-4.5686	3.2352
		CONTROL	-2.66667	1.75119	.159	-6.5686	1.2352
	PHRAG	influent	-6.00000*	1.75119	.006	-9.9019	-2.0981

SRP		Typha	-.66667	1.75119	.711	-4.5686	3.2352
		COMBINED	-1.33333	1.75119	.464	-5.2352	2.5686
		CONTROL	-3.33333	1.75119	.086	-7.2352	.5686
		influent	-4.66667*	1.75119	.024	-8.5686	-.7648
	COMBINED	Typha	.66667	1.75119	.711	-3.2352	4.5686
		PHRAG	1.33333	1.75119	.464	-2.5686	5.2352
		CONTROL	-2.00000	1.75119	.280	-5.9019	1.9019
		influent	-2.66667	1.75119	.159	-6.5686	1.2352
	CONTROL	Typha	2.66667	1.75119	.159	-1.2352	6.5686
		PHRAG	3.33333	1.75119	.086	-.5686	7.2352
		COMBINED	2.00000	1.75119	.280	-1.9019	5.9019
		Typha	3.66667	1.65999	.052	-.0320	7.3653
		PHRAG	4.00000*	1.65999	.037	.3013	7.6987
	Influent	COMBINED	3.00000	1.65999	.101	-.6987	6.6987
		CONTROL	1.33333	1.65999	.441	-2.3653	5.0320
		influent	-3.66667	1.65999	.052	-7.3653	.0320
		PHRAG	.33333	1.65999	.845	-3.3653	4.0320
	Typha	COMBINED	-.66667	1.65999	.696	-4.3653	3.0320
		CONTROL	-2.33333	1.65999	.190	-6.0320	1.3653

		influent	-4.00000*	1.65999	.037	-7.6987	-.3013
		Typha	-.33333	1.65999	.845	-4.0320	3.3653
	PHRAG	COMBINED	-1.00000	1.65999	.560	-4.6987	2.6987
		CONTROL	-2.66667	1.65999	.139	-6.3653	1.0320
		influent	-3.00000	1.65999	.101	-6.6987	.6987
		Typha	.66667	1.65999	.696	-3.0320	4.3653
	COMBINED	PHRAG	1.00000	1.65999	.560	-2.6987	4.6987
		CONTROL	-1.66667	1.65999	.339	-5.3653	2.0320
		influent	-1.33333	1.65999	.441	-5.0320	2.3653
		Typha	2.33333	1.65999	.190	-1.3653	6.0320
	CONTROL	PHRAG	2.66667	1.65999	.139	-1.0320	6.3653
		COMBINED	1.66667	1.65999	.339	-2.0320	5.3653
		Typha	65.33333	75.64537	.408	-103.2151	233.8817
		PHRAG	95.66667	75.64537	.235	-72.8817	264.2151
	Influent	COMBINED	89.33333	75.64537	.265	-79.2151	257.8817
BOD		CONTROL	89.33333	75.64537	.265	-79.2151	257.8817
		influent	-65.33333	75.64537	.408	-233.8817	103.2151
	Typha	PHRAG	30.33333	75.64537	.697	-138.2151	198.8817

			COMBINED	24.00000	75.64537	.758	-144.5484	192.5484
			CONTROL	24.00000	75.64537	.758	-144.5484	192.5484
			influent	-95.66667	75.64537	.235	-264.2151	72.8817
		PHRAG	Typha	-30.33333	75.64537	.697	-198.8817	138.2151
			COMBINED	-6.33333	75.64537	.935	-174.8817	162.2151
			CONTROL	-6.33333	75.64537	.935	-174.8817	162.2151
			influent	-89.33333	75.64537	.265	-257.8817	79.2151
		COMBINED	Typha	-24.00000	75.64537	.758	-192.5484	144.5484
			PHRAG	6.33333	75.64537	.935	-162.2151	174.8817
			CONTROL	.00000	75.64537	1.000	-168.5484	168.5484
			influent	-89.33333	75.64537	.265	-257.8817	79.2151
		CONTROL	Typha	-24.00000	75.64537	.758	-192.5484	144.5484
			PHRAG	6.33333	75.64537	.935	-162.2151	174.8817
			COMBINED	.00000	75.64537	1.000	-168.5484	168.5484
COD		influent	Typha	502.66667	257.39585	.079	-70.8470	1076.1804

