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OCCURRENCE OF ANTIBIOTIC RESISTANT BACTERIA IN THE LEGON SEWAGE TREATMENT PLANT AND THE RECEIVING ONYASIA STREAM; IMPLICATIONS FOR WASTEWATER REUSE

BY

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DECLARATION

I, Lady Asantewah Boamah Adomako hereby declare that this thesis was carried out entirely by me from research undertaken under the supervision of Dr. DzidzoYirenya-Tawiah and Dr. Daniel Nukpezah of the Institute for Environment and Sanitation Studies, University of Ghana. This thesis has never been presented, either in part or in whole for a degree in this university or any other institution. All cited works and assistance have been duly acknowledged

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ABSTRACT

Although wastewater treatment plants efficiently reduce the loads of pathogens in wastewater, the conventional treatment processes do not show significant removal of antibiotics, antibiotic resistant bacteria and antibiotic resistant genes, resulting in the introduction of these contaminants into the environment. This has human health implications for wastewater reuse and the use of effluent receiving water bodies as irrigation sources. Humans may be exposed to resistant bacteria and render treatment options for infections caused by resistant pathogens ineffective. In order to assess the occurrence of antibiotic resistance bacteria from the Legon Sewage Treatment Plant effluent and the receiving Onyasia stream, antibiotic resistance profiles of *Aeromonas hydrophila, Pseudomonas aeruginosa* and *Escherichia coli* isolated from wastewater and the Onyasia stream were evaluated for resistance to selected antibiotics. Wastewater and surface water samples were collected once per month in triplicate over a six-month period from two sampling sites in the Legon Sewage Treatment Plant (influent and effluent) as well as three sites from the Onyasia stream with reference to the treatment plant discharge point (upstream, outfall and downstream). *Aeromonas hydrophila, Pseudomonas aeruginosa* and *Escherichia coli* isolates were tested for resistance to Gentamicin, Amoxicillin clavulanate, Tetracycline, Ciprofloxacin, Cefuroxime, Aztreonam, Imipenem and Ceftazidime using the Kirby-Bauer disc diffusion method. *E. coli, Aeromonas hydrophila* and *Pseudomonas aeruginosa* isolates showed high resistance patterns to most tested antibiotics. *Escherichia coli* and *Aeromonas hydrophila* isolates were most resistant to Amoxicillin clavulanate (57% and 68% respectively), Cefuroxime (52% and 43% respectively), and Tetracycline (49% and 31% respectively). In contrast, they were susceptible to Imipenem (91% and 78% respectively) Gentamicin (83% and 91%) Aztreonam (74% and 73%

respectively), and Ciprofloxacin (71% and 78%). *Pseudomonas aeruginosa* isolates showed high resistance to Aztreonam (37%) and Ciprofloxacin (33%). *Pseudomonas aeruginosa* isolates sensitivity to Imipenem (96%), Gentamicin (22%) and Ceftazidime (89%) was high. Resistant rates were high in effluent, outfall and downstream isolates, Significant differences ($p < 0.05$) were observed between upstream and downstream sampling sites relative to the wastewater treatment plant discharge point (outfall) and the rate of *Escherichia coli* and *Aeromonas hydrophila* isolate resistance to Amoxicillin, Cefuroxime and Tetracycline and *Pseudomonas aeruginosa* isolate resistance to Aztreonam and Ciprofloxacin. There were also levels of multi-drug resistant isolates in downstream sampling site compared to upstream sampling site. Results show that the discharge of treated wastewater effluent into water bodies are potentially significant contributors to the dissemination and persistence of antibiotic resistance in the receiving watershed. These findings also have human health implications for effluent wastewater reuse and the use of the stream as a source of irrigation water.

DEDICATION

This work is dedicated to my dear husband Mr. Ofori Boamah Adomako, and children Michelle and Caleb Boamah Adomako.

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LIST OF ABBREVIATIONS

ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Genes
CFU	Colony Forming Unit
HPC	Heterotrophic Plate Count
WWTP	Wastewater Treatment Plant
WHO	World Health Organization
GN	Gentamicin
CXM	Cefuroxime
ATM	Aztreonam
IMP	Imipenem
AMC	Amoxicillin clavulanate
CIP	Ciprofloxacin
CAZ	Ceftazidime
TET	Tetracycline

CHAPTER ONE

INTRODUCTION

1.1 Background

Antibiotics are one of the breakthrough discoveries in human history. Antibiotics are substances that are able to prevent the growth of or kill other organisms (WHO, 2014). They are broadly used for improving human and animal health by preventing and treating infections caused by bacterial pathogens. In animal husbandry and aquaculture, antibiotics are used extensively as growth promoters. (Roca *et al*., 2015; Kummerer, 2009; Baquero *et al.*, 2008). Since their discovery antibiotics has saved many human lives, but the broadened range of antibiotic use for both medicinal and non-medicinal purposes has caused a surge in their use over the years (Bouki *et al*, 2013). The consequence of this includes the development and spread of antibiotic resistant bacteria (ARB) (Levy and Marshall 2004) due to the abuse and evolution through mutation.

Antibiotic resistance is defined as the capacity of bacteria to withstand the effects of antibiotics previously used in their treatment (WHO, 2014). The pervasive usage of antibiotics in humans and animals results in the development of resistant bacteria, which is a major concern as antibiotic resistance results in failure in the treatment of infections that were previously treated with these antibiotics.

Antibiotic resistance is one of the major health threats confronting humans (WHO, 2000). The O'Neill, 2014 Review on Antimicrobial Resistance, estimates that the 700,000 annual deaths caused by drug-resistant pathogen infections if left unchecked, will increase to 10 million by 2050 (O'Neill Commission, 2014).

Resistant bacteria that cause life-threatening infections are becoming increasingly prevalent, and this limits treatment options. The WHO Global Action Plan on

Antimicrobial Resistance which aims at reducing the rate at which antibiotic resistant bacteria evolve and spread (WHO, 2015). This action plan emphasizes the need to quantify environmental concentrations and total loads of antimicrobial resistant bacteria, antimicrobial resistant genes and antibiotic residues originating from humans and animals in the environment in order to provide needed evidence for decision-making and reduce exposure and human health risks (Wuijts *et al*., 2017). A strategic goal of the action plan is to increase surveillance and research into antimicrobial resistance.

Addressing the rising antimicrobial resistance threat requires a "One Health" approach. "One Health" approach involves the collaborative efforts of multiple sectors working together locally, nationally and globally to tackle antibiotic resistance and other health issues. The goal of "One Health" is to achieve optimal health through acknowledging that humans, animals, plants and the environment are interconnected (Centers for Disease Control,2018). The initiative recognizes that, the effects of antibiotic resistance on humans, animals as well as the environment must be addressed simultaneously. This is because antibiotics, which are used in treating various infections in animals, may be the same used for human infections. Antibiotic resistance bacteria originating from animals, humans and the environment may spread within each other as well, locally and globally. In addition, resistant pathogens found in clinical environments have obtained their resistant genes from environmental reservoirs (Wright, 2010).

Antibiotics enter the environment via different pathways including wastewater effluent discharge, surface runoff and leaching from agricultural lands applied with manure (Zhang *et al* 2009). The environment serves as potential reservoirs to antibiotic

resistance bacteria (Kummerer 2009; Zhang *et al*., 2009; WHO, 2014). Hotspots for antibiotic resistance are within environmental compartments where environmental bacteria mix and exchange genetic material with potential pathogenic bacteria. These hotspots include sewage, wastewater treatment plants (WWTPs), animal farms and aquaculture facilities (Kummerer, 2009; Rizzo *et al*, 2013, Wellington *et al*., 2013,).

Wastewater treatment plants (WWTPs) take and sewage from households, hospitals and other sources, which may contain antibiotics and antibiotic resistant bacteria. Hospital effluents, particularly, are very dangerous because they contain large amounts of residues of drugs and infectious pathogens; as such, they are an important source of antibiotics and multiple drug-resistant bacteria (Lorezo *et al*., 2018). During treatment of infections microbiota present in the human gut become exposed to high concentrations of antibiotics and which places selective pressure on microorganisms present and may stimulate the development of resistance phenotypes in the gut, which are then released into sewage through human excreta. A large percentage of most antibiotics administered for the prevention or treatment of microbial infections in humans and animals are excreted as an active substance, and are consequently released through sewage into the environment (Rizzo *et al*., 2013).

WWTPs are key points for the emergence and proliferation of antibiotic resistance in environmental settings (Rizzo *et al*., 2013). Antibiotics, antibiotic resistant genes and antibiotic resistant bacteria have been found in wastewater treatment plants (Zhang *et al*., 2009; Rizzo *et al*., 2013). The treatment of wastewater includes different processes, which include chemical, physical, biological, mechanical, and physicochemical that may affect the outcome of antibiotics, antibiotic resistant bacteria and genes.

Effluent may be discharged into the environment, and as such, they play a major role in antibiotic resistance development and proliferation (Rizzo *et al*., 2013). Urban wastewater treatment by biological treatment process creates a conducive environment that enables the development and spread of antibiotic resistant bacteria (Rizzo *et al*, 2013). This is as a result of the continuous mixing of antibiotics at low concentrations and bacteria present which leads to selective pressure resulting in the development of resistant bacteria. In most cases, although the concentrations of antibiotics may be low and not lethal to bacteria it is sufficient to select for resistance (Gullberg *et al*, 2011).

Wastewater treatment plants may discharge their effluent into receiving environments such as rivers, lakes, or oceans after treatment, and therefore has implications for wastewater reuse and the use of contaminated water bodies as irrigation water sources. Water bodies containing antibiotic resistant bacteria may overflow during heavy rains and results in the contamination of ground water sources.

Unfortunately, there are inadequate global and local regulations or guidelines for the permitted or desirable maximum limits for contaminants like antibiotics, antibiotic resistant bacteria and antibiotic resistance genes that can be discharged into the environment or reused (McConnell, 2016). Antibiotic resistant bacteria and genes which are still present after treatment end up in the environment where antibiotic resistance genes can spread among bacteria and can also be obtained by dangerous pathogens in the environment.

The reuse of wastewater is being encouraged in agriculture, aquaculture and other non-critical uses in order to manage aquatic resources to meet the United Nations Sustainable Development GOALS (SDG) goal 6 of ensuring the availability and sustainable management of water and sanitation for all. Nevertheless, there is a

potential risk to humans from consumption of wastewater irrigated produce as well as contact through recreational activities. As result of exposure, humans may acquire infections caused by antibiotic resistant pathogens thereby rendering treatment of infections ineffective. As such, antibiotic resistant bacteria and other contaminants in wastewater has to be monitored to protect human health if wastewater reuse is being encouraged.

This research seeks to investigate presence of antibiotic resistant *Pseudomonas aeruginosa P. aeruginosa*) *Escherichia coli* (*E. coli*) and *Aeromonas hydrophila* (*A. hydrophila*) isolates in the Legon Sewage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods. This will contribute to knowledge on antibiotic resistant levels in the wastewater treatment plant and the Onyasia Stream. The study will also provide evidence for the need for further treatment of wastewater to remove antibiotic resistance for the use of wastewater effluent in agriculture and other purposes. The study will also highlight the need to include antibiotic resistant bacteria in standards and guidelines for wastewater discharge and reuse.

The information from this study will also inform decision making regarding the reuse of wastewater from the Legon Sewage Treatment Plant for agriculture, aquaculture and other non-critical uses.

This study thus seeks to investigate the presence of antibiotic resistant *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods and the environmental health implications of the treatment plant discharge.

1.2 Problem statement

Inadequate sanitation and lack of access to safe and clean water threatens human health. Due to water scarcity, there has been recent efforts at considering the promotion of recycling and reuse of wastewater to reduce pressure on fresh water. Recycled wastewater is being recommended for irrigation, car washing and other non-critical uses. However, effluents discharged by wastewater treatment plants into the environment may contain antibiotics, antibiotic resistant bacteria and antibiotics, and may end up in water and soil (Aslan, 2018).

Wastewater and agricultural runoff are considered as sources of antibiotic resistance in the environment (Li *et al*., 2010). Water contaminated with antibiotic resistant bacteria can affect aquatic biodiversity (Aslan, 2018) and human health adversely (Hong *et al*., 2018). Antibiotic resistance bacteria could come into contact with drinking water resources, the food chain through irrigation as well as contact from recreational use rendering treatment options ineffective (Blaustein *et al*., 2016; Wang *et al*., 2014; Frey *et al*., 2015; Zhang *et al*., 2015). As such antibiotic resistance is a major health concern worldwide, and World Health Organization has recognized antibiotic resistant bacteria as an emerging water pollutant of concern (WHO, 2014).

In order to improve and expand sewerage and sanitation system for disposal of wastewater in Accra that meets environmental standards, the Legon Sewage Treatment Plant was constructed as part of the Accra Sewage Improvement Project (ASIP). The treatment plant is located in Legon, Accra and operates on the concept of waste stabilization ponds. Effluents from the plant is discharged into the Onyasia Stream, a nearby stream and part of the Odaw catchment. The Onyasia stream, which empties into the Odaw River, serves as a source of irrigation water to some vegetables farms along the stream in Dzorwulu, Airport and other parts of Accra (Drechsel and Keraita, 2014). It is also a water source for car washing in these communities. It is not known if it also serves other purposes in times of water shortages in the area.

Monitoring of antibiotic resistance in clinically relevant bacteria in treated effluent leaving the treatment plant and the receiving stream is essential to afford knowledge on the type of resistant bacteria being released into the environment. This information is necessary for the identification of public health risks to inform decision-making regarding improvement of removal rates of antibiotic resistant bacteria and genes to minimize exposure to humans.

1.3 Justification

Wastewater treatment plants reduce the concentrations of pathogens in wastewater, but the treatment processes are not able to substantially remove all antibiotic resistant genes and bacteria (WHO, 2014). Treatment of wastewater by use of stabilization ponds in particular have been shown to enhance the transfer of resistant genes among bacteria when compared to other treatment methods like chemical and mechanical treatment (Neudorf *et al*., 2017).

Wastewater treatment plants are therefore salient hotspots and risk environments for the emergence and distribution of antibiotic resistance (Kummerer, 2009; Rizzo *et al*., 2013). Surface waters are often the recipients of treated wastewater from treatment plants. These water bodies, which are also discharge points often, serve as irrigation water sources, posing a health risk to humans.

In a period of water scarcity, wastewater reuse is encouraged in agriculture, aquaculture and other non-critical uses in order to manage aquatic resources to meet the SDG 6 to ensure the availability and sustainable management of water and sanitation for all. The United Nations Educational Scientific and Cultural Organization (UNESCO) recommends treated wastewater reuse for irrigation in order to reduce

pressure on freshwater resources. This is because the use of water for agriculture makes up 70% of global water withdrawals.

There is a potential risk to humans from consumption of wastewater irrigated produce as well as contact through recreational activities. As result of exposure, humans may acquire infections caused by antibiotic resistant pathogens thereby rendering treatment of such infections ineffective. As such, antibiotic resistant bacteria and other contaminants in wastewater has to be monitored to protect human health.

Antibiotic resistance bacteria and genes are now considered as environmental pollutants and as such, severe measures need to be taken to prevent their further spread (WHO, 2014). Better understanding of antibiotic resistance reservoirs is needed to fight the resistance threat in the clinic and the environment.

There is limited knowledge on the antibiotic resistance occurrence as well as resistance types in the wastewater and wastewater affected environments. In the era of increasing antibiotic resistance and the potential reuse of wastewater, it is necessary to examine the contribution of wastewater treatment plants to resistance in the environment.

Mapping antibiotic resistance in wastewater environments in Ghana is essential in order to increase knowledge about the insights into extensive occurrence and range of antimicrobial resistance in non-clinical environments. This will also provide guidance for decision making regarding additional treatment of sewage needed to reduce the risk of development and spread of antibiotic resistance.

This thesis reports on a study designed to determine the presence of antibiotic resistant enterobacteria like *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewage

Treatment Plant and the receiving Onyasia stream, using bacterial resistance data from culture-dependent methods.

The aim is to determine the whether these resistant and potentially pathogenic microbes would likely compromise the quality of the effluent from the treatment plant. Further to this, the study also discussed the likely impact of the quality of the effluent on reuse in agriculture and other uses, as well as the general environmental health of the stream and its users.

1.4 General Objective

The primary objective of this study is to determine the presence of antibiotic resistant *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods, and assess the environmental health implications of the treatment plant discharge on wastewater and stream use.

1.5 Specific Objectives

1. Assess the level of occurrence of *E. coli*, *P.* aeruginosa and *A. hydrophila* in wastewater from the Legon Sewage Treatment Plant and the Onyasia stream.
2. To assess the occurrence of antibiotic resistant *E. coli*, *P. aeruginosa* and A. *hydrophila* isolated from the Legon Sewage Treatment Plant effluent.
3. To assess the occurrence of antibiotic resistant *E. coli*, *P. aeruginosa* and *A. hydrophila* isolated from sampling sites in the Onyasia stream relative to the wastewater treatment plant in order to determine the impact of the treatment plant effluent discharge on antibiotic resistance profiles of surface water bacteria.
4. To evaluate the safety of wastewater effluent for reuse options.

CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction

Antibiotics discovery is probably one of the greatest inventions of all times, as without them humankind would have been wiped out by diseases. Antibiotics are compounds that cause microbial cell death or inhibit cell growth due to specific interactions with bacterial targets. Antibiotics are essential in the treatment of infections in humans and animals. Antibiotic resistance occurs when bacteria are altered in such a way which makes medications that will otherwise kill or inhibit their growth ineffective in curing infections caused by the microorganisms. (Nordgård, *et al*., 2017).

The overuse of antibiotics has resulted in the selection of antibiotic resistant microorganisms. Resistance of microbes to antibiotics is a serious human and wildlife health threat in terrestrial or aquatic environments. The WHO, has asserted that humans may be entering a post-antibiotic era when simple bacterial infections that were previously treatable are no longer responding to antibiotic treatment resulting in death (O'Neill Commission, 2014). The 2014 O'Neill report commissioned by the UK government estimated that globally by 2050, antimicrobial resistant infections may become the leading cause of death (O'Neill Commission, 2014).

Although global increased levels of antibiotic resistance has been linked to the misuse of antibiotics in medical and agricultural practices, the contribution of the natural environment to the development and spread of resistance has not received a lot of attention until now. Human activities may contaminate the environment with antibiotic resistant bacteria and antibiotics which results in the acceleration of the development

and spread of resistance. Sources of antibiotic and antibiotic contamination in the environment include animal and human waste, and pharmaceutical manufacturing waste.

2.1 Antibiotic resistance

Antibiotic resistance may be intrinsic or acquired. Natural resistance bacteria have a natural ability for Deoxyribonucleic Acid (DNA) recombination and genetic mutation can occur in all bacterial isolates. However, intrinsic resistance occurs when the target of drug action does not exist or is inaccessible. For example, gram-negative bacteria have intrinsic resistance to some antibiotics, including erythromycin, because of the outer membrane being impermeable (Alkhaleefah, 2015). The genetic mechanisms underpinning acquired bacterial resistance development are gene transfer and mutation (Alkhaleefah, 2015).

Acquired resistance results from selective pressure exerted by antibiotic agents present in the environment (Osińska *et al.*, 2017).

2.2 Emergence of antibiotic resistance

There is a significant correlation between antibiotic consumption and antibiotic resistance (Karkman, 2015). Although the phenomenon of antimicrobial resistance was existent prior to mass production and clinical use of antibiotics, human activities have contributed massively in the development of environmental reservoirs of antibiotic resistance.

Large amounts of antibiotics manufactured since the 1940's have been used and released into the environment. A small proportion of antibiotics in clinical use is contributed by organisms that produce antibiotics naturally (Helt, 2012), with the bulk of commercial production accounting for the vast amount of antibiotics currently used. These antibiotics have spread widely providing continuous selective pressure on bacteria resulting in resistant strains in the environment (Davies and Davies, 2010). Clinically relevant pathogens resistant to penicillin were recorded not long after it was discovered. The first Tetracycline resistant *Shigella* strain was isolated five years later after the antibiotic was discovered in 1948. Horizontal transfer of genes has contributed to the evolution and transmission of resistant genes (Davies & Davies, 2010).

Superbugs are commensal and pathogenic bacteria that have acquired multiple resistance genes (Karkman, 2015). An example is Methicillin Resistant *Staphylococcus aureus* (MRSA). Some strains have in addition acquired increased virulence and enhanced transmissibility (Karkman, 2015). Due to massive antibiotic use, many pathogens associated with epidemics of human disease have acquired multiple resistance determinants. Multiple antibiotic resistant bacteria are causing serious problems in healthcare system worldwide. The World Health Organization (WHO) has raised concern about antibiotic resistance amidst fears the human race is heading back to pre-antibiotic era if serious action is not taken. (World Health Organization, 2014).

2.3 Mechanisms by which bacteria acquire resistance

Through evolution, some bacteria have developed various mechanisms to resist the effects of antibiotic compounds. Antibiotic resistance genes (ARGs) genetically code for resistant traits. Resistance can be acquired through mutation and gene transfer

(vertical and horizontal). Horizontal gene transfer occurs between microorganisms by means of use of mobile genetic elements including plasmids, integrons and transposons. These genetic elements contain genes that are transferred between bacteria (Marti *et al*., 2014). Resistant genes in mobile genetic elements control specific mechanisms, which fight an antibiotics ability to harm bacteria. Horizontal gene transfer mechanisms through which bacteria take foreign DNA are transformation, transposition, transduction or conjugation (Dantas and Sommer, 2014).

Transformation occurs when bacteria obtain free DNA from the environment, and transduction occurs when a bacteriophage (a virus that infects bacteria) carries DNA from one bacterial cell to another. Conjugation involves physical contact between a donor and a recipient cell via a conjugation pilus, through which genetic material is transferred, therefore involving live bacteria (McConnell, 2016).

Mobile genetic elements have a major role as vectors for accumulation and spread of antibiotic resistance genes among bacteria. This is due to their ability to encode conjugative transfer of genes, which enable the movement of genes between bacteria of different species. Resistance genes located on plasmids and other genetic elements can carry one or more genes. This enables various mechanisms of resistance to multiple antibiotics and other compounds like heavy metals.

The capacity of microorganisms to share genetic material through horizontal genes transfer implies that genes that occur in environment can potentially transfer resistant genes from animal to human pathogens within bacterial communities (Nordgård *et al*., 2017).

The environment is therefore a large reservoir of potential resistance genes and this can be referred to as environmental resistome (Wright, 2010). Resistome is a term used to describe the occurrence and transference of antimicrobial resistance genes between and

within different environments (Alkhaleefah, 2015). Figure 2.1 shows some mechanisms of action of antibiotic classes and the resistance mechanisms that exist to counteract them.

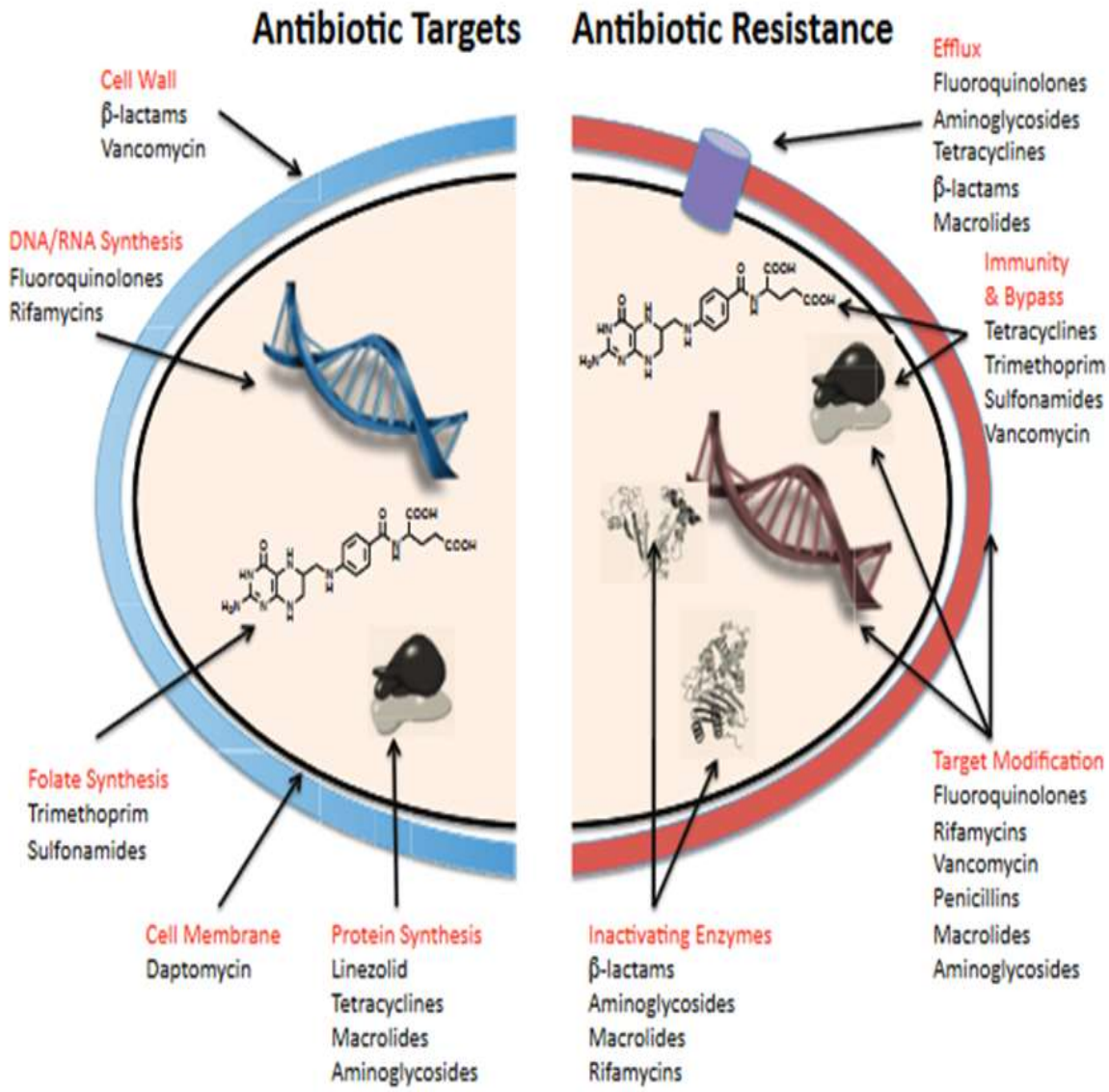

Figure 2.1: A diagram showing mechanisms of resistance and antibiotic targets (Adapted from Wright, 2010)

2.4 Antibiotic resistance in the environment

Antimicrobial resistance hotspots occur in clinical settings as well as environmental settings that have been impacted by anthropogenic activities**.** Antibiotic resistant

bacteria and genes and antibiotics have been found in different environmental niches including wastewater treatment plants, sediment, surface water, seawater and drinking water (Xi *et al*., 2009; Rizzo *et al*., 2013; Berglund *et al*., 2015; Berglund *et al*.,2014; Zhang *et al*., 2015; Zhang *et al*., 2016; Rizzo *et al*., 2013). Antibiotics and resistant genes may persist in the environment, after the use antibiotic has ended. Although resistant microorganisms can be found naturally in all environments, their occurrence has increased over the years, a phenomenon attributed to the increased usage of antibiotics in addition to exposure to other substances, which promote resistance for example, some heavy metals, biocides and disinfectants. (Martinez, 2009; Berglund *et al*., 2015).

The environment has different functions in the emergence and spread of antibiotic resistance. Firstly, the environment serves as a receiving receptacle of resistant bacteria from animals and humans that have ingested antibiotics. These bacteria may encounter other bacteria already present in the environment enabling the exchange of resistant genes between species. The environment also facilitates antibiotic resistant bacteria and resistance genes spread through air, groundwater and surface water (WHO, 2014). In addition, the environment serves a cache for natural resistance genes. Lastly, humans and animals are exposed to antibiotic resistance directly and indirectly through the environment (WHO 2014). Figure 2 depicts human activities and the environmental compartment interact in terms of spread of antibiotics, resistant bacteria and genes.

Hotspots are environmental compartments where environmental bacteria mix and exchange genetic material with potential pathogenic bacteria. These hotspots include sewage, wastewater treatment plants, animal farms and aquaculture (Kummerer, 2009; Wellington *et al*., 2013).

Municipal wastewaters are particularly an important environmental source for antibiotics, antibiotic resistant bacteria and genes (Rizzo *et al*., 2013). They also provide a conducive environment for the transfer of resistance genes between bacteria in the course of the treatment process or after discharge into the aquatic environment.

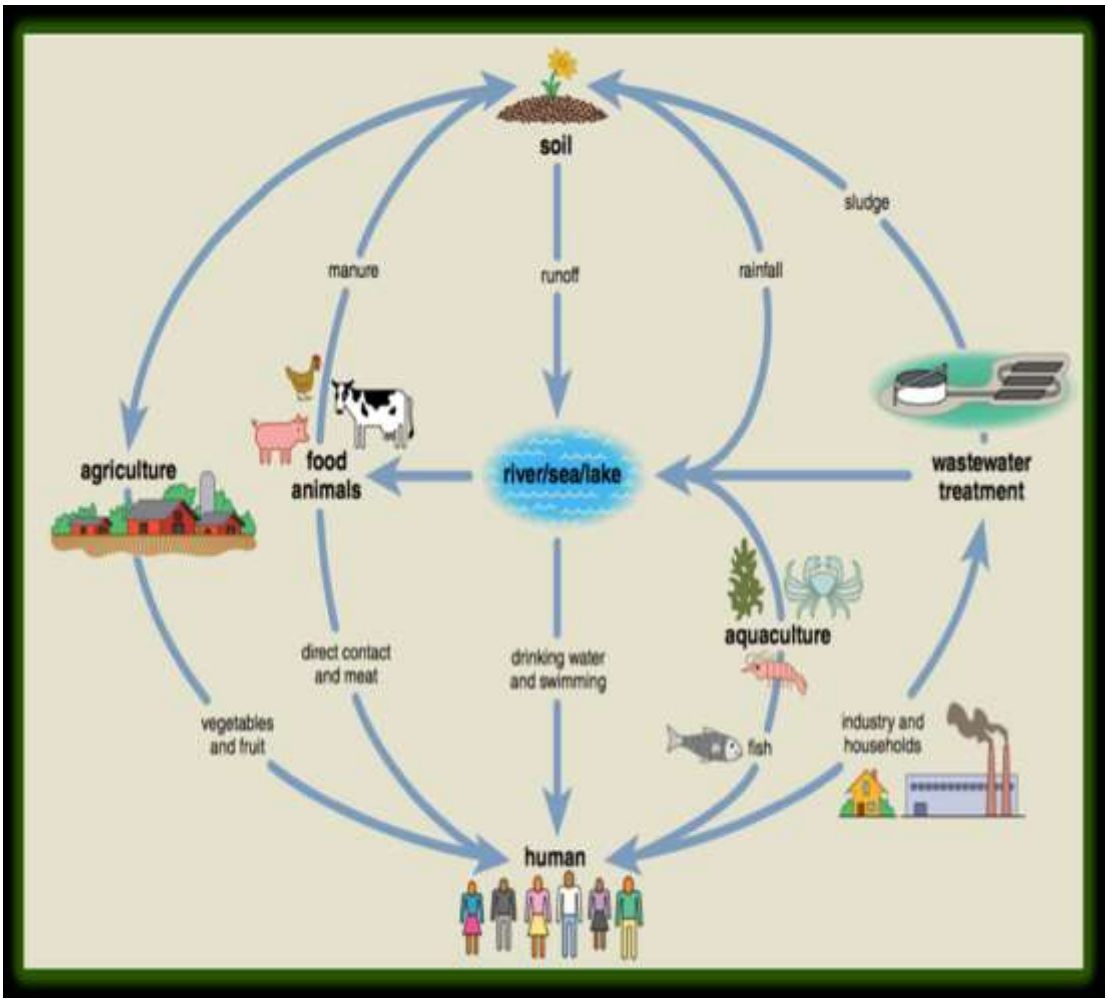

Figure 2.2: A diagram depicting how human activities and the environmental compartment intersect in terms of spread of antibiotics, antibiotic resistant bacteria and genes. (Adapted from Dantas and Sommers 2014).

