ASSESSING THE IMPACT OF WASTEWATER IRRIGATION ON THE DYNAMICS OF ANTIBIOTIC RESISTANCE IN AGRICULTURAL SETTINGS

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ABSTRACT

Water scarcity is one of the main challenges in sustainable development particularly in developing countries. The use of wastewater effluent for irrigation of crops is common in most water-stressed countries as this alleviates pressure on fresh water supply. Wastewater treatment plants (WWTPs) are regarded as hotspots for antibiotic resistance determinants; antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs) and mobile genetic elements (e.g. plasmids). These antibiotic resistance determinants are consequently introduced into the soil and fresh produce through irrigation with effluent wastewater. Microcosm and field surveillance studies in Palapye and Gaborone respectively, were carried out to evaluate the impact of wastewater effluent irrigation in soil and vegetables in agricultural settings. Culture-based, molecular (PCR), 16S rRNA gene metagenomics and shotgun metagenomics methods were used to determine the occurrence, abundance, diversity and overall dynamics of ARB and ARGs in effluent irrigated soil and vegetables. Clinically relevant bacteria (*Campylobacter, Listeria, Pseudomonas, E.coli, Enterobacter, Staphylococcus and Shigella* species) were targeted and isolated from wastewater effluent, effluent irrigated soils and selected vegetables. The results revealed a significant reduction in total viable bacterial quantities in the storage tank containing effluent used for microcosm irrigation. A shift in bacterial community profile was observed as notable reduction in proteobacteria and increase in firmicutes phyla from the microcosm soil following wastewater irrigation. Antibiotic resistance genes; beta-lactamase resistance gene (bla_{TEM}), tetracycline resistance gene (*tet*A), aminoglycoside resistance gene (*aad*A), sulfonamide resistance gene (*sul*1), trimethoprim resistant dihydrofolate (*dfr*A) were all identified by PCR in Gaborone wastewater treatment plant (GWWTP) effluent but only bla_{TEM}, *aad*A and *dfr*A were detected in the soil from an agricultural field irrigated using effluent from GWWTP. Shotgun metagenomics revealed diverse ARGs belonging to different classes of antibiotics; aminoglycoside, beta-lactam, trimethoprim, macrolide, glycopeptide, tetracycline, sulfonamide, quinolone and oxazolidinone in Palapye wastewater treatment plant (PWWTP) effluent used in the irrigation of the microcosm experiment. However only bla_{TEM} and *aad*A were identified in the microcosm soil, and only beta-lactamase gene bla_{TEM} was detected on vegetable surfaces following irrigation with PWWTP effluent wastewater. The results from this study demonstrated the short and long-term impact of wastewater irrigation which results in persistence and possible dissemination of wastewater-associated ARB and ARGs into agricultural soils and vegetables. Moreover, this study enhances our understanding of antibiotic resistance dynamics and highlights the importance of monitoring antibiotic

resistance in agro-systems, which is critical for informing policies aimed at sustainable use of wastewater effluent in water-stressed countries.

Keywords: wastewater effluent, antibiotic resistant bacteria, antibiotic resistance genes, soil, vegetables

DECLARATION AND COPYRIGHT

I, Onthatile Onalenna declare that this dissertation/thesis is my own original work and that it has not been presented and will not be presented to any other university for a similar or any other degree award.

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CERTIFICATION

The undersigned certifies that he/she has read and hereby recommends for acceptance by the Faculty of Science a thesis titled: Assessing the impact of wastewater irrigation on the dynamics of antibiotic resistance in agricultural settings, in fulfilment of the requirements for the degree of Master of Science in Biological Sciences of BIUST.

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(Supervisor)

Date: 09/12/2020

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DEDICATION

I would like to dedicate this work to my parents, Mr. & Mrs. Onalenna who held my hand every step of the way through my research ups and downs, and my son Kgosi Onalenna who continues to inspire and motivate me.

