DETECTION OF ANTIBIOTICS FROM WASTEWATER TREATMENT PLANTS IN ADDIS ABABA, ETHIOPIA

A thesis submitted to the School of pharmacy, Faculty of Health Sciences, Institute of Health, Jimma University in partial fulfillment of the Requirements for Degree of Masters of Science in Pharmaceutical Quality Assurance and Regulatory affairs

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Detection of antibiotics from wastewater treatment plants in Addis Ababa, Ethiopia

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DECLARATION

I, the undersigned declare that this thesis entitled "**Detection of antibiotics from wastewater treatment plants in Addis Ababa, Ethiopia**" prepared under the guidance of Prof. Sultan Suleman (PhD, professor) and Dr. Sileshi Belew (PhD, assistant professor) is an original work. All sources of materials used for the thesis have been dully acknowledged and the thesis has not been submitted either in part or in full to any other higher institutions for the purpose of earning similar or any other degree award.

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Abstract

Background: Antibiotics are the critical elements widely used to fight different bacterial infections. Amoxicillin, ciprofloxacin and sulfamethoxazole are relatively the most commonly prescribed antibiotics. Once consumed, most antibiotic drugs are excreted in unmetabolized forms via urines and feces and may contaminate the environment if not well treated. Thus, improperly disposed antibiotics are contributing for the development of bacterial resistances and environmental pollution. In urban areas, due to weak removal efficiency of wastewater treatment plants (WWTPs), municipal wastewater is considered as source for release of antibiotics into the environments. Therefore, investigating the concentration of antibiotics in municipal wastewater is critical to take appropriate mitigation strategies.

Objective: The aim of this study was to evaluate occurrence of selected antibiotics in the municipal wastewater (influent and effluent) in Addis Ababa, Ethiopia

Methods: The study was carried out from January to February, 2022 at Addis Ababa. A 1 L wastewater sample was collected from each sampling point (influent and effluent) of two WWTP using polyethylene bottles as grab samples in February, 2022, kept in the ice box and transported to the laboratory. Samples were appropriately stored until analysis. Before analysis, the PH of samples were adjusted using HCL, chelating agent (Na_2EDTA) was added then, and extracted by solid phase extraction using C18 cartridges. Identification and quantification of target antibiotics were performed using HPLC-UV. The laboratory analysis was conducted in Ethiopian food and drug authority drug quality laboratory.

Results: The results of this study revealed the presence of ciprofloxacin and sulfamethoxazole in influent wastewater of two treatment plants. Among investigated antibiotics, only sulfamethoxazole is detected in effluent sample of Site B. Amoxicillin is not detected in all wastewater samples of two WWTPs. The concentration (0.67 ±0.15 µg/L) of ciprofloxacin detected in influent sample of Site A treatment plant was relatively highest. The removal efficiency of WWTPs for the two detected antibiotics ranged from 36% to 100%. The removal efficiency was determined as the percentage of the concentrations difference between influents and effluents samples of each WWTP.

Highest removal percentages (100%) were recorded for both antibiotics in Site A treatment plant. The lowest removal percentage for sulfamethoxazole (36%) was recorded in Site B wastewater treatment plant.

Conclusion and recommendation: from three investigated antibiotics in this study, two were detected in influent municipal wastewater of two treatment plants with the concentration ranging from < LOQ-0.67ug/L for ciprofloxacin and < LOQ-0.06 ug/L for sulfamethoxazole respectively. In one treatment plant, sulfamethoxazole was still detected in final effluent at the concentration < LOQ. The concentration of ciprofloxacin (0.67±0.15 µg/L) measured in the present study is higher than the levels of Predicted No-Effect Concentrations (PNEC) to the environment for this compound (0.45 µg/L). This study recommends the more detailed studies of levels of these antibiotics in different water environments of Addis Ababa.

Key words: *Antibiotics residues, Occurrences, Wastewater, Detection, removal*

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List of abbreviations

API	Active Pharmaceutical Ingredient
CIP	Ciprofloxacin
DWTP	Drinking Water Treatment Plant
EFDA	Ethiopian Food and Drug Authority
GW	Ground Water
GC-MS	Gas Chromatography/Mass Spectroscopy
HLB	Hydrophilic Lipophilic Balanced
HPLC-DAD	High Pressure Liquid Chromatography/ Diode Array Detector
HPLC-UV	High Pressure Liquid Chromatography/ Ultraviolet
HWWTP	Hospital Wastewater Treatment Plant
LC-MS	Liquid Chromatography/Mass Spectroscopy
MWWTP	Municipal Wastewater Treatment Plant
PNEC	Predicted No-Effect Concentrations
RW	River Water
SMX	Sulfamethoxazole
SW	Surface Water
SPE	Solid Phase Extraction
UASB	Upflow anaerobic sludge blanket
WWTP	Wastewater Treatment Plant

Appendices

1. Introduction

1.1. Background

The prevalence of unwanted pharmaceuticals in the environment is increasing dramatically across the globe in recent years as a result of fast development in the pharmaceutical industries. The United Nations (U.N.) has estimated the pharmaceutical industry's annual growth rate as 6.5%. Furthermore, 10% of manufactured pharmaceutical products pose an environmental hazard. The Product Stewardship Council announced in 2019 that one-third of the 4 billion prescription products in the United States of America (USA) had become waste (1).

The use of pharmaceuticals is expected to increase by 43% by 2045 due to the large aging population in Germany. Active Pharmaceutical Ingredients (APIs) are administered globally via over-the-counter, prescription, and veterinary medicines. Animals and humans excrete 30% to 90% of oral pharmaceuticals as active substances via urine and feces. A high concentration of pharmaceuticals has been detected in rivers and industrial effluents in India, China, Korea, the USA, and Israel. Moreover, in the United Kingdom (U.K.), 13% of wastewater treatment plants (WWTP) contained high diclofenac, propranolol, ibuprofen, and ethinylestradiol concentrations (1).

A recent global review reported that out of the 713 pharmaceuticals tested, 631 were found above their detection limits in the environment (2). Several pharmaceuticals were also detected in wastewater in many African countries. The most studied groups of pharmaceuticals in Africa are non-steroidal anti-inflammatory drugs, antibiotics, antiretroviral drugs, and steroid hormones (3) and several major factors, including uncontrolled wastewater treatment, discharge of drug manufacturing processes, prescription rates, animal farm wastewater, the pharmacokinetics of pharmaceuticals and transformation are responsible for the occurrence in the environments (4).

Antibiotic pharmaceuticals are used around the world to treat and prevent bacterial infections in humans, animals, and even plants. According to a survey report from 76 countries, between 2000 and 2015 the total global antibiotic consumption rate grew by 39% to 42.3 billion defined daily doses (DDDs) (5). In low- and middle-income countries, antibiotic consumption increased 77%, from 7.6 to 13.5 DDDs per 1000 inhabitants per day between 2000 and 2015 (5). The use of antibiotics in livestock is projected to increase by 67% by 2030 in developing countries (6).

However, current antibiotic use in the hundreds of thousands of tons per annum and subsequent release of antibiotic residues into the environment produce a step-change in the magnitude of selection pressures that lead to the increase in antibiotic-resistant bacteria (6). Once consumed, most antibiotic drugs are excreted unmetabolized, along with resistant bacteria. They can then pass either through sewage systems or more directly into water and soils, and mix with environmental bacteria in the presence of other pollutants that may add further pressure to help select for antibiotic resistance, directly or indirectly. The extent to which the environment contributes to this problem depends on the level of environmental contamination, and how long antimicrobial residues persist in an active form (6).

Wastewater treatment plants (WWTPs) are considered to be among the most important source of pharmaceuticals contaminants in the water system. Many researches on the occurrence of these chemicals in WWTPs have been done in developed countries. In Ethiopia, limited or no researches have been conducted to understand the occurrence of antibiotics and other pharmaceuticals in WWTPs and aquatic environment. Therefore, this study was conceived in order to understand the occurrence and level of the antibiotics entering the WWTPs in Addis Ababa city.

1.2. Statement of problem

Because of their potential adverse effects on the ecosystem and human health, antibiotics are recognized as the emerging micro contaminants in water. Antibiotics are likely to be released into the aquatic environment via wastewater effluent and agricultural runoff as a result of incomplete metabolism, ineffective treatment removal, or improper disposal because large quantities of antibiotics are used annually in human therapy and in agriculture (7).

In developing countries like Ethiopia, pharmaceutical waste management is not given enough priority. Improper disposal of medication waste contributes to the appearance of their metabolites in the environment which eventually leads to serious personal and environmental health hazards. Environmental pollution is a well-known consequence of improper medication waste management (8) (9). The occurrence of pharmaceutically active compounds in the aquatic environment has been recognized as one of the serious and emerging problems. Most studies have given much emphasis on assessing the impact on groundwater resources. In some

investigations carried out in Europe and in the US, more than 80 pharmaceutical compounds and several drug metabolites have been detected in surface and groundwater samples (8) (9).

The occurrence of antibiotic residues in aquatic environment has raised important global health concerns. The major concerns have been increased bacterial resistance to antibiotics and interference with growth and reproduction not only in human echo systems but also inside aquatic organisms such as fish and frogs (8). Furthermore, exposure to non-target species leads to alteration in microbial diversity in aquatic ecosystem. Primary producers and decomposers appear to be particularly susceptible to the adverse effects of antibiotics which eventually results incorrect ecological functioning including change in nitrogen transformation, methanogenesis, sulfate reduction, nutrient cycling, and organic matter degradation (10).

About 24 antibiotics are frequently reported contaminants in surface water in Africa (4). There is limited information on the status of pharmaceutical residues in the environment in Ethiopia. This study aimed to fill the research gap in this area.

1.3. Significance of the study

Detecting and monitoring the level of antibiotics present in wastewater helps to prevent human and environmental hazards. It helps to prevent environmental pollution and antimicrobial resistance.

This study is useful to provide valuable information for local health policymakers, concerned bodies such as EFDA, pharmaceutical industries, and all other stakeholders involved to effectively plan, manage, and supervise the rational use and safe disposal of antibiotics. It will also provide baseline information and evidence for other concerned bodies such as environmental protection agencies and scientific communities for further follow-up of the problem.

2. Literature review

2.1. Antibiotics sources, occurrence and effects in the environments

Antibiotics can be defined as a class of pharmaceuticals used to fight different bacterial infections. There are several different kinds of antibiotics and they can be classified based on their chemical structure, action mechanism, action spectrum, and the route of administration. Out of these classifications, the most popular one is their mechanism of action, and based on it the most common groups are: b-lactams, sulfonamides, monobactams, carbapenems, aminoglycosides, glycopeptides, lincomycin, macrolides, polypeptides, polyenes, rifamycin, tetracyclines, chloramphenicol, quinolones, and fluoroquinolones (11).

Globally, antibiotics have been extensively and effectively used in human and veterinary medicines. Their benefits have been recognized in agriculture, aquaculture, beekeeping, and livestock as growth promoters. The high antibiotic consumption in humans and in farm animals resulted in increased release of partially metabolized antibiotics into the aquatic environment through wastewater (11). Human excreta and wastewater are recognized and documented as major sources of antimicrobial agents, their metabolites, antimicrobial-resistant bacteria, and their AMR genes because of the widespread and extensive use of antimicrobial agents by human populations. In many countries, facilities to treat municipal, community, and household wastes that may harbour antimicrobial agents, antimicrobial-resistant bacteria, and AMR genes are absent or inadequate (12). The dominant source of antibiotics in municipal sewage is households (about 75% in Europe and the US), followed by hospitals (5% - 20%) (10).

As a result, these contaminants are released directly into the environment where human exposures are likely and where antimicrobial-resistant bacteria and AMR genes are capable of persisting and spreading. Furthermore, human wastewater and excreta are used extensively in agriculture as sources of water and plant nutrients, and such use is encouraged by management practices such as ecological sanitation, municipal wastewater (re)use and water reclamation (12). The major entrance pathways for the occurrence of antibiotics in the environment are shown

below.

