Addis Ababa University

School of Graduate Studies

Institute of Biotechnology

Profiles of Physicochemical Characteristics, Antibiotic Residues, Antibiotic Resistance Genes and Bacteria Community Structure of Batu Tannery Wastewater Released to Little Akaki River

By: Tesfaye Admassu Abate

A Dissertation submitted to the Institute of Biotechnology, School of Graduate studies, Addis Ababa University, in partial fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

July, 2020
Addis Ababa, Ethiopia

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

Profiles of physicochemical, antibiotic residues, antibiotic resistance genes and bacteria community structure of tannery wastewater released to Little Akaki River

By

Tesfaye Admassu

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Approved by Examining Board:

Name	Signature	Date
1. Dr. Fassil Assefa (Advisor)		27/07/20
2. Dr. Adey Feleke (Advisor)		27/07/2020
4. Prof. Mulugeta Kibret (External Examiner)		27.07.2020
5. Dr. Diriba Muleta (Internal Examiner)		27-07-2020
6. Dr. Addis Simachew (Chairman)		27-07-2020

Addis Ababa University

School of Graduate Studies

Institute of Biotechnology

Profiles of Physicochemical Characteristics, Antibiotic residues, Antibiotic Resistance Genes and Bacteria Community Structure of Batu Tannery Wastewater released to little Akaki River

By: Tesfaye Admassu Abate

Email: tesadsanka@gmail.com/tesfaye.admassu@aau.edu.et

A Dissertation submitted to the Institute of Biotechnology, School of Graduate studies, Addis Ababa University in partial fulfillment of requirements for the Degree of Doctor of Philosophy in Biotechnology

Supervisors: Fasil Assefa (PhD)

Adey Feleke (PhD)

Nancy G. Love (Prof)

June, 2020

Addis Ababa, Ethiopia

Profiles of Physicochemical characteristics, antibiotic residues, antibiotic resistance genes and bacteria community structure of Batu Tannery wastewater released to Little Akaki River

Tesfaye Admassu[1], Dr Fasil Assefa[2], Dr Adey Feleke[2], Prof. Nancy G. Love[3]

[1]Institute of Biotechnology, Addis Ababa University, Ethiopia, [2] Molecular, Cellular and Microbial Biology, Addis Ababa University, [3]Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI, USA. E-mail: nglove@umich.edu

Abstract

Tanneries produce one of the highest COD wastewaters afterusing large volume of water and different chemicals to produce semi-finished and finished leather products. In Ethiopia, most of the tannery wastewater is discharged directly into water bodies with little or no treatment. These days, different treatment systems are constructed to treat tannery wastewaters that necessitate research to evaluate the efficiency. To this end, a research was conducted on Batu Tannery wastewater treatment plant to evaluate the physicochemical characteristics, bacteria community dynamics, antibiotic residues and antibiotic resistance genes in the different stages of the tannery wastewater treatment plant. The raw tannery wastewater showed variable strength from time to time, andthe highest mean COD (7204±8) mg/l, ammonia-N (190±3.6) mg/l, total nitrogen (308±2)mg/l, and total chromium (22±2.7)mg/lwere observed in April samples. The range of pH remains between 8.0 and 9.0 in raw tannery wastewater and 7.2 and 8.6 in the final effluent. The tannery effluent affected the physicochemical characteristics of the downstream river water of Little Akaki, except temperature and pH. The tannery effluent released to the Little Akaki River caused an increase in the physicochemical values by an average of 3.8%, 9% and 9.5% for COD; 23%, 10.4% and 12.5% for TN; 13.5%, 18.4% and 14.8% forammonia_N; and 31.5%, 28% and 52.8% for total chromium in the November, February, and April samples, respectively. However, only the COD, sulfate and total chromium cause significant ($p < 0.05$) difference in the downstream river water after tannery effluent was released. 16S rRNA amplicon-based bacterial community analysis from the treatment plant revealed that Firmicutes (48.5%), Bacteroidetes (32.6%) and Proteobacteria (11.5%) were the most represented bacterial phyla across the treatment plant. At the genus level, the dominant genera were Clostridium (15%), Synergistes (5%), Psychrobacter (4%), Acinetobacter (2.5%), Bacteroidetes (1.8%),

Anaerovorax (1.3%), Arcobacter (1.3%) and Shewanella (1%). Most of the core bacteria communities in the tannery wastewater were common signatures of gastrointestinal bacteria of ruminants. Some of the core bacteria arepotential pathogens tohuman including the Clostridium, Acinetobacter, Arcobacter and Shewanella. Antibiotic residues and antibiotic resistance genes were detected in the tannery wastewater. The antibiotic residues of tetracycline, oxytetracycline, erythromycin, penicillin G, trimethoprim, sulfadiazine, sulfadoxine, sulfamethoxazole and ciprofloxacin were present in all samples of the tannery wastewater, but penicillin V, amoxicillin, streptomycin, ampicillin and vancomycin were not detected in the same samples. The antibiotic resistance genes tet(A), tet(O), tet(M), erm(B), Sul(I), Sul(II) and Otr(A) were also detected in the tannery wastewater samples. An assessment on the use trend of antibiotics in livestock indicated that antibiotic contamination to tannery wastewater was highly likely to come from the misuse ofantibiotics in livestock disease treatment.

***Keywords**:Antibiotic residues, bacteria diversity, Illumina Miseq, Livestock, Potential pathogens, skin/hide, Tannery wastewater*

Acknowledgements

I would like to thank my supervisors' Dr Fasil Assefa, Dr Adey Feleke and Prof. Nancy G. Love for all their support and guidance. Your guidance helped me a lot and gave me opportunity to learn throughoutthe study. I am also grateful to Addis Ababa University, Institute of Biotechnology, for giving me the chance to study my PhD in such competitive and limited opportunities.

I would like to thank the Directors of Institute of Biotechnology for their administrative and academic supportduringthestudy.

I also owe special thanks to Dr Diriba Muleta and Dr Tesfaye Sisay who mentor me for the courses Seminar I and II, respectively.

I extend my appreciation and forward sincere thanks to the Batu tannery owners, management team, treatment plant operators, the office manager and all the staffs for allowing me to visit and collect samples from the tannery wastewater treatment plant.

I acknowledge the special support of my family especially my wife w/o Belaynesh Kefyalew, my sons Edomyas Tesfaye and Michael Tesfaye, my mother w/o BelayneshYimam, my father AtoAdmassu Abatebrothers, sisters and friends whose loud and bold encouragement energize me in those difficult times of the study particularly during times of departure for the sake of the study.

I dedicate all the sacrifices, ups, downs and final success of my study to my beautiful parents AtoKassaSeyoum and w/o Zewuditu Admassu. May your eternal rest is peaceful!!

I forward unreserved appreciation to my, staff, fellow PhD classmates of Institute of Biotechnology, and academic and administrative support staff of College of Natural and Computational Sciences for their direct and indirect support in my study.

I have special thanks to Prof. Nancy G. Love and her research team who hosted me to work part of my experiments in her laboratory at the University of Michigan, Ann Arbor. I also owe special thanks to Dr LutgardeRaskin and Dr Krista Rule Wigginton for their warm encouragement during my stay at the University of Michigan. I am also grateful to students working in the Civil and Environmental Engineering Laboratoriesfor their friendly approaches. I forward special thanks to Andrea McFarland and her family who were very kind for me during my stay in USA.

Finally, I thank Addis Ababa University for the partial support of my research visit at Michigan University. I also thank University of Michigan for hosting me in the research visit.

Above all, my praises and special thank is reserved tothe Almighty God and his blessed mother St. Merry!

Tesfaye Admassu

June, 2020

Table of Contents

List of Figures

List of Tables

List ofAbbreviation

APHA = American Public Health Association

ARB = Antibiotic Resistant Bacteria

ARG = Antibiotic Resistance Gene

CETP = Chemical Enhanced Treatment Plant

COD = Chemical Oxygen Demand

EC = Electrical Conductivity

EPA = Environment Protection Authority

HTS = High Throughput Sequencing

LAR = Little Akaki River

MGEs = Mobile Genetic Elements

NGS = Next Generation Sequencing

OTU = Operational Taxonomic Unit

PCR = Polymerase Chain Reaction

Ppt = Parts per thousand

Ppb = Parts per billion

SPE = Solid Phase Extraction

TDS = Total Dissolved Solid

TN = Total Nitrogen

UNIDO = United Nation Industrial Development Organization

WWTP =Wastewater Treatment Plant

This page is intentionally left blank!

Chapter One

1. General Introduction

1.1. Background

Leather industries are common in the low-income countries such as Ethiopia where there is abundant raw material, high demand for leather products and economic incentives (Azom et al., 2012). The process of tanning applies lengthy and wet steps of chemical treatments using several types of chemicals in order to change the skin and hide into imputrescible leather (Abdallh et al., 2016). Both traditional tanning and modern tanning activities can pollute water, but pollution is more aggressive in the latter since modern tanners use several types and massive amounts of tanning chemicals specific with the type of final products (Stefan et al., 2013).

According to Saxena et al. (2016), tanneries generate wastewater in the range of 30-35 m^3 per ton of raw hide/skin processed with variable pH, high concentration of suspended solids and chemical oxygen demand (COD). Thus, tanneries release high concentration of dissolved and suspended solids such as salts, nutrients, toxic chemicals, chlorine, lime, organic remains of skin, heavy metals and other pollutants to the environment. Consequently, leather tanning industries are one of the environmentally harmful industries and the wastewater produced is ranked as one of the most polluting industrial wastewaters (Goswami & Mazumder, 2013).

In addition to these conventional pollutants, there are emerging chemical pollutants of the tannery wastewaterassociated with the raw material inputsobtained from animals. These emerging pollutants in tannery wastewater are the antibiotic residues. Antibiotics are used to treat human and animal diseases by killing or inhibiting the growth of pathogenic bacteria at concentration low enough to avoid undesirable damage to the host (Dafale et al., 2016).However,

the antibiotics administered for disease treatment, prevention or growth promotion are not completely takenup and retained in the body of animals.Instead significant amount of the antimicrobials are released out of the animal body mainly through the feces and urine (Iglesias et al., 2013).One of the recipients of antibiotic residues are the tannery wastewater treatment plants (WWTPs) which have conducive environments for induction and propagation of antibiotic resistance by microorganisms. Once they are released to the environment, the antibiotic residues can have different fates including elicitingantibioticresistance genes (ARGs) by bacteria(Baquero et al., 2008). Due to the development of ARGs, the therapeutic efficiency of the antimicrobials is becoming compromised by the emergence of antibiotic resistant pathogens (Ahn & Choi, 2016).

Although the use of antibiotics in treatments of human and animal diseases is reasonably widespread, the spread of antibiotic resistance through animal husbandry is not recognized as it is in the human clinical settings (Michael et al., 2014). As a result, most of the hitherto research findings on antibiotic resistance by microorganisms are obtained from pharmaceutical industries and clinical discharges (Tuckwell, 2014; Le et al., 2016). This showed that the environmental significance of veterinary antibiotics is largely overlooked.

Wastewater treatment systems represent an important node for the spread of antibiotic resistance especially when treated wastewater is used as reclaimed water (Caucci et al., 2016). It can be hypothesized that antibiotic residues induce antibiotic resistance in tannery WWTP because of the use of more antibiotics in animal husbandry than in human(Holmes et al., 2016). Thus, antibiotics are emerging residual pollutants coming to the WWTP with manure, urine and animal tissues through the raw hide and skins used in the tanning process. When present in wastewater treatment systems, the antibiotics can enhance the emergence of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (Adefisoye & Okoh, 2017). Environmental sources of

antibiotic resistance are clinically relevant even if they are carried by the non-pathogenic bacteria. The resistance genes can spread from the environmental reservoirs to human pathogens and then to human hosts. Thus, recognizing the contribution of widespread use of antibiotics in animal husbandry and animal product-based industries to the emergence, amplification, persistence and dissemination of antimicrobial resistance is still a daunting task (Singer & Williams-Nguyen, 2014).

This initiates an interest in the current study to detect the presence of antibiotic residues and antibiotic resistance genes (ARGs) in the tannery wastewater. Since wastewater systems host large assemblages of microbial communities, the presence of large microbial biomass and high concentration of organic wastes in the tannery wastewater together with the antibiotic residues can make tanneries one of the hotspots for emergence and spread of antibiotic resistance. In WWTPs, the bacteria interact to each other and there is horizontal exchange of genetic elements between cells(Bellanger et al., 2014). Therefore, WWTPs can serve as a genetic reactors where genetic variability is enhanced as a result of the biological connectivity and the presence of special chemicals, such as the antibiotics which induce selective effect between bacteria in the treatment plants (Baquero et al., 2008).

The challenge of antibiotic resistance is a cross cutting agenda that must be taken seriously in the coming decades. An early forecast in human population showed that by 2050 world population will reach9 billion and in sub-Saharan Africa the population is estimated to grow by 1.2% each year (Thornton, 2010). This will enforce the use of more antimicrobials in animal agriculture in order to boost the productivity of animals and to improve the subsistence of farmers. This will result in the release of more antibiotic residues into the environment after the antimicrobials are

used for therapeutic, prophylactic and even for growth promotion purposes to improve the feed efficiency of animals (Aidara-kane et al., 2018).

The antibiotic residues and antibiotic resistance in bacteria reach to the environment from animal sources to the environment through different routes. Tannery wastewater is identified as one of the important routes for the distribution of antibiotic residues and antibiotic resistant bacteria into the environment mainly to surface water through the tannery effluent.In order to mitigate environmental pollution, leather factories are required to build WWTPs and treat the tannery wastewater before it is discharged into the water bodies. These treatment plants constitute a smaller aquatic ecosystem that involves many processes in microcosm constituting diverse communities of microorganism which play key roles in the removal of pollutants through nutrient recycling and energy flow. Bacteriaare the dominant microbial communities in the WWTPs and their diversity and dynamics is very important for the stability of the WWTPs(Giordano et al., 2016). Therefore, microbial ecology in WWTPs is an important causefor the functions and effectiveness of the treatment process.

For several years now, different studies have been undertaken on the microbial assemblage in the tannery wastewater systems in Ethiopia (Adey Feleke et al., 2014) and elsewhere (Lefebvre et al.,2006; Wang et al., 2014; Giordano et al., 2016; Liang et al,, 2017; Ma et al., 2018). These works showed that there is variation in the dominance and diversity of different groups of bacteria depending upon the treatment systems. A study on the microbial diversity at Modjo tannery WWTP in Ethiopiashowed that Firmicutes and Bacteroides were the dominant bacteria in the treatment system(Adey Feleke et al., 2014). In a study on different tannery wastewater systems in India, Lefebvre et al.,(2006)have also reported that Gammaproteobacteria (Proteobacteria) and Bacteroides were the most dominant bacteria in the tannery wastewater.For

instance, based on pyrosequencing of 16S rRNA genes on the full scale tannery WWTP, Wang et al., (2014)have showed that Proteobacteria and Synergistetes were dominant in the aerobic and anaerobic sludge. Ma et al., (2018)have also reported the dominance of Proteobacteria and Bacteroidetes in tannery wastewater involved in nitrification using Illumina Miseq sequencing.The same pattern of dominance of Proteobacteria and Bacteroidetes was reported in a study from leather sewage treatment plant in China(Liang et al,, 2017).

Thesestudies showed that the dynamics of bacteria community in tannery WWTPs vary and this has implications on the functioning of the microbial community in the treatment system. In addition, the studies carried out in different tannery WWTPs showed that the dominance of bacteria communities are different in different geographic locations and at different operating systems (Giordano et al., 2016). This shows that there is a need for more and continuous study of bacteria community dynamics in different tannery WWTPs at different times.

1.2. Statement of the problem

Ethiopia is endowed with enormous livestock population (2^{nd} in Africa and 8^{th} largest in the world) byhaving 53 million cattle, 27 million sheep and 23 million goats with 2.86% global share (Coppeaux et al., 2016). The country has an annual potential of supplying 20 million skins and hides each year for tanning industries (Mulat Alubel, 2015). Due to the abundant presence of raw materials, tanning is one of the oldest industries in Ethiopia which has been practiced for long time by traditional tanning process. It is one of the priority sectors in the country that there are more than 27 tanning industries in Ethiopia and some more are under construction to increase the tanning operators in the country (Mekonnen Birhanie et al, 2017).These tanneries have a total capacity of soaking more than 153,650 sheep and goat skins and 9,720 cow hides per day

(UNIDO, 2012).The tanneries discharge their wastewater into the nearby water bodies with or without treatment (Amde Eshete et al., 2016).

In Ethiopia, tanneries vary in their capacity of processing skin and hide each day and their capacity of treating the wastewater to the environmentally acceptable levels also vary to adhere the Ethiopian Environment Protection Authority (EPA) guidelines.The release of insufficiently treated tannery effluent to the rivers pollutes the environment by adding different chemical and microbial pollutants into the rivers. There are studies in Ethiopia which showed that the tannery wastewater has high pollutant load (Haile Reda, 2016; Mekonnen Birhanie et al., 2017) and the presence of diverse bacteria communities in tannery WWTPs is inevitable. The query is how diverse are the bacteria communities in the tannery WWTP and its implication for their biological roles in the treatment process. In most instances, Ethiopian tanneries release the tannery effluent into rivers which could affect the physicochemical characteristics and the general ecosystem of the river(Haile Reda, 2016). However, due to the variability in the strength of the tannery wastewater from tannery to tannery, the impact of wastewater on the specific river also vary and there is a need for unceasing study on the impact of the tannery effluents on the aquatic and terrestrial environments.

Moreover, most of the tanneries in and around Addis Ababa are established on the periphery of the river basins, and located in or near Addis Ababa city and Mojo town, 70 km away from the capital city. The tanneries are then one of the reasons for the pollution of the rivers in and around Addis Ababa city.Batu Tannery is amongst the different industries found on the periphery of Little Akaki River (LAR), which is one of the rivers in Addis Ababa with an estimated catchment area of 540 km^2. Despite the high pollution pressure on the LAR, the downstream suburban community uses the river water for irrigation of vegetable and fruit farm lands.

Tanneries convert raw hide into leather in almost wet processesby which large volume of wastewater is discharged after using water and several types of chemicals as a process input. Except few cases, most of the tannery wastewater treatment systems in Ethiopia are conventional types applying primary effluent treatment system. These treatment facilities target removals of common pollutants such as the Chemical Oxygen Demand (COD) and their efficiency is evaluated based on the removed amount of these pollutants by the wastewater treatment process. The hitherto studies, however, did not address the level ofthe ever-increasing trace pollutants such as antibiotic residues in the wastewater. The increase of antibiotic residues is due to the widespread use of the antimicrobials in animal health. However, most studies focus on sites contaminated with hospital (nosocomial) effluents where the release of antibiotics at a sub-lethal concentration in the environment could provoke antibiotic resistance through time(Le et al., 2016).

The presence of residual antibiotics in the WWTP not only elicits antibiotic resistance, but also builds background concentration by the gradual release of antibiotics into the environment.This can aggravate the problem through time as the fate of antibiotics is dependent on the physicochemical nature of the antibiotics often leading to slow degradation. Therefore, this study is important in order to generate a base line data on the presence and potential impact of antibiotic residues and ARGs which are the ‘hardest’ environmental problems dubbed as emerging genetic pollutants or “bad genes”.

Tannery wastewater treatment has another critical problem related with the presence of potential pathogens that are released to the environment, since the tannery wastewater can receive pathogenic bacteria of animal origin through the use of skin and hides. It is anticipated that the wastewater treatment system can reduce the mass of bacteria from the final effluent by a

combined flocculation and sedimentation processes, but the dynamics and composition of bacteria communities during the treatment has significant effect on the quality of the treated tannery effluent. To this end, there is limited information on the fate of the bacteria community during and after treatment.

Therefore, this study was initiated to describe the bacteria community dynamics and profiles during the treatment process. The study also focuses on the detection of the antibiotic residues and ARGs in the tannery WWTP, evaluation of the characteristics of the tannery wastewater and efficiency of tannery wastewater treatment system. The impact of the tannery effluent on the quality of the nearby Little Akaki River (LAR)was also assessed.

1.3. Research questions

Tannery wastewater is characteristically a strong wastewater which vary from tannery to tannery. Indeed, there is also variation in the tannery wastewater treatment facilities between tanneries in Ethiopia. This provokes a need to assess the performance of Batu tannery WWTPusing wastewater qualityparameters, and evaluation of the microbial dynamics in the treatment process. There is an empirical assumption that the community profiles of bacteria vary on spatial and temporal basis, and the antibiotic residues and ARGs can occur in the tannery WWTP. Based on these assumptions, the following research questions are formulatedfor this dissertation work.

1. How strong is the raw tannery wastewater and how much of the pollutants are removed by the treatment process in the wet and dry months?
2. Does the treated tannery effluent have impact on the physicochemical water quality characteristics of the nearby LAR when the ‘treated’ tannery effluent is released to the river?

3. Which bacteria communities are abundant in the Batu tannery WWTP and what is the fate of these dominant bacteria community in the treatment process?
4. Are there any antibiotic residues and ARGs in the Batu tannery WWTP, and what is the possible reason for the presence of antibiotic residues in the tannery wastewater?
5. Does the antibiotic use pattern in livestock have implication on the presence of antibiotic residues in the tannery wastewater?

1.4. Objectives of the study

1.4.1. General Objectives

The main objective of this research is to evaluatethe physicochemical characteristics of the Batu Tannery wastewater and assess the impact of tannery effluent on the quality of LAR water, and to detect the presence of antibiotic residues, ARGs in the tannery wastewater and to describe the dynamics and profiles of bacterial community in the tannery wastewater treatment process.

1.4.2. Specific objectives

The specific objectives of this study were:

- To characterize the strength of tannery wastewater before and after treatment based on the physicochemical properties of wastewater and water quality parameters in the wet and dry months.
- To evaluate the impacts of Batu Tannery effluent on water quality of LAR.
- To describe the abundance, diversity and dynamics of bacterial community in the different stages of the Tannery WWTP and estimate the relative abundance of potentially pathogenic bacteria in the tannery wastewater.

- To determine the presence of antibiotic residues and ARGs corresponding to the antibiotic residues in the tannery WWTP.
- To assess the antibiotic use pattern in selected livestock rearing areas and estimate the possibility of antibiotic misuse which possibly lead to the distribution of antibiotic residues to the environment.

Chapter Two

2.Literature Review

2.1. Leather tanning

In Ethiopia, tanning operation was started in 1920's (Behailu Amde, 2017), and for long time leather tanning is one of the manufacturing sectors which produce semi-finished and finished leather for export(Coppeaux et al., 2016).According to UNIDO (2012), leather tanning and leather product industries in Ethiopia contributed to 5.9% of the total export revenue for the country in 2011. Ethiopia is endowed with enormous livestock population with 53 million cattle, 27 million sheep and 23 million goats with 2.86% global share c.The country has an annual potential of supplying 20 million skins and hides from an off-take rate of 7% for cattle, 33% for sheep and 35% for goats which provide 3.1 million cattle, 7.8 million sheep and 8.2 million goat skins each year (Mulat Alubel, 2015). Skins/hides are mainly obtained from local collectors ofdifferent regions and slaughter houses found in different towns(Behailu Amde, 2017). Both traditional (Figure 2.1) and modern tanning use large amount of water in the tanning process and tanneries are among the highest wastewaterproducing industries which contain environmentally harmful substances (Hashem et al., 2016).

Processing each ton of raw hide/skin into leather consume about 300kg of different chemicals (Midha et al., 2008), 85% of which end up in the wastewater (Hashem et al., 2016). The major organic and inorganic pollutants of tannery wastewater are released with the soaking, pickling and chromium tanning streams (Stefan et al., 2013). The pollution load of tannery wastewater can be indicated by the physicochemical characteristics using wastewater quality parameters (Haydar & Aziz, 2009).

Figure 2.1. Traditional Soaking of dry hide in rural Ethiopia (source:Bisrat Gebremichael, 2016)

2.1.1. Tanning process

Leather production involve a series of unit operations consisting the pre-tanning, tanning and post-tanning stages all performed in a wet process, and converting skin/hide into leather use 34-56 m^3 water per ton of hide/skin(Haile Reda, 2016). In chrome tanning, leather is produced by the reaction between the carboxylic groups of protein fiber networks of collagen (skin dermis) and chrome tanning agent. In the process of converting raw hide and skin into leather, only about 20% of the initial skin is retainedin the final leather(Agrawal & Singh, 2016). For example, the tanning process of one ton raw hides produce approximately 250kg leather, 450-730kg solid waste, about 500,000liters of wastewater (Kolomaznik et al., 2008; Hu et al., 2011), 100-400mg/l chromium, 200-800 mg/l sulfide, high levels of fat and other solid wastes as well as pathogen contaminants (El-Bestawy et al., 2013). From the total chromium used in the tanning stages, nearly 40% of the chromium is not taken into the leather, instead released with the wastewater together with the different dyes used in the post-tanning stages (Chowdhury et al., 2013). Therefore, tannerywastewater is ranked as one of the strong and complex wastewaters with high loads of toxic pollutants (El-Sheikh et al., 2011; Agrawal & Singh, 2016).

2.1.2. Major steps of Leather tanning

Tanning convert raw skins and hides into stable leather which is resistant to microbial attack and has improved resistance to wet and dry conditions (Rajeswari, 2015). There are two common methods of tanning; the vegetable tanning and chrome tanning processes (Midha et al., 2008). Chrome tanning constitute almost 90% of the total tanning operations (Alvarez-bernal et al., 2006), which use chromium salts, mineral salts and different dyes to produce leather.

Major steps of tanning involve the following processes:

Curing: Curing is the first process that prevents the degradation of the skin/hide from the time they are flayed in the slaughter house until soaking in the beam house (Stefan et al., 2013). The preservation methods include salting, chilling, freezing and addition of some biocides(Saxena et al., 2016).

Soaking: Soaking is the bathing of the cured hide and skin in water for several hours. Soaking removes the preservatives used during the curing stage and allows the skin/hide to reabsorb the water lost during the curing step. Soaking dry hide require up to $20m^3$ water per ton and the most important pollutants of the soaking effluents are the salt, hide/skin surface impurities, dirt and globular protein substances dissolved in water (Islam et al., 2014). Water use is higher in the pre-tanning stages and the soaking alone contributes 50-55% of the pollution of tannery wastewater (Table 2.1).

Liming: The process of liming removes fats, hair and the skin keratin using the alkaline solutions called the ‘milk of lime’. Liming causes the swelling of the skin and splitting of fibers to the desired extent and this ideally prepares the collagen for tanning (Banani, 2013). Most of the organic pollutants in the pre-tanning stage come from the liming process in which proteins

(hair, skin, meat) and emulsified fats are removed from the skin and released with the wastewater. Liming wastewater has high sulfides, chemical oxygen demand (COD), total dissolved solids (TDS) and other solid wastes containing lime sludge, fleshing and hair (Hashem et al., 2016).

Table 2.1. The unit processes of tanning in leather production by chrome tanning operation and estimated wastewater discharge (Abdallh et al., 2016).

Unit processes of chrome tanning operation		Wastewater characteristics	Wastewater discharge volume
Pre-tanning	Curing: Salt preserved skin/hide	Contain high concentration of Cl, TDS, and soluble organics	Total 20-25 m^3/ton to end of beam house
	Washing and salting out	Highly saline wastewater	
	Soaking	Hypersaline wastewater	
	Liming/unhairing	Contain degradable pollutants	
	Deliming	Basic wastewater	
Tanning	Pickling	Acidic wastewater	Total 21-28 m^3/ ton
	Chrome tanning	Chromium containing, toxic wastewater	
Finishing	Shaving	High Soluble organics, TDS, COD	Total 34-40m^3/ton
	Fat-liquoring/Dyeing	Soluble organics, TDS, COD	
	Dried Crusting	Different dyes	
	Finishing		

Fleshing: Fleshing is the process of removing excessive organic matter such as the fat, meat remains, connective tissues and other tissue from the flesh side of the skin and hide. Fleshing can be carried out prior to soaking, after soaking, after liming or after pickling. Fleshing is called “green” when the flesh removal is done prior to liming and dehairing, but if it happens after

liming and dehairing, it is called “lime fleshing”. Fleshing operations produce effluent containing fatty and fleshy matter in suspension (Stefan et al., 2013).

Dehairing: The removal of hair from the surface of skin and hide is carried out by the dehairing chemical agents, the sodium sulfide and sodium hydrosulfides. The use of these chemicals result in the presence of up to 5000mg/lsulfide in the raw tannery wastewater (Midha et al., 2008).

Deliming: The deliming step involves addition of acidic chemicals in order to reduce the alkalinity of the skin and hide. Addition of acids shrink the swollen fibers of the skin and this prepare the skin for the bating stage. Deliming is thus the removal of lime carried out to avoid the interference of lime with the subsequent tanning stage (Brigden et al., 2000).

Bating: Bating is a reverse process to liming in which the skin or hide is treated with enzymes to soften the skin. Being an enzymatic process, bating removes lime, protein fibers and degradation products, thus improving the grain of the pelt and stretching of subsequent leathers. The bating material is typically composed of 50% wood flour or other carrier, 30% deliming agent (ammonium chloride) and 1-5% pancreatic enzyme (Brigden et al., 2000; Chowdhury et al., 2015).

Pickling: After bating, the next stage is treating the hides or skins first with salt and then acids. Addition of salt prevents the adverse effects of an increase in acidity added during the pickling stage (Figure 2.2). Pickling prepares the collagen materials for easy penetration by the tanning agent, the chromium sulfate. The raw hides and skins normally go through pickling using sulfuric acid and common salts (Chowdhury et al., 2015).Chromium (III) salts are used in tanning to give excellent properties for the leather by penetrating into the skin/hide, but most of

the chromium is released with the wastewater while some fraction of the original amount is absorbed into the leather (Ma et al., 2017).

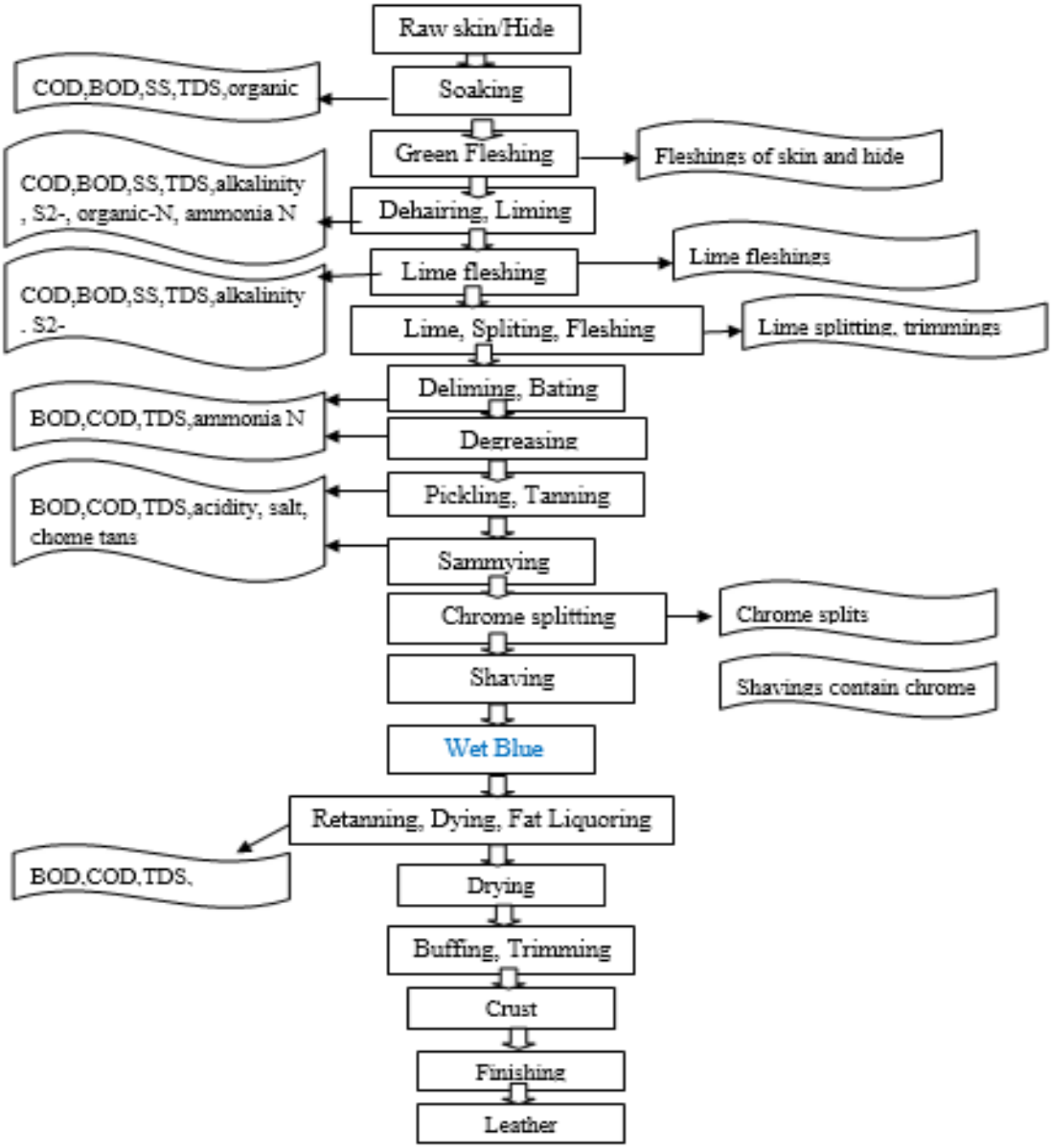

Figure 2.2. Tanning process and the pollutants generated at each stage **(**Source:UNIDO, 2016)

2.2. Tannery Wastewater and Water Pollution

Water pollution become an increasing environmental challenge in the 21st century that one out of ten people lack access to safe water (Ramírez-Castillo et al., 2015). The situation is worse in the developing countries as the population size is increasing while economic and social developments are not enough to safeguard access to safe water for all. Leather tanning is almost a wet process which generate large volume of often saline liquid waste (Lefebvre et al., 2006), and the wastewater generated from the tanning process contained large amount of organic matter, phenolics, tannins and toxic heavy metals mainly the chromium (Chowdhary et al., 2017). Tannery wastewater is distinguished by its high contents of organic compounds including proteins and fats, heavy metals and various salt ions (Sul et al., 2016). Tanning use large amount of chemicals including chromium salts, acids, alkalis, tannins, sulfates, phenolics, surfactants, dyes, auxiliaries, sulfonated oils and biocides (Stefan et al., 2013), but all of the chemicals used are not completely fixed by the hides and skins, but end up in the wastewater(Saxena et al., 2016).

Chromium occurs in several oxidation states from -2 to + 6, but two of the most common forms are the chromium (III) and chromium (VI), from which the latter is carcinogenic and mutagenic to human (Seiler & Berendonk, 2012) as a result of its higher solubility in water (Verma et al., 2009). The release of unabsorbed chromium residues into the environment result in the accumulation of the chromium in the environmental matrices and in the living tissues as well. At high concentration, heavy metals exert inhibitory effect to microorganisms by blocking functional groups, displacing essential metals or modifying active conformations of molecules (Alam et al., 2011). Exposure of microorganisms to chromium can also lead to development of heavy metal resistance that results in averting the sensitivity of the strains to high concentrations of chromium. Chromium is highly persistent in the environment and undergoes rapid sorption to

substrates and soluble chromium is found less abundant than sorbed chromium in the environment (Branco et al., 2005).

Tanneries are one of the major industries that cause river water pollution (Elabbas et al., 2016). In Ethiopia, most of the rural and suburban fresh water demand for human, domestic animals and agricultural activities are obtained from rivers water. However, industrialization, population explosion and waste disposal to the rivers is deteriorating the quality of river water. Amongst the industries causing river water pollution are the tanneries which discharge large volume of wastewater containing different types of chemical and biological pollutants (Elabbas et al., 2016; Nithya & Sudha, 2016). The tannery wastewaterisreleased to nearby water bodies which areused for irrigation and animal drinking (Figure2.3).

Figure 2.3. Cattle drinking at river water contaminated with tannery wastewater (Image taken in January 2017)

2.3. Tannery Wastewater treatment in Ethiopia

Tanneries are one of the highest wastewater generating industries which discharge environmentally harmful effluent to the environment (Hashem et al., 2016). Usually, tannery wastewater treatment is performed in several steps using physical, chemical and biological

mechanisms, and the latter is more effective in removing suspended and dissolved pollutants by the help of microorganisms mainly by bacteria, archaea, fungi and protists (Shchegolkova et al., 2016).Most of the tanning industries in Ethiopia have primary wastewater treatment systems which use sulfide oxidation, aeration, coagulation and sedimentation processes. This types of conventional tannery WWTPs mainly remove chemical oxygen demand (COD), biological oxygen demand (BOD) and suspended particles with faltering efficiencies. The biological processes in activated sludge systems depend on the ability of microorganisms to utilize organic materials as a source of energy and using minerals for growth. Therefore, microorganisms degrade organic materials and transformtoxic compounds into harmless products and stable operation of biological WWTPs rely upon the presence, abundance and performances of microbial population (Meerbergen et al., 2017).

Most leather manufacturing industries in Ethiopia are yet to build full-scale wastewater treatment systems for complete treatment of their wastewater produced after chemical intensive customary operations (Bosnic et al., 2003). Therefore, pollution problem associated with tanneries remain a pressing environmental challenge and the problem is exacerbated by the fact that most tanneries are located near river courses to which they discharge partially-treated or untreated effluent. Poorly treated wastewater can have profound influence on the receiving water shadeswhich are used by the downstream residents for various water needs (Joshua et al., 2017). The release of incompletely treated effluent to rivers can impair the ecosystem downstream from the site of contamination (Ayandiran et al.,2014).In the suburbs of Addis Ababa, the rivers which receive wastewater effluents are used for animal drinking, crop irrigation and other human uses(Samuel Abegaz, 2007). On the other hand, poor quality of effluents released from wastewater treatment plants can cause detrimental effect in the aquatic environment which is currently suffering from

eutrophication of lakes and river water pollution. Other than the environmental effects, tannery effluent can contaminate the public by releasing pathogens which cause an immediate negative health impact on people that use the water for different purposes (Joshua et al., 2017).

2.4. Antibiotic Residues in Tannery Wastewater

Tannery wastewater is largely known by the presence of high concentration of suspended and dissolved solids, nutrients and specific pollutants such as chromium and sulfides (Mandal et al., 2010). However, extensive use of agricultural chemicals such as antibiotics in animal production are becoming emerging chemical pollutants to the environment (Ayandiran et al., 2014). Antibiotics are used in disease protection and disease treatment as well for animal growth promotion since 1950's (Agga et al., 2015).After administered to the animals, the antibiotics are not fully metabolized inside the animal body; instead most of the antibiotics are released out of the animal gut. Thus antibiotics are introduced to the environment through a number of routes including through animal feces, urine, sewage, blood (Batt et al., 2008) and through wastewater effluents (Fent et al., 2006), land application of sewage sludge and animal manure (Kim & Aga, 2007). Drug manufacturing plants and hospital effluents are also another sources of antibiotic residues to the environment (Gothwal & Shashidhar, 2015).

According to van Boeckel et al(2015), by 2030 the global antibiotic consumption in food animals will grow by at least 67% with annual growth rate of 2.6% which is a comparable rate of antibiotic consumption by human (2.8%). Given the consumption rate of antibiotics by livestock is almost equal to human, the presence of antibiotic residues and ARGs in the environmentis a threat to human health.Moreover, some antibiotic classes used in animal treatment are also medically important in human (Schmitt et al., 2017).

WWTPs serve as ideal meeting places for antibiotic residues, bacteria community, nutrients and ARGs (Karkman et al., 2018). In the presence of antibiotic residues, antibiotic resistant bacteria can be favored, while the susceptible bacteria are eliminated. Therefore, tannery wastewater treatment systems can serve as the sources and routes for the transfer of antibiotic residues and antibiotic resistant bacteriato the surface water and this will result in gradual increase of antibiotic concentration in the environment. Once in the environment, antibiotic residues may not directly create antibiotic resistance in bacteria, but can enrich abundance of resistance genes by selective pressure in complex systems, and this enhance proliferation of ARGs in the microbiota(Gothwal & Shashidhar, 2015).

The presence of antibiotic residues and other virulence factors in the environment enforce the exchange of ARGs with pathogens and other environmental commensal bacteria to the human and animal microflora which is compounding the problem of drug resistance (Adefisoye & Okoh, 2017). In the environment, the persistence, bioaccumulation and toxicity of antibiotics could vary for different antibiotics, but all antibiotics can contribute to antibiotic resistance and have adverse effect on human and environmental health (Lundborg & Tamhankar, 2017).

Land application of wastewater sludgeand animal manure have environmental issue that theyserveas a source of antibiotic residuesto enter into the food chain by grazing animals and agricultural practices (Gothwal & Shashidhar, 2015). According to Kim et al.(2011), the effluences from land applications of livestock manure containing residual veterinary antibiotics are the dominant pathways for the releases of antibiotic to the environment. This happens when the animal manures, biosolids, sewages sludge and sediments are released to the terrestrial ecosystem and when antibiotic containing reclaimed water is used for irrigation of crop land

(Akimenko et al., 2015). Therefore, the release of tannery effluent into rivers remains an environmental challenge in developing countries (Adefisoye & Okoh, 2017).

2.5. Potential Impacts of Antibiotic Residues

2.5.1. Health Impacts of antibiotic residues

In Ethiopia, there is scarcity of data on the use pattern of antibiotics in livestock and there are instances of low quality and fake veterinary antimicrobials in the pharmaceutical market. The problem is similar in Africa as the economic estimate of substandard drugs in the continent is very high (Grace, 2015). Antimicrobial resistance is a significant health problem in both human and veterinary clinics with infections that were once readily treated now being resilient to antimicrobial therapy. The development and spread of ARGs in the environment is partly linked with the widespread use of antimicrobials in animal therapeutics, in food animal productions and other agriculture applications (Alexander et al., 2015). Diseases caused by antibiotic resistant pathogens registered an estimated 700,000 annual global deaths (Bengtsson-Palme & Larsson, 2016).

The relationship between antibiotic consumption and its impact in antibiotic resistant pathogens has been given more emphasis in hospital contexts, but the presence of sub-therapeutic levels of antibiotics in other environments can have similar effects on bacteria communities (Bellanger et al., 2014). Moreover, many of the antibiotics used in livestock disease control and human medicine are the same drug classes (Caudle et al., 2014). On the other hand, the use of antibiotics in health and agriculture applications is feared to cause genotoxicity in addition to promoting antibiotic resistant pathogens (Gueye et al., 2011). Some studies also showed that non-target

organisms are exposed to sub-lethal antibiotic concentrations which may induce toxicity at cellular or DNA level (Gothwal & Shashidhar, 2015).