	PHRAG	535.33333	257.39585	.064	-38.1804	1108.8470
	COMBINED	534.66667	257.39585	.064	-38.8470	1108.1804
	CONTROL	584.66667*	257.39585	.046	11.1530	1158.1804
	influent	-502.66667	257.39585	.079	-1076.1804	70.8470
Typha	PHRAG	32.66667	257.39585	.902	-540.8470	606.1804
	COMBINED	32.00000	257.39585	.904	-541.5137	605.5137
	CONTROL	82.00000	257.39585	.757	-491.5137	655.5137
	influent	-535.33333	257.39585	.064	-1108.8470	38.1804
PHRAG	Typha	-32.66667	257.39585	.902	-606.1804	540.8470
	COMBINED	-.66667	257.39585	.998	-574.1804	572.8470
	CONTROL	49.33333	257.39585	.852	-524.1804	622.8470
	influent	-534.66667	257.39585	.064	-1108.1804	38.8470
COMBINED	Typha	-32.00000	257.39585	.904	-605.5137	541.5137
	PHRAG	.66667	257.39585	.998	-572.8470	574.1804
	CONTROL	50.00000	257.39585	.850	-523.5137	623.5137

			influent	-584.66667*	257.39585	.046	-1158.1804	-11.1530
		CONTROL	Typha	-82.00000	257.39585	.757	-655.5137	491.5137
			PHRAG	-49.33333	257.39585	.852	-622.8470	524.1804
			COMBINED	-50.00000	257.39585	.850	-623.5137	523.5137
			Typha	45.33333*	19.24462	.040	2.4536	88.2130
			PHRAG	42.66667	19.24462	.051	-.2130	85.5464
		influent	COMBINED	41.66667	19.24462	.056	-1.2130	84.5464
			CONTROL	24.66667	19.24462	.229	-18.2130	67.5464
			influent	-45.33333*	19.24462	.040	-88.2130	-2.4536
TN			PHRAG	-2.66667	19.24462	.893	-45.5464	40.2130
		Typha	COMBINED	-3.66667	19.24462	.853	-46.5464	39.2130
			CONTROL	-20.66667	19.24462	.308	-63.5464	22.2130
			influent	-42.66667	19.24462	.051	-85.5464	.2130
		PHRAG	Typha	2.66667	19.24462	.893	-40.2130	45.5464
			COMBINED	-1.00000	19.24462	.960	-43.8797	41.8797

			CONTROL	-18.00000	19.24462	.372	-60.8797	24.8797
			influent	-41.66667	19.24462	.056	-84.5464	1.2130
		COMBINED	Typha	3.66667	19.24462	.853	-39.2130	46.5464
			PHRAG	1.00000	19.24462	.960	-41.8797	43.8797
			CONTROL	-17.00000	19.24462	.398	-59.8797	25.8797
			influent	-24.66667	19.24462	.229	-67.5464	18.2130
		CONTROL	Typha	20.66667	19.24462	.308	-22.2130	63.5464
			PHRAG	18.00000	19.24462	.372	-24.8797	60.8797
			COMBINED	17.00000	19.24462	.398	-25.8797	59.8797
			Typha	21.66667*	9.12384	.039	1.3375	41.9958
			PHRAG	21.33333*	9.12384	.041	1.0042	41.6625
		influent	COMBINED	20.66667*	9.12384	.047	.3375	40.9958
NH3			CONTROL	16.66667	9.12384	.098	-3.6625	36.9958
			influent	-21.66667*	9.12384	.039	-41.9958	-1.3375
		Typha	PHRAG	-.33333	9.12384	.972	-20.6625	19.9958
			COMBINED	-1.00000	9.12384	.915	-21.3292	19.3292