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CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1. Introduction

Antibiotics have transformed medicine and have become vital in many medical procedures (Munita & Arias 2016). However, a significant threat to the effectiveness of antibiotics in the treatment of infections is that bacteria are increasingly becoming resistant to most if not all antibiotics that have been developed (Adedeji 2016). The World Health Organization (WHO) has recognized antibiotic resistance as one of the top three public health threats of the 21st century. In the United States of America, the Centre for Disease Control and Prevention estimates that 23 000 lives are lost annually due to bacterial caused infections. Moreover, WHO predicts that antibiotic resistance will cause at least 300 million premature deaths by 2050 if development and dissemination of antibiotic resistance is not addressed (European Commission 2017).

Antibiotics are ubiquitous in clinical and non-clinical environments; the latter is because antibiotics are partially metabolized by the body and ultimately are excreted into the environment (Marti et al., 2013). Unused antibiotics in households are also disposed into sewage systems which leads to their accumulation in wastewater treatment plants (WWTPs) and downstream ecosystems (water and soil) (Hendriksen et al., 2019). WWTPs help in reducing the number of bacterial populations (quantified by targeting mainly indicator bacteria such as coliforms) before the effluent is discharged into downstream environments. The wastewater treatment is however not enough to remove the antibiotic resistance determinants (e.g. antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes) in the effluent before being released into the environment (Marti et al., 2013). Treated wastewater effluent frequently contain high concentrations of antibiotic residues and ARB even though the levels of indicator microorganisms are reduced (Esiobu et al., 2002). WWTPs are characterized by high bacterial densities and nutrient contents which is a conducive environment for horizontal transfer of ARGs among communities comprising of environmental microorganisms and clinically relevant pathogenic microorganisms (Rahube and Yost 2010).

Rapid human population growth and high urbanization rates have major socio-economic and ecological implications that include the need for increased food production, infrastructure improvement and sustainable use of resources. Using reclaimed water as an alternative water source in agriculture has become a practical alternative due to insufficient fresh water supply.

Reclaimed water is defined as water that has undergone the treatment processes that can be used for other purposes provided the water quality meets the set standards (Raschid-Sally & Jayakody 2008). The quality of reclaimed water is mainly based on the levels of biochemical oxygen demand (BOD) and indicator microorganisms. In developed countries, wastewater use follows international safety standards, however in most developing countries the use of wastewater is often unregulated, and this leads to increased health risks (Raschid-Sally & Jayakody 2008). Nutrients found in wastewater effluent are known to reduce the need for artificial fertilizers therefore reducing costs associated with fertilizer purchases while improving the crop yield. However, the use of wastewater for irrigation is a major contributing factor in contamination of vegetables with pathogens (Obinna & Destiny 2016). In recent years consumers are more health conscious and the search for healthier diet options have increased the consumption of raw vegetables and minimally cooked foods. Because raw vegetables are not subjected to any heat treatments such as cooking, they harbor different microorganisms including ARB which presents a serious health risk.

The Government of Botswana has implemented interventions as part of the vision 2036 pillar for sustainable economic development towards food security (Mogomotsi et al., 2018). Several horticultural farmers are allocated land near WWTPs to use wastewater effluent to cultivate fresh produce that can be supplied to government schools in an effort to combat under and malnutrition. Produce from these farms are also supplied to local supermarkets which empowers the farmers and contribute to the country's food security. The Government of Botswana also encourages its citizens to practice backyard gardening through one of its poverty eradication programs (Marumo et al., 2017). There is little research conducted on the environmental dimension of antibiotic resistance in Botswana, and these government initiatives present a potential risk for antibiotic resistance dissemination in agricultural environments and potentially to humans through consumption of contaminated vegetable produce. This study primarily focuses on the analysis of wastewater effluent, effluent-irrigated soils and vegetables from agricultural fields and microcosms using culture, molecular (PCR) and next generation sequencing techniques.

1.2. Problem Statement

Foodborne bacterial infections account for 48 million illnesses a year (European Commission 2017). Wastewater effluent is frequently used for crop irrigation, this practice provides a potential route for the spread of pathogens, ARB and ARGs in agro-systems and potentially to humans through consumption of raw or partially cooked produce. In many African countries including Botswana, there is lack of knowledge on the implications of antibiotic resistance to human health and the non-clinical environment dimension of antibiotic resistance is poorly understood. ARB and ARGs are not recognized as environmental contaminants and therefore not monitored in wastewater effluent that is discharged into the environment. Many developed countries have embarked on national research and surveillance programs to combat antibiotic resistance dissemination. From published literature, no evidence has been found to suggest that antibiotic resistance surveillance studies have been carried out in Botswana to determine occurrence, diversity, and overall dynamics of ARB and ARGs associated with agricultural soils and fresh produce irrigated with wastewater effluent.