2.5.2. Impact of antibiotics on Agriculture

In agricultural setups, the presence of bacterial pathogens specially the antibiotic resistant pathogens is one of the concerns in the production of safe foods for human consumption (Williams-Nguyen et al., 2016). Antibiotic residues enter into aquatic environment through different sewerage systems and by the wastewater released from livestock farms, and surface runoff water. When the antibiotic contaminated river water is used for irrigation, the antibiotic residues reach to the soils in the agro-ecosystems. Although soil is a habitat of indigenous antibiotics produced by soil microorganisms, the exogenous antibiotics incorporated in the soil through irrigation are persistent and cumulative. This result in an increasing residual concentrations of antibiotics ranging from few micrograms up to grams per kilograms of soil (Du & Liu, 2012).

2.5.3. Phytotoxicity of Antibiotic residues

Antibiotic residues in the environment are also sewage-derived which are partly eliminated in wastewater treatment processes, but still present in the final effluents and move to the ambient surface water bodies (Xu et al., 2015). Antibiotic residues and antibiotic resistance genes can be taken up by plants during wastewater reuse and the presence of antibiotics in water, soil and sludge facilitate a way for entry into biota (Gothwal & Shashidhar, 2015). In plants, antibiotics can have impact at different stages of development such as at seed germination (Du & Liu,

2012). For instance, tetracyclines, fluoroquinolones and macrolides are reported to have effect on the chloroplast and mitochondrial protein synthesis in plants. Fluoroquinolones inhibit DNA synthesis in eukaryotic cells and in plastid replication and have negative influences on plant morphology and photosynthesis. Streptomycin inhibit chlorophyll synthesis in *Hordeum vulgare*; sulfadimethoxine and enrofloxacin reduce growth significantly, and ciprofloxacin can reduce photosynthesis and hence growth in plants. Tetracyclines can also have phytotoxic effects which may lead to chromosomal aberrations and inhibition of plant growth. Beta-Lactams are considered to be less toxic, but they also affect the plastid division in lower plants. Tetracyclines, ciprofloxacin and erythromycin also reduce the content of photosynthetic pigments in plants. Penicillin, cephalosporins and tetracyclines again affect the photosynthetic electron transport rate (Gothwal & Shashidhar, 2015).

2.5.4. Environmental persistence of Antibiotic Residues

Antibiotics are grouped according to their chemical and structural properties, and those antibiotics having similar structures and belonging to the same class can have similar fate in the environment. Antibioticsbelonging to the β-lactams, tetracyclines, sulfonamides, quinolones and macrolides were detected in different environmental matrices (Zhang & Li, 2011).The presence of antimicrobial residues in the environment creates a condition to which microbes have to respond for survival. The ability of coping up environmental presence of antimicrobial chemicals by bacteria involves phenotypic and genotypic modifications stimulated by the presences of the antibiotics (Castiglioni et al., 2005).

Antibiotics are regarded as persistent or “pseudo-persistent” substances as the rate of entering into the environment is more than the rate of elimination. For example, tetracyclines could

persist in soil for over one year and only a moderate degradation of various tetracyclines can occur within 180 days (Du & Liu, 2012). Due to their persistence, the environmental presence of antibiotics has toxic effects on microorganisms, plants, animals and ultimately humans. The ecological hazards of antibiotics can be assessed on the basis of risk quotients. Ecological risk quotients can be calculated through the predicted environmental concentration or measured environmental concentration divided by the predicted non-effect concentration which can be obtained by the ratio of the half maximal effective concentration (EC_{50}) and the half maximal lethal concentration (LC_{50}) divided by an assessment factor(Mojica & Aga, 2011; Shashidhar, 2015).

2.6. Fates of Antibiotic residues in the wastewater treatment plant

Antibiotic contamination happens to soil, sediments and water bodies, but aquatic environment is the major medium for the movement of antibiotic residues. Environmental circulation of antibiotic residues is causing antibiotic resistance, which is a worldwide health concern and the problem is not limited to the clinical settings even though the consequences are clinical (Karkman et al., 2018). Antibiotics are predominantly hydrophilic, but the efficiency of human and animals to retain them in the body after consumption is low that 30% to 90% of them are released out into the environment (Blair et al., 2013). Non-discretionary use of antibiotics in animal medicine can be one of the reasons for the presence of antibiotic residues in the wastewater and residual antibiotics and ARGs can exist from the WWTP to the environment through effluent discharges (Cheng et al., 2015).Wastewater treatment systems can remove some of the antibiotics during the treatment process, but there is a possibility for the rest of the antibiotics to escape to the environment depending on the advances of the treatment system (Kim & Aga, 2007). The release of antibiotic residues into the environment can augment development

and propagation of the antibiotic resistant bacteria (ARB) in the environment (Gao et al., 2012). In aquatic environments, hydrophilic antibiotics can persist for long period and accumulate overtime to an amount as high as the prescribed doses. This happen by the interaction of functional groups such as the carboxylic moieties, amines and aldehydes which have a tendency to associate with organic substances in the suspension and build the concentration into higher amounts (Chen et al., 2013).

The environmental fates of antibiotics depends on their physicochemical properties, but the behavior of antibiotics in WWTPstill needs more elaboration(Akimenko et al., 2015). Depending on their physicochemical properties antibiotic residues can be removed from the wastewater by sorption, biodegradation, photodegradation and oxidation as major processes of elimination (Gothwal andShashidhar, 2015).

2.6.1. Adsorption

Most antibiotics have high sorption affinity to organic matter in the sludge and disposal of sludge can contribute for dissemination of antimicrobial residues to the environment (Chen et al., 2013). In WWTPs, antibiotics are either adsorbed to the sludge, remain unchanged or degraded to intermediate forms of active metabolites (Fent et al., 2006). Adsorption of antibiotic residues depend on their type and environmental parameters such as the pH, content of organic matter and by the presence of other abiotic factors (Du & Liu, 2012). For instance, tetracyclines can precipitate with cations such as calcium and their concentration can reduce in the effluent, but sulfonamides remain unchanged because they are non-degradable in conventional sewage treatment (Milić et al., 2013). Adsorption of tetracyclines toward humic substances and clay minerals is influenced by pH and ionic strength of the medium (Gothwal & Shashidhar, 2015).

The sorption phenomenon is useful for the removal of antibiotics in advanced wastewater treatment systems, like it is usable for the removal of toxic metals such as chromium from WWTPs(Saxena et al., 2016).

2.6.2. Degradation

One of the routes for the introduction of antibiotic residues from livestock operations into the environment is through the effluences released to the water bodies. In the water bodies, hydrolysis is an important degradation pathway for some antibiotics mainly the beta-lactams, macrolides and sulfonamides which appear to be the most susceptible to hydrolysis (Chee-Sanford et al., 2009). Hydrolysis is an important degradation pathway for organic pollutants in aquatic environment and at dissolved phase, biodegradation is the main elimination process in wastewater treatment carried out by aerobic and anaerobic process. Biological decomposition of antibiotics in activated sludge system increases with hydraulic retention time and sludge age (Tambosi et al., 2010), but the rate of antibiotic degradation in aerobic and anaerobic digestion vary with the antibiotic types and according to the abiotic and biotic conditions (Daghrir and Drogui, 2013). Degradation may change the structure of antibiotics, but degraded products of the antibiotics can still exhibit toxicity to organisms (Du & Liu, 2012). Degradation of antimicrobials can be intracellular or extracellular and microorganisms can degrade antibiotics under aerobic or anaerobic conditions by enzymatic processes, but biodegradation of antibiotics under aerobic conditions are rarely assisted by bacteria (Gothwal & Shashidhar, 2015).

2.7. Use trend of Veterinary Antimicrobials in Ethiopia

Antimicrobials in veterinary medicineare used to kill or inhibit growth of pathogens and promote the growth of animals, but most of theorally administered drugs are poorly absorbed in the

animal gut (Wei et al., 2016). Over the globe, there is lack of antibiotic use data in animals and it is a challenge in estimating the real environmental load of antibiotic residues (Kim & Aga, 2007). The problem is even worse in Ethiopia that there is lack of data on how many tones of antibiotics are used by animals each year. Even though, data is lacking, the massive population of livestock and animal extension activities in Ethiopia can be a good precedent to estimate that the environmental significances of veterinary antibiotics is massive. However, antibiotics used in veterinary medicines are almost equal or more than the antibiotics used in human medicine, and consequently veterinary use of antibiotics may contribute disproportionately to the prevalence of antibiotic resistance (Call et al., 2013). Antibiotic classes such as tetracyclines, penicillin, sulfonamides, trimethoprim, quinolones, lincosamides and aminoglycosides are primarily used in disease treatment or prevention (Chee-Sanford et al., 2009).

Besides to the lack of data on veterinary antibiotic use, antibiotic misuse such as inadequate dose, incomplete courses and indiscriminate drug usecan contribute to the emergence and spread of antimicrobial resistance (Awad et al., 2005). It is understood that the use of antibiotics in livestock is regulated by law, but there is unprecedented gap in implementing the laws. This causes challenges to combat the problem of antibiotic resistance by applying policy based prudent use of drugs in livestock farming which must be mandatoryto abide to the drug use guidelines on the purpose and dose (Call et al., 2013). The use of veterinary antibiotics as growth promoters in animal feeds are now strictly regulated in developed countries and even totally prohibited in the European Union since 2006 and partly in the United States (Wei et al., 2016). However, the situation in developing countries lacks practicality to implement the recommendations of reducing antibiotic use for growth promotion.

2.8. Antibiotic resistance Co-selection in wastewater treatment

Tannery WWTP is one of the ideal sites for co-selection of antibiotic and heavy metal resistant microorganisms in the presence of residual antibiotics and chromium(Seiler & Berendonk, 2012). Heavy metals are substances having a molecular weight grater than 55.8 and density of 5g/cm^3(Samanta et al. 2012; Coelho et al. 2015). Chromium has 51.99g molecular weight, but 7.15g/cm^3 density, higher thermal behaviours with a boiling and melting points of 2671^{o}C and 1907^{o}C respectively. As a heavy metal, chromium has high persistence and predominant occurrence in the environment. Long term exposures of heavy metals to bacteria can provoke resistance, and toxicity of heavy metals to microbes is related to their chemical affinity to the *thiol* groups(Younan et al., 2016). Bacteria evolved mechanisms to cope up the toxic effects of metal ions by complex formation, metal sequestration and by binding free metal ion to minimize their concentration in the cytoplasm (Seiler & Berendonk, 2012). Therefore, the probability of occurrence ofselective agents for ARGs in WWTPis highwhen the streams of influent wastewater contain antimicrobials residues in addition to heavy metals, detergents and other substances all of which have co-resistance and cross-resistance effect to antibiotics (Kim et al., 2010).

Resistance mechanisms to antibiotics by heavy metal tolerant microbes are modulated by co-selection and cross-resistance systems. Co-selection is an indirect genetic way that happens by coupling of antibiotic resistance against heavy metals, but cross-resistance is a physiological process to antibiotics which provide a means to resist more than one antimicrobial agent and heavy metals. According to Alam et al (2011)there is a correlation between heavy metal tolerance and antibiotic resistance by bacteria as shown by genome analysis of heavy metal resistant microorganisms. This is due to the simultaneous occurrence of the resistance genes on

the same genetic elements, the plasmids, transposons and integrons which specify resistance phenotypes.

Wastewater treatment systems are important environmental tools to remove pollutants from the effluent before released to the environment. Untreated or insufficientlytreated wastewater can induce anoxia, eutrophication and bad water quality in rivers (Atashgahi et al., 2015). One of the emerging pollutants present in wastewater is antibiotics which can inducethe development of ARGs at sub-therapeutic levels. Biologicalwastewater treatment systems can be suitable reservoir sites for ARB and for ARG transfer between the bacteria communities of the wastewater. Provided that the dynamics of ARB in wastewater treatment systems is high, the wastewater can be contained for a couple of days in the treatment plant and it is a favorite condition for the transfer of ARGs between bacteria (Karkman et al., 2018). The propagation of ARGs can happen in the presence of biologically active compounds especially when the compound present for sufficient duration with the bacterial community. After propagation in the wastewater treatment system, the ARB and ARG can leave from WWTPand move to the environment where animals are exposed to higher densities of resistant bacteria, and it is risk for contact transmission of antibiotic resistant pathogens (Williams-Nguyen et al., 2016; Yang et al., 2016).

Antibiotic resistance by bacteria is one of the current problems in human and animal medicine. There are 32 classes of antimicrobials and more than 260 drugs are used in human and animal treatment (Durso & Cook 2014). According to WHO, antibiotic resistance to these drugs is rising to high levels in all parts of the world at dangerous rate and it is fostered by misuse of antibiotics in humans and animals(Aidara-kane et al., 2018)**.** As shown in Table 2.2,antibiotics are

specifically used to kill or stop growth of bacteria, but in the process bacteria resist the effect of antibiotics by developing antibiotic resistance in differentmechanisms(Alanis, 2005)**.**

Table 2.2. Modes of actions of antibiotics, Source: (Alanis, 2005)

	Mechanism of resistance	Antibiotics
1	Cell wall synthesis inhibition	Beta-lactams, Glycopeptides, cyclic Lipopeptides
2	Protein synthesis inhibition	Tetracycline, Aminoglycosides, Streptogramins, Ketolides, Macrolides, Lincosamines
3	DNA synthesis inhibition	Floroquinolones
4	Inhibition of RNA synthesis	Rifampin
5	Competitive inhibition of folic acid synthesis inhibition	Sulfonamides, Trimethoprim
6	Membrane disorganization	Polymixins

2.9. Antibiotic Resistance Genes in Wastewater treatment

Wastewater treatment is one of the ideal sites for the proliferation and spread of ARGs (Gao et al., 2012; Bouki et al., 2013). The emergence and propagation of antibiotic resistance traits in wastewater treatment condition mainly result from the enrichment of ARB by the horizontaltransfer of ARGs (Rizzo et al., 2013). Therefore, presence of antibiotic residues combined with high microbial density makes wastewater treatment facilitiessuitable place for the spread of ARGs (Berendonk et al., 2015). In WWTPs bacteria, fungi, protozoa and archaea occur together and there is a possibility forhorizontal movement of ARGs across the different life domains (Bouki et al., 2013). Therefore, wastewater treatment systems bridge anthropogenic and natural environments allowing commensal and pathogenic bacteria, resistant and non-resistant

ones to reach the surface water ecosystems(Caucci et al., 2016). Once ARB enter theWWTPs, they can spread their resistance determinants to bacteria of the endogenous microbial community and those transiting through the WWTPs(Karkman et al., 2018).

Worldwide several hundreds of active antimicrobial substances escape to the environment in active forms from WWTPs and this condition is exacerbating the problems of antimicrobial pollutions (Castiglioni et al., 2005). It is understood that the wastewater treatment facilities cannot completely retain drug resistant bacteria from the final effluent, but can significantly lower the quantity of ARB from the discharged effluent though the proportion could still be very high compared to the raw wastewater influent (Cheng et al.2015). Disposal system of the sludge in the environments can also engineer the spread of different antimicrobial substances coming from different sources. Therefore, wastewater effluents can carry pharmaceuticallyactive substancesand contaminate soil and water after disposal and downstream use of reclaim wastewater for irrigation (Akimenko et al., 2015). In Ethiopia, reuse of effluent water is being promoted in order to meet the water demand of the suburban residents for small scale and urban agriculture, but there is a chance that antibiotic residues and ARGs could contaminate to human through the food chains(Kim & Aga, 2007).Thus,WWTPs serve as important reservoirs for commensal bacteria which potentially harbor antibiotic resistance determinants (Ferreira Da Silva et al., 2007).

Bacteria can acquire resistance genes against antibiotics vertically from chromosomal genes or horizontally from other strains by exchanging mobile genetic elements(Wang et al., 2013). As indicated in Figure 2.4, the mechanisms of horizontal gene transfer are alteration of target sites, change in membrane permeability, antibiotic efflux or expulsion, modification of the targets of

antibiotics and antibiotic inactivation by enzymes (Barbosa & Levy, 2000; Chroma & Kolar, 2010).

Mobile genetic elements conferring resistance to antibiotics are two types. Those introduced into the bacteria from external sources called the plasmids and conjugative transposons, and those elements moving from one location of the genome to the other in the same bacteria cell, including resistance plasmids, gene cassettes and insertion sequence common region (ISCR) promoted gene mobilization (Bennett, 2008). Plasmid transfer by conjugation (Figure 2.4) seems to be an evolutionarily conserved process. It starts with nicking the DNA by *relaxase* enzyme at a site called origin of transfer (*ori*T) which cause the single strand DNA pass to the receiving cell through a complex protein called the mating pair formation (*Mpf*) that serves as a channel for the DNA transfer. Some gram-negative bacteria such as *Enterococcus*, secret chemicals (pheromones) to stimulate the secretion of some adhesive substance by the plasmid donor cell (Berglund, 2014).

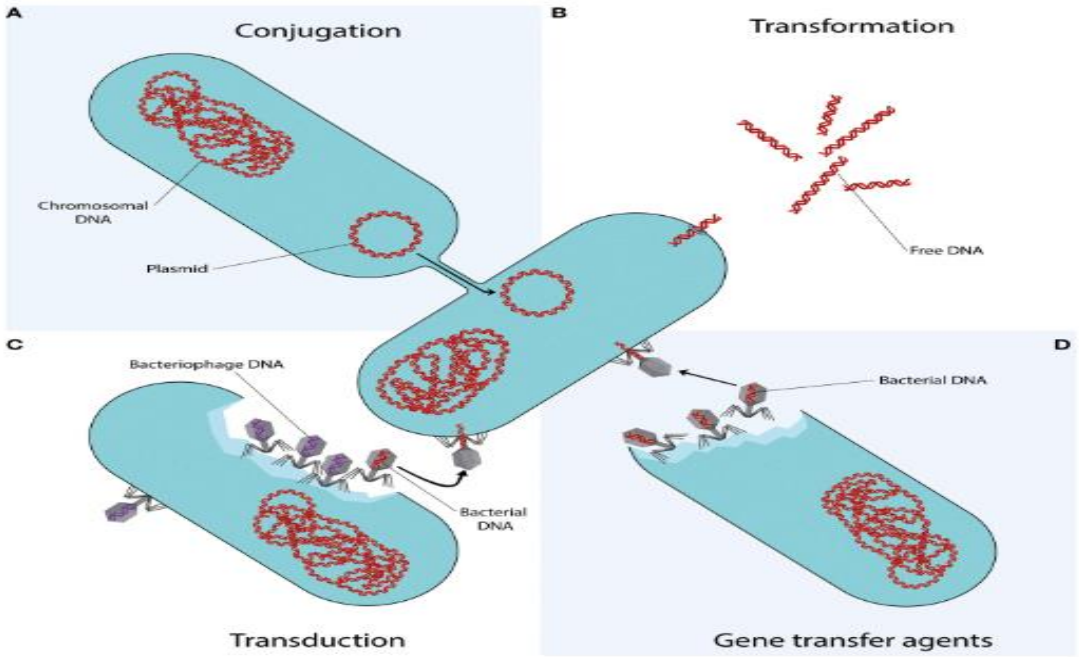

Figure 2.4. Mechanism of horizontal transfer of antibiotic resistance genes. (A) Conjugation is a process requiring cell to cell contact via cell surface pili or adhesins, through which DNA is transferred from the donor cell to the recipient cell. (B) Transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA. (C) Through specialized or generalized transduction, bacteriophages may transfer bacterial DNA from a previously infected donor cell to the recipient cell. During generalized transduction, bacterial DNA may be accidentally loaded into the phage head (shown as a phage with a red DNA strand). During specialized transduction, genomic DNA neighboring the prophage DNA is co-excised and loaded into a new phage (not shown). (D) Gene transfer agents (GTAs) are bacteriophage-like particles that carry random pieces of the producing cell's genome. GTA particles may be released through cell lysis and spread to a recipient cell (Source: Wintersdorff et al., 2016).

Horizontal movement of resistance determinants is carried out through mobile genetic elements mainly by plasmids, integrons, transposons or prophages, insertion elements, bacteriophages and genomic islands(Chroma & Kolar, 2010; D. Li et al., 2010). This happens between bacterial species in environments hosting large assembly of microbes such as the WWTPs and animal gut (Forslund et al., 2013). The location of ARGs on mobile genetic elements (MGEs) makes the transfer of resistance possible and easy to achieve among bacteria with the same or different origins (Karkman et al., 2018).

2.10. Methods of detecting Antibiotic resistance genes

There are different methods used for detection of ARB and ARGs in diverse environmental matrices using conventional culture-based and molecular culture-independent approaches. WWTPs are major reservoirs of ARB and ARGs to water bodies and the presence of ARGs in different environments can be detected by molecular and microbial techniques (Allen, 2014). Traditional culturing techniques, quantitative real time polymerase chain reaction, metagenomics and functional metagenomics are employed by several studies for resistance gene quantification. Molecular techniques provide rapid, sensitive and specific detection results without the need to carry out culturing and isolation. ARG detectionis important to investigate the predominance of

the genes in the environments and useful to correlate concentration of antibiotic residues with corresponding ARGS. DNA based methods gave advantage to detect the presence and abundance of ARB at the community level including the unculturable bacteria (Alexander et al., 2011). In the past few decades, DNA based techniques such as denaturing gradient gel electrophoresis (DGGE), Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Florescence in Situ Hybridization (FISH) were used to monitor the bacteria communities in WWTPs. In recent years the development of high throughput sequencing technologies such as the Illumina and Roche-454 provide remarkable throughput over the unculturable bacteria (Yang et al., 2016). These culture-independent systems are important to elucidate bacteria communities in different water types and it enable the recovery of bacteria having poor cultivability in aquatic environments(Vaz-Moreira et al., 2014).

2.11. Analytic methods for detection of antibiotic residues

Antibiotics are considered as emerging environmental contaminants even at low concentrations (ng/L to µg/L). Investigation on the presence and fate of antimicrobials in the environment needs to be carried out by accurate and sensitive methods (Batt et al., 2008). Antibiotic contamination to the environment is rising due to rapid population growth and increased health coverage. For instance, an estimated use of antibiotics between 100 and 200 thousand tons per year was published in 2002, but according to van Boeckel et al. (2015), global consumption of antibiotics increased by 36% between 2000 and 2010 and predict a further increase by 67% in 2030. As bioactive compounds, antibiotics are detectable at low concentrations in environmental samples,

but given their importance, continuous input and only partial degradation, the antibiotics are considered as emerging pollutants (Siedlewicz et al., 2016).

2.11.1. High Performance Liquid Chromatography–Mass Spectrometry

High performance liquid chromatography-mass spectrometry (HPLC–MS) and gas chromatography-mass spectrometry (GC–MS) have been used in determining antimicrobials in different environmental matrices. HPLC is a technique used for the purification, identification and quantification of individual components from a complex mixture in analytical chemistry. Antibiotics are special compounds in the analysis of pharmaceutical residuals in the environment due to their potential to cause drug resistance when contact with bacteria (Rossmann et al.,2014). Antibioticresidues can be quantified by HPLC-MS (Xu et al., 2015), which is the most frequently used technique for analyzing antibiotic residues coupled with mass spectrometry (MS). HPLC is a highly sensitive method that can separate a wide variety of antimicrobials. Analysis of contaminated wastewater samples can result in suppression of electrospray ionization and such problems can be solved by improving sample clean-up and quantification by internal standards or standard addition method (Babic et al., 2006). This sensitive analytical methods using solid phase extraction, followed by HPLC with fluorescence detection analysis have been developed to improve the current knowledge on the occurrence level of antibiotics in the water system (Ibraheem, 2012). HPLC is an attractive method of detecting antibiotic residues than the bioassay techniques in terms of the urgency, accuracy and precision of the results, and it is substituting cell based assays of antibiotic residue detection in different samples (Dafale et al., 2016).

Detecting typical environmental concentrations of antimicrobials from different water matrices is enhanced by using Solid Phase extraction (SPE) technique which is the method of choice for sample preparation. Therefore, the HPLC-MS is highly sensitive and selective tandem mass spectrometry analysis method for active antimicrobial residues from different water matrices. The highly sensitive and selective tandem mass spectrometry (MS/MS) is mostly used with HPLC to detect residues of the antimicrobials.The development and common use of sensitive analytical instruments like liquid or gas chromatographs coupled with mass spectrometers allowed the detection of trace concentrations of antimicrobial compounds in different environmental matrices (Siedlewiczet al., 2016). A combined SPE-LC-MS/MS method is therefore useful in the detection and analysis of antimicrobials from environmental water samples (Batt et al., 2008).

2.12. Bacterial community in wastewater treatment

Wastewater treatment processes use biological and physicochemical mechanismsdesigned to eliminate the pollutants from the wastewater(Shchegolkova et al., 2016). Biologicalwastewater treatment systems remove pollutants from the wastewater mainly by consortia of microorganisms (Giordano et al., 2016; Ranasinghe et al., 2012).The microorganisms consume nutrients from the wastewater as energy source and hence eliminate pollutants by transforming toxic compounds into harmless substances by biological oxidation and subsequent separation of microbial biomass from the treated effluent (Stalder et al., 2013). Effective and stable biological wastewater treatment system rely on the presence and functional diversity of microbial populations in the wastewater (Meerbergen et al., 2017). WWTPs receive raw influent containing divers species of bacteria which are presumed to be the main source of bacterial

diversity in activated sludge which is the most commonly used biological process of wastewater treatment (Lee et al., 2015).

Bacteria are the most important group of microorganisms in the biological wastewater treatment whose metabolic versatility and abundance can have useful impact to the efficiency and stability of the treatment process (Giordano et al., 2016). Understanding the diversity of bacteria communities in wastewater systems is useful as it gives insight on the bacteria dynamics in wastewater treatment bioreactors (Kim et al., 2013; Jabari et al., 2016). The diversity and dynamics of bacterial communities in wastewater has implications on the performance of the WWTP and thus effluent quality. However, most conventional wastewater treatment facilities are designed from the engineering perspectives often underestimating the dynamics and roles of microbial communities in the treatment process.

Wastewater treatment process removes oxygen-depleting organic materials, toxic substances, nutrients and reduce discharge of pathogens(Shah, 2014), including ARB from the WWTP to the environment and then to human and animal hosts(Vaz-Moreira et al., 2014). Hence, WWTPs are important biotechnological tools for prevention of environmental pollution by wastewater discharges which spread chemical and swage-borne pathogens (dos Santos et al., 2009). In tanneries, the physicochemical characteristics of the wastewater vary from tannery to tannery (Lee et al., 2015), and this can affect the diversity and dynamics of microbial community in the WWTPs(Liu et al., 2016).

Sewers and WWTPs are the principal collectors of commensal and pathogenic bacteria together with somefactors which can create suitable conditions for the spread of antibiotic resistant pathogens. The world is estimated to be inhabited by approximately 5×10^{30} bacteria, the vast majority of which are not pathogens (Finley et al., 2013). WWTPs host huge mass of

microorganisms including pathogens and some of these bacterial pathogens including those which come from fecal contaminations. One of the notable source of bacteria pathogens threatening public health is effluent discharged from WWTPs into the environment (Olaolu et al., 2014). This is mainly because primary sedimentation and secondary biological treatment process could only remove 20% and 80% of the pathogens from the wastewater, respectively (Lu et al., 2015). Wastewater is thus one of the routes for the transmission of zoonotic diseases from animals to human mainly through the fecalcontaminated water and foods. In tannery wastewater, animal skins and hides can carry fecal pathogens to the tannery WWTP and then reach the environment through tannery effluent. Thus, animal fecal microorganisms are sources of zoonotic pathogens including the antibiotic resistant ones(West et al., 2011).

2.13. Diversity of Bacteria community in Wastewater

The structure of bacteria community in WWTPcanindicatethe stability andperformance of the treatment process (Lee et al., 2015). Bacteria communities can be characterized by culture-based and culture-independent methods. Culture-based methods are still considered as “the gold standard” in microbial ecology studies, but almost 99% of the microbes found in the environment are yet to be identifiedby the culture system. Due to this limitation, the “complete” diversity of microbes in the environment cannot be described by the culture-based techniques (Kim et al., 2014). Most of the limitation of culture-based diversity studies are overcame by the emergence of high throughput culture-independent microbial technologies (Werner et al., 2011). Moleculartechniques including the clone-based libraries have provided insights in microbial community profile of complex environments such as the WWTP. However, these methods have difficulty of detecting low abundance microorganisms in wastewaterenvironments and may offer incomplete information about the diversity and structure of bacteria community (Wen et al.,

2015). The more recent 16S rRNA gene based metagenomic techniques are powerful option in the mining of bacteria community in WWTPs(Li et al., 2017).

2.13.1. 16S rRNA-based microbial community analysis

Environmental quality assessment using nucleic acids is expanding and enhance our understanding on the microbial structures beyond the minority of bacterial groups cultivable by classical microbial techniques (Vierheilig et al., 2015). Therefore, advances in molecular analysis of bacterial ecology are very important in exploring the diversity of bacteria in the wastewater treatment process by sequencing ribosomal RNA (rRNA) gene including the non-culturable ones (Jabari et al., 2016; Kim et al., 2014). Description of bacteria communities based on 16S rRNA gene sequencing is a good method in the study of bacterial communities in WWTPs(Allen et al., 2016). Thus, most culture-independent profiling of bacterial communities relies on the amplification and sequencing of the 16S rRNA genes and it improved ability of elucidation of the complex bacteria community in microbial consortia (Fadrosh et al., 2014).

The 16S rDNA gene based microbial analysis is one of the commonly used method in expounding bacteria since 1985 that it offers opportunity to design primers for amplification and sequence of hypervariable regions since the 16S rDNA genes contain both conserved and variable regions (Chakravorty et al., 2008; Fanning et al., 2017). As shown in Figure 2.5, the full length of 16S rRNA genes contain nine hypervariable regions inter-spaced with nine highly conserved regions (Yang et al., 2016).

Figure 2.5. Schematic location of the variable regions in the 16S rRNA gene (Barb et al., 2016)

2.13.2. High throughput sequencing and Data processing

High throughput sequencing (HTS) is one of thenew frontiers in microbial community analysis providing cost effective and rapid means of identifying the microbial phylotypes. Advances in sequencing technologies offered the opportunity to characterize microbial community by providing huge number of sequence reads at a lower cost (Nelson et al., 2014). Thus. HTS is very important in estimating the diversity of microbial community in different ecosystems(Caporaso et al., 2011).Compared to the classic Sanger sequencing methods, HTS has the advantage of generating multi-million sequences and thousands of Operational Taxonomic Units (OTUs) from environmental samples in a single run using instruments that enables sequencing of both abundant and rare community members(Shanks et al., 2013; Wan et al., 2017). However, the affordability of high throughput sequencing technologies came at the expenses of read length since most next generation sequencing (NGS) platforms generate short read (250 to 600 bp) from the nearly 1500 bp of the 16S rRNA gene in which increasing read length is generally accompanied by decrease in read accuracy (Burke & Darling, 2016).But, still advanced NGS has revolutionized the researchesapplied to describe the bacterial community and it became a new “gold standard” for bacterial diversity and composition studies (Ibarbalz et al., 2016). Illumina, Pacific Bioscience, Ion Torrent and SOLiDare some of the sequencing platforms which yield up to 600 giga bases of total sequence information and up to four million sequence reads per instrument run with read length of about 150 bases (Vierheilig et al., 2015).

2.13.3. Illumina sequencing

The Illumina sequencing methods involve the use of barcodes which are useful for the identification of large number of DNA sequences from environmental samples (Kim et al., 2014). Environmental studies have benefited from the advances of NGS technologies in terms of throughput, sequence read length and accuracy, and the diversity of bacterial community in such complex systems can be estimated using these sequencing technologies (Wu et al., 2015). Profiles of bacterial community in the environmental samples such as wastewater can be characterized by Illumina sequencing technologies which ease description of microbial ecology in such complex environments (Westcott & Schloss, 2015). Illumina Miseq technology, developed by Illumina Inc., sequencing surpass the 454-pyrosequencing technology (pyrotags) in terms of the cost, read quantity and read quality(Fadrosh et al., 2014). It can generate several giga bases of DNA sequence from environmental samples per each run using the chip-based bridge amplification procedure followed by sequencing by synthesis in the presence of reversible terminator dye nucleotides (Wen et al., 2015).

Illumina technology can generate 300 and 500 cycles of sequence data on HiSeq and MiSeq platforms respectively, and the cycles are commonly split into two paired reads of the same DNA fragments. The MiSeq platform can generate up to 8.5 Gbp using paired 250 nucleotide reads of about 17 million pairs of reads (Kozich et al., 2013). The Miseq platform has great flexibility, fast turnaround time, longer sequence reads and high accuracy which made the technology attractive in microbial ecology studies. Moreover, differentenvironmental samples can be sequenced together in a single run by using barcodes added during PCR amplification (Tremblay et al., 2015; Wu et al., 2015).

Illumina uses the reversible dye-terminator principle that allows addition of a single base to growing amplicons in each cycle and produce sequence reads of uniform lengths of paired 150

bp long sequencing which give a full length of about 254 bases with a 46 bp overlap (Figure 2.5). Sequencing runs can be monitored in real time using the Illumina sequencing viewer for cluster density, percentage of clusters passing filter, phasing/pre-phasing ratios, % of base, error rates, % reads with quality score ≥30 and other parameters (Wu et al., 2015). The paired-end sequencing is carried out using Illumina sequencing primers in two rounds of PCR, and the primers contain the Illumina adaptor sequences, an index sequence for the reverse primer, a 10 nucleotide pad to prevent hairpin formation, a two nucleotide linker that is non-complementary to the 16S rRNA gene specific primer (Kozich et al., 2013).

2.13.4. Post sequencing analysis and visualization techniques

Analysis of high throughput sequence data using bioinformatics tools is a basic step for elucidation of bacterial community in wastewater treatment. QIIME, Mothur, MEGAN, Vamps, RDP,etc.,are some of the most commonly used platforms in 16S rRNA gene sequence analysis (Ju & Zhang, 2015). The procedures of amplicon processing using bioinformatics platforms include data pre-treatment, OTU table construction and analysis of diversity using different data analysis tools (Figure 2.6).The complexity of environmental microbes and data sets generated from environmental samples can be simplified by clustering the NGS sequences into meaningful bins which are commonly called the OTUs. The OTUs are usable in comparing the diversity of microbes within and between environmental samples and this enable characterization of microbiota (Balvočiūtė, & Huson, 2017). One advantage of the OTU-based description is that the definition of the bins is operational and can be changed to suit the needs of the particular project (Westcott & Schloss, 2015).

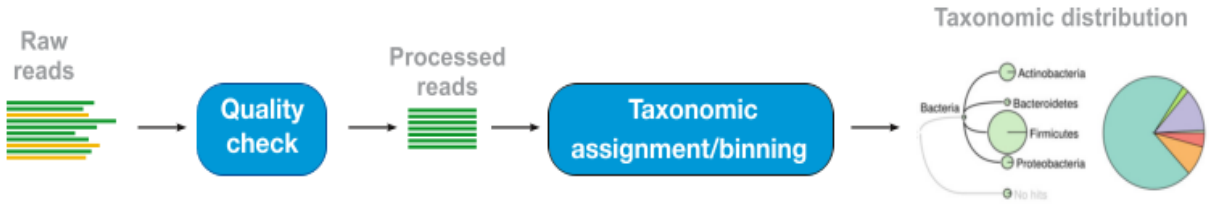

Figure 2.6. Illumina Miseq sequence read quality filter and binning workflow (Balvočiūtė & Huson, 2017).

Chapter Three

3. Materials and Methods

3.1. Description of Study Site

This study was carried out at Batu tannery WWTP located inside the compound of Batu tannery in Akaki Kality Sub City, Addis Ababa and at Little Akaki River (LAR). Batu Tannery is a medium size factory which produces finished leather from hides and skins. Batu Tannery has an average capacity of processing 8000sheep and goat skins, and 1000 cattle hide per day (UNIDO, 2012). The Tannery WWTP at the Batu Tannery applies a combined biological and chemical

enhanced coagulation processes performed in a series of pond systems (Figure 3.1). Raw Tannery influent enters to the treatment plant through three major channels. The largest volume of raw influent comes from the beam house operations and all the three channels finally mixed in sulfur oxidation-equalization pond which the operators called the general (G) wastewater. The deep blue colored chrome wastewater coming from tanyard operation is channeled into a separate pond from which the influent is then released to separate chromium recovery setup and after chromium recovery the wastewater is returned to the equalization (General)pond (Figure 3.1).

The general wastewater is released into aeration tank passing through small dye detection pond and after detention with aeration in the aeration pond the mixed liquor is pumped into coagulation pond. In the coagulation pond, the coagulants aluminum sulfate (alum) and ionic polymers are added and the wastewater flow to the sedimentation pond where flocculated biomass is separated from the effluents by gravitational force. Finally, the effluent is released to the LAR (Figure 3.3).

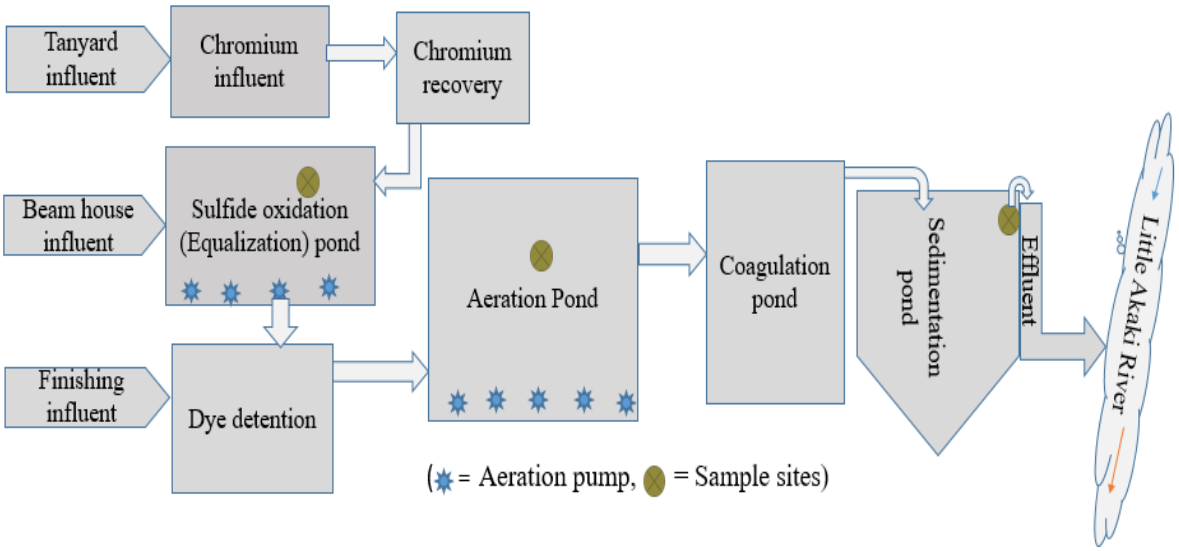

Figure 3.1. Schematic diagram of Batu Tannery wastewater treatment plant

3.1. Sample Collection and Preparation

Batu Tannery has wastewater treatment facility consisting sulfur oxidation, aeration and sedimentation ponds as well as separate chromium recovery system. The tannery WWTP receives raw tannery influent coming from segregated sections of the beam house, chrome tanning and finishing rooms. Samples of tannery wastewater were collected from the general (equalization) pond, aeration tank and at the discharge point of tannery effluent from sedimentation pond. LAR water samples were collected upstream from tannery effluent discharge point and downstream about 250 meters where the tannery effluent and river water effectively mixed after the tannery effluent discharge point (Figure 3.2 and Figure 3.3). From each site, grab samples were collected at different sampling points and the grab samples were mixed into composite samples. Wastewater samples from tannery were collected in the wet (August, 2015) and dry months of the year 2016-2017 namely in November, February and April months (2016/17) which corresponded to the wet and dry seasons, respectively. LAR water

samples were collected during the dry months. The samples were brought to Institute of Biotechnology and Bioinstrumentation laboratories, Addis Ababa University. Samples were stored at -21^{o}C until analysis was done, and duplicate samples were stored at the same freezing condition for molecular analysis.

Figure 3.2. A Satellite Map showing the location of Batu Tannery and the nearby Little Akaki River, Image date 1/19/2020, 8^{o}55’54.35” N, 38^{o}45’29.78 E, Elev. 7094 ft

The general influent wastewater (denoted as G) was sampled from the equalization pond, the aerated mixed liquor sample (denoted as S) was taken from aeration pond and the final effluent sample (denoted as E) was collected from tannery effluent after released from sedimentation pond (Figure 3.1). The sample sites henceforth are named as G, S and E, respectively and the numbers in the subscript showed the order of sample collection time during the sampling campaigns. Temperature, pH and conductivity (EC) were measured onsite using digital portable pH meter with separate probes for EC and pH (Thermo AP85 meter, Fisher Scientific, Singapore).

Figure 3.3. The upstream and downstream condition of LAR (Picture was taken in January 2017).

3.2. Physicochemical characterization of tannery wastewater &LAR water

The physicochemical characteristics of Batu Tannery wastewater was analyzed in wet month (August 2015) and dry months of 2016/17(November, February and April). Samples were analysed in triplicate following standard procedures of American Public Health Association (APHA) for the examination of water and wastewater(APHA, 1999; Krishnan et al., 2016). Selected physicochemical parameters were used to determine the characteristics of the tannery wastewater and LAR water. The parameters were pH, temperature, electrical conductivity (EC), Chemical Oxygen Demand (COD), Total Nitrogen (TN), sulfide, sulfate, total dissolved solids

(TDS), ammonia-N, nitrate and total chromium and analysis was done following the standard procedures of APHA.

3.2.1. Measurement of pH, Temperature and Conductivity

The pH, conductivity and temperature were measured on site by using portable multi-probe pH meter (Thermo AP85 meter, Fisher Scientific, Singapore) which has separate probes for the pH and conductivity detection.

3.2.2. Determination of COD and TDS

The TDS was measured to describe the concentration of dissolved inorganic and organic ions of tannery wastewater and LAR water. The TDS was determined by the thermogravimetric method (method 2540C). The wastewater was filtered using the No1 Whatman filter paper, and the filtrate (50ml) was poured onto the weighed crucible and dried at 180^{o}C for one hour and the crucible was weighed again after desiccation to determine the TDS.

The COD is used to estimate amount of degradable organic substances in the water and wastewater samples(Guasch et al., 2010). COD was determined by the modified closed reflux method (method5220). COD analysis was performed by mixing 2.5ml of diluted wastewater samples with 1.5ml of 0.25N K_2CrO_4, mercuric sulfate (Hg_2SO_4) and 3.5ml of COD reagent in a test tube and putting it on the COD digestion reactor at 150^{o}C for two hours. Digested samples were titrated with 0.1N standard FAS (ferrous ammonium sulfate) and ferroin is used as indicator and the saturation point was marked by the formation of reddish-brown color. Distilled water was used as blank with similar volume to the experimental samples(Islam et al., 2014).