2.5 Antibiotic resistance and wastewater treatment plants

Wastewater treatment plants are key antibiotic resistant bacteria sources (Kümmerer, 2009; Figueira *et al*., 2011a; Lupo *et al*., 2012 Rizzo *et al*., 2013). Antibiotics, antibiotic resistant genes and bacteria have been found in wastewater and activated sludge (Dröge *et al*., 2000; Auerbach *et al*., 2007; Zhang *et al*., 2009).

Antibiotics consumed by humans are released into municipal treatment plants partly metabolized or unused (Bouki *et al*, 2013). These antibiotics present in wastewater, even at low concentrations, apply selective pressure on bacteria present, which results in emergence of antibiotic resistant bacteria (Kummerer 2009).

Most wastewater treatment processes have primary, secondary, and tertiary treatment stages. Physical operations to remove solids in the primary treatment process, where the use of bar screens to remove large particles like sand, gravel and other heavy particles are found.

Biological and chemical processes are used in the secondary treatment to remove organic matter. In tertiary treatment, other processes are used to take out other components such as nutrients and toxic materials, which are not removed during the secondary treatment. In the course of wastewater treatment distribution of bacterial populations are altered. Due to the constant mixing of antibiotics at sub lethal concentrations with bacteria, the treatment process provides favorable conditions for resistance development and spread (Rizzo *et al*., 2013).

Although there is a remarkable reduction in the quantity of bacteria in treated wastewater, wastewater treatment process may not remove all antibiotics present and may contain higher amounts of resistant bacterial populations when compared with the

corresponding proportions contained in surface water (Huang *et al*., 2012). In addition, antibiotics present in final effluents even after treatment may persists and exert selective pressure on bacteria present (Varela and Manaia, 2013).

β-lactams, a class of antibiotics are easily broken down in the presence of water but have been found in the environment albeit at low concentrations and some b-lactams resistant genes have been detected in WWTPs (Bouki *et al*., 2013). High Tetracycline resistant gene prevalence has been found in all the processes that take place within a wastewater treatment plant that were assessed, suggesting treatment plants as potential sources for the dissemination of resistance into the environment (Auerbach *et al*., 2007). Antibiotic resistant bacteria are also abundant in activated sludge (Ge *et al*. 2013), and could enter the environment by the application of sludge as manure for growing crops. There is therefore the need to ensure removal of antibiotics and antibiotic resistant bacteria in wastewater to prevent their discharge into the environment.

2.6 Wastewater reuse

Climate change, socio-economic transformations and increase in human population over the years have resulted in the concerns regarding the sustainability of water use for humans. Wastewater reuse in agriculture is now considered as a viable solution to reduce water scarcity. In 2010 the African Water Facility proposed an initiative for the effective treatment of wastewater and faecal sludge as it can be a valuable resource with potential for reuse and also can serve to finance and provide incentive for strong sanitation systems as well as reduce surface water contamination

Currently, process water for industrial cooling, irrigation and aquifer recharge are some of the accepted and applied wastewater reuse activities (Maryam and Büyükgüngör 2019). Countries like Switzerland, Australia, Singapore, South Africa, Tunisia, Cyprus,

Saudi Arabia, Qatar, and Kuwait run successful wastewater reuse projects (Maryam and Büyükgüngör 2019).

In the United States of America, Texas, California and Arizona use reclaimed water for irrigation due to water scarcity. In addition, these states have adopted some wastewater reuse regulations (Aslan *et a*l 2018). However, recycled water is routinely screened for faecal coliform bacteria levels but not antibiotic resistant indicator bacteria (Aslan *et al.*, 2018).

The concern with wastewater reuse is the contamination of the environment as well as the risk of acquiring infections due to exposure of antibiotic resistant bacteria and antibiotics from wastewater-irrigated produce. Although there have been many advancements in wastewater treatment technologies over the years, there are concerns on wastewater discharge and reuse safety (Aslan *et al*., 2018). Many personal care products and pharmaceutical products have been detected 30 cm below soil where turf-grass fields were irrigated with treated effluent (Xu *et al*., 2009)

Currently there are guidelines and critical values for indicator bacteria for effluent discharge and for reuse of wastewater and greywater for agriculture (EPA, 2010; WHO 2006) and other non-critical uses. However, these guidelines do not have recommended antibiotic resistant bacteria limits in wastewater for discharge into the environment nor reuse for irrigation and other purposes. In addition, most studies that have evaluated the suitability of wastewater for agriculture have mostly focused on indicator bacteria and not pathogenic bacteria. There is the need for the quantification of antibiotic resistance bacteria in addition to other indicator organisms to facilitate monitoring wastewater

effluent discharge and use in agriculture in order to reduce the potential risks to protect human and environmental health.

2.7 Human health and ecological impacts of discharge of antibiotic resistant bacteria from wastewater treatment plants

Enteric diseases such as cholera and diphtheria are attributed to poor sanitation, the use of contaminated water and poor hygiene practices.

Depending on their origin, wastewater may contain microorganisms, organic compounds, metals and other substances. Non-degradable pharmaceutical contaminants like hormones and antibiotics found in wastewater persist in the water cycle and have become of greater concern over the years. Wastewater contains a rich diversity of microorganisms including pathogenic and non-pathogenic viruses and bacteria.

The purpose of wastewater treatment is to remove contaminants and reduce the undesirable impacts on the aquatic environment and public health. Studies have demonstrated that these microorganisms (including antibiotic resistant bacteria) and other compounds may survive the treatment processes and can be detected in final effluents (Rizzo *et al*., 2013). Antibiotics present in wastewater may apply pressure on bacteria which resulting in the development of resistant genes, which persist in the environment and have harmful effects on humans and animals. The release of waste containing human bacteria into environments already laden with bacteria and are enriched in resistance elements increases the chances of acquiring novel resistance by pathogens that have clinical significance (Martinez, 2009; Zhang *et al*., 2009). Non-pathogenic and pathogenic bacteria may proliferate or acquire virulence or resistance genes.

This is a major public health, as resistant bacteria and genes that are released into the environment could be transferred to humans through surface water used for irrigation, recreation and potable water production (Xi *et al*., 2009; Figueira *et al*., 2012).

Vegetables irrigated with surface water sources that receive wastewater may transfer antibiotic resistance bacteria onto the human food chain. Multiple drug resistant *Salmonella sp* have been found in water used for irrigation of vegetables (Al-Jassim *et al*., 2015; Parvathi *et al*., 2011). Antibiotic-resistant *Enterococcus sp* has been detected in reclaimed water used for spray irrigation leaving farm workers exposed to Enterococci during spray irrigation activities (Carey 2013). In Europe 2011, an epidemic was caused by multiple antibiotic resistant enterohaemorrhagic *E. coli* on vegetables which came from contaminated irrigation water.

Soil and water may also be contaminated by antibiotic resistance bacteria resulting in major changes in the population and physiology of microbiota present and as well as their activities when irrigated with wastewater (Martinez, 2009). Dalkmann *et al*. (2012) compared effects of wastewater irrigation in soils and found a rise in total microbiota and a rise in the abundance of antibiotic resistant gene in soil irrigated with wastewater in Mexico.

This study seeks to investigate the presence of antibiotic resistant *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods and the environmental health implications of the treatment plant discharge.

2.8 Tools for detecting antibiotic resistant bacteria in waste water and surface water

To recognize hotspots for the possibility of spreading of antibiotic resistant genes and resistance bacteria, their relative abundance has to be determined. Two main approaches are used for determining antibiotic resistant bacteria and genes, namely culture-based and molecular-based methods for clinical and environmental samples. Environmental samples which include wastewater and surface water contain a myriad of bacterial species as compared to clinical bacteria, as such in order to achieve accurate results modifications need to be made which, can be applied to wastewater and surface waters antibiotic resistance surveillance testing.

Microbial analysis of water is done using the membrane filtration method or spread plate method after which bacteria are isolated for further identification and then characterization of antibiotic resistance. Coliforms and other enteric bacteria are mostly cultured because they serve as indicators of microbial contamination (APHA, 2012). Isolates are identified, counted and confirmed using biochemical tests after which they are screened for their resistance to selected antibiotics. Common methods for determining resistance patterns are disc diffusion and micro dilution methods. The dilution method is based on determining the minimum concentration (MIC) of antibiotics that inhibit the tested isolate by using agar or broth dilutions. The disc diffusion method includes the determination of zones of inhibition on a plate covered with Mueller Hinton agar seeded with the isolate being tested. The zones of inhibitions are determined at the minimum inhibitory concentration for a specific antibiotic. Standardized performance values (e.g. from Clinical Laboratory Standards Institute (CLSI), 2017) are used in the distinction between organisms that are susceptible, resistant or intermediate.

Studies have been done that involved modifications to the culture-based methods due to the laborious nature of culture-based methods. Selective media are augmented with antibiotics at varied concentrations for the enumeration of bacteria in environmental samples (Rizzo *et al*., 2013). Percentage resistance is then computed as the ratio between bacteria that grow with and without target antibiotic (Li *et al*., 2010; Novo and Manaia, 2010; Figueira *et al*., 2011). However, this addition of antibiotics to selective media has not been standardized. Mueller-Hinton agar is the ideal medium for routine antibiotic susceptibility testing because it has shown reproducibility for repeated antibiotic susceptibility and data has been collected with regard to its use in susceptibility tests (Hardy diagnostics, 1996).

Molecular methods have also been used for the examination of antibiotic resistance genes in water and wastewater. Molecular methods such as Polymerase Chain Reaction (PCR), whole-genome sequencing deoxyribonucleic microarray, metagenomics, and whole genome sequencing have been used to detect presence of microorganisms, which are fastidious (McConnell 2016). They are used on the basis that an isolate carrying a gene for resistance implies resistance. Therefore, genetic based tests focus on the finding resistant genes or genetic elements that help in the transfer of genes in bacteria. There is lack of standardization with these molecular methods for detecting resistant genes (Rizzo *et al*., 2013). However, the use of genes in molecular tests have an advantage over culture-based methods because they are more useful in epidemiological studies that trace the spread of important resistance genes in wastewater-impacted environments. This is possible because molecular methods enable the detection of genetic markers that code for antibiotic resistance as well as virulence genes in tandem. Molecular and culture-based methods can be used together for analyzing pathogens and

the genes that encode resistance in the target organism (McConnell, 2016). Figure 3 illustrates how both methods are complementary.

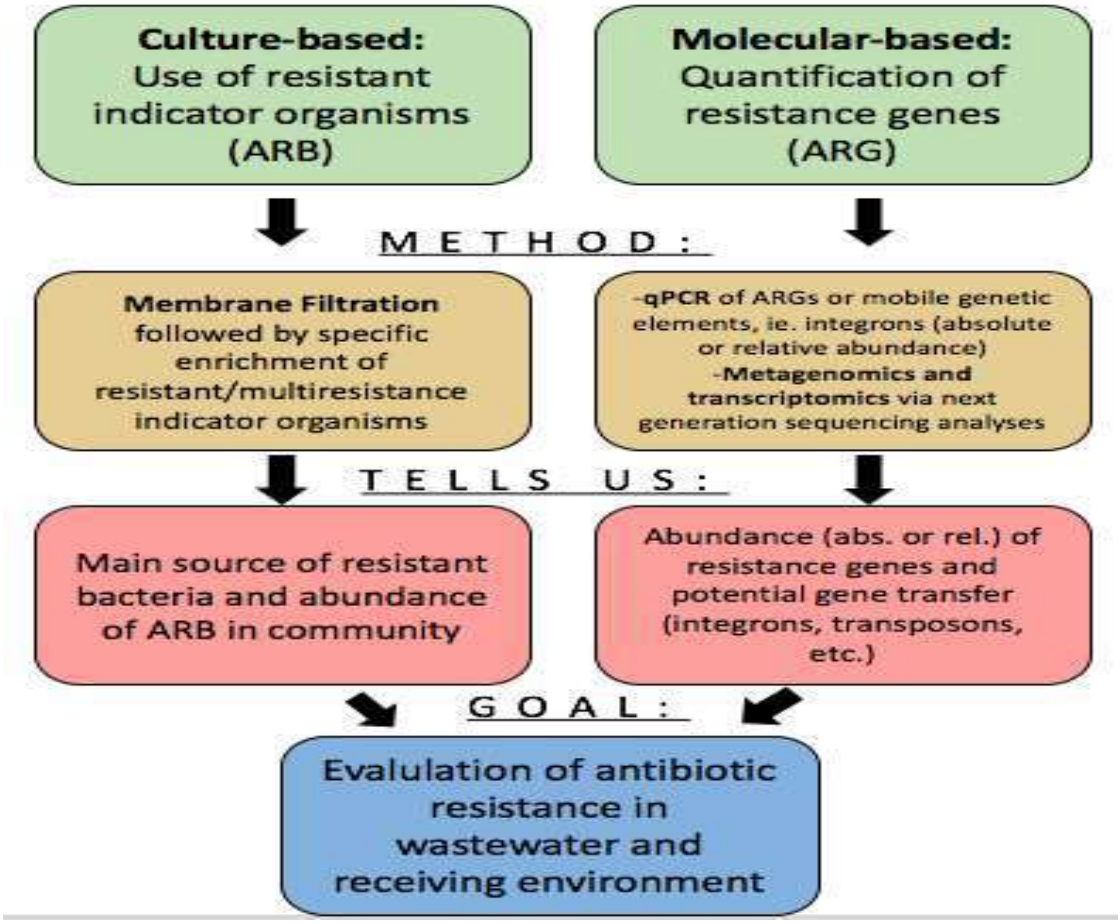

Figure 2.3: Methods used in assessing antibiotic resistance genes and antibiotic resistance bacteria present in wastewater and surface water (Adapted from McConnell, 2016).

2.9 Current Knowledge on antibiotic resistant bacteria and antibiotic resistance genes in wastewater treatment plants and surface waters.

Studies have shown that wastewater treatment plants have a significant role in the development and proliferation of antibiotic resistance, and can therefore serve as major reservoirs or hotspots (Szczepanowski *et al*., 2009; Rizzo *et al*., 2013; Luczkiewicz *et*

al., 2010; Al-Jassim, *et al*., 2015; Kim *et al*., 2016; Rafraf *et al*., 2016; Nordgård, *et al*., 2017).

Wastewater treatment plants are considered as hotspots because of the potential presence of substances like antibiotics, heavy metals and biocides which exert selective pressure on bacteria (Xi *et al*., 2009; Plosz *et al*., 2010; Michael *et al*., 2013) and the presence of other favorable microbial growth conditions (such as nutrients) during treatment. These conditions may favor horizontal transfer of resistance genes between bacteria present. Table 2.1 shows results from antibiotic resistance prevalence studies in wastewater treatment plant and the receiving environment.

Studies in antibiotic resistant bacteria have mostly been done on bacteria that are used as water quality indicators, for example coliforms and enterococci (Sabate *et al*., 2008; Figueira *et al*., 2011; Amaya *et al*., 2012). Thus, coliforms and enterococci are the most investigated bacterial groups in wastewater treatment plants. There have also been studies on resistance in human pathogens such as *Staphylococcus aureus* and other gram-negative bacteria (Thompson *et al*., 2013). Standardization of methods for testing resistance in environmental bacteria is limited compared to clinical settings that have been standardized extensively.

Currently, most studies on antibiotic resistance in have been focused on clinical settings, as such data on antibiotic resistant bacteria in wastewater and wastewater receiving environments is lacking especially in Ghana. Regulations or guidelines for the permitted or desirable maximum limits for contaminants like antibiotics, antibiotic resistant bacteria and antibiotic resistance genes that can be discharged into the environment and reused are inadequate. This study therefore seeks to help fill this knowledge gap by providing data on antibiotic resistance in treated effluent from the

Legon Sewerage Treatment Plant and the receiving water body. This will provide evidence for decision making regarding improvement in treatment options in order to reduce exposure and human health risks.

Table 2.1. Studies quantifying antibiotic resistance bacteria in urban wastewater treatment and receiving rivers

Guadeloupe	Enterobactereaceae	Third generation cephalosporin (50.4%), Ciprofloxacin (51.6%), Tetracycline (40.2%), Cotrimoxazole (34.1%) and Gentamicin (11.8%)	Guyomard-Rabenirin *et al*., 2017
Saudi Arabia	*Acinetobacter sp, Aeromonas sp, Legionella sp, Mycobacterium sp, Streptococcus sp, Neisseria sp, Pseudomonas sp, , Enterococcus sp*	Ampicillin (72%), Erythromycin (51%), Chloramphenicol (46%), Kanamycin (41%), Ceftazidime 32%), Tetracycline (21%), Meropenem (12%), Ciprofloxacin (6.6%)	Al-Jassim *et al*. (2015)
Ireland	*E. coli*	Ampicillin (12.5%), Streptomycin (10%), Sulfamethoxazole (12.5%), Tetracycline (39%), Ciprofloxacin 0%), Cefoxitin (2.6%)	Galvin *et al*., (2010)
Poland	*E. coli*	Ampicillin (34%), Piperacillin (24%), Tetracycline (23%), Levofloxacin (15%), Nitrofurantoin (53%), Ciprofloxacin (10%), Trimethoprim/Sulphamethoxazole (11%), Erythromycin (44%), Ciprofloxacin (29%), Tetracycline (20%),	Luczkiewicz *et al*. (2010)
USA	*Acinetobacter*	Trimethoprim (100%), Rifampin (77.5%), Chloramphenicol (35%), Amoxicillin clavulanate (20%), Ciprofloxacin (11.3%)	Zhang *et al*. (2009)

CHAPTER THREE

MATERIALS AND METHODS

3.1 The Study Area

The study was carried out at the Legon Sewage Treatment Plant and the receiving Onyasia Stream located near the University of Ghana Botanical garden, Legon, and Accra.

Accra is the capital city of Ghana, covering an area of 200 square kilometers with a population of about 2.5 million (World Population Review, 2020). Legon, a suburb of Accra located 12 kilometers northeast of the city center in the Accra Metropolitan District Low ridges characterize the landscape of Accra, 20 m above sea level separated by small river courses running in a southerly direction towards the sea. Accra has a gentle sloping terrain, which favors natural drainage by gravity. The Odaw River and its tributaries, the Nima, Onyasia, Dakobi and Ado Streams drain the catchment area.

Currently, conventional sewerage network covers close to 15% of Accra with areas that are not covered using public toilets, septic tanks, pit latrines and pan latrines (Mohammed *et al*, 2017). The Legon, Kotoku and Mudor Sewage Treatment plants are currently the main plants functioning in Accra,

The Legon Sewage Treatment Plant was chosen for the study because effluents from the plant are discharged directly into the Onyasia stream, which serves as an irrigation source for vegetable farms along the stream. In addition, the design of treatment plant and its operation influence the fate of resistant bacteria and resistant genes found in wastewater (Kim and Aga, 2007). Wastewater stabilization ponds have shown to enhance the transfer

of resistance genes among bacteria when compared to other methods of treatment (Neudorf *et al*., 2017). Figure 3.1 shows a map of the study area.

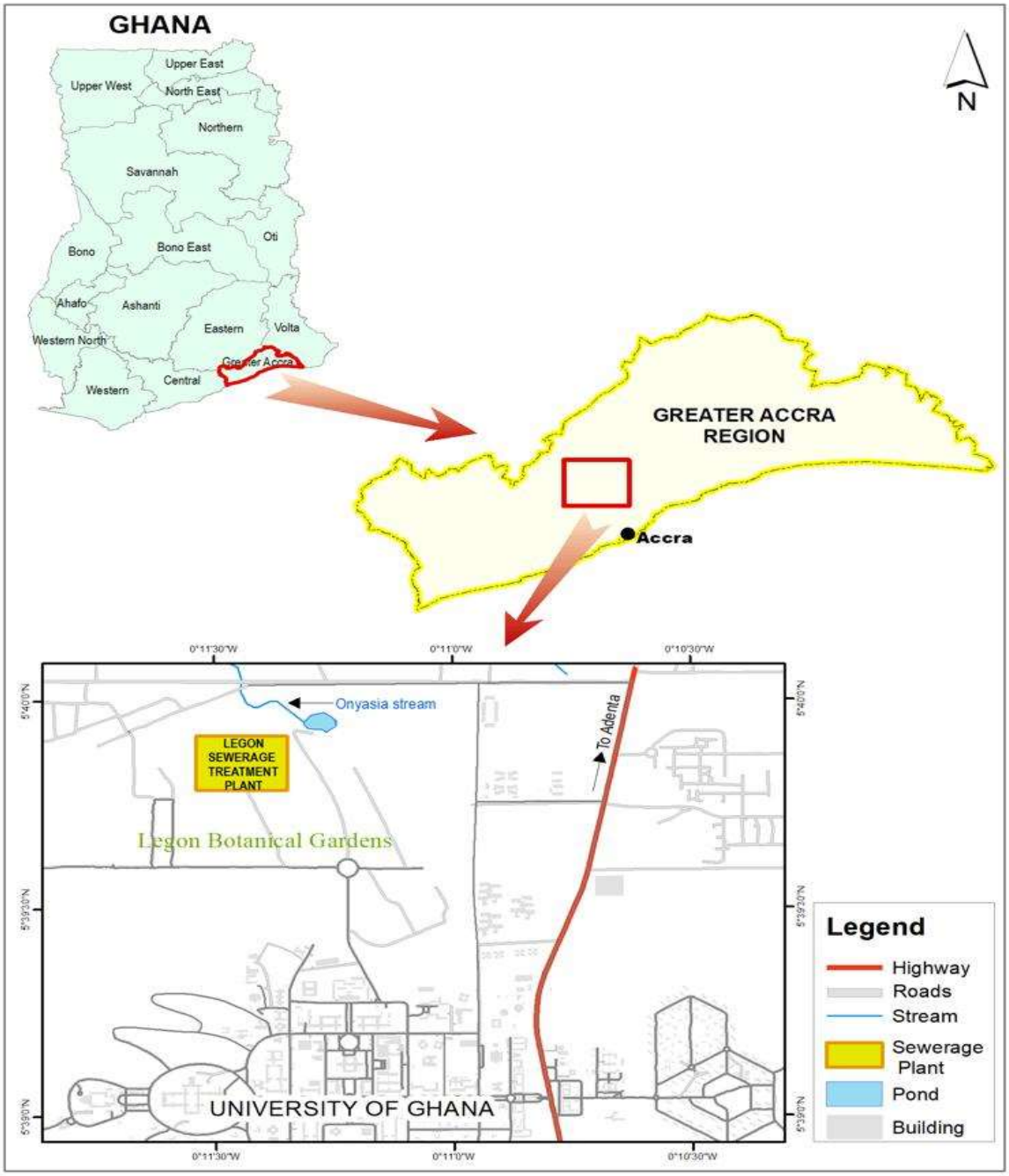

Figure 3.1. A map of study Area

3.1.1 Description of study site

Legon Sewage Treatment plant

The Legon Sewage Treatment plant was constructed and commissioned in 2012 as part of the Accra Sewerage Improvement Project (ASIP), to extend sewage treatment in order to improve sanitation and sewage treatment in Accra. The treatment plant uses waste stabilization ponds which consist of open basins including anaerobic, facultative and maturation ponds, and the effluent discharged directly into the Onyasia Stream.

The Legon Sewage Treatment Plant consists of 3 anaerobic ponds, 3 facultative ponds and 6 maturation ponds with an outlet after which the treated effluent is discharged into the Onyasia Stream.

The plant has the capacity to treat (9,000 m^3) of sewage inflow per day and was expected to serve 33,000 residents (ASIP appraisal report) but is currently operating at 3606 m^3 per day (Manager, Legon sewage treatment plant). The treatment plant treats wastewater from the University of Ghana, Presbyterian Senior High School, University of Professional Studies, Achimota Senior High School and Achimota Hospital. (African development fund, 2005).

Volumes of liquid waste from these institutions are channelled through various pumping stations into a receiving tank at the treatment plant daily. The liquid waste first goes through a wide screen which removes large particles and other non–biodegradable materials. The liquid waste then flows into a distribution chamber and from there it flows into three grit channels each connecting to a treatment stream. Primary treatment occurs in the anaerobic pond, where suspended solids and soluble parts of organic matter Biological Oxygen Demand (BOD)) are removed. The secondary treatment which occurs in the facultative pond where most of the remaining BOD is removed with the help of

heterotrophic bacteria and algae. The main function of the maturation pond which is a tertiary treatment process is to remove pathogens and nutrients. Figure 3.2 below shows a schematic diagram of the Legon Sewage Treatment plant with the different ponds.

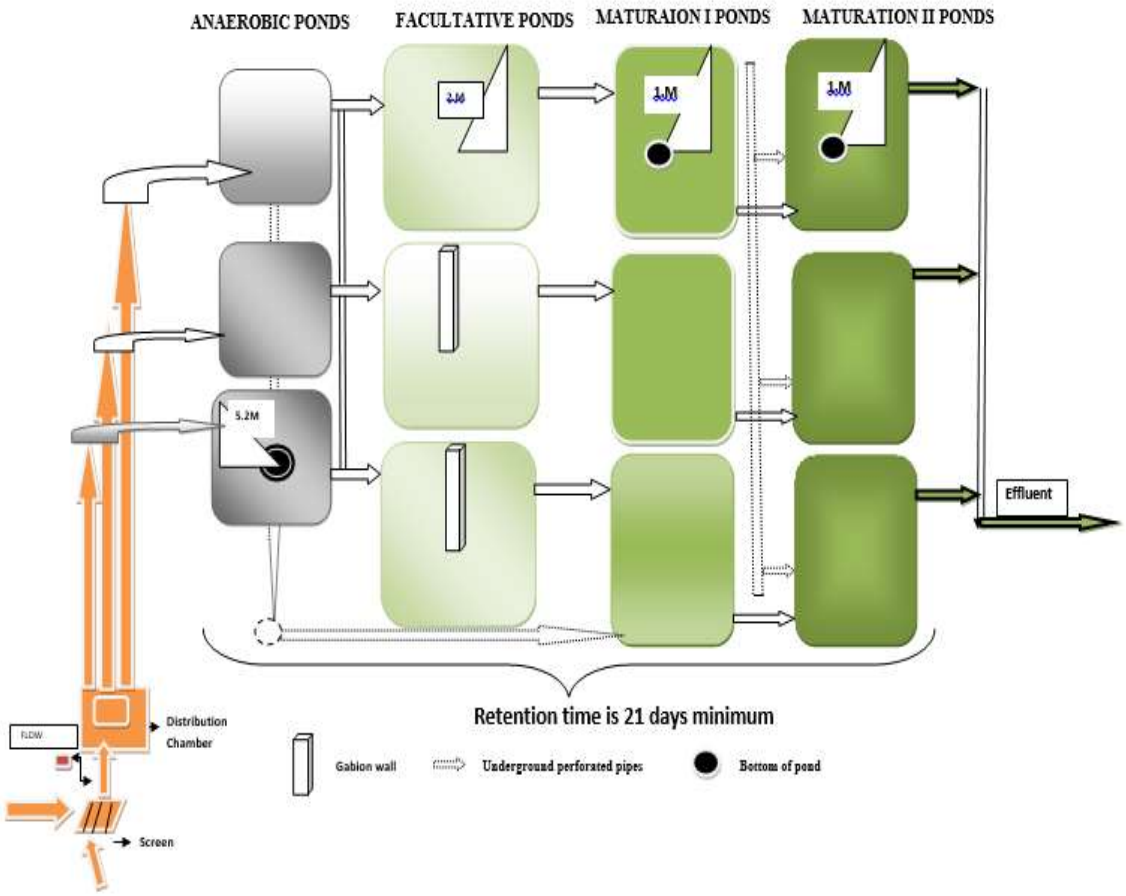

Figure 3.2: Schematic representation of the Legon Sewage treatment plant. (Source Legon Sewage Treatment Plant)

Onyasia Stream

Effluent from the Legon Sewage Treatment Plant is discharged into the Onyasia Stream. The Onyasia Stream runs within the Odaw catchment, stretching from Akuapem Mountains into the sea at Korle Gonno.

The Onyasia stream serves as a source of irrigation water for nearby vegetable farms. Human activities such as farming, settlements, dumping of refuse, littering has resulted in the pollution of the stream.

3.2 Study design

The study assessed the extent to which the Legon Sewage Treatment Plant contributes to the increase of antibiotic resistance in the Onyasia stream. The study employed a descriptive method that involved the assessment of antibiotic resistant *E. coli*, *P. aeruginosa* and *A. hydrophila* in wastewater and the receiving stream over a six-month period.

Permission was sought from the manager of the Legon Sewage Treatment Plant prior to data collection. The study involved collection of wastewater from two points in the Legon Sewage Treatment Plant influent i.e. where the plant receives wastewater from the University of Ghana, Achimota Senior High School catchment area and effluent i.e. treated water in the final maturation point that discharges into the Onyasia stream.

Three points along the Onyasia stream course were selected relative to the treatment plant discharge point; outfall i.e. the point of wastewater discharge into the stream, upstream i.e. 500 meters before the treatment plant discharge point (outfall) and downstream i.e. 500 meters down the treatment plant discharge point).