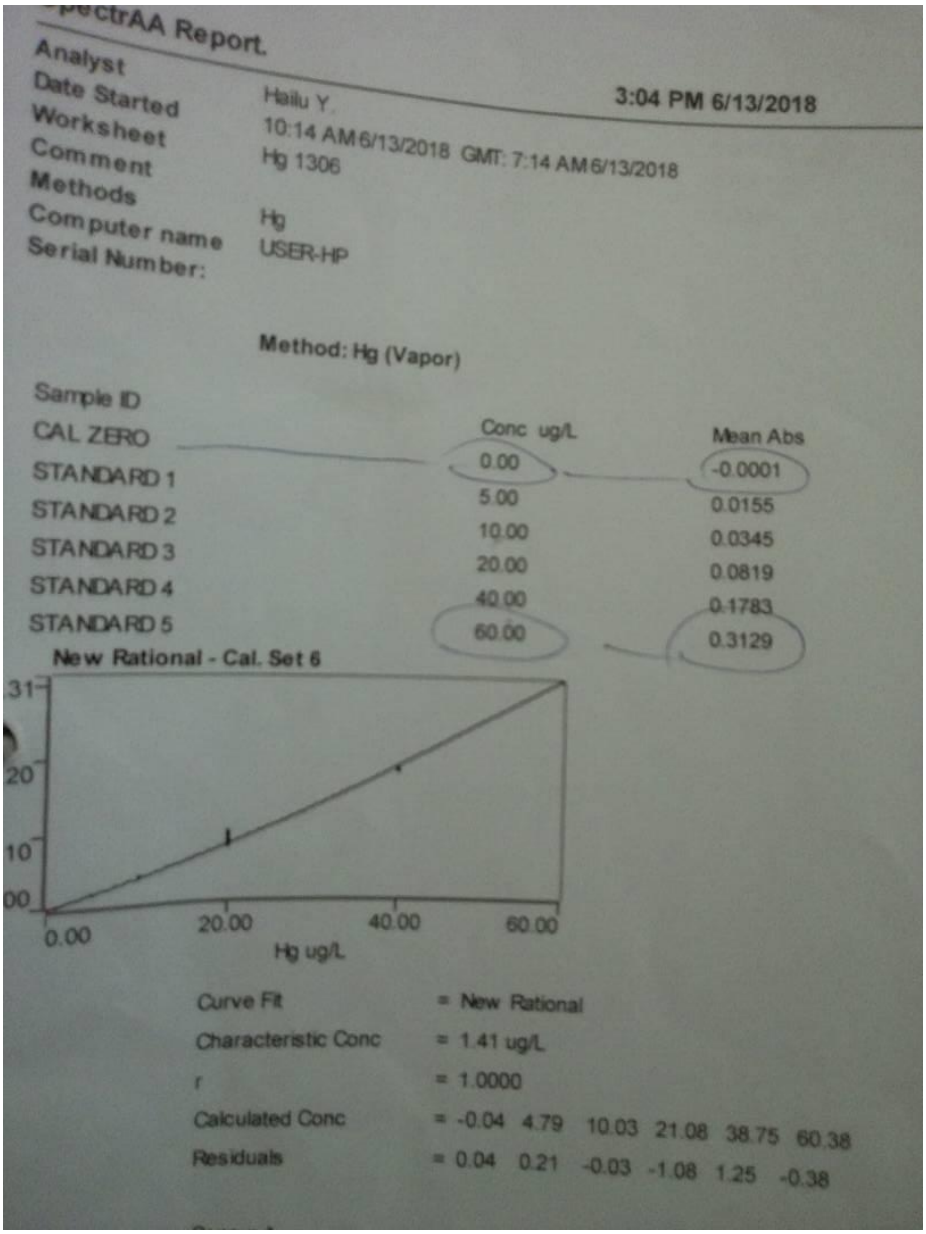
			CONTROL	-5.00000	9.12384	.596	-25.3292	15.3292
			influent	-21.33333*	9.12384	.041	-41.6625	-1.0042
			Typha	.33333	9.12384	.972	-19.9958	20.6625
		PHRAG	COMBINED	-.66667	9.12384	.943	-20.9958	19.6625
			CONTROL	-4.66667	9.12384	.620	-24.9958	15.6625
			influent	-20.66667*	9.12384	.047	-40.9958	-.3375
			Typha	1.00000	9.12384	.915	-19.3292	21.3292
		COMBINED	PHRAG	.66667	9.12384	.943	-19.6625	20.9958
			CONTROL	-4.00000	9.12384	.670	-24.3292	16.3292
			influent	-16.66667	9.12384	.098	-36.9958	3.6625
			Typha	5.00000	9.12384	.596	-15.3292	25.3292
		CONTROL	PHRAG	4.66667	9.12384	.620	-15.6625	24.9958
			COMBINED	4.00000	9.12384	.670	-16.3292	24.3292
			Typha	.27667	.15766	.110	-.0746	.6279
			PHRAG	.28333	.15766	.103	-.0679	.6346
		influent	COMBINED	.21667	.15766	.199	-.1346	.5679
Zinc			CONTROL	.11233	.15766	.492	-.2389	.4636
			influent	-.27667	.15766	.110	-.6279	.0746
			PHRAG	.00667	.15766	.967	-.3446	.3579
		Typha	COMBINED	-.06000	.15766	.711	-.4113	.2913