3.2.3. Determination of ammonia-N, Total Nitrogen and Nitrate

Ammonia-N was determined by Nessler method adapted from standard methods(APHA, 1999)for the examination of water and wastewater (method 4500-NH_3 B & C). For testing ammonia-N, diluted 25 ml samples of tannery wastewater and water were added to sample cells (bottles) and the same volume of distilled water was used as a control. Three drops of mineral stabilizer were added to each sample cell and mixed thoroughly after which three drops of polyvinyl alcohol dispersing agent and finally 1.0 ml of Nessler reagent (potassium tetraiodomercurate, K_2HgI_4) was added. After one minute of reaction time, the mixture was read on spectrophotometer at 380 N for ammonia-N and recorded in mg/l.

Nitrate was determined by cadmium reaction method by taking 10ml wastewater and water samples into a standard sample cells and mixed with the powder pillow reagent. After a minute of reaction, the concentration of nitrate was determined on the spectrophotometer at a program of 355 N for nitrate and the result was recorded in mg/l. TN was determined by persulfate digestion method (method 4500 N) by digesting the sample at 105^oC for 30 minutes using persulfate reagent and the result was recorded in mg/l N by reading the sample mixture at 395N program on the spectrophotometer.

3.2.4. Determination of Sulfate, Sulfides andChromium

The concentration of sulfide was determined by the methylene blue method (method 4500 S2- D) and sulfate was measured by the turbidimetric method (method4500-SO_4^{2-} E). Sulfide was measured using sulfide 1 and sulfide 2 reagents and reading was done at 690 sulfide programs.

The presence of sulfide was indicated by the formation of blue color after addition of the sulfide reagents and the result was recorded in mg/l S^{2-}. Total chromium was determined by inductively coupled plasma mass spectrometry (Agilent 7900) method and partly by atomic absorption spectrometric method. All analyses were conducted in triplicateand the results were reported in mean and the standard deviations reflect triplicate analyses. All protocols were adapted from standard water and wastewater analysis methods (APHA, 1999).

3.3. DNA Extractionfor Bacteria community Analysis

Genomic DNA was extracted from the tannery wastewater samples. The tannery wastewater samples were prepared in duplicates for DNA extraction. DNA extraction was carried out using Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's protocol. Briefly, 300mg of sediment samples were added to lysing matrix tube to which 978 μl sodium phosphate buffer was added and vortexed for 15 seconds. A 122 µl MT buffer was added and samples were homogenized by a bead beater (BioSpec, Bartlesville, OK, USA) for 40 seconds. Sample homogenate was centrifuged at 14000g for 10 minutes and supernatants were transferred to clean microcentrifuge tube to which 250µl PPS (protein precipitating solution) was added, shaken ten times, incubated at room temperature for 10 minutes and centrifuged for 5 minutes. The supernatant (800µl) was transferred to new sterile tubes and equal volume of binding matrix was added, gently shaken and inverted five times. Finally, the DNA was eluted using 100µl DES solution. The integrity of DNA extract was verified on 1% agarose gel electrophoresis using 1xTAE buffer and quality and concentration of DNA extract was verified at 260/280nm by NanoDrop1000 UV-Visible spectrophotometer (ND-1000 Thermo Fisher Technologies, USA). By the same procedure blank water was used during

DNA extraction as a negative control and no detectable DNA concentration was recovered from the water sample.

3.3.1. DNA Extraction for ARG Detection

Total genomic DNA was extracted from tannery wastewater samples collected at the three different sampling sites of the treatment plant and wastewater samples were concentrated by centrifugation at 5000 rpm for 10 minutes and 300mg concentrated sludge pellets was used for DNA extraction from each sample. DNA extraction was performed using Fast DNA SPIN kit for soil (MP Biomedicals, Solon, USA) according to the manufacturer's protocol. In each sample, 100µl of elution buffer was used for DNA dissolution. The DNA from each sample was extracted in duplicate and the integrity of the DNA extract was verified by 1% agarose (Thermo Scientific) gel electrophoresis.The concentration of extracted DNA was checked using NanoDrop spectrophotometer, ND-1000 (Nano Drop Technologies, INC. Wilmington, DE, USA) and by QuBit fluorometer (Invitrogen, Carlsbad, California, USA). After quality and quantity determination, the DNA extract was stored at -21^{o}C in 2ml Eppendorf for antibiotic resistance genes (ARGs) assay using standard Polymerase chain Reaction (PCR) method.

3.4. Illumina Sequencing of 16S rRNA Genes

Sequencing of the genomic DNA extract obtained from tannery wastewater samples was carried out by Illumina Miseq technologyat the sequencing core in the University of Michigan (Medical College, An Arbor, Michigan, USA). Sequencing was performed using 2x250 paired-end chemistry (Figure 3.4).Amplicon library was generated from the V_4 region of the 16S rRNA gene after two step amplification of the DNA fragment using universal dual index primers 515F/ 806R

(Mwaikono etal.,2015; Saunders et al., 2015; Hien et al., 2017),and DNA was amplified in two stage PCR with the primers (Wen et al. 2015). The amplicons were indexed by barcodes and adaptors which allow sequencing on the same flow cell and easier demultiplexing during sequence data analysis (Fouhy et al., 2015).

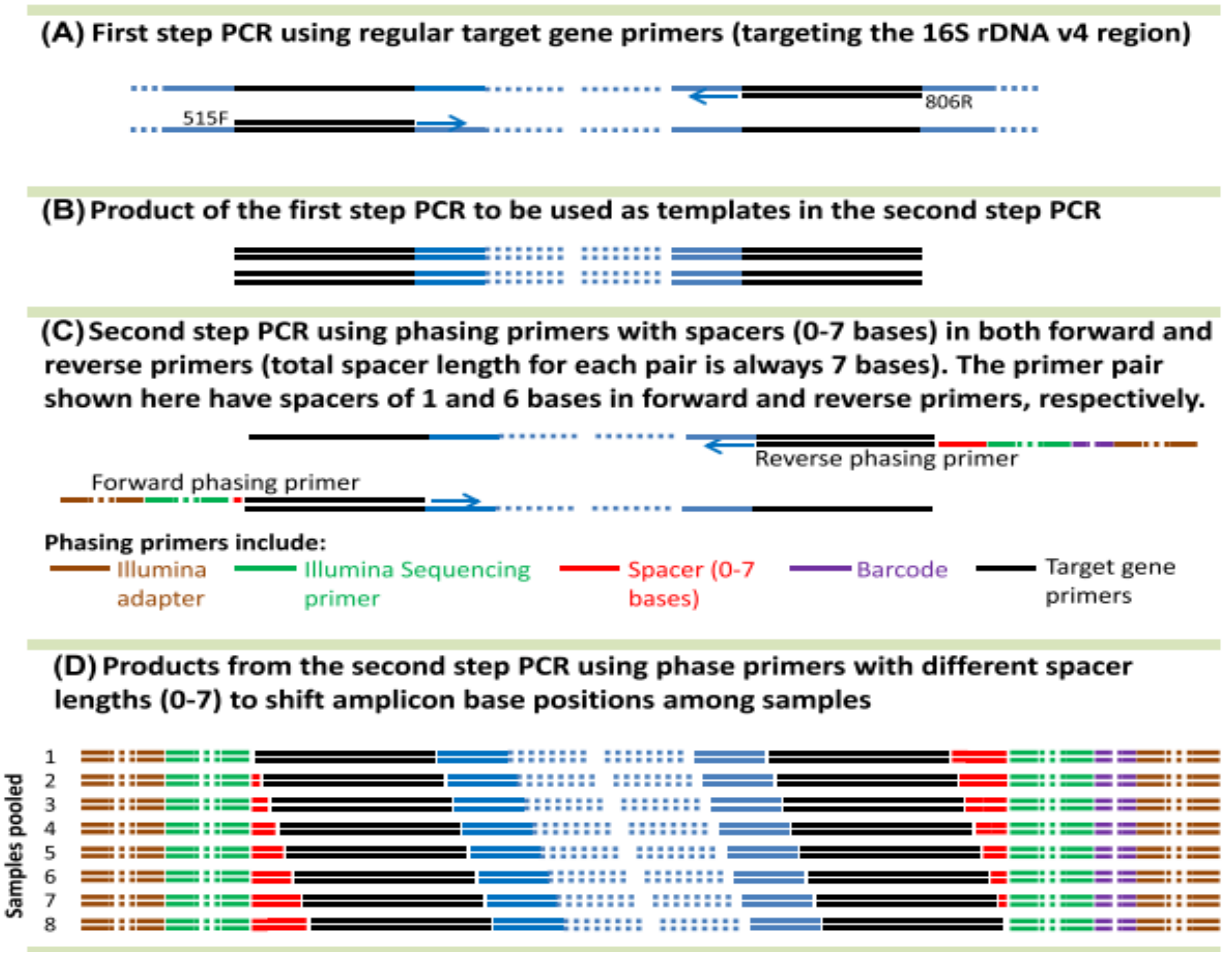

Figure 3.4. Amplification process of the V_4 region of 16S rRNA using 515F and 806R primers (Wu et al., 2015).

The primer sets and the V_4 region of 16S rRNA was chosen for sequencing as they give reasonably good length of paired-end Miseq sequence reads (253 bp long) that can provide ample information for taxonomic assignments of bacteria (Fadrosh et al., 2014; Wu et al., 2015).

The second set of PCR amplification was carried out using the first amplification product as a template. After sequencing, the sequence reads were collected in the fastq format.

3.5. Antibiotic residue detection in the tannery wastewater

3.5.1. Sample preparation for Solid phase extraction

Antibiotic residues and ARG detectionswere carried out from tannery wastewater samples. The tannery wastewater samples were collected from three sites in the treatment plant by sterile plastic bottles. The samples were general raw tannery wastewater (G), aerated mixed liquor (S) and treated effluent (E) collected from general (equalization and sulfur oxidation) pond, aeration pond and at the final tannery effluent discharge point after sedimentation, respectively. The samples were prepared by centrifugation and filtration for solid phase extraction (SPE) and DNA extraction. Centrifugation was done at 5000 rpm for 10 minutes by which suspended solid materials were separated as a pellet while dissolved substances remain in the supernatant. The supernatant was filtered by 0.22 μm glass filters (Millipore, Billerica, MA, USA), and 250ml of the filtrate was used for SPE and samples were stored at -21°C until further molecular analysis was carried out.

3.5.2. Solid Phase Extraction and Recovery of Antibiotics

Residual antibiotics were extracted from tannery wastewater samples by SPE techniques following the standard methods. Briefly, 250ml tannery wastewater filtrates were extracted using Oasis Hydrophilic-Lipophilic balanced (HLB) cartridges (60ml, 3ml, Waters, Milford MA, USA) from each sample (Kulkarni et al., 2017). The purpose of SPE was to retain antibiotic residues by adsorbing to the Oasis HLB cartridges compatible with samples of all pH ranges and adsorb both polar and non-polar compounds simultaneously.The Oasis HLB cartridges contain

both hydrophobic and lipophilic balance of polymers for a reversed phase interaction and strong cation exchange for selective retention of the antibiotics as analyte. Before loading the filtrate samples, the HLB cartridges were preconditioned with 2 x 6ml acetonitrile by gravity and after conditioning the sample was loaded to the cartridges at controlled flow rate. The HLB cartridge was washed with 5% methanol to remove adsorbed substances other than antibiotics and allowed to dry on the manifold for 30 minutes by pulling air through them.

The antibiotic analytes were eluted from the cartridges using HPLC grade 2 x 6 ml acetonitrile on SupelcoVisiprep 12 port Manifold (Figure 3.5, a). Blank Cartridges were also washed twice by acetonitrile (2 x 6ml). After elution, the extracted acetonitrile-antibiotic solution was concentrated to 0.2ml by evaporating the elution with gentle nitrogen steam using the Nitrogen Evaporator (Figure 3.5, b). After evaporation of acetonitrile, Nano pure water was added to antibiotic extract to 1ml volume on the pre-calibrated test tube and vortexed to dissolve and suspend any analyte dried on the side of the tube.

a) SupelcoViseprep, SPE Vacuum Manifold b) N-Evaporator

Figure 3.5. Antibiotic extraction and recovery apparatus using (a) Supelco, Viseprep Manifold and (b) concentrating antibiotic residues by using N-Evaporator apparatus

3.5.3. HPLC-MS Analysis of Antibiotic residues

Detection of antibiotic residues was carried out by liquid chromatography mass spectrometry (LC-MS) using Thermo Scientific Exactive Plus Orbitrap™ HRAM (high resolution accurate mass) spectrometer. Calibration solutions were prepared from the stock solutions provided by Dr. Aga Group (SUNY, Buffalo) and purchased from Sigma-Aldrich resulting in 6 levels of antibiotics for quantitative analysis. Dilutions were made in laboratory water (Millipore-grade) to create six different calibration levels. The antibiotic calibration samples were acidified with formic acid to a concentration of 0.1% formic acid. The concentration range varied for each compound but was in the approximate range of 1 ppt to 10 ppb.

A validated chromatographic method was used for the detection of antibiotic residues in the tannery wastewater. The antibiotic analytes considered in this study were tetracycline, oxytetracycline, penicillin G, streptomycin, penicillin V, trimethoprim, sulfonamides, amoxicillin and ampicillin. Detection and analysis of antibiotic residues was carried out from influent (G), aerated mixed liquor (S) and effluent (E) tannery wastewater samples by using high performance liquid chromatography (HPLC) techniques. HPLC was performed using the Thermo Scientific EQuan MAX system which consists of two high pressure liquid chromatography (HPLC) pumps, autosampler and switching valves (Figure 3.6). The first HPLC pump, a Thermo Scientific Ultimate 3000 pump, was used to transfer the 1ml sample volume from the autosampler loop to the loading column (Thermo Scientific Hypersil Gold Q column,

20 x 2.1 mm, 12 µm) at a flow rate of 1.0 ml/min. After 1.2 minutes, a six-port valve was switched to back-flush the loading column onto the analytical column (Thermo Scientific Accucore Q column, 50 x 2.1mm, 1.9µm).

The analytes were eluted using a reversed-phase gradient from the second HPLC pump, called the Thermo Scientific Ultimate 3000 RS pump. The mobile phases were water and methanol, both containing 0.1% formic acid and the total run time was 16 minutes. Result acquisition and quantitation was carried out using Trace Finder™ software. The theoretical mass of each protonated antibiotic compound was used as the mass for quantitation in this analysis. Calibration curves were created for each compound, using either a linear or quadratic curve and fit with either a linear or quadratic curve.

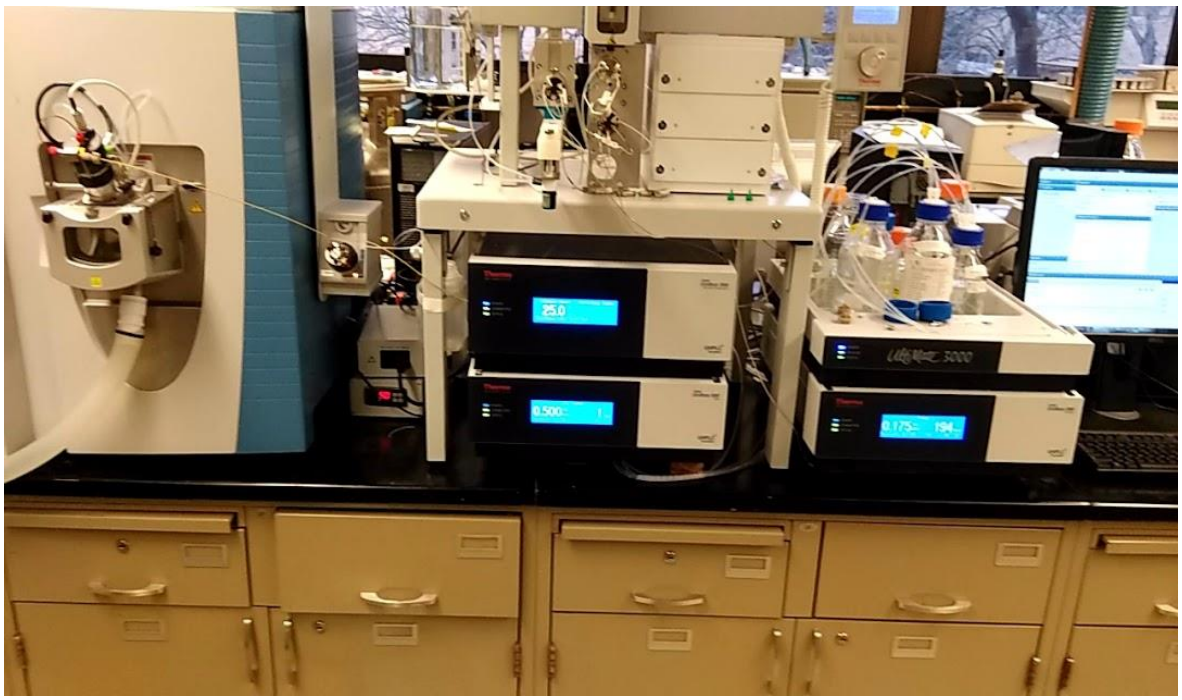

Figure 3.6. High pressure Liquid chromatography-MS(HPLC-MS) determination of Antibiotic residues (University of Michigan, water and environment engineering laboratory)

3.6. ARG assay by Polymerase Chain Reaction

The presence of antibiotic resistance genes in the Batu tannery wastewater was detected by qualitative PCR assay using DNA extracted from raw general influent (G), aerated mixed liquor (S) and final effluent (E_1 and E_2) tannery wastewater samples. ARGs assay in the tannery wastewater includedthetetracycline (*tetA, tetO, tetQ, tetM),*Oxytetracycline *(OtrA),* sulfonamide (*SulI, SulII),* quinolone (*qnrA*) and macrolides(*ermB*) resistance genes using different primers.

Table 3.1. The PCR primers used in the detection of ARGs from Batu Tannery wastewater samples.

ARGs	**Primers**	**Oligo Sequences (5'→3')**	****T_a**	***Size**	**References**
tet(A)	Tet(A)- F	TTGGCATTCTGCATTCACTC	53	125	(Veslemoy et al., 2015)
	Tet(A)-R	GAAGGCAAGCAGGATGTAGC	53		
tet(M)	Tet(M)-F	CAACGAGGACGGATAATACGC	56	191	(Veslemoy et al., 2015)
	Tet(M)-R	CCATCTTTTGCAGAAATCAGTAGA	56		
tet(O)	Tet(O)-F	AAGAAAACAGGAGATTCCAAAACG	57	75	(Veslemoy et al., 2015)
	Tet(O)-R	CGAGTCCCCAGATTGTTTTTAGC	57		
tet(Q)	Tet(Q)-F	AGAATCTGCTGTTTGCCAGTG	55.9	169	(Auerbach et al., 2007)
	Tet(Q)-R	CGGAGTGTCAATGATATTGCA	55.9		
Otr(A)	Otr(A)- F	GGCATYCTGGCCCACGT	60	212	(Awad et al., 2015)
	Otr(A)-R	CCCGGGGTGTCGTASAGG	60		
Sul(I)	Sul(I)-F	CGCACCGGAAACATCGCTGCAC	66.8	163	(Negreanu et al., 2012)
	Sul(I)-R	TGAAGTTCCGCCGCAAGGCTCG	66.8		
Sul(II)	Sul(II)-F	GATATTCGCGGTTTTCCAGA	52.7	141	(Veslemoyet al., 2015)
	Sul(II)-R	CGCAATGTGATCCATGATGT	52.7		
QnrA	qnrAf-RT(F)	ATTTCTCACGCCAGGATTTG	53.3	124	(Negreanu et al., 2012)
	qnrAr-RT(R)	GCAGATCGGCATAGCTGAAG	53.3		
erm(B)	erm(B)-91fc	GATACCGTTTACGAAATTGG	49	364	(Negreanu et al., 2012)
	erm(B)-54rc	GAATCGAGACTTGAGTGTGC	49		

*Size refers to length of amplicons, ** T_a = Annealing Temperature

The genomic DNA used in PCR reaction was diluted 10 times by DNase and RNase free Molecular Biology grade PCR water (Water ultra-pure, Quality Biological, USA). For detection of ARG, pairs of primer sets supplied by the Integrated DNA Technologies (Coralville, Iowa, USA) were specifically used for each ARG. The PCR reaction was carried out in a 20 μl reaction volume of the following reaction mixture: 2µl DNA template (50ng), 0.4µM primers (20µM), 10µl 2x Phusion Flash High-Fidelity PCR master mix, 0.25µl Bovine Serum Albumin (BSA, 50mg/ml) and 6.95µl nuclease free PCR water. PCR amplification was performed using Applied Biosystems thermocycler according to the following modified programs of (Rintala et al., 2017): Initial denaturation at 98^{o}C for 1min, 35 cycles of denaturation at 98^{o}C for 1sec, different annealing temperature for each primer pairs presented in Table 3.1 and extension at 72^{o}C for 4 seconds and final extension at 72^{o}C for 1 min, and holding temperature of 4^{o}C.The PCR products of each ARG was run on 1.5% agarose gel electrophoresis stained with SYBR safe DNA Gel stain (Invitrogen, Thermo Fisher Scientific, USA) and the gel was visualized by UV-transIlluminator (Bio-Rad Laboratories, 6000 Alfrednovel, Hercules, CA, USA) and the images were captured.

3.7. Assessment ofAntibiotic use Pattern in Livestock

Cross sectional survey on the use pattern of antibiotics in livestock was carried out in eight selected districtsin Amhara region. The study area was selected based on the economic activities, agro-ecology and absence of similar studies in the region and data was collected using structured questionnaires. The region is one of the major producers and supplier oflive animals, skin and hides to the leather producing industries. This survey was carried out from June 2017 to February

2018 to evaluate the antibiotic use system in the rural areas for livestock treatment. The surveillance was carried out to estimate the carryover effect of antibiotic use in animals to the environment through the use of animal skin and hides in the tanneries.

The study population consisted of 150 participants from which 55% (n = 83) were farmers involved in animal fattening and45% (n = 67) were veterinary clinicians selected by purposive sampling method.The purposes and confidentiality of the data were disclosed to respondents and data were collected after consent was made by each respondent. Farmer respondents were separately interviewed using questionnaires, but veterinary clinicians were given the questionnaires containing open and close end items about the use of antimicrobials in livestock treatment. The questionnaire also included items about the location of animal fattening centers and the animal waste disposal approaches in the study areas.

3.8. Data analysis

Illumina Miseq sequence reads were analyzed using Mothur (version 1.36.1) platform. Sequence reads were filtered and denoised to remove low quality and ambiguous reads using the filter and screen codes. The two sets of reads (R_1 and R_2) were overlapped and combined to form contigs using the function make.contigs. Chimeric sequences were removed using the UCHIME algorithm embedded in Mothur by checking against chimera free data bases of 16S rRNA gene sequences following the sequence binning work flow. Sequence alignment was carried out using Silva reference database (www.arb-silva.de, version 123). Quality filtered sequences were assigned to taxonomic identities by reference database project (RDP) classifiers (Wan et al., 2017), and sequences were clustered into OTUs at 97% similarity threshold level using UCLUST algorism embedded in Mothur.

Bacterial community richness was compared using rarefaction analysis after sub-sampling (Vrieze et al., 2016). Diversity indices (Shannon, Simpson, and Inverse Simpson) were calculated and the number of OTUs in each sample was used in estimating the abundance, diversity and evenness of bacteria community (Wen et al., 2017). Principal coordinate analysis (PCoA) was also generated from OTU table. The PCoA calculates the distance matrix for each pair of samples based on the sampling sites and turn the distances into points in a space of few dimensions. The observed core bacteria in each sample wereplotted in heatmap at genera level using *clustvis* (https://biit.cs.ut.ee/clustvis/) software (Metsalu & Vilo, 2015), and OTU identification was done using BLASTn (www.ncbi.org).

Descriptive statistics was used to determine the mean and standard values of physicochemical data, and the standard deviations were computed as a measure of variance from triple sample values. Statistical data analysis was performed using statistical package for social studies (SPSS) software version 23.0 and Microsoft excel (2016). Physicochemical data were calculated from triplicate sample analysis and the results are given as mean values with standard deviations. One-way analysis of variance (ANOVA) was done to test the mean difference between the upstream and downstream LAR water parameters before and after receiving tannery effluent at alpha level of 0.05.

Chapter Four

4. Results and Discussion

4.1. Physicochemical characteristics of tannery wastewater in the wet month

The physicochemical characteristics of tannery wastewaterbased on the analysis of composite raw tannery influentand 'treated' effluent samples collected in the wet (rainy) and dry months is presented in Table 4.1 and Table 4.2, respectively. Thedata showed that the raw tannery wastewaterhasobservable variations in COD, ammonia-N and chromium between G1 and G2 samples, but the difference in the amount of sulfide, TDS and total nitrogen (TN)concentration among the G1 and G2 tannery wastewater samples was not significant ($p > 0.05$).On average, the tannery WWTPreduces the COD by58%, TDS by 49.5%, sulfides by 68.5%, ammonia-N by 87%, TN by 62.5%, and chromium by 71% in the wet month. In all cases, it was observed that almost all parameters in the G2 samples were higher than concentration in the G1 samples, except total chromium. This showed that there was temporal variability in the strength of raw tannery influent. The temporal variation in the strengths of raw tannery wastewater could result from the type of raw material (skin or hide), amount and type of chemicals used in the tanning processes and types of final product (Jahan et al., 2014).

ThepH and temperature of the tannery wastewater were significantly ($p < 0.05$)reduced in the treated tannery effluent (E1 and E2) samples and bothcomplied with the minimum discharge limit permitted by the Ethiopian Environmental Protection Authority (EPA) for the discharge of tannery effluent.However, the concentration of COD, TDS, TN, sulfides and total chromium

werehigher than thestandards for the discharge of tannery effluent into the surface water (Table 4.1). For these parameters, thequality of the treated tannery wastewater were 1.5 to 63 times higher than the provisional discharge limit ofEthiopian EPA for tanneries(EPA, 2003). This showed that the treatment plant requires additional effluent polishing systems either before (such as Imhoff) or after the secondary treatment system (such as the use of constructed wetland).

Despite the incompliance to the Ethiopian EPA discharge limit, the tannery WWTP showed encouraging performance for pH, COD, TN, ammonia-N, sulfide and chromiumthat the concentrations of these parameters significantly ($p < 0.05$) reduced after the treatment process. This can be demonstrated by the apparent difference in pollution load between the general influent (G1 and G2) wastewater and treated effluent (E1and E2)in the wetmonth samples.However, sulfate was an exception to this trend that its amount in the E1 and E2 samples showed contrasting fate as it decreased in the E1 sample while itwas slightly increased in the E2 sample (Table 4.1).

Table 4.1. Physicochemical characteristics of Batu tannery wastewatersampled in wet (August 2015) month (mean ± STD). Sources for discharge limit is EPA(2003).

Parameters	G1	E1	G2	E2	Discharge Limit
pH	7.97±0.1	7.5±0.1	8.3±0.1	7.9±0.1	6.0-9.0
Temp(°C)	20.3±0.5	18.3±0.5	20.0	17.3±0.5	40
EC (mS/cm)	21.1±0.1	14.4±0.1	25.5±0.1	13.5±0.1	NA
COD (mg/l)	2103±30	1066±58	3994±942	1333±67	500
TDS (mg/l)	4307±5	2223±72	4646±11	2254±133	NA
Sulfide(mg/l)	191±3.9	58±1.7	212±1.3	68±3.4	1
Sulfate(mg/l)	571±13	98±1.3	646±4.3	669±5.1	NA
Nitrate(mg/l)	0.9±0.8	1.1±0.1	7±0.8	1.6±0.1	NA
Ammonia(mg/l)	32±2.6	1.4±0.2	59±1.9	12±1.3	30

TN (mg/l)	289±3	110±1.7	321±3	118±4	60
Total Cr(mg/l)	37±0.9	4.8±0.2	13±1	5.8±0.3	2

G_1= raw 1st round, S_1= mixed liquor 1st round, E_1 =effluent 1st round, G_2= raw 2nd round, S_2=mixed liquor 2nd round, E_2= effluent 2nd round, NA= Not Available, Temp = Temperature, EC = Electrical conductivity, COD = Chemical Oxygen Demand, TDS = Total Dissolved Solid, TN =Total Nitrogen

As presented in Table 4.1, the tannery effluent did not meet the discharge limits for most parameters in the wet month. However, the physicochemical values of raw tannery wastewater in the Batu tannery in this study was comparable to previous studies at Mojo tannery (Haile Reda, 2016), Dire tannery (Mekonnen Birhanie et al., 2017), Bahir Dar tannery (Assefa Wosenie and Ayalew Wondie, 2014), Sheba tannery (Abrha Gebrekidan et al., 2009). However, theperformance of Batu tanneryWWTP was lower than the integrated tannery wastewater treatment at Modjo tannery which meet the discharge limits for most the studied parameters(Adey Feleke et al., 2014). These studies demonstrated that the physicochemical data in the tannery effluent still cancause pollution to surface water since some of these tanneries release insufficiently treated effluent to the nearby rivers. The release of insufficiently treated tannery wastewater to rivers is damaging the ecosystem and impair the river water quality for downstream use(Haile Reda, 2016).

4.2. Characteristics of the tannery wastewaterin the dry months

The physicochemical properties of Batu Tannery wastewater were analyzed before and after treatment in the dry months of the year 2016/17, and the result of the tannery wastewater is presented in Table 4.2. The results showed that the tannery wastewater was laden with high concentrations of pollutantsin the drymonths, compared to the wet month presented in Table 4.1.The same physicochemical parameters were analyzed for the LAR water in order to evaluate the impact of tannery effluent on the physicochemical characteristics of the LAR water.

Comparative analysis was made between upstream (before receiving) and downstream LAR water before and after receiving tannery effluent, respectively.

Table 4.2. Physicochemical characteristics of raw and treated tannery wastewater in the dry monthsof the year 2016/17, Data presented in Mean±Stdev. Sources for discharge limit is EPA(2003).

Parameters	November		February		April		Discharge Limit
	Raw (G3)	Treated (E3)	Raw (G4)	Treated (E4)	Raw (G5)	Treated(E5)	
pH	8.4±0.4	7.2±0.2	8.6±0.2	8.1±0.1	9.0±0.2	8.6±0.1	6 - 9
Temp (°C)	19.3±0.6	17.8±0.3	21.5±0.5	20.2±0.3	22.8±0.3	21.2±0.3	40
EC (mS/cm)	26.7±0.2	22.6±0.1	36.8±0.2	31.5±0.2	18.8±0.1	16.3±0.2	-
COD (mg/l)	5979±281	3322±87	7084±56.7	3075±37	7204±8.1	3529±1	500
TDS (mg/l)	5812±397	3092 ±63	4320 ±5.5	2173±46	4342 ±13.4	2126±7.2	-
Ammonia-N(mg/l)	111.0±8.5	87.3±6.1	222.0±5.3	103.7±7.4	190.0±3.6	82.7±4.0	30
TN (mg/l)	249.7±5.7	102.0±2.0	275.0±3.6	122.3±3.5	308.0±2.0	141.7±2.5	60
Nitrate	3.9±2.6	8.3±0.6	0.0	0.5±0.0	0.0	5.3±1.5	-
Sulfide (mg/l)	185±23.8	50.3±4.0	280±14.5	104±12.7	192±9.3	91 ±4.6	1
Sulfate (mg/l)	126.7±5.0	212.7±7.1	344.7±9.5	310.3±8.6	250.0±5.6	281.3±4.2	-
Total Cr(mg/l)	16.3±2.1	5.7±2.1	23.7±2.1	12.0±2.7	22.0±2.7	11.9±0.8	2

Raw = tannery wastewater before treatment, Treated = tannery wastewater after treatment, Temp = Temperature, EC = Electrical Conductivity, COD = Chemical Oxygen Demand, TDS = Total Dissolved Solid, TN = Total Nitrogen, Total Cr = Total Chromium, G3 = Raw tannery wastewater collected in November, G4 = Raw tannery wastewater collected in February, G5 = Raw tannery wastewater collected in April

4.2.1. pH, Temperature and Conductivity

In the current study, the mean temperature and pH of treated Tannery wastewaterwas within the range of discharge limit for tannery effluent to the rivers according the discharge limit set for

tanneries(EPA, 2003). At relatively higher temperature (21.5-22.8^{o}C) of the tannery influent, better COD, ammonia-N and TDS removal were observed in the treatment process, but total-N and sulfide removal was higher at relatively lower temperature (19.3±0.6). This corroborates with the notion that temperature influencesthe performance of WWTP and affect quality of the discharged effluent (Hoque & Khan, 2016).

The mean pH of raw tannery wastewater in the dry months was between 8.0 and 9.0. The highest pH (9.0±0.21) was recorded in the April (G5) month, but the lowest mean pH (8.4±0.4) was observed in the November (G3) sample (Table 4.2). In the treated effluent, the pH was between 7.5 and 8.6, and the highest pH was recorded in April (E5). The data showed that pH of tannery effluent in the dry months was generally ranged with in the tolerance limit of the tannery effluent in accordance with the Ethiopia EPA guidelines (EPA, 2003). Mean EC (mS/cm) of raw tannery wastewater was in the range of 18.8±0.1 in April (G5) to 36.8±0.15 in February (G4) months, and EC was in the range of 16.3±0.2 (E5) to 31.5±0.2) EC (E4) in treated wastewater showing 13.3% (E5) to 15.4% (E4) reduction of the EC after the tannery wastewater treatment operation (Figure 4.1).The range of mean temperature in the raw tannery wastewater was between 19.3±0.6 and 22.8±0.3 and between 17.8±0.3 and 21.2±0.3 for the treated tannery effluent in the three dry months.

The pH of raw tannery wastewater in the current study was generally in the range of 6.0-9.0 and the final effluent met the discharge limit for pH of tannery effluent. Comparable pH to this study was reported in combined tannery wastewater treatment system (Agrawal & Singh, 2016; Krishnan et al., 2016), composite raw tannery wastewater in India (Goswami & Mazumder, 2016), but higher than the pH from tannery wastewater (pH of 4.1) treated by an electrocoagulation process in Turkey (Deghles & Kurt, 2016). The pH and EC results of the

current study was also comparable to the physicochemical characteristics reported at Mojo tannery wastewater , Ethiopia (pH 8.33 ± 0.01 to 9.33 ± 0.06 and EC in μScm^{-1} of 14,496.67 ± 40.41 to 15,670.00 ± 20) (Haile Reda, 2016).

4.2.2. COD and TDS

As presented in Table 4.2, the range of COD (mg/l) value in the raw tannery wastewater was between 5674 and 7213 in the dry months. After treatment, the COD was reduced by 44.4%, 56.6% and 51%in the treated tannery effluent samples of the November, February and April months, respectively. Similarly, the TDS (mg/l) value of the raw tannery wastewater was in the range of 4315 and 6236, but reduced in the treated effluent to the range of 2118and3144in the dry months, showing the TDS was removedby 46.9%, 49.7% and 51%in the November, February and April months, respectively (Figure 4.1).This indicated that the COD and TDS content of treated tannery effluent exceeded the discharge limit for tannery effluentpermitted by the Ethiopian EPA guidelines. The COD in both the dry and wet month samples was lower than the COD (12,913±6874.7 mg/l) of raw tannery wastewater reported in the Dire tannery (Mekonnen Birhanie et al., 2017), in the RMM Leather industries Ltd (12,840mg/l) (Jahan et al., 2014) and in composite chrome tannery wastewater (11,981 mg/l COD) of common effluent treatment unit(Goswami & Mazumder, 2016).

Likewise, the COD in the final effluent was reduced from 44.4% to 56.6%, but it was still above the permissible discharge limit for tanneries. In contrast to tannery wastewater treatment by using yeast (Stanley et al., 2017) and by UASB reactor (El-Sheikh et al., 2011), the COD and

TDS removal efficiency of Batu tannery wastewater treatment plant was reasonably lower in this study. A similar 40% to 70% COD removal from comparable tannery influent strength of 7255mg/l was reported by El-Sheikh et al(2011). This demonstrates the COD removed in the tannery effluent was lower than the total theoretical biodegradable COD (57.4%) of the wastewater (Pire-Sierra et al., 2016).

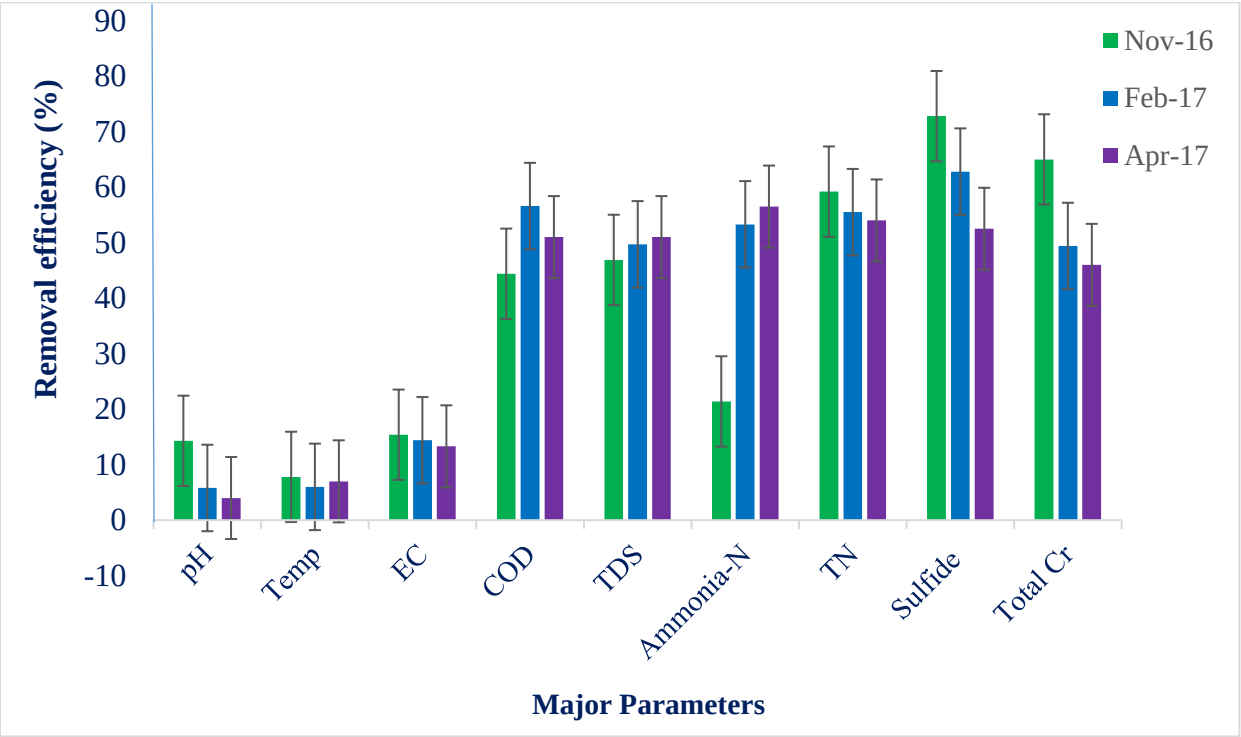

Figure 4.1.Removal efficiency of the tannery wastewater treatment for some of the wastewater quality parameters analysed in the dry months (Nov = November, Feb = February, Apr = April).

As shown in Figure 4.1, the TDS (mg/l) in the treated effluent was reduced by 46.9% to 51% in the treated tannery samples of the dry months. The TDS removal efficiency of the tannery WWTP might be influenced by the slight alkalinity (8.0 - 9.0) of the raw tannery wastewater (Hashem et al., 2016). On the other hand, high concentrations of the TDS in the tannery wastewater attributes to the presence of soluble substances mainly the sulfates and chlorine

(Sugasini & Rajagopal, 2015).A closeTDS valueof 5557 mg/l tannery wastewater to thecurrent TDS value was reported inchemical enhanced treatment plant (CETP)(Bhatnagar et al., 2013), and in the TDS of raw tannery wastewater of 5966.1 to 7068.2mg/l treated by coagulation (Shegani, 2014). TDS observed in the raw and treated tannery in this study was three times lower when compared to the TDS in the raw (34,200mg/l) and treated (28,600mg/l) tannery wastewater(Mangal et al., 2013). The TDS in this study was also lower when compared to the TDS value in the raw tannery wastewater (14000 ±59.99mg/l) of complete leather production (Chowdhury et al., 2013). Although, the TDS removed in the Batu tannery WWTP is higher than these reports, it was still above the permissible discharge limits for the tannery effluent to the river according to EPA(2003) guidelines cited in(Solomon Sorsa et al., 2015).

4.2.3. Ammonia-N, Total Nitrogen and Nitrate

The mean concentration of ammonia-N(mg/l)was ranged between 102 and 228 in the raw (untreated) tannery wastewater samples of the dry months (Table 4.2). After the treatment process, the amount of ammonia-N was reduced by 21.3% in E3to 56.5% in E5samples resulting in the range of 79 to 112mg/l ammonia-N in the treated tannery effluent. As presented in Figure 4.1, total nitrogen (TN) was also reduced by 54% to 59.2% after the treatment process, but a range of 100 to 144 mg/l TN was still remainedin the treated tannery effluent (Table 4.2).In all cases, the ammonia-N and TN remained above the permissible discharge limit of the tannery effluent to discharge into inland surface water. The presence of high concentration of ammonia and TN in the treated tannery effluent showed lower performance of the treatment plant in removing these nitrogen pollutants when compared to moving-bed biofilm reactor (MBBR) which removed 97% of the TN that was in the range of 126mg/l to 626mg/l in the raw tannery wastewater(Ding et al., 2016). In the raw tannery wastewater, mean nitrate concentration of

7.5±4 mg/l was recorded in one of the dry month's (G3) sample, but low concentration of nitrate was present in the treated effluent samples with mean value of 8.3±0.6 (in E3) and 5.3±1.5 (in E5). The presence of low concentration of nitrate in the raw tannery wastewater is not surprising, but its occurrence at low concentration in the treated effluent suggested that there is unsuitable condition in the aeration pondfor the functioning of ammonia oxidizing and nitrite oxidizing bacteria in the treatment process.

4.2.4. Sulfide, Sulfate and Total Chromium

Sulfides are one of the harmful pollutants associated with the tannery wastewater (Table 4.2). The range of sulfide (mg/l) in the raw tannery wastewater was from 168 in G3 to 294mg/l in G4. After going through the treatment stages, sulfide was reducedby 72.8%, 62.8% and 52.5% in theE3, E4 and E5tannery effluent samples respectively. As shown in the Figure 4.2, the lowest sulfide removal was observed in the April (E5) sample. The removal of sulfide can be attributed to the oxidation processes particularly in the equalization pond where sulfide was treated by air stripping (Midha et al., 2008).

Sulfate is another pollutant in the tannery wastewater (Table 4.2). In the current study, the range of sulfate (mg/l) concentration in the raw tannery wastewater was between 122mg/l and352mg/lin the dry months. After going through the treatment process the fates of sulfate was variable as it was increased by 39.5% in the E3 and by 11% in E5 samples, but reduced by 10% in the E4 sample (Table 4.3). A higher mean sulfate value (1240mg/l) than this study, but comparable sulfide (156mg/l) to the current study was reported in similar chemically enhanced primary tannery wastewater treatment system(Haydar & Aziz, 2009). However, the mean sulfate and sulfide contents of raw tannery wastewater in this study was lower when compared to

previously reported data at the Dire tannery (Mekonnen Birhanie et al., 2017) and Mojo tannery wastewater in Ethiopia (Tadesse Alemu and Seyoum Leta, 2015).