This study hypothesizes that irrigation of soil and vegetables with wastewater effluent will result in a significant shift in bacterial community structure following introduction of viable bacterial population including ARB into the soil. Higher occurrence, abundance and diversity of viable ARB and ARGs is predicted from agricultural soils and vegetables exposed to wastewater effluent over long periods.

1.3. Objectives

The general objective of this study is to determine the impact of wastewater irrigation on antibiotic resistance dynamics (occurrence, abundance and diversity) in established agricultural fields and microcosm settings.

The specific aims are;

• To isolate and enumerate groups of indicator bacteria, potentially pathogenic and ARB (e.g. *Escherichia coli, Salmonella* spp*., Listeria* spp*., Campylobacter* spp*., Pseudomonas* spp*, Enterobacter* spp*., Staphylococcus* spp*., Shigella* spp) associated with water-borne and food-borne illness in soil and fresh vegetables using culture-based methods.

• To detect the presence of common ARGs associated with resistance to different classes of antibiotics in wastewater effluent, effluent irrigated soils and vegetables using conventional PCR method.

• To determine the changes in bacterial community profiles and ARGs in wastewater effluent and irrigated soils using 16SrRNA gene and shotgun metagenomics sequencing methods.

• To assess the potential dissemination of ARB and ARGs from wastewater effluent to soil and subsequently vegetables.

1.4. Significance of study

The rapid global increase in antibiotic resistance probes action from both local and global communities. It is therefore crucial that the health risks associated with antibiotic resistance are highlighted and the public is sensitized on antibiotic resistance spread from non-clinical environments and implications to global health. Consumption of fresh produce provides a pathway for direct exposure of ARB and ARGs to humans, therefore it is critical that changes in antibiotic resistance profiles in soil and food crops are understood. Furthermore, the study is important to the many stakeholders; scientists, medical practitioners, policy makers and farmers in Botswana as they should work together towards the development of a national antibiotic resistance surveillance/monitoring schemes and implementation of evidence-based policies on the sustainable and safe use of wastewater effluent in agriculture. Moreover, this study will contribute to the World Health Organization (WHO) mandate for combatting antibiotic resistance development and spread at a global level.

CHAPTER 2: LITERATURE REVIEW

The long-term impact of wastewater irrigation in the era of global concern of antibiotic resistance: a perspective from a developing country

Portions of this chapter have been published previously as a review in the *Journal of Experimental Biology and Agricultural Sciences Volume 7, Issue 5, October Issue – 2019, Pages: 481-488.*

2.1. The role of wastewater treatment plants in antibiotic resistance development

Water scarcity is a global challenge, especially Sub-Saharan Africa having higher number of water-stressed countries such as Botswana, Zimbabwe, and Kenya (Ozturk 2017). Zimbabwe has a semi-arid climate with recurrent drought and wastewater effluent is being used for irrigation of covo (*Brassica oleracea* variety, *acephala*) sugar beans (*Glycene max*) and maize (*Zea mays*) (Mutengu et al., 2007). The government of Botswana introduced a wastewater irrigation scheme in 2003, initially wastewater effluent was used to irrigate Lucerne in golf courses (Arntzen & Setlhogile, 2007). Now, vegetable crops are also grown and irrigated with wastewater effluent, the irrigation scheme is aimed at diversifying the economy and empowering youth farmers (Arntzen & Setlhogile 2007).