		CONTROL	-.16433	.15766	.322	-.5156	.1869
		influent	-.28333	.15766	.103	-.6346	.0679
		Typha	-.00667	.15766	.967	-.3579	.3446
	PHRAG	COMBINED	-.06667	.15766	.681	-.4179	.2846
		CONTROL	-.17100	.15766	.304	-.5223	.1803
		influent	-.21667	.15766	.199	-.5679	.1346
		Typha	.06000	.15766	.711	-.2913	.4113
	COMBINED	PHRAG	.06667	.15766	.681	-.2846	.4179
		CONTROL	-.10433	.15766	.523	-.4556	.2469
		influent	-.11233	.15766	.492	-.4636	.2389
		Typha	.16433	.15766	.322	-.1869	.5156
	CONTROL	PHRAG	.17100	.15766	.304	-.1803	.5223
		COMBINED	.10433	.15766	.523	-.2469	.4556
		Typha	.05433	.09364	.575	-.1543	.2630
		PHRAG	.06767	.09364	.486	-.1410	.2763
	influent	COMBINED	-.07233	.09364	.458	-.2810	.1363
Nickel		CONTROL	.03767	.09364	.696	-.1710	.2463
		influent	-.05433	.09364	.575	-.2630	.1543
	Typha	PHRAG	.01333	.09364	.890	-.1953	.2220

		COMBINED	-.12667	.09364	.206	-.3353	.0820
		CONTROL	-.01667	.09364	.862	-.2253	.1920
		influent	-.06767	.09364	.486	-.2763	.1410
		Typha	-.01333	.09364	.890	-.2220	.1953
	PHRAG	COMBINED	-.14000	.09364	.166	-.3486	.0686
		CONTROL	-.03000	.09364	.755	-.2386	.1786
		influent	.07233	.09364	.458	-.1363	.2810
		Typha	.12667	.09364	.206	-.0820	.3353
	COMBINED	PHRAG	.14000	.09364	.166	-.0686	.3486
		CONTROL	.11000	.09364	.267	-.0986	.3186
		influent	-.03767	.09364	.696	-.2463	.1710
		Typha	.01667	.09364	.862	-.1920	.2253
	CONTROL	PHRAG	.03000	.09364	.755	-.1786	.2386
		COMBINED	-.11000	.09364	.267	-.3186	.0986

*. The mean difference is significant at the 0.05 level.

Appendix 2. Calibration curve for mercury



pectrAA Report

Analyst	Hailu Y.	3:04 PM 6/13/2018
Date Started	10:14 AM 6/13/2018 GMT: 7:14 AM 6/13/2018	
Worksheet	Hg 1306	
Comment		
Methods	Hg	
Computer name	USER-HP	
Serial Number:		