The refractory fate of sulfate in the treatment process may be related with the scheme of the Batu tannery wastewater treatment system which lacks anaerobic stages. In the absence of anaerobic pond, sulfate is unlikely to reduce into sulfide, but in the aerobic condition sulfide can be oxidized into elementary sulfur that is removable during flocculation. The treatment set up at Batu tannery wastewater treatment system seems to be suitable for sulfide removal by the combined sulfide stripping and coagulation steps. But sulfide occurred in the treated effluent at concentrations more than the recommended discharge limit. This can be the effect of pH variability across the treatment plant which has impact on the sulfide removal. According to Santos et al (2014), the alkaline pH condition in raw tannery wastewater could facilitate the generation and elimination of sulfide, but at pH greater than 9.0 sulfide may remain in the dissolved state.

Chromium is another serious pollutant associated with tannery wastewater (Table 4.2). In this study, the mean concentration of total chromium (mg/l) in the G3, G4 and G5 samples was 16.3±2.1, 23.7±2.1 and 22.0±2.7 respectively. In the final effluent total chromium was reduced by 67.5%, 49.4% and 46% after treatment.At these values, the amount of total chromium in the tannery effluent was up to five times higher than the permissible discharge limitof chromium in tannery effluent set by Ethiopian EPA (2003). An average comparable chromium concentration (9±0.11) to this study was reported in a complete leather production wastewater (Chowdhury et al., 2013), and from common effluent treatment plant (Bhatnagar et al., 2013). Nevertheless, the total chromium in the raw Batu tannery wastewater was relatively lower when compared with the

total chromium of Modjo tannery (Adey Feleke et al., 2014), and Dire tannery wastewater(Mekonnen Birhanie, et al., 2017).

4.3. Physicochemical impactof Tannery effluent on the LAR water

The physicochemical characteristic of LAR water was analyzed before and after receiving tannery effluent in order to evaluate the impact of tannery effluent on the river water (Table 4.3). The water quality data showed that most of the physicochemical parameters of LAR waterwere very higheven before receiving the tannery effluentexcept the temperature and pH.As presented in Table 4.3, after receiving tannery effluent released periodically, the pH, temperature and EC of the downstream river waterincreased little in the dry months. Low disturbance in temperature of the LAR water after receiving tannery effluent showed that there is no thermal pollution of the LAR water associated with the discharge of tannery effluent from the Batu Tannery WWTP during the study months. However, the EC of LAR increased by 5.5% in November, 6.9% in February and 15.9% in April months after receiving tannery effluent showing that average contribution of tannery effluent to the EC of the LAR was 9.4% in dry months (Figure 4.2).

Table 4.3. Physicochemical characteristics of Little Akaki River (LAR) before and after receiving Batu Tannery effluent in the dry months of the year 2016/17(mean value ± standard deviation)

Parameters	November 2016		February 2017		April 2017		P-value
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream	
pH	8.1±0.1	8.7±0.1	8.7±0.1	9.2±0.2	7.2±0.2	7.8±0.3	0.073
Temp (^{o}C)	21.5±0.6	21.3±0.1	20.8±0.1	20.7±0.1	22.4±0.6	22.7±0.3	0.794
EC (μS/cm)	57.7±1.5	61.1±0.9	41.5±0.8	44.6±0.4	35.3±3.1	42±1.0	0.347
COD (mg/l)	1102.1±95	1146.4±16	1210.4±69	1330.5±52	1023±9.5	1130.4±43	0.047
TDS (mg/l)	9875±7.0	11204±8.7	7364±11.2	8157±39.3	8248±18.0	9099±32.5	0.108
Ammonia-N	137±6.2	158.3±6.5	86.3±2.5	105.7±3.2	43.7±1.5	51±2.1	0.213

(mg/l)							
TN (mg/l)	168±12.5	218.3±6.0	122.7±6.1	137±4.6	92.7±1.5	106±1.5	0.445
Nitrate (mg/l)	46±2.0	48.3±1.5	59±4.4	59.5±4.4	13.7±2.5	16.7±0.6	0.838
Sulfide (mg/l)	33±4.6	42.7±1.5	84.3±2.5	94.7±3.2	54.3±7.2	61±4.6	0.422
Sulfate (mg/l)	467±7.0	539±21.2	347±4.4	449±8.3	420±2.6	549±7.5	0.001
Total Cr (mg/l)	21.3±2.1	31 ±1.7	11.3±2.5	15.7±1.5	9.3±1.5	19.7±2.1	0.001

Upstream = Before receiving tannery effluent, Downstream = After receiving tannery effluent, Temp = Temperature, EC = Electrical Conductivity, COD = Chemical Oxygen Demand, TDS = Total Dissolved Solid, TN = Total Nitrogen, Tot Cr = Total Chromium

The pH of LAR water slightly increased after receiving the tannery effluent, showing the tannery effluent had no notable impact on the pH of the LAR water as the pH of LAR water remained unaffected after receiving tannery effluent in all study months. This can be associated with the volume of the LAR water which has a dilution factor and the presence of other chemicals that could reduce the pH of the slightly alkaline tannery effluent. On the other hand, the slight increase in the pH of the LAR water after receiving the tannery effluent seems to result from the presence of free ammonia in the tannery effluent (Prabu et al., 2011). In addition to the tannery effluent, the LAR receives effluents from several residential locations and industries such as the breweries, wineries, pharmaceuticals, distillers and alcohol liquor industries all of which are on the peripheries of the LAR (Yared Worku and Giweta, 2018).

4.3.1. The COD and TDS

The mean COD (mg/l) concentration in the upstream LAR water was in the range of 1022 to and 1212 in the dry months, but the downstream COD concentration of the LAR increased by 3.8%, 9% and 9.5% in the November, February and April months, respectively after receiving tannery effluent (Figure 4.2). Similarly, the TDS (mg/l) concentration in the upstream LAR water was in the range of7356mg/l in Februaryand 9872mg/l recorded in November months. TheTDS of LAR

water increased by 11.8%, 9.7% and 9.4% in the November, February and April months,respectively after receiving treated tannery effluent. In river waters, TDS is an important parameter for assessing the suitability of the river water for irrigation.When evaluated by this principle, theTDS of the LAR waterwas high that it is not suitable for irrigation. The high TDS content of the LAR water is suspected to account for the severely turbid physical appearance of the LAR water which wasdarker during the sampling times.

By average, the tannery effluent causes an increase in the LAR water quality by about 10% for most parameters (Table 4.2). This showed that the impact of tannery effluent on the quality of the LAR water seems to be insignificant ($p > 0.05$)for most of the parameters at study months. However, the pollutants can accumulate over time in the river sediment and affect the ecosystem as some of the chemicals such as the chromium has environmental persistence and longer half-life (Tadesse Alemu et al., 2019).

4.3.2. Ammonia, Total Nitrogen and Nitrate

The concentration of ammonia-N (mg/l) in the LAR after receiving tannery effluent increased by 13.5%, 18.4% and 14.8% in the November, February and April months, respectively (Figure 4.2). Similarly, TN of LAR water increased by 23%, 11% and 13% in the November, February and April samples, respectively after receiving the tannery effluent. Unlike the ammonia-N and TN, the nitrate concentration in the LAR was not significantly ($p > 0.05$)increasedafter the tannery effluent was released that the downstream nitrate content of the LAR water remains unchanged (Table 4.3). This shows the nitrate content of tannery effluent was low to cause change in the nitrate content of the downstream river water, but the ammonia-N and TN contents

of the downstream river showed consistent increase, although is was statistically insignificant ($p > 0.05$).

4.3.3. Sulfides, Sulfate and Chromium

In the upstream LAR water samples, the mean sulfide concentration was 84.3±2.5mg/l in the February, but it was lower (33±4.6 mg/l) in the November. The sulfide concentration of downstream LAR water was also proportionally higher in the February with about 11% increase after the tannery effluent was discharged into the river. The sulfate concentration in the LAR water was also increased by 13.4%, 22.8% and 23.5% in the downstream samples of the November, February and April months, respectively (Figure 4.2). The amount of total chromium in the upstream LAR samples was 21.3±2.1, 11.3±2.5 and 9.3±1.5 in the November, February and April months respectively. This resulted in the increment of total chromium concentration in the downstream LAR water by 31%, 28% and by 52.8% in November, Februaryand April months, respectively (Figure 4.2).

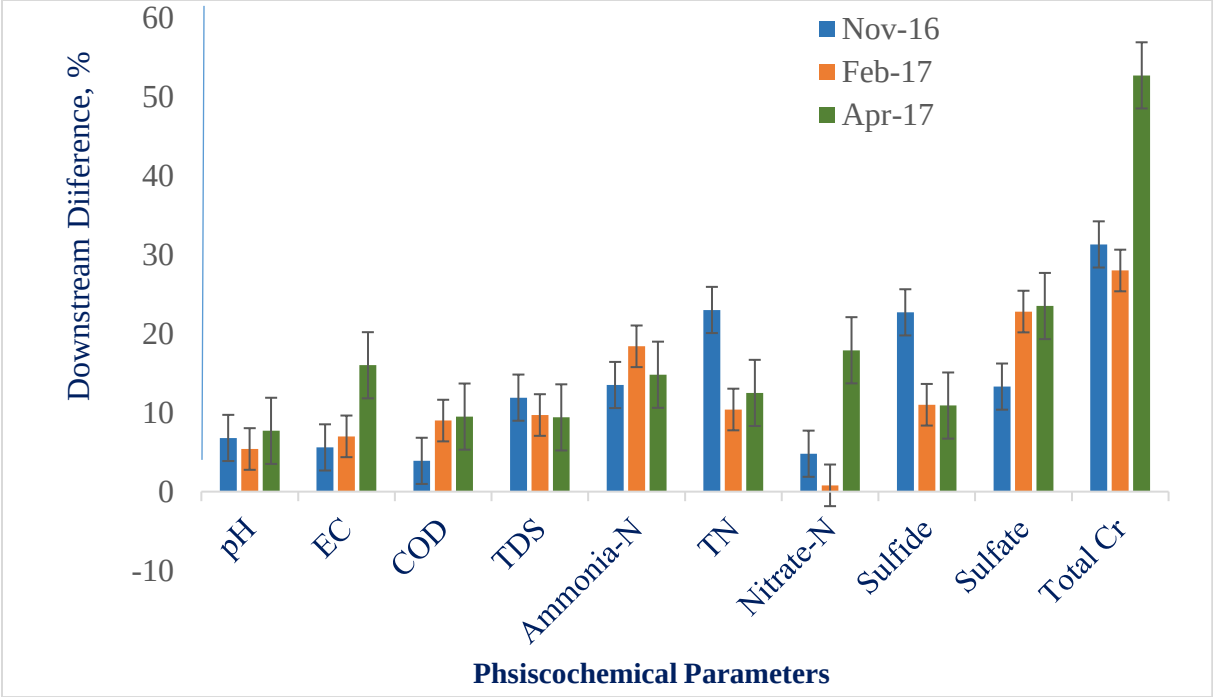

Figure 4.2. The percentage difference in the physicochemical characteristics of downstream LAR water after receiving tannery effluent in the dry months (Nov = November, Feb = February, and Apr = April).

In the current study, the EC, TDS, and COD of LAR water was 36, 13 and 2 times higher than the report in 2007 (Samuel Abegaz, 2007), but the pH and temperature reports remain very close. This showed that the quality of LAR water is deteriorating very rapidly. The sources of pollutants for the LAR water are suspected to be industrial and domestic effluents discharged to the river since the river is flowing across the residential and industrial sites of the city (Ferezer Eshetu , 2012). As shown in Table 4.3, the average COD (mg/l), TDS, ammonia-N, total-N, sulfide, sulfate and total chromium of the river water increased by 7.5%, 10%, 14.7%, 17%, 13.5%, 19.7% and 51.6% in the downstream river water, respectively. This showed that the release of tannery effluent into the river exacerbate the pollution problem that the river is suffering from the upstream sources.

The TN, ammonia-N, pH, and temperature were very close to the water quality reports of river contaminated by tannery effluent, but the TDS, COD and sulfides were higher in the current study than the report on Modjo river (Haile Reda, 2016). The nitrate concentration in the LAR water in the current study wasalso lower than the previous report (189 ± 319 mg/l) in the dry seasons (Frezer Eshetu, 2012). On the other hand, the increase in the water quality parameters of the LAR water may bepronounced by the decreasing volume of the river water in thedry months. The physicochemical characteristics of LAR water were generally higher in February and lower in the April months. This can be linked with the seasonal variation in the flow rate, volume of the river water and variance in the volume of sewer streams released from residential and commercial centers of Addis Ababa city to the river (Taddese Animaw, 2011).

Although the averageimpact of tannery effluent on the river water was about 10% on average for most of the water quality parameters on the LAR water, continuous release of tannery effluent to the river in the long term can severely affect the river ecology and exacerbates the ongoing pollution problems that the river faces. The LAR water in the dry months of the study year has unpleasant physical appearance showing the current condition of the river is not fit for human, animal and irrigation uses. The aesthetically displeasing appearance of the river water result from high concentration of dissolved and suspended pollutants and this is affecting the sustainability of the river (Gupta et al., 2014).

In order to evaluate the statistical significances of tannery effluent on the LAR water, One-way ANOVA analysis was done. One-way ANOVA determine the relationship or differences of water quality parameters between the upstream and downstream sampling sites of the LAR water at 95% significance level ($\alpha = 0.05$). The ANOVA analysis revealed that there are significant ($p < 0.05$) difference in the mean concentrations of the LAR water for the COD, total chromium

and sulfate between the upstream and downstream samples (Table 4.3). However, the ANOVA does not show significant ($p > 0.05$) difference in the water quality parameters of pH, temperature, EC, sulfides, NH_3-N, Nitrate, TN and TDS between the upstream and downstream samples.

Although there was significance difference ($p < 0.05$) in three parameters only, there is still increase in the percentage of the other parameters in the downstream of LAR water after receiving tannery effluent. In the upstream LAR water, unexpected high amount of nitrate was observed, but there was no significant ($p > 0.05$) increase in nitrate of the downstream river water after tannery effluent was released. This is likely due to the low nitrate profile of the tannery effluent that was too low to cause difference in the amount of the nitrate in the LAR water. A more concerning observation was the presence of relatively high concentration of total chromium in the LAR water which increased by up to 51% after tannery effluent is released to the river. The data revealed the presence of significant difference ($p < 0.05$) between the mean values of total chromium in the upstream and downstream LAR water (Table 4.3). The increase in the amount of chromium could be pronounced by the decreasing volume of the river water in the dry months when there is a reduction in the flow rate and volume of the river water. For most of the studied parameters the impact of tannery effluent to the physicochemical condition of the river water was not statistically ($p > 0.05$) significant. However, the physical and chemical conditions of LAR water is below the recommended standard quality of surface water to use for human, animal and agricultural purposes.

4.4. Structure of Bacteria community in the tannery wastewater

The Illumina Miseq sequencing produced an initial 2.29 million reads from the tannery wastewater samples that was processed and filtered using Mothur (version 1.36.1) pipeline. Low quality sequence reads were removed and the surviving forward and reverse reads (R1 and R2) were assembled generating 1,791,453 initial contigs which later reduced to 1,433,740 good contigs, and final high quality 1,387,715 reads withzero ambiguity and maximum of 253 bp parameters. The Illumina sequenced reads showed a minimum 99.7% sequence coverage in all samples with a range of 142,198 to 308,999and average of 231,286 reads (Table 4.4).

The purified reads were assigned into the taxonomic ranks using the RDP classifier from phylum to the genus levels at the threshold of 97% similarity. A total of 2466 bacterial operational taxonomic units (OTUs) were obtained that are classified into 28 phyla, 62 classes, 123 orders, 242 families and 441 genera.Nearly 99% of the total sequences were annotated to the phyla, 83% to the class, 67% to the order, 56% to the family and 39% to the classified genera taxonomic ranks. The highest numbers of OTUs were found in the aerated mixed liquors samples (S1=1059 and S2=1008), followed by the effluent wastewater (E1=874 and E2=873). Low numbers of OTUs were obtained in the raw (G1) tannery wastewater samples (Table 4.4).

The sequence data showed that 96% of the total sequence reads of the domain eubacteria obtained from the treatment belong to the four phyla namely Firmicutes, Bacteroidetes, Proteobacteria and Synergistetes (Figure 4.3). Among them the Firmicutes was the most abundant phylum constituting a total of 48% in all sample points. The second most abundant phyla wereBacteroidetes with relative abundance of 32% followed by Proteobacteria (11.5%) and Synergistetes (5%) in decreasing order. In the E1 and E2 samples, Synergistes was the third dominant bacteria, but it was Proteobacteria which was the third prevalent phylum in the other samples (Figure 4.3).

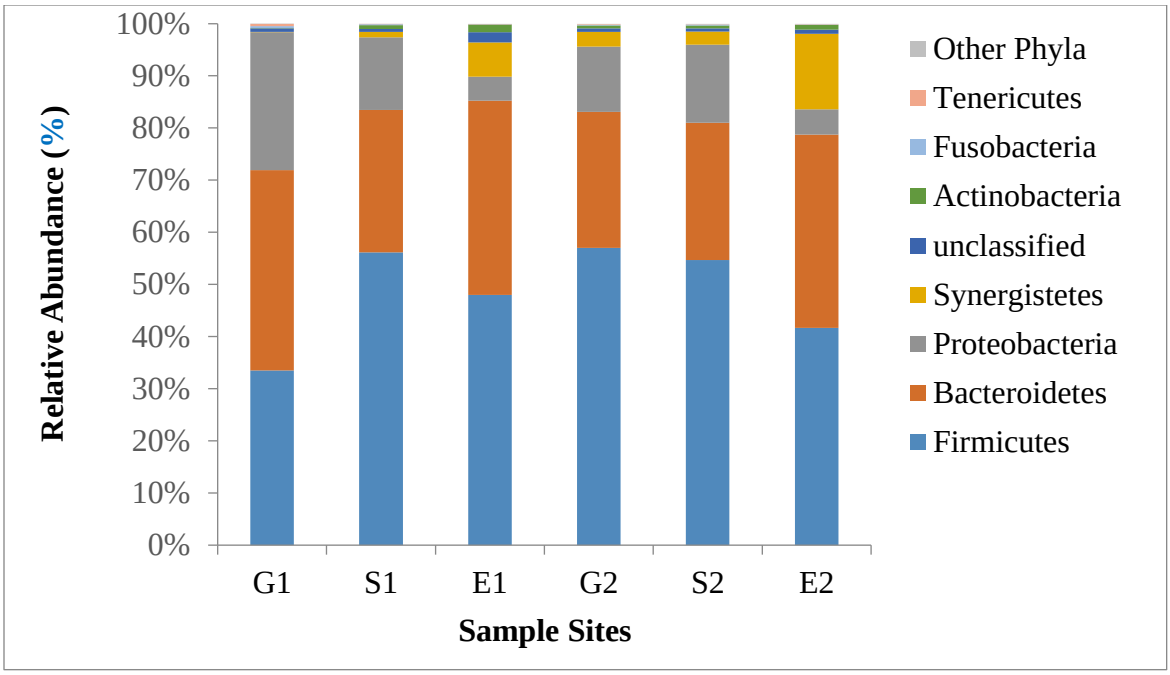

Figure 4.3. Relative abundance of bacteria at the Phylum level in the tannery wastewater treatment plant (Other phyla refers to all other phyla identified in the study but not presented in the graph).

Firmicutes and Bacteroidetes alone make over 80% of the total sequences showing their exclusive dominance in the treatment plant. At phylum level, only 61% of the phyla were found in G1 (n=17) sample, but the G2 sample possessed 75% of the total phyla (n=21). The difference in the abundance of bacteria phyla between G1 and G2 samples suggested that there is temporal variation in the microbial richness of raw influent tannery wastewater (Sheik et al., 2012).

The pattern of bacterial phyla dominance with Firmicutes, Bacteroidetes and Proteobacteria was similar with clone library based 16S rRNA sequence analysis of an integrated tannery wastewater treatment system in Ethiopia (Adey Feleke et al., 2014). Firmicutes was also indicated to be the most dominant phylum inthe tannery wastewater treatment bacteria communitystudy conducted by Krishnan et al.(2016) and in the Up-flow anaerobic sludge

blanket (UASB) membrane bioreactor (MBR) process (Qiu et al., 2013). In another study on the bacteria community of the tannery sludge in Italy, Proteobacteria and Bacteroidetes were the most abundant phyla (Giordano et al., 2016).

Relative abundance of Firmicutes in the chrome tannery wastewater in this study was also close to the study carried out in soil bacteria community contaminated with chromium (Desai et al., 2009), in coal-mine wastewater (Ma et al., 2015) and untreated municipal wastewater in the USA (Shanks et al., 2013). Similar dominance of Firmicutes, Bacteroidetes and Actinobacteria was also reported in anaerobic biogas plant(Sun et al., 2016). The dominance of these bacteria in the tannery wastewater may be related with the high organic load and high inorganic nitrogen species in the tannery wastewater and these bacteria could have important roles in carbon and nitrogen metabolism.

The structure of bacteria communities in the tannery wastewater was further analysed at the lower taxonomic levels (Figure4.4).Accordingly, at the class level, Clostridia (33%), Gammaproteobacteria (10%) Lactobacillales (9%), Bacteroidia (5.5%) and Synergistes (5%) were the most dominant groups in the tannery wastewater (Figure 4.4). Under the Proteobacteria, the Gammaproteobacteria, and Epsilonproteobacteria were dominant in all stages of the treatment process, and within the Firmicutes;Clostridia, Lactobacillus, and Bacillales were more dominant in the same stages of the tannery wastewater treatment. Among the Firmicutes, the Clostridia alone constitute 67% of the class level dominance in the tannery WWTP, and among the Bacteroidetes and Proteobacteria the Bacteroidia and Gammaproteobacteria represent 17% and 83.6% of the within the phylum abundance, respectively. Next to the Gammaproteobacteria, Epsilonproteobacteria and Deltaproteobacteria were the other abundant bacteria lineages of the phylum Proteobacteria.

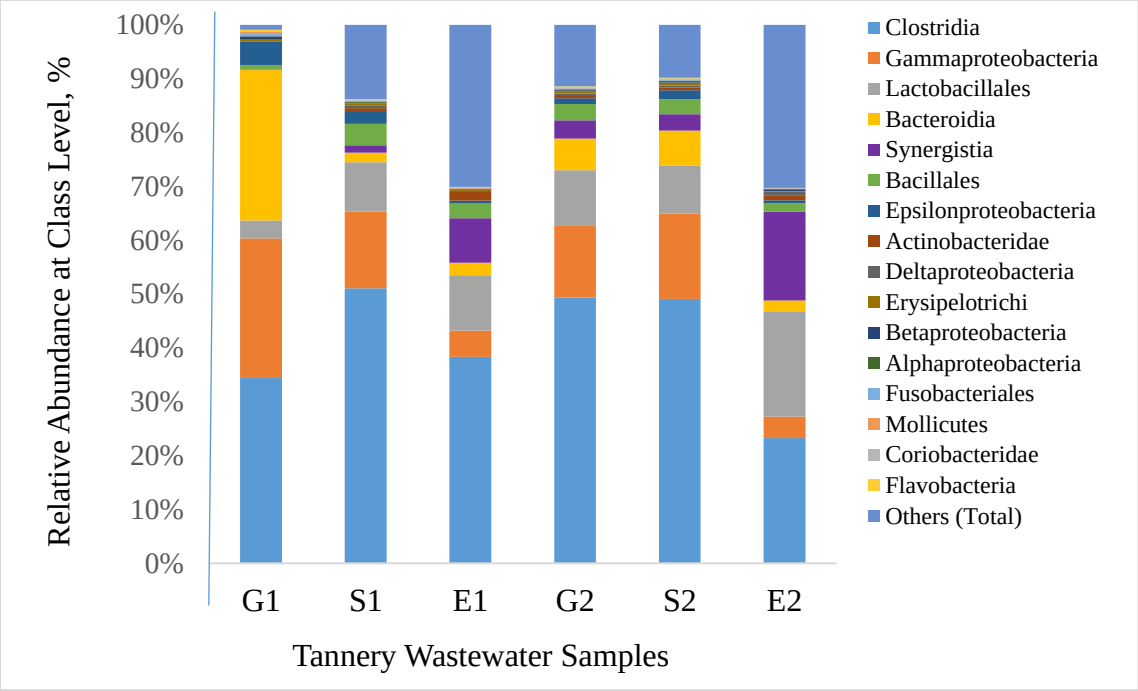

Figure 4.4. Relative abundance of bacteria at class level in the Tannery wastewater. Color coded bars illustrate the percentages of reads for each class (Others refer to total bacteria identified at class level, but not presented in the graph).

Similar dominance of the class Clostridia in tannery wastewater was reported in clone libraries based study at Modjo integrated tannery wastewater treatment system (Adey Feleke et al., 2014). The dominance of Clostridia and Gammaproteobacteria in the tannery wastewater suggested that these bacteria have important roles in the treatment process (Marathe et al., 2016), or came to the tannery wastewater treatment plant from consistent source. The other possible explanation is that the tannery wastewater environment may have a selective advantage for the dominant groups of bacteria.

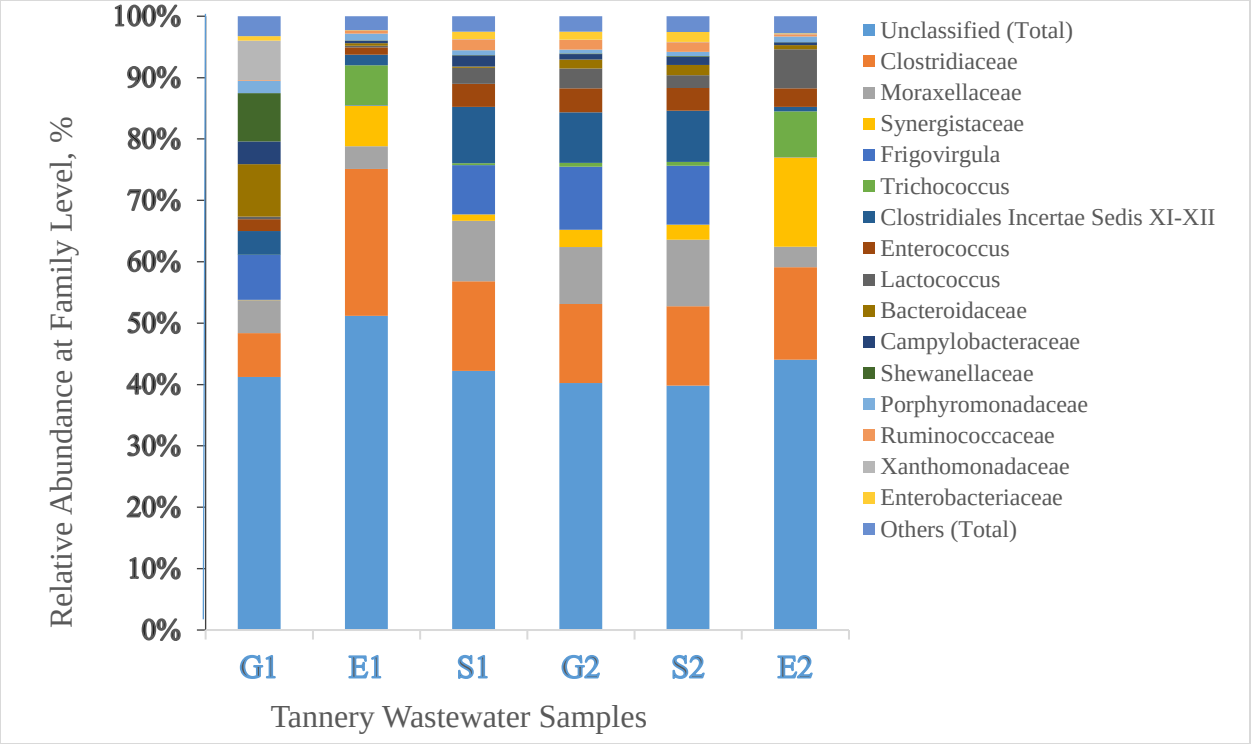

Figure 4.5. Relative abundance of bacteria at Family level in the Batu Tannery wastewater. Color coded bars illustrate the percentages of reads for each Family (Others refer to total bacteria identified at family level, but not presented in the graph).

The same trend of dominance was observed at the family level in which Clostridiaceae was the most predominant classified group in all the samples, except in the G1 sample where the Bacteroidaceae was more dominant. At the family level, a total of 242 taxa (160 classified and 82 unclassified) were identifies in the tannery wastewater samples, and the majority of the sequences belong to the 13 families. The families Clostridiaceae, Moraxellaceae, Synergistaceae, Frigovirgula, Trichococcus, Enterococcaceae, Lactococcus, Bacteroidaceae, Campylobacteriaceae, Prophyromonadaceae, Shewanellaceae, Ruminococcaceae and Xanthomonadaceae have greater than or equal to 1% relative abundance (Figure 4.5). The families Clostridiaceae, Moraxellaceae, Synergistaceae and Frigovirgula were more dominant in all the sample sites of the tannery WWTP (Figure 4.5).

At the genera level, 441 (254 classified and 187 unclassified) genera were identified. The most abundant genera in the tannery WWTP are presented in Figure 4.6. Overall, *Clostridium*, *Synergistes*, *Psychrobacter*, *Acinetobacter*, *Bacteroides*, *Anaerovorax* and *Shewanella*were the most dominant bacteria at genera level with relative abundance of more than or equal to 1%. These ten most abundant genera constituted 33% of the total reads, and 85% of the reads were affiliated to classified genera. The relative abundance of classified bacteria identified in the Batu tannery wastewater are summarized and presented in the Appendix VIII.

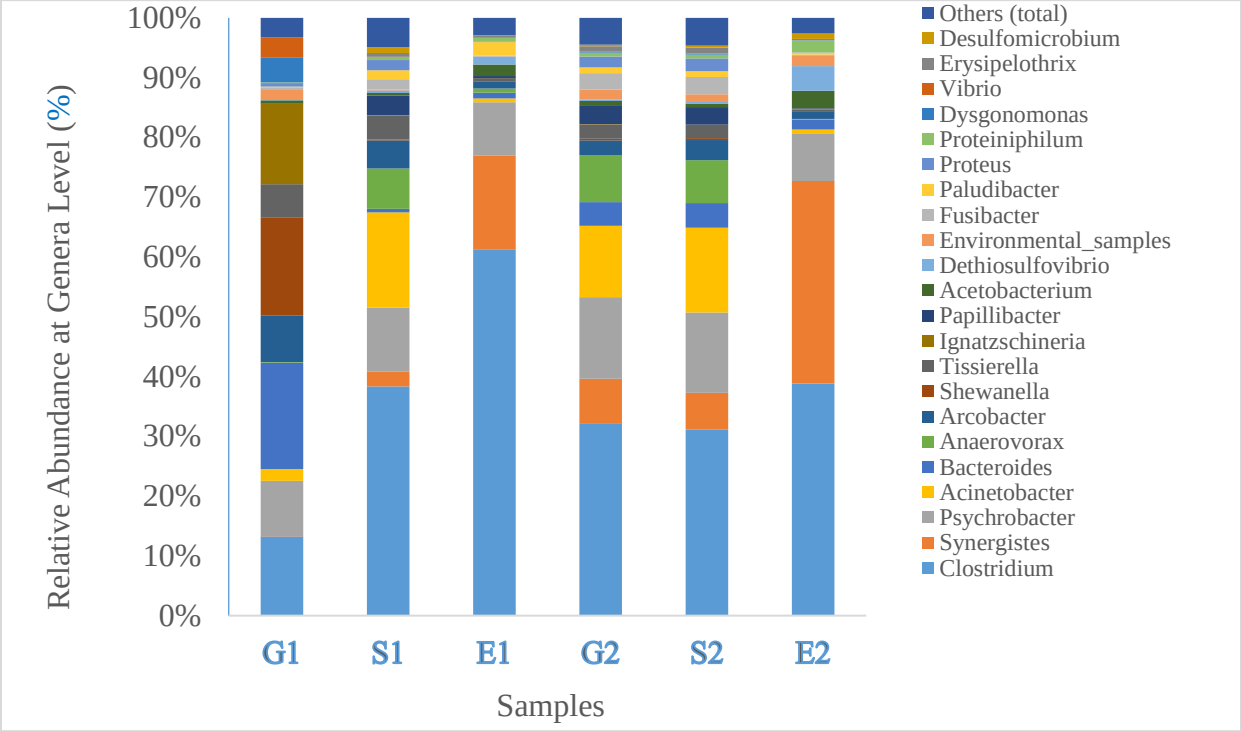

Figure 4.6. Relative abundance of bacteria at genus level in the tannery wastewater treatment plant. Color coded bars illustrate the percentages of sequence for each genus detected in the six samples (Others refer to total bacteria identified at genera level, but not presented in the graph).

The *Bacteroides*, *Shewanella*, *Acinetobacter*, *Ignatzschineria*, *Arcobacter*, *Tisseriella*, *Anaerovorax*, *Dysgonomonas*, *Vibrio* and *Papillobacter* were more dominant in the raw tannery wastewater (G1 and G2) samples (Figure 4.6). But the relative abundance of these bacteria

reduced by 84%, 99.7% 87.5%, 99.6%, 68.8%, 87%, 85.7%, 100%, 100% and 77%, respectively in the treated effluent. However, the abundance of *Clostridium, Synergistes, Acetobacterium, Paludibacter, Dethiosulfovibrio,* and *Proteiniphilum* increased by 66%, 88%, 81%, 69.6%, 97% and 99%, respectively in the treated effluent (Figure 4.6). This showed that there is high dynamics of bacteria communities in the tannery wastewater treatment process (Figure 4.6).

4.4.1. Dominant Bacteria showed resemblance to rumen gut bacteria

The Illumina-based bacteria community revealed that Firmicutes, Bacteroidetes, Proteobacteria and Synergistetes were the most abundant bacteria in the tannery wastewater (Figure 4.3). The dominant bacteria observed in this study were consistent with the studies on the bacteria communities in the gastrointestinal tract of shoats (sheep and goat) and bovines (Jami & Mizrahi, 2012; Han et al., 2015; Tapio et el., 2016; Granja-Salcedo et al., 2017; Liu et al., 2017; Tanca et al., 2017). The dominance of Firmicutes, Bacteroidetes and Proteobacteria was also reported in the gastrointestinal tract of equine (Shepherd et al., 2011), rabbit (Monteils et al., 2008), milk cows (Mao et al., 2015; Liu et al., 2016). Another study also indicated that Firmicutes (46%) and Bacteroidetes (36%) were dominant in animal manure (Ozbayram et al., 2018).

The predominant occurrence of Firmicutes, which are mostly anaerobic bacteria (Wang et al., 2014)in the tannery wastewater treatment system which is apparently without anaerobic stage indicated that these dominant bacteria came to the tannery WWTP through the influent wastewater.Moreover, Firmicutes is one of the dominant enteric bacteria which can remain viable and able to survive treatment systems such as anaerobic stabilization (Hong et al., 2013). Similar abundance of these bacteria between the tannery wastewater and gastrointestinal tract of animals suggested that there was a link between rumen bacteria community and tannery WWTP

and that are most likely through the skins and hides used as a raw material in the leather production.

On the other hand, the occurrence of Bacteroidetes and Proteobacteria as the second and third most abundant bacteria can be associated with the presence of high organic load and inorganic nitrogen species (Assefa Wosnie and Ayalew Wondie, 2014) in the tannery wastewater and these bacteria are important in carbon and nitrogen metabolism, respectively. The dominant families such as Bacteroidaceae, Clostridiaceae, Ruminococcaceae and the genus *Acinetobacter* detected in this study at the tannery wastewater werereported as common sewer and animal fecal signatures (Wan et al., 2017).

4.4.2.Dominant bacteria constitute the Core bacteria communities

At phylum level, 13(46.4%) out of the total 28 phyla were uniformly detected in all sampling sites. The G1, S1 and E1 samples shared 14 (50%) phyla, whereas the G2, S2 and E2 samples shared 16 (57%) of the phyla. At class level 41.7% (n = 20) among the classified classes were shared by all the samples. At family level 36.3% (n = 58) were uniformly identified in all samples, and at the genus level22.8% (n = 57) were shared by all the samples. Out of the total 441 generaidentified in the tannery wastewater,254 genera were classified accounting 39% of the total sequences and the remaining sequences were affiliated to the unclassified genera. The genera *Clostridium* (14.7%),*Synergistes* (4.8%),*Psychrobacter* (4%),*Acinetobacter* (2.5%),*Bacteroides* (1.8%),*Anaerovorax* (1.3%),*Arcobacter* (1.3%),*Shewanella* (1%),*Tissierella* (0.9%) and *Ignatzschineria* (0.8%)were abundant (Figure 4.6). *Clostridium* was found in large proportion in all samples with a range of 5.5% in G1 to 35.6% in E1 and average of 14.7% relative abundance across all sample sites. In the treated effluent *Acinetobacter*, *Bacteroides* and

Arcobacter were relatively lower(Figure 4.6), but in the G1 sample, *Bacteroides*werethe most abundant classified genera.

Although there was variation in abundance between different sample sites, the raw influent (G1, G2), aerated mixed liquor (S1, S2) and final effluent (E1, E2) samples shared 57 classified and 58 unclassified core bacteria at the genera level from which the top 44 classified genera were analysed using heatmap (Figure 4.7). The relative abundances of the core classified bacteria in the tannery wastewater showed difference in the different parts of the tannery WWTP. All taken together, the study indicatedthe dominance of core bacteria (bacteria occurring in all samples) groups although their distribution varied at the different sites of the tannery wastewater treatment system. Majority of the sequence reads belonged to the core bacteria taxa affiliated to the phylum Firmicutes, Bacteroidetes and Proteobacteria in decreasing order. At the class level Gammaproteobacteria, Clostridia, Lactobacilales, and Bacteroidia were the dominant core bacteria but, at family and genus levels, the pattern of dominance varied from sample to samples. In the S1 and S2 samples, Clostridiaceae, Moraxellaceae, and Frigovirgula were the three most dominant families. In the E1 and E2 samples, Synergistaceaeand Enterococcaceae were the dominant families next to Clostridiaceae. In the G1 and G2 samples, Clostridiaceae is the most dominant family, but Bacteroidaceae, Frigovirgula, Shewanellaceae and Xanthomonadaceae were the next abundant families in G1 in decreasing order. In G2, Frigovirgula, Moraxellaceae and Enterococcaceae were the second, third and fourth abundant families next to Clostridiaceae. Although *Clostridium* is the overall dominant genus,the next abundant core genera were variable in the tannery effluent since *Synergistes* was the second abundant genus in the E1 and E2 samples next to *Clostridium*. *Psychrobacter* and *Bacteroidetes* were the second and third abundant genera in the G2 sample, but in G1 the Bacteroides and *Shewanella* were the second

and third abundant classified core genera. In the S1 and S2 samples,*Acinetobacter* and *Psychrobacter* were the second and third abundant core genera (Figure 4.6). The abundance of core bacteria at the family and genera level showed that the treatment plant shaped the dominance of the bacteria although there is a difference in the abundance of the core bacteria in the raw (G1 and G2) tannery wastewater. The dominant presence of some the bacteria such as the *Acinetobacter* in the effluent has a concern of potential pathogenicity and can develop antibiotic resistance in environment where there are antibiotic residues and ARGs (Hong et al., 2013).

Among the core bacteria, *Clostridium, Synergistes, Psychrobacter* and *Acinetobacter* were the most omnipresent genera. Similar abundance of *Clostridium* (14.67%) and *Bacteroides* (1.8%) was reported in a study on the bacteria communities of bovine rumen and feces (Callaway et al., 2010). The abundance of these core taxa across the treatment plant suggested that these bacteria have important functions in the treatment process or they come from sources that have the same core bacteria community profiles and these are most likely animal sources whose skin and hides are used as a raw material for leather production. Moreover, *Clostridium* species have the ability of forming spores in the time of unsuitable environment for several months and they can resume normal replications when the conditions are restored (Hensgens et al., 2012). This special ability of the *Clostridium* might account for their abundance in the tannery effluent which is released from the sedimentation pond.

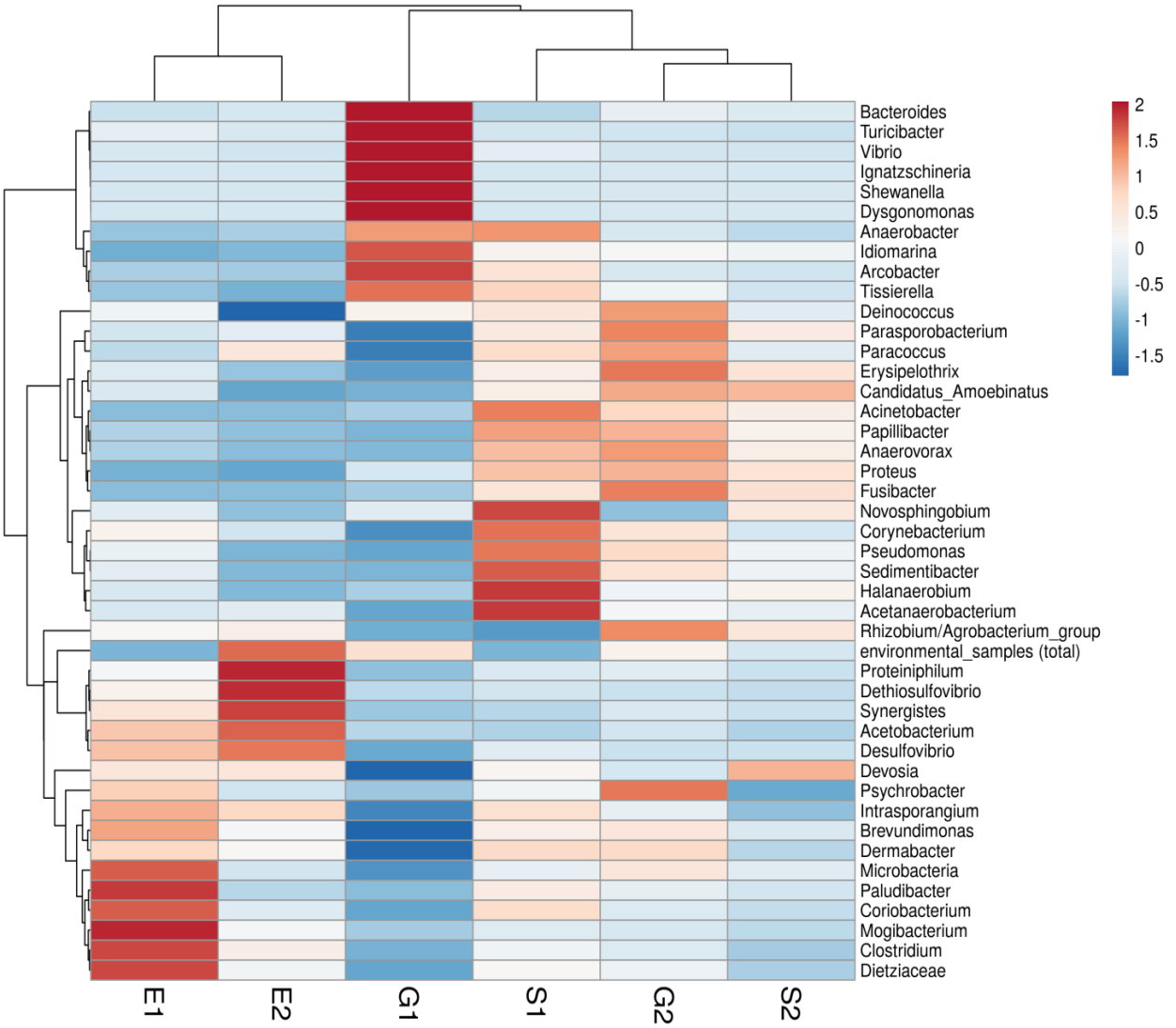

Figure 4.7. Heatmap showing relative abundance of core bacteria at genera level in the Batu tannery wastewater.The color codes showed relative prevalence of sequences for tannery samples (G = General raw wastewater, S = aerated mixed liquor, E = treated final effluent).