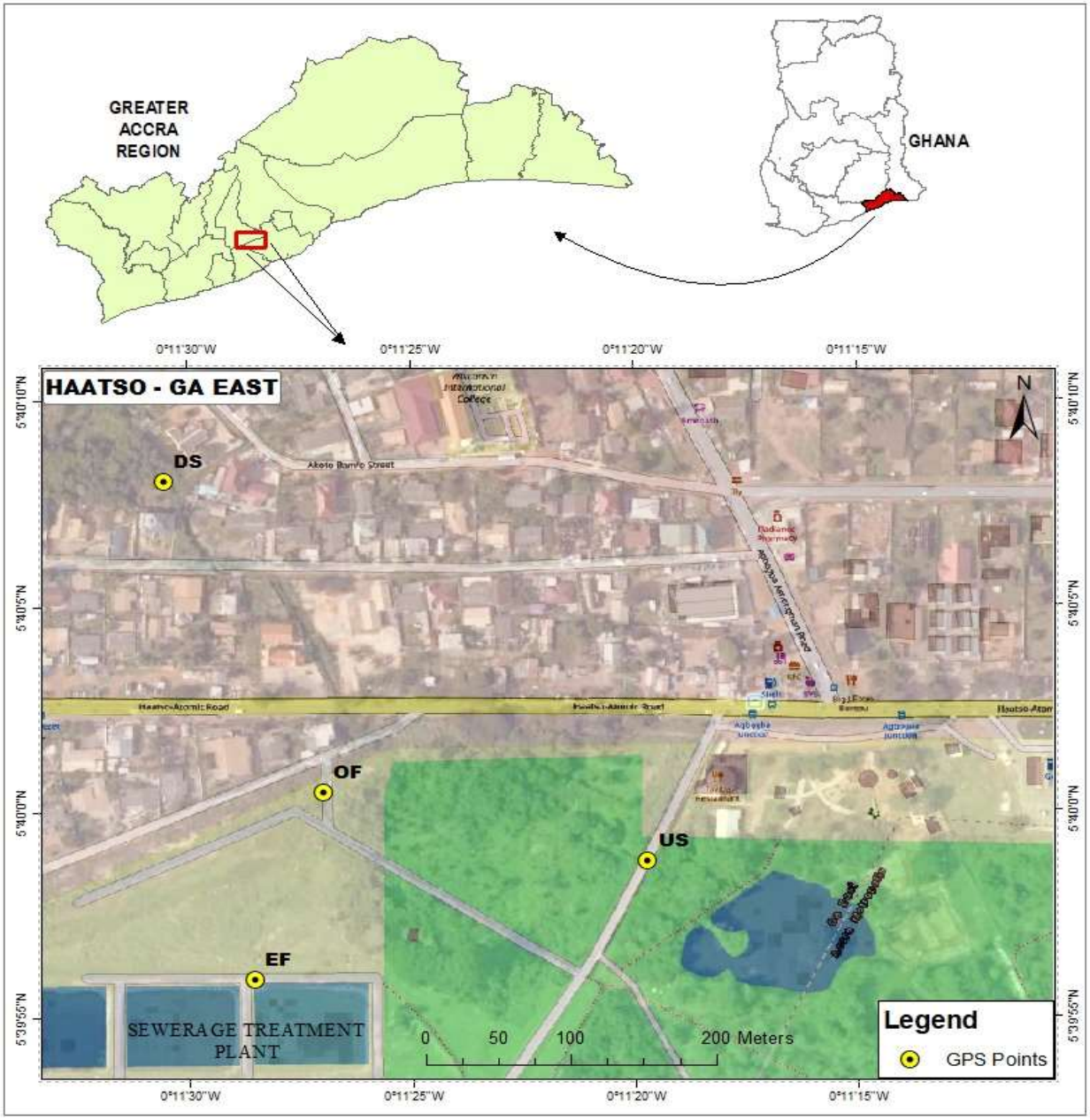

Figure 3.3: Map showing sampling points

EF: Effluent**, US:** Upstream**; OF:** Outfall**; DS:** Downstream

E. coli, *A. hydrophila* and *P. aeruginosa* are the organisms chosen for this study. These bacteria were selected because they are pathogens that cause diseases in humans. The choice of bacteria is based on the fact that they are pathogens which cause human diseases

and their resistance to antibiotics can pose a serious health threat to humans, also WHO listed *P. aeruginosa* and *Enterobactereaceae* some of the priority pathogens for antibiotic resistance research (Shrivastava *et al*., 2018). Surveillance of *A. hydrophila* in wastewater is also important due to their ubiquity in aquatic environments making it able for other bacteria to transfer resistance to this organism.

Culture-dependent methods were used to quantify *E. coli, A. hydrophila and P. aeruginosa* resistant to selected antibiotics.

3.3 Field data collection

Prior to sampling a transect walk was carried out in December 2017 in order to observe activities along the course of Onyasia stream.

Sampling was done over a 6-month period between January 2018 and June 2018. Both wastewater influent and effluent were sampled. Wastewater and water sampling was done between 8am to 9 am and immediately transported to the laboratory for further analysis. All the samples were collected in triplicate and pooled together as one composite sample per sampling location. Sampling site, location, and the Global Position System (GPS) readings of the locations are presented in appendix VII.

All sampling procedure followed standard protocols in the Standard Methods for Examination of Water and Wastewater (APHA, 2012). Below is a detailed description of how samples were collected.

3.3.1 Wastewater collection from treatment plant

Influent and effluent samples from the treatment plant were collected aseptically in 500 millilitres (ml) Whirl Pak bags (eNasco, Fort Atkinson, WI) with a sampling pole. Influent and final effluent wastewater samples were collected from 0.2-0.3meters (m) below the surface. Samples were collected in triplicate from influent and mixed as a composite sample in a sterile bottle. Effluent samples were also collected in triplicate mixed as a composite sample in a sterile bottle. Samples were kept on ice and transported in a cooler box to the CSIR-Water Research Institute Microbiology Laboratory for analyses. All samples were analysed within 24 hours of sample collection.

3.3.2 Surface water collection from Onyasia stream

Upstream, outfall and downstream water samples were taken 0.2-0.3m below the surface aseptically with a sampling pole into sterile 500ml bottles. Samples were collected in triplicate from each sampling location and mixed as a composite sample per sampling site in a sterile bottle. Samples were kept on ice transported in a cooler box to the CSIR-Water Research Institute Microbiology Laboratory for analyses. All samples were analysed within 24 hours of sample collection.

3.4 Laboratory analysis

Laboratory analyses were conducted following protocols in the Standard Methods for Examination of Water and Wastewater (APHA, 2012) and Clinical Laboratory Standards Institute Guidelines (CLSI, 2017; CLSI, 2018). Bacterial analysis of the wastewater and water samples was determined by membrane filtration method.

3.4.1 Wastewater analysis

Ten-fold serial dilutions of wastewater in phosphate-buffered saline solution was carried out. One ml of each dilution as well as 1ml undiluted influent wastewater was then filtered through a 0.45 µm filter membrane (Millipore, Billerica, MA) membrane filters by vacuum filtration. Membrane filters were put on prepared selective 47mm agar plate in sterile petri plates aseptically. Each sample was plated on *Aeromonas* agar base (OXOID) supplemented with ampicillin for the isolation of *A. hydrophila*, Cetrimide agar for isolation of *P. aeruginosa*, Chromo cult coliform agar (MERCK) for *E. coli,* and total coliforms and M-FC agar for faecal coliforms. Inoculated plates were incubated for 24 hours at 37°C for *A. hydrophila* , *P. aeruginosa, E. coli,* total coliforms and 44°C for faecal coliforms.

Following incubation, characteristic colonies that grew on the plates were counted and recorded. Colonies were identified as dark green colonies with opaque centers and translucent peripheries for *A. hydrophila,* green fluorescing colonies for *P. aeruginosa* and blue colonies for *E. coli* and counted as colony forming units (CFU) per 100ml of the water sample. Counts were recorded as mean values.

Five presumed *A. hydrophila*, *P. aeruginosa*, and *E. coli* colonies for each of the five sampling site sets were randomly selected and streaked by means of a sterile wire loop over nutrient agar for further purification. Isolates were sub cultured thrice and the purity confirmed by microscopy. Pure colonies were transferred onto Tryptic Soy agar (DIFCO) slants for further identification and antibiotic sensitivity testing.

Physical and biochemical confirmatory tests such as gram reaction, catalase, indole, citrate, oxidase, triple sugar iron, glucose fermentation and sulphide indole motility were carried out to confirm isolates according to procedures outlined in Cheesbrough (2005).

3.4.2 Surface water analysis

Ten-fold serial dilutions of surface water in phosphate-buffered saline solution was carried out. One ml of each dilution and well as 1ml and 10ml undiluted each of the undiluted surface water samples were separately filtered through gridded 0.45 μm (Millipore, Billerica, MA) membrane filters by vacuum filtration. Membrane filtration procedure was used to analyse surface water as described in section 3.4.2. Isolation and confirmatory tests were carried out for surface water as outlined in section 3.4.2.

3.4.4 Physical confirmatory test

Gram reaction test was carried out for all isolates. A smear was prepared by placing a loop full of bacteria isolate on a slide applying heat. The slide was flooded with crystal violet for 1 minute. The slide was gently rinsed under an indirect stream of tap water for 2 seconds. Iodine was added to the slide for 1 minute and rinsed. The slide was then flooded with Ethyl alcohol (95%) for 15 seconds to decolorize the smear and flooded with a counter stain, safranin for 30 seconds. The slide was washed and dried with absorbent paper and then observed under a bright field microscope (Nikon eclipse 90i microscope) to determine to determine the morphology of cells.

3.4.5 Biochemical confirmatory tests

Biochemical tests were carried out on 24-hour culture plates, including oxidase and catalase for *A. hydrophila* and *P. aeruginosa* motility, citrate and glucose fermentation for *A. hydrophila*, Indole, and Triple Sugar Iron agar tests for *E. coli*.

3.4.5.1 Catalase test

A loopful of bacteria isolate was placed on a dry slide. A drop of hydrogen peroxide solution (3%) was put on the culture. Rapid bubble formation after 5-10 seconds indicated positive for catalase test.

3.4.5.2 Indole test

A loopful of bacteria isolate was inoculated into 4 millilitres (ml) of tryptone water and incubated for 24 hours at 37°C. A drop of Kovacs reagent was added after incubation and observed after 20 minutes. A pink to red ring formation indicated a positive indole test.

3.4.5.3 Citrate test

A tests tube with 4 ml of Simmons Citrate agar was lightly inoculated by touching the tip of a straight wire loop to a 24 hour bacteria culture and incubated at 37°C for a period of 24 hours. Blue colour formation indicated a citrate positive and green colour citrate negative.

3.4.5.4 Oxidase test

Bacteria isolates were smeared on filter papers impregnated with oxidase reagent 1% (w/v) aqueous solution of oxidase reagent (tetramethyl-p phenylenediamine). Oxidase positive isolates were observed by the formation of a purple colour within 5 to 10 seconds.

3.4.5.5 Sulphide indole motility test

A test tube with 5ml Sulphide Indole Motility (SIM) Medium was inoculated by stabbing the centre of the medium to a depth of half an inch. The test tube was incubated for 24 hours at 35°C. Hydrogen sulphide (H_2S) production and motility was observed. After H_2S and motility reaction was observed three drops of Kovac's reagent was added to the solution in the test tube. Motility is seen as a diffused zone of growth arising from the inoculation line. The formation of a pink to red band ring on the top of the SIM after addition of Kovac's reagent represented a positive for indole.

3.4.5.6 Glucose fermentation test

A loopful of *A. hydrophila* isolate was inoculated in a 4 ml test tube of Phenol Red Glucose broth with a Durham tube and incubated for 24 hours at 37°C. Color change and acid production was observed. Positive glucose fermentation was seen as a change in the color of broth from purple to yellow. Gas production was observed by the formation of a bubble in the inverted Durham tube.

3.4.5.7 Triple sugar iron test

A loopful *E. coli* isolate was inoculated by stabbing the butt of a 20 mL triple sugar iron test agar slant and then streaking upwards along the surface of the slant. The neck of the test tube was capped, incubated for 24 hours at 37°C and observed colour change; a yellow colouration indicated acid production due to fermentation of glucose, lactose or sucrose and a red colouration indicated alkaline production due to non-fermentation of the sugars;

glucose, lactose or sucrose on butt and slant. Hydrogen Sulphide production was observed as black precipitate on the medium. (Cheesbrough, 2005).

3.4.6 Antibiotic sensitivity testing

The antibiotic susceptibility test was carried out on five biochemically confirmed isolates taken randomly for each sample using the Kirby Bauer Disc Diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar as recommended by Clinical Laboratory Standards Institute guidelines (CLSI 2017; CLSI, 2018). Antibiotics used for antibiotic susceptibility test were selected based on CLSI recommendations (CLSI, 2017; CLSI, 2018). Antibiotics tested represent seven different classes of antibiotics including β lactams (Amoxicillin clavulanate), Monobactams (Aztreonam), Carbapenems (Imipenem), Aminoglycosides (Gentamicin), Tetracyclines (Tetracycline) and Quinolones (Ciprofloxacin), Cephalosporin (Cefuroxime and Ceftazidime). These antibiotics also constitute the major classes of drugs commonly used in the treatment of infections. Mueller Hinton agar was prepared according to manufacturer's instructions and sterilized at 121°C for 15 minutes at 1 bar pressure in an autoclave. Thirty-one mL of Mueller Hinton Agar (MHA) was aseptically dispensed into 100mm plastic petri dishes and made to dry under a laminar flow hood.

Cell suspensions of bacteria were prepared by transferring twenty-four hour colonies of each bacteria isolate into tubes with 5 ml of sterile saline (0.85%) water and turbidity adjusted to correspond to 0.5 McFarland standard. Within 15 minutes of inoculation, the bacterial suspension was then streaked on Mueller Hinton agar plates by means of a sterile cotton swab. Antibiotic discs for Gentamicin, Amoxicillin clavulanate, Tetracycline, Ciprofloxacin, Imipenem, Cefuroxime, Ceftazidime and Aztreonam (OXOID) were placed

on inoculated plates with a sterile forcep. Inoculated Mueller Hinton plates were incubated for 16 hours at 35°C.

Following incubation, a ruler was used to measure the zones of inhibition diameters. To determine the response i.e. resistant, intermediate resistance or susceptible of each isolate to test antibiotics, measured zone of inhibition were compared with CLSI zone diameter interpretive charts. The percentage for an antibiotic resistance prevalence was calculated as the proportion of the number of strains resistant to the antibiotic over the total number of strains tested, multiplied by 100.

3.4.6.1 Multiple resistance patterns

Multiple antibiotic resistance may be regulated by multiple resistance mechanisms in both related an unrelated strain (Hayford, 2016). Isolate resistance to different antibiotic groups were identified. Binomial resistance values, which was determined by the number of responses of each isolate, were tailed and sorted into five groups, which were isolates resistant to 0, 1, 2, 3, or ≥4 antibiotics.

3.5 Quality assurance

All samples collected for laboratory analysis were transported to the laboratory on ice on the day of collection and analysed within 4 hours while observing strict aseptic techniques. Negative control was done by plating 0.1ml of sterile distilled water and incubated concurrently with cultural samples. This was done to ensure bacteria loads recovered from samples were not influenced by laboratory conditions.

Reference organisms *P. aeruginosa* ATCC 29213 and *E. coli* ATCC 25922 were used as controls in accordance with CLSI guidelines to ensure that antibiotic disc diffusion process was consistent. Positive control was done by plating spiked reference *P. aeruginosa* ATCC 27853 and *E. coli* 25922 strains in sterile distilled water to test the efficacy of bacteriological media.

Antibiotics were stored at -20°C for long term storage and tested for potency using reference *P. aeruginosa* ATCC 27853 and *E. coli* 25922 strains.

All glassware used for bacteriological analyses were sterilized in a hot-air oven for 2hours at 170°C. All bacteriological media, Phosphate Buffered Saline, saline and pipette tips were sterilized in an autoclave at a temperature of 121°C and pressure of 1 bar for 15 min.

All wire loops and forceps were flamed with a Bunsen burner before and after usage. The neck of media bottles was also flamed before and after dispensing. All laboratory analyses were conducted in a laminar flow hood to ensure aseptic environment. Seventy percent ethanol and ten percent bleach used to disinfect work areas before and after use. Gloves were worn for all laboratory procedures.

3.6 Data analysis

Data processing was carried out with Statistical Package for Social Science (SPSS. Chicago, Illinois, USA) Version 23 and Microsoft Excel and analysed using Kruskal-Wallis One-way Analysis of Variance (ANOVA).

The differences in bacterial levels across sampling sites as well as differences across months were determined using the Kruskal-Wallis one-way ANOVA with significance level set at $P < 0.05$.

Bacterial isolate responses to antibiotic susceptibility disc diffusion were categorized as either susceptible or resistant i.e. resistant and intermediate resistance and assigned a binary value for each response: 0 for susceptible and 1 for resistant. Isolates and isolate responses were then grouped and significant associations by Chi-square analysis at significance level, $p<0.05$ with 95% confidence interval was determined. Percentage resistance to antibiotics was calculated and results presented in tabular and graphical forms.

CHAPTER FOUR

RESULTS

4.1 Occurrence of total coliforms, *E. coli*, *A. hydrophila*, *P. aeruginosa* in treatment plant

All *E. coli* isolates were confirmed as Gram-negative rods after performing gram reaction test. All isolates tested were positive for indole and triple sugar iron test.

All *Aeromonas hydrophila* isolates were confirmed as Gram-negative rods after performing gram reaction test. All isolates tested were positive for glucose fermentation, motility and citrate tests. Isolates were negative for oxidase test.

All *Pseudomonas aeruginosa* isolates were seen as pigmented greenish colonies with fluorescence when cultured on Cetrimide agar. *Pseudomonas aeruginosa* strains were identified as Gram-negative rods. All Pseudomonas *aeruginosa* isolates were oxidase positive. *E. coli*, *A. hydrophila* and *P. aeruginosa* were present in all wastewater and stream water samples. Table 4.1 presents level of *E. coli*, *A. hydrophila*, *P. aeruginosa* in the treatment plant. Mean count of *E. coli* in the treatment plant over the study period was 7.88 $\log_{10}$ CFU/100ml in influent and 2.58 $\log_{10}$ CFU/100ml in final effluent wastewater. Mean count of *A. hydrophila* was 5.57 $\log_{10}$ CFU /ml in influent and 3.90 $\log_{10}$ CFU /100ml in effluent. Mean count for *P. aeruginosa* was 6.44 $\log_{10}$ CFU/100ml and 3.17 $\log_{10}$ CFU/100ml for influent and effluent wastewater respectively. Results show the mean abundance of bacteria decreased after treatment (effluent). Figures 4.1, 4.2 and 4.3 show bar chats of monthly *E. coli*, *A. hydrophila* and *P. aeruginosa* counts in the treatment plant.

Kruskal-Wallis statistical test showed significant differences between occurrence of bacteria isolated from influent and effluent wastewater during the study period.

Table 4.1 Levels of *P. aeruginosa, E. coli,* and *A. hydrophila* in influent and effluent wastewater

	Mean Log $_{10}$ CFU/100ml of bacteria					
Sample site	***E.coli***	**Standard error**	***P. aeruginosa***	**Standard error**	***A. hydrophilla***	**Standard error**
Influent	7.88	0.17	6.44	0.24	5.57	0.03
Effluent	2.58	0.23	3.17	0.06	3.90	0.12

Table 4.2 represents the mean values of total coliform levels in both treated and untreated wastewater effluent over the period of study. The highest levels of total coliforms in influent and effluent samples was recorded in March. There was a significant decrease in mean total and faecal coliform counts in effluent after treatment (8.32 Log_{10} CFU/100ml and 7.72 Log_{10} CFU/100ml mean total coliform and faecal coliform for influent and 4.64 Log_{10} CFU/100ml and 3.22 Log_{10} CFU/100ml mean total and faecal coliform for effluent respectively). Figures 4.4 and 4.5 show bar chats of monthly levels.

Table 4.2 Mean levels of total and faecal coliforms in influent and effluent wastewater.

	Mean Log_{10} CFU/100ml of bacteria			
Sample site	**Total Coliform**	**Standard error**	**Faecal Coliform**	**Standard error**
Influent	8.32	0.09	7.92	0.20
Effluent	4.64	0.16	3.22	0.08

4.2 Occurence of *E. coli, A. hydrophila, P. aeruginosa* in Onyasia Stream

E. coli was present in both influent and effluent wastewater samples. Mean counts of *E. coli* in the Onyasia Stream were 2.83 log_{10} CFU/100ml, 3.58 log_{10} CFU/100ml and 6.02 log_{10} CFU/100ml in upstream, outfall and downstream water respectively (Table 4.3). Mean counts of *A. hydrophila* in upstream, outfall and downstream were 4.34 log_{10} CFU/100ml, 4.68 log_{10} CFU/100ml and 5.96 log_{10} CFU/ml respectively (Table 4.3). Counts for *P. aeruginosa* in upstream, outfall and downstream water samples was 3.34 log_{10} CFU/100ml, 3.68 log_{10} CFU/100ml and 5.49 log_{10} CFU/100ml respectively (Table 4.3). Results show the mean abundance of bacteria tended to increase downstream.

Kruskal-Wallis one-way ANOVA statistical test showed significant differences between mean *E. coli*, *A. hydrophila* and *P. aeruginosa* counts in upstream and downstream sampling sites.

Table 4.3 Levels of *P. aeruginosa*, *E. coli*, and *A. hydrophila* in the Onyasia stream

	Mean Log_{10} CFU/100ml of bacteria					
Sample site	***E.coli***	**Standard error**	***P. aeruginosa***	**Standard error**	***A. hydrophilla***	**Standard error**
Outfall	3.58	0.25	3.68	0.18	4.68	0.09
Upstream	2.83	0.17	3.34	0.07	4.34	0.11
Downstream	6.02	0.34	5.49	0.26	5.96	0.23

4.3 Monthly variations in bacteria within sample sites

4.3.1 Treatment plant

Figure 4.1 shows the monthly variations of *E. coli* in the treatment plant. The highest monthly level of *E. coli* in influent was 8.41 log_{10} CFU/100ml recorded in March. May recorded the lowest monthly level of *E. coli* of 7.13 log_{10} CFU/100ml. Effluent recorded the highest *E. coli* of 3.30 log_{10} CFU/100ml in February and the lowest (2.0 log_{10} CFU/100ml) in June.

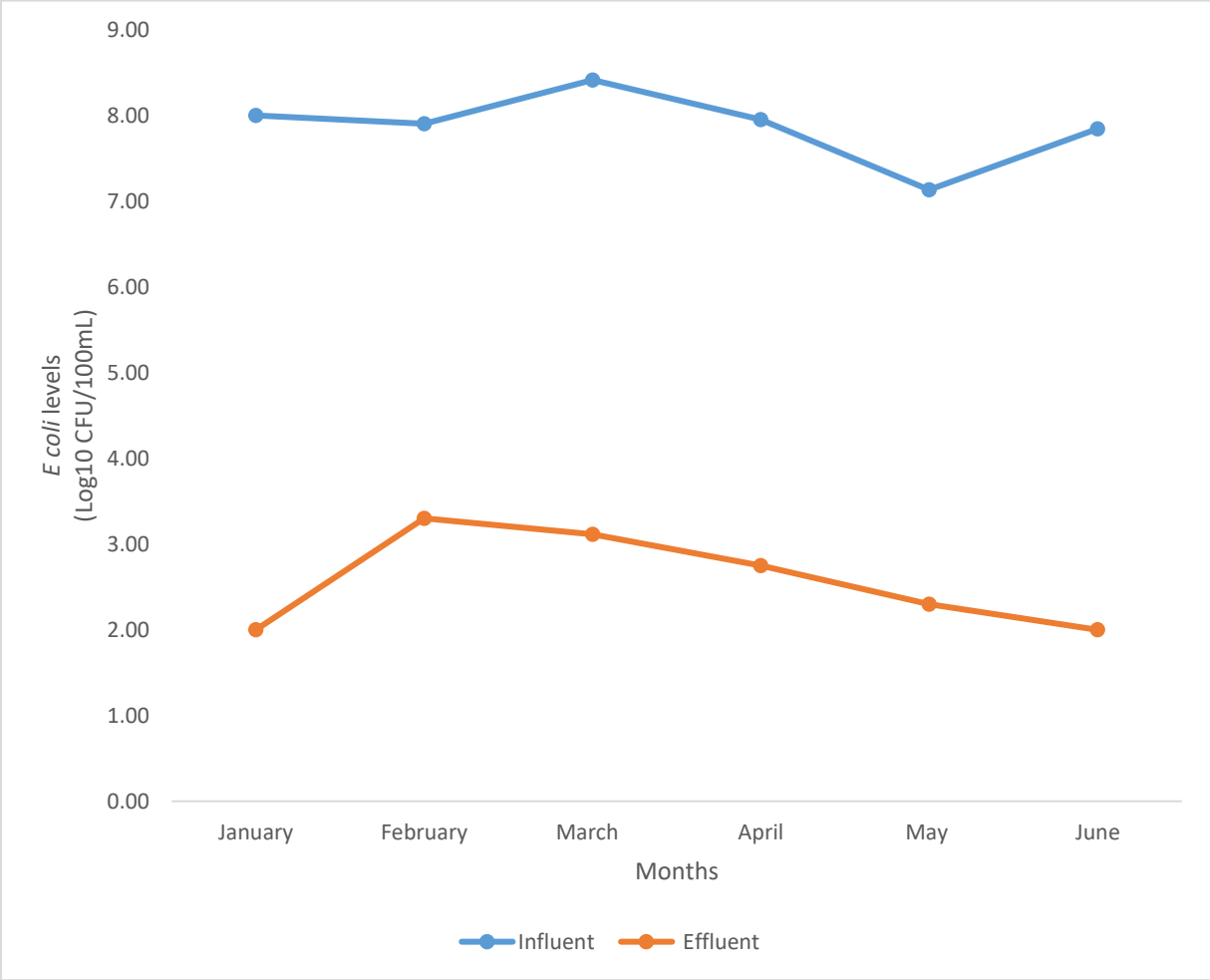

Fig 4.1: A line chart showing levels of *E. coli* in treatment plant

Figure 4.2 shows the monthly variations of *P. aeruginosa* in the treatment plant. The highest monthly level of *P. aeruginosa* in influent was 7.34 $\log_{10}$ CFU/100ml, which was recorded in January. April recorded the lowest monthly level of *P. aeruginosa* of 5.48 $\log_{10}$ CFU/100ml. Effluent recorded the highest *P. aeruginosa* of 3.30 $\log_{10}$ CFU/100ml in

February, March and April. The lowest *P. aeruginosa* count in effluent of 3.00 $\log_{10}$ CFU/100ml recorded in January.

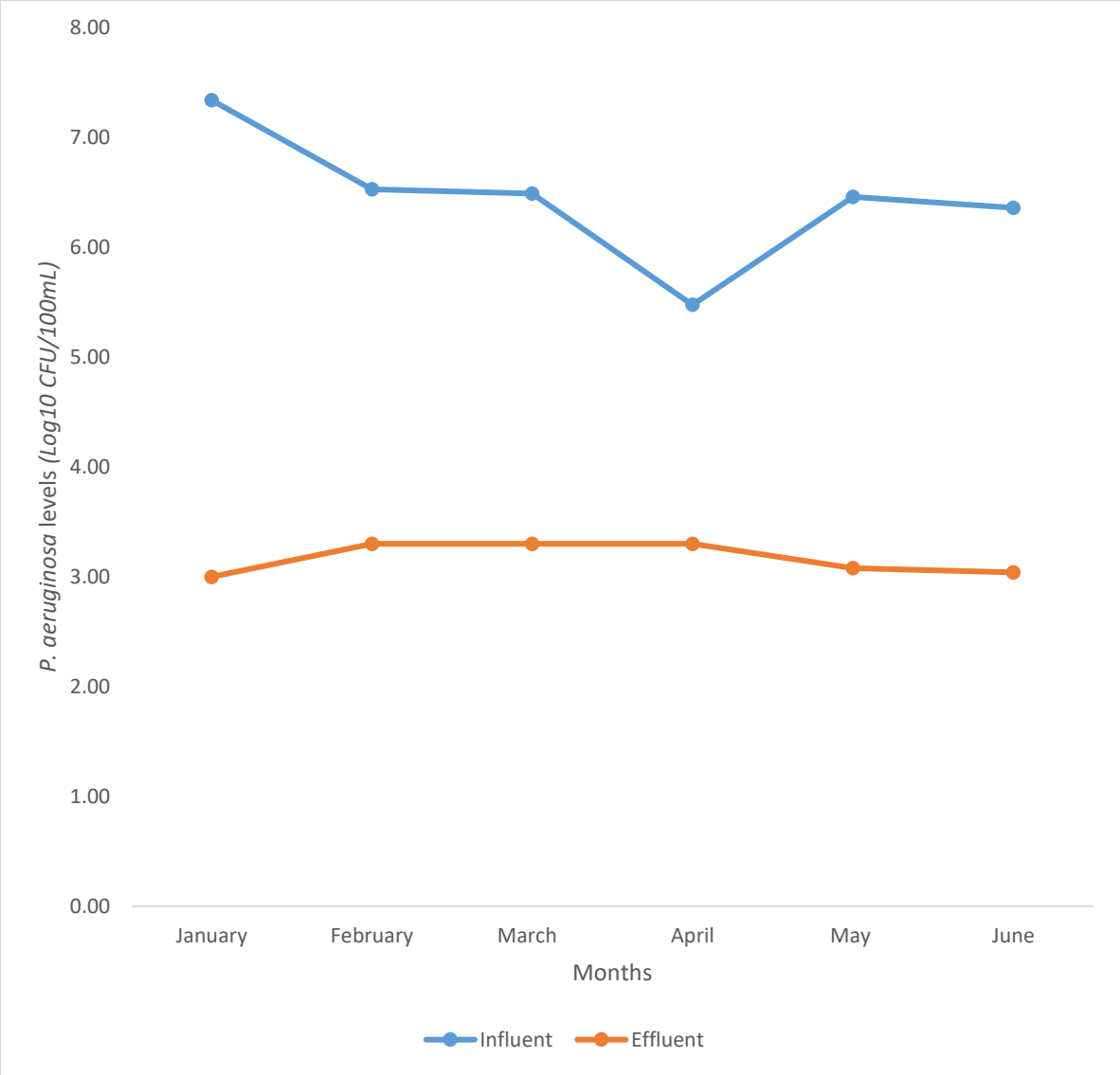

Fig 4.2: A line chart showing levels of *P. aeruginosa* in treatment plant

Figure 4.3 depicts the monthly variations of *A. hydrophila* in the treatment plant. The highest monthly level of *A. hydrophila* in influent was 5.64 $\log_{10}$ CFU/100ml, which was

recorded in April. April recorded the lowest monthly level of *A. hydrophila* of 5.43 $\log_{10}$ CFU/100ml in influent. Effluent recorded the highest *A. hydrophila* of 4.24 $\log_{10}$ CFU/100ml in February. The lowest effluent *A. hydrophila* count of 3.60 $\log_{10}$ CFU/100ml was recorded in May and June.