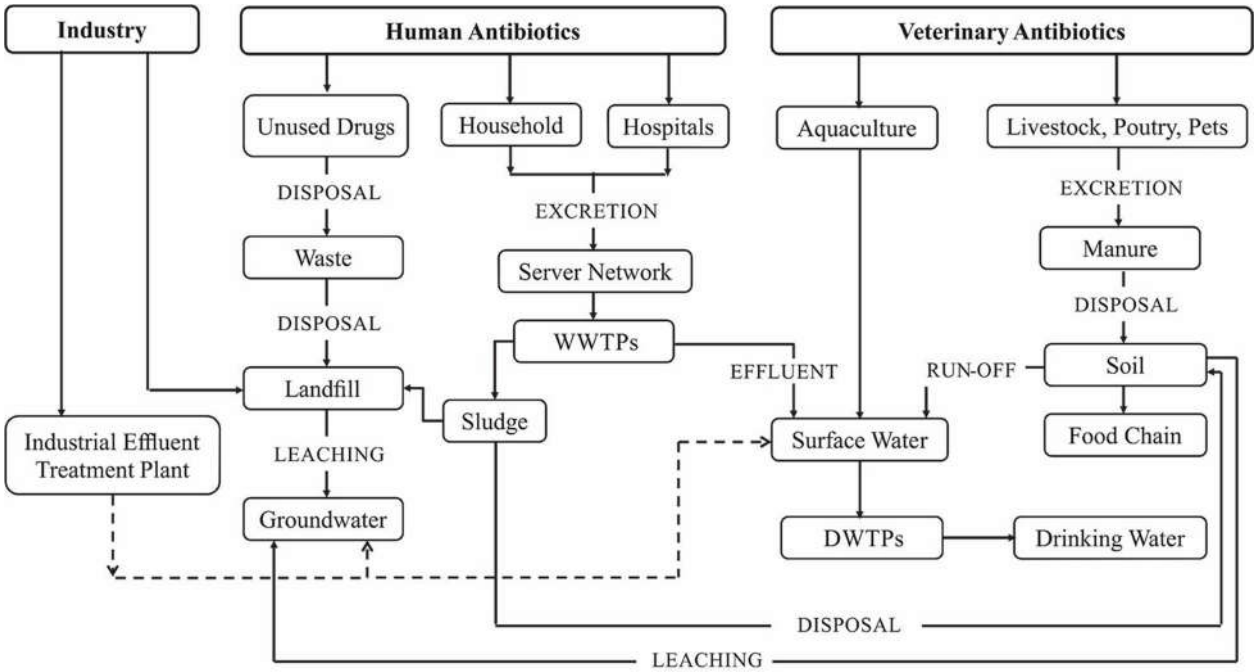


Figure 1: Pathways of entry for antibiotics into Environment (13)

Municipal wastewater contains a different kind of contaminants: pharmaceuticals and personal care products from households; hospital waste with high concentrations of antibiotics and disinfectants; and compounds from industrial activity, including heavy metals (6). Removal efficiencies of conventional sewage treatment are found to vary substantially and they are not designed to deal with emerging pollutants like antibiotics (6) (11). The antibiotic concentration found in waste is too low to be lethal to exposed bacteria, but sufficient to select for resistance. Human exposure to environmental bacteria and to antibiotic resistance genes can take place through drinking water, food consumption or through direct contact with the environment (6).

This practices leads to bacterial resistance to commonly used antibiotic and pollutions. According to Drewes JE et al, several antibiotics are detected in different water bodies globally (14).

Table 1: Occurrence of antibiotics in water bodies (14)

Water sample	Antibiotic	Concentration range
Surface	Trimethoprim, dehydro-erythromycin, roxytromycin, novobiocin, clarithromycin, tylosin, chloramphenicol, ionophores, chlortetracycline, oxytetracycline, tetracycline, sulfadimethoxine, sulfamethoxazole, sulfadiazine, sulfamethazine, sulfathiazole, N4-acetylsulfamethoxazol	7–15,000 ng/L
Groundwater	Chloramphenicol, sulfamethoxazole, sulfadimethoxine, sulfamethazine, oxytetracycline, tetracycline, lincomycin, dehydro-erythromycin	0.05–1.4 mg/L
Drinking water	Sulfamethoxazole	< 25 ng/L
STPs/WWTPs effluents	Sulfamethoxazole, trimetoprim, N4-acetylsulfamethoxazol, sulfadiazine, sulfacetamide, sulfisoxazole, sulfamethazine, sulfapyridine, atorvastatin, roxythromycin, novobiocin, ciprofloxacin, clarithromycin, azythromycin, ofloxacin, norfloxacin, chloramphenicol, dehydro-erythromycin, lincomycin, doxycycline, tetracycline, cephalexin, spiramycin, amoxicillin, tylosin	10–6000 ng/L
Hospital wastewater	Gentamycin, ciprofloxacin, metronidazole, sulfamethoxazole, trimetoprim, doxycycline	0.4–125 mg/L

According to recent reports, only few countries in Africa investigated the presence of antibiotics in environment including Nigeria, Egypt, Ghana, Tunisia, South Africa, Cameroon and Kenya (15) (3). Among the investigated antibiotics, sulfamethoxazole had the highest concentration of up to 2.9 μg L 1 in the river samples, followed by ciprofloxacin and erythromycin with their levels reaching 1.2 μg Lin Ghana (15).

In Ethiopia, several studies reported on the misuse, overuse and mismanagement of pharmaceutical wastes. These factors are repeatedly recognized as predisposing causes for appearance of several important antibiotics in the environments. Recent study reported inappropriate antibiotic use is a huge problem in Ethiopia, and there are many bacteria that are resistant to commonly used antibiotics and similarly, multidrug-resistant bacterial strains are numerous (16). According to this investigation, there is a high burden of multidrug-resistant bacteria which would make empiric antibiotic use challenging. Resistant E. coli and S. aureus to common antibiotics like ceftriaxone, ciprofloxacin, and norfloxacin were high. These antibiotics are used as a mainstay of treatment for various severe bacterial infections in Ethiopia (16).

Another investigation conducted in Ethiopia revealed that Enterobacteriaceae in wastewater from hospitals, abattoir and downstream water bodies are resistant to 11 standard antimicrobials (17). Hospital effluents contained more of MDR bacteria, posing significant public health threat through dissemination to the downstream water bodies. This study assessed antimicrobial susceptibility of bacteria belonging to the family Enterobacteriaceae isolated from wastewater samples (WWS) of two hospitals: Tikur Anbessa Specialized Hospital (TASH) and Minilik II hospital, a wastewater treatment plant (WWTP) and an abattoir, and downstream rivers in Addis Ababa (17).

According to study reported in 2019, there is inadequate knowledge and inappropriate practice toward rational use of antibiotics among community residents in Addis Ababa, Ethiopia (18). This revealed the self-medication with antibiotics may cause significant antibiotic resistance, which is predominant in developing countries (18).

2.2. Antibiotics utilization and bacterial resistance in Ethiopia

Several studies reported irrational consumption of antibiotic in Ethiopia. A Study reported in 2018 revealed a high rate of antibiotics prescribing in the health centers often empirically which

might exacerbate the antimicrobial resistance situation in the country (19). This study reported that Amoxicillin was the most frequently prescribed antibiotics (44.8%) followed by Ciprofloxacin (13.6%) and Cotri-moxazole (11.2%) of all antibiotics in primary health facilities found in Addis Ababa (19). In other study reported in community pharmacies of Addis Ababa revealed nonprescription sales of antibiotics were common for Amoxicillin, Ciprofloxacin and Cotri-moxazole (20).

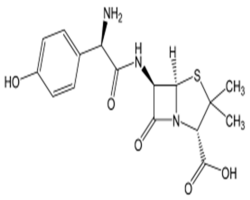
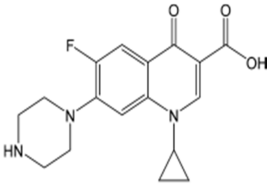
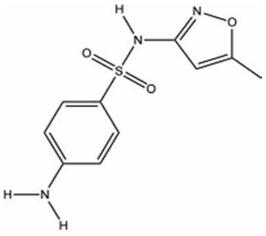
Amoxicillin is a semi-synthetic drug, which belongs to a class of antibiotics called the penicillins (β-lactam antibiotics). Amoxicillin is a broad-spectrum antibiotic widely used for treating both human and animal diseases, and it belongs to a group that are excreted unchanged within urine and faeces; therefore, it is possible to find traces of this drug or its degradation products in environmental water bodies. In water, it is rapidly degraded by biotic and abiotic factors, yielding different intermediate products; these are suspected of being more resistant to degradation, and potentially more toxic, than the parent compound. In the water bodies, these compounds may produce toxic effects on the aquatic organisms from different trophic levels and produce an ecological imbalance (21). It has been chosen as subject of this study because of its heavy predicted environmental load, as it is one of the most commonly used antibiotics in Ethiopia.

Ciprofloxacin is most widely prescribed fluoroquinolone antibiotic in Ethiopia which is active against a broad spectrum of Gram-negative and Gram-positive bacteria. It is frequently detected in the environment and proven to be genotoxic (22). The administered dose of CIP in humans, 45–62% is excreted unmetabolised via urine and 15–25% via faeces (22). Study reported that CIP is persistent and affects the microbial communities and activities in soil (23). In this work, it was demonstrated that ciprofloxacin is more degradable in soil than in aqueous system. The fate and persistence of antibiotics depends on physicochemical properties of the drugs and environmental conditions. Ciprofloxacin is more stable in environmental conditions: less prone to hydrolysis, high temperature and but susceptible to UV degradation (23). Amoxicillin is readily degraded in aqueous medium so that it cannot persist for long in water bodies (24).

Sulfamethoxazole is the sulfonamide antibiotic most commonly used around the world in combination with trimethoprim or pyrimethamine for the treatment of various systemic infections. It is an antibacterial drug which has been used since the 1960s in the treatment of

various systemic infections in humans and other species. SMX is rapidly absorbed on oral administration; metabolism is mainly hepatic, with the formation of predominantly N4-acetylSMX (NAcSMX) and glucuronide conjugates (GluSMX) (25). Excretion is renal with a half-life of 7–12 hours, most of the excreted substance being NAcSMX (30–70% of administered), followed by SMX (10–40%) and GluSMX. The proportion of SMX and NAcSMX excreted is dependent on the urine pH; up to 25% of the dose is excreted unchanged when the urine is acid, rising to 40% or more in alkaline urine (25). Like Ciprofloxacin, SMX is more stable in environmental conditions: less prone to hydrolysis and but susceptible to photo degradation (25).The physicochemical properties of antibiotics are shown as following (**Table2**).

Table 2: physicochemical properties of selected antibiotics (26)

Compound	Molecular structure	M.W.	log KO W	pKa	Water solubility(mg/ml)
Amoxicillin		(g/mol) 365.4	0.87	3.2	0.96
Ciprofloxacin		331.34	0.28	5.9	1.35
Sulfamethoxazole		253.28	0.89	5.4	0.46

Partially metabolized antibiotics in the environment may increase the development of antibiotics resistant microbes and eco-toxic effects (24). These drugs are associated with algal toxicity in reported studies (24) (15) (10). Algae and cyanobacteria, as primary producers, play an important role as the base of the food chain in aquatic ecosystems. Their roles also include oxygen production and nitrogen fixation. Any alteration to the community of photoautotrophic organisms may result in severe bottom-up effects on other organisms at higher trophic levels. Therefore, determination of the toxicity to non-target species is crucial to understand the ecosystem effects of antibiotics (10). Several studies reported the occurrence of amoxicillin, ciprofloxacin and sulfamethoxazole in different wastewater bodies of Africa (27) (28) (26) (29) (30) (31) (32) (33). SPE-LC-MS is the commonly used analytical method for detection and quantification of these antibiotics.