At genera level, among the 254 classified genera, only 57 genera were common in all samples, and these core classified bacteria genera in the tannery wastewater treatment samples comprise 38% of the sequences affiliated to the classified genera. This showed that relative abundance of core bacteria at the rank of genera for the six samples consisted 98% of the classified reads. The remaining rare genera constitute only 2% of the total sequences assigned to the classified core

genera. *Clostridium* (14.7%),*Synergistes (4.8%), Psychrobacter* (4%),*Acinetobacter* (2.5%),*Bacteroides* (1.8%),*Anaerovorax* (1.3%),*Arcobacter* (1.26%)and *Shewanella* (1%) were the most prevalent core genera detected in all tannery samples (Figure 4.6). There are also 58 unclassified taxa which were commonly present in all samples showing that these taxa were also core bacteria but they are unidentified bacteria in the tannery wastewater. These core genera are supposed to play important roles in the tannery wastewater treatment process (Liu et al., 2016).

The most dominantly identified classified bacteria at the genus level was *Clostridium* (14.7%), but the highest number of unique sequences assigned to a single genus were affiliated to unclassified OTUs which account 14.8% relative abundancefrom the entire general level sequences. Comparable high proportion of unclassified bacterial community at the genus level (44.6%) was reported in the gastrointestinal tract of cattle (Kim et al., 2014), feces of milk cows (Liu et al., 2016) and pond water samples (Qin et al., 2016). The presence of abundant unclassified OTUs showed limitation in microbiome studies of tannery wastewater and lack of reference databases for 16S rRNA gene (Han et al., 2015), which contain only certain of the bacteria identified in this study. On the other hand, the resemblance of unclassified bacteria community between the tannery wastewater and rumen bacteria reported in previous studies at the genera level reiterates that the dominant bacteria community in the tannery wastewater may have animal sources. This is notable considering that the source of nearly 80% of raw hides and skins are obtained from rural areas and 20% of these are collected from abattoirs and slaughter houses in large cities and towns (Coppeaux et al., 2016), and there is a possibility to bring diverse group of bacteria from a wide range of the agro-ecological zones to the tannery WWTP through skin and hide.

4.4.3. Diversity of Bacteria in the tannery wastewater

Alpha Diversity

Alpha diversity values of the bacteria community in the tannery wastewater was determined using different diversity indices (Table 4.4). These diversity indexes are good to compare and characterize the bacteria community profile of the tannery wastewater at different treatment stages. The alpha diversity was analyzed based on the OTU outputs generated at the 97% similarity threshold (Table 4.4). The alpha diversity of bacteria in the tannery wastewater treatment was estimated by using diversity and richness parameters (observed OTUs, Shannon index, Inverse Simpsonand evenness). In addition,rarefaction curve was generated from the OTU table using the minimum sample depth of samples which is the lower sequence count (Figure 4.8).

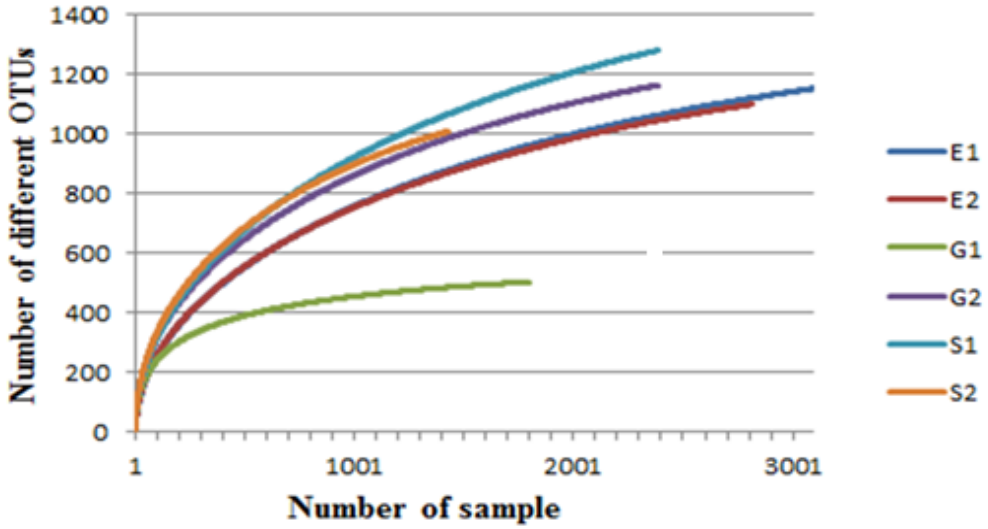

Figure 4.8. Rarefaction analysis for the raw (G), aerated mixed liquor (S) and final effluent (E) tannery wastewater depicting the OTUs and number of sampled reads

The rarefaction curves for the samples approached saturation showing that the bacteria communities are represented and the Illumina sequenced data was reasonable (Figure 4.7). Moreover, the rarefaction curve showed that our sampling effort provided sufficient coverage of OTUs in order to describe the tannery wastewater bacteria community structure and composition. Average Goods' coverage of the sequence reads at 97% similarity threshold was 99.7% for all samples showing that the sequences retrieved at sufficient depth and represent the diversity of bacteria community in the tannery wastewater (Table 4.4).Rarefaction curves did not approach full saturation for samples S1 and G1 showing some bacteria taxa are not retrieved.

The observed OTUs, Chao1 and other diversity estimators (Shannon, Invsimpson) are presented in Table 4.4. The Shannon index (H)indicated that the diversity and evenness of the bacteria community in the tannery wastewater was higher in the aerationpond samples.The S1 and S2 samples have high Shannon index, followed by the G2 and the treated effluent (E1 and E2) samples (Table 4.4).A relatively higher value of the Shannon index in the S1 and S2 samples demonstrate that the aerated mixed liquor samples (S1 and S2) had richer diversity of bacteria communities than the treated (E1 and E2)tannery wastewater. However, the values of Shannonindex were in close range of 2.5 to 3.17 across all samples and similar modest bacteria community evenness index was observed in the samples (Table 4.4).

The range of Shannon index falls in the typical values expected in microbial ecology studies (generally between 1.5 and 3.5). The Shannon index was lower in the E1 and E2 samples, but the Simpson index was higher (> 0.9) and remains linear for all samples that only little variations

were witnessed in the different samples. Lower Shannon index in the treated effluent demonstrate both the richness and evenness of the bacteria community decrease. However, small difference in the evenness index suggested that the tannery WWTP was dominated by few groups of the bacteria phylotypes (Table 4.4). The E1 and E2 samples had the lowest evenness index compared to the upper treatment plant samples. The OTUs clustered at 97% identity level showed that average relative richness of OTUs in the samples was 879 and the lower number of OTUs wasobserved in the G1 tannery sample, and the highest number of OTUs were obtained in the S1 sample (Table 4.4).

Table 4.4. Alpha diversity measures of the tannery wastewater bacteria communities at 3% dissimilarity level

Alpha diversity	Samples					
	G_1	S_1	E_1	G_2	S_2	E_2
Individual Sequences	179,994	237,711	308,999	238,071	142,198	280,742
Taxa_S	192	277	263	263	246	258
Number of OTUs	485	1059	874	977	1008	873
Good's coverage (%)	99.94	99.70	99.75	99.75	99.78	99.76
Shannon	2.905	3.032	2.537	3.17	3.17	2.602
Invsimpson	18.56	15.95	8.62	20.18	19.79	8.72
Phylodiversity	33.8	79.8	72.6	74.5	63.8	68.3
Simpson	0.92	0.93	0.92	0.93	0.87	0.87
Evenness	0.095	0.075	0.048	0.091	0.097	0.052
Chao 1	195.7	326	296	286.2	283	285.4

G1 = Raw tannery wastewater collected in first round, S1 = aerated mixed liquor collected in first round, E1 = Tannery effluent collected in first round, G2 = Raw tannery wastewater collected in second round, S1 = aerated mixed liquor collected in second round, E2 = Tannery effluent collected in second round, Taxa_S = taxa sums

As presented in Table 4.4, the bacteria community diversity in the aerated mixed liquor was higher than the bacteria diversity in the treated effluent (E1 and E2) samples.Comparable alpha diversity indices showing variation in bacteria community richness in influent wastewater was

reported by Tang et al(2016). The high number of OTUs in the influent tannery wastewater was almost similar to the aerated sludge samples in the tannery wastewater suggesting that there was continuous supply of the treatment plant with new load of microbial community in the raw influent tannery wastewater. Bacteria community richness demonstrated by numbers of OTUs in the E1 and E2 samples was lower than the raw and aerated sludge samples. The bacteria communities in the tannery wastewater were abundant, but discreetly diverse (average Invsimpson = 12.3). The diversity of bacteria indicated by average Invsimpsonin this study was lower than reported diversity of bacteria community (Invsimpson value 44.21±1.4) of abattoir and fecal material contaminated solid dumpsite (Mwaikono et al., 2016). The tannery WWTP had also lower bacteria community richness than the bacteria community of the solid waste dump site contaminated with tannery wastes (Sheik et al., 2012).The Shannon index value of this study showed that there is moderate diversity of bacteria in the wastewater (Jena et al., 2015). Interestingly, most of the alpha diversity indices decrease in the treated effluent suggesting that the bacteria community is less diverse than the upstream S and G sites.

The presence of abundant but less diverse bacteria in the treated effluent (E1 and E2) could be related with problems in the flocculation and sedimentation process of the mixed liquor which probably does not retain some of the dominant bacterial community from the final effluent. On the other hand, inefficient flocculation of the activated sludge could be associated with the alkalinity (slightly high pH) of the tannery wastewater which can cause weak aggregation (Nithya & Sudha, 2016), hence poor sedimentation and low effluent microbial quality. Therefore, poor microbial quality of treated effluent is a concern to public and environmental health (Fredriksson et al., 2012), and more specifically the ecological composition of the natural bacteria community in the effluent receiving river. The abundant presence of bacteria in the

effluent of tannery wastewater treatment plant showed the essence of biomonitoring (Naidoo & Olaniran, 2013) for potential pathogens in the treated tannery effluent before discharged to the receiving surface water.

At the phylum level, most of the OTUs were affiliated to Firmicutes (40%), unclassified bacteria (19%), Proteobacteria (14%) and Bacteroidetes (11%) (Figure 4.9). The OTU abundance data supplement the Shannon index value supporting the fact that the S1 (3.032) and S2 (3.17) samples have higher bacteria diversity than the E1 (2.54) and E2 (2.6) samples (Table 4.4). The invsimpson index also showed that the bacteria community in the raw tannery influent (G1 and G2) and aerated sludge (S1 and S2) samples were more diverse than the bacteria community in the E1 and E2 samples.

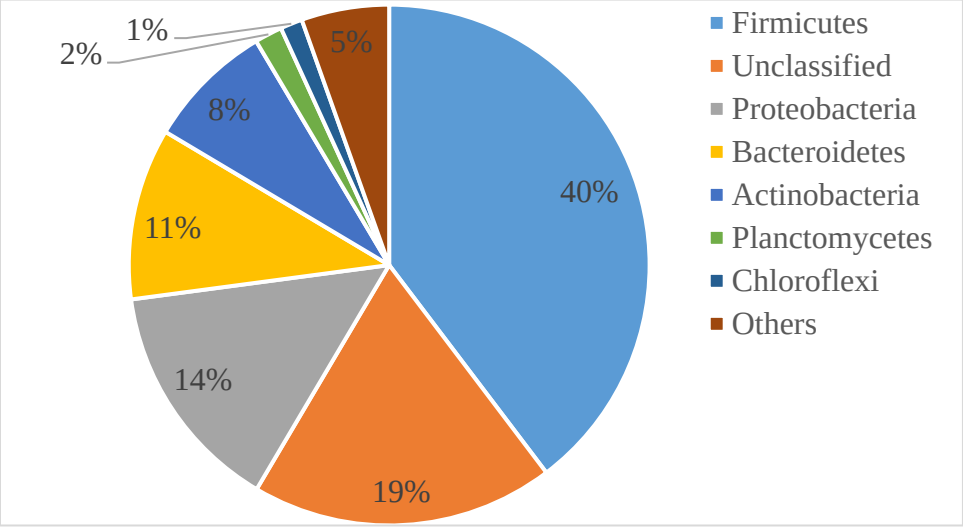

Figure 4.9. Relative abundances of bacteria at the phylum level revealed by the percentages of the OTUs in the Batu tannery wastewater treatment plant(Others refer to 23 phyla denoted by few numbers of OTUs)

As a measure of taxonomic uniqueness, phylogenetic diversity is one of the proper measures of distance among the taxa. The values of phylogenetic diversity denote the sum of the lengths of branches in a phylogenetic tree that connect the taxa identified in the samples. Bacteria

communities with high phylogenetic diversity values must contain phylogenetically diverse bacteria communities (Chiarucci et al., 2011). Like the other alpha diversity indices, the phylogenetic diversity index of mixed liquor (S1) samples has the highest diversity, but the G1 sample has low phylogenetic diversity. These values reaffirm that there is temporal variability in the diversity of bacteria communities of the raw tannery wastewater, but this variability reduces in the mixed liquor and treated tannery wastewater samples.

Sample sites were compared in terms of the similarity and differences of the bacteria community by comparing the numbers of shared and unique OTUsusing the Venn diagram (Figure 4.10). In two or more communities, the uniqueness of bacteria communities can be shownby using shared or unique OTUs (Lemos et al., 2011). Accordingly, the shared bacterial taxa should have overlapping unit of OTUs, and those OTUs which are not shared by the different sample sites are considered as unique OTUs. As shown in Figure 4.10, the fraction of shared OTUs werehigh between the tannery wastewater samples. Temporal comparison of bacteria community in the treatment process showed that the G1, S1 and E1 samples had 167, 261 and 444 unique OTUs, respectively and relatively high numbers of unique OTUs were observed in the G2 (298), S2 (279) and E2 (309) samples. The G1, S1 and E1 samples shared 208 (10.4%) OTUs from possible total 2002 OTUs and the G2, S2 and E2 samples shared 398(19.3%) OTUs from possible total 2026 OTUs (Figure 4.10). The numbers of shared OTUs between the samples calculated from OTU table also showed that G1 and G2 samples shared 288 OTUs, S1 and S2 samples shared 568 OTU's and E1 and E2 samples shared 467 OTU's. The E1 and G1 samples shared 244 OTU's, whereas the E2 and G2 samples shared 468 OTU's. The shared OTU's between G1 and G2 samples and the treated effluent (E1 and E2) samples showed that 50% of G1 and 52% of the G2 OTU's present in the respective E1 and E2 treated effluent samples. In

general, the Venn diagram showed that there is much overlap between the bacteria phylotypes of the tannery wastewater sample sites (G, S and E).

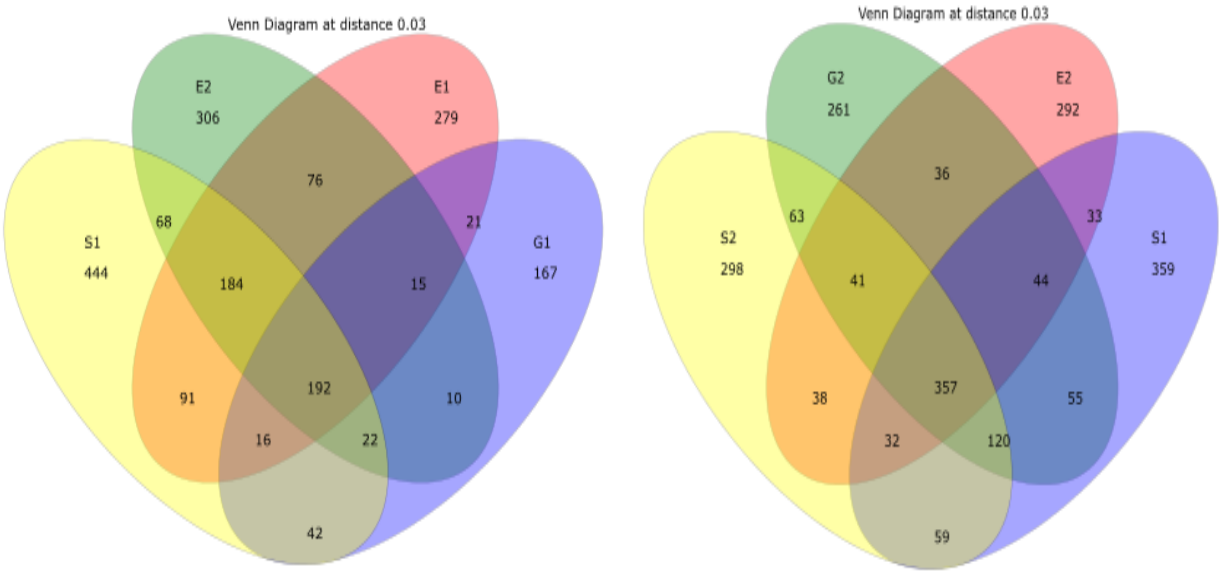

Figure 4.10. Venn diagram showing shared and unique OTUs between sample locations in the tannery wastewater treatment plant (G=General raw wastewater, S=aerated mixed liquor, E = final effluent)

Beta diversity

In microbial ecology studies, beta diversity is important diversity indicator to reveal how close the bacteria communities are between the different data sets or sample sites. Principal Coordinate Analysis (PCoA) was carried out to visualize the differences in bacteria community structures between samples (Figure 4.11). PCoA is thus used to determine the clusters of samples denoting similarity of the bacteria communities in space, and the clusters are said to be close when the sequences are representative of the bacteria communities between the samples (Lemos et al.,

2011). Thecluster analysis of deeply sequenced data by the PCoA showed that the S1 and S2 samples of the aerated mixed liquor clustered together (Figure 4.11). Similarly, the E1 and E2 samples from the effluent were clustered together, but the G1 and G2 samples of raw general influent bacteria communities were not clustered together. Spatial trend of clustering of the bacteria community in the tannery WWTP showed that the tannery wastewater treatment process has shaped the bacteria community once receiving the raw tannery influent which contains variable bacterial community. The PCoA plot portraited that the bacteria community in the G1 and G2 samples didn't cluster together (Figure 4.11). The bacteria community in the G2 sample showed closeness to the bacteria community in the mixed liquor (S1 and S2) samples, but the PCoA plot showed that the bacteria community in the G1 sample remains different from the other samples (Figure 4.11). The variability of bacteria communities could happen due to the geographical differences in the sources of skins and hides collected from a different region (s)compared to the previous batches of the soaking outlets.

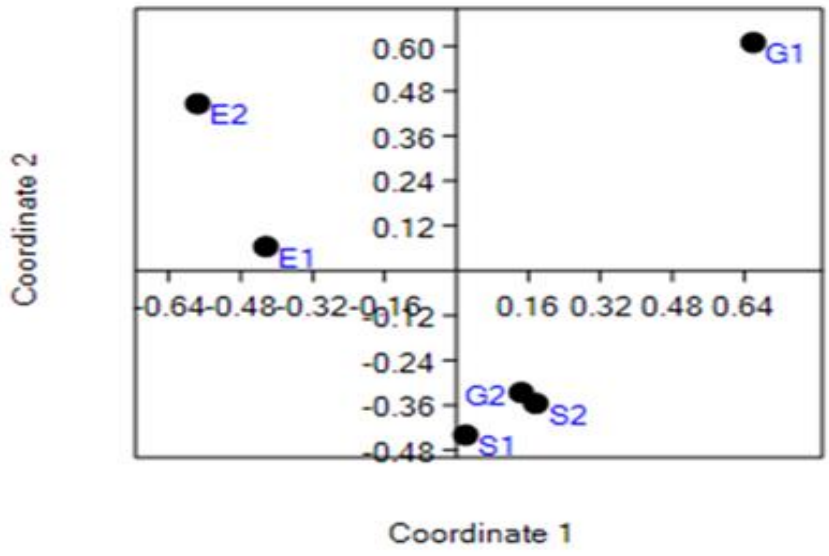

Figure 4.11. Principal Coordinate Analysis of the tannery wastewater treatment plant by unweighted UniFrac distance as a measure of similarity distance between the samples

The PCoA plot showed that there is spatial difference in the bacteria community composition between the sample locations in the tannery wastewater treatment. As shown in Figure 4.11, the PCoA plot of the aerated mixed liquor (S1 and S2) bacteria communities were distantly related with the treated tannery effluent (E1 and E2) bacteria communities. However, the influent raw tannery wastewater (G1 and G2) samples showed nonconformity to this pattern as the G1 and G2 samples were not clustered close to each other (Figure 4.2).On the other hand, the PCoA plot revealed that there waslittle or no temporal variation in the bacteria community of the S1 and S2 samples since they werevery close to each other (Figure 4.11). The same trend was observed in the E1 and E2 samples that the bacteria community in the two samples showed closeness by being clustered together, but the bacteria communities in the G1 and G2 samples are exceptions to this trend. This showsthat there is temporal variation in the bacteria community of the raw tannery wastewater, but there was no such type ofdifferencebetween the bacteria communities in the aerated mixed liquor and in the treated tannery effluent samples. Instead the bacteria community in the G2 sample was closer to S1 and S2 samples than the G1 bacteria communities. This was consistent with the heatmap analysis of core bacteria community which depicted that the bacteria community in the G1 sample was distantly related to the other samples.

4.4. Potential bacterial pathogens in the tannery wastewater

Potential bacterial pathogens were identified at genera level based on the Illumina sequenced 16S rRNA gene from tannery wastewater samples (Figure 4.12). The analysis revealed that there are potential bacteria pathogens which could be released from the tannery WWTP into the environment since many potential bacteria pathogens were detected in the treated effluent. These

potential bacterial pathogensidentified in the tannery WWTP affiliated to 26 bacteria genera, 24 families, 7 orders, 9 classes and 3 phyla (Table 4.5). The primary reason for the design and operation of wastewater treatment is to reduce the pollutant nutrients (organic and inorganic) and microorganisms particularly the pathogens often through the sequential physical, biological and chemical treatment steps (Xu et al., 2014).

Table 4.5. Potential bacterial pathogens detected in the tannery wastewater samples and their affiliation to the different levels of taxonomic ranks.

Phylum	**Class**	**Order**	**Family**	**Genus**
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*
	Erysiopelothrica	Erysiopelothricales	Erysiopelothricaceae	*Erysipelothrix*
	Mollicutes	Acholeplasmatales	Acholeplasmataceae	*Acholeplasma*
	Bacilli	Bacillales	Bacillaceae	*Bacillus_cereus_group*
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*
			Pseudomonadaceae	*Pseudomonas*
			Moraxellaceae	*Moraxella*
		Alteromonadales	Shewanellaceae	*Shewanella*
		Vibrionales	Vibrionaceae	*Vibrio*
		Aeromonadales	Aeromonadaceae	*Aeromonas*
		Enterobacteriales	Enterobacteriaceae	*Escherichia*
		Legionellales	Legionelaceae	*Legionella*
		Xanthomanadales	Xanthomonadaceae	*Stenotrophomonas*
		Enterobacterales	Enterobacteriaceae	*Klebsiella*
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*
			Helicobacteriaceae	*Helicobacter*
			Campylobacteraceae	*Campylobacter*
	Alphaproteobacteria	Rhiozobiales	Rhizobiaceae	*Candidatus*
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*
Actinobacteria	Actinobacteridae	Actinomycetales	Corynebacteriaceae	*Corynebacteria*
			Nocardioidaceae	*Nocardioides*
		Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*
		Actinomycetales	Nocardiopsaceae	*Nocardiopsis*
			Nocardiaceae	*Nocardia*
			Streptomycetaceae	*Streptomyces*
			Mycobacteriaceae	*Mycobacterium*

At the genus level, 27% and 15.8% of the potential pathogens were identified in the E1 and E2 samples, respectively. From the entire potential pathogen affiliated sequences, *Clostridium* (73%) had the highest relative abundance followed by *Acinetobacter* (12.6%), *Arcobacter* (6.3%) and *Shewanella* (5%) in the tannery wastewater samples. Previous studies reported similar potential pathogens in the tannery wastewater such as the *Clostridium, Arcobacter, Campylobacter, Pseudomonas, Acinetobacter, Bulkholderia, Bacillus, Legionella, Mycobacterium*, *Arcobacter* and *Aeromonas* consist pathogenic species (Ibekwe et al., 2013; Zhang & Wang, 2017).

Some of the potential pathogens particularly the *Clostridia species*were more abundant in the effluent (E1 and E2) samples than in the raw influent (G1 and G2) samples. However, the *Acinetobacter, Arcobacter* and *Shewanella* had high relative abundance in the raw tannery influent (G1 and G2) samples than in the mixed liquor and treated effluent samples(Figure 4.12). The same trend of decreasing in the relative prevalence of *Arcobacter* in the wastewater effluent was reported in wastewater treatment (Tang et al., 2016), showing that thebacteria were retained from the aerated mixed liquor in the sedimentation process. Many of the potential pathogens affiliated to the orders Burkholderiales, Campylobacterales, Enterobacteriales and Xanthomonadales belonged to the lineage of Proteobacteria (Auffret et al., 2017; Lüneberg et al., 2018).

At the genera level, 20% of the sequences from the entire reads and 51.7% of the reads from the classified genera were affiliated to potential bacterial pathogens. Among the most dominant bacterial pathogens in the treatment plant were the*Clostridium, Acinetobacter, Arcobacter* and *Shewanella* whichcomprised 97% of the sequences associatedto the potential bacterial pathogens. *Acinetobacter, Arcobacter* and *Shewanella* were relatively higher in abundance in the

raw tannery wastewater (G1 and G2) samples. The relative abundance of potential pathogenic bacteria in the tannery wastewater treatment was quite different between the raw influent and treated effluent wastewater samples.

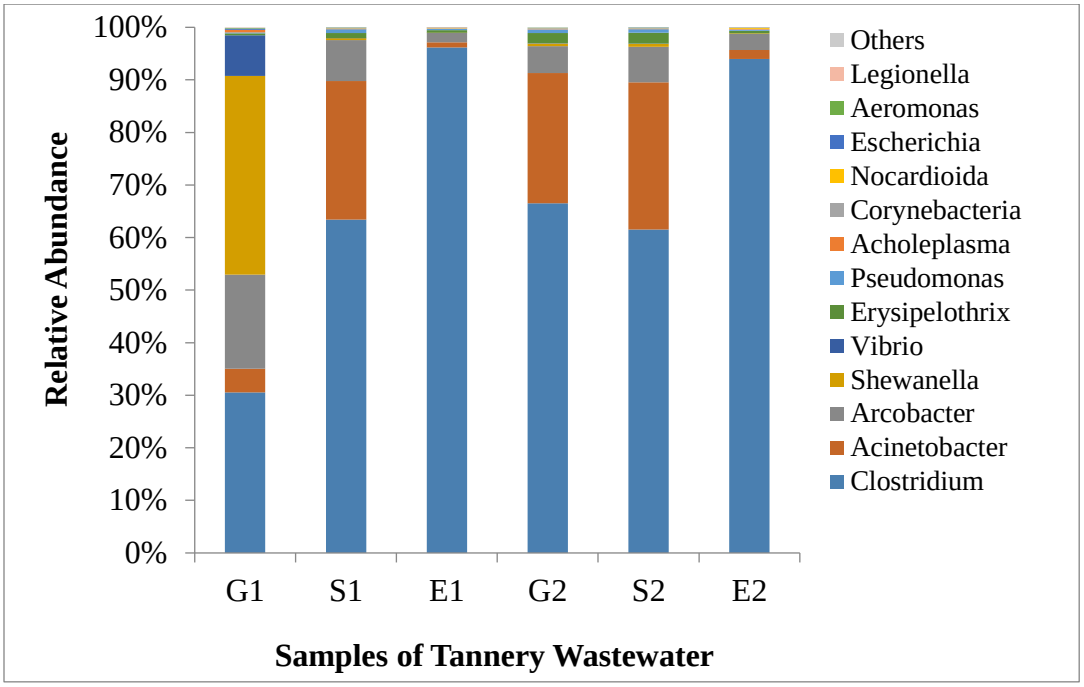

Figure 4.12. Relative abundance of potential pathogenic bacteria at genera level in the tannery wastewater treatment plant (G1 = Raw tannery wastewater collected in first round, S1 = aerated mixed liquor collected in first round, E1 = Tannery effluent collected in first round, G2 = Raw tannery wastewater collected in second round, S1 = aerated mixed liquor collected in second round, E2 = Tannery effluent collected in second round)

Thepotential bacteria pathogen in the treated effluent, raw general wastewater and aerated mixed liquor accounted for 42.5%, 28.7% and 28.8% of the total reads observed across the tannery WWTP (Figure 4.12). As indicated before, the *Acinetobacter*, *Arcobacter* and *Shewanella*have relatively higher abundance in the raw tannery wastewater (G1 and G2) samples, but *Legionella* and *Norcardioides* were relatively higher in the final effluent (E1 and E2) samples like *Clostridium* (Figure 4.12). From the total reads affiliated to potential pathogens,55.5% of them

belonged to the phylum *Proteobacteria*, 29.6% belonged to the *Actinobacteria*and 38.3% belonged to the *Firmicutes*. As shown in Table 4.5, at the class level,*Gammaproteobacteria*was representedby12 potential pathogen families andthey represent 9.6% from the entire class level sequences followed by *Epsilonproteobacteria* which contained three families of potential pathogens, but there was one potential pathogen family under the *Betaproteobacteria*. Orders *Pseudomonadales* and *Enterobacterales* were the most dominant members of the potential pathogens under the *Gammaproteobacteria*(Table 4.5).

Potential pathogens identified by Illumina sequencing in this study showed similarity to the pathogens revealed by 454 pyrosequencing which reported the occurrence of *Arcobacter*, *Aeromonas*, *Klebsiella*, *Clostridium* and *Corynebacterium* in the raw sewage samples (Brown & Gast, 2015).Interestingly, the well-known pathogens like the *Rickettsia*, *Helicobacter*, *Enterobacter*, *Legionella*, *Treponema*, *Leptospira* and *Pseudomonas*(Zhou et al., 2017)belonged to the least abundant groups identified in this study. Many of the potential pathogen identified in the tannery wastewater, such as the *Clostridium* and *Campylobacter,*were reported to be common colonizers of animal gastrointestinal tract and the former being more common than the latter (Cameron & McAllister, 2016).

The abundance of some potential pathogens in the tannery effluent suggested that these bacteria (e.g. *Clostridium* and *Arcobacter)* remain with the effluent instead of settling with the activated sludge (Callaway et al., 2010). This could allow thebacteria to escape out into the nearby river through the tannery effluent. The dominance of *Clostridium* in the tannery wastewater is relevant because of its importance as a potential pathogen of increasing public health concern(Freeman et al., 2010),and also its prospects as an indicator organism (Steele & Odumeru, 2004; Stelma, 2018).

The analysis of microbial ecology of tannery wastewater treatment system from influent to effluent using next generation sequencing methods showed the universal dominance of *Clostridium*. The dominance of *Clostridium*in the tannery wastewater is consistent with earlier studies of a tannery effluent using 16S rRNA gene clone library analysis at Mojo tannery(Adey Feleke et al., 2014). Although the physiological form of *Clostridium*was not determined at species level, the increase in the relative abundance of *Clostridium*across treatment suggests that planktonic forms of *Clostridium* may have been abundant in the effluent. Depending on the environmental condition, the *Clostridium* species can change from planktonic to sessile and vice versa forms, and are more likely to take on a pathogenic phenotype when existing in the sessile forms(Crowther et al., 2014). Its possible relevance as an indicator of tannery wastewater treatment system performance wasreported in previous reports which showed the dominance of *Clostridium* DNA in sediments downstream of a tannery industries in Morocco (Essahale et al., 2010), and evidence that toxigenic forms can survive in river sediments.

The spores of *Clostridia* would enable the bacterium to survive the treatment with the potential to grow during the anaerobic condition probably created during the sedimentation process (Xu et al., 2014). The persistence of *Clostridium* in Tannery effluents suggests that its profile can be considered as indicator for tannery effluent microbial water quality. Therefore, sequence-based analysis of bacteria community in the tannery wastewater revealed that bacteria with pathogenic members could escape out of the treatment plant into the river water. The origin of these potential pathogens in the tannery wastewater are most likely animals since food producing animals are the primary reservoirs of zoonotic pathogens (Sharma et al., 2018). The release of tannery effluent containing potential pathogens into river water has real health concern for the

downstream community who are using the river water for agricultural (irrigation) and domestic purposes.

4.5. Antibiotic residues in the tannery wastewater

The antibioticsof interest were selected based on the trends of antibioticuse inlivestock treatment in Ethiopia with the assumption that tanneries could receive residual antibiotics from animal sources through the skins and hides. Tetracycline, oxytetracycline, ampicillin, amoxicillin, penicillin G, erythromycin, penicillin V, sulfonamides (sulfamethoxazole, sulfadiazine and sulfadoxine), trimethoprim, streptomycin, ciprofloxacin and vancomycin were considered for detection in the tannery wastewater (Table 4.6).

Table 4.6. Antibiotic residues assessed in the Batu Tannery wastewater, August 2015

Antibiotic residues	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**
Oxytetracycline	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+
Penicillin G	+	+	+	+	+	+
Penicillin V	–	–	–	–	–	–
Trimethoprim	+	+	+	+	+	+
Amoxicillin	–	–	–	–	–	–
Sulfamethoxazole	+	+	+	+	+	+
Sulfadiazine	+	+	+	+	+	+
Sulfadoxine	+	+	+	+	+	+
Erythromycin	+	+	+	+	+	+
Streptomycin	–	–	–	–	–	–
Ampicillin	–	–	–	–	–	–
Ciprofloxacin	+	+	+	+	+	+
Vancomycin	–	–	–	–	–	–

(+) = in the MDL, (-) = below MDL, MDL = Method Detection Limit, G1 = Raw tannery wastewater collected in first round, S1 = aerated mixed liquor collected in first round, E1 = Tannery effluent collected in first round, G2 = Raw tannery wastewater collected in second round, S1 = aerated mixed liquor collected in second round, E2 = Tannery effluent collected in second round

The LC/MS analysis showed that 64.3% of the targeted antibiotic residues were present in the tannery wastewater samples (Table 4.6). This means from the 14 targeted antibiotics, nine antibiotic residues were present in all tannery samples, and all of the nine antibiotic residues were uniformly detected (+) in all tannery wastewater samples (Table 4.6). However, the antibiotic residues of ampicillin, streptomycin, penicillin V, amoxicillin and vancomycin were below the method detection limit (MDL) and described as absent (-). As a trend, the antibiotics detected in the raw influent (G) samples were detected in the downstream tannery effluent samples, and those antibiotic residues not detected in the raw tannery samples were also absent in the treated tannery effluent samples.

Theproblems of antibiotic pollution to surface water and soil is an existing problem since the advent of antibiotic use for human and animal health care(Sharma et al., 2018). The occurrence of antibiotic residues in tannery wastewater indicates that tanneries are one of the routes from which antibiotics escape to the environment. Besides, the antibiotic residues can exert some sort of selective effect among bacteria and can enhance the predominance of antibiotic resistance bacteria(Lundborg & Tamhankar, 2017).

Batu Tannery WWTP employs conventional treatment system mainly to remove nutrients, suspended solids, sulfides and chromium from the tannery wastewater before discharged to the environment. However, the tannery wastewater wasalso found to contain antibiotic residues which are environmentally dangerous pollutants from the public health point of view(WHO,

2011). The removal of antibiotics in WWTP can happen by chemical and bio-adsorption (physical) processes with subsequent separation of the adsorbed sludge in the sedimentation units (Hong et al., 2013). But, in this study the antibiotic residues not only present in the mixed liquor, but also in the final treated effluent. The presence of antibiotic residues all the way from raw influent to the final treated effluent shows that the treatment system is not retaining antibiotic residues.This causes the release of antibiotic residues into the environment through the tannery effluent, and the release of antibiotic residues into the environment is supposed to be the major driver of antibiotic resistance (Novo et al., 2013).

The antibiotic residues detected in the tannery wastewater belonged to the class beta-lactams, sulfonamide, tetracycline, macrolides and quinolones, all of which are used in treating livestock (WHO, 2011). The presence of similar antibiotic residuesidentified in this study was reported in the wastewater effluent, soil and sediment samplesin agro-ecosystem(Awad et al., 2015),in effluent of hospital and pharmaceutical industries(Le et al., 2016), andin municipal WWTP (Hong et al., 2013). Among the antibiotics, tetracycline was one of the antibiotics used in veterinary medicine forlivestock treatment such as forcattle treatment(Al-Bahry et al., 2016). The occurrence of tetracycline and oxytetracycline in the raw and treated tannery wastewater can be related with their stability and long half-life in aquatic environments which is up to 419 days under different environmental factors (Pikkemaat, et al., 2016).

Sulfonamides and macrolides are also among the commonly used antibiotics in livestock treatment (Wegst-Uhrich et al., 2014). Thesulfonamides (sulfamethoxazole, sulfadoxine and sulfadiazine) and trimethoprim were detected in the raw influent and treated tannery effluent (Table 4.6). The presence of trimethoprim in the treated effluent could be connected with its resistance to biodegradation at short retention time(Hong et a., 2013). Trimethoprim isused

together with sulfamethoxazole inratio of 1:5 for therapeutic agent known as co-trimoxazole (Le et al., 2016). The occurrence of sulfonamides in the treated effluent samples (E1 and E2) imply that these antibioticshave longer half-life andmay not be degraded by bacteria due to the presence of complex ρ-aminobenzene sulfonamide rings, as well as their low sorption potentials (Hong et al., 2013).

Another broad-spectrum antibiotic was theciprofloxacin (fluoroquinolone) detected in the tannery wastewater (Table 4.6).The occurrence of ciprofloxacin in the tannery wastewater samples including in the treated tannery effluent can be related with its slow degradability and ability to persist in the environment for up to 80 days in its parent form (Massé et al., 2014). The presence of ciprofloxacin in the tannery effluent could raise a concern of fluroquinolone resistance transmission from animal to human since there is resemblancebetween veterinary and human uses(Hao et al., 2016).

On the other hand, mostof the penicillin families (penicillin V, amoxicillin and ampicillin) belonging to the class β-*Lactams* were not detected in the raw and treated tannery wastewater samples (Table 4.6), even ifsome of these antibiotics are used in treating animals in combination with other antibiotics. The absence of most of the β-*Lactams* in the tannery WWTP could be linked with the instability of β-*lactam* ring in environmental matrices (Milić et al., 2013), or their short half-life or rapid degradation under the abiotic conditions in the tannery WWTP. This is because the β-lactam ring caneasily be cleaved off by the ubiquitousβ-lactamase enzymes of bacteria (Zeng & Lin, 2013).

On the other hand, the presence of penicillin G in the tannery wastewater can be a result of its widespread use in animal treatment and its low biodegradability like the sulfamethoxazole(Diwan et al., 2018). The sulfonamides, macrolides, β-lactams are susceptible

to hydrolysis and other abiotic transformations, but for sulfonamides and macrolides hydrolysis at the neutral pH is relatively slow and negligible (Huang et al., 2011). Therefore, continuous release of effluent from tanneries has a probability ofleadingto the accumulation of antibiotic residues to a toxic level in the effluent receiving environments(Zhang et al., 2014). The presence of the antibiotic residues in the final effluent after going through aerobic treatment and sedimentation stages suggested that removal of these antibiotics under aerobic condition by biodegradation was low or not happening in the wastewater treatment process.

Thepresence of antibiotic residues in the tannery wastewater hintedthat the tannery WWTPis one of the suitable places for antibiotic resistance and spread of ARGs between bacteria. The issue of ARGs in the tannery WWTP has serious public health concern since the contributing factors are available in the tannery WWTP. As discussed in section 4.4, many potential bacteria pathogens of zoonotic origins were identified in the same tannery wastewater.The occurrence of antibiotic residues togetherwith the potential bacterial pathogens in the tannery wastewater may allow the pathogens to develop antibiotic resistance. For instances, *Clostridium*and *Campylobacter*were detected in tannery effluentand these bacteria arezoonotic pathogens reported in food animals develop antibiotic resistance in the presence of antibiotic residues(Hong et al., 2013; Lekshmi et al., 2017).

Almost all of the antibiotics detected in the raw tannery wastewater were also present in the outgoing tannery effluent after treatment. This suggests that the release of antibiotic residues through the tannery effluent into the environment is imminent and this may affect the diversity and functionality of the organisms in the aquatic ecosystem. For example, sulfamethoxazole can delay cell growth, limit denitrification and affect the bacterial community composition even at low concentrations(Groot & Hooft, 2018). This indicates that theantibiotic residues in the

tannery wastewater may slow down denitrification and upsets the removal of nitrogen by the biological process. Therefore, the low removal efficiency of the tannery WWTP to the total nitrogen could belinked with the presence of antibiotic residues.

In addition, the release of antibiotic residues in to the river with the tannery effluent could lead to their accumulationsin sediments by some important sorption mechanisms (e.g., cation bridging, cation exchange, and hydrogen bonding) and this can be a secondary source of antibiotics for aquatic environment (Siedlewicz et al., 2016). When antibiotic residues enter to the river, the persistence of antibiotic residues could be enhanced by being sorbed to the organic particles based on the physicochemical properties of the antibiotics(Pikkemaat et al., 2016). On the other hand, the use of antibiotic contaminated river for irrigation is supposed to play important rolesby transmitting antibiotic residues and antibiotic resistant bacteria intosoil environment (Shashidhar, 2015). Given the antibiotic classes used in livestock are also used in human, the presence of antibiotic residues in the tannery effluent can be a concern of cross-resistance in bacterial pathogens (Hong et al., 2013). Once in the environment, antibiotic residues could pose public health issue and environmental toxicity concerns.