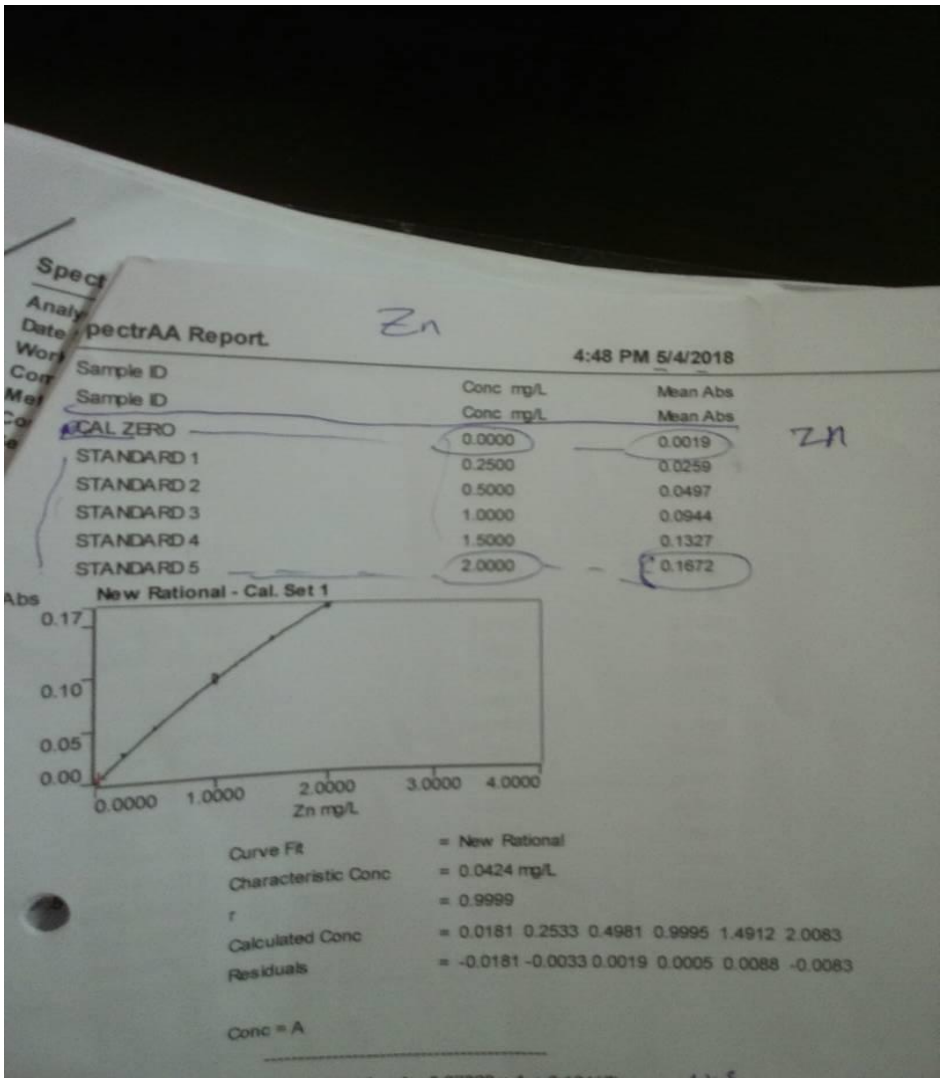
Method: Hg (Vapor)

Sample ID	Conc ug/L	Mean Abs
CAL ZERO	0.00	-0.0001
STANDARD 1	5.00	0.0155
STANDARD 2	10.00	0.0345
STANDARD 3	20.00	0.0819
STANDARD 4	40.00	0.1783
STANDARD 5	60.00	0.3129

New Rational - Cal. Set 6

Curve Fit	= New Rational
Characteristic Conc	= 1.41 ug/L
r	= 1.0000
Calculated Conc	= -0.04 4.79 10.03 21.08 38.75 60.38
Residuals	= 0.04 0.21 -0.03 -1.08 1.25 -0.38

Appendix 3 Calibration curve for zinc



SpectrAA Report

Zn

4:48 PM 5/4/2018

Sample ID	Conc mg/L	Mean Abs
Sample ID	Conc mg/L	Mean Abs
CAL ZERO	0.0000	0.0019
STANDARD 1	0.2500	0.0259
STANDARD 2	0.5000	0.0497
STANDARD 3	1.0000	0.0944
STANDARD 4	1.5000	0.1327
STANDARD 5	2.0000	0.1672