WWTPs collect wastewater from different environments for controlling environmental pollution by reducing biological oxygen demand, nutrients (nitrogen and phosphorus) and environmental contaminants such as pesticides and heavy metals. In treatment plants wastewater undergoes different stages of treatment to remove as much contaminants as possible, the stages include; preliminary, primary, secondary and tertiary treatment (Qasim 2017). Preliminary water treatment is the removal of waste using screens of any material that floats or readily settles, this prevents blockage of pipes throughout the process. Following pre-screening, primary water treatment involves the removal of suspended solids, through sedimentation. Once the water has passed through primary screening, dissolved solids and nutrients are removed by biological processes such as activated sludge. Secondary treatment converts complex organic compounds into simple volatile compounds such as water, carbon dioxide and methane (Emongor et al., 2005). Tertiary treatment is the last process before water is discharged into the environment; it involves removal of inorganic substances and pathogens through physical removal such as filtration, chemical removal or irradiation methods (Emongor et al., 2005). WWTPs are widely recognized as reservoirs for ARGs that are associated with

pathogenesis (Rahube &Yost 2010). WWTPs promote bacteria proliferation because of high bacterial abundance and nutrient density (Zhang et al., 2009). WWTPs have also been shown to promote the incorporation of chromosomally encoded antibiotic resistance genes into plasmids which are then transferred between bacteria (Cattoir et al., 2008). The global gene diversity of antibiotic resistance has been shown to have wide variation as per regions; the gene abundance corresponds to the regional environmental factors, socio-economic and health status (Hendriksen et al., 2019). A trans-European surveillance of antibiotic resistance in discharged wastewater effluent showed that antibiotic resistance profiles of effluent mirrored the pattern of antibiotic resistance prevalence in clinical settings (Karkman et al., 2019).

The current wastewater treatment infrastructure in Botswana is not enough to remove the antibiotic residues and ARB in effluent before being released to receiving rivers or environment. Treated wastewater may contain antibiotic resistance determinants even though the levels of indicator microorganisms comply (Lood et al., 2017). A previous study carried out in Gaborone wastewater treatment plant (GWWTP) has shown that ARB and ARG accumulate in the treatment plant and are consequently released into the receiving river, 85.1% of isolates from GWWTP effluent were found to be resistant to more than one antibiotic tested. Antibiotic resistance genes *tet*A, *mph*A, *sul*1, *dfr*A, *int*1, and *str*B were detected in the final effluent from GWWTP. These results suggest that antibiotic resistance determinants from WWTPs disseminates to downstream environments (Tapela & Rahube 2019).

2.2. Impact of wastewater irrigation on soil microbiome

There is still no consensus regarding the horizontal gene transfer and the potential dissemination of ARGs from wastewater effluent-borne bacteria to soil microbiome. However, irrigation of agricultural soils with wastewater effluent have been shown to alter the physiological properties of the soil (Becerra-Castro et al., 2015). It has been reported that the pH of the soil increases with long-term wastewater effluent irrigation, therefore promoting selection of certain microorganisms such as Actinomycetes (Sun et al., 2015). The availability of organic matter in soil is an important property of soil, it is increased by wastewater irrigation of soil. The availability of organic matter also affects soil structure, soil fertility and microbial communities inhabiting the soil, (Sun et al., 2015). Wastewater effluent also contains high concentrations of dissolved inorganic substances such as salts which results in soil salination following long-term wastewater effluent irrigation. Increase in soil salinity has been shown to reduce microbial biomass and diversity in the soil (Ke et al., 2013). Microbial communities in

the soil may also be affected by interactions with contaminants such as metals, phenolic compounds and pharmaceuticals from wastewater effluent (Becerra-Castro et al., 2015).

Soil is a natural reservoir for antibiotic producing bacteria and approximately 50% of Actinomycetes microorganisms isolated from soil synthesize antibiotics therefore providing a natural antibiotic residue in soil (Popowska et al., 2012). Anthropogenic activities have been proved to accelerate the development and spread of ARGs in the environment, it is also becoming more evident that ARB and ARGs are widespread in natural untreated soils (Aminov & Mackie 2007). Factors influencing ARGs dissipation rates from bacterial hosts introduced into the soil include the transport of bacteria hosting ARGs, the binding of ARGs to soil and the decline of the bacterial hosts (Thanner et al., 2016). The stability of mobile genetic elements (MGEs) is affected by a wide range of parameters such as nutrient availability, temperature, oxygen, pH and soil type (Rahube & Yost 2010).