4.6. Antibiotic Resistance Genes in the tannery wastewater

The presence of antibiotic residues in the wastewater treatment systemsis thought to exert selective effect on commensal bacteria and can spread resistance amongthe pathogens. This is because the antibiotic residues have substantial effect on the bacteria physiology even at sublethal concentrations (Holmes et al., 2016). As presented in Table 4.6, many of the targeted antibiotics were detected in the Batu Tannery WWTP. In the same treatment plant, antibiotic resistance genes (ARGs)corresponding to the antibiotic residues were detected in the tannery

wastewater samples (Figure4.13). The ARGs considered for the qualitative PCR assessment were*tet*(A), *tet*(O), *tet*(M), *tet*(Q), *erm*(B), *Sul*(I), *Sul* (II), *Otr*(A) and*qnrA*. The PCR assay revealed that all of the targeted ARGs weredetectedat least in some of thetannery wastewater samples, except the*tet*(Q) genewhichwasnot detected in one of the mixed liquor (S2) sample, while it was very lightly observed in the raw general (G1) sample (Figure 4.13).

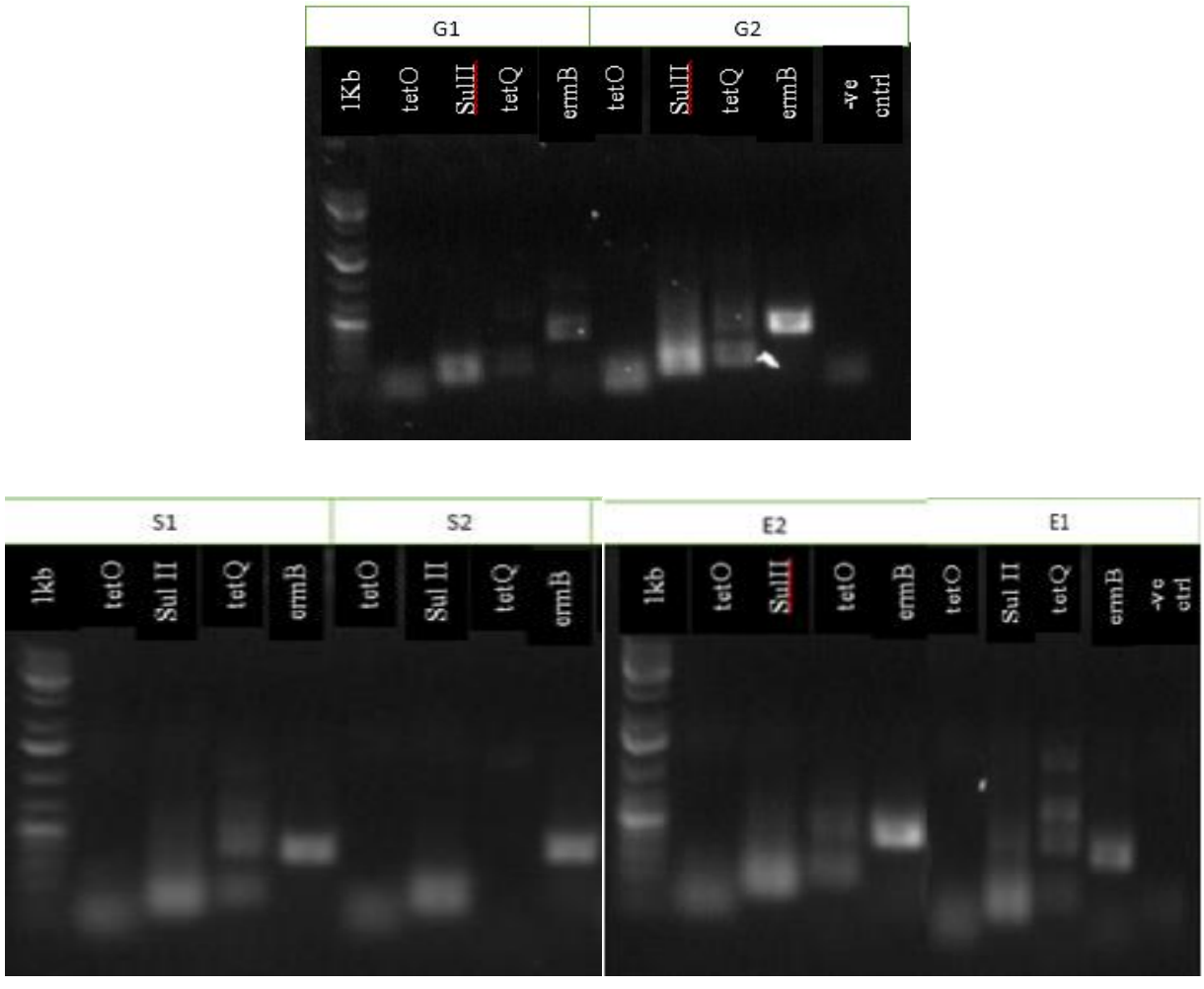

Figure 4.13. Agarose gel electrophoresis images showing PCR amplicons of the ARGs detected from the tannery wastewater samples (G1 = Raw tannery wastewater collected in first round, S1 = aerated mixed liquor collected in first round, E1 = Tannery effluent collected in first round, G2 = Raw tannery wastewater collected in second round, S1 = aerated mixed liquor collected in second round, E2 = Tannery effluent collected in second round)

In all the tannery wastewater samples, there was positive correspondence between the presence of antibiotic residues and ARGs that the antibiotic residues and ARGs were detected at the same timeand in thesame samples of the tannery wastewater.Being animal husbandry is most common economic activity and antibiotic misuse is believed to be the major driving factors for antibiotic resistance (Sharma et al., 2018); presence of antibiotic residues and ARGs in tannery effluent is a seriousissueof public health. The presence of antibiotic residues in the tannery effluent together with ARGs shows that the current tannery wastewater treatment scheme is seemingly unable to retain ARGs and antibiotics.The presence of ARGs and antibiotic residues together with abundant potential bacterial pathogens may expose the bacteria to situationsthat impose selection in favor of the resistant bacteria (Hong et al., 2013).

The detection of ARGs in the tannery wastewater together with the corresponding antibiotic residues in the same environment suggested that the treatment plant provide suitable conditions for the selective propagation of antibiotic resistant bacteria and transmission of ARGs. For example, the presence of sulfonamide resistance genes in the wastewater was reported to have positive correlation with the presence of sulfonamide residues, but similar correlation was not observed between tetracycline resistance genes and tetracycline antibiotic residues (Lupan et al., 2017). However, this contradicts with the report by Hong et al.(2013) which showed correlation between abundance of tetracycline resistance genes with concentration of tetracycline residues and constant exposure of the antibiotic residue that lead to selective pressure for the resistance genes. The occurrence of antibiotic residues in the tannery effluent means that the antibiotic residues and ARGs enter to the river water.

Tetracycline is one of the most commonly used antibiotics in livestock treatment. However, resistances to tetracyclines are rampantly reported that resistance is mediated by efflux pump,

ribosome protection proteins and enzyme inactivation genes(Hong et al., 2013). In this study, the efflux pump *tet*A and target modification ribosomal protection protein *tet* resistance genes (*tet*M, *tet*O and *tetQ)* were detected in the raw (G1 and G2) and treated tannery wastewater (E1 and E2) samples (Figure 4.13). The t*etM*, *tetQ* and *tetW* genesareoften associated with conjugative transposonsthatare ubiquitously detected in various environments showing that they are widespread in the bacteria communities of different WWTPs (Tehrani & Gilbride, 2018). For example, tetracycline resistance mediated by efflux proteins have been reported in more than 50% of the *Burkholderiales*, *Xanthomonadales*, *Enterobacteriales* and *Aeromonadales* (Walsh & Duffy, 2013), all of which were identified in the tannery WWTP in this study.

According to Scott et al.(2000), *tetW* was common in *Acinetobacter* and*Clostridium*,but*Enterococcus* and *Bulkholderia* were found to host the *tetM and OtrA*in additionto *tetW*genes (Kobashi et al., 2007). The t*et*Agenes are also common broad host range resistance genes often presentin pathogenic bacteria such as *Acinetobacter*, *Chrysobacterium*, *Pseudomonas*, *Vibrio*, *Clostridium*, *Pseudomonas* and *Aeromonas* species(Van Hoek et al., 2011). All of these bacteria are identified in the same tannery wastewater with significant abundance. *TetM* is also widely distributed tetracycline resistance gene reported in many (18 gram-positive and 8 gram-negative) bacteria genera(Hedayatianfard et al., 2014).The*tetM* resistant bacteria includes *Enterococcus*, *Streptococcus*, *Bifidobacterium*, *Legionella*, *Pseudomonas* and other bacteria and wide spread occurrence of *tetM*is attributed to mobile genetic elements. The*tetW* and *tetO* genes werereported to be prevalent in animal gastrointestinal environments and have been considered promiscuous in their ability for horizontal gene transfer among bacteria communities(Sullivan et al., 2013). However, at the higher taxonomic ranks, the most dominant bacteria identified at the tannery wastewater (Firmicutes, Bacteroidetes and

Proteobacteria)were reported to have negative correlations with resistance to the antibiotics tetracyclines, penicillin and sulfonamides(Novo et al., 2013).

In thisstudy,*ermB* resistance genes were also detected in thetannery wastewater samples. The *ermB* genes encode resistance against macrolides, lincosamide and streptogramin antibiotics and the *ermB*gene is generally associated with conjugative transposons. Rafraf et al., (2016)have reported that members of the Firmicutes such as *Enterococcus*, S*taphylococcus* and S*treptococcus* and gram-negative bacteria belonging to *Bacteroidetes* have developed *ermB*resistance in the presence of erythromycin. Erythromycin has reputation of human clinical use and associated erythromycin resistance genes were detected in clinical wastewaters (Rahube et al., 2014). There are also reports which showed that the presence of erythromycin and penicillin G in the tannery wastewater can enrich antibiotic resistance by pathogenic *Campylobacter* and *Enterococcus* evenat smaller quantities (Economou & Gousia, 2015; Ge et al., 2017).

The occurrence of erythromycin residue together with these bacteria can favora selective condition suitable for the proliferation of *ermB* resistant bacteria in the tannery wastewater treatment systems. Moreover, the macrolide resistance gene *ermB* and tetracycline resistance genes *tetO* and *tetQ* can spread among the phylogenetically different gram-negative and gram-positive bacteria in the wastewater (Van Schaik, 2015). The *qnrA*gene encoding resistance against quinolone was also detected in all samples of the tannery wastewater (Figure 4.13). Previousreport showed that this gene was detected in aquatic bacteria such as in*Escherichia*, S*almonella (Enterobacteriaceae*), *Shewanella, Aeromonas*species and *Vibrionaceae*families(Finley et al., 2013), all of which are identified in the current study site.

Based on the antibiotic residue, ARGs and microbial data, the tannery WWTPis suitable environment for the development and propagation of ARGs and can be considered as one of the most important hotspots for the spread of ARB and ARGs to the environment(Kim & Aga, 2007; Bouki et al., 2013). Therefore, the release of insufficiently treated tannery wastewater to the river can transmit the antibiotic residues and ARGs from animal sources to the tannery wastewater and then into the river water and soil environment. This is because the bacteria or mobile elements can linger on animal skins and in animal feces (Holmes et al., 2016), which can be transferred to the tannery WWTP through the skin and hides. The problem can be exacerbated by the physicochemical characteristics of the antibiotics which may have longer half-life in environment and persist for long time(Kulkarni et al., 2017).

Theoccurrence of antibiotic residues in concert with heavy nutrient load and diverse bacteria community can have public health concern associated withthe antibiotic resistant pathogens(Miller et al., 2016).Moreover, since thetannery effluent is released to the nearby river and the river water is used for small scale irrigation by downstream community, the presence of ARGs and antibiotic residuesin the tannery effluent haveapparentenvironmental risks. In the long-term,there is ecological concern that the antibiotic residues and ARGs can affect the structure of bacteria community in the river sediment and irrigated soil. The presence of ARGs in the tannery effluent demonstrate that the current tannery wastewater treatment scheme is one of the hot spot for the bacteria resistance(Hong et al., 2013).

Co-occurrence of antibiotic residues and ARGs in the tannery wastewater and the fact that bacteria communities always compete for survival means selective pressure is inevitable in the tannery WWTP. According toHolmes et al.(2016), antibiotic resistance is at least in part emerged as a consequence of the presence of antibiotics which exert selective pressure. The presences of

antibiotic residues not only exert selective effect, but also induce the horizontal mobility of antibiotic resistance determinants in bacteria. Therefore, the detection of both ARGs and antibiotic residues in the same artificial environment, the WWTP, can enhance the emergence and spread of ARGs among bacteria. Although thedischarge of ARGs and antibiotic residues with the tannery effluent is evident, the environmental fates of ARGs and antibiotic resistant bacteria in the environment are liable to several factors. This is because the fate and persistence of resistance genes would depend on the ability of the bacteria host to survive in the discharged environment where there is competitive condition (Hong et al., 2013).Resistancefactors mobilized through mobile genetic elements are likely to have increased fitness cost which is a burden of keeping multiple copies of the same genes and difficulty of maintaining expression control of the gene.

In addition to the selective effect of antibiotic residues in favor of antibiotic resistant bacteria, the presence of antibiotic residues in the tannery wastewater can have negative impact on the removal of pollutants as the presence of antibiotic residues in the wastewater could affect nitrogen and phosphorus metabolisms and their removal (Novo et al., 2013). Therefore, it must be emphasized that the tannery WWTP has uniquely favorable conditions due to the presence of large microbial communities, different nutrients, chromium and antibiotic residues all of them considered as contributing stress factors.

4.7. Antibiotic use trend in livestockshowed low prudence

In the current study, nine antibiotic residues were detected in the Tannery wastewater samples (Table 4.6). This elicit question why these antibiotic residues occur in the Tannery wastewater, and survey was carried out in order to gather information on the use trend of antimicrobials in

animals. Therefore, antibiotic use trend in animal health systems for therapeutic and non-therapeutic uses was assessed in rural areas. The assessment on antibiotic use trend in livestockinvolved83(55.3%) farmer informants and 67 (44.7%) veterinary clinicians as key informants. From the total respondents, 76.7% were males and 92% of the age of the informants was between 18 and 50 years. With regard to educational background, 42.7% of the veterinary clinicians were trained at diploma level, 51.2% at Bachelor degree and 6.1% at MSc level. Nearly 40% of the farmer informants involved in animal fattening were college graduates in different disciplines, whereas most of the informant farmers were not formally educated. Work experience of 44.6% animal clinicians was less than 5 year, 33.7% have from 5 to 10 years of experience and the remaining 21.7% of the workers have more than 10 years of experience in government and private organizations.

Antimicrobial misuse is not uniquely associated with human health alone, rather there is also rampant use and abuse of antibiotics in livestock for disease treatment, and at subtherapeutic levels for prophylaxis and growth promotion purposes (Hong et al., 2013). Therefore, the use trend of antimicrobials in livestock treatment can incite concern on the environmental fate of the veterinary antimicrobials after used by the animals since significant amount of the antimicrobials are not retained in the animal body(Blair et al., 2013).

The survey revealed that there was possibility for misuse of the veterinary antibiotics and poor animal waste management which allow the transmission of antibiotic residues to the environment after used by the animals (Figure 4.6). The antibiotic use trendby animal fatteners showed several flawed practices. Among the problems noticed in the surveywasinappropriate withdrawal of food animals after administration of antimicrobials and lack of awareness on the environmental side effects of antimicrobials by the farmers. Associated with veterinary medicine

uses, there are different routes of exposing the antibiotics residues to the environment (Figure 4.14). One of these routes is through the tannery wastewater after the raw hides and skins are used in leather production.

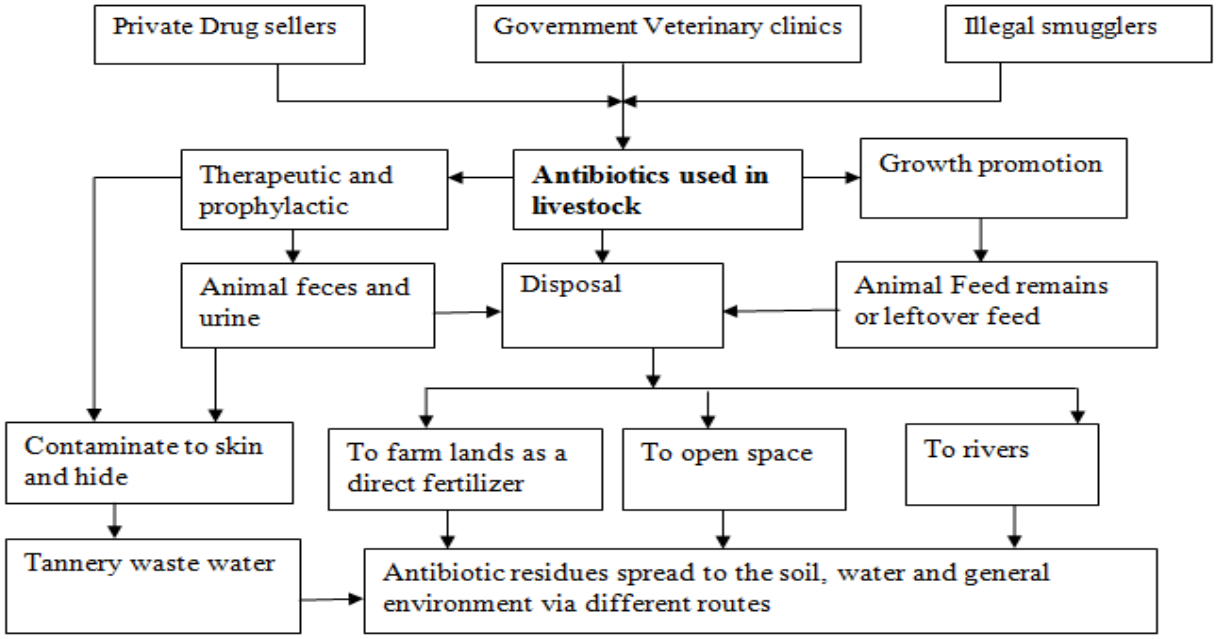

Figure 4.14. Summary of veterinary antibiotic use pattern in animals and theirpossible environmental fate

Antibiotic use trend model in the Figure 4.14 showed that the antibiotics spread to the environment in different routes. The antibiotics used in livestock treatment are obtained from private drug sellers, government clinics and from Illegal sellers. Veterinary clinicians in the observed clinics (September 2017 to Jun 2018) indicated that many domestic animals were admitted to the veterinary clinics and treated with different antimicrobials including the antibiotics (Table 4.7). The animals treated by antimicrobials consists the cattle, sheep, goats, chickens, pigs, horses, camels, mule and donkeys, from which the cattle, sheep and goats constitutedthe largest number of animals treated withthe antimicrobials (Table 4.7).

Table 4.7. Antimicrobials commonly used for the treatment of cattle, sheep and goats in eight districts of the east Amhara region, Ethiopia

Antimicrobials used	Livestock		
	Cattle (%)	Sheep (%)	Goat (%)
Oxytetracycline	29.7	35.8	34.5
Sulfonamides	33.6	36	30.4
Penstrep	34.6	36.4	29
Penicillin	33.3	30.2	36.5
Procaine penicillin	37.2	26.8	36
Ivermectin	35.3	29.8	34.9
Albendazole	43.4	30.6	26
Tinidazoles	33	31	36
Multi-vitamins	32.7	32	35.3
Acaricides	16.7	45	38.3

An observation in the antimicrobial use tendency also showed that there was low prudence of antimicrobial use in livestock. From the existing use trend in the visited areas, veterinary antimicrobials are observably exposed to misuse unknowingly by the farmers and carelessly by the drug sellers. This was demonstrated by the informants who indicated that farmers can buy drugs from animal health centers often without physical witnessing of the diseased animals for observing disease symptoms and physical conditions of the animals. Such erroneous use of antibiotics in livestock treatment in Ethiopia can have impact to the public health since the wide spectrum antibiotics are used in both human and animal medicine (Groot & Hooft, 2018).The antibiotics detected in the tannery wastewaterinclude tetracyclines, penicillin, macrolides, sulfonamides and macrolides which are also the dominant antibiotic used in livestock production

(Hong et al., 2013). The lack of prudent use of antibiotic in animals can lead to public health problems by enhancing antibiotic resistant pathogens (Lekshmi et al., 2017).

In thisstudy, the tannery wastewater samples were confirmed to contain antibiotic residues and ARGs. The source of antibiotic residues and ARGs present an intriguing question of why the antibiotic residues are there in the tannery wastewater, and the field observation during the survey revealed that it is likely that the antibiotic residues have animal origins. This was implicated by many instances of antimicrobial misuse observed in the visited areas such as the needless prescriptions to farmers to buy antimicrobials whichenhance fattening of the animals, albeit the guidelines recommend for prudent use (Holmes et al., 2016). Hence, the tannery WWTPcan link the route between antibiotics use in animal treatment and the aquatic environment through the use of skin and hide in tanneries. The tannery wastewater can also serve as a connection site for zoonotic bacteria between animals and aquatic environment.

Animal farming globally relies on the use of antimicrobials in order to improve animal health and increase animal food products (Sharma et al., 2018). In using antibiotics for animal treatment, many fake and low-quality antimicrobialsare suspected to be circulating in the pharmaceutical market.There is also a concern of circulating fake and low-quality antibiotics in the rural and boarder areas where the farmers use these antibiotics for their animals without prescription. An illegal import of fake and substandard drugs in Ethiopia can be indicated by the repeated mainstream media out letsreporting cases of unlawful drugs in Ethiopia (FBC, 2019).

Although the farmer informants did not confessthe problem of fake antimicrobials, but they observed that the efficiency of antimicrobials is not as good as it was few years back. This implied that the antimicrobials are either adapted by the pathogens or they are not of the right quality to threat animal diseases. Although data is lacking in Ethiopia, Sahoo et al.,

(2010)showed that low quality drugs are distributed in the market due to defective drug control practices in developing countries where there are many instances of obtaining prescription only antimicrobials without prescription. In Ethiopia, the presence of similar problem can be anticipated from the frequent quest of the government which is calling for cordial participation of the community to fight against fake and low-quality drugs.

4.8. Antibiotic use trend in Livestock treatment

Thequestionnaire-baseddatashowed that different antimicrobials areused for livestock treatments (Table 4.7). As presented in Table 4.7, 54.5% of the antimicrobials used livestock treatment waseither antibiotics or antibiotic mixturesused for treatment of livestockmainly the cattle, sheep and goats. The tetracyclines, sulfonamides, beta-lactams, quinolones and macrolides are commonly used antibiotic forpoultry, cattle, sheep, goat and pigs treatment(GARP, 2011). The antimicrobials are administered to the livestock in different methods. For instance, in almost all visited areas, oral administration was carried out by mixing the antimicrobials with drinking water and animal feeds.This method of administering antibiotics to livestock was noticed to have possibility of misuse since the farmersadminister the antibiotics to more than one animaltogether in the same feeding vessel and this type of practice is common in rural farmers. Thisallow for under or over dose consumption of theantibiotics by animals depending on the efficiency of competing for the feed and drink, and the required dose may not be given to the animals in the specified period of time. The other rampant problem in rural areas was lack of access to nearby professionals for diagnosis and this is enforcing farmers to buydrugs from drug stores to administer for animals without prescription.

The use trend of antimicrobials in livestock showed that most of the antibiotics are used for the treatment of cattle, sheep and goat (Table 4.7). This is in agreement with to a study on the rational use of veterinary antibiotics and other antimicrobials in the central Ethiopia where oxytetracycline, penicillin-streptomycin, sulfadrugs, albendazole and ivermectin (antihelminths) were the most frequently prescribed drugs (Takele Beyene et al., 2015). The result was also consistent with findings oversea where penicillin, amoxicillin, ciprofloxacin and tetracyclines were among the most frequently used antibiotics for prophylactic and growth promotion purposes in dairy and poultry farms (Sahoo et al., 2010). However, the use trend of antibiotics in livestock is likely to vary from region to region and the use of antibiotics in different phases of the livestock production use different types and amounts of antibiotics (Hong et al., 2013).

Another concern associated with the use of antimicrobials in rural livestock health is management of animal wastes. Many of the respondents (76%) showed that there was little concern to the environmental consequences of animal wastes with which residual antibiotics are released after therapeutic or other applications. The release of antibiotic residues after use by animals has environmental problems related to antibiotic resistance linked with misuse of the antibiotics (Groot & Hooft, 2018). Almost all (99%) animal farmers have used antibiotics for their livestock, but their awareness waslowon the side effects of antibiotic misuse in livestock.

Extensive use of antimicrobials in livestock health care coupled with low education level of farmers (62.7%) means the misuse probability of antimicrobials was high in animal husbandry. The misuse problems were more pronounced by the inappropriate withdrawal time of food animals after given the antibiotics to which farmers showed little awareness to the proper guidelines for withdrawal time after antimicrobial treatments. According to Goutard et al. (2017) this types of antibiotic misuse in livestock are major drivers for the development and propagation

of the antibiotic resistance in pathogenic bacteria. Moreover, the rumen of food animals naturally harbor large population of bacteria and oral administration of antibiotics could enhance development of antibiotic resistance by the bacteria which later enter to the environment through animal wastes and other products including the flesh and hides. Of the orally administered antibiotics, the rumen bacteria can be exposed to the antibiotics in the way through gastrointestinal tractwhich is eliminated out of the animal body with the feces and urine (Lekshmi et al., 2017; Schmitt et al, 2017).

The result of this study agreed to the use pattern of antibiotics in 27 African member countries which showed that sheep, goats, cattle and poultry (food producing animals) use most of the antibiotics from which tetracyclines (63%) and macrolides (17%) were used in large proportion. In addition, sulfonamides (2%), penicillin (3%) and fluoroquinolones (6%) were among the reported antimicrobial classes (Baxter et al., 2008). The oxytetracycline, tetracycline, amoxicillin, erythromycin, penicillin, chlortetracycline, sulfamethazine, streptomycin, sulfathiazole and trimethoprim are approved for growth promotion in animals (Baxter et al., 2008).

The use of antibiotics for the treatment of animal diseases caused by infectious pathogens should be in line with the guidelines as it aids the decision for what type of antimicrobials have to be used (Daniel Teshome, 2018). However, most of the veterinary clinician respondents showed that antimicrobials are still obtainable without prescription from some drug stores and illegal sellers. Similar trend of antibiotic use trend was reported in Kenya where nearly 78% of the veterinary medicine outlets are operated by people not legally considered qualified(GARP, 2011). This shows that there is misuse of antibiotics in cattle and shoats (sheep and goats) which are the second and third most food producing animals next to poultry in developing countries

(Baxter et al., 2008). The same trend of antibiotic use was reported oversea showing that the amoxicillin, ampicillin, oxytetracycline, erythromycin, sulfonamides, penicillin G, trimethoprim, tetracycline and quinolones were used in sheep, goat and cattle treatments (Economou & Gousia, 2015).

Thesimilarity in the antibiotics used for livestock treatment and antibiotic residues detected in the tannery wastewaterimplied that the antibiotic residues and ARGsdetected in the tannery wastewater may came from animal sources with animal tissues(Abbasi et al., 2012). Similar antibiotics identified in the cattle tissue (beef) obtained from small holder and semi-intensive farms have shown that oxytetracycline (tetracycline), penstrip (beta-lactam) and sulfadiazine (sulfonamides) were present in different cattle tissues (Birhan Agmas & Mulugojjam Adugna, 2018). Therefore,the use of antibiotics in livestock together with inaccurate use trend is suspected to be thecause for the presence of antibiotic residues and ARGs in animal tissues transmitted to the tannery wastewater.

Chapter Six

6. Conclusions and Recommendations

6.1. Conclusions

The physicochemical results in this study showed that major pollutants (COD, TN, TDS, ammonia-N, sulfides and total chromium) remain above the permissible discharge limit in the tannery effluent. The pH and temperature of the treated effluent abideto the standards for the discharge of tannery effluent.

Up on the discharge of the tannery effluent to the nearby LAR water, the physicochemical characteristics of the downstream river water increased for most parameters, but significant changewas observedfor COD, total chromium and sulfate.It can be concluded that the pollution impact of Batu Tannery effluent to the LAR water cannot be overlooked and the current conditions of LAR water is severely polluted.

The most dominant bacteria communities at the higher taxonomic ranks in the Batu Tannery WWTP were Firmicutes, Bacteroidetes and proteobacteria. At the genera level, *Clostridia* was dominant in the mixed liquor and treated tannery effluent, but the dominance of *Clostridium* in the raw tannery samples show temporal difference.

Interestingly, the structure of most dominant bacteria identified in the tannery wastewater showresemblance to the dominant gastrointestinal bacteria of ruminants.Bacteria community in

the treated effluent showed lower diversity than the bacteria community in the mixed liquor and raw tannery wastewater samples.

Some of the dominant bacteria communities detected in the tannery wastewater are potential pathogensto human(e.g., *Clostridium spp.*). However, more confirmatory culture-based work is required to conclude on the actual pathogenicity of these bacteriasincethere are possibilities of similarity of the 16S rRNA sequences between the pathogenic and non-pathogenic phylotypes.

Antibiotic residues and antibiotic resistance genes were detected in tannery wastewater samples. Thesimultaneous presence of antibiotics and ARGs in the tannery wastewater samples has implications that the presence of antibiotic residues can enhance the preponderance of ARBs in the WWTP.

Antibiotic use trendby livestock has shownseveral issues of misusein rural areas and this implicate for the possible transfer of antibiotic residues from animals to the tannery wastewaterthrough animal tissues, manure and urine contaminated to the skins and hides.

6.2.Recommendations

- The efficiency of Batu Tannery WWTP in removing priority pollutants is encouraging, but the final tannery effluent need to be polished in order to fulfill the effluent quality standards indicated in the EPA guidelines.
- The current condition of LARwater requires more attention by the governmentthat upstream source of effluents discharges must be traced and immediate mitigation is recommended before it is too late to restore the river ecology. Therefore, appropriate interventions are advised to all stakeholders in order to alleviate the pollution problem of the river.

- The presence of antibiotic residues in the raw and treated tannery wastewater shows that antibiotic residues are another environmental pollution problem. The author recommends more studies on antibiotic residues in other tanneries in order to generate national data which can enforce inclusion of these pollutants in the national tannery wastewater quality guidelines.
- Morequantitative study is alsorecommended in order to determine the antibiotic resistance genes in the tannery effluent using quantitative assessment techniques. Thereis a need to investigate on technologies that avoid or containthe resistance gene from escaping out with the final effluent.
- The widespread use of antimicrobials in animal husbandry has inevitable consequence of enhancing antibiotic resistance. So, there is a need for integrated approach of adopting policies and guidelines which require the control and monitoring of antibiotic residue levels in the industrial and domestic WWTP by the environmental and health sectors.
- The author also recommends for more antibiotic use surveillance study in livestock at the country level in order to generate a national data on antibiotic use in animals. Strong national policy and law enforcement is advised in order to minimize the misuse of antibiotics in animal production.
- In the tannery wastewater, many bacteria groups were identified that can to be potential pathogens to human and animals. We strongly recommend a final disinfection treatment for tannery effluent and sludge before discharged to the environment.

7. Reference

Abbasi, M. M., Nemati, M., Babaeia, H., Ansarin, M., & Nourdadgar, A. O. S. (2012). Solid-phase extraction and simultaneous determination of tetracycline residues in edible cattle tissues using an HPLC-FL method. *Iranian Journal of Pharmaceutical Research*, ***11***(3), 781–787.

Abdallh, M. N., Abdelhalim,W. S., and Abdelhalim, H. S. (2016). Biological Treatment of Leather Tanneries Wastewater Effluent- Bench Scale Modeling. *International Journal of Engineering Science and Computing*, ***6***(9), 2271–2286.

Abrha Gebrekidan, Gebrekidan Gebresellasie, & Afework Mulugeta. (2009). Environmental impacts of Sheba tannery (Ethiopia) effluents on the surrounding water bodies. *Bulletin of the Chemical Society of Ethiopia*, ***23***(2), 269–274.

Adey Feleke, Fasil Assefa, Seyoum Leta, Stomeo, F., Wamalwa, M., Njahira, M., & Appolinaire, D. (2014). Microbial community structure and diversity in an integrated system of anaerobic-aerobic reactors and a constructed wetland for the treatment of tannery wastewater in Modjo, Ethiopia. *PLoS ONE*, ***9***(12), 1–22.

Adefisoye, M.A. & Okoh, A.I. (2017). Ecological and Public Health Implications of the Discharge of Multidrug-Resistant Bacteria and Physicochemical Contaminants from Treated Wastewater Effluents in the Eastern Cape. *Water*, **9** (562), 1–18.

Agga, G. E., Arthur, T. M., Durso, L. M., Harhay, D. M., & Schmidt, J. W. (2015). Antimicrobial-Resistant Bacterial Populations and Antimicrobial Resistance Genes

Obtained from Environments Impacted by Livestock and Municipal Waste. *PLOS ONE*, ***10***(7), 1–19.

Agrawal, N., & Singh, R. (2016). Study of Heavy and Transition Elements in Tannery Effluents and its Impact on Soil around Kanpur, India. *International Journal of Scientific & Engineering Research*, ***7***(3), 3–6.

Ahn, Y., & Choi, J. (2016). Bacterial Communities and Antibiotic Resistance Communities in a Full-Scale Hospital Wastewater. *Water*, ***8***(580), 1–11.

Aidara-kane, A., Angulo, F. J., Conly, J. M., Minato, Y., Silbergeld, E. K., & Mcewen, S. A. (2018). World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. *Antimicrobial Resistance and Infection Control*, ***7***(7), 1–8.

Akimenko, Y. V., Kazeev, K. S., & Kolesnikov, S. I. (2015). Impact assessment of soil contamination with antibiotics (For example, an ordinary chernozem). *American Journal of Applied Sciences*, ***12***(2), 80–88.

Al-bahry, S., Al-sharji, N., Yaish, M., Al-musharafi, S., & Mahmoud, I. (2016). Diversity of Tetracycline Resistant Genes in Escherichia coli from Human and Environmental Sources. *The Open Biotechnology Journal*,**10**, 289–300.

Alam, M. Z., Ahmad, S., & Malik, A. (2011). Prevalence of heavy metal resistance in bacteria isolated from tannery effluents and affected soil. *Environmental Monitoring and Assessment*, ***178***(4), 281–291.

Alanis, A. J. (2005). Resistance to antibiotics: Are we in the post-antibiotic era? *Archives of Medical Research*, ***36***, 697–705.

Alexander, J., Bollmann, A., Seitz, W., & Schwartz, T. (2015). Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria. *Science of the Total Environment*, ***513***, 316–325.

Alexander, T. W., Yanke, J. L., Reuter, T., Topp, E., Read, R. R., Selinger, B. L., & Mcallister, T. A. (2011). Longitudinal characterization of antimicrobial resistance genes in feces shed

from cattle fed different subtherapeutic antibiotics.*BMC Microbiology*, **19** (11), 1-12.

Allen, H. K. (2014). Antibiotic resistance gene discovery in food-producing animals. *Current Opinion in Microbiology*, ***19***(1), 25–29.

Allen, H. K., Bayles, D. O., Looft, T., Trachsel, J., Bass, B. E., Alt, D. P., Bearson, S.M.D, Nicolson, T. & Casey, T. A. (2016). Pipeline for amplifying and analyzing amplicons of the V1 – V3 region of the 16S rRNA gene. *BMC Research Notes, **9***(380), 1–6.

Alvarez-bernal, D., Contreras-ramos, S. M.,Trujillo-tapia, N., Olalde-Portugal, V., Frıas-Herna´ndez, J.T. & Dendooven, L. (2006). Effects of tanneries wastewater on chemical and biological soil characteristics. *Applied Soil Ecology, **33***, 269–277.

Amde Eshete, Haiku Fekadu, Solomon Tejineh & Mesfin Tafa. (2016). The Pollution Profile of Modjo River Due to Industrial Wastewater Discharge, in Modjo Town, Oromia, Ethiopia. *Journal of Environmental and Analytical Toxicology*, ***6***(3), 1-5

APHA. (1999). *Standard Methods for the Examination of Water and Wastewater*. *American Public Health Association*. Washington D.C.

AssefaWosenie& AyalewWondie. (2014). Assessment of downstream impact of Bahir Dar tannery effluent on the head of Blue Nile River using macroinvertebrates as bioindicators. *International Journal of Biodiversity and Conservation*, ***8***(6), 342–350.

Atashgahi, S., Aydin, R., Dimitrov, M. R., Sipkema, D., Hamonts, K., Lahti, L., Maphosa F., Kruse, T., Saccenti, E., Springael, D., Dejonghe, W. &Smidt, H. (2015). Impact of a wastewater treatment plant on microbial community composition and function in a hyporheic zone of a eutrophic river. *Nature Publishing Group*, ***5 (17284)***, 1–13.

Auerbach, E. A., Seyfried, E. E., & Ã, Katherine, D. M. (2007). Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research*, ***41***(5), 1143–1151.

Auffret, M. D., Dewhurst, R. J., Duthie, C.A., Rooke, J. A., John Wallace, R., Freeman, T. C., Stewart, R., Watson, M. &Roehe, R. (2017). The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. *Microbiome*, ***5***(1), 159.

Awad, A., Eltayeb, I., Matowe, L., & Thalib, L. (2005). Self-medication with antibiotics and antimalarials in the community of Khartoum State, Sudan. *Journal of Pharmacy and Pharmaceutical Sciences*, ***8***(2), 326–331.

Awad, Y. M., Kim, K. R., Kim, S. C., Kim, K., Lee, S. R., Lee, S. S., & Ok, Y. S. (2015). Monitoring antibiotic residues and corresponding antibiotic resistance genes in an agroecosystem. *Journal of Chemistry*,**974843**, 1 - 7.

Ayandiran, T. A., Ayandele, A. A., Dahunsi, S. O., & Ajala, O. O. (2014). Microbial assessment and prevalence of antibiotic resistance in polluted Oluwa River, Nigeria. *Egyptian Journal of Aquatic Research*, ***40***(3), 291–299.

Azom, M. R., Mahmud, K., Yahya, S. M., Sontu, A., & Himon, S. B. (2012). Environmental Impact Assessment of Tanneries: A Case Study of Hazaribag in Bangladesh. *International Journal of Environmental Science and Development*, ***3***(2), 152–156.

Babic, S., Asperger, D., Mutavdzic, D., Horvat, A.J.M. & Kastelan-Macan, M. (2006). Solid phase extraction and HPLC determination of veterinary pharmaceuticals in wastewater. *Talanta, ScienceDirect*, ***70(4)***, 732–738.

Balvočiūtė, M., & Huson, D. H. (2017). SILVA, RDP, Greengenes, NCBI and OTT- How do these taxonomies compare? *BMC Genomics*, ***18*** *(*114), 1–8.

Banani, B. (2013). Political Economic Study on Tannery Industry Development and Subsequent Effect. Doctoral Dissertation, Tokyo University of Marine Science and Technology.

Baquero, F., Martinez, J. L., & Cantin, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*, ***19***(3), 260–265.

Barb, J. J., Oler, A. J., Kim, H., Chalmers, N., Wallen, R., Cashion, A., Munson, P.J &Ames, N. J. (2016). Development of an Analysis Pipeline Characterizing Multiple Hypervariable Regions of 16S rRNA Using Mock Samples. *PLOS ONE*, ***11***(2), 1–18.

Barbosa, T. M., & Levy, S. B. (2000). The impact of antibiotic use on resistance development and persistence. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, ***3***(5), 303–311.

Batt, A. L., Kostich, M. S., & Lazorchak, J. M. (2008). Analysis of ecologically relevant pharmaceuticals in wastewater and surface water using selective solid-phase extraction and UPLC-MS/MS. *Analytical Chemistry*, ***80***(13), 5021–5030.

Baxter, R., Hastings, N., Law, A., & Glass, E. J. (2008). A rapid and robust sequence-based genotyping method for BoLA-DRB3 alleles in large numbers of heterozygous cattle. *Animal Genetics*, **39** (5), 561–563.

Behailu Amde. (2017). Major Factors Affecting Hide and Skin Production , Quality and the Tanning Industry in Ethiopia. *Advances in Biological Research*, ***11***(3), 116–125.

Bellanger, X., Guilloteau, H., Bonot, S., & Merlin, C. (2014). Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. *Science of the Total Environment*, ***493***, 872–882.

Bengtsson-Palme, J., & Larsson, D. G. J. (2016). Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. *Environment International*, ***86***, 140–149.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, ***153***, 347–S357.

Berendonk, T. U., Manaia, C. M., Merlin, C. Fatta-Kassinos, D. Cytryn, E. Walsh, F. Bürgmann, H., Sørum, H., Norström, M. Pons, M. N., Kreuzinger, N., Huovinen, P. Stefani, S., Schwartz, T., K., Baquero, F., & Martinez, J. L. (2015). Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology*, ***13***(5), 310–317.

Berglund, B. (2014). Deliberations on the impact of antibiotic contamination on dissemination of antibiotic resistance genes in aquatic environments. Medical Dissertation, Linköping University, Sweden

Bhatnagar, M. K., Raviraj, S., Sanjay, G., Prachi, B., Shambhu, P., Shukla, N., & College, G. P. G. (2013). Study of Tannery Effluents and Its Effects on Sediments of River Ganga in Special Reference To Heavy Metals At Jajmau , Kanpur , India. *Journal of Environmental Research and Development*, ***8***(1), 56–59.

Birhan Agmas & Mulugojjam Adugna (2018). Antimicrobial residue occurrence and its public health risk of beef meat in Debre Tabor and Bahir Dar, Northwest Ethiopia. *Veterinary World*, ***11***(7), 902–908.

Bisrat Gebremichael. (2016). Traditional Leather Processing , Production and Marketing in Amhara Regional State of Ethiopia. *Open Access Library Journal*, ***3***(e2751), 1–17.

Blair, B. D., Crago, J. P., Hedman, C. J., & Klaper, R. D. (2013). Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere*, ***93***(9), 2116–2123.

Bosnic, M., Buljan, J., Daniels, R. P., & Rajamani, S. (2003). Pollutants in Tannery Effluent: International Scenario on Environmental Regulations and Compliance. Technical Information on Industrial Processes, United Nations Industrial Development Organization, Vienna.

Bouki, C., Venieri, D., & Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicology and Environmental Safety*, **91**, 1–9.

Branco, R., Chung, A. P., Veríssimo, A., & Morais, P. V. (2005). Impact of chromium-contaminated wastewaters on the microbial community of a river. *FEMS Microbiology Ecology*, ***54***(1), 35–46.

Brigden, K., Labunska, I., Stringer, R., Johnston, P. Santillo, D. & Ashton, J. (2000). Organic pollutants and heavy metal contaminants in wastewater discharges and sediments from the Riachuelo river. Greenpeace Research Laboratories, Department of Biological Sciences, University of Exeter, UK.

Brown, M. V., & Gast, R. (2015). Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. *Frontiers in Microbiology*,***6***(1027), 1–20.

Burke, C. M., & Darling, A. E. (2016). A method for high precision sequencing of near full-length 16S rRNA genes on an Illumina MiSeq. *PeerJ*, ***4*** (e2492), 1-20.