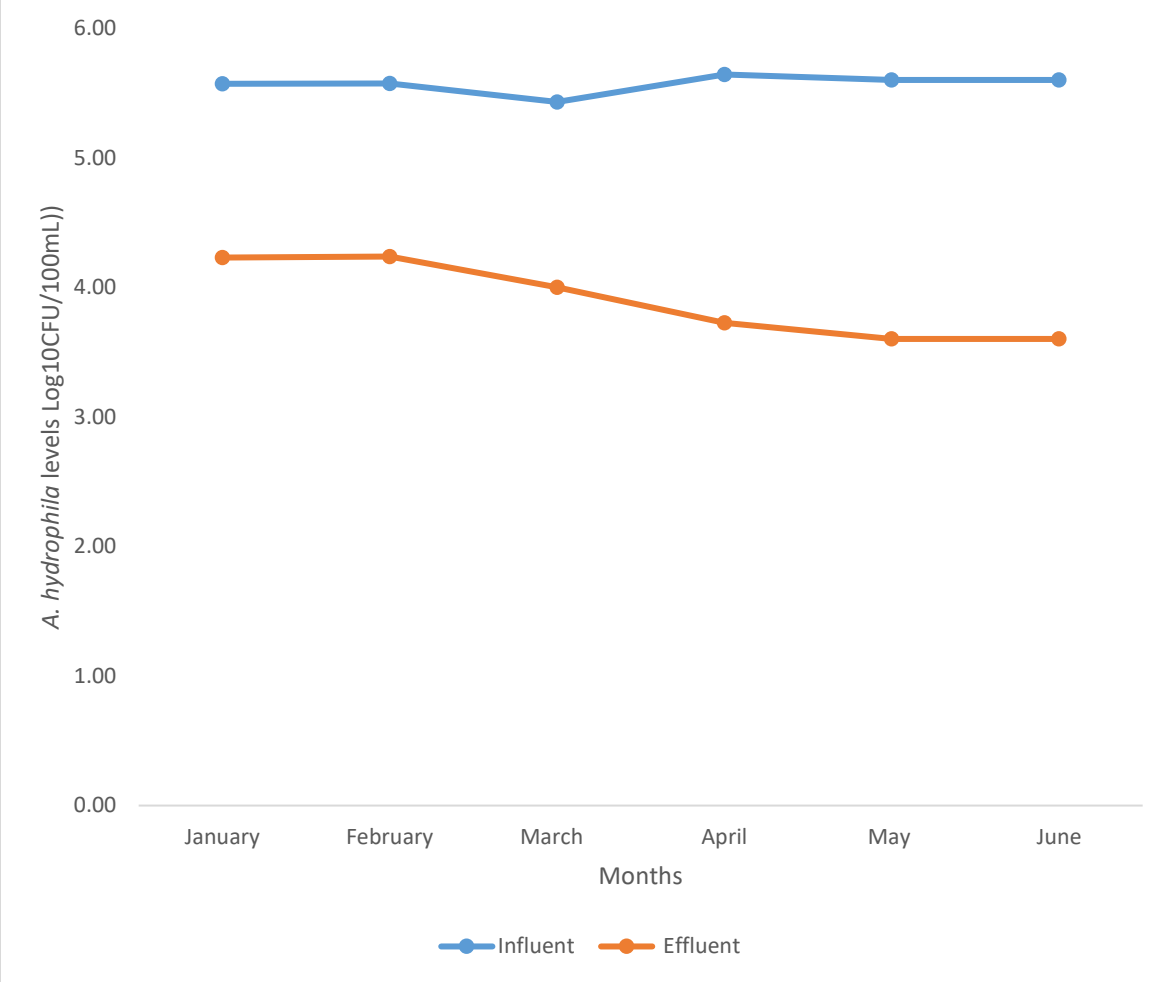

Fig 4.3: A line chart showing levels *A. hydrophila* in treatment plant.

Figure 4.4 and 4.5 depict the monthly levels of total and faecal coliforms in the treatment plant. The highest monthly level of total coliform in influent was recorded in March ($8.72\log_{10}$ CFU/100ml) and the lowest recorded in June ($8.11\log_{10}$ CFU/100ml). The highest monthly level of total coliform counts in effluent was recorded in March at $5.57\log_{10}$ CFU/100ml and the lowest recorded in June i.e. $4.23\log_{10}$ CFU/100ml. March recorded the highest faecal coliform in influent at $8.45\log_{10}$ CFU/100ml and the lowest in May at $7.20\log_{10}$ CFU/100ml. The highest faecal coliform count was recorded in effluent was recorded in February ($3.57\log_{10}$ CFU/100ml) and the lowest recorded in May ($3.00\log_{10}$ CFU/100ml /100ml).

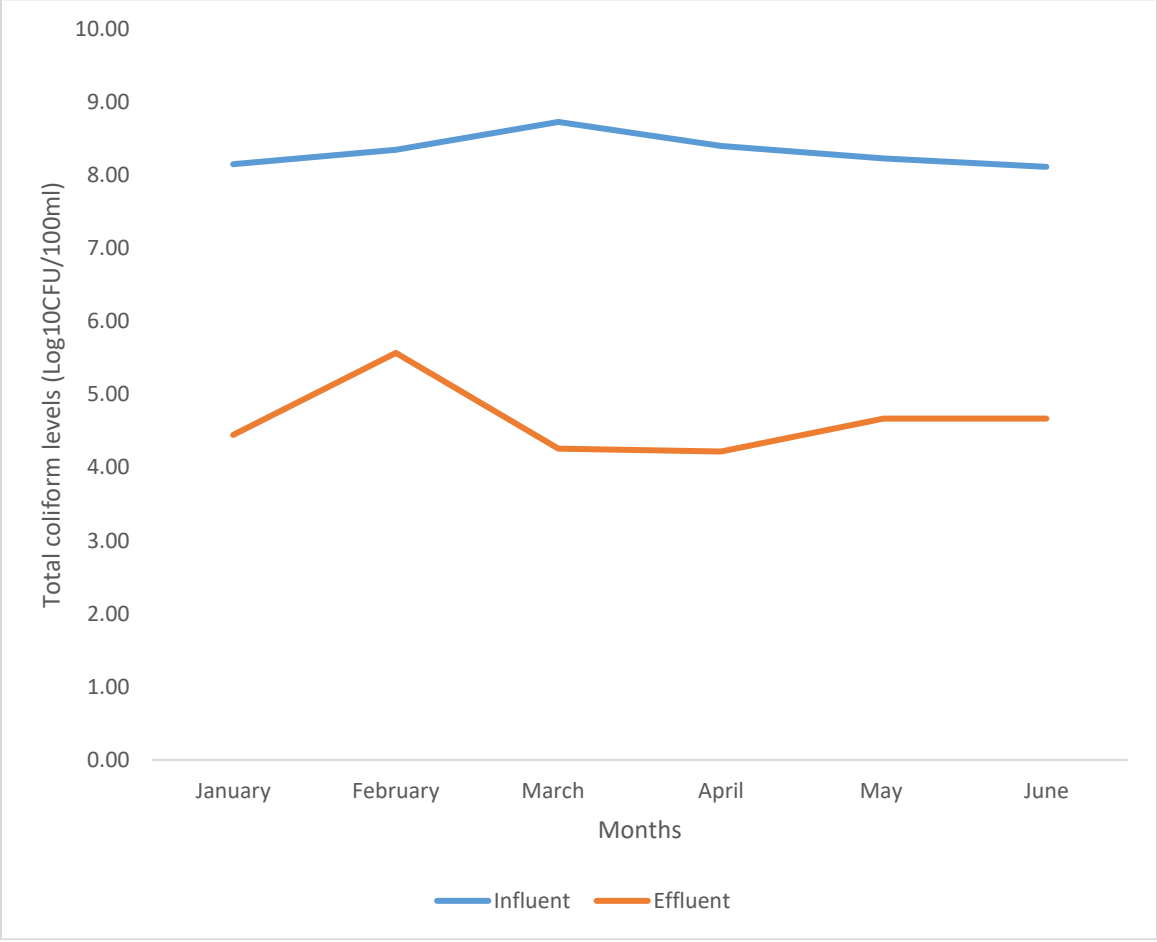

Fig 4.4: A line chart showing levels of total coliforms in treatment plant

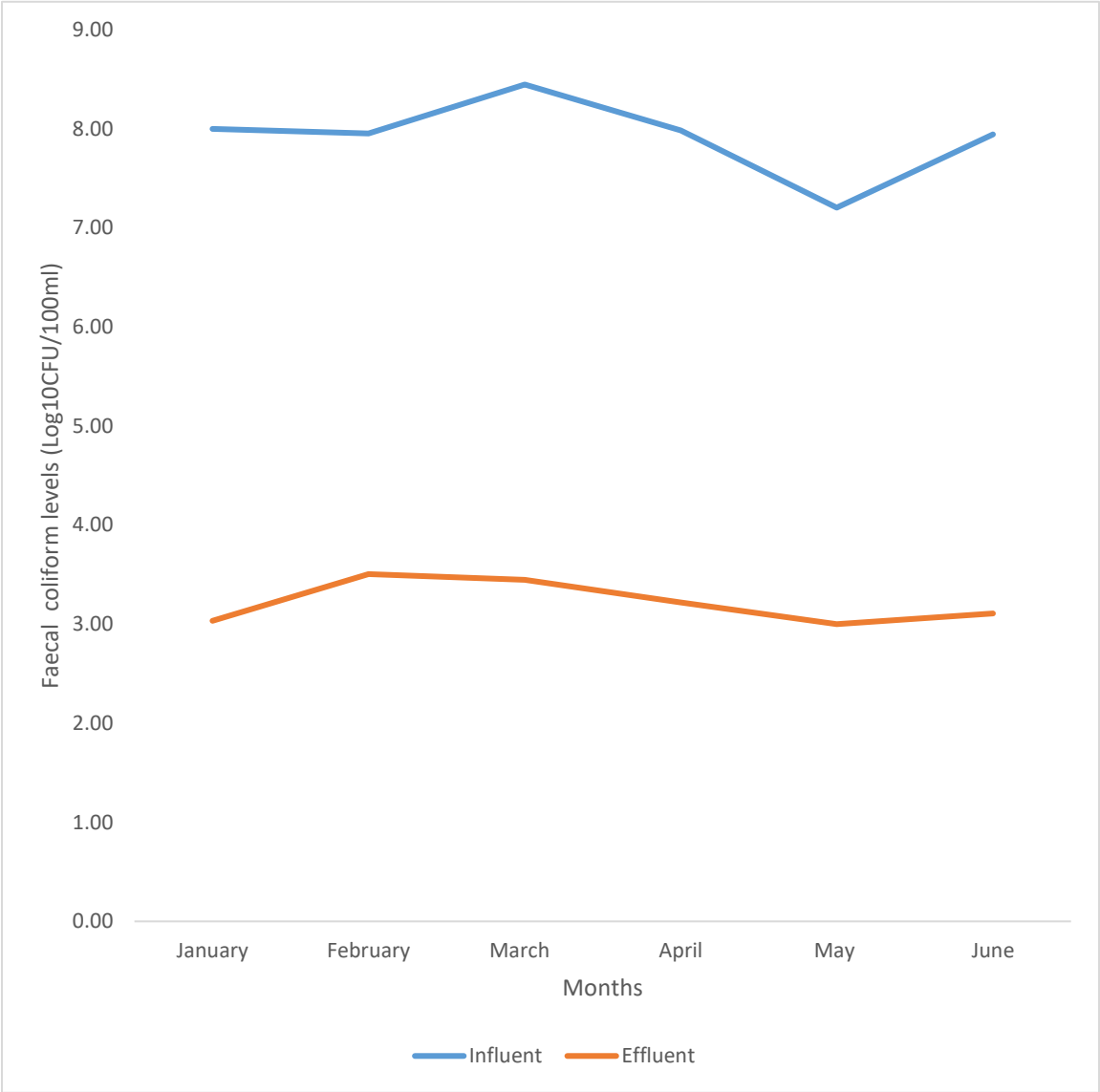

Fig 4.5: A line chart showing levels of faecal coliforms in treatment plant

Kruskal-Wallis statistical test showed that the distribution of total coliforms, faecal coliforms, *E. coli*, *P. aeruginosa* and *A. hydrophila* over months in both influent and effluent was not significantly different at $p>0.05$.

4.3.2 Onyasia Stream

Figure 4.6 shows the monthly variations of *E. coli* in the Onyasia Stream. The highest monthly level of *E. coli* was 6.9 $\log_{10}$ CFU/100ml, which was recorded downstream in March. The lowest monthly level of *E. coli* was 2.30 $\log_{10}$ CFU/100ml, which was recorded upstream in June.

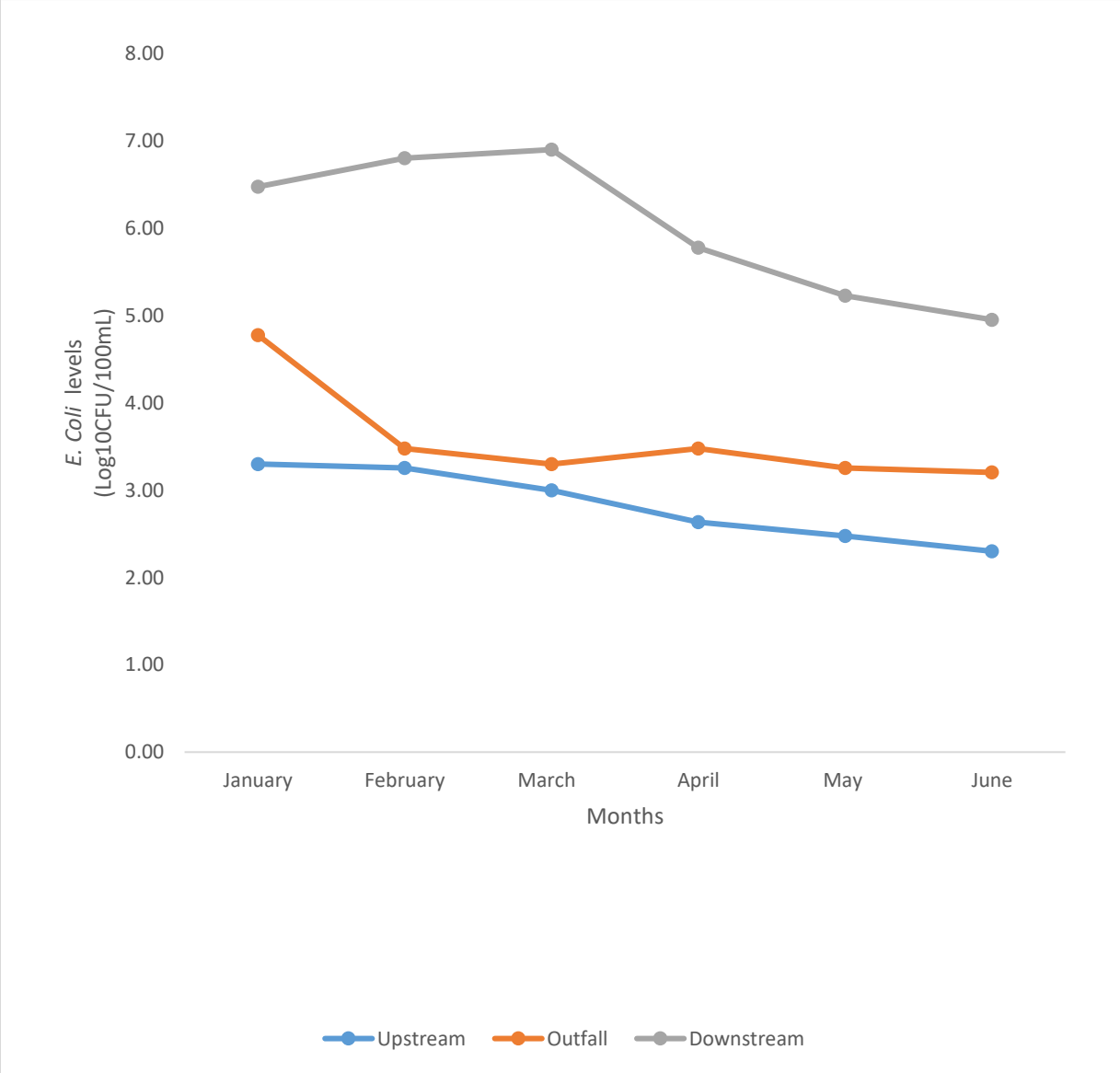

Fig 4.6: A line chart showing levels *E. coli* in Onyasia Stream

Figure 4.7 depicts the monthly variations of *A. hydrophila* in the Onyasia stream water samples. The highest monthly level of *A. hydrophila* was 6.97 $\log_{10}$ CFU/100ml, which was recorded downstream in February. The lowest monthly level of *A. hydrophila* was 3.88 $\log_{10}$ CFU/100ml, which was recorded upstream in June.

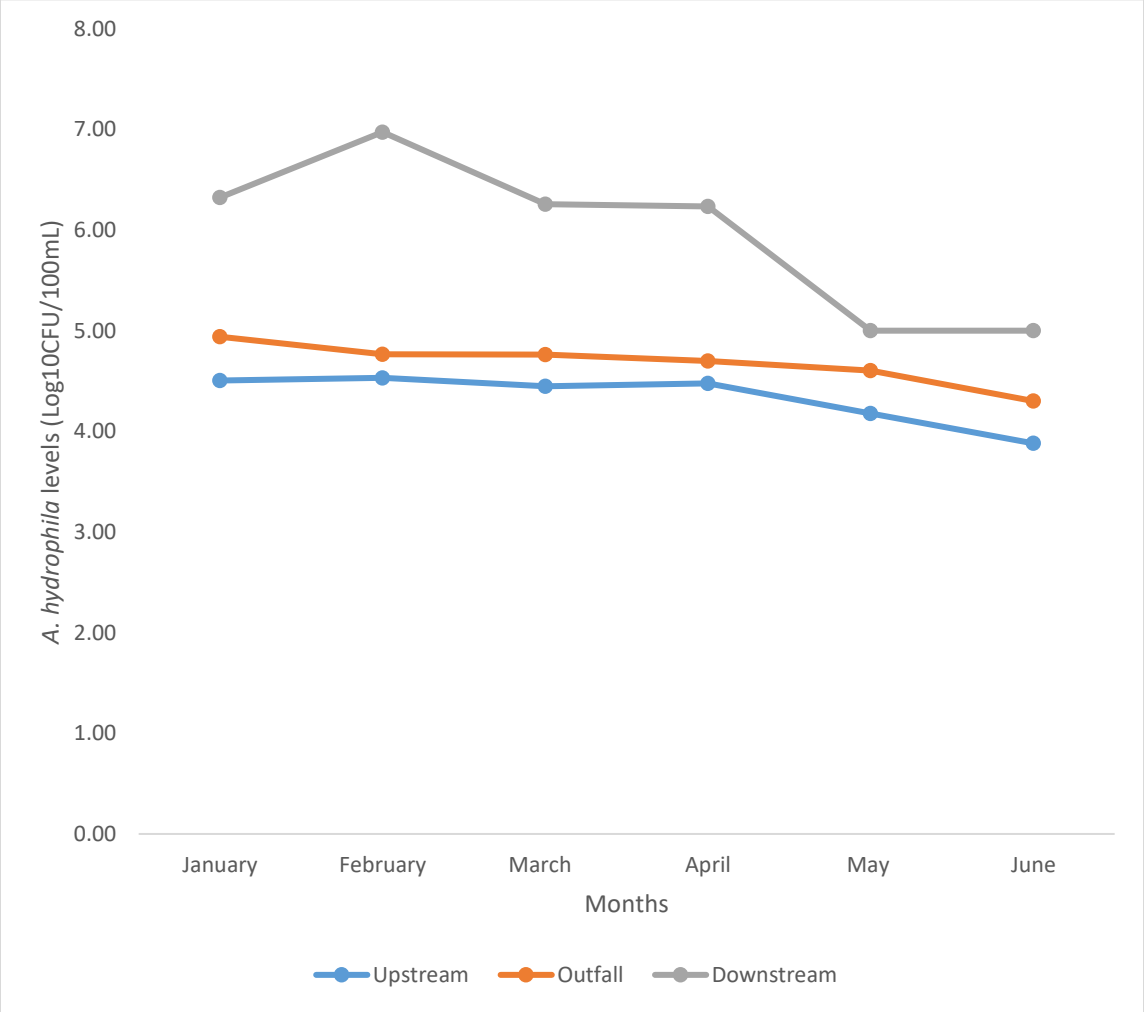

Fig 4.7: A line chart showing levels *A. hydrophila* in Onyasia Stream

Figure 4.8 depicts the monthly variations of *P. aeruginosa* in the Onyasia stream water samples. The highest monthly level of *P. aeruginosa* was 6.20 $\log_{10}$ CFU/100ml, which was

recorded downstream in January. The lowest monthly level of *P. aeruginosa* was 3.11 $\log_{10}$ CFU/100ml, which was recorded upstream in June.

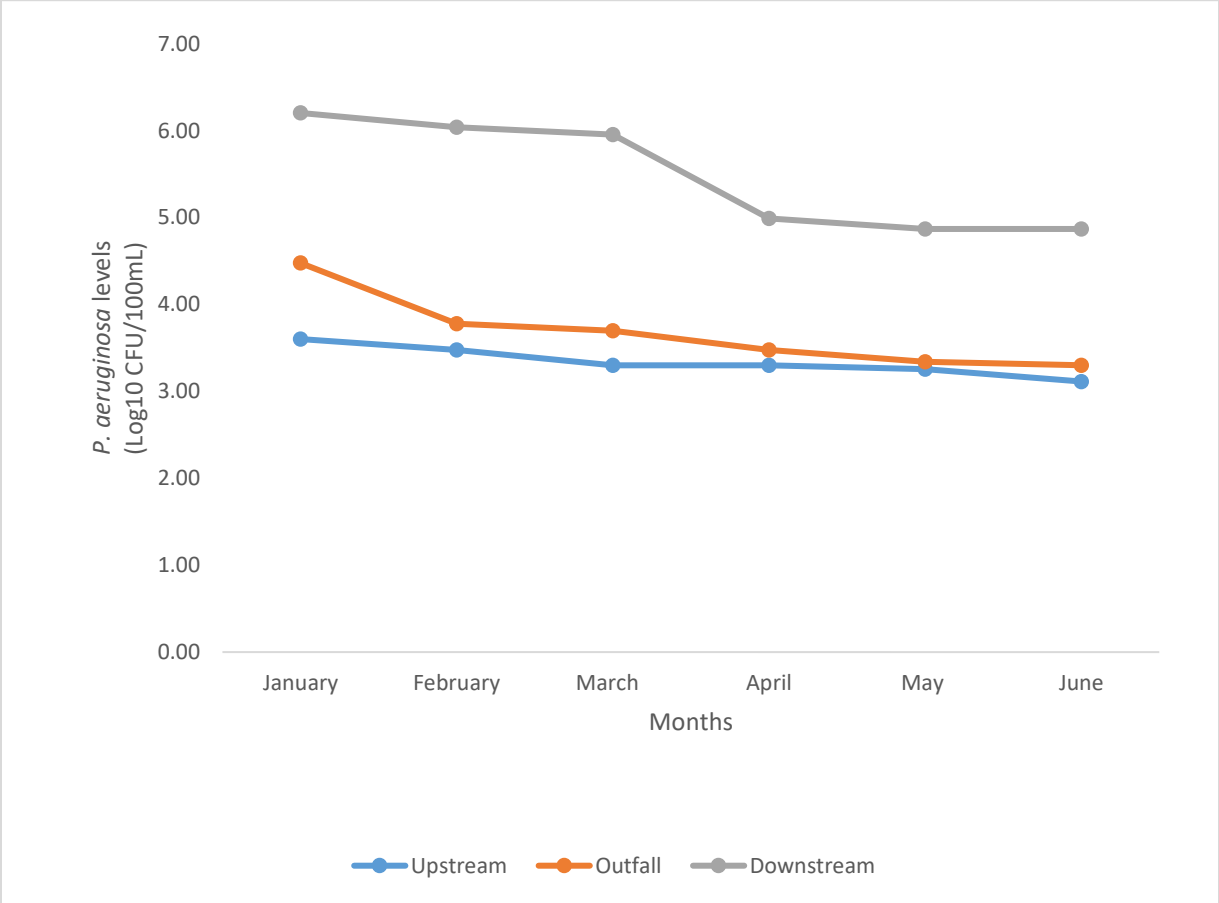

Fig 4.8: A line chart showing levels of *P. aeruginosa* in Onyasia Stream

Kruskal-Wallis statistical test showed that distribution of *E. coli, P. aeruginosa and A. hydrophila* levels across months was not significantly different at $p>0.05$.

4.4 Field observations

Prior to water quality assessment for antibiotic resistance, a field observation exercise was conducted along the environs of the Onyasia stream to observe and human activities that may influence the occurrence of anti-biotic resistance. The survey revealed vegetable farms

along the Onyasia stream course beyond the downstream sampling point which abstract water directly from the stream for irrigation. Plates 1 to 3 below are pictures of some of these observations

Plate 1: A photograph showing lettuce farm located at downstream sampling site

Plate 2: A photograph showing vegetable farm abstracting water for irrigation (note arrow showing water hose for abstraction)

Plate 3: A photograph showing a farmer abstracting water for irrigation of vegetables

It was also observed that there were sewage pipes connected from households discharge wastewater into the stream. This shows other possible sources of antibiotic resistance in the Onyasia stream. Plates 4 and 5 below are pictures of some of these observations.

Plate 4: A photograph showing wastewater connections from a household into the Onyasia stream (note arrow showing PVC waste pipe)

Plate 5: A photograph showing wastewater connections into the Onyasia stream (note arrow showing PVC waste pipe)

4.5 Determination of antibiotic resistant *E. coli* isolates

4.5.1 Occurrence of all antibiotic resistant E. *coli* isolates

The number of all *E. coli* isolates expressing resistance to individual antimicrobial agents is indicated in Table 4.4.

Sixteen percent of all isolates were found to be susceptible to all 7 antibiotics. All isolates showed low resistance prevalence to Imipenem (9%), Gentamicin (17%), Aztreonam (26%) and Ciprofloxacin (29%). Isolates showed high resistance prevalence to Amoxicillin clavulanate (57%), Cefuroxime (52%) and Tetracycline (49%).

Table 4.4 Percentage number of all *E. coli* resistant to antibiotics tested

Antibiotics	Percentage(%) resistance and susceptibility of isolates	
	Resistant	Susceptible
GN-10	17	83
AMC-20	57	43
TE-30	49	51
CIP-5	29	71
IMP-10	9	91
CXM-30	54	44
ATM-30	26	74
CAZ-30	28	72

TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM**-30: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN**-10: Gentamicin

4.5.2 Occurrence of antibiotic resistant *E. coli* isolates across sampling sites

Figures 4.9 and 4.10 show percentage occurrence of antibiotic resistance in effluent, outfall and upstream and downstream sampling sites for *E. coli* isolates. Resistance prevalence in effluent isolates was high for Amoxicillin clavulanate (50%), Cefuroxime (47%) and Tetracycline (37%). On the contrary, resistance in effluent isolates was low for ciprofloxacin (10%), Imipenem (17%), Aztreonam (20%) and Gentamicin (27%).

Resistance rates in outfall isolates were high for Amoxicillin clavulanate (60%), Cefuroxime (50%) and Tetracycline (40%) but low for Ciprofloxacin (30%), Aztreonam (27%), Gentamicin (20%) and Imipenem (13%). Downstream had the highest resistance rates in all stream isolates with Amoxicillin clavulanate (83%), Cefuroxime (73%), Tetracycline (56%) and being the highest. Downstream isolates however recorded lower resistance rates for Aztreonam (40%), Ciprofloxacin (30%), Gentamicin (30%) and Imipenem (13%). Upstream isolates showed lower resistance rates compared to outfall and downstream isolates. Resistance prevalence rates for upstream isolates were 43%, 40%, 33%, 13%, and 10% for Amoxicillin clavulanate, Cefuroxime, Tetracycline, Gentamicin, Aztreonam and Ciprofloxacin respectively. All upstream isolates were susceptible to Imipenem.

In general, isolate resistance to antibiotics tested was higher downstream compared to upstream sampling sites. Chi-square tests for isolate resistance by individual sampling site showed significant differences ($p < 0.05$) between upstream and downstream sampling sites for all seven antibiotics. Chi-square tests for isolate resistance by individual sampling site showed significant differences ($p > 0.05$) between effluent and downstream sampling sites for all seven antibiotics. Figure 4.7 and 4.8 show prevalence of resistant *E. coli* in the treatment plant and stream.