All experiments are dependent on the quality of sampling (26). Sample collection and preparation (preservation, filtration and extraction) are the first steps and an essential part of the analytical procedure, followed by chromatographic separation, detection and data analysis. Proportionately, 80% of the analytical time is used for sampling and sample preparation. Factors such as frequency of sampling, temperature, sampling method and sampling equipment must be critically considered in order to have a good sample for antibiotics analysis. A good knowledge of the physicochemical properties of the analytes (antibiotics) is an important precondition in sample preparation (26).

2.3. Analytical methods for determination of antibiotics in the environments

Among a wide diversity of pharmaceutical compounds, antimicrobials are of particular interest; their environmental occurrence and fate has raised scientific and public concern due to the potential spread and maintenance of bacterial resistance through continuous exposure, which can result in untreatable microbial infectious diseases (34) (14). This issue increased the need for selective and sensitive analytical techniques and methods in order to determine and monitor these trace emerging contaminants in the environments.

Table 3: Occurrence of selected antibiotics and analytical methods used in Africa

Compound	Country	Sample	Analytical method	LoD, LoQ	Reference
Amoxicillin	Egypt	WWTP,SW	SPE-LC-MS/TOF-MS	1.1-3.67ng/ml	(29)
Ciprofloxacin	S. Africa	HWWTP	SPE-LC-MS/MS	0.06-0.18ug/L	(30)
Ciprofloxacin and Sulfamethoxazole	S. Africa	MWWTP	SPE-HPLC-DAD	0.31-2.34ug/L	(32)
	Kenya	RW, MWWTP	SPE–LC–ESI–MS/MS	3 – 12ng/L	(33)
	Tunisia	WWTP	SPE-UPLC-MS/MS	1.1-23.1ng/L	(31)

2.3.1. Sample preparation techniques

The environmental analysis of trace pollutants constitutes a difficult task because of both the complexity of the matrices and the normally very low concentrations of the target compounds. Therefore, in essentially all cases analyte enrichment is necessary to isolate the target compounds from the matrix and to achieve the LODs required (34). A typical analytical procedure includes various sample preparation steps, such as filtration, extraction, purification, concentration and the final determination is performed by chromatography and mass spectroscopy (34).

Sampling techniques plays a very important step in determination of these traces pollutants (35). Judgmental (grab), systematic, or random pattern approaches can be employed for sample collection (35). A judgmental approach focuses the sampling points around a predetermined spot such as a known point source. A systematic approach involves taking samples from locations identified by a consistent grid pattern. The random approach has no defined locations for sample collection (35). Samples are mostly collected using grab sampling approach and transported to the laboratory where they are stored at 4 C until further processing where analyte extraction and pre-concentration is performed. The suspended matter is removed by filtration prior to the extraction process (35) (3).

2.3.2. Sample extraction

In recent studies, solid phase extraction (SPE) has replaced liquid−liquid extraction (LLE) for analyte concentration and purification steps (36) (37). SPE advantages over LLE include shorter analytical times, reduced solvent use, and improvements in method selectivity, specificity, and reproducibility. Because of frequent very low analyte concentrations, the method concentration factor is an important parameter, generally ranging from 100 to 2000. SPE based methods have proven to be excellent for analyte extraction, preconcentration, and cleanup from complex aqueous matrixes, allowing for multiple analyte extraction and good target compound recoveries (36).

Solid phase extraction removes the dissolved pharmaceutical from a mobile phase by passing it through a cartridge where it is bound to a solid stationary phase. This is often done in four steps. First, the cartridge is conditioned with a suitable solvent which wets the stationary phase surface. The sample is then loaded onto the cartridge where target analytes are retained while unwanted matrix chemicals pass through with the solvent (mobile phase). The cartridge is then washed to further remove impurities before the target pharmaceuticals are eluted in a solvent, which is often buffered to an appropriate pH. Following elution, the solvent is adjusted to a known volume and is ready for instrumental analysis (36). Oasis HLB is the most commonly used SPE sorbent for extraction of pharmaceuticals in water samples (37). This could be due to its ability to extract a wide range of compounds from water.

2.3.3. Analytical detection and quantification methods

LC–MS is becoming more extensively used in the identification and quantification of antibiotics because of its high sensitivity and ability to provide compound confirmation as compared to conventional LC-UV detection or LC-fluorimetric detection (LC-FD) (37) (36) (35). Due to poor volatility of most pharmaceuticals, liquid chromatography (LC) is most used rather than gas chromatography (GC) (34). The combination of LC-MS and suitable sample preparation method result in a sensitive analytical method with detection limits in low ng L -1 to μg L -1 levels (34). These low detection limits are usually sufficient for the environmental monitoring of pharmaceuticals.

2.4. Conceptual Framework of the study

The main purpose of this study was to make a detailed analysis on occurrences of antibiotics in two municipal wastewater treatment plants. Based on the aforementioned review of studies and concepts on sources of antibiotics residues, the conceptual frame work for this study has been developed. (See Fig. 2.)

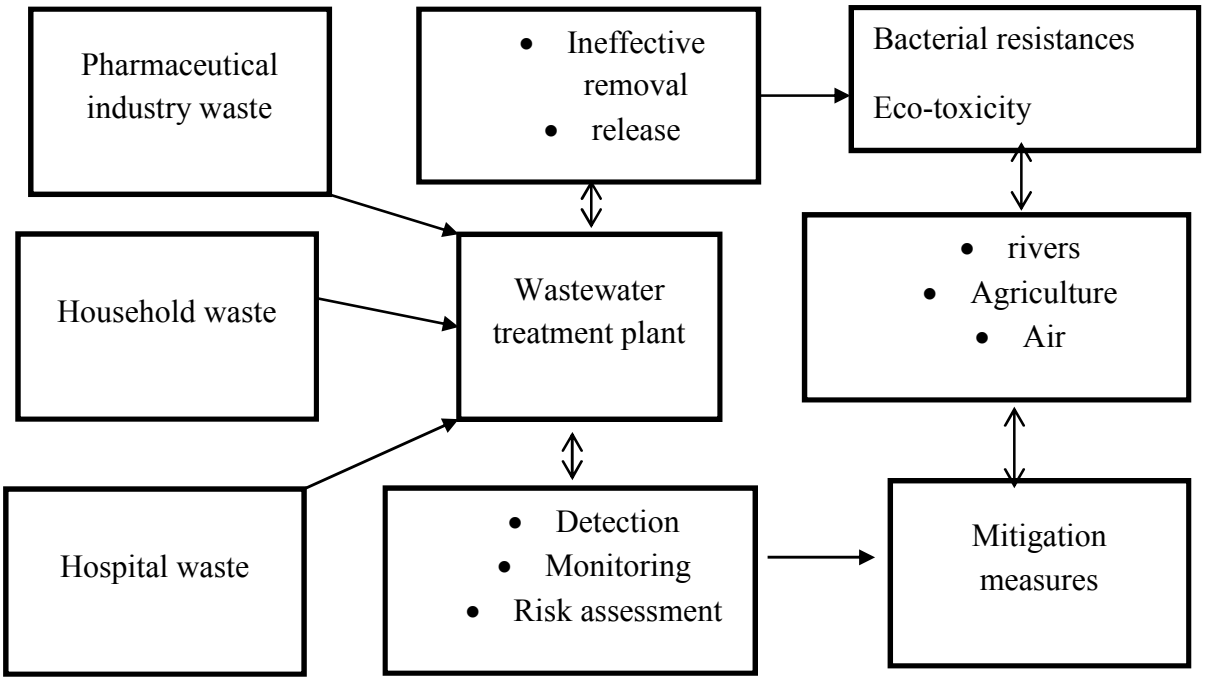


Figure 2: Conceptual framework of the study

Based on studies reported, human antibiotics reach wastewater treatment plants and WWTPs are not specifically designed for antibiotic removal. Consequently, these contaminants are released directly into the environments.

3. Objective of the study

3.1. General objective

- To determine the occurrences of selected antibiotics in municipal wastewater treatment plants founds in Addis Ababa, Ethiopia

3.2. Specific objectives

- To determine the presences of selected antibiotics from municipal wastewater treatment plants
- To estimate the level of selected antibiotics from municipal wastewater treatment plants
- To determine removal efficiencies of selected antibiotics by these municipal wastewater treatment plants found in Addis Ababa, Ethiopia

3.3. Research hypothesis

The hypothesis of this study is:

- There are antibiotic residues/ active metabolites in municipal wastewater treatment plants in Addis Ababa and
- Treatment plants may release these emerging trace contaminants without efficient treatment into the environments.

4. Methods and materials

4.1. Study area and period

The study was carried out at Addis Ababa City Administration. Addis Ababa is the capital city of Ethiopia and diplomatic capital of Africa. It is located in the heart of the country surrounded by mountains and 2355 m above sea level. The city covers about 527 km2, and approximately 4 million populations live in 10 sub-cities and 116 districts (38) (**figure 3**). From secondary data that was collected as part of this study, it was observed that there are 17 MWWTPs in Addis Ababa, and they are owned by Addis Ababa Water and Sewerage Authority (39). Of the 17 MWWTPs, 13 are operational while the remaining plants are still under construction. The MWWTPs that are currently operational form the sampling frame (**Appendix 9**). The treatment plants are of different capacities. There is one large and 12 small MWWTPs. Accordingly, a stratified sampling was adopted. Considering the homogeneity in terms of capacity and operational status, the largest MWWTP and one of the smallest WWTP were selected for this study. The study was conducted starting from, January 2022- February 2022/ 2014 E.C in EFDA Drug quality laboratory.

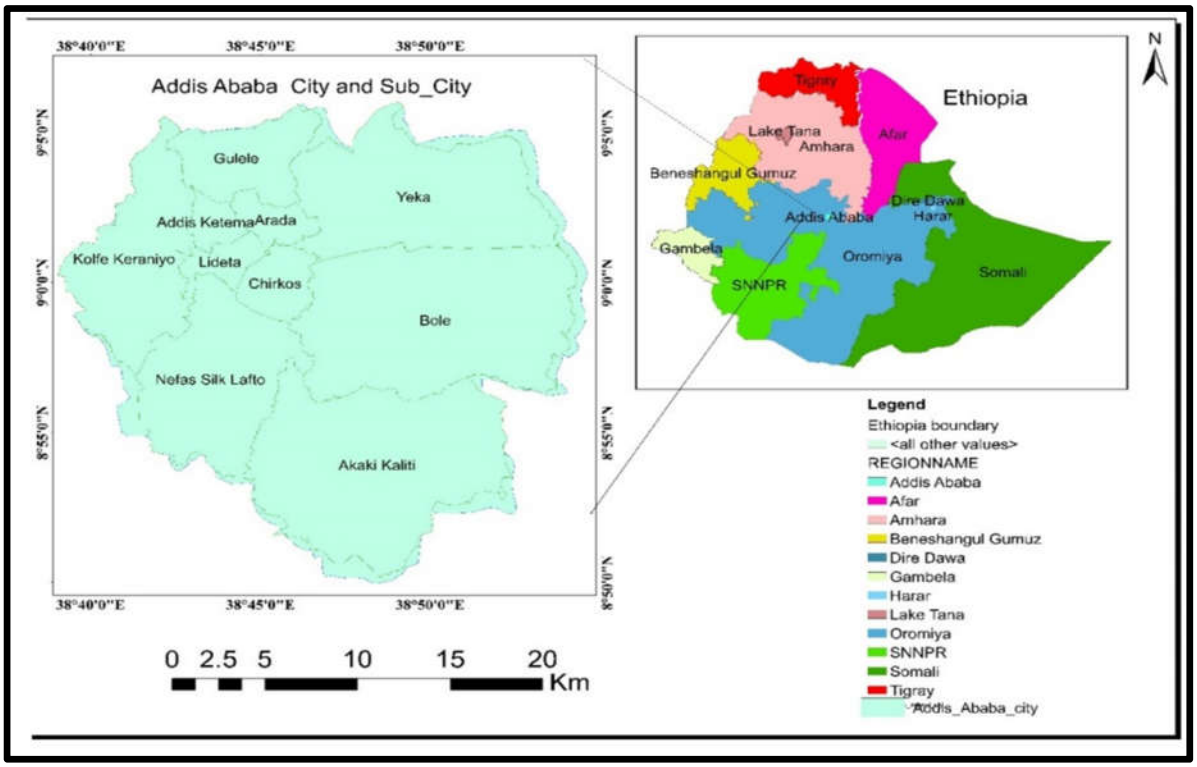


Figure 3: Map of Addis Ababa city (source: Arc-GIS software)

4.2. Study design

Experimental study design was used.