Irrigation of crops using wastewater effluent has been adapted by many African countries such as South Africa, Tunisia, Zimbabwe and Botswana, this reduces the need for fresh water while improving food security (Khalid et al., 2018). Surface irrigation is the recommended method for effluent irrigation of crops because it is more efficient as it allows water to drip from soil surface into the soil minimizing evaporation and contact of wastewater effluent with crops. Nonetheless overhead irrigation is still practiced and there is direct contact of wastewater effluent with crops which could transfer ARB from effluent directly to crops (Ait-Mouheb et al., 2018). From a study by Solomon et al., (2003) *E.coli* was found to be more persistent in lettuce irrigated with effluent using overhead irrigation when compared to surface irrigated lettuce, this therefore suggest that irrigation methods have a profound effect on the dynamics of the microorganisms in fresh produce. Several studies have shown that irrigation with wastewater effluent increases the nutritive value of the soil which provides a conducive environment for bacterial proliferation (Wafula et al., 2015). Bacteria from wastewater effluent accumulate in soil and can survive for extended periods of time because of nutrient abundance through long-term irrigation. In a study carried out to assess rapid stabilization of the antibiotic resistome in receiving freshwater bodies from wastewater effluent, persistence of wastewater effluent irrigation into the receiving environment was shown to promote the stabilization of resistome from wastewater effluents to the newly formed microbial communities in that environment (Corno et al., 2019).

2.3. Impact of wastewater irrigation on the dynamics of antibiotic resistance determinants in vegetables

Fresh fruits and vegetables have high fiber content and are low calorie yielding, these have become a preferred option as more people are becoming health conscious. The global consumption of fresh produce has significantly increased, from ~10g to ~110g in individuals per day in sub-Saharan Africa (Mensah et., al 2020). Fruits and vegetables irrigated with effluent are highly exposed to microbial contamination through contact with irrigated soil and wastewater effluent. Some leafy green vegetables require no heat treatment before consumption therefore there is an increased risk of ARB and ARGs exposure to humans through consumption of fresh produce (Holvoet et al., 2013). The emergence of antibiotic resistance in vegetables is proving to be a serious concern affecting human and environmental health. Previous studies have revealed the abundance of antibiotic resistant coliforms and pathogenic bacteria as well as persistence of ARGs linked to wastewater/sewage isolated from soil and vegetables at harvest as well as in retail (Kilonzo-Nthenge & Mukuna 2018, Rahube et al., 2014; 2016). Antibiotic resistance pool in the human gut can be increased by multi-drug resistant (MDR) bacteria that are carried by raw vegetables (Walia et al., 2013). This therefore elevates the likelihood of plasmid conjugal transfer between bacteria on vegetables and human gut flora (Schjorring & Krogfelt 2011).

Increasing reports have also shown that plants have the ability to passively uptake water soluble contaminants through the roots which can be translocated and concentrated into other parts of the plant such as leaves, although this more so in hydroponic cultures compared to conventional crops in soil (Pullagurala et al., 2018, Madikizela et al., 2018). Uptake of ARB and ARGs by plants is determined by several factors including, the physiochemical properties of the contaminant, the plant genotype, physiological state of the plant and stress effects on the plant such as weather conditions (Madikizela et al., 2018). *Salmonella* is considered among the causative agents associated with common foodborne infections. It is therefore a major concern that some species of *Salmonella* are increasingly becoming resistant to a wide range of antibiotics (Kilonzo-Nthenge & Mukuna 2018). From the studies carried out by Wadamori et al., (2016), multi-drug resistant Salmonella expressing resistance to vancomycin, erythromycin, ampicillin and penicillin was isolated from vegetables. Globally *Bacillus cereus* is also considered an important pathogen in foodborne poisoning (Park et al., 2018). In another study , *B. cereus* exhibiting multi-drug resistance was isolated in raw vegetables. (Park et al., 2018). In a study carried out to assess the occurrence of bacterial species in vegetables and