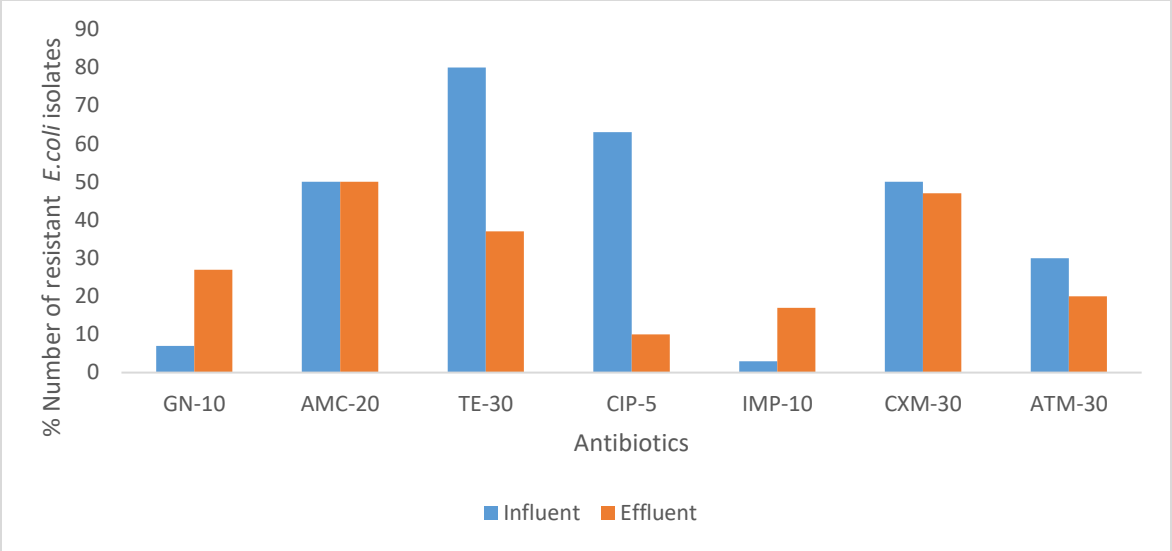

Figure 4.9: A bar chart showing percentage occurrence of resistant *E. coli* isolates in treatment plant

TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM**-30: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN-10**: Gentamicin

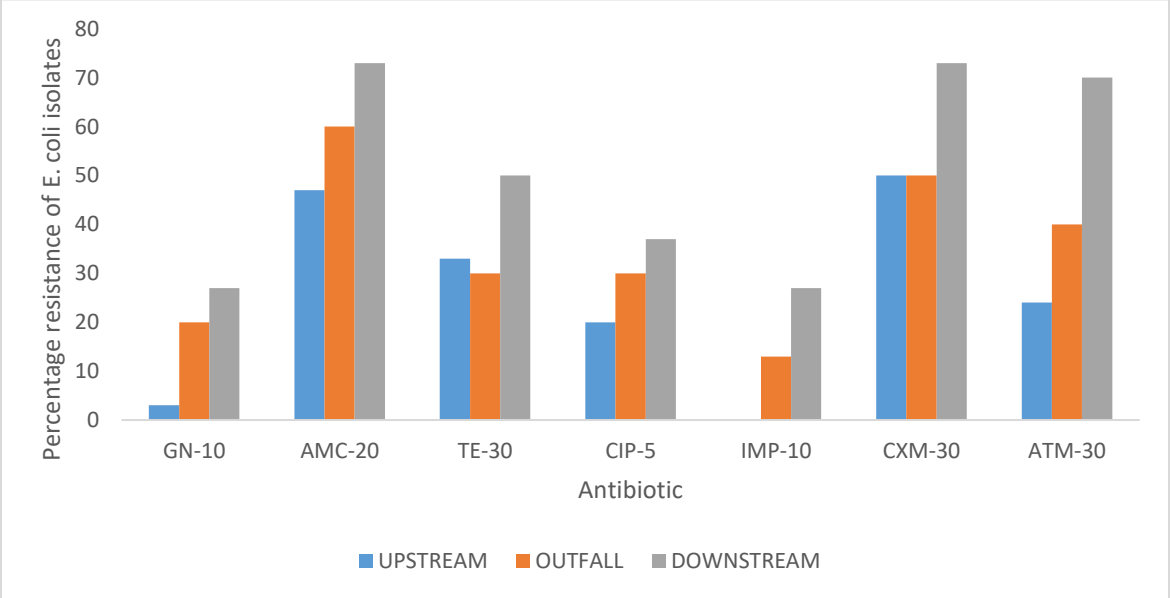

Figure 4.10: A bar chart showing percentage occurrence of resistant *E. coli* isolates in the Onyasia Stream

TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM**-30: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN-10**: Gentamicin

4.5.3 Multi-drug resistance in *E. coli* isolates

Out of the 150 confirmed isolates, 83% showed resistance to a minimum of 1 out of 7 tested antibiotic agent. Seventy percent of all isolates showed resistance to not less than 2 antibiotic agents. Thirty-two isolates (21 %) were resistant to 2 agents, 24 (16%) of isolates tested were resistant to 3 agents and 48(32%) of isolates tested were resistant to 4 or more agents. Out of 105 multi-drug resistant isolates collected (resistant to a minimum of 2 antibiotics), 22 isolates were obtained from effluent,18 isolates from upstream, 29 isolates from outfall and 30 from downstream. Table 4.5 and 4.6 show multi-drug resistance of *E. coli* isolates in the treatment plant and stream.

Table 4.5: Multi-drug resistance of *E. coli* isolates in the treatment plant.

	Number of isolates with Resistance to n antibiotics:									
Sample site	**n=0**	**(%)**	**n=1**	**(%)**	**n=2**	**(%)**	**n=3**	**(%)**	**n≥4**	**(%)**
Influent	4	13	3	10	5	17	5	17	13	43
Effluent	8	27	6	20	4	13	4	13	8	27

n=0: number of isolates not resistant to all antibiotics tested; n=1: number of isolates resistant to 1 antibiotic; n=2; number of isolates resistant to 2 antibiotics, n=3: number of isolates resistant to 3 antibiotics; n=4: number of isolates resistant to 4 or more antibiotics

Table 4.6: Multi-drug resistance of *E. coli* isolates in the Onyasia stream

Sample site	Number of isolates with Resistance to n antibiotics:									
	n= 0	**(%)**	**n= 1**	**(%)**	**n= 2**	**(%)**	**n= 3**	**(%)**	**n≥ 4**	**(%)**
Outfall	1	3.33	7	23.33	7	23.33	1	3.33	14	46.67
Upstream	12	40	1	3	8	27	8	27	1	3
Downstream	0	0	3	10	8	27	7	23	12	40

n=0: number of isolates not resistance to any of the antibiotics tested; n=1: number of isolates resistant to 1 antibiotic; n=2; number of isolates resistance to 2 antibiotics, n=3: number of isolates resistance to 3 antibiotics; n=4: number of isolates resistance to 4 or more antibiotics

4.6 Determination of antibiotic resistant *A. hydrophila* isolates

4.6.1 Occurrence of all antibiotic resistant *A. hydrophila* isolates

The number of all *A. hydrophila* isolates expressing resistance to individual antimicrobial agents are indicated in Table 4.7.

Fifteen percent of all isolates were susceptible to all 7 antibiotics. Sixty-eight percent of all isolates expressed resistance to Amoxicillin clavulanate out of the isolates collected followed by Cefuroxime (43%) and Tetracycline (31%) of all isolates. Lower resistance rates to Aztreonam, Ciprofloxacin and Imipenem in all isolates were at 27%, 22% and 17%

respectively. Resistance to Gentamicin was found in fewer isolates, at rates of 9% for all isolates.

Table 4.7 Percentage number of all *A. hydrophila* resistant to antibiotics tested

Antibiotic	Percentage(%) resistance and susceptibility of isolates	
	Resistant	Susceptible
GN-10	9	91
AMC-20	68	32
TE-30	31	69
CIP-5	22	78
IMP-10	22	78
CXM-30	43	57
ATM-30	27	73
CAZ-30	20	80

TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM-30**: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN-10**: Gentamicin

4.6.2 Occurrence of antibiotic of *A. hydrophila* isolates across sampling sites

Figures 4.11 and 4.12 show percentage occurrence of antibiotic resistance in influent, effluent, outfall and upstream and downstream sampling sites in *A. hydrophila* isolates. Amoxicillin, Cefuroxime and Tetracycline resistance prevalence was high in effluent, outfall and downstream sampling sites. Upstream isolates expressed much lower resistance rates compared to other sampling sites.

Resistance prevalence in effluent isolates was high for Amoxicillin clavulanate (97%) and Cefuroxime (50%), Aztreonam (37%) and Imipenem (33%). Resistance in effluent isolates was low for Tetracycline (23%), Gentamicin (17%), and Ciprofloxacin (13%).

Resistance rates in outfall isolates were high for Amoxicillin clavulanate (77%) and Cefuroxime (37%). Resistance rates were low for Aztreonam (17%), Imipenem (16%), Ciprofloxacin (13%) and Gentamicin (7%) and in outfall isolates. Downstream had the highest resistance rates in all stream isolates with Amoxicillin clavulanate (80%), Cefuroxime (47%) Aztreonam (40%) and Tetracycline (33%). Downstream isolates however recorded lower resistance rates for Imipenem (27%), Ciprofloxacin (17%), and Gentamicin (10%). Upstream isolates showed lower resistance rates compared to outfall and downstream isolates, with resistance rates of 27%, 10% and 7% for Amoxicillin clavulanate, Tetracycline and Cefuroxime respectively. Upstream isolates were all susceptible to Imipenem, Gentamicin, Ciprofloxacin, and Aztreonam.

Isolate resistance to antibiotics tested was higher in downstream isolates compared to upstream isolates. Chi-Square tests for isolate resistance by individual sampling site showed significant differences ($p < 0.05$) between upstream and downstream sampling sites for all antibiotics tested except Gentamicin.

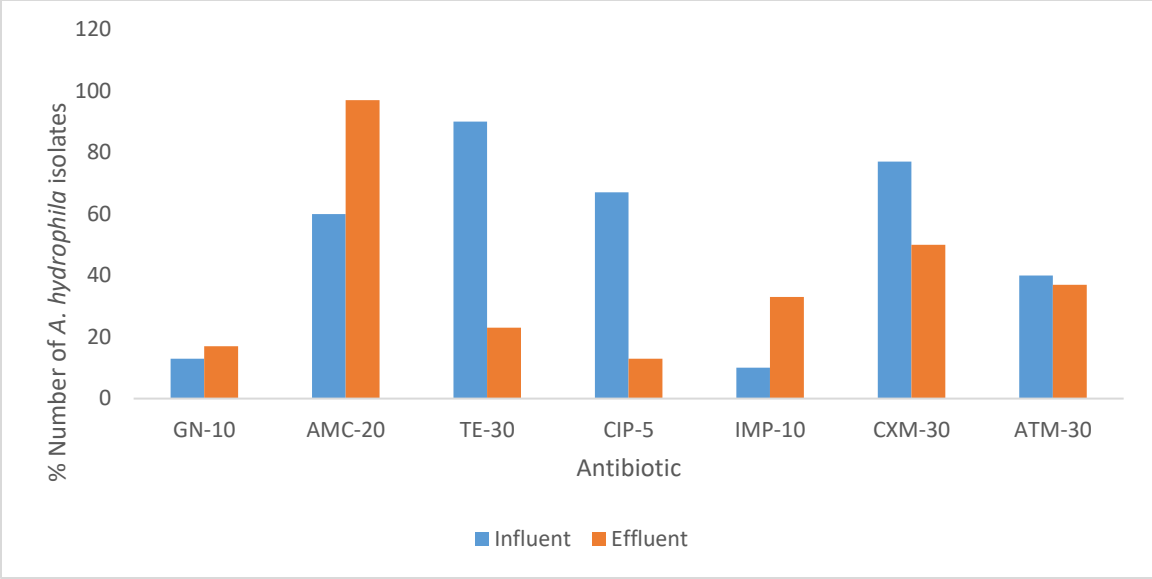

Figure 4.11: A bar chart showing percentage resistance of *A. hydrophila* isolates in treatment plant
TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM**-30: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN-10**: Gentamicin

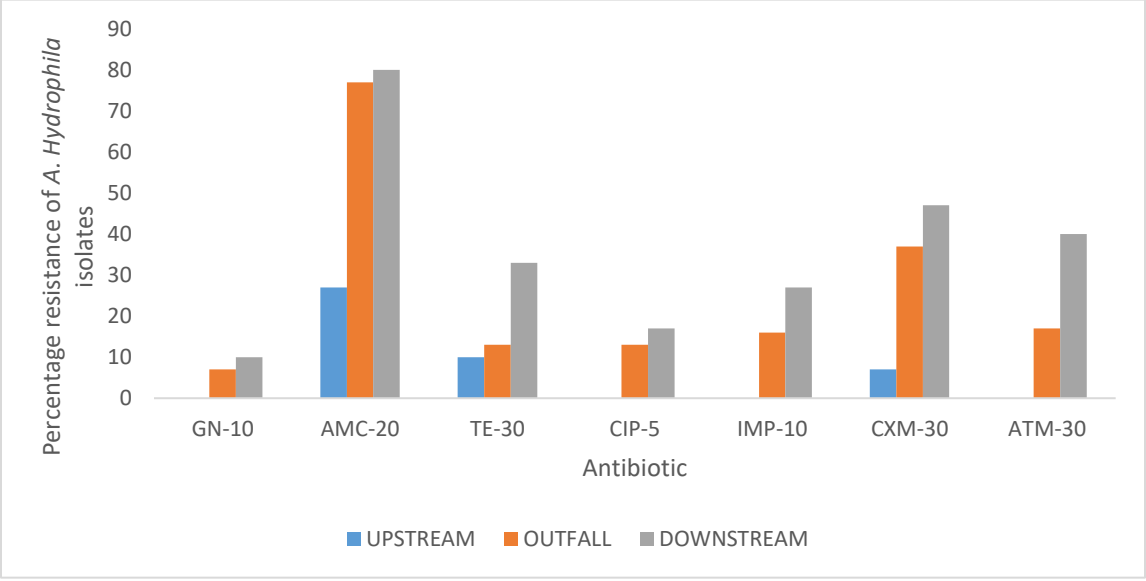

Figure 4.12: A bar chart showing percentage resistant *A. hydrophila* isolates in the Onyasia Stream
TET-30: Tetracycline 30 µg; **AMC-20**: Amoxicillin Clavulanate 20 µg; **CIP-5**: Ciprofloxacin 5µg; **ATM-30**: Aztreonam 30 µg; **CXM**-30: Cefuroxime- 30 µg: **IMP-10**: Imipenem 10µg; **GN-10**: Gentamicin

4.6.3 Multi-drug Resistance in *A. hydrophila* isolates

Out of the 150 isolates, 84% showed resistance to at least one of 7 tested antibiotic agent. Sixty-one (98 of total) of isolates showed resistance to not less than 2 antibiotic agents. Twenty-five (26%) isolates were resistant to 2 agents, 25 were resistant to 3 agents and 42 were resistant to 4 or more agents. Out of 98 multi-drug resistant isolates collected .i.e. those resistant to a minimum of 2 antibiotics, 27 isolates were obtained from influent wastewater, 27 isolates from effluent,1 isolate from upstream, 15 isolates from outfall and 22 from downstream. Table 4.8 and 4.9 shows tables of multiple resistant isolates per sampling site.

Table 4.8: **Multi-drug resistance of *A. hydrophila* isolates by sampling site in the treatment plant**

Sample site	**Number of isolates with Resistance to n antibiotics:**									
	n=0	(%)	n=1	(%)	n=2	(%)	n=3	(%)	n≥4	(%)
Influent wastewater	1	3	2	7	3	10	1	3	23	77
Final effluent	0	0	3	10	10	33	10	33	7	23

n=0: number of isolates not resistant to all antibiotics tested; n=1: number of isolates resistant to 1 antibiotic; n=2; number of isolates resistant to 2 antibiotics, n=3: number of isolates resistant to 3 antibiotics; n=4: number of isolates resistant to 4 or more antibiotics

Table 4.9: **Multi-drug resistance of *A. hydrophila* isolates by sampling site in the Onyasia stream**

Sample site	Number of isolates with Resistance to n antibiotics:									
	n=0	**(%)**	**n=1**	**(%)**	**n=2**	**(%)**	**n=3**	**(%)**	**n≥4**	**(%)**
Outfall	2	7	13	43	5	17	6	20	4	13
Upstream	19	63.33	10	33.33	1	3.33	0	0	0	0
Downstream	1	3	7	23	6	20	8	27	8	27

n=0: number of isolates not resistant to all antibiotics tested; n=1: number of isolates resistance to only one antibiotic; n=2; number of isolates resistance to two antibiotics, n=3: number of isolates resistance to three antibiotics; n=4: number of isolates resistance to four or more antibiotics

4.7 Determination of antibiotic resistant *P. aeruginosa* isolates

4.7.1 Occurrence of antibiotic resistant of all *P. aeruginosa* isolates

The number of all *P. aerugi**nosa*** isolates expressing resistance to individual antimicrobial agents are indicated in Table 4.7.

Forty-four percent of all isolates were susceptible to all 5 antipseudomonal agents. Resistance rates were generally low for antipseudomonal agents. All Isolates expressed resistance to Aztreonam (37%), Ciprofloxacin (33%) and Gentamicin (22%). Resistance to Ceftazidime and Imipenem was lower compared to other antipseudomonal agents with

resistance rates of 11% and 5% respectively. Figure 4.13 shows resistant profiles of all *P. aeruginosa* isolated

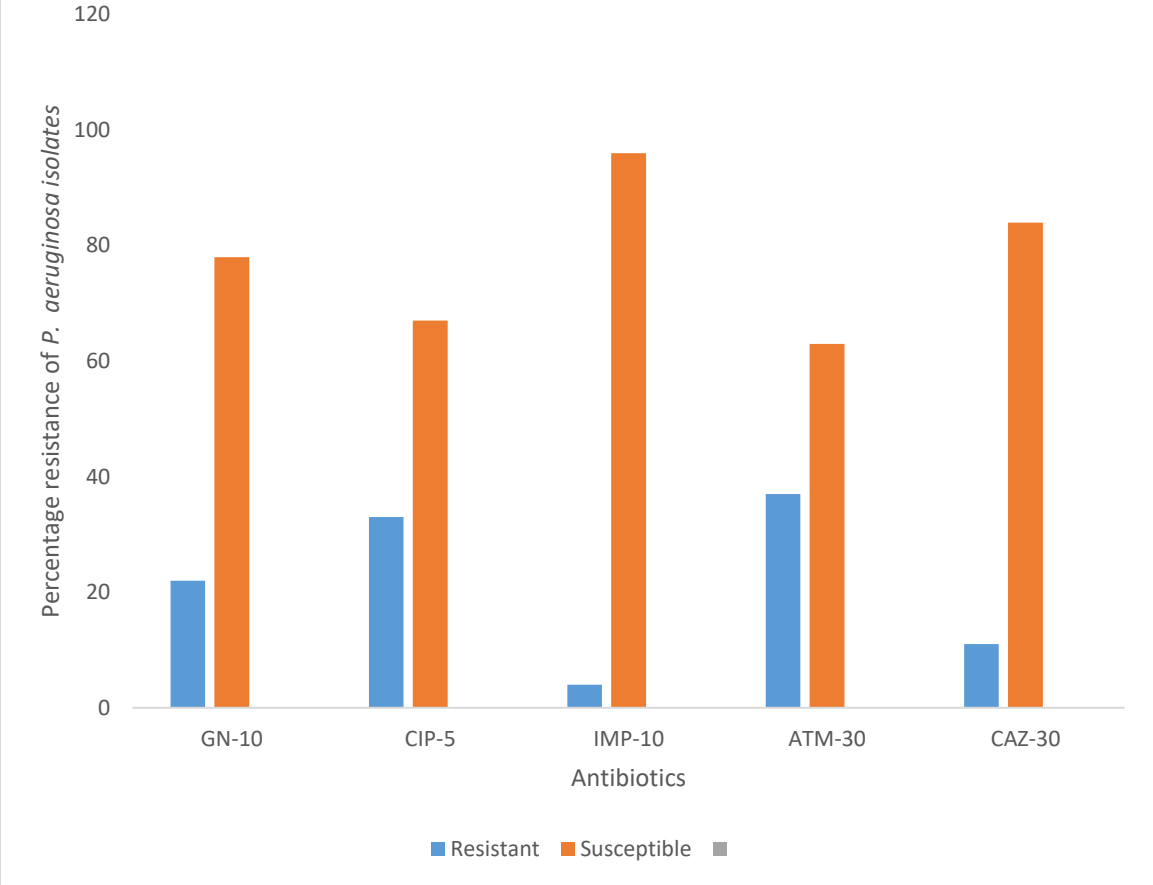

Figure 4.13: A bar chart showing percentage occurrence of resistance of all isolated *P. aeruginosa*.
ATM; Aztreonam 30 µg; **CAZ**: Ceftazidime 10 µg; **CIP**: Ciprofloxacin 5µg; **GN**: Gentamicin 10 µg; **IPM**: Imipenem 10 µg

4.7.2 Occurrence of antibiotic resistance *P. aeruginosa* isolates across sampling sites

Figures 4.14 and 4.15 show isolate resistance responses by sampling site and antibiotics. High resistance is seen in final effluent wastewater, outfall and downstream isolates. Upstream isolates showed low resistance rate to antipseudomonal agents. Upstream

isolates were all susceptible to Imipenem and resistance to Ceftazidime was 3%. Resistance to Ciprofloxacin was the highest with resistance rates of 40%, 20%, and 13% at downstream, outfall and final effluent sampling sites respectively. After Ciprofloxacin, Aztreonam had the next highest resistance at 33%, 17% and 13% at downstream, outfall and final effluent sampling site respectively.

In general, isolate resistance to antibiotics tested was higher downstream than upstream. Chi-square tests for isolate resistance by individual sampling site showed significant differences ($p < 0.05$) between resistance to Ciprofloxacin, Aztreonam and Gentamicin at all sampling sites for upstream and downstream isolates.

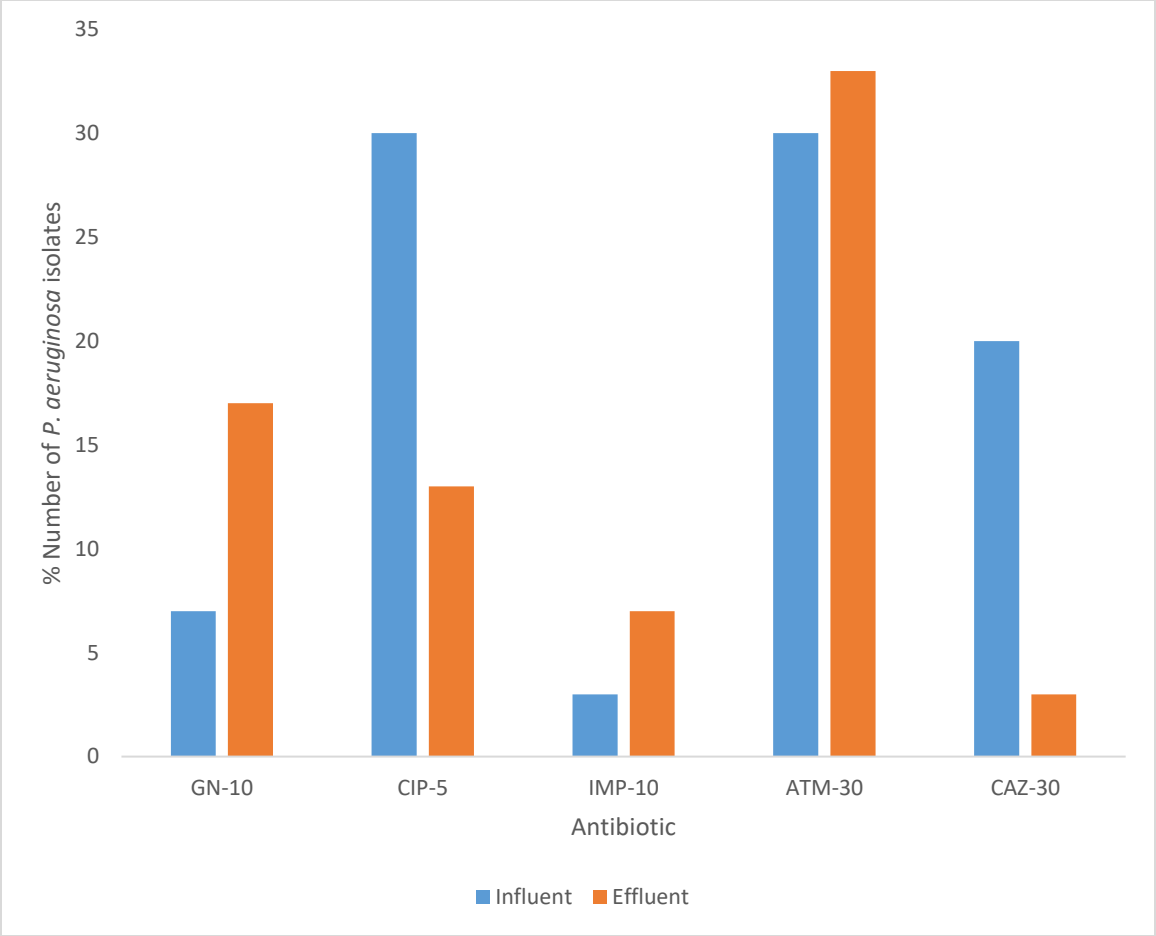

Figure 4.14: A bar chart showing percentage resistance of *P. aeruginosa* isolates in treatment plant
ATM; Aztreonam 30 µg; **CAZ**: Ceftazidime 10 µg; **CIP**: Ciprofloxacin 5µg; **GN**: Gentamicin 10 µg; **IPM**: Imipenem 10 µg

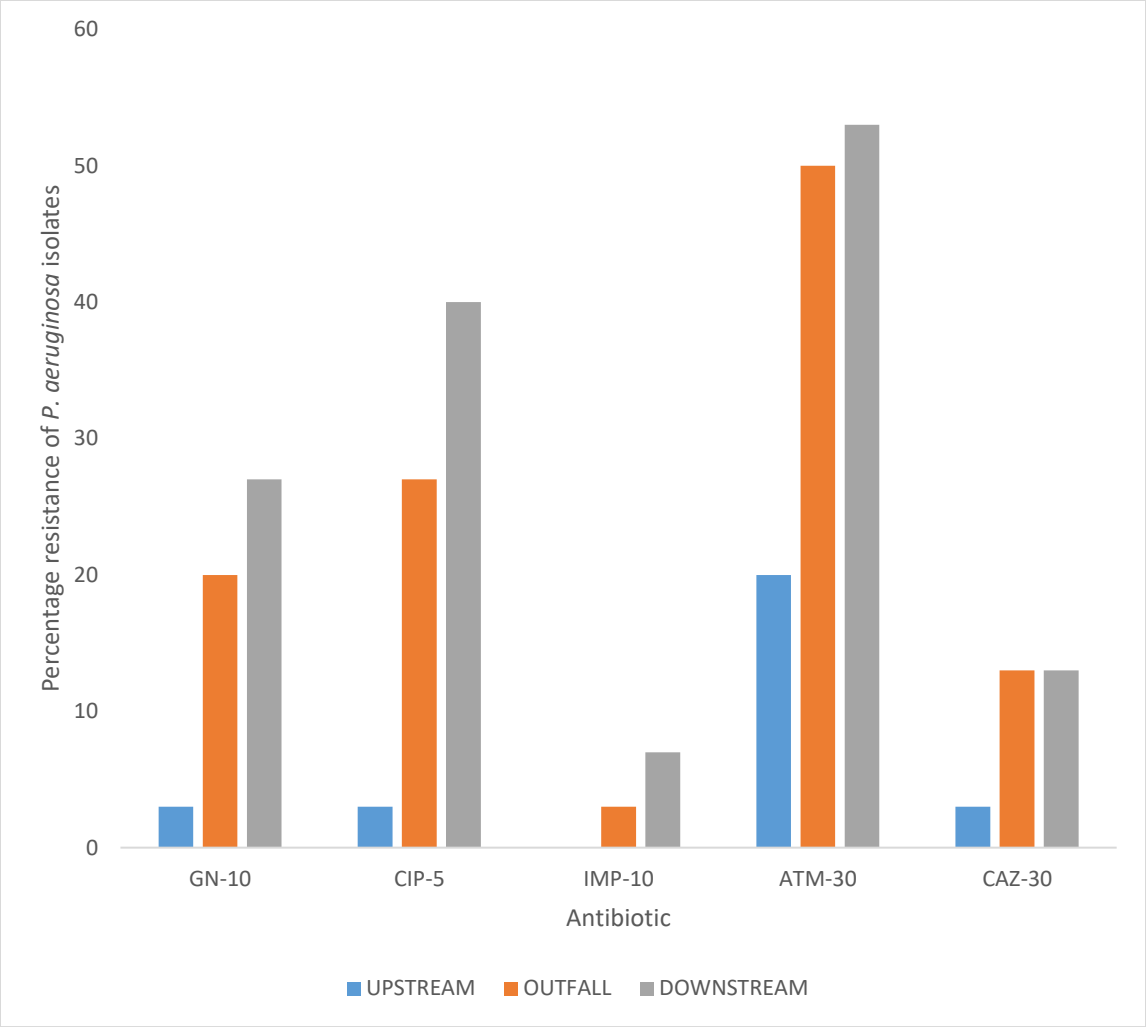

Figure 4.15: A bar chart showing percentage occurrence of resistant *P. aeruginosa* isolates in the Onyasia Stream
ATM; Aztreonam 30 µg; **CAZ**: Ceftazidime 10 µg; **CIP**: Ciprofloxacin 5µg; **GN**: Gentamicin 10 µg; **IPM**: Imipenem 10 µg

4.7.3 Multi-drug resistance in *P. aeruginosa* isolates

Thirty-six percent (122 of total) of all 150 *P. aeruginosa* isolates showed resistance to at least 1 antibiotic agent. Twenty-one percent of all isolates showed resistance to not less than 2 antibiotic agents. Nineteen (13% of total) of all isolates were resistant to 2 agents, 9 (6% of total) were resistant to 3 agents and 4 (3% of total) were resistant to 4 or more

agents. Out of 32 multi-drug resistant isolates collected (resistant to at least 2 antibiotics), 6 isolates were obtained from effluent, 9 isolates from outfall and 12 from downstream sampling sites.

In general, multiple resistance to antibiotics was highest in downstream sites, followed by outfall and effluent. Multiple resistant *P. aeruginosa* isolates were not recorded in the upstream sampling site. Table 4.10 and 4.11 records tables of multiple resistant isolates per sampling site.

Table 4.10: **Multi-drug resistance of *P. aeruginosa* isolates by sampling site.**

Sample site	**Number of isolates with Resistance to n antibiotics:**									
	n=0	**(%)**	**n=1**	**(%)**	**n=2**	**(%)**	**n=3**	**(%)**	**n≥4**	**(%)**
Influent	1	3	2	7	3	10	4	13	20	67
Final effluent	0	0	4	13	11	37	7	23	8	27

n=0: number of isolates not resistant to all antibiotics tested; n=1: number of isolates resistant to 1 antibiotic; n=2; number of isolates resistant to 2 antibiotics, n=3: number of isolates resistant to 3 antibiotics; n=4: number of isolates resistant to 4 or more antibiotics

Table 4.11: **Multi-drug resistance of *P. aeruginosa* isolates by sampling site.**

Sample site	Number of isolates with Resistance to n antibiotics:									
	n=0	**(%)**	**n=1**	**(%)**	**n=2**	**(%)**	**n=3**	**(%)**	**n≥4**	**(%)**
Outfall	2	7	12	40	8	27	4	13	4	13
Upstream	19	63	10	33	1	0	0	0	0	0
Downstream	1	3	8	27	6	20	9	30	6	20

n=0: number of isolates not resistant to all antibiotics tested; n=1: number of isolates resistance to only one antibiotic; n=2; number of isolates resistance to 2 antibiotics, n=3: number of isolates resistance to 3 antibiotics; n=4: number of isolates resistance to 4 or more antibiotics

This study sought to investigate the presence of antibiotic resistant *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods and the environmental health implications of the treatment plant discharge.

In general, *P. aeruginosa*, *E. coli* and *A. hydrophila* was recorded in all sampling sites. Although the Legon Sewage Treatment Plant significantly reduced the levels of bacteria in influent wastewater, levels of bacteria in effluent wastewater was very high. Downstream

bacterial levels was significantly high than upstream. Resistance to Amoxicillin Clavulanate, Cefuroxime and Tetracycline was the highest. Downstream isolates displayed the highest resistance and well as multiple resistance.