4.3. Selection of antibiotics and sample collection

Study was conducted on sample collected from two municipal wastewater treatment plants found in Addis Ababa, Ethiopia. A detailed characteristic of these treatment plants is shown in table 7.

Selected antibiotics (amoxicillin, ciprofloxacin and sulfamethoxazole) were investigated in this study. The selection of these antibiotics is based their prescription pattern in Addis Ababa (most frequently prescribed antibiotics) (19) (20). In 2018, two antibiotics are included under substances watch list in water (40). The growing awareness of the potential for antibiotic residues to damage aquatic organisms has led to the placement of these antibiotic compounds (amoxicillin and ciprofloxacin) on the European Union (EU)" Watch List" of emerging water pollutants (40).

4.4. Materials

4.4.1. Chemical and reagents

Reference standard of (Amoxicillin, Ciprofloxacin and Sulfamethoxazole >99%), hplc grade water (from EFDA quality control laboratory), Acetonitrile (Assay=99.9%, Alpha chemika) and methanol (99.8%, Alpha chemika), Formic acid (99%, Honeywell), Hydrochloric acid (37%, Sigma-Aldrich), Ascorbic acid (99%, Guanghus Sci-Tech) and Na_2EDTA (98.5-101%, Carlo Erba) were used. Amoxicillin and ciprofloxacin reference working standards were obtained from Epharm Ethiopia plc and Sulfamethoxazole working standard was obtained from Julphar Ethiopia plc.

4.4.2. Apparatus and Instruments

Filter paper (pore size 0.45um), C18 SPE cartridges (500 mg, 6 mL, J & K Scientific), polyethylene bottles, digital PH meter, Analytical balance, Ultrasonicator, ice box and HPLC-UV were used.

4.5. Protocols

All water samples were collected, transported and preserved in accordance to US EPA and EU water directives guidelines (41) (40).

4.5.1. Sample collection and pretreatment

Influent and effluent water samples were collected from two wastewater treatment plants as grab samples in February, 2014 E.C (**figure 4**). A 1L wastewater samples were collected from each sampling point (influent and effluent) of two WWTPs. At each sampling point, two water samples were collected using precleaned 500ml polyethylene bottles and these samples was kept in the ice box during transportation to the laboratory. Before sampling, polyethylene bottles were washed with detergent, rinsed by ultrapure water and dried under vacuum to avoid possible cross contamination. The bottles were appropriately labeled with identification and storage instruction information's. The labeling information contains name of sampling location, sampling point, sample quantity, number, date of sample collection and storage instruction.

In the laboratory, two of 500ml samples collected at each sampling point were transferred to 1000ml glass flask. Then, 50 mg of ascorbic acid was weighed using analytical balance and added to 1000ml of wastewater sample and the samples were kept in refrigerator at 4∘C (40). All water samples were extracted within less than 7day after the date of collection. Ascorbic acid is used as preservative to inhibit microbial growth and as dechlorinating agent if residual chlorine is present in wastewater samples (42).

a.  b.  c.

d. 

Figure 4:Site A (a:influent;b:effluent) and Site B(c:influent;d:effluent)

Before extraction, all samples were filtered by 0.45 µm nylon membrane filters and then, the pH of filtered samples was adjusted to 3 using hydrochloric acid (HCL). Then, 500mg of Na_2EDTA was weighed and added to 1000ml of wastewater sample as a chelating agent to prevent antibiotics from forming complexes with metallic ions (41).

4.5.2. Sample extraction procedure

The samples were extracted using solid phase extraction procedure. Concentration of antibiotics was carried out using C18 SPE cartridges (500 mg, 6 cm3) (43). The following the steps were used for extraction. First, cartridges were conditioned with 6 mL of MeOH, followed by 6 mL of HPLC water which was acidified at a pH of 3 using hydrochloric acid. Secondly, wastewater samples (200 mL) were passed individually through the cartridges at a flow rate of 5–10 mL/min.

Then, the cartridges were washed with 5 mL of HPLC water and dried under vacuum for 30 sec. Then, pre-concentrated analytes were eluted with 6 mL MeOH at a flow rate of 1 mL/min by gravity in a 10 mL vial. Finally, the extract was evaporated near to dryness in water bath at 30 °C

and was reconstituted with 2ml of mobile phase (MeOH/HPLC grade water with 0.1% formic acid (50:50, v/v). The extracts were then stored in refrigerator at 4∘C and analyzed within 1 week. All samples were filtered using 0.45-µm nylon microfilters prior to injection to LC instrument.

4.5.3. Preparation of analytical standards

Individual stock standard solutions were prepared in a concentration of 1 mg/mL for each antibiotic separately. Amoxicillin was dissolved in hplc grade water. Ciprofloxacin and Sulfamethoxazole were dissolved in hplc grade methanol. Mixed stock solution (100ug/ml) was prepared by dissolving with ultra-pure water and methanol (50:50, v/v). Then the standard solutions were stored in -20 °C freezer.

Working standard solutions in concentration (0.05, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 25 µg/mL) were prepared by dissolving with mixture of hplc water and MeoH (90:10 v/v) from mixed stock solution by serial dilution.

Solvents (mobile phase) used for HPLC analysis were prepared as follows. Solvent A: (0.1% formic acid in HPLC water) was prepared by adding 1ml of formic acid into ultrapure water in 1000ml volumetric flask and mixing thoroughly. Solvent B**;** HPLC-grade acetonitrile/methanol **(**1:1): was prepared by mixing 500 mL of methanol and 500 mL of acetonitrile in 1000ml volumetric flask. Then, all the solution was filtered and sonicated for 30 min.

4.5.4. HPLC Analysis

Analytical method for target antibiotics was adapted from previous study with modifications (43). The identification and the quantification of the analytes were conducted using a HPLC (Shimadzu, Japan) equipped with ultraviolet visible (UV) detector at 250-nm wavelength (43). A waters C18 column ((3.9-mm i.d. × 150mm, 5-µm particle) was used for separation of the target analytes. Column temperature was set at 30^{o}c and injection volume was 20µL. Gradient elution was carried out using a mobile phase consisting of HPLC-grade water with 0.1 % formic acid (A) and (50: 50 v/v) HPLC-grade acetonitrile/methanol (B) at a flow rate of 0.8 mL/min.

The gradient program was set as follows: (0min), A = 95 %; 1min, A=95%; 5 min, A = 12 %; 12 min, A = 0 %; and 20 min, A = 95 %. Twenty µL of the reconstituted solution was injected into HPLC for analysis. Analyte identification was based on comparison of chromatograms of

unknowns with those of standards. Standards and blanks were measured periodically throughout the analysis for quality assurance. Quantitative analysis of antibiotics was achieved through the integration of selected HPLC chromatograms. It is based on linear regression calibration curves using external calibration method. All analyses were carried out in triplicate.

4.6. Method validation

Validation of analytical method in terms of system suitability, linearity, sensitivity, accuracy, repeatability and specificity was evaluated. Validation studies were carried out in order to demonstrate the performance of the method. The calibration curves and linearity of the detector response were evaluated by analyzing standard solutions of target antibiotics within the range of 0.05–25 μg/mL. The sensitivity (limit of detection and limit of quantification) were determined from the standard deviation of the response and slope of the calibration curve of target compounds. The accuracy of the analytical method was evaluated through recovery studies for two kinds of water samples (QC spiked samples) and precision was determined based on repeatability in recoveries.

4.7. Data analysis

The data obtained from the experimental part of the investigation were analyzed using Microsoft Excel 2010 for statistical and graphical evaluation. Descriptive statistics' such as mean values and measurement uncertainty (standard deviations, %relative standard deviations) were evaluated.

4.8. Ethical considerations

This study was reviewed and approved by the Ethical Review Committee of Jimma University, Institute of health. The study was conducted after receiving permission from Addis Ababa office of municipal water and sewage authority. Confidentiality of the treatment plant was assured. Every sites and samples were coded.

5. Results

5.1. Validation results

I. System suitability

In order to perform system suitability study, six replicate injections of 10µg/ml mixed standard solution were injected and analyzed with HPLC. Standard solution (10µg/mL) was prepared by transferring and dissolving 1ml of mixed stock solution (100ug/ml) in 10ml volumetric flask and filled to volume with mixture of hplc water and methanol (90:10 v/v).

Then, System suitability test parameters such as tailing factor, theoretical plate and mean value of peak area with % RSD were determined and compared to the reference USP guidelines as indicated below (**Table 4**) (44).

Table 4: System suitability test results

Drug	Retention time	Tailing factor	Theoretical plate	mean area	RSD%, n=6
Amoxicillin	4.142	1.16	3563	35694.8	0.73
Ciprofloxacin	5.060	1.39	32601	196822.8	0.72
Sulfmethoxazole	5.955	1.08	34320	219171.3	0.17
USP limit	-	≤2-2.5	≥2000	-	≤1.5-2

According to the result shown above, the system is found suitable in respect of these system suitability parameters as per USP requirements. Acceptance range for amoxicillin, ciprofloxacin and sulfamethoxazole for ; tailing factor (TF) ≤2.5,2,2, %RSD ≤ 2,1.5,2,respectively and TPN≥2000 for all antibiotics. %RSD value for all antibiotics working standard (WS) was less than 2% for prepared concentration measurement which satisfy requirement of USP for repeatability.

II. Linearity and sensitivity

Calibration curves of the three antibiotics were constructed using six points' standard solution over concentrations ranging 0.05 up to 25ug/ml to determine linearity of analytical method depending pharmaceuticals response. Calibration curve for each antibiotic was plotted using six

concentration levels against peak response and the obtained data was subjected to regression analysis. Linearity was evaluated through the linearity coefficients (R2) of the obtained calibration curves. For amoxicillin standards the calibration curve was linear over the range of 1–25µg /mL solutions. For ciprofloxacin and sulfamethoxazole standards the calibration curve was linear over the range of 0.05–5µg /mL solutions.

The limits of detection (LOD) and limits of quantification (LOQ) of the method were calculated from the standard deviation of the response and slope of the calibration curve of pharmaceutical compounds using the formula as per ICH guideline, 3.3σ/s and 10σ/s, respectively, where σ is the standard deviation of the response and s the slope of the calibration curve (45). The standard deviation of the response was calculated using "LINEST" function on Microsoft excel 2010.

The overall performance of the method is presented below (**Table4**).

Table 5: Performance characteristics of analytical method

Drug	**Regression Equation**	**R^2**	**LOD(µg/L)**	**LOQ(µg/L)**
Amoxicillin	y=3470.2x+124.31	0.999	0.7	2.25
Ciprofloxacin	y=18849x-332.77	0.9987	0.14	0.42
Sulfamathoxazole	y=21235x-1853.44	0.998	0.17	0.5

The analytical method showed good linearity over the concentration range for target antibiotics. The correlation coefficients (R2) for the calibration curves of the targeted compound were within the range of 0.998–0.999. The limit of detection ranged from 0.1 to 0.7µg/L while the limit of quantification ranged from 0.4 to 2.25µg/L indicating the sensitivity of the method.

III. Accuracy and precision

Accuracy and precision of the analytical method were further assessed by spiking 500uL of 100ug/ml of mixed antibiotics standard solution into 200 mL influent and effluent wastewater samples, extracted and finally analyzed by HPLC. These samples were collected using two 200ml polyethylene bottles separately from test samples. Then, appropriately stored and pretreated using the same procedure.