Zn

New Rational - Cal. Set 1

Abs

0.17
0.10
0.05
0.00

0.0000 1.0000 2.0000 3.0000 4.0000

Zn mg/L

Curve Fit = New Rational

Characteristic Conc = 0.0424 mg/L

r = 0.9999

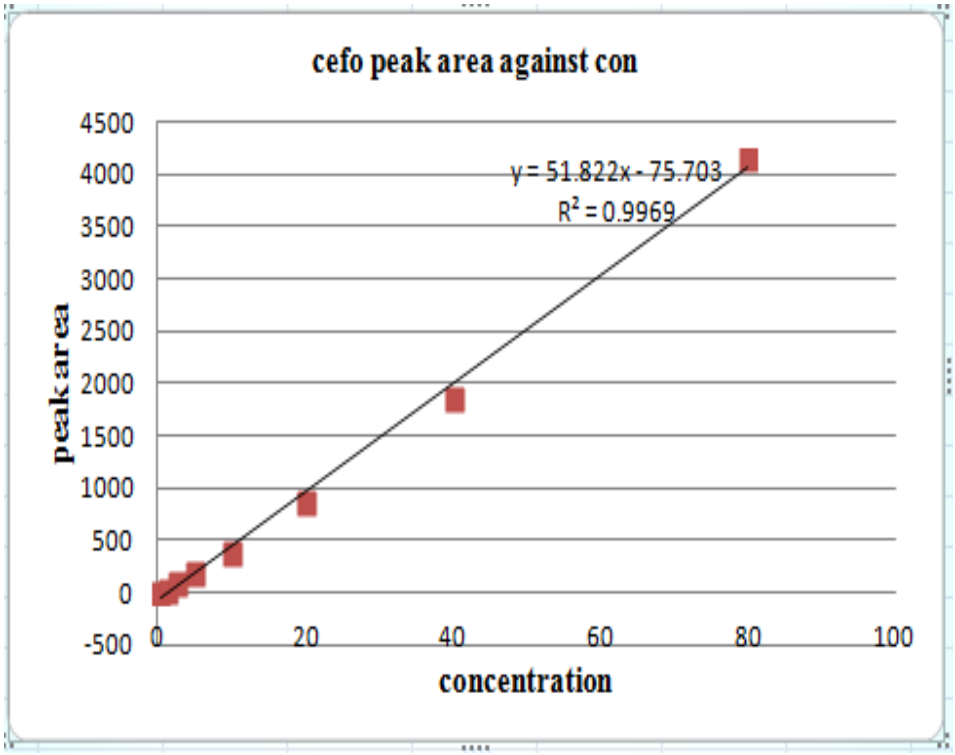
Calculated Conc = 0.0181 0.2533 0.4981 0.9995 1.4912 2.0083