DISCUSSION

5.0 Introduction

Aquatic environments are considered suitable reservoirs for antibiotic resistance distribution, because of constant discharge of antibiotics and antibiotic resistant bacteria into the environment (Ramírez Castillo *et al*., 2013). Aquatic systems may therefore be contaminated by the release of sewage and serve as wastewater point sources and sources of antibiotics and resistant microflora. Most water bodies that receive these contaminants are often used in irrigating crops with and without treatment and this may pose potential health risks to humans and animals through the food chain (Zhang *et al*., 2009; Mazari-Hiriart *et al*., 2008). As such monitoring pathogens and their resistance patterns present in effluents and the receiving surface waters is important.

Wastewater treatment plants are recognized as major hotspots and risk environments for the development and dissemination of antibiotic resistance in aquatic environments (Kummerer, 2009; Rizzo *et al*., 2013). Studies conducted in various countries have detected the presence of antibiotics in hospital effluents, municipal effluents and sewage treatment stations (Rizzo *et al*., 2013). These antibiotics in effluent contribute to the selection of antibiotic bacteria in the environment. In addition, antibiotic resistant bacteria in effluent may further transfer resistance to other non-infectious and infectious bacteria through mobile genetic elements like plasmid and transposons.

In the present study, antibiotic resistance levels present in final effluents from the Legon Sewage Treatment Plant as well in the receiving Onyasia stream were assessed.

5.1 Occurrence of *E. coli, A. hydrophila* and *P. aeruginosa*

E. coli, A. hydrophila and P. aeruginosa were recorded in all sampling sites. Abundance of *E. coli, A. hydrophila and P. aeruginosa* loads in effluent was lower compared to influent water; as such, there was improved wastewater quality after treatment. There was up to a fivefold reduction of mean bacterial loads. However, the levels exceed the Ghana Environmental Protection Agency (EPA) permissible limits of *E.coli* (10CFU/100ml and total coliform (400CFU/100ml) for wastewater discharge. This could be as a result of the high influx of bacteria in influent from the catchment population as such although there was reduction appreciable reduction in bacterial counts, levels remained high.

Mean total and faecal coliform counts were much lower compared to previous studies carried out by Kwabla (2017) in the Legon Sewage Treatment Plant. Results of this study is consistent with a study conducted by Abuenyi (2010) which recorded *E. coli* values in final effluents from a treatment plant above Environmental Protection Agency's permissible levels. Although wastewater treatment plants processes typically reduce the number of heterotrophic bacteria, high numbers of bacteria may still be discharged into surface water (Kwak *et al*., 2014).

Comparatively *E. coli* counts in final effluent were much lower than *P. aeruginosa* and *A. hydrophila.* This may be due to the fact that *E. coli* has a shorter die-off period compared to *P. aeruginosa* and *A. hydrophila. P. aeruginosa* are able to colonize biofilms making their survival rate higher in water (Mena and Gerba, 2009).

Mean *A. hydrophila* counts in influent wastewater and final effluent were much higher in this study compared to a study conducted by Hassani *et al*. (1992) which had mean counts of 5.63×10^4 and 1.04×10^3 for influent wastewater and final effluent respectively.

Although the wastewater treatment plant tested was in the expected range for their ability to reduce the loads of bacteria in influent wastewater, *E. coli*, *P. aeruginosa* and *A. hydrophila* counts found close to the discharge point in the Onyasia Stream were high.

E. coli, *P. aeruginosa* and *A. hydrophila* counts increased significantly from upstream to downstream sites in the Onyasia stream. Similarly, Lorenzo *et al*. (2018) reported an increase in *E. coli* from upstream to downstream in the Zenne River, Belgium. Downstream bacterial counts were higher than in final effluents samples. This suggests that the treatment plant is not the only source of *E. coli, A. hydrophila* and *P. aeruginosa* in the stream. This is consistent with field observations where wastewater was seen to be discharged into the Onyasia stream at downstream sampling points.

5.2 Antibiotic resistance in *E. coli* isolates

Susceptibility of all *E. coli* isolates to antibiotics classes including β- lactam/β-lactamase inhibitors (Amoxicillin clavulanate), Cephalosporin (Cefuroxime and Ceftazidime), Monobactam (Aztreonam), Carbapenem (Imipenem), Aminoglycosides (Gentamicin), Tetracycline (Tetracycline) and Quinolones (Ciprofloxacin) were determined.

Isolates displayed resistance to between one and seven antibiotic agents. Imipenem, Gentamicin, Aztreonam and Ciprofloxacin residence rates were low. High activity of

Imipenem may be due to the fact that there is infrequent use clinically and in the community compared to other antibiotics tested. Imipenem is a group 2 Carbapenem used as a last resort antibiotic against particularly resilient Gram-negative pathogens. As such, Imipenem is not frequently prescribed which implies resistance to the agent would not be expected to be high. Aztreonam is also not frequently prescribed and the mode of prescription for Gentamicin is through injections, which is not preferred as such usage may be low in the population. Accordingly, a large proportion of the isolates collected from all sampling sites showed low resistance to Imipenem, Gentamicin, Aztreonam, and Ciprofloxacin.

High resistance to Amoxicillin clavulanate, Cefuroxime and Tetracycline was observed in *E. coli* isolates from effluent and downstream isolates. Resistance to Amoxicillin clavulanate (50%), Cefuroxime (47%) and Tetracycline (37%) in effluent isolates were lower compared to downstream isolates. Resistance to tetracycline was lower compared to a study conducted in South Africa by Olayinka and Okoh (2017), which recorded higher Tetracycline resistance (74.1%) in final effluents from two waste treatment plants.

Downstream isolates recorded the high resistance to Amoxicillin clavulanate (83%), Cefuroxime (73%) and Tetracycline (56%). A study conducted by Sullivan & Karthikeyan (2013), found a substantial occurrence of bacteria resistant to tetracycline and tetracycline resistant genes in downstream sediment and surface water samples from Carter's Creek watershed, a watershed influenced by waste discharge and other anthropogenic activities. The study observed that the occurrence of Tetracycline resistance genes increased downstream of WWTPs.

Resistance was higher in downstream compared to final effluent samples, which may suggest that there may be other sources of antibiotic resistant bacteria other than the Legon Sewage Treatment Plant in the Onyasia stream. Similarly, high levels of antibiotic resistant coliforms were reported downstream a wastewater treatment plant discharge in surface water (Goni-Urriza *et al*., 2000; Zhang *et al*., 2009; Akiyama and Savin, 2010; Guyomard-Rabenirina *et al*., 2017; Akiyama and Savin, 2010).

In this study, a significant association ($p < 0.05$) was found to exist between the location of sampling sites relative to the waste treatment plant .i.e. upstream and downstream, and isolates expressing resistance to Amoxicillin clavulanate, Cefuroxime and Tetracycline. This result is similar to a study conducted by Laird (2016) which found an increase in *E. coli* resistant isolates downstream in an urbanized watershed impacted by wastewater treatment plants, which lends support to the hypothesis that WWTPs effluent may be contributing to the conveyance of antibiotic resistance bacteria downstream from discharge points. Upstream isolates expressed resistance to Amoxicillin clavulanate, Cefuroxime and Tetracycline although comparatively lower to other sites, further suggesting that there may be some unknown sources of antibiotics or resistance strains discharged further upstream of the treatment plant.

Seventy percent of all *E. coli* isolates expressed resistance to not less than 2 antibiotics. This result is similar to a study by Odonkor and Addo (2012) which recorded 63% of multiple resistant *E. coli* strains in drinking water sources in Accra. Blaak *et al*. (2015) reported a high presence of multiple drug resistant *E. coli* in surface waters.

E. coli isolates from upstream were resistant to 2 or more antibiotic agents, although only one isolate was resistant to more than 4 antibiotics. Fifty-three percent of *E. coli* isolates from final effluent from the treatment plant were resistant to not less than 2 antibiotic agents. Downstream recorded the highest number of isolates resistant to 2 or more antibiotics with 93% multiple resistant isolates, which is similar to results from a study by Blaak *et al*., 2015 which reported high multiple resistant strains downstream a WWTP. Multi-drug resistance was found to increase downstream the treatment plant for isolates resistant to ≥ 2, ≥ 3, and ≥ 4 agents. Studies have observed high rates in the development of multi-drug resistance in *E. coli* isolates in WWTPs (Korzeniewska *et al.*, 2013; Amador *et al.*, 2015). This is found to be primarily driven by the transfer of conjugative plasmids and are shown in other studies to persist and propagate through the environment once discharged. (Laird, 2016). Hence high resistance found in *E.coli* downstream.

5.3 Antibiotic resistance in *A. hydrophila*

Ubiquitous bacteria, which are capable of colonizing different water types, are of particular importance when determining antimicrobial resistance dissemination in the environment. As a result of their ubiquity and resistance patterns, *Aeromonas sp* can grow in different sources of water; as such, they are important in assessing possible forms of antimicrobial resistance dissemination. *Aeromonas sp* have been shown to develop and spread antibiotic resistance in the aquatic systems (Figueira *et al*., 2011) rendering surveillance of resistance in this organism is therefore very imperative.

Susceptibility of all *A. hydrophila* isolates to antibiotics classes, which include β- lactam/β-lactamase inhibitors (Amoxicillin clavulanate), Cephalosporin (Cefuroxime and

Ceftazidime), Monobactam (Aztreonam), Carbapenem (Imipenem), aminoglycosides (Gentamicin), Tetracycline (Tetracycline) and Quinolone (Ciprofloxacin), were determined. Isolates exhibited resistance to between one and seven antibiotic agents. Among all the antibiotics, Gentamicin and Ceftazidime were active against 91% and 80 % of all *A. hydrophila* isolates. Also, susceptibility rates of all isolates to Aztreonam, Ciprofloxacin, Imipenem was 73 %, 78% and 78% respectively.

High resistance to Amoxicillin clavulanate (68%), Cefuroxime (43%) and Tetracycline (31%) was observed in all isolates. Final effluent recorded resistance of 97% for Amoxicillin clavulanate, 50% for Cefuroxime and 23% Tetracycline. The rate of isolate resistance to tetracycline in effluent was found to be lower than expected when compared to similar research (Igbinosa and Okoh, 2012) which recorded 77.8% in effluent samples. Resistance to tetracycline was 10% and 33% upstream and downstream respectively. Tetracycline resistance has also been reported in *Aeromonas sp* isolated from a river that receives wastewater discharge (Goñi-Urriza, 2000). High Amoxicillin clavulanate and Cefuroxime resistance (80% and 47% respectively) was recorded downstream.

Resistance rates were higher in downstream than in final effluent samples suggesting that there are other sources contributing to high resistance in the Onyasia stream other than the waste treatment plant. Other external sources may include untreated wastewater from households and abattoirs, which are directly discharged into the stream.

Upstream isolates recorded lower Amoxicillin clavulanate (23%) and Cefuroxime (23%) resistance. A significant association ($p < 0.05$) was found to exist between the location of sampling sites relative to the waste treatment plant .i.e. upstream, downstream) and isolates

expressing resistance to Amoxicillin clavulanate, Cefuroxime and Tetracycline. This result further corroborates effluents from wastewater treatment plant as contributors to the conveyance of antibiotic resistance bacteria downstream from discharge points (McConnell 2016).

Sixty-one percent of all *Aeromonas hydrophila* isolates expressed resistance to 2 or more antibiotics, 99% of *A. hydrophila* isolates from final effluent from the treatment plant were resistant to 2 or more antibiotic agents. Fifty-six percent of *A. hydrophila* isolates in final effluent expressed resistance to 3 or more antibiotics. This result is similar to results from a study conducted by Figueira *et al*. (2011), which recorded high multiple resistance to at least three different antibiotic classes (Gentamycin, Tetracycline and Sulphamethoxazole/Trimethoprim) in *Aeromonas punctata* and *Aeromonas media* isolated from final effluents.

The Downstream sampling site recorded the highest number of isolates resistant to 2 or more antibiotic with 73% multiple resistant isolates. Upstream recorded 3% isolates resistant to 2 or more isolates. Multi-drug resistance was found to increase downstream the treatment plant for isolates resistant to ≥ 2, ≥ 3, and ≥ 4 agents. Studies on antibiotic resistance in multiple resistance of *Aeromonas sp* using culture dependent methods in waste water and surface water is limited.

5.4 Antibiotic resistance in *P. aeruginosa* isolates

P. aeruginosa is a ubiquitous member of the genus *Pseudomonas*. *P. aeruginosa* causes nosocomial infections in immunocompromised patients, which are often difficult to treat (Hayford, 2016). Treatment of these infections are limited to a few class of antibiotics usually referred to as antipseudomonal antibiotics due to the fact that *Pseudomonas sp* are resistant to common antibiotics (Hayford, 2016). There is the need for routine surveillance of the distribution, resistance pattern of *P. aeruginosa* as well as other pathogenic bacteria from wastewater in order to provide local data on resistance pathways especially the contribution from wastewater.

Susceptibility of all *P. aeruginosa* isolates to antibiotics classes, which include Cephalosporin (Ceftazidime), Monobactam (Aztreonam), Carbapenem (Imipenem), Aminoglycosides (Gentamicin), and Quinolone (Ciprofloxacin) were determined. Forty-five percent of all *P. aeruginosa* isolates were susceptible to all antipseudomonal agents. Among all the antibiotics tested, Imipenem and Ceftazidime were active against 96% and 89 % of all *P. aeruginosa* isolates. The high activity of these antibiotics in *P. aeruginosa* may be due to infrequent use of these antibiotics community and clinical settings in Ghana.

High resistance to Aztreonam (37%), Ciprofloxacin (33%) and Gentamicin 22%) was observed in all isolates, a result is found similar to a study conducted by Hayford (2016), which reported resistance of *P. aeruginosa* to Aztreonam (22%) in clinical and environmental samples in Accra. Ciprofloxacin resistance (54%) and Gentamicin resistance (60%) was also higher in Hayford, 2016 compared to this study. High resistance

rate of *P. Aeruginosa* to Aztreonam have also been observed in Brazil (Pitondo-Silva *et al*., 2014).

Final effluent recorded high resistance for Aztreonam (33%), Ciprofloxacin (13%) and Gentamicin (17%). This result is in contrast with a study conducted by Igbinosa *et al*. (2012) which found higher resistance rates for Gentamicin (22%) of *P. aeruginosa* isolated from effluent of a wastewater treatment plant in South Africa. Imipenem susceptibility was lower in this study when compared to Igbinosa *et al*., 2012, which recorded 100% susceptibility in *P. aeruginosa* isolates to Imipenem. Results of this study in contrasted with a study carried out by Basri *et al.* (2017) which recorded slightly higher resistance rates for ciprofloxacin (15%) in *P. aeruginosa* in effluents from a wastewater treatment plant. In this current study, effluent isolate resistance to gentamicin was higher compared to Basri *et al.* (2017) which recorded no resistance (0%) to *P. aeruginosa* in effluent of a wastewater treatment plant.

There was an increase in the percentages of resistant strains downstream from the wastewater discharge for Aztreonam (50%) and Ciprofloxacin (40%). Resistance rates in effluents were lower than downstream samples, and similar resistant patterns were seen in effluent and downstream isolates.

P. aeruginosa isolated from the upstream also showed some resistance to Aztreonam (20%) and Ciprofloxacin (3%) albeit at low rates. This observation suggests that the source of the resistance was located further upstream from the discharge and that it was not related to treatment plant discharge. However, possible sources of antibiotic resistance were not observed at the upstream sampling site of this study, the reason might also be attributed to

natural resistance. Studies have shown that indigenous bacteria in unpolluted environments can also show some antibiotic resistance (Laird 2016).

Nevertheless, a significant association ($p < 0.05$) was found to exist between the location of sampling site relative to the waste treatment plant (upstream, outfall and downstream) and isolates expressing resistance to Aztreonam and Ciprofloxacin. This shows that the discharge of treated wastewater contributes to antibiotic resistant *P. aeruginosa* in the receiving Onyasia stream.

Studies have shown that multiple resistant strains persist and propagate in the aquatic environment once discharged (Laird, 2016). In this study, however resistance to Aztreonam, Ciprofloxacin, and Gentamycin was more frequently found in multi-drug resistant isolates, which implies that resistance to these antibiotic agents is mostly driven by similar mechanisms of defense coded by resistance genes to other agents, or that the acquisition of resistance to these agents usually occurs in tandem with other antibiotic resistances. High Quinolone-Aminoglycoside (Ciprofloxacin-Gentamicin) cross-resistance in this study is similar to results from Hayford, 2016 study which characterized multiple resistant *P. aeruginosa* from environmental sources in Kumasi, Ghana.

Survey along the course of Onyasia stream sampling points did not show other possible sources of antibiotic resistance close to the upstream sampling point. On the contrary, it was observed that there were many wastewater connections directly from households, which possibly discharge untreated wastewater into the stream.

Vegetable farms were found along the Onyasia stream and close to the downstream sampling points. The stream water was abstracted for irrigation in these vegetable farms.

This finding has human health implications, as humans could be exposed to resistant strains of *E. coli, Pseudomonas aeruginosa, Aeromonas hydrophila* and other resistant organisms through the food chain or contact with contaminated water.

Globally, antibiotic resistant bacteria have been found on fresh fruit and vegetables (O' Flaherty *et al*., 2019). O'Flaherty *et al*. (2019) created a quantitative human exposure assessment model using scenario analysis to investigate the potential human exposure to antibiotic resistant *E. coli* through the consumption of lettuce irrigated with surface water. Results from their study showed the mean human exposure levels ranged between 1.00×10^{-2} and 1.35×10^{6} colony forming units antibiotic resistant *E. coli* per 100 g of surface water irrigated lettuce for the different scenarios investigated. This finding has human health implications, as humans could be exposed to resistant strains of *E. coli, P. aeruginosa, A. hydrophila* and other resistant organisms through the food chain or contact with contaminated water due to the fact that the Onyasia stream serves as an irrigation water source.

Generally, this study shows there is a significant increase in antibiotic resistance prevalence of *E. coli, A. hydrophila* and *P. aeruginosa* downstream of the treatment plant compared to upstream of the treatment plant. This finding is consistent with a study conducted by Guyomard-Rabenirina *et al*. (2017) which observed high resistant rates of *E. coli* downstream a wastewater treatment plant in Guadeloupe. High resistance downstream could also be attributed to wastewater discharge from households as observed in the field. Resistant strains were also found upstream, which suggest other sources of antibiotic

resistance other than the treatment plant. Possible sources could be wastewater from households and abattoirs. Nonetheless, it seems likely that waste treatment plant discharge may be contributing to some degree to the spread of antibiotic resistant *E. coli*, *A. hydrophila* and *P. aeruginosa.*

High resistance rates found in effluent isolates in this study is consistent with results recorded in a study conducted by Neudorf *et al*. (2017), which showed that wastewater stabilization ponds enriched the relative antibiotic resistance genes due to the long holding periods resulting in higher antibiotic resistance rates. This highlights the role of treatment plants in the spread of antibiotic resistance in the environment.

5.5 Suitability of treatment plant effluent for reuse in agriculture

Monitoring wastewater for antibiotic resistance bacteria is important to understand the spread of resistance. Treated wastewater is recommended for use in agriculture in order to achieve sustainable management of water resources, which however this presents a health risk as wastewater treatment plants are unable to significantly reduce antibiotics, antibiotic resistant bacteria and genes which may end up in the food chain when contaminated produce are consumed.

Mean *E. coli*, *A. hydrophila* and *P. aeruginosa* reduced significantly in effluent compared to influents showing improved wastewater quality after treatment, with up to a fivefold reduction of mean bacterial loads.

According to the WHO standards for wastewater, excreta and greywater for agriculture, the effluent from the Legon Sewage Treatment Plant meets the requirement for unrestricted ($<10^5$ *E.coli*/100 ml) and unrestricted irrigation ($<10^3$ *E.coli*/100 ml).

Kwabla (2017) recommended reuse of treated wastewater from the Legon Sewage Treatment Plant in light of the effluents meeting the WHO guidelines for the reuse of wastewater and grey water for agriculture. However, *E. coli*, and *A. hydrophila* resistance rates to Amoxicillin clavulanate, Cefuroxime, Tetracycline, Aztreonam and Imipenem, as observed in this study were high in effluent isolates even though bacterial counts were lower. Multiple antibiotic resistant isolates were also very high in effluent isolates. This has implications for wastewater reuse in agriculture as antibiotic resistance bacteria may enter the food chain. Results from this study is consistent with a study conducted by Aslan *et al*., 2018 in wastewater treatment effluents used an irrigation water source. Similar to this study, high resistance to antibiotics tested .i.e. Ampicillin, Sulfamethoxazole, Ciprofloxacin and Tetracycline was recorded in *E. coli* isolated from effluents. Studies have shown that consumers are at risk of being exposed to and consuming antibiotic resistant bacteria in raw produce irrigated with treated wastewater (Pina *et al*., 2018).

There is also a major health risk to farm workers as they may be exposed to antibiotic resistant bacteria through contact with wastewater effluent. A study conducted by Goldstein (2013) detected Vancomycin Resistant *Enterococci* and Methicillin Resistant *Staphylococcus aureus* were found in 26% and 29% of spray irrigation workers respectively.

High antibiotic resistance found in this study is of great concern and emphasises the importance of monitoring the quality of wastewater for irrigation purposes with respect to antibiotic resistance bacteria and the need to improve the wastewater treatment process to remove antibiotic resistant bacteria.

5.6 Implications for guidelines and standards for compliance in effluent quality discharge into the environment and agriculture.

This study shows the presence of bacterial isolates with high resistance to antibiotics tested as well as multiple resistant *E. coli*, *A. hydrophila* and *P. aeruginosa*. Currently microbiological guidelines on wastewater discharge and the safe reuse of wastewater in agriculture exists for indicator microorganisms (Ghana EPA, 2010; WHO, 2006). However, these guidelines do not include critical values for antibiotic resistance bacteria for wastewater discharge or reuse in agriculture. Results from this study confirms the need for revision of these guidelines to include monitoring of antibiotic resistance bacteria due to the possible risk of antibiotic resistant bacteria entering the food chain from contaminated irrigation water. *E. coli* has been suggested for monitoring antibiotic resistance in wastewater and surface water (Gekenidis *et al*., 2018).

This study sought to investigate the presence of antibiotic resistant *P. aeruginosa*, *E. coli* and *A. hydrophila* in the Legon Sewerage Treatment Plant and the receiving Onyasia stream using bacterial resistance data produced by culture-dependent methods and the environmental health implications of the treatment plant discharge. Findings show although there was a significant reduction in levels of *P. aeruginosa*, *E. coli* and *A.*

hydrophila in effluent being discharged from the treatment plant as well high isolate resistant to antibiotics tested. The general objective of the study was achieved.

This study contributes to the knowledge gap about antibiotic resistance in wastewater discharge and wastewater impacted environment in Ghana.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study has provided a basis for the need for further treatment of wastewater before discharge and reuse as well as surveillance of antibiotic resistant bacteria in treated effluent and the receiving stream.

The following major conclusions were reached during the conduct of this study.

- Legon Sewage Treatment plant has been proven to significantly reduce bacterial loads after treatment. However, effluent quality did not meet Ghana EPA guideline limit for wastewater discharge.

- Findings show treated wastewater from the Legon Sewage Treatment Plant meets the WHO guideline limit for unrestricted ($<10^5$ *E.coli*/100 ml) and unrestricted irrigation ($<10^3$ *E.coli*/100 ml). However, it contains *E. coli*, *P. aeruginosa and A. hydrophila* resistant to most of antibiotics tested as well as high multiple resistant bacteria.
- Farm hands are therefore at risk of acquiring resistant infections during irrigation. Antibiotic resistant bacteria could also enter the food chain through effluent reuse for irrigation.

- The relative location of sampling points upstream and downstream of the Legon Sewage Treatment Plant discharge points had a significant impact on the

occurrence of antibiotic resistant *P. aeruginosa*, *E. coli*, and *A. hydrophila* as resistance was higher downstream compared to upstream.

- This finding shows that the Legon Sewerage Treatment Plant potentially contributes to the spread and persistence of antibiotic resistant bacteria in the Onyasia stream.

- Resistant *E. coli*, *P. aeruginosa and A. hydrophila* were found upstream of the treatment plant suggesting other sources of antibiotic resistance in the stream before treatment. As such results do not conclusively identify the Legon Sewage treatment plant discharge as the only source of increased rates of resistance seen in this study.

- There are human health implications on the use of the Onyasia stream as an irrigation water source by farmers downstream as high bacterial resistance was detected in water samples.

- Farmers who come in contact with the irrigation water as well as consumers of raw vegetables irrigated with contaminated water are at risk of exposure to resistant pathogens.

6.2 RECOMMENDATIONS

To help preserve the quality of water and mitigate the spread of antibiotic resistance in the receiving Onyasia Stream, the efficacy of Legon Sewerage Treatment Plant needs to be improved.

- There is a need for continuous surveillance of antibiotic resistance bacteria in Legon Sewerage Treatment Plant by the Ghana Environmental Protection Agency and the Water Research Institute as faecal bacteria in wastewater can be used as an early warning system to detect emerging resistance trends in the population, to provide insights into the burden of faecal carriage.

- Culture based methods and molecular tools may be used in tandem to ensure more accurate characterization of the abundance and transfer of antibiotic resistance genes.

- In order to help reduce the risk associated with exposure of antibiotic resistant bacteria in treated wastewater to farmers and through the food chain, farmers should be trained on good agricultural and handling practices.

- The Onyasia Stream is used untreated for irrigation and can pose a significant health risk to farmers and consumers through direct contact with contaminated wastewater, breathing in aerosols and consumption of vegetables. The Ministry of Agriculture, should encourage farmers through education campaigns, to use

protective equipment when handling stream water. Stream water should be used to irrigate vegetables not consumed raw.

- The WHO Guidelines for the Safe Use of Wastewater, Excreta and Greywater: Wastewater Use in Agriculture and Ghana Environmental Protection Agency standards for wastewater discharge should be revised to include critical values for antibiotic resistant bacteria.
- *E. coli* could serve as one of the organisms used an indicator for screening antibiotic bacteria in wastewater treatment plants.

Further Research

- Further research is needed to investigate health risks associated with wastewater harbouring antibiotic resistant bacteria used in agriculture, aquaculture, recreational and other non-critical uses.

- A quantitative microbial risk assessment (QRMA) is needed to highlight the risks associated with consuming produce irrigated with treated wastewater from the treatment plant as well as water from the Onyasia Stream.

- Further research is needed to be conducted to identify other possible sources of antibiotic resistant bacteria and antibiotics in the Onyasia Stream as some resistant bacteria were found upstream of the treatment.

- Resistance profile of bacteria in surface water and sediment may vary, as such further research is needed on prevalence of resistant bacteria in the sediments of the Onyasia stream.

- There is also the need for standardization of techniques for evaluation of antibiotic resistance in the environment. This will be useful in understanding the mechanism of resistance in wastewater treatment plants and the receiving environment.