Accuracy was evaluated as percent average recovery of spiked antibiotic from wastewater samples and precision was calculated as relative standard deviation (RSD) for each wastewater samples analyzed in triplicate. The relative recovery of each antibiotic was calculated by comparing the peak areas for extracted target antibiotics from spiked water and a standard solution of antibiotic in distilled water. The precision was determined based on repeatability in recoveries (n=3, %RSD) and evaluated by carrying out the extraction and analysis of the fortified samples at spiking concentration of (0.25µg/mL). The result of extraction recovery and precision study is presented below (**Table 5**).

Table 6: relative recoveries (% RR) of selected antibiotic compounds using the extraction method with wastewater samples

Drug	Sample	Spiked Conc.(µg/ml) (n=3)	Found Conc.(µg/mL)	RSD (n=3)	Recovery (%)
Amoxicillin	Influent	0.25	0.03	0.6	12
	Effluent	0.25	0.09	0.8	36
Ciprofloxacin	Influent	0.25	0.15	0.8	60
	Effluent	0.25	0.18	1.07	72
Sulfamethoxazole	Influent	0.25	0.32	0.46	128
	Effluent	0.25	0.1825	0.3	73

The result of validation study showed good precision for all antibiotics (RSD) < 2% at spiked concentration. Recovery results of these antibiotics ranged from (12-128%). Even though the recovery result for amoxicillin in this study (12-36%) is low, the result consistent to previous published a studies result which ranged from (nr- 20% recovery) (46) (47).

The specificity of the HPLC method was established by injecting the blank (solvent) without active pharmaceutical ingredient, into the HPLC system using the optimized conditions. No

peaks were detected at the retention times corresponding to any of the target analytes considered in this study.

5.2. Description of studied sites

Addis Ababa is the capital and largest city in Ethiopia. The capital generates an estimated annual volume of 49Mm3 total wastewater from which about 4Mm3 is industrial wastewater (48). Addis Ababa Water and Sewerage Authority (AAWSA) is the only institution in Addis responsible for all aspects of water supply of the city residences and sewerage control and wastewater treatment.

In this study, two municipal wastewater treatment plants located in different parts of Addis Ababa city were investigated. Treatment plants were coded as Site A and Site B for this investigation to keep the confidentiality of the results. WWTP 1(Site A) is the largest treatment plant that serves a population of more than 1 million population and which represent the major part of the municipal wastewater derived from the capital. These include Bole, "Ledeta", Old Airport, "Arada", "Kirkos", Mekanisa and "Kera" areas. WWTP 2 (Site B) receives only a part of wastewater derived from the capital and newly constructed condominium site residing around the plant. Site A municipal wastewater treatment plants was built in the late 1970's and commissioned in 1983 (48).

The general characteristics of these treatment plants are summarized in the table (Table 7). Each treatment plant uses different technology to treat wastewater received from residential, institutions and treated industrial wastes.

Table 7: Characteristics of municipal wastewater treatment plants investigated

Code	Location	Source of waste	Capacity (m3/d)	Treatment methods	Downstream river
Site A	Akaki kalati sub city, Addis Ababa	Hospitals, Institutions, residential	10,0000	Physical, biological and chemical	Akaki river
Site B	Between yeka and Bole sub city, Addis Ababa	institutions, residential, Industries	9,260	Physical and biological	Bulbula river

Site A treatment plant has a capacity of 100,000 m3/d and uses up-flow anaerobic sludge blanket digestion (UASB) based technology. It is UASB + Trickling Filter-based plant with sludge dewatering with drying beds.

Site B treatment plant was established in early 1990 E.C and uses older technology to treat wastewater. In Site B, only two steps of treatment processes are used which involve physical screening and sedimentation of wastewater in larger surface ponds (reservoirs) until it forms algae, and then treated sewage effluent are released from plants. In site A, the Conventional treatment method mainly involves three treatment processes is used. Primary treatment includes pre-treatment of raw wastewater intake by coarse and fine screens for grit removal. This process uses sedimentation tanks to allow the heavier organic particles to settle. Secondary treatment of raw water using activated sludge. This process uses aerated biological digestion by bacteria to remove the remaining suspended and dissolved material.

Then, the wastewater enters the secondary sedimentation tank to allow the separation of the liquid and solid phases. After secondary sedimentation, the wastewater enters maturation ponds for further pathogen removal. Tertiary treatment is the final step used by sewage treatment plant A. Addition of Chlorine and Dechlorination by sodium metabisulfite (NaHSO3) (used only at

sewage treatment plant A) are the disinfection processes used before the treated sewage effluent are released from plants.

5.3. Occurrence of selected antibiotics in wastewater treatment plants

Influent and effluent water samples were collected from both Site A and Site B in February month to investigate the presence and the level of amoxicillin, ciprofloxacin and sulfamethoxazole. From these antibiotics, amoxicillin was not detected in influent and effluent water samples of both sites. Ciprofloxacin and sulfamethoxazole were detected in influent samples of both sites and sulfamethoxazole was detected in effluent water of Site B. The concentration of ciprofloxacin measured in influent wastewater of Site A (o.67±o.15ug/L) is the highest detected concentration in all wastewater samples. The concentration of detected antibiotics is shown in table below.

Table 8: Concentrations of the selected antibiotics found in real samples

Sampling location		Water sample (Con.in µg/L, n = 3, %RSD)	
Sampling site	Sampling point	Ciprofloxacin	Sulfamethoxazole
Site A	Influent	0.67(0.15)	<LOQ
	Effluent	ND	ND
Site B	Influent	<LOQ	<LOQ
	Effluent	ND	<LOQ

Reporting level: nd: not detected; <loq and for concentration >loq= in (ug/L)

5.4. Removal of selected antibiotics by treatment plants

In this study, aqueous samples removal efficiency of each target antibiotics from treatment plants was evaluated using the following equation (30).

$$Removal\ efficiency(\%) = (CInfluent - CEffluent)/CInfluent\ x\ 100$$

Where C $_{influent}$ and C $_{effluent}$ represent the mean concentrations in influent and effluent, respectively and the result shown as indicated below. By using the above formula, the removal

of two detected antibiotics; Ciprofloxacin and sulfamethoxazole were calculated from each wastewater treatment plants as indicated in table below.

Table 9: Removal of antibiotics in aqueous phases from treatment plants

code	Drug	Influent concentration	Effluent concentration	Removal (%)
site A	Ciprofloxacin	0.67µg/L	ND	100
	Sulfamethoxazole	0.06ug/L	ND	100
site B	Ciprofloxacin	0.0112ug/L	ND	100
	Sulfamethoxazole	0.0352ug/L	0.0224ug/L	36

As indicated in table above, the highest removal percentages (100%) were recorded for both antibiotics in Site A treatment plant. The lowest removal percentage for sulfamethoxazole (36%) was recorded in Site B wastewater treatment plant.

6. Discussion

In this study, samples (influent and effluent) collected from two municipal wastewater plants located in Addis Ababa were investigated. The occurrence and level of three antibiotics compounds: amoxicillin, ciprofloxacin and sulfamethoxazole measured in wastewater samples from two MWWTPs are summarized above (**Table 7**). Among investigated antibiotics, Amoxicillin is not detected in both municipal wastewater samples despite it is prescription pattern in Addis Ababa. This is maybe due to its physicochemical instability towards biotic and abiotic factors in the environments. The β-lactam ring of amoxicillin has poor stability and can be unlocked by chemical hydrolysis or by βlactamase enzyme which results in rapid degradation in water (21).

Ciprofloxacin and sulfamethoxazole are detected in both treatment plants. The concentration of ciprofloxacin measured in influent wastewater of Site A treatment plant (o.67ug/L) is the highest detected concentration in all wastewater samples. Ciprofloxacin is detected in a higher concentration (o.67ug/L) than sulfamethoxazole which is 0.06ug/L in influent sample of site A. Both antibiotics are not detected in effluent wastewater of Site A treatment plant. According to a Study reported from Ghana, a higher concentration of ciprofloxacin (2.371ug/L) and sulfamethoxazole (7.194ug/L) than this study was found in influent sample in WWTP (15).

Both antibiotics are detected in influent wastewater of Site B treatment plant. But the concentrations of sulfamethoxazole (0.0352ug/L) found in influent samples of site B was higher than ciprofloxacin (0.0112ug/L). In this study, only sulfamethoxazole (0.0224ug/L) is detected in effluent sample of site B treatment plant. Results of this study (**Table 8**) showed that concentrations of sulfamethoxazole, and ciprofloxacin in influents and effluents samples varied among locations. This variation can be attributed to antibiotics prescription patterns in different locations of Addis Ababa city, which have an effect on influent concentrations.

In a study reported from Kenya, the concentration of sulfamethoxazole in effluent water is higher than this study which is 3.3ug/L (33). Another study reported from Kenya also showed the presence of quantifiable levels of both antibiotics in wastewater samples (43). A study reported from Egypt showed the presence of both antibiotics with the concentration of: 0.98ug/L for sulfamethoxazole; 0.48ug/L for ciprofloxacin in influent sample and in effluent sample: for SMZ

(1.72 μg/L), followed by CIP (0.31 μg/L) respectively (49). The removal efficiency for the investigated antibiotics from the WWTP ranges was -30.6% to 80.9% (49).

The release of quantifiable level of antibiotic residues through effluent of municipal wastewater treatment plant indicates incomplete removal of this contaminant which eventually poses negative human and environmental hazards. The high concentration of ciprofloxacin (0.67ug/L) and sulfamethoxazole (0.06 ug/L) found in municipal samples in my investigation were compared to Minimum Inhibitory Concentration (PNEC-MIC) values and Predicted No-Effect Concentrations (PNEC-ENV) limit of standard guideline to assess risk with respect to antimicrobial resistance and eco-toxicity (50). The PNEC values are 0.06 & 0.45 ug/L for ciprofloxacin and 16 & 0.6 ug/L for sulfamethoxazole respectively (50). The concentration of CP measured in this study poses higher risk than sulfamethoxazole for antimicrobial resistance and eco-toxicity when we compared with the discharge limit.

The removal efficiency of selected antibiotics in this study is summarized above (**Table 8**). The removal efficiency of the target antibiotics in the treatment process by the plant was measured by the ratio of the drop in concentration between the influent and the effluent prior to discharge to the initial concentration in the influent.

The efficiency of the treatment process on the antibiotic was in the range of 36-100% with ciprofloxacin having the highest removal percentage and sulfamethoxazole having the least. This variation can possibly be associated with specific treatment processes occurring in individual WWTP and chemical properties of the antibiotics. Site B uses the conventional treatment of municipal wastewater by (wastewater stabilization ponds) WSP technology and Site A uses up-flow anaerobic sludge blanket digestion (UASB) based technology to treat waste originated from domestic and different institutions.

The occurrences and removal of antibiotics in wastewater depends on physicochemical properties of individual antibiotics (51). Amoxicillin is less likely to occur and persist in aquatic environment due to low stability toward extremely high temperature and biodegradation. Inversely, ciprofloxacin and sulfamethoxazole are more likely to occur in aquatic environment because of their strong chemical stability toward extreme environmental conditions. Ciprofloxacin have higher sorption capacity to organic matter and solid matrixes so that

significant amount can be removed with sludge samples in wastewater. Sulfonamides including sulfamethoxazole are the most detected antibiotic in all type of water samples owing to their high water solubility, nearly weak chelating ability, and low sorption to the soil (51).

From different literature, we can understand that the occurrence of APIs in aquatic environments can adversely affect living organisms on different organizational levels and lead to alteration in the ecological function of rivers and lakes. Further contamination of drinking water and food sources with these compounds may lead to unintended human exposures and potential effects on health. Therefore continuous monitoring of these emerging contaminants is very crucial.