Call, D. R., Matthews, L., Subbiah, M., & Liu, J. (2013). Do antibiotic residues in soils play a role in amplification and transmission of antibiotic resistant bacteria in cattle populations? *Frontiers in Microbiology*, ***4***(193), 1–8.

Callaway, T. R., Dowd, S. E., Edrington, T. S., Anderson, R. C., Krueger, N., Bauer, N., Kononoff, P.J. & Nisbet, D. J. (2010). Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing 1. *American Society of Animal Science*, ***88***, 3977–3983.

Cameron, A., & McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. *Journal of Animal Science and Biotechnology*, ***7***(1), 1–22.

Caporaso, J .G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N. & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *PNAS*, ***108***, 4516–4522.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., & Zuccato, E. (2005). A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters, ***1092****(2)*, 206–215.

Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M. & Berendonk, T. U. (2016). Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. *FEMS Microbiology Ecology*, ***92***(5), 1–10.

Caudle, L. R., Knowlton, K. F., Strickland, M. S., & Akers, R. M. (2014). Metagenomic analysis of antibiotic resistance genes in the fecal microbiome following therapeutic and prophylactic antibiotic administration in Dairy Cows. Masters Thesis, Virginia Polytechnic Institute and State University, USA

Chakravorty, S., Helb, D., Burday, M., & Connell, N. (2008). Adetailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *NIH Public Access*, ***69***(2), 330–339.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y., Yannarell, A. C., Maxwell, S., & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality*, ***38***(3), 1086–1108.

Chen, Y., Yu, G., Cao, Q., Zhang, H., Lin, Q., & Hong, Y. (2013). Occurrence and environmental implications of pharmaceuticals in Chinese municipal sewage sludge. *Chemosphere*, ***93***(9), 1765–1772.

Cheng, V. C., Wong, S. C., Ho, P.-L., & Yuen, K.-Y. (2015). Strategic measures for the control of surging antimicrobial resistance in Hong Kong and mainland of China. *Emerging Microbes & Infections*, ***4***(1), 1 - 13.

Chiarucci, A., Bacaro, G., & Scheiner, S. M. (2011). Old and new challenges in using species diversity for assessing biodiversity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, ***366***(1576), 2426–2437.

Chowdhary, P., More, N., Raj, A., & Bharagava, R. N. (2017). Characterization and identification of pathogens from treated tannery wastewater bacterial. *Microbiology Research International*, ***5***(3), 30–36.

Chowdhury, M. Mostafa, M G, Biswas, T.K, Mandal, A, Saha, A. (2015). Characterization of the Effluents from Leather Processing Industries. *Environmental Processes*, ***2***, 173–187.

Chowdhury, M., Mostafa, M. G., Kumar, T., & Kumar, A. (2013). Treatment of leather industrial ef fluents by filtration and coagulation processes. *Water Resource and Industry*, ***3***, 11–22.

Chroma, M., & Kolar, M. (2010). Genetic Methods for Detection of Antibiotic resistance : Focus on Extended-spectrum β-Lactamases.*Biomed Pap Med Fac Univ Palacky Olomouc Czech Rep*, ***154***(4), 289–296.

Coelho, L. M., Rezende, H. C., Coelho, L. M., Sousa, R. D, Melo, D. O., & Coelho, N. M. (2015). Bioremediation of Polluted Waters Using Microorganisms.In: Advances in Bioremediation of Wastewater and Polluted Soil, *Naofumi Shiomi, Intech Open*,Pp.1–22.

Coppeaux, Z., Corral-Collière, C., Ilhami, A., Laigle, R., Savu, N., Soudan, M., Kyle, M. & Bomsel, O. (2016). Does Ethiopia have a comparative advantage in the leather industry? (February, 2016). Mines ParisTech Working Paper Industrial Organization, Ethiopia.

Crowther, G. S., Chilton, C. H., Todhunter, S. L., Nicholson, S., Freeman, J., Baines, S. D., & Wilcox, M. H. (2014). Comparison of planktonic and biofilm-associated communities of Clostridium difficile and indigenous gut microbiota in a triple-stage chemostat gut model. *Journal Antimicrobial Chemotherapy*, ***69****(8)*, 2137–2147.

Dafale, N. A., Semwal, U. P., Rajput, R. K., & Singh, G. N. (2016). Selection of appropriate analytical tools to determine the potency and bioactivity of antibiotics and antibiotic resistance $. *Journal of Pharmaceutical Analysis*, ***6***(4), 207–213.

Daghrir, R. and Drogui, P. (2013). Tetracycline antibiotics in the environment : A review, Environmental Chemistry Letters, **11** (3), 209–227.

Daniel Teshome. (2018). Review on Rational Use of Veterinary Antimicrobials and Anthelmintics. *Austin Journal of Veterinary Science & Animal Husbandary*, ***5***(2), 1–6.

Deghles, A., & Kurt, U. (2016). Treatment of tannery wastewater by a hybrid electrocoagulation/electrodialysis process. *Chemical Engineering and Processing: Process Intensification*, ***104***, 43–50.

Desai, C., Parikh, R. Y., Vaishnav, T., Shouche, Y. S., & Madamwar, D. (2009). Tracking the influence of long-term chromium pollution on soil bacterial community structures by comparative analyses of 16S rRNA gene phylotypes. *Research in Microbiology*, ***160***(1), 1–9.

Ding, Y., Qian, Y., Li, L., Ren, H., & Wang, Q. (2016). Total nitrogen removal of synthetic leather wastewater treated by MBBR. *2nd International Conference on Machinery, Materials Engineering, Chemical Engineering and Biotechnology*, *Atlantis Press*, pp. 565–569.

Diwan, V., Hanna, N., Purohit, M., Chandran, S., Riggi, E., Parashar, V., Tamhankar, A.J. & Stålsby Lundborg, C. (2018). Seasonal variations in water-quality, antibiotic residues,

resistant bacteria and antibiotic resistance genes of *Escherichia coli* isolates from water and sediments of the Kshipra River in Central India. *International Journal of Environmental Research and Public Health*, ***15***(6), 1–16.

dos Santos, A. L., Peixoto, R., & Rosado, A. S. (2009). New Aproaches to understanding microbial diversity in wastewater, Land fill and leachate treatment. *Oecologia Australis*, ***13***(4), 631–648.

Du, L., & Liu, W. (2012). Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agronomy for Sustainable Development*, ***32***(2), 309–327.

Durso, L. M., & Cook, K. L. (2014). Impacts of antibiotic use in agriculture: What are the benefits and risks? *Current Opinion in Microbiology*, ***19***(1), 37–44.

Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infection and Drug Resistance*, ***8***, 49–61.

El-bestawy, E., Ranya, F. A., & Reham, A. (2013). Biological Treatment of Leather-Tanning Industrial Wastewater Using Free Living Bacteria. *Advances in Life Science and Technology*, ***12***, 46–65.

El-Sheikh, M.A Saleh, H.I, Flora, J.R. & Abdel-Ghany, M. R. (2011). Biological tannery wastewater treatment using two stage UASB reactors. *Desalination*, ***276***, 253–259.

Elabbas, S., Ouazzani, N., Mandi, L., Berrekhis, F., Perdicakis, M., Pontvianne, S.,Leclerc, J. P. (2016). Treatment of highly concentrated tannery wastewater using electrocoagulation: Influence of the quality of aluminium used for the electrode. *Journal of Hazardous Materials*, ***319***, 69–77.

EPA. (2003). Provisional Standards for industrial pollution control in Ethiopia, United Nations Industrial Development Organization. Ethiopian Environment Protection Authoority (EPA), Addis Ababa, Ethiopia.

Essahale, A., Malki, M., Mar, I., & Moumni, M. (2010). Bacterial diversity in Fez tanneries and Morocco ' s Binlamdoune River , using 16S RNA gene based fingerprinting. *Journal of Environmental Sciences*, ***22***(12), 1944–1953.

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., & Ravel, J. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome*, ***2***(1), 1–7.

FBC. (2019). Fana Broadcasting corporation S.C. (FBC), @ fanabroadcasting.Media.News Company.

Fanning, S., Proos, S., Jordan, K., & Srikumar, S. (2017). A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Frontiers in Microbiology*, ***8***(1829), 1–16.

Fent, K., Weston, A. A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology*, ***76***, 122–159.

Ferezer Eshetu. (2012). Physico-chemical pollution pattern in Akaki River basin , Addis Ababa , Ethiopia. Master's thesis, Physical Geography and Quaternary Geology, 30 HECs, Stockholm University, Sweden.

Ferreira Da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., & Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiology Ecology*, ***60***(1), 166–176.

Finley, Rita L., Collignon, P., Larsson, D. J., Mcewen, S. A., Li, X. Z., Gaze, W. H., Reid-Smith, R., Timinouni, M., Graham, D. W. & Topp, E. (2013). The scourge of antibiotic resistance: The important role of the environment. *Clinical Infectious Diseases*, ***57***(5), 704–710.

Forslund, K., Sunagawa, S., Kultima, J. R., Mende, D. R., Arumugam, M., Typas, a, & Bork, P. (2013). Country-specific antibiotic use practices impact the human gut resistome. *Genome Research*, ***23***(7), 1163–1169.

Fouhy, F., Deane, J., Rea, M. C., Sullivan, Ó. O., Ross, R. P., Callaghan, G. O., Plant, B.J. & Stanton, C. (2015). The Effects of Freezing on Faecal Microbiota as Determined Using MiSeq Sequencing and Culture-Based Investigations. *PLOS ONE*, ***10***(3), 1–12.

Fredriksson, N. J., Hermansson, M., & Wilén, B. (2012). Diversity and dynamics of Archaea in an activated sludge wastewater treatment plant. *BMC Microbiology*, ***12***(140), 1–18.

Freeman, J., Bauer, M. P., Baines, S. D., Corver, J., Fawley, W. N., Goorhuis, B., Kuijper, E. J. & Wilcox, M. (2010). The Changing Epidemiology of Clostridium difficile Infections. *Clinical Microbiology Reviews*, ***23***(3), 529–549.

Gao, P., Munir, M., & Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of the Total Environment*, ***422***, 173–183.

GARP. (2011). Antibiotic Use and Resistance in Kenya. Global Antibiotic Resistance Partnership (GARP), Nairobi.

Ge, B., Domesle, K. J., Yang, Q., Young, S. R., Rice-Trujillo, C. L., Jones, S. B., Gaines, S.A., Keller, M.W., Li, H., Pineiro, S.A., Whitney, B.M., Harbottle, H.C. & Gilbert, J. M. (2017). Effects of low concentrations of erythromycin, penicillin, and virginiamycin on bacterial resistance development *in vitro*. *Scientific Reports*, ***7***(1), 1–11.

Giordano, C., Boscaro, V., Munz, G., Mori, G., & Vannini, C. (2016). Summer holidays as break-point in shaping a tannery sludge microbial community around a stable core microbiota. *Nature Publishing Group*, ***6***(30376), 2–10.

Goswami, S., & Mazumder, D. (2013). Treatment of Chrome Tannery Wastewater by Biological Process - A Mini Review, ***7***(11), 798–804.

Goswami, S., & Mazumder, D. (2014). Scope of biological treatment for composite tannery wastewater, ***5***(3), 607–622.

Goswami, S., & Mazumder, D. (2016). Comparative study between activated sludge process (ASP) and moving bed bioreactor (MBBR) for treating composite chrome tannery wastewater. *Materials Today: Proceedings*, ***3***(10), 3337–3342.

Gothwal, R., & Shashidhar, T. (2015). Antibiotic Pollution in the Environment: A Review. *Clean - Soil, Air, Water*, ***43***(4), 479–489.

Goutard, F. L., Bordier, M., Calba, C., Erlacher-Vindel, E., Góchez, D., De Balogh, K., Benigno, C., Kalpravidh, W., Roger, F. & Vong, S. (2017). Antimicrobial policy interventions in food animal production in South East Asia. *British Medical Journal*, ***358***, 31–41.

Grace, D. (2015). Review of evidence on antimicrobial resistance and animal agriculture in developing countries. International Livestock Research Institute (ILRI), Nairobi.

Granja-Salcedo, Y. T., Ramirez-Uscategui, R. A., Machado, E. G., Messana, J. D., Kishi, L. T., Dias, A. V. L., & Berchielli, T. T. (2017). Studies on bacterial community composition are affected by the time and storage method of the rumen content. *PLOS ONE*, ***12***(4), 1–15.

Groot, M. J., & Hooft, K. E. Van. (2018). The Hidden Effects of Dairy Farming on Public and Environmental Health in the Netherlands , India , Ethiopia , and Uganda , Considering the Use of Antibiotics and Other Agro-chemicals. Frontiers in Public Health, **4**(12), 1- 9.

Guasch, H., Serra, A., Corcoll, N., Bonet, B., & Leira, M. (2010). Metal Ecotoxicity in Fluvial Biofilm: Potential Influence of Water Scarcity. **In**: Water scarcity in Meditranean. Hdb Environmental Chemistry, **8**, 41–53.

Gueye, M. T., Seck, D., Ba, S., Hell, K., Sembene, M., J-P.Wathelet, & Lognay, G. (2011). Insecticidal activity of *Bosciasenegalensis* (*Pers*.) Lam ex Poir. on *Caryedon serratus* (*Ol*.) pest of stored groundnuts. *African Journal of Agricultural Research*, ***6***(30), 6348–6353.

Gupta, P., Asthana, M., Kumar, A., & Barun, S. (2014). Physicochemical Analysis and Microbial Diversity of Yamuna Water and Industrial Effluents. *International Journal of Applied Sciences and Biotechnology*, ***2***(2), 199–205.

Haile Reda. (2016). Physico Chemical Characterization of Tannery Effluent and Its Impact on the Nearby River. *Open Access Library Journal*, ***3***(3), 1–8.

Han, X., Yang, Y., Yan, H., Wang, X., Qu, L., & Chen, Y. (2015). Rumen bacterial diversity of 80 to 110-day- Old goats using 16S rRNA sequencing. *PLoS ONE*, ***10***(2), 1–12.

Hao, H., Sander, P., Iqbal, Z., Wang, Y., & Cheng, G. (2016). The Risk of Some Veterinary Antimicrobial Agents on Public Health Associated with Antimicrobial Resistance and their Molecular Basis. *Frontiers in Microbiology*, ***7***(1626), 1–11.

Hashem, M. A., Nur-A-Tomal, M. S., & Bushra, S. A. (2016). Oxidation-coagulation-filtration processes for the reduction of sulfide from the hair burning liming wastewater in tannery. *Journal of Cleaner Production*, ***127***, 339–342.

Haydar, S., & Aziz, J. A. (2009). Characterization and treatability studies of tannery wastewater using chemically enhanced primary treatment (CEPT) - A case study of Saddiq Leather Works. *Journal of Haza, **163***, 1076–1083.

Hedayatianfard, K., Akhlaghi, M., & Sharifiyazdi, H. (2014). Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction.*Veterinary Research Forum, **5***(4), 269–275.

Hensgens, M., Keessen, E., Squire, M., Riley, T., Koene, M., Boer, E., Lipman, L. & Kuijper, E. (2012). *Clostridium difficile* infection in the community: Azoonotic disease ? *Clinical Microbiology and Infection, **18***(7), 635–645.

Hien, P. T., Tadashi, T., & Kazuhiro, M. (2017). Changes in Tetracycline Resistance Gene Populations and Microbial Community in Tetracycline-Contaminated and Uncontaminated Wastewater via Constructed Wetland Treatment. *Journal of Aquatic Pollution and Toxicology*, ***2***(1:20), 1–8.

Holmes, A. H., Moore, L. S. P., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. V. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet, **387***, 176–187.

Hong, P.-Y., Al-Jassim, N., Ansari, M. I., & Mackie, R. I. (2013). Environmental and Public Health Implications of Water Reuse: Antibiotics, Antibiotic Resistant Bacteria, and Antibiotic Resistance Genes. *Antibiotics, **2(3)***, 367–399.

Hoque, M. A., & Khan, M. (2016). Seasonal Variation of Physicochemical Properties of Water in the Buriganga River, Bangladesh. *World Applied Sciences Journalciences Journal*, ***34***(1), 24–34.

Hu, J., Xiao, Z., Zhou, R., Deng, W., Wang, M., & Ma, S. (2011). Ecological utilization of leather tannery waste with circular economy model. *Journal of Cleaner Production*, ***19***(2–3), 221–228.

Huang, C.-H., Renew, J. E., Smeby, K. L., Pinkston, K., & Sedlak, D. L. (2011). Assessment of potential anbibiotic contaminants in water and preliminary occurrence analysis. *Journal of*

Contemporary Water Research and Education, ***120***(1), 30 -40.

Ibarbalz, F. M., Orellana, E., Figuerola, E. L. M., & Erijman, L. (2016). Shotgun metagenomic profiles have a high capacity to discriminate samples of activated sludge according to wastewater type. *Applied and Environmental Microbiology*, ***82***(17), 5186–5196.

Ibekwe, A. M., Leddy, M., & Murinda, S. E. (2013). Potential human pathogenic bacteria in a mixed urban watershed as revealed by pyrosequencing. *PLoS ONE*, ***8***(11). 1- 9

Ibraheem, J. A. (2012). Detection of Tetracycline , Doxycycline , Chlortetracycline , and Oxytetracycline Antibiotics in Nineveha Drug Wastewater. *Nathrain University, College of Engineering Journal*, ***15***(2), 215–221.

Iglesias, A., Nebot, C., Miranda, J., Vázquez, B., Abuín, C., & Cepeda, A. (2013). Determination of the Presence of Three Antimicrobials in Surface Water Collected from Urban and Rural Areas. *Antibiotics*, ***2***(1), 46–57.

Islam, B. I., Musa, A. E., Ibrahim, E. H., Sharafa, S. A. A., & Elfaki, B. M. (2014). Evaluation and Characterization of Tannery Wastewater. *Journal of Forest product and Industries*, ***3***(1), 141–150.

Jabari, L., Gannoun, H., Khelifi, E., Cayol, J. L., Godon, J. J., Hamdi, M., & Fardeaub, M. L. (2016). Bacterial ecology of abattoir wastewater treated by an anaerobic digestor. *Brazilian Journal of Microbiology*, ***47***(1), 73–84.

Jahan, M. A. A., Akhtar, N., Khan, N. M. S., Roy, C. K., & Islam, R. (2014). Characterization of tannery wastewater and its treatment by aquatic macrophytes and algae. *Bangladesh Journal of Science Industry Research*, ***49***(4), 233–242.

Jami, E., & Mizrahi, I. (2012). Composition and Similarity of Bovine Rumen Microbiota across Individual Animals. *PLOS Medicine*, ***7***(3), 1–8.

Jena, J., Kumar, R., Dixit, A., Pandey, S., & Das, T. (2015). Evaluation of simultaneous nutrient and COD removal with polyhydroxybutyrate (PHB) accumulation using mixed microbial consortia under anoxic condition and their bioinformatics analysis. *PLOS ONE*, ***10***(2), 1–20.

Joshua N. Edokpayi, J. O. O. and O. S. D. (2017). - Impact of Wastewater on Surface Water Quality in Developing Countries : A Case Study of South Africa. *Water Quality*, Intech, London, pp. 401–417.

Ju, F., & Zhang, T. (2015). 16S rRNA gene high-throughput sequencing data mining of microbial diversity and interactions. *Applied Microbiology and Biotechnology*, ***99*** *(10)*, 4119–4129.

Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. J. (2018). Antibiotic-Resistance Genes in Waste Water. *Trends in Microbiology*, ***26***(3), 220–228.

Kim, K., Owens, G., Kwon, S., So, K., Lee, D., & Ok, Y. S. (2011). Occurrence and Environmental Fate of Veterinary Antibiotics in the Terrestrial Environment. *Water Air Soil Pollut*, ***214*** *(4)*, 163–174.

Kim, M., Kim, J., Kuehn, L. A., Bono, J. L., Berry, E. D., Kalchayanand, N., Freetly, C., Benson, A.,K. &Wells, J. E. (2014). Investigation of bacterial diversity in the feces of cattle fed different diets. *Journal of Animal Science*, ***92****(2)*, 683–694.

Kim, M., Yoon, H., Kim, Y. E., Kim, Y. J., Kong, W. S., & Kim, J. G. (2014). Comparative analysis of bacterial diversity and communities inhabiting the fairy ring of Tricholoma matsutake by barcoded pyrosequencing. *Journal of Applied Microbiology*, ***117***(3), 699–710.

Kim, S., & Aga, D. S. (2007). Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*, ***10***(8), 559–573.

Kim, S., Park, H., & Chandran, K. (2010). Propensity of activated sludge to amplify or attenuate tetracycline resistance genes and tetracycline resistant bacteria : A mathematical modeling approach. *Chemosphere*, ***78***, 1071–1077.

Kim, T., Jeong, J., Wells, G. F., & Park, H. (2013). General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. Applied Microbiology and Biotechnology,**97**(4) 1755–1765.

Kobashi, Y., Hasebe, A., Nishio, M., & Uchiyama, H. (2007). Diversity of tetracycline resistance

genes in bacteria isolated from various agricultural environments. *Microbes and Environments*, ***22***(1), 44-51.

Kolomaznik, K., Adamek, M., Andel, I., & Uhlirova, M. (2008). Leather waste-Potential threat to human health, and a new technology of its treatment. *Journal of Hazardous Materials*, ***160***(3), 514–520.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology*, ***79***(17), 5112–5120.

Krishnan, M., Suganya, T., & Pandiarajan, J. (2016). Bacterial Community Exploration through Ion Torrent Sequencing from Different Treatment Stages of CETP for Tannery. *Expert Opinion on Environmental Biology*, ***5***(3), 1–13.

Kulkarni, P., Olson, N. D., Raspanti, G. A., Goldstein, R. E. R., Gibbs, S. G., Sapkota, A., & Sapkota, A. R. (2017). Antibiotic concentrations decrease during wastewater treatment but persist at low levels in reclaimed water. *International Journal of Environmental Research and Public Health*, ***14***(6), 1–13.

Le, T., Ng, C., Chen, H., Yi, X. Z., Koh, T. H., Barkham, T. M. S., Zhou, Z. & Gin, K. Y. (2016). Occurrences and Characterization of Antibiotic Resistant Bacteria and Genetic Determinants of Hospital Wastewaters in a Tropical Country. *Antimicrobial Agents and Chemotherapy*, ***60***(12), 7449-7456

Lee, S., Kang, H., & Park, H. (2015). Influence of influent wastewater communities on temporal variation of activated sludge communities. *Water Research*, ***73***, 132–144.

Lefebvre, O., Vasudevan, N., Thanasekaran, K., Moletta, R., & Godon, J. J. (2006). Microbial diversity in hypersaline wastewater: The example of tanneries. *Extremophiles*, ***10***(6), 505–513.

Lekshmi, M., Ammini, P., Kumar, S., & Varela, M. F. (2017). The Food Production Environment and the Development of Antimicrobial Resistance in Human Pathogens of

Animal Origin. *Microorganisms*, ***5***(1), 1–15.

Lemos, L. N., Fulthorpe, R. R., Triplett, E. W., & Roesch, L. F. W. (2011). Rethinking microbial diversity analysis in the high throughput sequencing era. *Journal of Microbiological Methods*, ***86***(1), 42–51.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., & Qi, R. (2010). Antibiotic Resistance Characteristics of Environmental Bacteria from an Oxytetracycline Production Wastewater Treatment Plant and the Receiving River. *Applied and Environental Microbiology, **76***(11), 3444–3451.

Li, R., Tun, H. M., Jahan, M., Zhang, Z., Kumar, A., Fernando, D.,Farenhorest, A & Khafipour, E. (2017). Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Scientific Reports*, ***7***(1), 1–11.

Liang, H., Ye, D., & Luo, L. (2017). Unravelling diversity and metabolic potential of microbial consortia at each stage of leather sewage treatment. *RSC Advances*, ***7***(66), 41727–41737.

Liu, J., Zhang, M., & Zhang, R. (2016). Comparative studies of the composition of bacterial microbiota associated with the ruminal content , ruminal epithelium and in the faeces of lactating dairy cows. *Microbial Biotechnology*, ***9***(2), 257–268.

Liu, K., Xu, Q., Wang, L., Wang, J., Guo, W., & Zhou, M. (2017). The impact of diet on the composition and relative abundance of rumen microbes in goat. *Asian-Australian Journal of Animal Science*, ***30***(4), 531–537.

Liu, T., Liu, S., Zheng, M., Chen, Q., & Ni, J. (2016). Performance Assessment of Full-Scale Wastewater Treatment Plants Based on Seasonal Variability of Microbial Communities via High-Throughput Sequencing. *PLOS ONE*, ***11***(4), 1–15.

Lu, X., Zhang, X. X., Wang, Z., Huang, K., Wang, Y., Liang, W., Tan, Y., Liu, B. & Tang, J. (2015). Bacterial pathogens and community composition in advanced sewage treatment systems revealed by metagenomics analysis based on high-throughput sequencing. *PLOS ONE*, ***10***(5), 1–15.

Lundborg, C.S. & Tamhankar, A. (2017). Antibiotic residues in the environment of South East

Asia. *British Medical Journal*, ***358****(j2440)*, 42–45.

Lupan, I., Carpa, R., Oltean, A., Simona Kelemen, B., & Popescu, O. (2017). Release of Antibiotic Resistant Bacteria by a Waste Treatment Plant from Romania. *Microbes and Environments*, ***32***(3), 219–225.

Ma, H., Zhou, J., Hua, L., Cheng, F., Zhou, L., & Qiao, X. (2017). Chromium recovery from tannery sludge by bioleaching and its reuse in tanning process. *Journal of Cleaner Production*, ***142****(4)*, 2752–2760.

Ma, Q., Qu, Y., Zhang, X., Shen, W., Liu, Z., Wang, J., Zhang, Z., &Zhou, J. (2015). Identification of the microbial community composition and structure of coal-mine wastewater treatment plants. *Microbiological Research*, ***175***, 1–5.

Ma, X., Wu, C., Jun, H., Zhou, R., & Shi, B. (2018). Microbial community of tannery wastewater involved in nitrification revealed by illumina MiSeq sequencing. *Journal of Microbiology and Biotechnology*, ***28***(7), 1168–1177.

Mandal, T., Dasgupta, D., Mandal, S., & Datta, S. (2010). Treatment of leather industry wastewater by aerobic biological and Fenton oxidation process. *Journal of Hazardous Materials*, ***180***(1–3), 204–211.

Mangal, M., Agarwal, M., & Bhargava, D. (2013). A Case Study of Impacts of Tannery Effluent of Leather Industry of Manpura Machedi on Ground Water Quality of. *Journal of Pharmacognosy and Phytochemistry*, ***2***(2), 229–233.

Mao, S., Zhang, M., Liu, J., & Zhu, W. (2015). Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle : membership and potential function. *Nature Publishing Group*, **5**(16116), 1–14.

Marathe, N. P., Shetty, S. A., & Shouche, Y. S. (2016). Limited Bacterial Diversity within a Treatment Plant Receiving Antibiotic- Containing Waste from Bulk Drug Production. *PLOS ONE*, ***11***(1), 1–12.

Massé, D. I., Saady, N. M. C., & Gilbert, Y. (2014). Potential of Biological Processes to Eliminate Antibiotics in Livestock Manure: An Overview. *Animals*, ***4***, 146–163.

Meerbergen, K., Van Geel, M., Waud, M., Willems, K. A., Dewil, R., Van Impe, J., Appels, L. &Lievens, B. (2017). Assessing the composition of microbial communities in textile wastewater treatment plants in comparison with municipal wastewater treatment plants. *MicrobiologyOpen*, ***6***(1), 1–13.

Mekonnen Birhanie, Seyoum Leta, & Khan, M. M. (2017). Removal of hazardous pollutants from tannery wastewater by naval filter medium (Pumice) through adsorption and filtration method. *IOSR Journal of Environmental Science, Toxicology and Food Technology*, ***11***(9), 38–45.

Metsalu, T., & Vilo, J. (2015). ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Research*, ***43***, 566–570.

Michael, C. A., Dominey-Howes, D., & Labbate, M. (2014). The antimicrobial resistance crisis: causes, consequences, and management. *Frontiers in Public Health*, ***2***(145), 1- 8.

Midha, V., & Dey, A. (2008). Biological treatment of tannery wastewater for sulfide removal. *International Journal of Chemical Science*, ***6***(2), 472–486.

Milić, N., Milanović, M., Letić, N. G., Sekulić, M. T., Radonić, J., Mihajlović, I., & Miloradov, M. V. (2013). Occurrence of antibiotics as emerging contaminant substances in aquatic environment. *International Journal of Environmental Health Research*, ***23***(4), 296–310.

Miller, J. H., Novak, J. T., Knocke, W. R., & Pruden, A. (2016). Survival of Antibiotic Resistant Bacteria and Horizontal Gene Transfer Control Antibiotic Resistance Gene Content in Anaerobic Digesters. *Frontiers in Microbiology*, ***7***(263), 1–11.

Mojica, E., & Aga, D. S. (2011). Antibiotics Pollution in Soil and Water : Potential Ecological and Human Health Issues. *Encyclopedia of Environmental Health*, Elsevier B.V, 97–110.

Monteils, V., Cauquil, L., Combes, S., Godon, J. J., & Gidenne, T. (2008). Potential core species and satellite species in the bacterial community within the rabbit caecum. *FEMS Microbiology Ecology*, ***66***(3), 620–629.

Mulat Alubel. (2015). Revealed Comparative Advantage of Ethiopian Leather Industry with

Selected African Economies. *International Journal of Business and Economics Research*, ***4***(5), 229–237.

Mwaikono, K. S., Maina, S., Sebastian, A., Kapur, V., & Gwakisa, P. (2015). 16S rRNA Amplicons Survey Revealed Unprecedented Bacterial Community in Solid Biomedical Wastes. *American Journal of Microbiological Research*, ***3***(4), 135–143.

Mwaikono, K. S., Maina, S., Sebastian, A., Schilling, M., Kapur, V., & Gwakisa, P. (2016). High-throughput sequencing of 16S rRNA Gene Reveals Substantial Bacterial Diversity on the Municipal Dumpsite. *BMC Microbiology*, ***16***(145), 1–12.

Naidoo, S., & Olaniran, A. O. (2013). Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*, ***11***(1), 249–270.

Negreanu, Y., Pasternak, Z., Jurkevitch, E., & Cytryn, E. (2012). Impact of Treated Wastewater Irrigation on Antibiotic Resistance in Agricultural Soils.*Environmental Science and Technology*, **46**, 4800 - 4808

Nelson, M. C., Morrison, H. G., Benjamino, J., Grim, S. L., & Graf, J. (2014). Analysis, optimization and verification of illumina-generated 16S rRNA gene amplicon surveys. *PLOS ONE*, ***9***(4), 1–14.

Nithya, R., & Sudha, P. N. (2016). Removal of heavy metals from tannery effluent using chitosan-g-poly(butyl acrylate)/bentonite nanocomposite as an adsorbent. *Textiles and Clothing Sustainability*, ***2***(7), 1–8.

Novo, A., Andre, S., Viana, P., Nunes, O. C., & Manaia, C. M. (2013). Antibiotic resistance, Antimicrobial residues and bacterial community composition in urban wastewater. *Water Research*,***47***(5), 1875–1887.

Olaolu, Tomilola Debby , Oghenerobor Benjamin Akpor, C. O. A. (2014). Pollution Indicators and Pathogenic Microorganisms in Wastewater Treatment: Implication on Receiving Water Bodies. *International Journal of Environmental Protection and Policy*, ***2***(6), 205-212.

Ozbayram, E., Ince, O., Ince, B., Harms, H., & Kleinsteuber, S. (2018). Comparison of rumen

and manure microbiomes and implications for the inoculation of anaerobic digesters. *Microorganisms*, ***6***(15), 1–110.

Pikkemaat, M. G., & Yassin, H. van der F.-K. and B. J. A. B. (2016). Antibiotic Residues and Resistance in the Environment. Wageningen, RIKILT Wageningen UR (University & Research Centre)

Pire-Sierra, M.C. Cegarra-Badell, D.D. Carrasquero-Ferrer, S.J. Angulo- Cubillan, N.E. & Diaz-Montiel, A.R. (2016). Nitrogen and COD removal from Tannery wastewater using Biological and physicochemical treatments. *Revista Facultad de Ingenieria*,**80**, 63 - 73

Prabu, P. C., Wondimu, L., & Tesso, M. (2011). Assessment of water quality of Huluka and Alaltu Rivers of Ambo, Ethiopia. *Journal of Agricultural Science and Technology*, ***13***(1), 131–138.

Qin, Y., Hou, J., Deng, M., Liu, Q., Wu, C., Ji, Y., & He, X. (2016). Bacterial abundance and diversity in pond water supplied with different feeds. *Scientific Reports*, ***6***(1), 1–13.

Zhou, Q., T. C. and S. H. (2017). Characteristics of bacterial communities in Cyanobacteria-blooming aquaculture wastewater influenced by the phytoremediation with water hyacinth. *Water*, ***9***(956), 1–11.

Qiu, G., Song, Y., Zeng, P., Duan, L., & Xiao, S. (2013). Characterization of bacterial communities in hybrid upflow anaerobic sludge blanket (UASB) membrane bioreactor (MBR) process for berberine antibiotic wastewater. *Bioresource Technology*, ***142***, 52–62.

Rafraf, I. D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C. M., & Balcázar, J. L. (2016). Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia. *Environmental Pollution*, ***219***, 353–358.

Rahube, T. O., Viana, L. S., Koraimann, G., & Yost, C. K. (2014). Characterization and comparative analysis of antibiotic resistance plasmids isolated from a wastewater treatment plant. *Frontiers in Microbiology*, ***5***(558), 1–9.

Rajeswari, A. (2015). Efficiency of effluent treatment plant and assessment of water quality parameters in tannery wastes. *Environmental journal of Experimental Biology*, ***5***(8), 49–55.

Ramírez-Castillo, F., Loera-Muro, A., Jacques, M., Garneau, P., Avelar-González, F., Harel, J., & Guerrero-Barrera, A. (2015). Waterborne Pathogens: Detection Methods and Challenges. *Pathogens*, ***4***(2), 307–334.

Ranasinghe, P.D, Satoh H, Oshiki M, Oshima K, Suda W, Hattori M, &Mino. T. (2012). Revealing Microbial Community Structures in Large and Small Scale Activated Sludge Systems by Barcoded Pyrosequencing of 16S rRNA gene. *Water Science & Technology*, ***66 (10)***, 2155–2161.

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J. P., Laiho, A., Pekkala, S. &Huovinen, P. (2017). Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. *Journal of Biomolecular Techniques*, ***28***(1), 19–30.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., Michael, I., & Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment*, ***447***, 345–360.

Rossmann, J., Schubert, S., Gurke, R., Oertel, R., & Kirch, W. (2014). Simultaneous determination of most prescribed antibiotics in multiple urban wastewater by SPE-LC-MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, ***969***, 162–170.

Row, W., Verner-jeffreys, D.W., Baker-Austin, C., & Ryan, J.J., Maskell, D.j. & Pearce, G.P. (2016). Compatrative metagenomics reveals a divers range of antimicrobial resistance genes in effluents entering a river catchment. *Water Science and Technology*, **73** (7), 1-9

Sahoo, K. C., Tamhankar, A. J., Johansson, E., & Lundborg, C. S. (2010). Antibiotic use , resistance development and environmental factors : a qualitative study among healthcare professionals in Orissa , India. *BMC Public Health*, ***10***(629), 1-10.

Samanta, A., Bera, P., Khatun, M., Sinha, C., Pal, P., Lalee, A., & Mandal, A. (2012). An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus sp* . isolated from municipal waste. *Journal of Microbiology and Biotechnology*

Research Scholars, ***2***(1), 178–189.

Samuel Abegaz. (2007). Pollution status of Tinishu Akaki River and its tributaries (Ethiopia) evaluated using physicochemical parameters, major ions, and nutrients. Columbus State University CSU ePress, Faculty Bibliography. Paper 910

Santos, T. F., & Camacho, L. A. (2014). Dynamic Water Quality Modelling Of Tannery Effluents – Bogotá River (Colombia). *11thInternational Conference on Hydroinformatics*, CUNY, Acadamic Works, Pp. 1- 9.

Saunders, A. M., Albertsen, M., Vollertsen, J., & Nielsen, P. H. (2015). The activated sludge ecosystem contains a core community of abundant organisms. *The ISME*, ***10*** *(1)*, 11–20.

Saxena, G., Chandra, R., & Bharagava, R. N. (2016). Environmental Pollution , Toxicity Profile and Treatment Approaches for Tannery Wastewater and Its Chemical Pollutants. In:*Review of Environmental contaminationn and Toxicology*. **240**, Pp. 32–69).

Schmitt, H., Laak, T. & Karen, D. (2017). Development and Dissemination of antibiotic resistance in the environment under environmnetally relevant concentrations of antibiotics and its risk assessment. IRAS, Institute for Risk Assessment Sciences, Utrecht University, TD Utrecht.

Scott, K. P., Melville, C. M., Barbosa, T. M., & Flint, H. J. (2000). Occurrence of the new tetracycline resistance gene tet(W) in bacteria from the human gut. *Antimicrobial Agents and Chemotherapy*, ***44***(3), 775–777.

Seiler, C., & Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology*, **3** (339), 1–10.

Shah, M. P. (2014). Exploited Application of Pyrosequencing in Microbial Diversity of Activated Sludge System of Common Effluent Treatment Plants. *American Journal of Microbiological Research*, ***2***(5), 157–165.

Shanks, O. C., Newton, R. J., Kelty, C. A., Huse, S. M., Sogin, M. L., Mclellan, L., & Lake, C. (2013). Comparison of the Microbial Community Structures of Untreated Wastewaters from

Different Geographic Locales. *Applied and Environmental Microbiology*, ***79***(9), 2906–2913.

Sharma, C., Rokana, N., Chandra, M., Singh, B. P., Gulhane, R. D., Gill, J. S., Ray, P, Puniya, A.K. &Panwar, H. (2018). Antimicrobial resistance: Its Surveillance, Impact, and Alternative Management Strategies in Dairy Animals. *Frontiers in Veterinary Science*, ***4***(237), 1–27.

Shashidhar, R. G. T. (2015). Antibiotic Pollution in the Environment: A Review Antibiotics. *Clean Soil, Air, Water*, ***43***(4), 479–489.

Shchegolkova, N. M., Krasnov, G. S., Belova, A. A., Dmitriev, A. A., Kharitonov, S. L., Klimina, K. M., Melnikova, N. V. & Kudryavtseva, A. V. (2016). Microbial community structure of activated sludge in treatment plants with different wastewater compositions. *Frontiers in Microbiology*, ***7***(90), 1–15.

Shegani, G. (2014). Treatment of Tannery Effluents by the Process of Coagulation. *International Journal of Environmental and Ecological Engineering*, ***8***(4), 240–244.

Sheik, C. S., Mitchell, T. W., Rizvi, F. Z., Rehman, Y., Faisal, M., Hasnain, S., McInerney, M.J &Krumholz, L. R. (2012). Exposure of soil microbial communities to chromium and arsenic alters their diversity and structure. *PLOS ONE*, ***7***(6), 1–13.

Shepherd, M. L., Jr, W. S. S., Jensen, R. V, & Ponder, M. A. (2012). Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. *FEMS Microbiology Letters*, ***326***, 62–68.

Siedlewicz, G., Borecka, M., Białk-Bielińska, A., Sikora, K., Stepnowski, P., & Pazdro, K. (2016). Determination of antibiotic residues in southern Baltic Sea sediments using tandem solid-phase extraction and liquid chromatography coupled with tandem mass spectrometry. *Oceanologia*, ***58***(3), 221–234.

Singer, R. S., & Williams-Nguyen, J. (2014). Human health impacts of antibiotic use in agriculture: A push for improved causal inference. *Current Opinion in Microbiology*, ***19***(1), 1–8.

Solomon Sorsa, Yadessa Chibssa, Girma Tilahun, & Daniel Fitamo. (2015). Environmental & Analytical Toxicology Heavy Metal Concentrations and Physicochemical Characteristics of Effluent along the Discharge Route from Hawassa Textile Factory, Ethiopia. *Journal of Environmental and Analytical Toxicology*, ***5***(4), 1–7.

Stalder, T., Alrhmoun, M., Louvet, J. N., Casellas, M., Maftah, C., Carrion, C.,Pons, M. N., Pahl, O., Ploy, M.&Dagot, C. (2013). Dynamic assessment of the floc morphology, bacterial diversity, and integron content of an activated sludge reactor processing hospital effluent. *Environmental Science and Technology*, ***47***(14), 7909–7917.

Stanley, I., Okoduwa, R., Igiri, B., Udeh, C. B., Edenta, C., & Gauje, B. (2017). Tannery Effluent Treatment by Yeast Species Isolates from Watermelon. *Toxics*, ***5***(6), 1–10.

Steele, M., & Odumeru, J. (2004). Irrigation Water as Source of Foodborne Pathogens on Fruit and Vegetables. *Journal ofFood Protection*, ***67***(12), 2839–2849.

Stefan, R. Michael, B. Bianca, M. S. & Michele, C. (2013). Best Available Techniques (BAT) Reference Document for the Tanning of Hides and Skins. Integrated Pollution Prevention and Control, Seville, Spain.

Stelma Jr, G. (2018). Use of bacterial spores in monitoring water quality and treatment. *Journal of Water Health*, ***16***(4), 1–13.

Sugasini, A., & Rajagopal, K. (2015). Original Research Article Characterization of Physicochemical Parameters and heavy metal Analysis of Tannery Effluent. *International Journal of Current Microbiology Applied Science*, ***4***(9), 349–359.

Sul, W., Kim, I., Ekpeghere, K. I., Song, B., Kim, B., Kim, H., Kim, J. &Koh, S. (2016). Toxic / Hazardous Ssorsaubstances and Environmental Engineering Metagenomic insight of nitrogen metabolism in a tannery wastewater treatment plant bioaugmented with the microbial consortium BM-S-1. *Journal of Environmental Science and Health, Part A*, ***51***(13), 1164–1172.

Sullivan, B. A., Gentry, T., & Karthikeyan, R. (2013). Characterization of tetracycline-resistant bacteria in an urbanizing subtropical watershed. *Journal of Applied Microbiology*, ***115***(3),

774–785.

Sun, L., Liu, T., Müller, B., & Schnürer, A. (2016). The microbial community structure in industrial biogas plants influences the degradation rate of straw and cellulose in batch tests. *Biotechnology for Biofuels*, ***9***(128), 1-20.

Tadesse Alemu and Seyoum Leta. (2015). Evaluation of selected wetland plants for removal of chromium from tannery wastewater in constructed wetlands , Ethiopia. *African Journal of Environmental Science and Technology*, ***9***(5), 420–427.

Tadesse Alemu, Andualem Mekonnen, & Seyoum Leta. (2019). Journal of Water Process Engineering Integrated tannery wastewater treatment for effluent reuse for irrigation: Encouraging water efficiency and sustainable development in developing countries. *Journal of Water Precess Engineering*, **30**, 1-8.