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APPENDICES

APPENDIX I

AEROMONAS ISOLATION MEDIUM COMPOSITION

Ingredients	Grams/Litre
Peptone, special	5.0
Yeast extract	3.0
L-Lysine hydrochloride	3.5
L-Arginine hydrochloride	2.0
Inositol	2.5
Lactose	1.5
Sorbose	3.0
Xylose	3.75
Bile salts	3.0
Sodium thiosulfate	10.67
Sodium chloride	5.0

Ferric ammonium citrate	0.8
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	12.5

Final pH 7.0 ± 0.2 at 25°C

APPENDIX II

E. COLI ISOLATION MEDIUM COMPOSITION

Ingredients	Grams/Litre
Chromocult® Coliform Agar	
Enzymatic Digest of Casein	1
Yeast Extract	2
NaCl	5
NaH2PO4 x 2 H2O	2.2
Na2HPO4	2.7
Sodium Pyruvate	1
Sorbitol	1
Tryptophane	1
Tergitol® 7	0.15
6-Chloro-3-indoxyl-beta-Dgalactopyranoside	0.2
5-Bromo-4-chloro-3-indoxyl-β-Dglucuronic acid	0.1
Isopropyl-beta-Dthiogalactopyranoside	0.1

Agar-agar*	10

APPENDIX III

PSEUDOMONAS ISOLATION MEDIUM COMPOSITION

Ingredients	Grams / Litre
Gelatin peptone	20.000
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000

APPENDIX IV

PLATES SHOWING SAMPLE COLLECTION AND ANALYSIS

Plate 6: A photograph showing sample collection

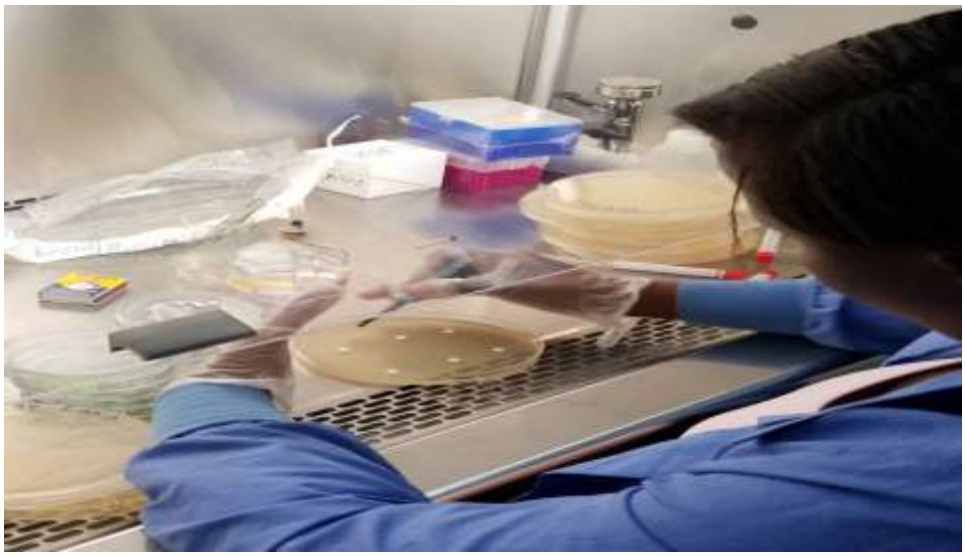

Plate 7: A photograph showing antibiotic sensitivity test being carried out using Kirby Bauer Disc diffusion method

APPENDIX V

PLATES SHOWING BACTERIAL CULTURE PLATES

Plate 8: A photograph showing culture plates showing *P. aeruginosa* (left) and *E. coli* colonies (right)

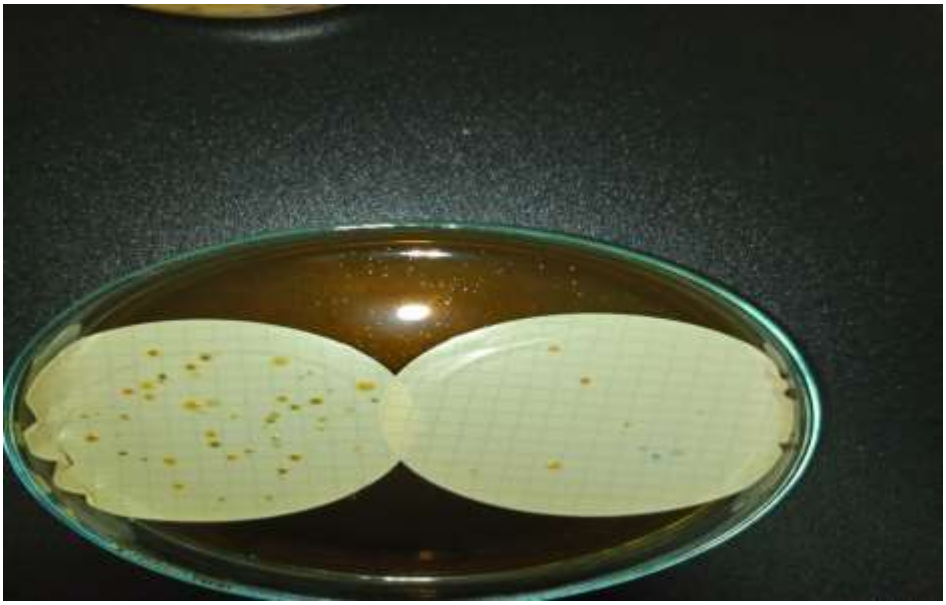

Plate 9: A photograph of culture plates showing *A. hydrophila* colonies

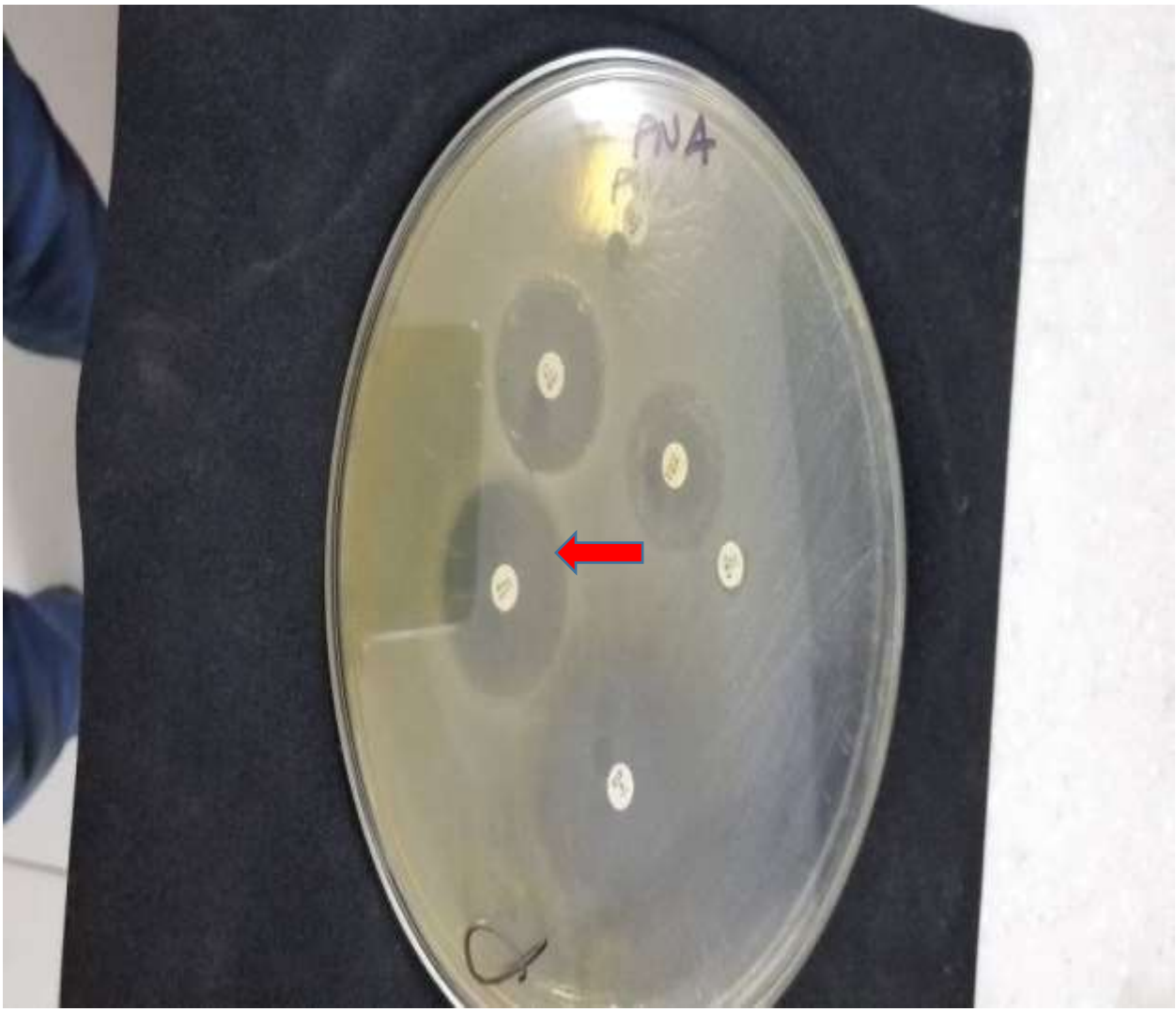

Plate 10: A photograph showing antibiotic sensitivity tests of *A. hydrophila.* Note the zones of inhibition

APPENDIX VI

SOME BIOCHEMICAL TESTS CONDUCTED ON ISOLATES

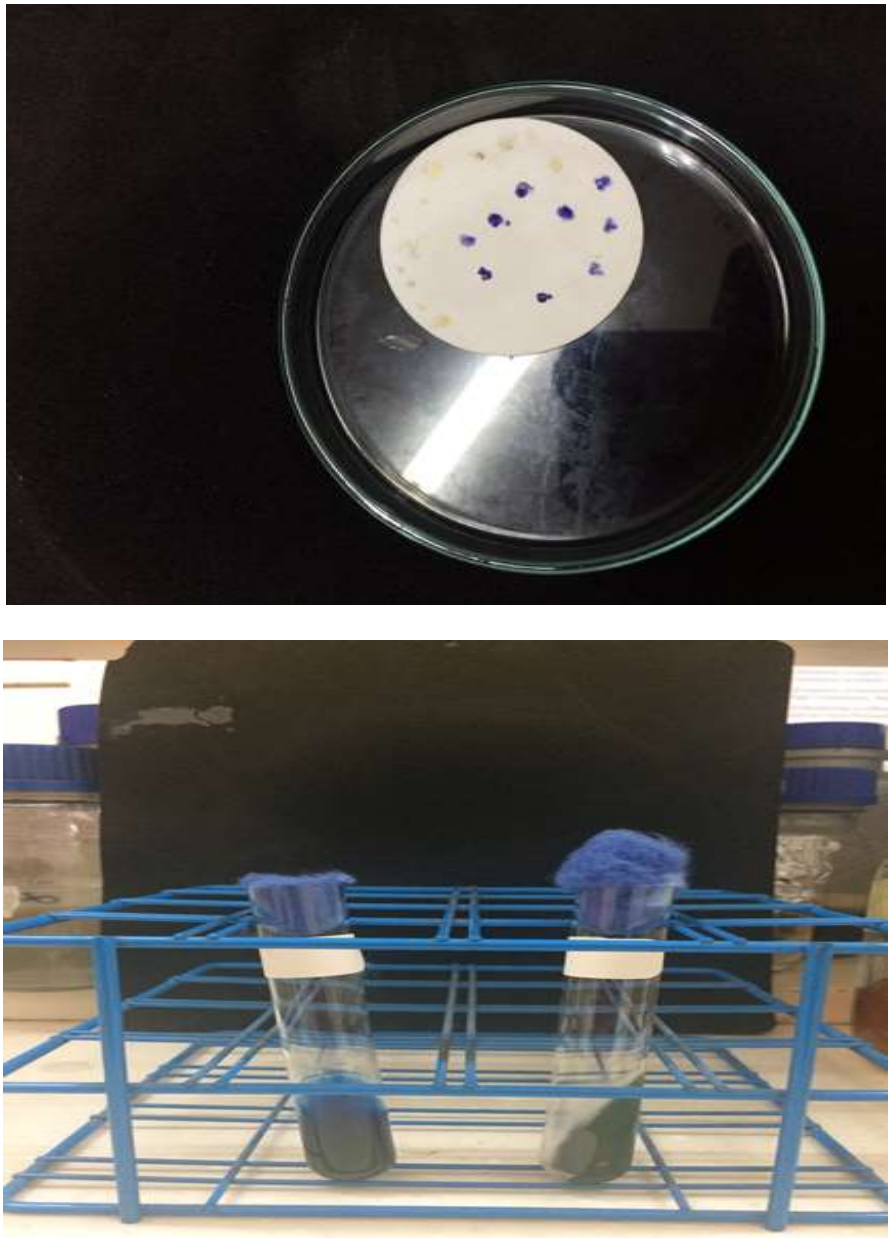

Plate 11: A set up showing Oxidase positive (Left) and Citrate positive (Right) tests

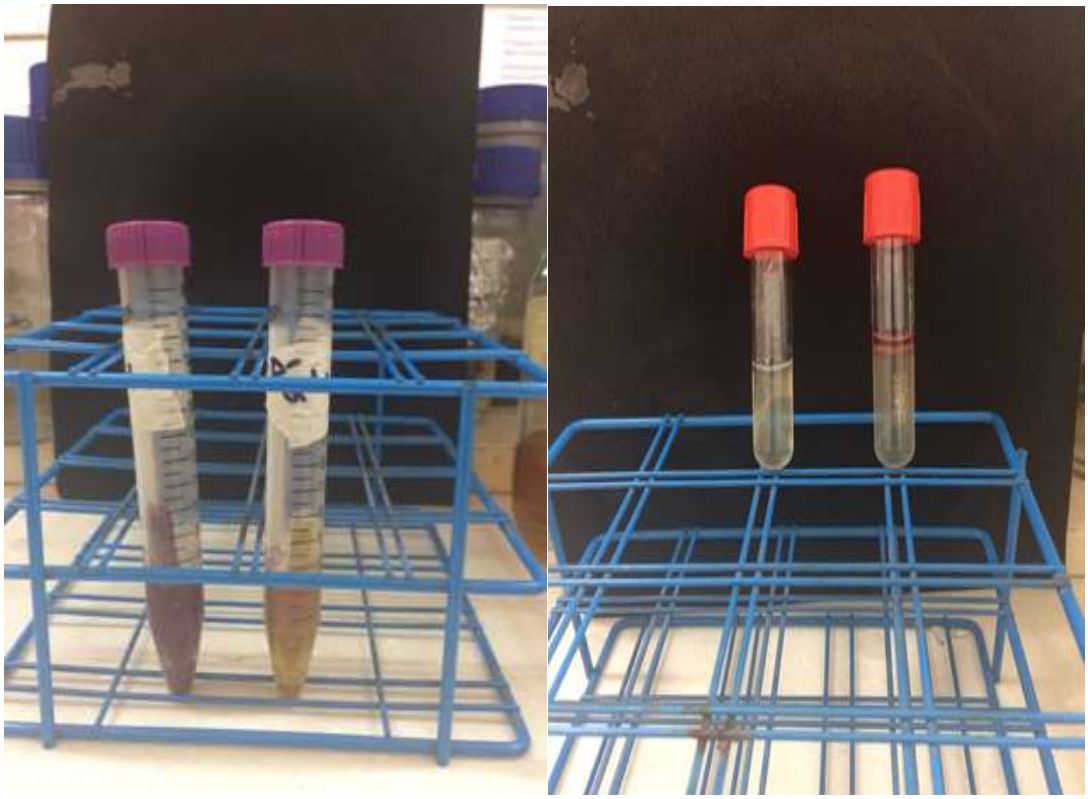

Plate 12: A set up showing glucose fermentation (Left) and Sulphide Indole Motility (SIM) positive (Right) tests

APPENDIX VII

WATER SAMPLING SITES AND COORDINATES

SITE NUMBER	SAMPLE SITE	COORDINATES
1	Treatment plant Influent	N 05° 39’ 44.9’’ W 000° 11.28. 0’’
2	Treatment plant effluent	N 05° 39’ 56. 0’’ W 000° 11 28. 7’’
3	Upstream (500 meters before the treatment plant discharge point)	N 05° 39’ 58. 8’’ W 000° 11 20. 1’’
4	Outfall (Discharge point)	N 05° 40’ 00. 5’’ W 000° 11 27. 2’’
5	Downstream(500 meters after the treatment plant discharge point)	N 05° 40’ 08. 4’’ W 000° 11 31. 0’’

APPENDIX VIII

BACTERIAL COUNTS, ANTIBIOTIC SENSITIVITY PATTERNS AND BIOCHEMICAL IDENTIFICATION

Negative control (blanks) analysed alongside water and wastewater samples

MONTH	Blank		
	Chromocult agar	*Aeromonas* agar	Cetrimide agar
JANUARY	>1	>1	>1
FEBRUARY	>1	>1	>1
MARCH	>1	>1	>1
APRIL	>1	>1	>1
MAY	>1	>1	>1
JUNE	>1	>1	>1

Levels of *E. coli*, *A. hydrophila* and *P. aeruginosa* in wastewater and surface water at the sampling sites.

	E.coli CFU/100ml							
SAMPLE ID	**January**	**February**	**March**	**April**	**May**	**June**	**Mean**	**Mean log**
Influent	100,000,000	80,000,000	260,000,000	90,000,000	13,600,000	70,000,000	102,266,667	7.88
Effluent	100	2,000	1,300	560	200	100	710	2.58
Outfal	60,000	3,000	2,000	3,000	1,800	1,600	11,900	3.58
Upstream	2,000	1,800	1,000	430	300	200	955	2.83
Downstream	3,000,000	6,400,000	8,000,000	600,000	170,000	90,000	3,043,333	6.02
***P. aeruginosa* CFU/100ml**								
Influent	22,000,000	3,400,000	3,100,000	300,000	2,900,000	2,300,000	5,666,667	6.44
Effluent	1,000	2,000	2,000	2,000	1,200	1,100	1,550	3.17
Outfal	30,000	6,000	5,000	3,000	2,200	2,000	8,033	3.68
Upstream	4,000	3,000	2,000	2,000	1,800	1,300	2,350	3.34
Downstream	1,600,000	1,100,000	900,000	98,000	74,000	74,000	641,000	5.49
***A. hydrophila* CFU/100ml**								
Influent	372,000	376,000	270,000	440,000	400,000	400,000	376,333	5.57
Effluent	17,000	17,300	10,000	5,320	4,000	4,000	9,603	3.90
Outfall	87,000	58,400	58,000	50,000	40,000	20,000	52,233	4.68

Upstream	32,000	34,000	28,000	30,000	15,000	7,600	24,433	4.34
Downstream	2,100,000	9,400,000	1,800,000	1,720,000	100,000	100,000	2,536,667	5.96

Levels of total coliform in wastewater

Total coliforms CFU/100ml								
Sample ID	January	February	March	April	May	June	Mean	Mean Log
Influent	100000000	90000000	280000000	96000000	16000000	88000000	111666667	7.92
Effluent	1080	3200	2800	1650	1000	1280	1835	3.26

***A. hydrophila* breakpoint values for interpretation of zone diameters as stated by European Committee on Antimicrobial Susceptibility Testing CLSI (2018)**

INFERENCE	GEN	AMC	TET	CIP	IPM	CXM	ATM
	Diameter of zones of inhibition						
S ≥	15	18	15	21	16	18	21
R <	12	13	11	15	13	14	17

TET: Tetracycline 30 µg; AMC: Amoxicillin Clavulanate 20 µg; CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CXM: Cefuroxime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

***E. coli* breakpoint values for interpretation of zone diameters as stated by European Committee on Antimicrobial Susceptibility Testing CLSI (2017)**

INFERENCE	GEN	AMC	TET	CIP	IPM	CXM	ATM
	Diameter of zones of inhibition						
S ≥	15	18	15	21	23	23	21
R <	12	13	11	15	19	14	17

TET: Tetracycline 30 µg; AMC: Amoxicillin Clavulanate 20 µg; CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CXM: Cefuroxime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

P. aeruginosa **breakpoint values for interpretation of zone diameters as stated by European Committee on Antimicrobial Susceptibility Testing CLSI (2017)**

INFERENCE	GN	CIP	IMP	ATM	CAZ
	Diameter of zones of inhibition				
S ≥	15	21	19	22	18
R <	12	15	15	15	14

CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µ; IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

E. coli **antibiotic sensitivity quality control analysis**

MONTH	ISOLATE	ANTIBIOTICS						
		GN	AMC	TET	CIP	IPM	CXM	ATM
JANUARY	*E. coli* ATCC 25922	18	20	23	32	26	22	29
FEBRUARY	*E. coli* ATCC 25923	19	20	24	32	26	22	28
MARCH	*E. coli* ATCC 25924	19	19	23	31	26	22	28

APRIL	*E. coli* ATCC 25925	19	19	23	31	27	21	28
MAY	*E. coli* ATCC 25926	19	20	23	31	27	21	28
JUNE	*E. coli* ATCC 25927	19	20	24	31	26	21	28

TET: Tetracycline 30 µg; AMC: Amoxicillin Clavulanate 20 µg; CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CXM: Cefuroxime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

***P. aeruginosa* antibiotic sensitivity quality control analysis**

MONTH	ISOLATE	ANTIBIOTICS				
		GN	CIP	IMP	ATM	CAZ
JANUARY	***P. aeruginosa*** ATCC 29214	19	31	20	24	25
FEBRUARY	***P. aeruginosa*** ATCC 29215	19	31	21	24	25
MARCH	***P. aeruginosa*** ATCC 29216	20	31	21	24	24
APRIL	***P. aeruginosa*** ATCC 29217	20	32	20	23	25
MAY	***P. aeruginosa*** ATCC 29218	20	32	20	23	25
JUNE	***P. aeruginosa*** ATCC 29219	20	31	21	24	25

JUNE	***P. aeruginosa*** ATCC 29220	20	30	21	23	24

CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CAZ: Ceftadzidime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg

Susceptibility patterns of *Aeromonas hydrophila* isolates against the selected antibiotics

Aeromonas hydrophila zones of inhibition measured in millimeters (mm)																	
	INFLUENT ISOLATES																
ISOLATE ID	ANTIBIOTICS/INTERPRETATION																
	GN	IN	AMC	IN	TET	IN	CIP	IN	IMP	IN	CXM	IN	ATM	IN	CAZ	IN	MULTIPLE DRUG RESISTANCE
JBAA	20	S	25	S	10	R	18	I	26	S	12	R	17	R	6	R	4
JBBA	19	S	11	R	6	R	21	S	25	S	17	I	20	I	7	R	3
JBCA	24	S	10	R	6	R	26	S	24	S	12	R	10	R	12	R	4
JBGA	18	S	6	R	7	R	27	S	22	S	6	R	21	S	17	R	4
JBQA	26	S	11	R	7	R	25	S	22	S	6	R	9	R	25	S	4
FBAA	14	R	13	R	11	R	20	I	13	R	26	S	32	S	31	S	4
FBBA	17	S	20	S	14	I	24	S	20	S	33	S	25	S	20	I	0
FBCA	14	R	16	I	6	R	25	S	20	S	15	S	31	S	18	I	2
FBFA	20	S	21	S	11	R	25	S	20	S	30	R	25	S	31	S	2
FBGA	18	S	7	R	11	R	20	I	21	S	25	S	19	I	22	S	2
MBA	18	S	16	I	12	I	20	I	13	R	24	R	17	I	7	R	3
MBB	14	R	13	R	12	I	20	I	13	R	20	R	22	S	17	R	4
MBC	17	S	6	R	6	R	10	R	23	S	6	R	17	S	7	R	4
MBD	18	S	6	R	6	R	8	R	23	S	6	R	22	R	17	R	4
MBE	19	S	22	S	6	R	10	R	25	S	6	R	22	R	17	R	4
ABA	20	S	24	S	7	R	8	R	25	S	6	R	20	I	11	R	4
ABB	19	S	25	S	6	R	10	R	25	S	6	R	19	I	8	R	4

ABC	20	S	15	S	25	S	19	I	27	S	23	S	32	S	25	S	0
ABD	18	S	12	R	6	R	9	R	24	S	6	I	9	S	13	S	3
ABE	15	R	6	S	7	R	19	I	23	S	6	S	24	S	15	S	2
MBA	26	S	17	I	30	S	30	S	35	S	6	R	25	S	30	S	1
MBB	20	S	21	S	23	S	23	S	30	S	23	S	30	S	26	S	0
MBC	17	S	18	S	14	I	14	R	25	S	8	R	29	S	26	S	2
MBD	19	S	20	S	21	I	22	S	31	S	21	S	31	S	25	S	0
MBE	17	S	17	I	14	I	14	R	27	S	7	R	28	S	24	S	2
JBAA	17	S	6	R	6	R	10	R	23	S	6	R	17	S	7	R	4
JBBA	18	S	6	R	6	R	8	R	23	S	6	R	22	S	17	R	4
JBCA	19	S	22	S	6	R	10	R	25	S	6	R	22	S	17	R	4
JBDA	18	S	12	R	6	R	9	R	24	S	6	R	9	R	13	R	4
JBEA	15	S	6	R	7	R	14	R	20	S	6	R	16	R	15	R	4
ISOLATE ID	EFFLUENT																
	ANTIBIOTICS/INTERPRETATION																
	GN	IN	AMC	IN	TET	IN	CIP	IN	IMP	IN	CXM	IN	ATM	IN	CAZ	IN	
JAA	23	S	15	I	15	I	27	S	26	S	6	R	17	R	25	S	2
JAB	24	S	18	I	15	I	29	S	27	S	9	R	20	I	24	S	1
JAC	20	S	14	I	11	R	30	S	29	S	6	R	32	S	21	S	2
FAA	29	S	17	I	20	S	30	S	30	S	7	R	21	S	30	S	1
FAB	18	S	14	I	11	R	25	S	31	S	28	S	22	S	10	R	2
FAC	17	S	14	I	11	R	26	S	18	S	31	S	13	R	31	S	2
AAE	20	S	16	I	20	S	30	S	20	S	6	R	22	S	25	S	1
JAD	21	S	14	R	17	S	30	S	23	S	6	R	21	S	13	R	3
FAB	20	S	7	R	23	S	15	R	20	S	25	S	23	S	12	R	3
FAD	19	S	13	R	25	S	30	S	19	S	27	S	30	S	30	S	1
MAA	18	S	11	R	20	S	30	S	21	S	25	S	31	S	31	S	1
MAB	13	R	13	R	25	S	26	S	17	S	25	S	18	I	27	S	2
MAC	15	R	11	R	23	S	30	S	19	S	22	S	21	S	28	S	2
MAD	16	S	7	R	25	S	27	S	18	S	30	S	17	I	32	S	1
MAE	17	S	9	R	20	S	20	I	8	R	13	R	21	S	17	R	4

AAA	14	I	10	R	25	S	31	S	13	R	6	R	20	S	25	S	3
AAB	20	S	12	R	24	S	30	S	12	R	6	R	17	I	25	S	3
AAC	23	S	12	R	23	S	32	S	14	S	6	R	13	R	27	S	3
AAD	14	I	10	R	20	S	32	S	12	S	6	R	15	R	30	S	3
MAA	20	S	11	R	22	S	30	S	15	S	23	S	22	S	27	S	1
MAB	20	S	12	R	27	S	30	S	18	S	27	S	35	S	30	S	1
MAC	20	S	10	R	30	S	35	S	13	R	20	S	12	R	30	S	3
MAD	14	I	8	R	11	R	20	I	8	R	14	R	21	S	25	S	4
MAE	20	S	8	R	20	S	20	I	9	R	13	R	29	S	14	R	4
JAA	21	S	10	R	28	S	30	S	13	R	23	S	15	R	29	S	3
JAB	15	S	9	R	28	S	25	S	14	S	24	R	30	S	28	S	2
JAC	21	S	8	R	27	S	29	S	11	R	20	S	11	S	27	S	2
JAD	22	S	7	R	30	S	35	S	11	R	18	S	20	S	26	S	2
JAE	21	S	12	R	29	S	40	S	13	R	22	S	21	S	28	S	2
JAE	17	S	13	S	15	I	25	S	30	S	6	R	19	I	23	S	1
	UPSTREAM ISOLATES																
ISOLATE ID	ANTIBIOTICS/INTERPRETATION																
	GN	IN	AMC	IN	TET	IN	CIP	IN	IMP	IN	CXM	IN	ATM	IN	CAZ	IN	
JOAA	20	S	13	R	11	R	32	S	19	S	12	R	30	S	30	S	3
JOBA	15	S	20	S	21	S	30	S	18	S	25	S	32	S	30	S	0
JOCA	18	S	26	S	27	S	24	S	12	R	25	S	33	S	28	S	1
JOD	26	S	24	S	21	S	24	S	22	S	24	S	17	R	26	S	1
JOE	21	S	12	R	25	S	25	S	23	S	29	S	30	S	28	S	1
FOA	20	S	17	I	30	S	26	S	20	S	30	S	34	S	30	S	0
FOB	20	S	19	S	25	S	30	S	20	S	30	S	17	R	26	S	1
FOC	20	S	24	S	26	S	22	S	17	S	30	S	32	S	29	S	0
FOD	15	S	25	S	23	S	20	I	11	R	30	S	30	S	26	S	1
FOE	20	S	8	R	25	S	26	S	13	R	35	S	30	S	30	S	2
MOA	18	S	8	R	18	S	21	S	25	S	30	S	34	S	26	S	1
MOB	20	S	20	S	26	S	25	S	12	R	28	S	30	S	28	S	3
MOC	14	I	13	R	11	R	20	I	24	S	21	S	35	S	30	S	2

MOD	22	S	6	R	35	S	17	I	18	S	6	R	25	S	26	S	2
MOE	13	I	9	R	25	S	18	I	19	S	28	S	33	S	28	S	1
AOA	16	S	13	R	27	S	35	S	22	S	14	R	35	S	28	S	2
AOB	18	S	15	I	28	S	32	S	10	R	6	R	30	S	30	S	2
AOC	15	S	9	R	28	S	35	S	20	S	6	R	30	S	30	S	2
AOD	6	R	6	R	10	R	28	S	17	S	6	R	32	S	27	S	4
AOE	20	S	11	R	29	S	34	S	24	S	7	R	20	I	28	S	2
MOA	17	S	11	R	28	S	33	S	25	S	29	S	34	S	26	S	1
MOB	19	S	14	I	28	S	34	S	27	S	25	S	35	S	28	S	0
MOC	18	S	11	R	30	S	38	S	14	S	6	R	17	R	30	S	3
MOD	18	S	12	R	27	S	34	S	27	S	6	R	23	S	30	S	2
MOE	18	S	16	I	30	S	38	S	14	S	9	R	17	R	28	S	2
JOA	22	S	11	R	15	I	25	S	26	S	30	S	30	S	30	S	1
JOB	21	S	14	I	17	S	30	S	28	S	30	S	35	S	29	S	0
JOC	23	S	11	R	19	S	25	S	22	S	23	S	30	S	29	S	1
JOD	20	S	14	I	17	S	32	S	30	S	20	S	31	S	32	S	0
JOE	24	S	12	R	20	S	25	S	22	S	13	R	30	S	17	R	3
	OUTFAL ISOLATES																
ISOLATE ID	ANTIBIOTIC/INTERPRETION																
	GN	IN	AMC	IN	TET	IN	CIP	IN	IMP	IN	CXM	IN	ATM	IN	CAZ	IN	
JUAA	20	S	15	S	24	S	30	S	13	S	25	S	30	S	29	S	1
JUBA	19	S	20	S	27	S	34	S	20	S	30	S	35	S	27	S	0
JUCA	20	S	19	S	29	S	35	S	20	S	28	S	27	S	30	S	0
JUD	18	S	16	R	20	S	25	S	16	S	21	S	36	S	32	S	0
JUE	16	S	20	S	25	S	29	S	17	S	22	S	30	S	28	S	0
FUA	15	S	20	S	24	S	30	S	15	S	26	S	29	S	34	S	0
FUB	20	S	20	S	22	S	30	S	19	S	30	S	28	S	28	S	0
FUC	20	S	16	S	23	S	25	S	15	S	21	S	36	S	29	S	0
FUD	21	S	7	R	22	S	26	S	15	S	22	S	35	S	24	S	1
FUE	15	S	22	S	20	S	26	S	20	S	30	S	32	S	29	S	0
MUA	16	S	20	S	22	S	30	S	30	S	29	S	36	S	30	S	0

MUB	20	S	24	S	24	S	35	S	35	S	21	S	32	S	26	S	0
MUC	21	S	16	S	25	S	29	S	22	S	22	S	28	S	27	S	0
MUD	19	S	18	S	29	S	29	S	20	S	25	S	27	S	32	S	0
MUE	22	S	20	S	30	S	31	S	18	S	29	S	32	S	31	S	0
AUA	16	S	12	R	25	S	30	S	18	S	29	S	30	S	27	S	1
AUB	17	S	13	R	28	S	29	S	25	S	30	S	30	S	30	S	1
AUC	18	S	15	S	25	S	32	S	29	S	30	S	29	S	27	S	0
AUD	16	S	14	R	25	S	32	S	17	S	29	S	35	S	30	S	0
AUE	17	S	16	S	30	S	25	S	26	S	31	S	35	S	28	S	0
MUA	21	S	15	R	30	S	40	S	20	S	30	S	40	S	35	S	0
MUB	20	S	6	R	35	S	23	S	26	S	6	R	35	S	32	S	2
MUC	21	S	14	S	28	S	35	S	21	S	30	S	35	S	28	S	0
MUD	20	S	14	S	32	S	29	S	26	S	7	R	30	S	25	S	1
MUE	21	S	14	S	28	S	35	S	21	S	30	S	35	S	28	S	0
JUAA	17	S	13	R	15	I	26	S	21	S	30	S	36	S	30	S	1
JUBA	17	S	15	S	34	S	41	S	20	S	30	S	40	S	29	S	0
JUCA	20	S	13	S	34	S	35	S	15	S	30	S	20	S	29	S	1
JUD	17	S	15	S	11	R	26	S	22	S	32	S	38	S	29	S	1
JUE	17	S	15	S	11	R	26	S	22	S	32	S	38	S	29	S	1
DOWNSTREAM ISOLATES																	
ISOLATE ID	ANTIBIOTICS/INTERPRETATION																
	GN	IN	AMC	IN	TET	IN	CIP	IN	IMP	IN	CXM	IN	ATM	IN	CAZ	IN	
JDA	25	S	14	I	7	R	20	I	13	R	12	R	28	S	30	S	2
JDB	24	S	6	R	20	S	21	S	20	S	10	R	29	S	28	S	2
JDC	25	S	11	R	20	S	25	S	24	S	25	S	30	S	26	S	1
JDD	24	S	8	R	20	S	30	S	26	S	8	R	31	S	33	S	2
JDE	20	S	13	R	28	S	34	S	24	S	25	S	32	S	31	S	1
FDA	17	S	8	R	17	S	25	S	26	S	30	S	30	S	29	S	1
FDB	20	S	13	R	15	S	25	S	25	S	25	S	27	S	25	S	1
FDC	23	S	8	R	20	S	35	S	30	S	25	S	35	S	27	S	1
FDD	19	S	12	R	20	S	23	S	30	S	20	S	16	R	17	R	3