7. Conclusion and recommendations

The occurrence of antibiotics in environment is serious problem because of their harmful effects on human health and the ecosystem. The major pathway in which antibiotics residues enter aquatic environment is through municipal sewage system. This study was conducted to determine the occurrence of three commonly used antibiotics from influent and effluent samples of two municipal sewage treatment plants found in Addis Ababa city.

The finding of this study revealed the presence of two antibiotics (Ciprofloxacin and Sulfamethoxazole) in influent wastewater of two treatment plants. Among investigated antibiotics, only sulfamethoxazole was detected in effluent sample. Amoxicillin was not detected in all wastewater samples. The concentration of detected antibiotics ranges from <LOQ-0.67µg/L. The concentration of ciprofloxacin (0.67±0.15µg/L) measured in the present study is higher than the levels of Predicted No-Effect Concentrations (PNEC) to the environment for this compound (0.45 µg/L) (50). This could pose the harmful environmental impact. This study also identified that SMX was still detected in final effluent indicating the releases of antibiotics into environment as a result of inadequate treatment. The high concentrations found in municipal wastewaters and the low removal rates achieved during conventional wastewater treatment indicate that urban wastewater is an important source of antibiotic pollution in the aquatic environment.

Therefore, further comprehensive studies on environmental fate of these antibiotics, ecological and human health effects and risk control measures are recommended. From this study, it is anticipated that many other pharmaceuticals might occur at concentration levels exceeding the PNEC; thus, further studies are also required for other emerging pharmaceutical contaminants such as: NSAIDs, steroids, and antipsychotic drugs which are frequently reported in other studies.

7.1. Limitation of the study

Due to resource constraints, the data for present study was collected in a limited period of time and the finding may not represent the occurrence and level of investigated antibiotics in different seasons of the year. Grab sampling may not be representative because samples are collected at a specific spot at a site over a short period of time. Therefore, further analysis on this municipal water by more refined sampling technique both in locations and seasonally varied period of sample collection is recommended for future research.

Furthermore, the target antibiotic not detected in present study is may be due to the detection limit of analytical method used for the investigation. Thus, a more sensitivity analytical method like LC-MS should also be considered for future research works.

8. Reference

1. Organisation for Economic Co-operation and Development (OECD). Pharmaceutical Residues in Freshwater: Hazards and Policy Responses. IWA (International WATER ASSN); 2019.

2. aus der Beek T, Weber FA, Bergmann A, Hickmann S, Ebert I, Hein A, Küster A. Pharmaceuticals in the environment—Global occurrences and perspectives. Environmental toxicology and chemistry. 2016 Apr;35(4):823-35.

3. Madikizela LM, Ncube S, Chimuka L. Analysis, occurrence and removal of pharmaceuticals in African water resources: A current status. Journal of Environmental Management. 2020 Jan 1;253:109741.

4. Fekadu S, Alemayehu E, Dewil R, Van der Bruggen B. Pharmaceuticals in freshwater aquatic environments: A comparison of the African and European challenge. Science of the total Environment. 2019 Mar 1;654:324-37.

5. Klein, E.Y., Van Boeckel, T.P., Martinez, E.M., Pant, S., Gandra, S., Levin, S.A., Goossens, H., Laxminarayan, R., 2018. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proceed. Nat. Acad.Sci. U. S. A. 115, E3463-eE3470.

6. Gaze W, Depledge M. Antimicrobial Resistance: Investigating the Environmental Dimension-Frontiers 2017: Emerging Issues of Environmental Concern. Frontiers 2017: Emerging Issues of Environmental Concern, pp. 12-22. 2017.

7. Huang CH, Renew JE, Smeby KL, Pinkston K, Sedlak DL. Assessment of potential antibiotic contaminants in water and preliminary occurrence analysis. Journal of contemporary water research and education. 2011;120(1):4.

8. Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquatic toxicology. 2006 Feb 10;76(2):122-59.

9. Bronstein AC, Spyker DA, Cantilena Jr LR, Green JL, Rumack BH, Heard SE. 2007 Annual report of the American Association of Poison Control Centers' National poison data system (NPDS): 25th annual report. Clinical Toxicology., 2008 Jan 1;. 46(10):927-1057..

10. Kovalakova P, Cizmas L, McDonald TJ, Marsalek B, Feng M, Sharma VK. Occurrence and toxicity of antibiotics in the aquatic environment: A review. Chemosphere. 2020 Jul 1;251:126351.

11. Gothwal R, Shashidhar T. Antibiotic pollution in the environment: a review. Clean–Soil, Air, Water. 2015 Apr;43(4):479-89.

12. World Health Organization. Briefing note: antimicrobial resistance: an emerging water, sanitation and hygiene Issue. World Health Organization; 2014.

13. Frade VM, Dias M, Teixeira AC, Palma MS. Environmental contamination by fluoroquinolones. Brazilian Journal of Pharmaceutical Sciences. 2014 Jan;50:41-54.

14. JE, Drewes. Analysis, fate and removal of pharmaceuticals in the water cycle. Amsterdam : Wilson & Wilsons. Elsevier, 2007. 427-46.

15. Azanu D, Styrishave B, Darko G, Weisser JJ, Abaidoo RC. Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. Science of the Total Environment. 2018 May 1;622:293-305.

16. Muhie OA. Antibiotic use and resistance pattern in Ethiopia: systematic review and meta-analysis. International journal of microbiology. 2019 Aug 1;2019.

17. Tesfaye H, Alemayehu H, Desta AF, Eguale T. Antimicrobial susceptibility profile of selected Enterobacteriaceae in wastewater samples from health facilities, abattoir, downstream rivers and a WWTP in Addis Ababa, Ethiopia. Antimicrobial Resistance & Infection Control. 2019 Dec;8(1):1-1.

18. Bogale AA, Amhare AF, Chang J, Bogale HA, Betaw ST, Gebrehiwot NT, Fang Y. Knowledge, attitude, and practice of self-medication with antibiotics among community residents in Addis Ababa, Ethiopia. Expert review of anti-infective therapy. 2019 Jun 3;17(6):459-66.

19. Worku F, Tewahido D. Retrospective assessment of antibiotics prescribing at public primary healthcare facilities in Addis Ababa, Ethiopia. Interdisciplinary perspectives on infectious diseases. 2018 Feb 28;2018.

20. Gebretekle GB, Serbessa MK. Exploration of over the counter sales of antibiotics in community pharmacies of Addis Ababa, Ethiopia: pharmacy professionals' perspective. Antimicrobial resistance and infection control. 2016 Dec;5(1):1-7.

21. Elizalde-Velázquez A, Gómez-Oliván LM, Galar-Martínez M, Islas-Flores H, Dublán-García O, SanJuan-Reyes N. Amoxicillin in the aquatic environment, its fate and environmental risk. Environmental Health Risk-Hazardous Factors to Living Species. 2016 Jun 16;1:247-67.

22. Golet EM, Xifra I, Siegrist H, Alder AC, Giger W. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environmental science & technology. 2003 Aug 1;37(15):3243-9.

23. Girardi C, Greve J, Lamshöft M, Fetzer I, Miltner A, Schäffer A, Kästner M. Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. Journal of hazardous materials. 2011 Dec 30;198:22-30

24. Andreozzi R, Caprio V, Ciniglia C, De Champdoré M, Lo Giudice R, Marotta R, Zuccato E. Antibiotics in the environment: occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. Environmental science & technology. 2004 Dec 15;38(24):6832-8.

25. Straub JO. Aquatic environmental risk assessment for human use of the old antibiotic sulfamethoxazole in Europe. Environmental toxicology and chemistry. 2016 Apr;35(4):767-79.

26. Abou-Elela SI, El-Khateeb MA. Performance evaluation of activated sludge process for treating pharmaceutical wastewater contaminated with β-lactam antibiotics. Journal of Industrial Pollution Control. 2015 Jun 1;31(1).

27. Hendricks R, Pool EJ. The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. Journal of Environmental Science and Health, Part A. 2012 Jan 15;47(2):289-97.

28. Faleye AC, Adegoke AA, Ramluckan K, Bux F, Stenström TA. Identification of antibiotics in wastewater: current state of extraction protocol and future perspectives. Journal of Water and Health. 2017 Dec;15(6):982-1003.

29. Abdallah MA, Nguyen KH, Ebele AJ, Atia NN, Ali HR, Harrad S. A single run, rapid polarity switching method for determination of 30 pharmaceuticals and personal care products in waste water using Q-Exactive Orbitrap high resolution accurate mass spectrometry. Journal of Chromatography A. 2019 Mar 15;1588:68-76.

30. Kanama KM, Daso AP, Mpenyana-Monyatsi L, Coetzee MA. Assessment of pharmaceuticals, personal care products, and hormones in wastewater treatment plants receiving inflows from health facilities in North West Province, South Africa. Journal of toxicology. Oct 30;2018.

31. Moslah B, Hapeshi E, Jrad A, Fatta-Kassinos D, Hedhili A. Pharmaceuticals and illicit drugs in wastewater samples in north-eastern Tunisia. Environmental Science and Pollution Research. 2018 Jul;25(19):18226-41.

32. Agunbiade FO, Moodley B. Pharmaceuticals as emerging organic contaminants in Umgeni River water system, KwaZulu-Natal, South Africa. Environmental monitoring and assessment. 2014 Nov;186(11):7273-91

33. Ngumba E., Gachanja A., Tuhkanen T. Occurrence of selected antibiotics and antiretroviral drugs in Nairobi River Basin, Kenya. s.l. : Sci. Total Environ., 2016, Vols. 539, 206-213.

34. Madikizela LM, Tavengwa NT, Chimuka L. Status of pharmaceuticals in African water bodies: occurrence, removal and analytical methods. s.l. : Journal of environmental management, 2017 May 15;, Vols. 193:211-20.

35. Alvarez DA, Jones-Lepp TL. Sampling and analysis of emerging pollutants. Water Quality Concepts, Sampling, and Chemical Analysis. 2010:199-226.

36. Patel M, Kumar R, Kishor K, Mlsna T, Pittman Jr CU, Mohan D. Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. s.l. : Chemical reviews, 2019 Mar 4;, Vols. 119(6):3510-673.

37. Sadutto D, Picó Y. Sample Preparation to Determine Pharmaceutical and Personal Care Products in an All-Water Matrix: Solid Phase Extraction. s.l. : Molecules., 2020 Jan;, Vol. ;25(21):5204.

38. Amsalu MW, Kemal J, Gebru MW, Kassu DT. Quality and safety of municipal drinkingwater in Addis Ababa City, Ethiopia. s.l. : Environ Health Prev Med., 2020, Vols. 25(9):1–6.

39. Agizew Nigussie, Assie Kemal,Zerihun Getahun,ZerihunAbate. The Study of Water Use and Treated Wastewater. Addis Ababa : School of Civil and Environmental Engineering,Addis Ababa Institute of Technology, Addis Ababa University, October, 2018.

40. Loos R, Marinov D, Sanseverino I, Lettieri T. Analytical methods for substances in the Watch List under the Water Framework Directive. s.l. : InWorkcshop Report JRC, 2018 Mar 1.

41. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil,Sediment, and Biosolids by HPLC/MS/MS. Englert, B. s.l. : US Environmental Protection Agency (EPA), 2007, pp. pp. 1–72.

42. EPA, US. Stability of Pharmaceuticals, Personal Care Products, Steroids, and Hormones in Aqueous Samples, POTW Effluents, and Biosolids. s.l. : U.S. Environmental Protection Agency, Office of Water (4303T), 1200 Pennsylvania Avenue, NW, Washington DC 20460, EPA-820-R-10-008, 2010.

43. Kimosop SJ, Getenga ZM, Orata F, Okello VA, Cheruiyot JK. Residue levels and discharge loads of antibiotics in wastewater treatment plants (WWTPs), hospital lagoons, and rivers within Lake Victoria Basin, Kenya. Environmental Monitoring and Assessment. 2016 Sep;188(9):1-9.