Residuals = -0.0181 -0.0033 0.0019 0.0005 0.0088 -0.0083

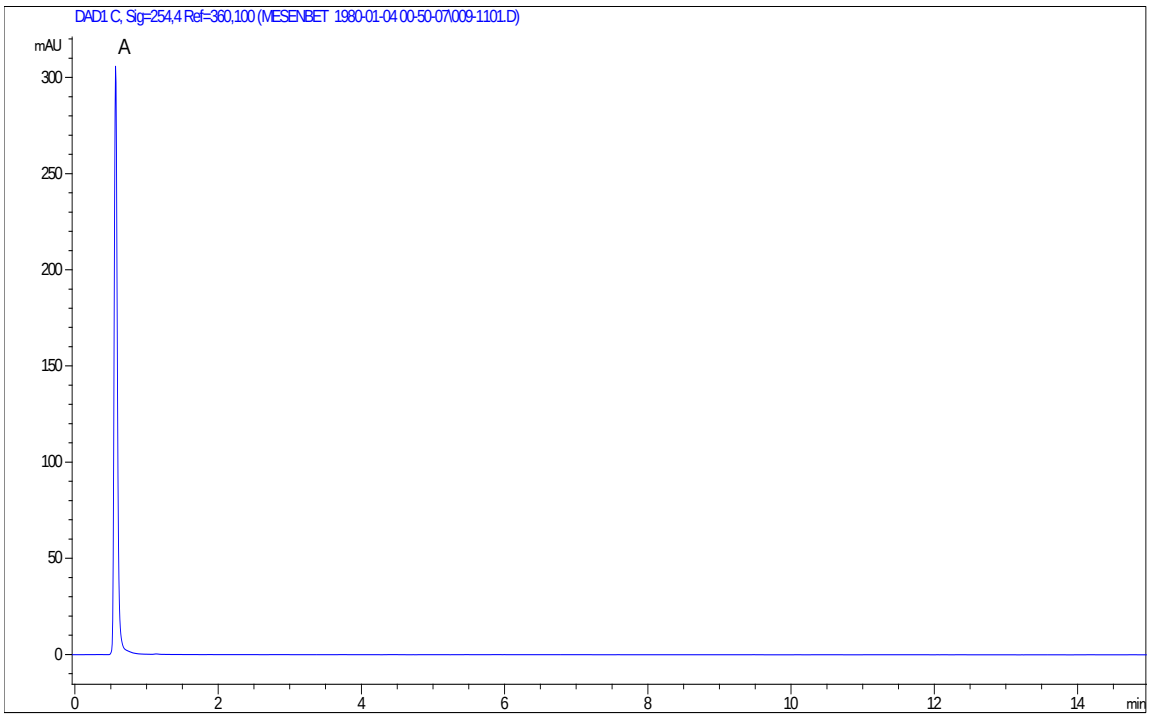
Conc = A

Appendix 4. Concentration of ciprofloxacin and cefotaxime and their calibration curves

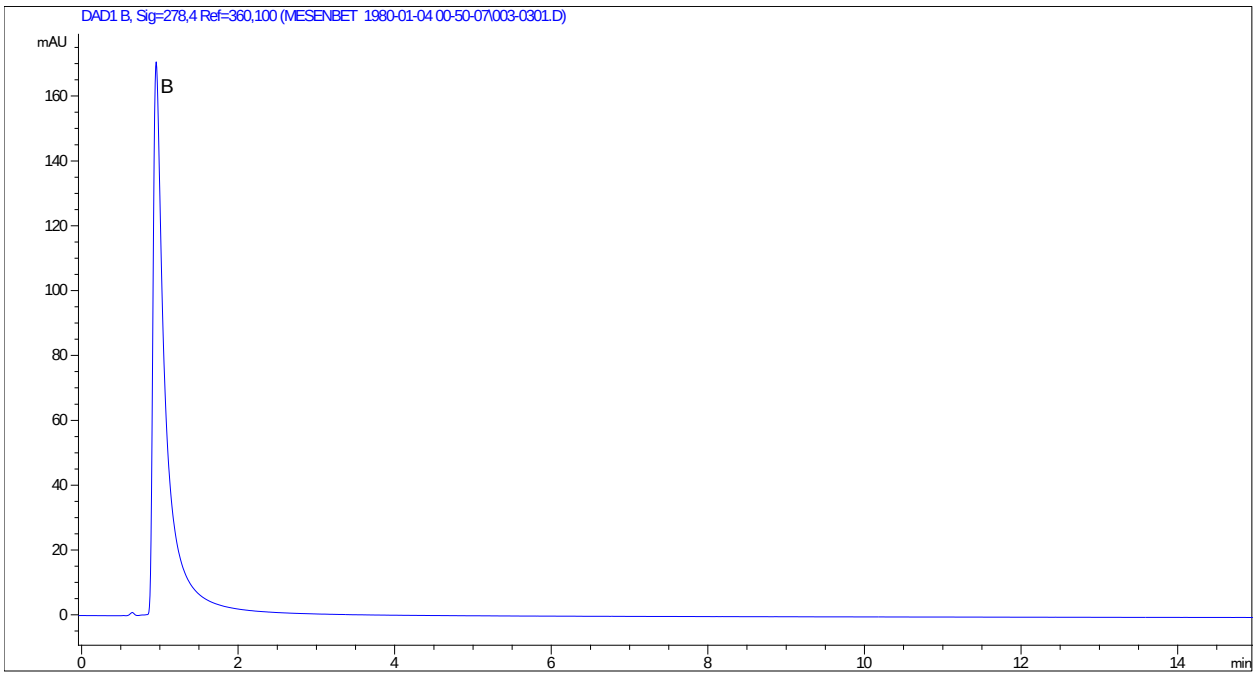
Concentration		Peak area	
Ciprofloxacin	Cefotaxime	Ciprofloxacin	Cefotaxime
0.625	0.3215	10.8	8.6
1.25	1.25	30	41.1
2.5	2.5	69.4	92.8
5	5	156	201.7
10	10	342.7	398.3
20	20	798.2	868.9
40	40	1704.9	1865.4
80	80	3310.4	4161



Appendix 5 Typical chromatogram of Cefotaxime at 40 ppm



Appendix 6 Typical chromatogram of Ciprofloxacin at 40 ppm



Appendix 7 Typical chromatogram of raw waste water at wave length of 278 nm (left) and 254 nm (Right) and Peaks; A (Cefo) and B (Cipro)