Taddese Animaw. (2011). Understanding the situation of wastewater irrigation in community based irrigation schemes: The case of Akaki catchment , Ethiopia. M.Sc. Thesis, Wageningen University

Takele Beyene, Dagnachew Endalamaw, Yonas Tolossa, & Ashenafi Feyisa. (2015). Evaluation of rational use of veterinary drugs especially antimicrobials and anthelmintics in Bishoftu, Central Ethiopia Veterinary Research. *BMC Research Notes*, ***8***(1), 1–8.

Tambosi, J. L., Yamanaka, L. Y., José, H. J., De Fátima Peralta Muniz Moreira, R., & Schröder, H. F. (2010). Recent research data on the removal of pharmaceuticals from sewage treatment plants (STP). *Quimica Nova*, ***33***(2), 411–420.

Tanca, A., Fraumene, C., Manghina, V., Palomba, A., Abbondio, M., Deligios, M., Pagnozzi, D., Addis, M.F. &Uzzau, S. (2017). Diversity and functions of the sheep faecal microbiota: a multi-omic characterization. *Microbial Biotechnology*, ***10***(3), 541–554.

Tang, J., Bu, Y., Zhang, X., Huang, K., He, X., Ye, L., Ye, L., Shan, Z. &Ren, H. (2016). Ecotoxicology and Environmental Safety Metagenomic analysis of bacterial community composition and antibiotic resistance genes in a wastewater treatment plant and its receiving surface water. *Ecotoxicology and Environmental Safety*, ***132***, 260–269.

Tapio, I., Shingfield, K. J., Mckain, N., Bonin, A., & Fischer, D. (2016). Oral Samples as Non-Invasive Proxies for Assessing the Composition of the Rumen Microbial Community. *PLOS ONE*, ***11***(3), 1–15.

Tehrani, A. H., & Gilbride, K. A. (2018). A closer look at the antibiotic-resistant bacterial community found in urban wastewater treatment systems. *Microbiology open*, **7** (4), 1–13.

Thornton, P. K. (2010). Livestock production: Recent trends, future prospects. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences*, ***365***(1554), 2853–2867.

Tremblay, J., Singh, K., Fern, A., Kirton, E. S., He, S., Woyke, T., Lee, J., Chen, F., Dangl, J.L. &Tringe, S. G. (2015). Primer and platform effects on 16S rRNA tag sequencing. *Frontiers in Microbiology*, ***6***(771), 1–15.

Tuckwell, B. R. (2014). The impact on receiving waters of pharmaceutical residues and antibiotic resistant faecal bacteria found in urban waste water effluents. PhD Dissertation, Middlesex University, London, UK.

UNIDO. (2012). Technical assistance project for the upgrading of the Ethiopian leather and leather products industry. *Independent Evaluation Report*, Vienna.

UNIDO. (2016). Pollutants in tannery effluent: Sources, description, environmental impact. United Nations Industrial Development Organization (UNIDO), Vienna.

van Boeckel, T. P. Van, Brower, C., Gilbert, M., Grenfell, B. T., & Levin, S. A. (2015). Global trends in antimicrobial use in food animals. *PNAS*, ***112***(18), 5649–5654.

Van Hoek, A., Mevius, D., Guerra, B., Mullany, P., Roberts, A. & Aarts, H. (2011). Acquired antibiotic resistance genes: An overview. *Frontiers in Microbiology*, ***2***(203), 1–27.

Van Schaik, W. (2015). The human gut resistome. *Philosophical Transactions of the Royal Society B*, ***370***, 1–9.

Vaz-Moreira, I., Nunes, O. C.& Manaia, C. M. (2014). Bacterial diversity and antibiotic resistance in water habitats: Searching the links with the human microbiome. *FEMS*

Microbiology Reviews, ***38***(4), 761–778.

Verma, T., Garg, S. K., & Ramteke, P. W. (2009). Genetic correlation between chromium resistance and reduction in Bacillus brevis isolated from tannery effluent. *Journal of Applied Microbiology*, ***107***(5), 1425–1432.

Veslemoy, G., Mellerup, A., Christiansen, L. E., Stahl, M., Olson, J. E. and, & Angen, O. (2015). Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches. *PLOS ONE*, ***10***(6), 1–22.

Vierheilig, J., Savio, D., Ley, R. E., Mach, R. L., Farnleitner, A. H., & Reischer, G. H. (2015). Potential applications of next generation DNA sequencing of 16S rRNA gene amplicons in microbial water quality monitoring. *Water Science and Technology*, **72** (11), 1962–1972.

Vrieze, J. De, Regueiro, L., Props, R., Vargas, R. V., Jáuregui, R., Pieper, D. H., Lema, J.M. & Carballa, M. (2016). Biotechnology for Biofuels Presence does not imply activity : DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. *Biotechnology for Biofuels*, ***9***(244), 1–13.

Walsh, F., & Duffy, B. (2013). The Culturable Soil Antibiotic Resistome: A Community of Multi-Drug Resistant Bacteria. *PLoS ONE*, ***8***(6), 1–11.

Wan, Y., Ruan, X., Zhang, Y. & Li, R. (2017). Illumina sequencing-based analysis of sediment bacteria community in different trophic status freshwater lakes. *Willey Microbiology Open*, ***6***(e450), 1–15.

Wang, Z. H., Yang, J. Q., Zhang, D. J., Zhou, J., Zhang, C. D., & Su, X. R. (2014). Composition and structure of microbial communities associated with different domestic sewage outfalls. *Genomics and Molecular Research*, ***13***(3), 7542–7552.

Wang, Z., Zhang, X-X., Huang, K., Miao, Y., Shi, P., Liu, B., Long, C. &Li, A. (2013). Metagenomic Profiling of Antibiotic Resistance Genes and Mobile Genetic Elements in a Tannery Wastewater Treatment Plant. *PloS One*, ***8***(10), 1- 9.

Wang, Z., Zhang, X-X., Lu, X., Liu, B., Li, Y., Long, C., & Li, A. (2014). Abundance and diversity of bacterial nitrifiers and denitrifiers and their functional genes in tannery

wastewater treatment plants revealed by high-throughput sequencing. *PLOS ONE*,***9***(11), 1-19.

Wegst-Uhrich, S. R., Navarro, D. A. G., Zimmerman, L., & Aga, D. S. (2014). Assessing antibiotic sorption in soil : A literature review and new case studies on sulfonamides and macrolides. *Chemistry Centeral Journal*, **8** (1), 1–12.

Wei, R., Ge, F., Zhang, L., Hou, X., Cao, Y., Gong, L., Chen, M., Wang, R. & Bao, E. (2016). Occurrence of 13 veterinary drugs in animal manure-amended soils in Eastern China. *Chemosphere*,***144***, 2377–2383.

Wen, C., Wu, L., Qin, Y., Nostrand, J. D. Van, Ning, D., Sun, B., Xue, K., Liu, F., Deng, Y., Liang, Y. &Zhou, J. (2017). Evaluation of the reproducibility of amplicon sequencing with Illumina MiSeq platform. *PLOS ONE*, ***12***(4), 1–20.

Wen, Y., Jin, Y., Wang, J., & Cai, L. (2015). MiSeq Sequencing Analysis of Bacterial Community Structures in Wastewater Treatment Plants. *Polish Journal of Environmental Studies*, ***24***(4), 1809–1815.

Werner, J., Koren, O., Hugenholtz, P., Desantis, Z., Walters, A., Caporaso, G., Angenent, L., Knight, R. &Ley, R. (2011). Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *The ISME Journal*, ***6***(1), 94–103.

West, B. M, Liggit, P., Clemans, D. L., & Francoeur, S. N. (2011). Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near cafo farms and wastewater treatment facilities. *Water, Air, and Soil Pollution*, ***217***(1–4), 473–489.

Westcott, S. L., & Schloss, P. D. (2015). De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *Peer J*, **3** (e1487), 1–23.

WHO. (2011). Tackling antibiotic resistance from a food safety perspective in Europe. World Health Organization Regional Office for Europe, Denmark.

Williams-Nguyen, J., Sallach, J., Bartelt-Hunt, S., Boxall, A., Durso, M., McLain, J., Singer, S., Snow, D. & Zilles, J. (2016). Antibiotics and antibiotic resistance in agroecosystems: State

of the science. *Journal of Environmental Quality*, ***45***(2), 394–406.

Wintersdorff, C. J. H. Von, Penders, J., & Niekerk, J. M. Van. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, ***7***(173), 1–10.

Wu, L., Wen, C., Qin, Y., Yin, H., Tu, Q., Nostrand, J. D. Van, & Yuan, T. (2015). Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. *BMC Microbiology*, ***15***(125), 1–12.

Xu, C., Weese, J. S., Flemming, C., Odumeru, J., & Warriner, K. (2014). Fate of *Clostridiumdifficile* during wastewater treatment and incidence in Southern Ontario watersheds. *Journal of Applied Microbiology*, ***117***(3), 891–904.

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X. &Meng, W. (2015). Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere*, ***119***, 1379–1385.

Yang, B., Wang, Y., & Qian, P.Y. (2016). Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics*, ***17***(1), 135.

Yang, Q., Zhang, H., Guo, Y., & Tian, T. (2016). Influence of chicken manure fertilization on antibiotic-resistant bacteria in soil and the endophytic bacteria of pakchoi. *International Journal of Environmental Research and Public Health*, ***13***(7), 1–12.

Yared Worku and Giweta M. (2018). Can we imagine pollution free rivers around Addis Ababa city , Ethiopia ? What were the wrong-doings? What action should be taken to correct them? *Journal of Pollution Effects and Control*, ***6***(3), 1–9.

Younan, S., Sakita, G. Z., Albuquerque, T. R., Keller, R., & Bremer-neto, H. (2016). Chromium (VI) bioremediation by probiotics. *Journal of Science Food and Agriculture*, ***96,*** 3977–3982.

Zeng, X., & Lin, J. (2013). Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Frontiers in Microbiology*, ***4***(128), 1–9.

Zhang, L., & Wang, S. (2017). Bacterial community diversity on in-shell walnut surfaces from six representative provinces in China. *Scientific Reports*, ***7***(1), 1–9.

Zhang, T., & Li, B. (2011). Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. *Critical Reviews in Environmental Science and Technology*, ***41***(11), 951–998.

Zhang, X., Li, Y., Liu, B., Wang, J., Feng, C., Gao, M., & Wang, L. (2014). Prevalence of Veterinary Antibiotics and Antibiotic- Resistant *Escherichia coli* in the Surface Water of a Livestock Production Region in Northern China. PLOS ONE,***9***(11), 1-11.

Appendices

Appendix I:

Table A1.Total number of bacterial taxa recovered from Batu tannery wastewater treatment plant by taxonomic ranks from August 2015 sample.

Taxa	Samples									
	G1	**S1**	**E1**	**G2**	**S2**	**E2**	**Total**	**Range**	**Mean**	**Stdev**
Phyla	17	23	18	21	18	20	28	17-21	19.5	2.26
Class	32	49	43	44	44	42	62	32-49	42.3	5.61
Order	69	93	85	86	84	84	123	69-93	83.5	7.87
Family	130	130	165	152	150	155	242	130-165	147	14.14
Genus	193	278	264	264	247	259	441	193-278	250.8	30.03

Table A2. Relative abundance of the bacterial Phyla in the different stages of Batu tannery wastewater treatment plant

Phyla (n=28)	Sample Site					
	G1(n=17)	S1(n= 23)	E1(n=18)	G2(n=21)	S2(n=18)	E2(n=20)
Bacteroidetes	15.3	14.3	25.4	13.7	8.3	22.9
Actinobacteria	1.0	15.6	40.4	12.5	7.1	23.4
Firmicutes	8.9	19.8	22.0	20.2	11.6	17.4
Fusobacterium	39.0	25.6	0.4	20.1	12.9	1.9
Proteobacteria	29.8	20.8	8.9	18.6	13.3	8.6
Synergistetes	0.01	3.5	27.5	9.1	4.8	55.0
Unclassified	8.9	9.3	45.8	12.1	7.0	16.9
Remaining phyla	30.3	8.9	18.6	15.7	8.4	18.1
% read coverage	12.9	17.1	22.3	17.2	10.3	20.2

*n refers number of phyla

Appendix II

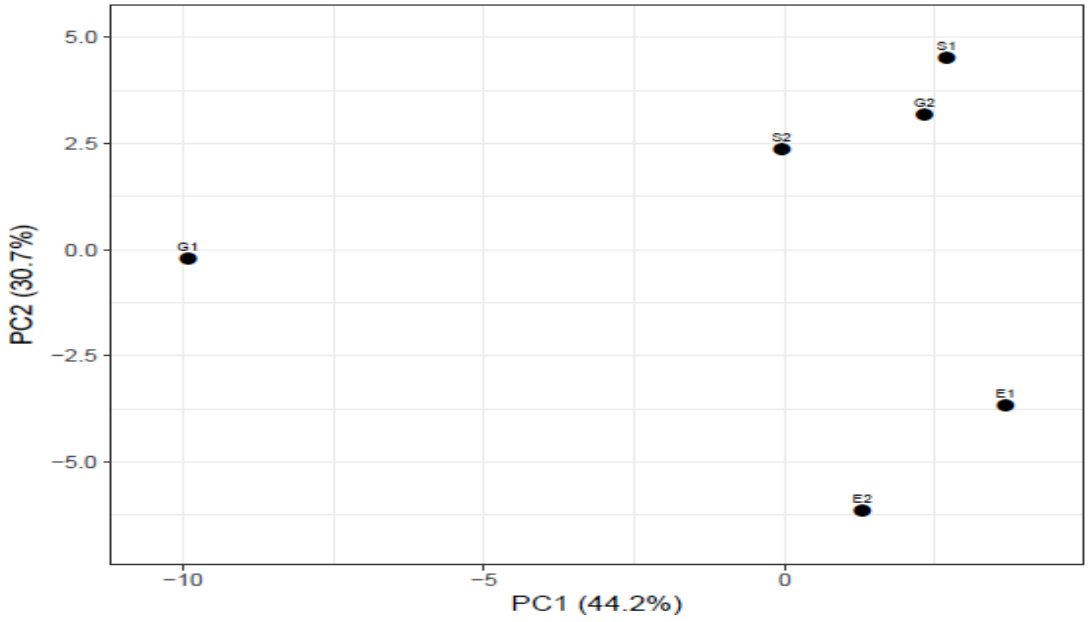

Figure A1.Principal Component Analysis of the bacteria communities at Batu tannery wastewater treatment plant as a measure of similarity distance between samples(G= General raw wastewater, S = aerated mixed liquor, E = final effluent).

Appendix III

Table A3. Alpha diversity indices

Parameters	Samples					
	G1	S1	E1	G2	S2	E2
Taxa_S	192	277	263	263	246	258
Individuals	179994	237711	308999	238071	142198	280742
Dominance (D)	0.08002	0.08101	0.1331	0.06711	0.06944	0.1261
Simpson (1-D)	0.92	0.919	0.8669	0.9329	0.9306	0.8739
Shannon (H)	2.905	3.032	2.537	3.17	3.2	2.602
Evenness (e^H/S)	0.09509	0.07485	0.04805	0.09054	0.09677	0.0523
Brillouin	2.902	3.029	2.535	3.168	3.166	2.6
Menhinick	0.4526	0.5681	0.4731	0.539	0.6524	0.4869
Margalef	15.78	22.3	20.73	21.16	20.65	20.49
Equitability (J)	0.5525	0.5391	0.4552	0.5689	0.5758	0.4686
Fisher_alpha	21.23	30.96	28.28	29.2	28.94	28
Berger-Parker	0.1351	0.1762	0.2348	0.1447	0.1568	0.2563
Chao-1	195.7	326	296	286.2	283	285.4

Appendix IV

Sample based abundances of the bacteria phyla identified from the tannery wastewater treatment plant

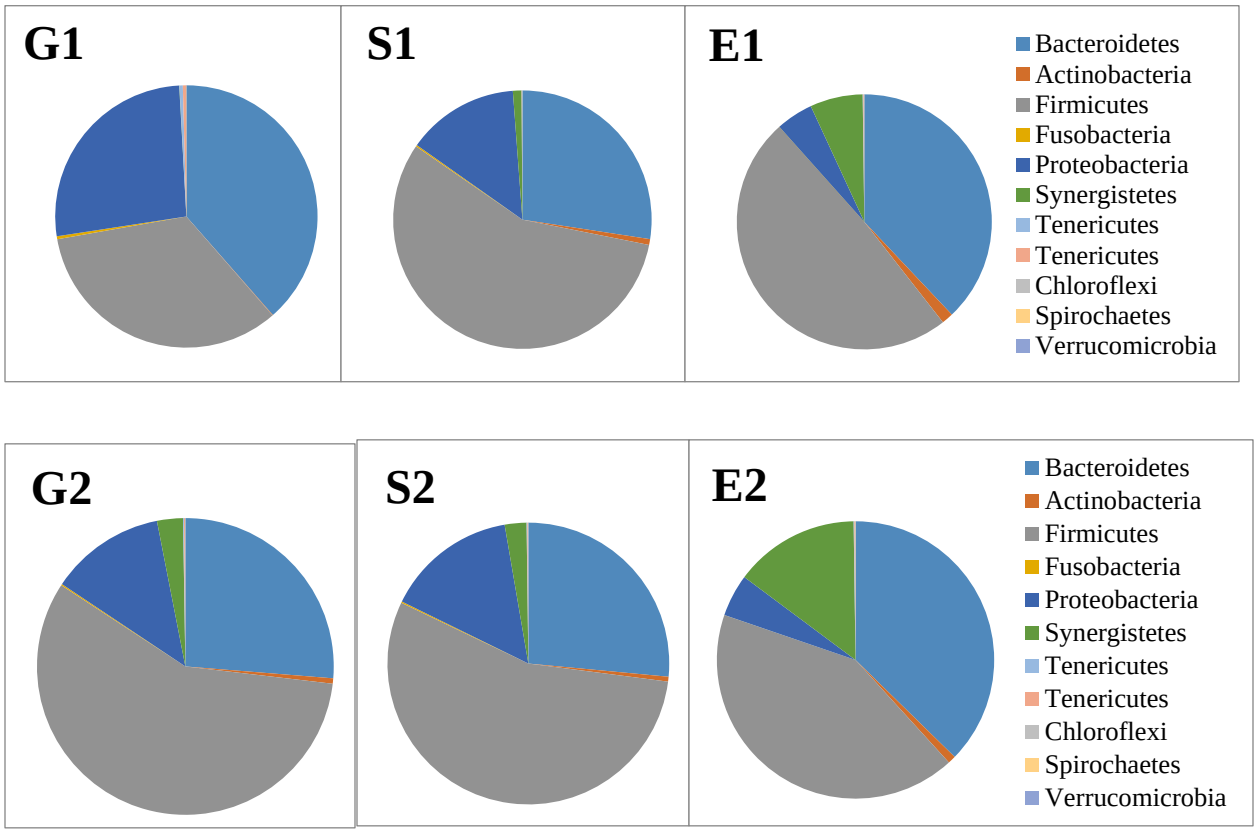

Figure A2. Sample based abundances of the bacteria phyla illustrated by the number of effective reads in each sample site of the tannery wastewater treatment plant

Appendix V. Common Animal diseases in the study area

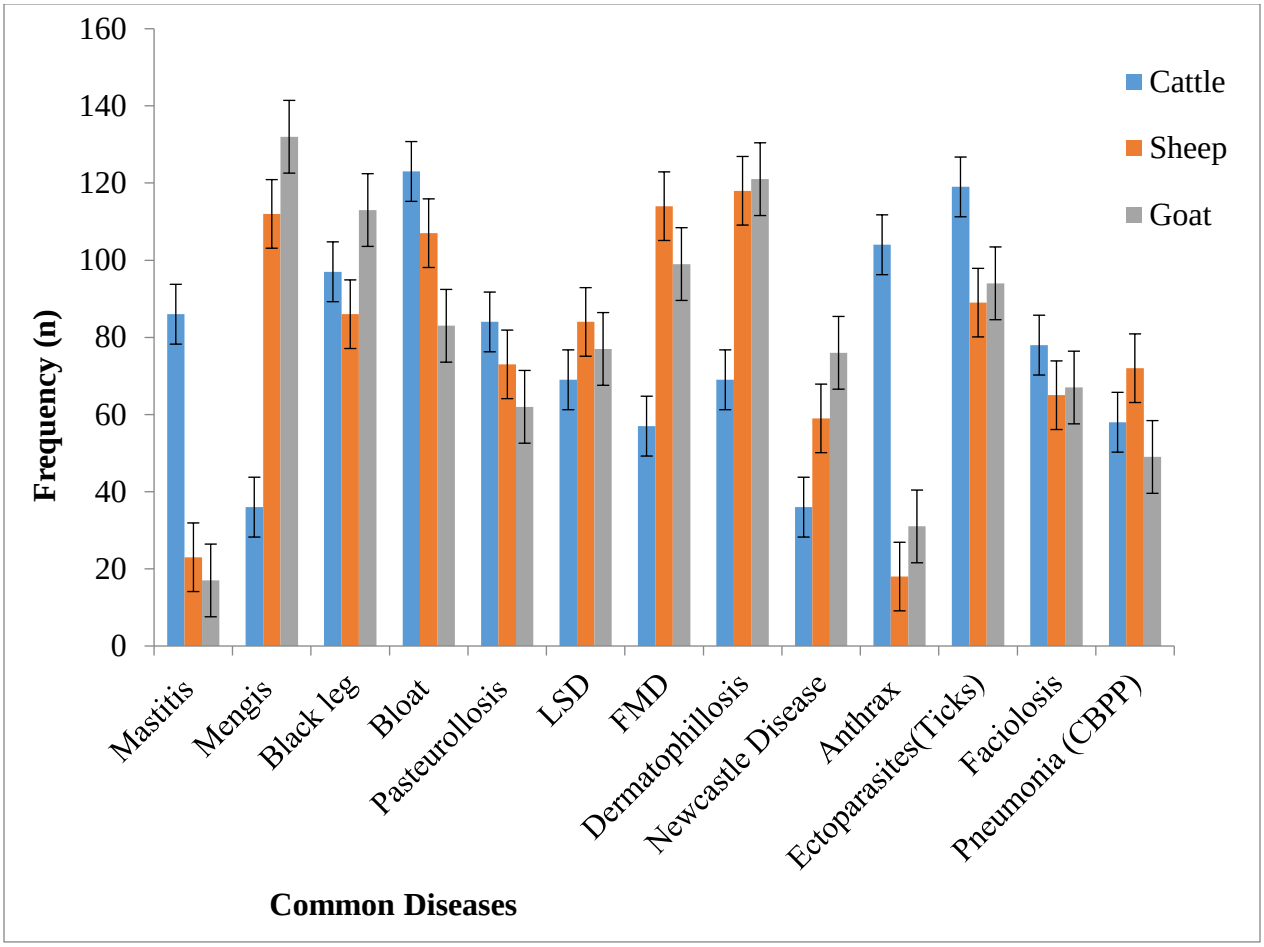

Figure A3. Most common livestock diseases in the study area (LSD = Lumpy skin Disease, FMD = Foot and mouth disease, CBPP = contagious bovine pleura pneumonia)

Appendix VI

Table A4. One-way Analysis of Variance of water quality parameters between upstream and downstream sample sites of LAR

Parameters		Sum of Squares	df	Mean Square	F	p-value
pH	Between Groups	1.45	1	1.45	3.675	0.073
	Within Groups	6.3	16	0.39		
	Total	7.74	17			
Temp	Between Groups	0.067	1	0.067	0.07	0.794
	Within Groups	15.3	16	0.957		
	Total	15.4	17			
EC	Between Groups	86.2	1	86.24	0.94	0.347
	Within Groups	1467.618	16	91.73		
	Total	1553.9	17			
COD	Between Groups	36964.8	1	36964.81	4.632	0.047
	Within Groups	127689.9	16	7980.62		
	Total	164654.7	17			
TDS	Between Groups	4421346.7	1	4421346.72	2.907	0.108
	Within Groups	24337347.8	16	1521084.24		
	Total	28758694.5	17			
TN	Between Groups	3068.1	1	3068.06	1.679	0.213
	Within Groups	29240.4	16	1827.53		
	Total	32308.5	17			
NH3	Between Groups	1168.1	1	1168.06	0.613	0.445
	Within Groups	30482.9	16	1905.18		
	Total	31650.9	17			
NO3	Between Groups	17.21	1	17.21	0.043	0.838
	Within Groups	6337.8	16	396.11		
	Total	6355	17			
Sulfide	Between Groups	355.6	1	355.56	0.678	0.422
	Within Groups	8390.4	16	524.4		
	Total	8746	17			
Sulfate	Between Groups	46005.6	1	46005.56	17.746	0.001
	Within Groups	41478.4	16	2592.4		
	Total	87484	17			
TotCr	Between Groups	589.4	1	589.39	21.784	0.001
	Within Groups	432.9	16	27.06		
	Total	1022.3	17			

df = degree of freedom, F = F-statistics (ratio of mean squares)

Appendix VII

Questionnaire

Dear respondent:

This questionnaire is designed to be used as a basic tool to collect information from respondents working in animal feeding sites, animal clinics, and veterinary drug store to assess the use of veterinary pharmaceuticals in animal farming in Ethiopia. The information obtained from this assessment will be used to complement the antibiotic detection in the tannery wastewater of PhD dissertation work.

I am grateful to your cooperation in advance!

Instruction: Please encircle your choice(s) when it applies and put the best of your information you have to fill in the blank items.

I. Respondent type:

a) Farmer
b) Community animal health worker
c) Veterinary clinician (DVM)
d) Pharmacist
e) Animal health assistant
f) Worker in animal farm
g) Drug seller
h) Administrator

Any other: _____

II. Name of animal farm _____

Farm owned by:

a) government
b) small scale farmers
c) private company

III. Informants background Information

1. Sex: a) Male b) Female
2. Education: a) Non-formal education b) Formal education
3. If 'formal education', what is the highest level?
   a) Diploma (2 years training)
   b) First Degree
   c) 2nd Degree
4. Area of training or specialization _____

IV. Information on animals and location of the feeding center

5. Location of animal farm center is
   a) Near river, lake, stream, irrigation canal
   b) Near or inside farm land, floriculture,
   c) In the farmers house

   Any other site: _____
6. What types of animals are there in the feeding center?
   a) Cattle
   b) Sheep
   d) Poultry
   e) Mixed

c) Equines f) Pigs (if any other _____)

7. How many of the animals (mentioned above) are there in the feeding center?
   a) Less than 10 c) 11 - 100
   b) 101-500 d) More than 500
8. How long the animals stay in the feeding center before withdrawal?
   a) Less than three months c) More than six months
   b) Between three and six months
9. How do you dispose animal wastes (feces, urine, food remain …)?
   a) Discharge to the nearby open field d) Public sewage
   b) Used as fertilizers e) River/streams
   c) Used as a feed for biodigester
   Any other: _____
10. What is the major waste produced in the animal feeding center?
   a) Animal feces and urine
   b) Feed remains
   c) Wastewater

   Any other: _____

11. Is there any waste treatment process before discharged to the environment?
   a) Yes b) No

12. Do you have any information on the environmental effect of animal waste?
   a) Yes b) No
13. What are major health problems for the animals in the farming center?
   a) Malnutrition c) Infection with endoparasites
   b) Infection with ectoparasites
   Any other: _____
14. What are the most prevalent infectious diseases infecting animals in your region?
   a) Bacteria d) Helminthes
   b) Fungi e) external parasites (Ticks, Flies, Lice, Mange mites)
   c) Protozoa f) Viruses
   Any other: _____
15. What are the most commonly prescribed drugs for animal treatment?
   a) Antibiotics d) Antihelminthis
   b) Antifungal e) Acaricides
   c) Antiprotozoans
   Any others: _____
16. List the most commonly prescribed drugs in this treatment center/ region?

AntibioticsAntihelminthes

a) _____ a) _____
b) _____ b) _____
c) _____ c) _____
d) _____ d) _____

Other: _____ Other _____

AntiprotozoaAcarcids

a) _____ a) _____
b) _____ b) _____
c) _____ c) _____
d) _____ d) _____

Others: _____ Other _____

17. How the antimicrobials are given to the animals?
    a) Through injection
    b) Via oral routes
    c) External body spray
    Any other: _____
18. Who approve the purchase and administration of these antimicrobials?

_____

19. How long is the withdrawal time after the last time of drug administration?

_____

20. How much veterinary drugs your company/office buy annually?

_____

21. Where do you buy the drugs?

_____

22. How long the drugs are stored?

_____

23. What is the storage condition?

_____

24. Disposal mechanisms of expired drugs if any?

_____

25. Length of the distribution channel?

_____

26. Your opinion about illegal trade?

_____

Appendix VIII

Table A5. Relative Abundance of classified genera identified from Batu tannery wastewater

Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Clostridium	11269	33484	72547	27538	17251	41443	203532
Synergistes	5	2189	18676	6499	3406	36205	66980
Psychrobacter	7950	9333	10624	11711	7447	8443	55508
Acinetobacter	1657	13903	726	10237	7855	759	35137
Bacteroides	15159	506	1154	3414	2291	1766	24290
Anaerovorax	78	5892	841	6762	3967	140	17680
Arcobacter	6615	4121	1386	2136	1889	1346	17493
Shewanella	13951	174	15	201	160	30	14531
Tissierella	4789	3471	605	2086	1224	278	12453
Ignatzschineria	11468	44	6	56	32	40	11646
Papillibacter	79	2880	442	2682	1641	191	7915
Acetobacterium	400	363	2295	621	330	3131	7140
Dethiosulfovibrio	52	353	1584	177	122	4408	6696
Environmental_samples	1574	158	52	1460	737	1977	5958
Fusibacter	259	1483	121	2352	1577	99	5891
Paludibacter	96	1329	2691	832	539	362	5849
Proteus	517	1472	83	1551	1196	14	4833
Proteiniphilum	63	475	782	557	340	2156	4373
Dysgonomonas	3507	54	34	106	86	34	3821
Vibrio	2851	14	0	16	16	0	2897
Erysipelothrix	143	520	363	794	575	233	2628
Desulfomicrobium	0	822	103	239	173	991	2328
Sedimentibacter	35	694	234	423	275	46	1707
Coriobacteriaceae	7	358	543	167	116	176	1367
Parasporobacterium	3	253	137	379	244	171	1187
Pseudomonas	58	380	189	284	196	77	1184
Desulfobulbus	0	119	188	243	142	446	1138
Micrococcaceae	11	292	233	190	115	134	975
Dietziaceae	26	141	286	125	62	133	773
Halomonas	33	252	10	225	189	7	716
Providencia	78	97	3	256	180	4	618
Halanaerobium	45	219	68	96	113	31	572
Intrasporangiaceae	18	117	141	82	46	125	529
Desulfovibrio	7	58	126	71	40	159	461
Turicibacter	316	11	51	8	4	20	410
Acetanaerobacterium	10	139	43	63	54	49	358
Environmental_samples	335	4	1	4	3	0	347

Paracoccus	2	81	35	100	49	74	341
Dermabacteraceae	7	73	74	73	36	57	320
Environmental_samples	45	24	43	58	28	108	306
Acholeplasma	171	15	31	26	3	54	300
Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Corynebacteria	2	93	53	62	32	30	272
Microbacteria	5	41	94	60	38	31	269
Myroides	160	29	3	35	33	3	263
Pseudochrobactrum	0	41	45	73	29	44	232
Brevundimonas	17	41	51	43	33	38	223
Faecalibacterium	11	25	26	35	24	101	222
Ralstonia	202	0	14	0	0	0	216
Nocardioida	0	25	59	26	17	70	197
Sulfurospirillum	0	103	16	33	30	10	192
Anaerobacter	136	5	3	32	10	4	190
Yaniella	11	30	55	31	5	46	178
Brevibacteria	0	20	88	11	4	41	164
Alcaligenes	77	9	10	26	21	13	156
Escherichia	91	13	4	19	18	8	153
Comamonas	107	8	0	8	7	2	132
Helcococcus	0	24	54	6	8	30	122
Actinomycetace	0	18	34	21	11	20	104
Parvimonas	61	10	8	1	8	4	92
Peptostreptococcus	72	0	4	6	8	2	92
Aeromonas	60	5	0	19	5	1	90
Devosia	3	16	18	12	22	18	89
Candidatus_Amoebinatus	7	17	12	23	22	6	87
Idiomarina	27	16	6	15	14	7	85
Wautersiella	52	4	0	6	11	0	73
Mogibacterium	1	8	40	6	3	13	71
Dermatophila	0	6	14	15	6	18	59
Deinococcus	10	11	9	14	8	2	54
Marinobacter	9	16	2	6	15	4	52
Morganella	24	14	0	11	3	0	52
Salinivibrio	31	8	0	5	6	2	52
Rhizobium/Agrobacterium_group	2	1	9	16	11	10	49
Desulfobacter	0	1	0	16	8	23	48
Sphingobacterium	11	9	9	12	3	4	48
Azorhizobium	0	0	15	14	3	15	47
Sphingomonas	0	18	2	10	1	15	46
Desulfococcus	0	9	21	4	2	1	37
Pirellula	0	3	19	2	8	5	37
Oceanisphaera	30	3	0	0	3	0	36
Erythrobacter	0	9	3	10	5	4	31
Legionella	1	0	13	0	0	17	31

Planctomyces	0	5	3	7	1	15	31
Succiniclasticum	0	7	1	5	9	8	30
Environmental_samples	0	2	7	4	2	14	29
Mesorhizobium	0	0	0	13	4	12	29
Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Akkermansia	28	0	0	0	0	0	28
Micromonosporaceae	0	2	13	5	0	8	28
Geodermatophilaceae	11	2	1	4	0	9	27
Marinobacterium	14	4	0	1	0	8	27
Methylobacterium	17	0	2	2	0	6	27
Nocardiopsaceae	0	6	7	6	0	7	26
Novosphingobium	4	7	4	3	5	3	26
Syntrophomonas	0	12	2	8	4	0	26
Petrobacter	0	1	0	11	12	1	25
Luteimonas	0	7	2	3	10	2	24
Nocardiaceae	1	3	8	8	1	3	24
Pelomonas	24	0	0	0	0	0	24
Streptomycete	0	7	8	0	0	9	24
Bosea	0	4	5	1	8	5	23
Environmental_samples	10	4	1	2	0	6	23
Glycomycetaceae	0	8	6	0	3	6	23
Mycobacteriaceae	0	6	5	4	6	2	23
Dermacoccaceae	0	8	3	6	1	4	22
Stappia	15	1	0	0	6	0	22
Castellaniella	14	0	1	0	0	5	20
Burkholderia	19	0	0	0	0	0	19
Rhodobacter	8	8	0	1	2	0	19
Soehngenia	10	7	1	1	0	0	19
Stenotrophomonas	0	6	1	6	2	4	19
Laribacter	0	2	0	7	9	0	18
Empedobacter	9	2	0	6	0	0	17
Guggenheimella	0	7	4	1	1	4	17
Oscillibacter	0	2	0	3	5	7	17
Phascolarctobacterium	0	5	0	3	9	0	17
environmental_samples	14	0	1	0	0	0	15
Bacillus_cereus_group	9	0	4	0	0	0	13
Bifidobacterium	0	6	6	0	0	1	13
Brachymonas	0	1	0	3	6	3	13
Oligella	0	4	2	3	3	1	13
Thermomonosporaceae	0	5	3	2	2	1	13
Dehalobacter	0	0	1	7	1	2	11
Desulfobotulus	0	7	0	3	1	0	11
Flavisolibacter	3	6	2	0	0	0	11
Gordoniaceae	0	1	5	3	2	0	11
Phenylobacterium	0	1	1	5	1	3	11

Pseudaminobacter	0	5	0	2	1	3	11
Rickettsiella	0	0	5	5	1	0	11
Solirubrobacter	0	4	3	2	0	2	11
Caloramator	10	0	0	0	0	0	10
Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Chryseobacterium	0	7	0	1	2	0	10
Promicromonosporaceae	0	0	6	0	0	4	10
Ruminococcus	0	7	1	0	0	2	10
Spermatophyta	10	0	0	0	0	0	10
Acidaminococcus	8	0	1	0	0	0	9
Jonesiaceae	0	2	1	0	2	4	9
Nevskia	0	0	4	0	0	5	9
Peredibacter	9	0	0	0	0	0	9
Prevotella	3	5	0	0	1	0	9
Rhodopirellula	0	0	4	2	2	1	9
Thauera	0	3	0	2	4	0	9
Thermus	3	0	0	6	0	0	9
Xanthomonas	0	3	0	3	1	2	9
Diaphorobacter	0	1	1	5	0	1	8
Salegentibacter	0	5	2	1	0	0	8
Thioalkalimicrobium	7	0	0	0	1	0	8
Caldilinea	0	1	0	5	1	0	7
Flavobacterium	3	0	0	1	3	0	7
Pantoea	0	0	6	0	0	1	7
Anaerobranca	0	4	0	0	0	2	6
Bogoriellaceae	0	4	0	0	2	0	6
Lysobacter	0	3	0	2	1	0	6
Myxococcaceae	0	4	1	0	0	1	6
Ochrobactrum	1	3	1	1	0	0	6
Rubrobacteraceae	0	0	2	0	1	3	6
Acidithiobacillus	0	0	3	0	1	1	5
Alkalibacter	0	2	0	1	1	1	5
Bradyrhizobium	0	1	4	0	0	0	5
Cystobacteraceae	0	0	2	1	2	0	5
Klebsiella	0	0	0	1	4	0	5
Luteolibacter	0	1	2	2	0	0	5
Methylophilus	5	0	0	0	0	0	5
Opitutus	1	0	4	0	0	0	5
Porphyrobacter	3	1	0	0	0	1	5
Actinosynnemataceae	0	0	1	0	0	3	4
Angulomicrobium	0	0	4	0	0	0	4
Butyrivibrio	0	0	0	2	2	0	4
Cellulomonadaceae	0	0	0	0	2	2	4
Conexibacter	0	1	3	0	0	0	4
Helicobacter	0	0	1	0	3	0	4

Holophaga	0	0	0	4	0	0	4
Lampropedia	0	0	0	0	4	0	4
Pseudoalteromonas	2	0	2	0	0	0	4
Sanguibacteraceae	0	0	3	0	0	1	4
Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Sporanaerobacter	0	0	0	4	0	0	4
Symbiobacterium	0	0	0	0	4	0	4
Beijerinckia	0	0	3	0	0	0	3
Bergeyella	0	0	3	0	0	0	3
Caulobacter	0	0	2	1	0	0	3
Chitinophaga	0	1	0	0	2	0	3
Chromohalobacter	0	0	0	3	0	0	3
Desulfocapsa	0	0	0	0	3	0	3
Desulforhopalus	0	0	0	0	0	3	3
Dyella	3	0	0	0	0	0	3
Environmental_samples	3	0	0	0	0	0	3
Environmental_samples	0	0	0	0	2	1	3
Mannheimia	0	2	1	0	0	0	3
Methylocystis	2	0	0	0	0	1	3
Polyangiaceae	0	0	0	0	0	3	3
Rhodobaca	0	0	0	3	0	0	3
Roseomonas	0	0	0	2	1	0	3
Salicola	0	1	0	2	0	0	3
Thermomonas	0	1	0	0	0	2	3
Williamsiaceae	0	0	0	0	0	3	3
Zobellella	0	0	0	3	0	0	3
Acidaminobacter	0	2	0	0	0	0	2
Afipia	0	0	2	0	0	0	2
Alistipes	0	0	0	0	1	1	2
Arenimonas	0	1	0	0	0	1	2
Cytophaga	0	2	0	0	0	0	2
Environmental_samples	0	1	0	0	0	1	2
Environmental_samples	0	0	2	0	0	0	2
Geosporobacter	0	0	0	0	0	2	2
Hydrogenophilus	0	0	0	0	2	0	2
Hyphomicrobium	0	0	0	2	0	0	2
Isosphaera	0	0	0	0	0	2	2
Kaistella	0	0	0	2	0	0	2
Labrys	0	0	0	0	0	2	2
Methylobacter	0	0	0	1	0	1	2
Oscillospira	0	0	0	0	0	2	2
Paracraurococcus	0	2	0	0	0	0	2
Peptoniphilus	0	0	0	2	0	0	2
Perlucidibaca	2	0	0	0	0	0	2
Pigmentiphaga	0	0	2	0	0	0	2

Propionibacteriaceae	0	0	1	0	1	0	2
Prosthecobacter	0	0	1	0	0	1	2
Pseudonocardiaceae	1	1	0	0	0	0	2
Rhodopseudomonas	0	0	0	2	0	0	2
Taxon	**G1**	**S1**	**E1**	**G2**	**S2**	**E2**	**total**
Salinimicrobium	0	1	0	1	0	0	2
Sarcina	0	0	0	0	2	0	2
Shinella	0	0	0	0	0	2	2
Simplicispira	0	0	0	2	0	0	2
Sphingobium	0	0	1	0	0	1	2
Sphingopyxis	0	2	0	0	0	0	2
Sporacetigenium	0	0	0	2	0	0	2
Acetivibrio	0	0	0	1	0	0	1
Acidothermaceae	0	0	0	0	0	1	1
Actinobacillus	0	0	0	0	1	0	1
Anaerobaculum	0	1	0	0	0	0	1
Arenibacter	0	0	0	1	0	0	1
Azospirillum	0	1	0	0	0	0	1
Balneola	0	0	0	1	0	0	1
Barnesiella	0	0	1	0	0	0	1
Bdellovibrio	0	0	0	0	1	0	1
Campylobacter	0	1	0	0	0	0	1
Coprobacillus	0	0	0	1	0	0	1
Desulfatirhabdium	0	1	0	0	0	0	1
Desulfomonile	0	1	0	0	0	0	1
Desulfotignum	0	0	0	0	0	1	1
Environmental_samples	0	0	0	0	0	1	1
Environmental_samples	1	0	0	0	0	0	1
Environmental_samples	0	0	0	0	0	1	1
Environmental_samples	0	0	0	1	0	0	1
Environmental_samples	0	1	0	0	0	0	1
Eubacterium	0	0	0	0	0	1	1
Gelidibacter	0	1	0	0	0	0	1
Geobacter	0	1	0	0	0	0	1
Hyphomonas	0	1	0	0	0	0	1
Kineosporiaceae	0	1	0	0	0	0	1
Mahella	0	0	0	1	0	0	1
Malikia	0	1	0	0	0	0	1
Moraxella	0	0	0	0	1	0	1
Nitratireductor	0	0	1	0	0	0	1
Streptosporangiaceae	0	0	0	0	0	1	1
Thiobacillus	0	1	0	0	0	0	1

Declaration

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented for similar purpose in any other university, and all sources of materials used for the Dissertation have been duly acknowledged.

Candidate: Tesfaye Admassu, Addis Ababa University, Ethiopia

Signature: _____

This Dissertation has been submitted for examination with my approval as the research supervisor of the candidate.

Supervisors: Fasil Assefa (PhD), Addis Ababa University, Ethiopia

Signature: _____

Adey Feleke (PhD), Addis Ababa University, Ethiopia

Signature: _____

Nancy G. Love (Prof), Michigan University, USA

Signature: _____