44. U.S.P.C.I. United States Pharmacopeia, The National Formulary USP, 2009.

45. EMA (1995). ICH Topic Q 2 (R1)Validation of Analytical Procedures:Text and Methodology. Vol. CPMP/ICH/381/95.

46. da Silva DC, Oliveira CC. Development of micellar HPLC-UV method for determination of pharmaceuticals in water samples. Journal of analytical methods in chemistry. Mar 1;2018.

47. Gros M, Rodríguez-Mozaz S, Barceló D. Rapid analysis of multiclass antibiotic residues and some of their metabolites in hospital, urban wastewater and river water by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. Journal of Chromatography A. 2013 May 31;1292:173-88.

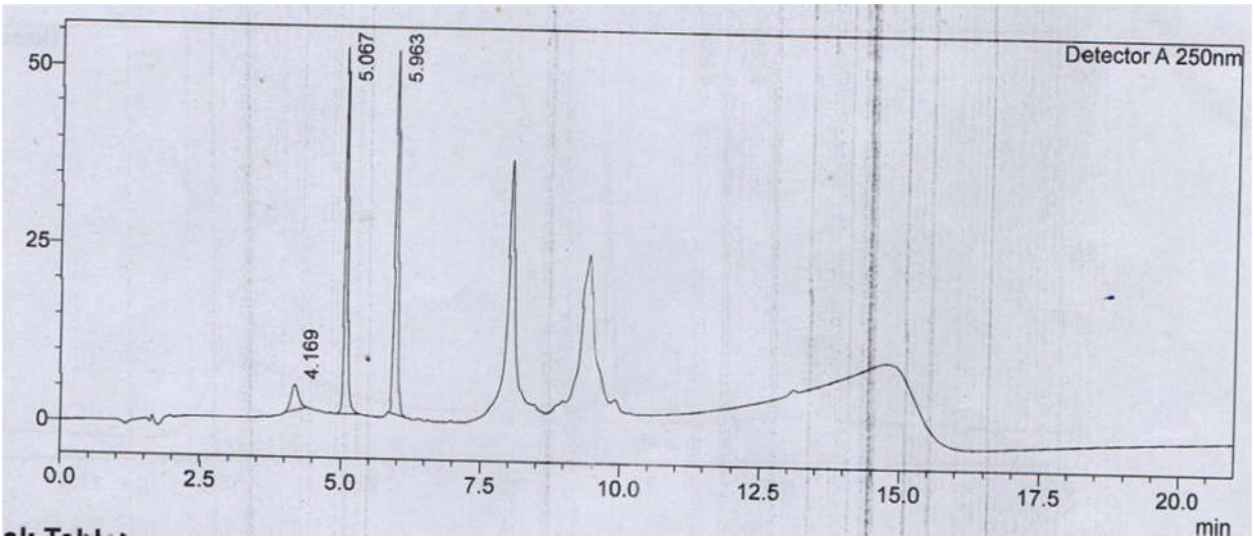
48. (AAWSA), Addis Ababa Water and Sewerage Authority. Environmental and social impact assessment of the wastewater treatment plant and sewer lines expansion and rehabilitation in the kaliti catchment. Addis Ababa, Ethiopia. October 2014.

49. Younes HA, Mahmoud HM, Abdelrahman MM, Nassar HF. Seasonal occurrence, removal efficiency and associated ecological risk assessment of three antibiotics in a municipal wastewater treatment plant in Egypt. Environmental Nanotechnology, Monitoring & Management. 2019 Dec 1;12:100239.

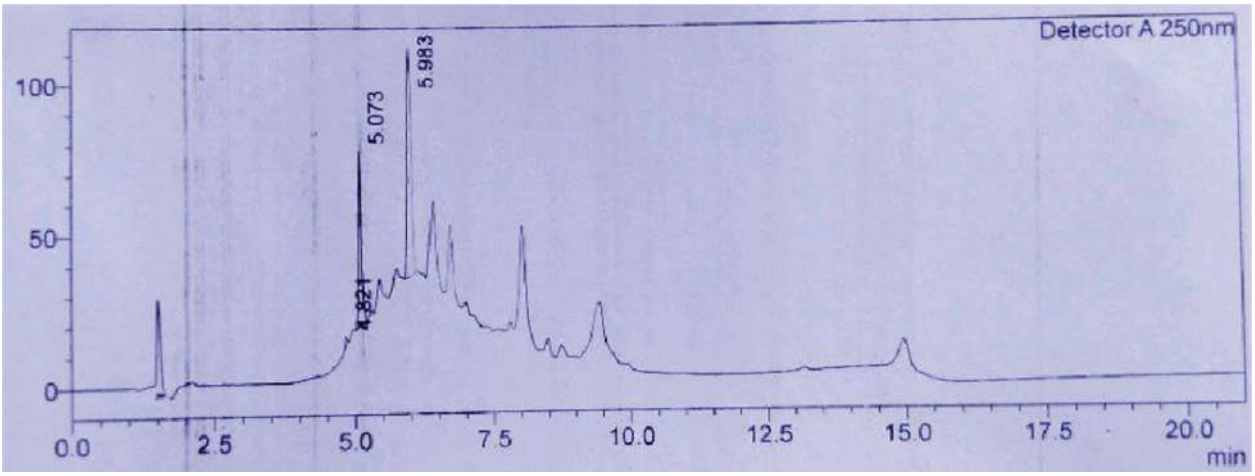
50. AMR Industry Alliance Antibiotic Discharge Targets. List of Predicted No-Effect Concentrations (PNECs) (2018).

51. Jafari Ozumchelouei E, Hamidian AH, Zhang Y, Yang M. Physicochemical properties of antibiotics: A review with an emphasis on detection in the aquatic environment. Water Environment Research. 2020 Feb;92(2):177-88.

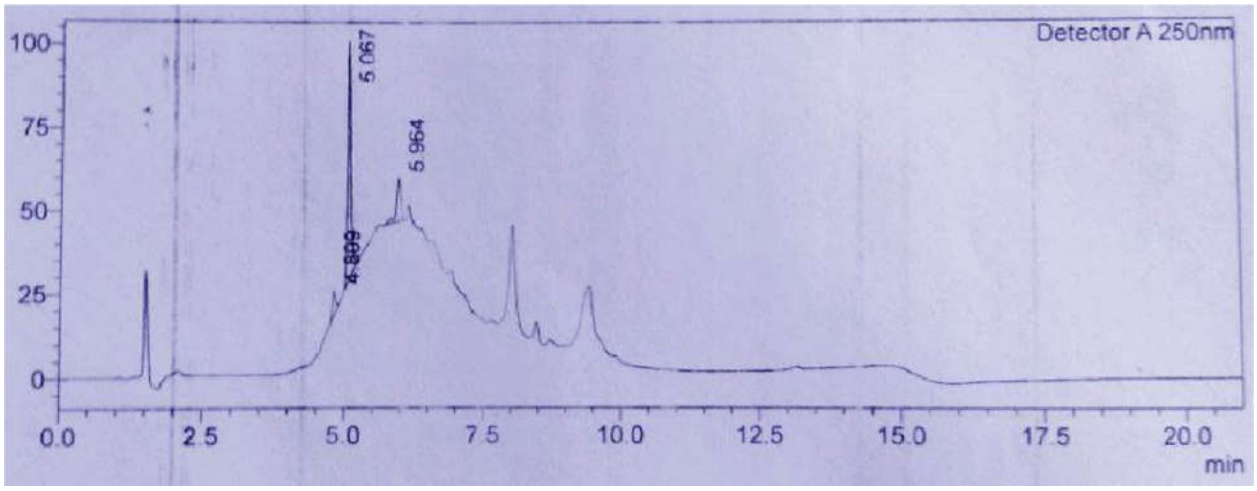
Appendices



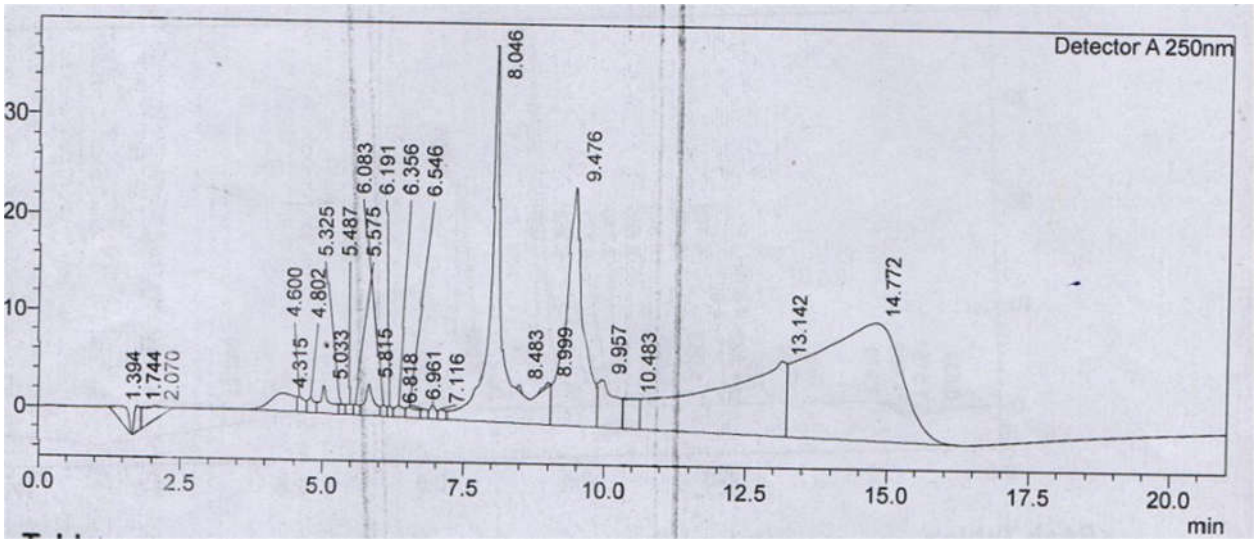
Appendix 1: Chromatogram of standard mixture solution (peak 1retention time (4.146) represent: Amoxacillin;2 (5.060): Ciprofloxacin;3(5.958): Sulfamethoxazole)



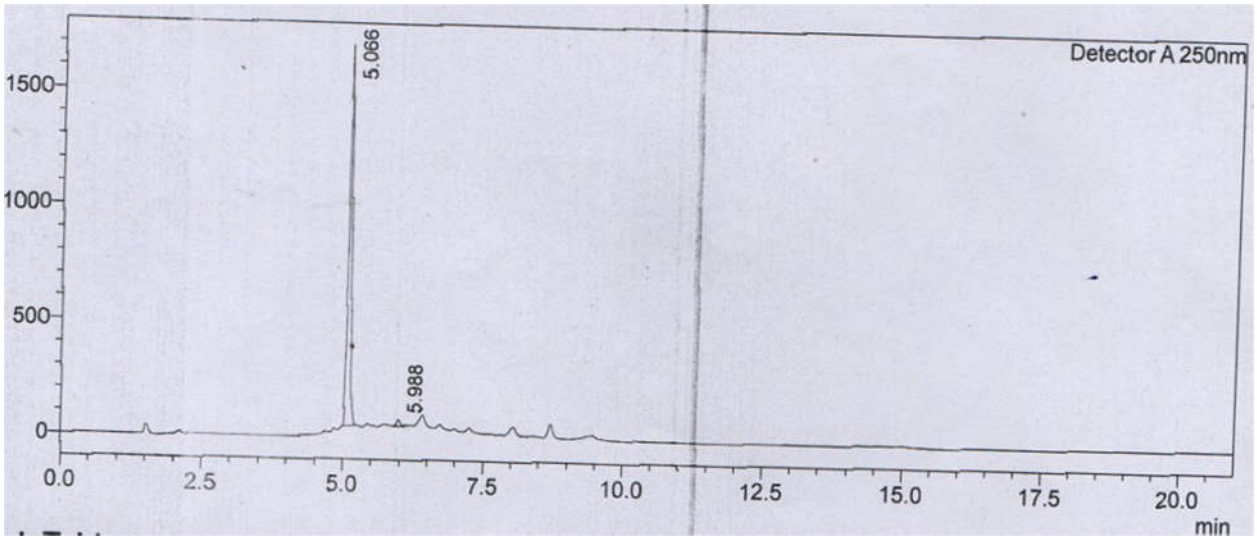
Appendix 2: Chromatogram of standard spiked, extracted influent wastewater using SPE