FDE	20	S	7	R	26	S	25	S	23	S	30	S	35	S	30	S	1
MDA	22	S	9	R	18	S	23	S	20	S	6	R	16	R	31	S	3
MDB	22	S	10	R	25	S	30	S	25	S	25	S	32	S	16	R	2
MDC	20	S	12	R	25	S	32	S	20	S	30	R	14	R	28	S	3
MDD	15	S	11	R	11	R	33	S	23	S	6	R	30	S	29	S	3
MDE	18	S	6	R	26	S	21	S	14	R	10	R	25	S	31	S	2
ADA	20	S	10	R	9	R	33	S	20	S	28	S	28	S	30	S	2
ADB	18	S	11	R	10	R	30	S	20	S	22	S	14	R	27	S	3
ADC	18	S	12	R	9	R	31	S	13	R	30	S	25	S	25	S	3
ADD	19	S	11	R	8	R	26	S	20	S	18	S	16	R	30	S	3
ADE	19	S	10	R	8	R	17	I	14	R	26	S	28	S	25	S	2
MDA	15	S	10	R	10	R	18	I	13	R	25	S	27	S	24	S	3
MDB	20	S	9	R	33	S	40	S	21	S	30	S	25	S	27	S	1
MDC	25	S	19	S	28	S	27	S	40	S	10	R	16	R	31	S	2
MDD	16	S	17	S	24	S	40	S	30	S	30	S	25	S	27	S	0
MDE	24	S	20	S	29	S	35	S	34	S	11	R	16	R	32	S	2
JDA	15	S	18	S	13	I	25	S	15	R	6	R	15	R	24	S	2
JDB	16	S	12	R	13	I	20	I	15	R	6	R	17	R	17	R	3
JDC	21	S	13	R	25	S	30	S	18	S	8	R	20	I	20	I	1
JDD	12	R	20	S	22	S	22	S	22	S	9	R	18	I	17	R	3
JDE	12	R	20	S	26	S	21	I	13	R	8	R	20	I	20	I	3

TET: Tetracycline 30 µg; AMC: Amoxicillin Clavulanate 20 µg; CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CXM: Cefuroxime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

Susceptibility of *P. aeruginosa* isolates against the selected antibiotics

***P. aeruginosa* Zones of inhibition measured in millimeters (mm)**											
INFLUENT ISOLATES											
ISOLATE ID	**ANTIBIOTICS/INTERPRETATION**										**MULTIPLE DRUG RESISTANCE**
	GN	**IN**	**CIP**	**IN**	**IMP**	**IN**	**ATM**	**IN**	**CAZ**	**IN**	
JBA	20	S	27	S	35	S	27	S	29	S	0
JBB	23	S	16	I	27	S	30	S	31	S	0
JBC	23	S	16	I	27	S	25	S	30	S	0
JBD	18	S	26	S	20	S	30	S	14	I	0
JBE	6	R	6	R	30	S	20	S	15	I	2
FBA	17	S	7	R	30	S	23	S	35	S	1
FBB	18	S	8	R	29	S	27	S	14	I	1
FBC	18	S	30	S	23	S	24	S	30	S	0
FBD	8	R	6	R	30	S	15	R	24	S	3
FBE	20	S	6	R	30	S	29	S	27	S	1
MBA	19	S	30	S	18	S	20	I	25	S	1
MBB	18	S	13	R	20	S	8	R	14	R	3
MBC	18	S	29	S	24	S	20	I	22	S	0

MBD	18	S	30	S	19	S	20	I	25	S	0
MBE	18	S	30	S	20	S	21	I	25	S	0
ABA	20	S	31	S	26	S	25	S	25	R	1
ABB	15	S	26	S	24	S	24	S	24	R	1
ABC	15	S	27	S	27	S	25	S	25	S	0
ABD	21	S	30	S	27	S	27	S	24	S	0
ABE	15	S	26	S	25	S	23	S	23	S	0
MABA	16	S	28	S	21	S	22	S	22	S	0
MABB	17	S	29	S	21	S	23	S	29	S	0
MABC	17	S	31	S	24	S	22	S	21	S	0
MABD	18	S	29	S	20	S	24	S	22	S	0
MABE	17	S	30	S	21	S	23	S	31	S	0
JBA	20	S	20	S	20	S	32	S	27	S	0
JBB	18	S	13	R	23	S	20	S	23	S	1
JBC	20	S	30	S	17	R	14	R	22	S	2
JBD	17	S	27	S	25	S	18	I	25	S	0
JBE	22	S	30	S	30	S	14	R	26	S	1
FINAL EFFLUENT ISOLATES											

ISOLATE ID	ANTIBIOTICS/INTERPRETATION										
	GN	IN	CIP	IN	IMP	IN	ATM	IN	CAZ	IN	
JAA	19	S	30	S	20	S	23	S	25	S	0
JAB	24	S	35	S	25	S	26	S	23	S	0
JAC	20	S	34	S	24	S	27	S	29	S	0
JAD	18	S	23	S	20	S	23	S	23	S	0
JAE	19	S	30	S	24	S	27	S	22	S	0
FAA	20	S	15	R	28	S	10	R	25	S	1
FAB	21	S	26	S	29	S	30	S	24	S	0
FAC	20	S	25	S	25	S	31	S	27	S	0
FAD	19	S	35	S	25	S	26	S	28	S	0
FAE	15	S	26	S	26	S	23	S	28	S	0
MAA	15	S	15	R	20	S	22	S	25	S	1
MAB	20	S	14	R	21	S	10	R	28	S	2
MAC	16	S	25	S	21	S	18	I	22	S	0
MAD	15	S	29	S	23	S	22	S	23	S	0
MAE	15	S	25	S	23	S	21	I	20	S	0
AAA	27	S	30	S	24	S	21	I	28	S	0

AAB	20	S	25	S	22	S	30	S	25	S	0
AAC	8	R	26	S	6	R	6	R	14	R	4
AAD	23	S	30	S	8	R	10	R	29	S	2
AAE	8	R	25	S	21	S	25	S	27	S	1
MAA	13	R	30	S	24	S	20	I	24	S	1
MAB	25	S	35	S	20	S	21	I	23	S	0
MAC	14	I	30	S	21	S	22	S	26	S	0
MAD	28	S	35	S	20	S	22	S	21	S	0
MAE	14	I	30	S	21	S	21	I	28	S	0
JAA	18	S	23	S	20	S	22	S	22	S	0
JAB	19	S	30	S	24	S	23	S	29	S	0
JAC	20	S	13	R	28	S	22	S	21	S	1
JAD	21	S	26	S	29	S	23	S	22	S	0
JAE	20	S	25	S	25	S	22	S	20	S	0
	OUTFAL ISOLATES										
ISOLATE ID	**ANTIBIOTICS/INTERPRETATION**										
	GN	**IN**	**CIP**	**IN**	**IMP**	**IN**	**ATM**	**IN**	**CAZ**	**IN**	
JOA	15	S	30	S	25	S	33	S	30	S	0

JOB	19	S	34	S	24	S	30	S	31	S	0
JOC	19	S	33	S	19	S	31	I	25	S	0
JOD	16	S	34	S	29	S	29	S	24	S	0
JOE	29	S	31	S	27	S	27	S	29	S	0
FOA	31	S	32	S	21	S	28	S	27	S	0
FOB	30	S	35	S	29	S	22	S	30	S	0
FOC	31	S	38	S	27	S	24	S	32	S	0
FOD	17	S	37	S	26	S	29	S	31	S	0
FOE	17	S	37	S	26	S	28	S	29	S	0
MOA	15	S	34	S	22	S	21	I	33	S	0
MOB	13	S	25	S	16	S	18	I	25	S	1
MOC	12	R	17	I	19	S	20	I	24	S	1
MOD	15	S	19	I	22	S	21	I	21	S	0
MOE	15	S	25	S	21	S	22	S	23	S	0
AOA	16	S	13	R	25	S	22	S	24	S	1
AOB	18	S	15	R	23	S	10	R	17	I	2
AOC	16	S	14	R	23	S	11	R	17	I	2
AOD	11	R	24	S	23	S	21	I	20	S	1

AOE	17	S	30	S	23	S	20	I	20	S	0
MOA	14	I	27	S	23	S	23	S	21	S	0
MOB	11	R	34	S	24	S	21	I	26	S	1
MOC	16	S	34	S	26	S	24	S	25	S	0
MOD	10	R	34	S	24	S	20	I	27	S	1
MOE	16	S	30	S	25	S	24	S	25	S	0
JOA	17	S	30	S	23	S	22	S	23	S	0
JOB	8	R	10	R	10	R	14	R	13	R	4
JOC	17	S	30	S	23	S	15	R	23	S	1
JOD	15	S	6	R	6	R	6	R	13	R	4
JOE	15	S	15	R	20	S	20	I	20	S	1
	UPSTREAM ISOLATES										
ISOLATE ID	**ANTIBIOTICS/INTERPRETATION**										
	GN	**IN**	**CIP**	**IN**	**IMP**	**IN**	**ATM**	**IN**	**CAZ**	**IN**	
JUSA	25	S	31	S	24	S	23	S	24	S	0

JUSB	21	S	30	S	30	S	23	S	26	S	0
JUSC	23	S	31	S	23	S	24	S	25	S	0
FUSA	18	S	28	S	30	S	27	S	27	S	0
FUSB	26	S	33	S	26	S	30	S	29	S	0
FUSC	22	S	31	S	25	S	31	S	31	S	0
FUSD	18	S	30	S	29	S	24	S	25	S	0
FUSE	29	S	39	S	23	S	30	S	26	S	0
FUSF	25	S	36	S	22	S	29	S	25	S	0
FUSG	21	S	30	S	30	S	25	S	29	S	0
MUSA	15	S	30	S	19	S	20	I	24	S	0
MUSB	16	S	33	S	21	S	23	S	25	S	0
MUSC	17	S	29	S	22	S	22	S	25	S	0
MUSD	16	S	32	S	24	S	20	I	23	S	0
MUSE	16	S	35	S	24	S	22	S	25	S	0
AUSA	18	S	30	S	23	S	23	S	26	S	0
AUSB	18	S	30	S	25	S	20	I	29	S	0
AUSC	17	S	30	S	24	S	22	S	25	S	0
AUSD	17	S	30	S	25	S	23	S	31	S	0

AUSE	17	S	31	S	24	S	22	S	25	S	0
MUSB	17	S	30	S	23	S	20	I	20	S	0
MUSC	18	S	29	S	28	S	22	S	25	S	0
MUSD	17	S	30	S	23	S	23	S	20	S	0
MUSE	18	S	27	S	30	S	24	S	24	S	0
JUSA	17	S	25	S	21	S	14	R	23	S	1
JUSB	15	S	26	S	23	S	22	S	15	I	0
JUSC	16	S	25	S	19	S	22	S	24	S	1
JUSD	20	S	26	S	28	S	17	I	25	S	0
JUSE	25	S	31	S	27	S	25	S	28	S	0
MUSA	11	R	24	S	23	S	22	S	25	S	1

	DOWNSTREAM ISOLATES										
ISOLATE ID	**ANTIBIOTICS/INTERPRETATION**										
	GN	**IN**	**CIP**	**IN**	**IMP**	**IN**	**ATM**	**IN**	**CAZ**	**IN**	
JDSA	19	S	14	R	22	S	25	S	25	S	1

JDSB	20	S	16	R	26	S	26	S	23	S	1
JDSC	15	S	30	S	24	S	27	S	25	S	0
JDSD	23	S	11	R	20	S	23	S	27	R	2
JDSE	12	R	11	R	31	S	10	R	29	S	3
FDSA	15	S	30	S	32	S	28	S	25	S	0
FDSB	15	S	25	S	23	S	26	S	23	S	0
FDSC	23	S	11	R	14	R	23	R	27	R	4
FDSD	24	S	32	S	36	S	20	S	25	S	0
FDSE	12	R	12	R	34	S	15	R	25	S	3
MDSA	23	S	11	R	20	S	15	R	25	S	2
MDSB	15	S	32	S	25	S	8	R	25	S	1
MDSC	15	S	26	S	19	S	20	I	25	S	0
MDSD	10	R	30	S	21	S	10	R	19	S	2
MDSE	12	R	30	S	22	S	11	R	20	S	2
ADSA	16	S	26	S	23	S	20	I	23	S	0
ADSB	18	S	10	R	26	S	26	S	11	R	2
ADSC	15	S	31	S	23	S	23	S	26	S	0
ADSD	16	S	29	S	24	S	21	I	27	S	0

ADSE	10	R	9	R	27	S	25	S	11	R	3
MDSA	27	S	8	R	31	S	15	R	25	S	2
MDSB	24	S	15	R	23	S	13	R	25	S	2
MDSC	19	S	13	R	25	S	30	S	26	S	1
MDSD	22	S	31	S	30	S	14	R	25	S	1
MDSE	20	S	20	S	30	S	30	S	30	S	0
JDSA	12	R	30	S	24	S	21	I	23	S	1
JDSB	11	R	30	S	26	S	23	S	22	S	1
JDSC	15	S	25	S	21	S	21	I	20	S	0
JDSD	16	S	28	S	20	S	21	I	25	S	0
JDSE	12	R	30	S	26	S	23	S	26	S	1

TET: Tetracycline 30 µg; AMC: Amoxicillin Clavulanate 20 µg; CIP: Ciprofloxacin 5µg; ATM: Aztreonam 30 µg; CXM: Cefuroxime 30 µg: IMP: Imipenem 10µg; GN: Gentamicin 10 µg; R: Resistant; I: Intermediate S: Susceptible

Biochemical and physical confirmatory tests for *E. coli isolates*

BIOCHEMICAL TEST AND PHYSICAL TESTS						
ISOLATE ID	**GRAM $^{-}$/+**	**INDOLE**	**TSI**			
			SLANT	BUTT	GAS	H2S
JBAA	−	+	ACID	ACID	+	−
JBBA	−	+	ACID	ACID	+	−
JBCA	−	+	ACID	ACID	+	−
JBGA	−	+	ACID	ACID	+	−
JBQA	−	+	ACID	ACID	+	−
FBAA	−	+	ACID	ACID	+	−
FBBA	−	+	ACID	ACID	+	−
FBCA	−	+	ACID	ACID	+	−
FBFA	−	+	ACID	ACID	+	−
FBGA	−	+	ACID	ACID	+	−
MBA	−	+	ACID	ACID	+	−
MBB	−	+	ACID	ACID	+	−
MBC	−	+	ACID	ACID	+	−
MBD	−	+	ACID	ACID	+	−
MBE	−	+	ACID	ACID	+	−
ABA	−	+	ACID	ACID	+	−
ABB	−	+	ACID	ACID	+	−
ABC	−	+	ACID	ACID	+	−
ABD	−	+	ACID	ACID	+	−
ABE	−	+	ACID	ACID	+	−
MBA	−	+	ACID	ACID	+	−
MBB	−	+	ACID	ACID	+	−
MBC	−	+	ACID	ACID	+	−

MBD	–	+	ACID	ACID	+	–
MBE	–	+	ACID	ACID	+	–
JBAA	–	+	ACID	ACID	+	–
JBBA	–	+	ACID	ACID	+	–
JBCA	–	+	ACID	ACID	+	–
JBDA	–	+	ACID	ACID	+	–
JBEA	–	+	ACID	ACID	+	–
JAA	–	+	ACID	ACID	+	–
JAB	–	+	ACID	ACID	+	–
JAC	–	+	ACID	ACID	+	–
FAA	–	+	ACID	ACID	+	–
FAB	–	+	ACID	ACID	+	–
FAC	–	+	ACID	ACID	+	–
AAE	–	+	ACID	ACID	+	–
JAD	–	+	ACID	ACID	+	–
FAB	–	+	ACID	ACID	+	–
FAD	–	+	ACID	ACID	+	–
MAA	–	+	ACID	ACID	+	–
MAB	–	+	ACID	ACID	+	–
MAC	–	+	ACID	ACID	+	–
MAD	–	+	ACID	ACID	+	–
MAE	–	+	ACID	ACID	+	–
AAA	–	+	ACID	ACID	+	–
AAB	–	+	ACID	ACID	+	–
AAC	–	+	ACID	ACID	+	–
AAD	–	+	ACID	ACID	+	–
MAA	–	+	ACID	ACID	+	–
MAB	–	+	ACID	ACID	+	–
MAC	–	+	ACID	ACID	+	–

MAD	–	+	ACID	ACID	+	–
MAE	–	+	ACID	ACID	+	–
JAA	–	+	ACID	ACID	+	–
JAB	–	+	ACID	ACID	+	–
JAC	–	+	ACID	ACID	+	–
JAD	–	+	ACID	ACID	+	–
JAE	–	+	ACID	ACID	+	–
JAE	–	+	ACID	ACID	+	–
JOAA	–	+	ACID	ACID	+	–
JOBA	–	+	ACID	ACID	+	–
JOCA	–	+	ACID	ACID	+	–
JOD	–	+	ACID	ACID	+	–
JOE	–	+	ACID	ACID	+	–
FOA	–	+	ACID	ACID	+	–
FOB	–	+	ACID	ACID	+	–
FOC	–	+	ACID	ACID	+	–
FOD	–	+	ACID	ACID	+	–
FOE	–	+	ACID	ACID	+	–
MOA	–	+	ACID	ACID	+	–
MOB	–	+	ACID	ACID	+	–
MOC	–	+	ACID	ACID	+	–
MOD	–	+	ACID	ACID	+	–
MOE	–	+	ACID	ACID	+	–
AOA	–	+	ACID	ACID	+	–
AOB	–	+	ACID	ACID	+	–
AOC	–	+	ACID	ACID	+	–
AOD	–	+	ACID	ACID	+	–
AOE	–	+	ACID	ACID	+	–
MOA	–	+	ACID	ACID	+	–

MOB	–	+	ACID	ACID	+	–
MOC	–	+	ACID	ACID	+	–
MOD	–	+	ACID	ACID	+	–
MOE	–	+	ACID	ACID	+	–
JOA	–	+	ACID	ACID	+	–
JOB	–	+	ACID	ACID	+	–
JOC	–	+	ACID	ACID	+	–
JOD	–	+	ACID	ACID	+	–
JOE	–	+	ACID	ACID	+	–
JUAA	–	+	ACID	ACID	+	–
JUBA	–	+	ACID	ACID	+	–
JUCA	–	+	ACID	ACID	+	–
JUD	–	+	ACID	ACID	+	–
JUE	–	+	ACID	ACID	+	–
FUA	–	+	ACID	ACID	+	–
FUB	–	+	ACID	ACID	+	–
FUC	–	+	ACID	ACID	+	–
FUD	–	+	ACID	ACID	+	–
FUE	–	+	ACID	ACID	+	–
MUA	–	+	ACID	ACID	+	–
MUB	–	+	ACID	ACID	+	–
MUC	–	+	ACID	ACID	+	–
MUD	–	+	ACID	ACID	+	–
MUE	–	+	ACID	ACID	+	–
AUA	–	+	ACID	ACID	+	–
AUB	–	+	ACID	ACID	+	–
AUC	–	+	ACID	ACID	+	–
AUD	–	+	ACID	ACID	+	–
AUE	–	+	ACID	ACID	+	–

MUA	–	+	ACID	ACID	+	–
MUB	–	+	ACID	ACID	+	–
MUC	–	+	ACID	ACID	+	–
MUD	–	+	ACID	ACID	+	–
MUE	–	+	ACID	ACID	+	–
JUAA	–	+	ACID	ACID	+	–
JUBA	–	+	ACID	ACID	+	–
JUCA	–	+	ACID	ACID	+	–
JUD	–	+	ACID	ACID	+	–
JUE	–	+	ACID	ACID	+	–
JDA	–	+	ACID	ACID	+	–
JDB	–	+	ACID	ACID	+	–
JDC	–	+	ACID	ACID	+	–
JDD	–	+	ACID	ACID	+	–
JDE	–	+	ACID	ACID	+	–
FDA	–	+	ACID	ACID	+	–
FDB	–	+	ACID	ACID	+	–
FDC	–	+	ACID	ACID	+	–
FDD	–	+	ACID	ACID	+	–
FDE	–	+	ACID	ACID	+	–
MDA	–	+	ACID	ACID	+	–
MDB	–	+	ACID	ACID	+	–
MDC	–	+	ACID	ACID	+	–
MDD	–	+	ACID	ACID	+	–
MDE	–	+	ACID	ACID	+	–
ADA	–	+	ACID	ACID	+	–
ADB	–	+	ACID	ACID	+	–
ADC	–	+	ACID	ACID	+	–
ADD	–	+	ACID	ACID	+	–

ADE	–	+	ACID	ACID	+	–
MDA	–	+	ACID	ACID	+	–
MDB	–	+	ACID	ACID	+	–
MDC	–	+	ACID	ACID	+	–
MDD	–	+	ACID	ACID	+	–
MDE	–	+	ACID	ACID	+	–
JDA	–	+	ACID	ACID	+	–
JDB	–	+	ACID	ACID	+	–
JDC	–	+	ACID	ACID	+	–
JDD	–	+	ACID	ACID	+	–
JDE	–	+	ACID	ACID	+	–

Biochemical and physical confirmatory tests for *Aeromonas hydrophila*

BIOCHEMICAL TEST AND PHYSICAL TESTS					
ISOLATE ID	**GRAM -/+**	**MOTILIY**	**CITRATE**	**GLUCOSE**	**OXIDASE**
JBAA	–	+	+	+	+
JBBA	–	+	+	+	+
JBCA	–	+	+	+	+
JBGA	–	+	+	+	+
JBQA	–	+	+	+	+
FBAA	–	+	+	+	+
FBBA	–	+	+	+	+
FBCA	–	+	+	+	+
FBFA	–	+	+	+	+
FBGA	–	+	+	+	+
MBA	–	+	+	+	+
MBB	–	+	+	+	+
MBC	–	+	+	+	+
MBD	–	+	+	+	+

MBE	-	+	+	+	+
ABA	-	+	+	+	+
ABB	-	+	+	+	+
ABC	-	+	+	+	+
ABD	-	+	+	+	+
ABE	-	+	+	+	+
MBA	-	+	+	+	+
MBB	-	+	+	+	+
MBC	-	+	+	+	+
MBD	-	+	+	+	+
MBE	-	+	+	+	+
JBAA	-	+	+	+	+
JBBA	-	+	+	+	+
JBCA	-	+	+	+	+
JBDA	-	+	+	+	+
JBEA	-	+	+	+	+
JAA	-	+	+	+	+
JAB	-	+	+	+	+
JAC	-	+	+	+	+
FAA	-	+	+	+	+
FAB	-	+	+	+	+
FAC	-	+	+	+	+
AAE	-	+	+	+	+
JAD	-	+	+	+	+
FAB	-	+	+	+	+
FAD	-	+	+	+	+
MAA	-	+	+	+	+
MAB	-	+	+	+	+
MAC	-	+	+	+	+

MAD	-	+	+	+	+
MAE	-	+	+	+	+
AAA	-	+	+	+	+
AAB	-	+	+	+	+
AAC	-	+	+	+	+
AAD	-	+	+	+	+
MAA	-	+	+	+	+
MAB	-	+	+	+	+
MAC	-	+	+	+	+
MAD	-	+	+	+	+
MAE	-	+	+	+	+
JAA	-	+	+	+	+
JAB	-	+	+	+	+
JAC	-	+	+	+	+
JAD	-	+	+	+	+
JAE	-	+	+	+	+
JAE	-	+	+	+	+
JOAA	-	+	+	+	+
JOBA	-	+	+	+	+
JOCA	-	+	+	+	+
JOD	-	+	+	+	+
JOE	-	+	+	+	+
FOA	-	+	+	+	+
FOB	-	+	+	+	+
FOC	-	+	+	+	+
FOD	-	+	+	+	+
FOE	-	+	+	+	+
MOA	-	+	+	+	+
MOB	-	+	+	+	+

MOC	-	+	+	+	+
MOD	-	+	+	+	+
MOE	-	+	+	+	+
AOA	-	+	+	+	+
AOB	-	+	+	+	+
AOC	-	+	+	+	+
AOD	-	+	+	+	+
AOE	-	+	+	+	+
MOA	-	+	+	+	+
MOB	-	+	+	+	+
MOC	-	+	+	+	+
MOD	-	+	+	+	+
MOE	-	+	+	+	+
JOA	-	+	+	+	+
JOB	-	+	+	+	+
JOC	-	+	+	+	+
JOD	-	+	+	+	+
JOE	-	+	+	+	+
JUAA	-	+	+	+	+
JUBA	-	+	+	+	+
JUCA	-	+	+	+	+
JUD	-	+	+	+	+
JUE	-	+	+	+	+
FUA	-	+	+	+	+
FUB	-	+	+	+	+
FUC	-	+	+	+	+
FUD	-	+	+	+	+
FUE	-	+	+	+	+
MUA	-	+	+	+	+

MUB	-	+	+	+	+
MUC	-	+	+	+	+
MUD	-	+	+	+	+
MUE	-	+	+	+	+
AUA	-	+	+	+	+
AUB	-	+	+	+	+
AUC	-	+	+	+	+
AUD	-	+	+	+	+
AUE	-	+	+	+	+
MUA	-	+	+	+	+
MUB	-	+	+	+	+
MUC	-	+	+	+	+
MUD	-	+	+	+	+
MUE	-	+	+	+	+
JUAA	-	+	+	+	+
JUBA	-	+	+	+	+
JUCA	-	+	+	+	+
JUD	-	+	+	+	+
JUE	-	+	+	+	+
JDA	-	+	+	+	+
JDB	-	+	+	+	+
JDC	-	+	+	+	+
JDD	-	+	+	+	+
JDE	-	+	+	+	+
FDA	-	+	+	+	+
FDB	-	+	+	+	+
FDC	-	+	+	+	+
FDD	-	+	+	+	+
FDE	-	+	+	+	+

MDA	-	+	+	+	+
MDB	-	+	+	+	+
MDC	-	+	+	+	+
MDD	-	+	+	+	+
MDE	-	+	+	+	+
ADA	-	+	+	+	+
ADB	-	+	+	+	+
ADC	-	+	+	+	+
ADD	-	+	+	+	+
ADE	-	+	+	+	+
MDA	-	+	+	+	+
MDB	-	+	+	+	+
MDC	-	+	+	+	+
MDD	-	+	+	+	+
MDE	-	+	+	+	+
JDA	-	+	+	+	+
JDB	-	+	+	+	+
JDC	-	+	+	+	+
JDD	-	+	+	+	+
JDE	-	+	+	+	+

Biochemical and physical confirmatory tests for *P. aeruginosa* isolates

BIOCHEMICAL TEST AND PHYSICAL TESTS			
ISOLATE ID	**GRAM -/+**	**OXIDASE**	**CATALASE**
JBAA	-	+	+
JBBA	-	+	+
JBCA	-	+	+
JBGA	-	+	+
JBQA	-	+	+
FBAA	-	+	+
FBBA	-	+	+
FBCA	-	+	+
FBFA	-	+	+
FBGA	-	+	+
MBA	-	+	+
MBB	-	+	+
MBC	-	+	+
MBD	-	+	+
MBE	-	+	+
ABA	-	+	+
ABB	-	+	+
ABC	-	+	+

ABD	-	+	+
ABE	-	+	+
MBA	-	+	+
MBB	-	+	+
MBC	-	+	+
MBD	-	+	+
MBE	-	+	+
JBAA	-	+	+
JBBA	-	+	+
JBCA	-	+	+
JBDA	-	+	+
JBEA	-	+	+
JAA	-	+	+
JAB	-	+	+
JAC	-	+	+
FAA	-	+	+
FAB	-	+	+
FAC	-	+	+
AAE	-	+	+
JAD	-	+	+
FAB	-	+	+
FAD	-	+	+

MAA	-	+	+
MAB	-	+	+
MAC	-	+	+
MAD	-	+	+
MAE	-	+	+
AAA	-	+	+
AAB	-	+	+
AAC	-	+	+
AAD	-	+	+
MAA	-	+	+
MAB	-	+	+
MAC	-	+	+
MAD	-	+	+
MAE	-	+	+
JAA	-	+	+
JAB	-	+	+
JAC	-	+	+
JAD	-	+	+
JAE	-	+	+
JAE	-	+	+
JOAA	-	+	+
JOBA	-	+	+

JOCA	-	+	+
JOD	-	+	+
JOE	-	+	+
FOA	-	+	+
FOB	-	+	+
FOC	-	+	+
FOD	-	+	+
FOE	-	+	+
MOA	-	+	+
MOB	-	+	+
MOC	-	+	+
MOD	-	+	+
MOE	-	+	+
AOA	-	+	+
AOB	-	+	+
AOC	-	+	+
AOD	-	+	+
AOE	-	+	+
MOA	-	+	+
MOB	-	+	+
MOC	-	+	+
MOD	-	+	+

MOE	-	+	+
JOA	-	+	+
JOB	-	+	+
JOC	-	+	+
JOD	-	+	+
JOE	-	+	+
JUAA	-	+	+
JUBA	-	+	+
JUCA	-	+	+
JUD	-	+	+
JUE	-	+	+
FUA	-	+	+
FUB	-	+	+
FUC	-	+	+
FUD	-	+	+
FUE	-	+	+
MUA	-	+	+
MUB	-	+	+
MUC	-	+	+
MUD	-	+	+
MUE	-	+	+
AUA	-	+	+

AUB	-	+	+
AUC	-	+	+
AUD	-	+	+
AUE	-	+	+
MUA	-	+	+
MUB	-	+	+
MUC	-	+	+
MUD	-	+	+
MUE	-	+	+
JUAA	-	+	+
JUBA	-	+	+
JUCA	-	+	+
JUD	-	+	+
JUE	-	+	+
JDA	-	+	+
JDB	-	+	+
JDC	-	+	+
JDD	-	+	+
JDE	-	+	+
FDA	-	+	+
FDB	-	+	+
FDC	-	+	+

FDD	-	+	+
FDE	-	+	+
MDA	-	+	+
MDB	-	+	+
MDC	-	+	+
MDD	-	+	+
MDE	-	+	+
ADA	-	+	+
ADB	-	+	+
ADC	-	+	+
ADD	-	+	+
ADE	-	+	+
MDA	-	+	+
MDB	-	+	+
MDC	-	+	+
MDD	-	+	+
MDE	-	+	+
JDA	-	+	+
JDB	-	+	+
JDC	-	+	+
JDD	-	+	+
JDE	-	+	+