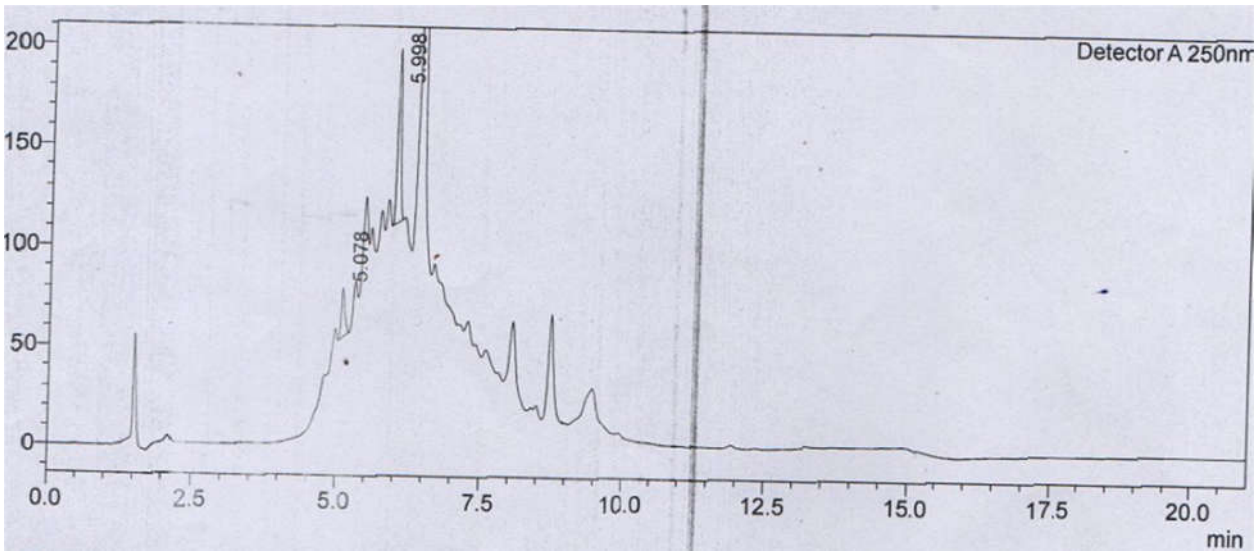
Appendix 3: Chromatogram of standard spiked, extracted effluent wastewater using SPE



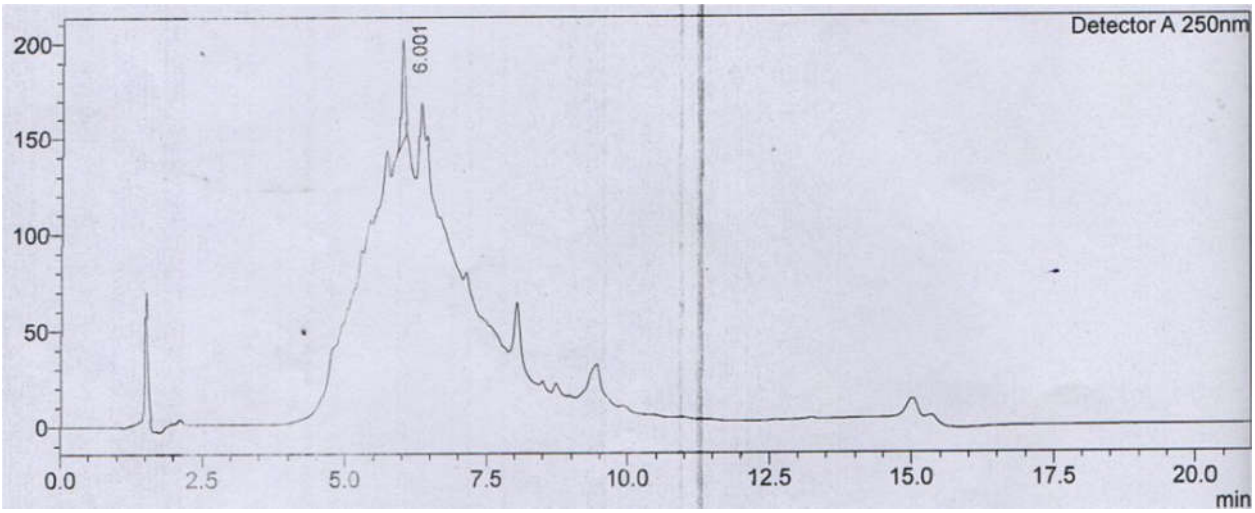
Appendix 4: Chromatogram of blank solution



Appendix 5: Chromatogram of target antibiotics detected in influent wastewater of Site A

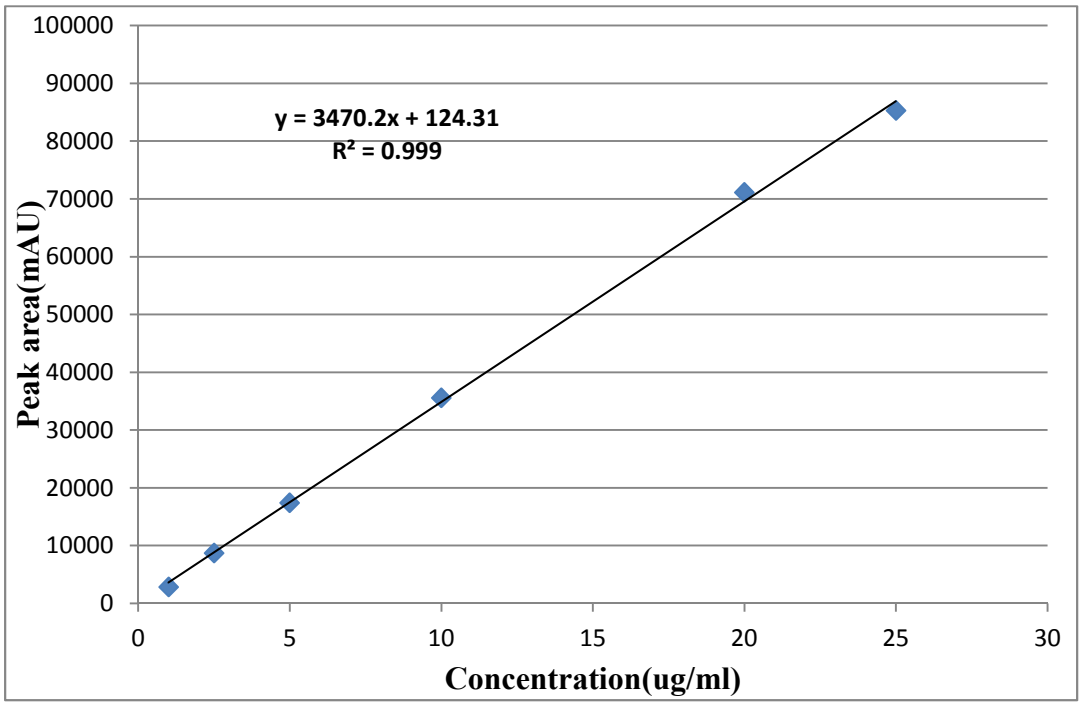


Appendix 6: Chromatogram of target antibiotics detected in influent wastewater of Site B

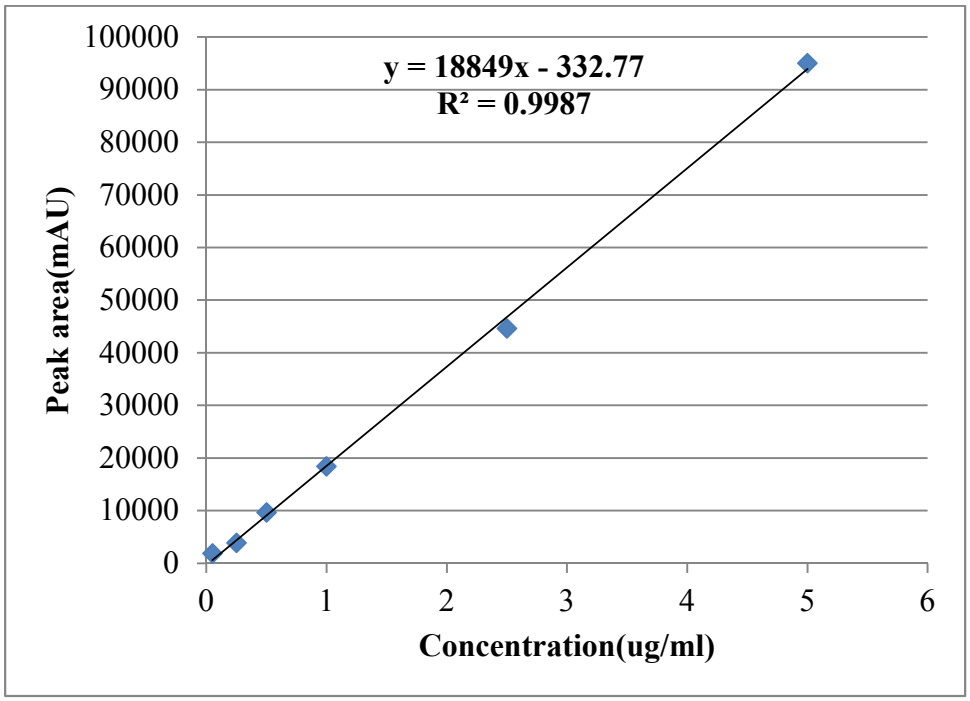


Appendix 7: Chromatogram of target antibiotics detected in effluent wastewater of Site B

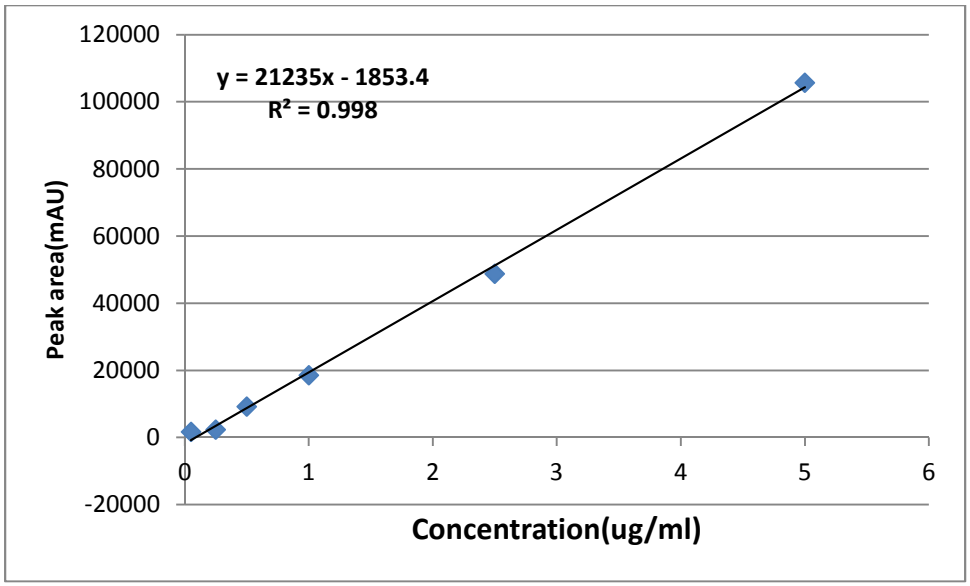
Appendix 8: calibration curves of three antibiotics standards



Calibration curve of Amoxicillin standard



Calibration curve of ciprofloxacin standard



Calibration curve of sulfamethoxazole standard

Appendix 9: sampling frame