their antibiogram to antibiotics in Nigeria, vegetables were shown to harbor antibiotic resistance genes which could potentially be introduced to humans (Obinna & Destiny 2016). Furthermore, in recent studies it has been reported that plants grown in sulfonamide-contaminated soil showed uptake of ARB and ARGs in leaves (Piña et al., 2020). Another study was done to investigate occurrence of antibiotic resistance in raw salad vegetables. Tomato, cucumber and beetroots showed high contamination of coliforms with 98% of the isolates being resistant to ampicillin, erythromycin, streptomycin, gentamycin, ciprofloxacin, cephalexin and chloramphenicol (Rashmi et al., 2017). *Listeria monocytogenes* was found to be present in salad vegetables (cabbage, cucumber, lettuce, and tomato) and antibiotic susceptibility testing showed that 92.9% of the isolates were resistant to ampicillin, 85.7% to oxacillin and 14.3% of isolates were resistant to ciprofloxacin (Rashmi et al., 2017). In a study where pakchoi (Brassica chinensis) was exposed to tetracycline, cephalexin, and sulfamethoxazole at minimum inhibitory levels, *tet*X, *bla*$_{CTX-M}$, *sul*1 and *sul*2 genes were detected in the plant endophytic system which highlights the absorption of ARGs by plants and potential risk of ARGs dissemination from vegetable crops to human beings. Risk assessment studies on the microbial hazards introduced by raw vegetable consumption has previously been carried out, however quantitative risk assessment for antibiotic resistant bacteria (ARB) in vegetables is yet to be done (Hölzel et al., 2018).

2.4. Human health risks of antibiotic resistance genes

Human health implications of consuming produce with ARB and ARGs is mostly unknown. The question then becomes whether antibiotic resistant bacteria from the environment and that in the human gut consists of a common pool. It has been speculated that ARGs cause potential health impacts such as disrupted digestive system functions, allergic reactions and chronic toxic effects (Berglund et al., 2014). Even at low abundance these bacteria may be transmitted to humans in an asymptomatic long-term colonization which may only surface when the immunity is compromised (Christou et al., 2017). The human gut is considered a reservoir for ARGs, because of dense and diverse microbial population found in the human gut, resistance genes may be transferred between bacteria (Salyers et al., 2004). Studies have shown evidence to support the reservoir hypothesis and ARG transfer in both in vivo and in vitro models (Schjorring and Krogfelt 2011, Moubareck et al., 2003). Under normal conditions, it is known that DNA is broken down into small non-functional fragments upon uptake by humans. However, studies on mice have shown that under certain conditions DNA can be taken up without complete breakdown (Hohlweg & Doerfler 2001). Research conducted at the State

Institute for Quality Control of Agricultural Products in Netherlands indicates that antibiotic resistance genes can be transferred from genetically engineered bacteria to bacteria in the colon and persist for prolonged time in the gut (Speksnijder et al., 2015). Many in vitro experiments have been carried out to characterize antibiotic resistant gene transfer between environmental and human microflora, however the frequency of transfer under natural environments is yet to be determined (Phillips et al., 2004). It is however still difficult to assess the human health risks associated with ARB and ARGs, this may be due to the insufficient data on the number of bacteria that may be required to start a successful colonization in the human body as well as insufficient information on routes of dissemination of ARB and ARGs into the human body (Amarasiri et al., 2020).

2.5. Methods for assessing antibiotic resistance dissemination in agricultural soils

Whole-ecosystem studies are most ideal in research as they better reflect the microbial diversity and changing dynamics in the environment (Tanentzap et al., 2017). On the other hand, microcosm studies are mostly preferred for short-term studies because parameters involved can be manipulated and can be maintained under defined conditions (Eller et al., 2005). Because of higher capacity of experimental controls, microcosm experiments have been successfully used to investigate tetracycline resistance in agricultural soils, the impacts of amoxicillin on bacteria in manure treated soil and the dissemination of multi-drug resistance plasmids in wastewater sludge (Binh et al., 2007; Schmitt et al., 2006).

Further, culture-based methods are globally recognized as conventional methods of surveillance of antibiotic resistance in viable bacterial communities across different environments and have been shown in multiple studies to provide reproducible results (Pachepsky et al., 2011). It can be challenging to target environmental microorganisms using culture given that matrices like soil have an abundance of about 10^8 to 10^{10} microorganism per gram (Pachepsky et al., 2011). It is for this reason that the use of selective media is a preferred solution to counteract this challenge. However, selective media might associate with high false positive rates which lead to inaccurate identification and quantification of bacterial species (Pachepsky et al., 2011). Consequently, confirmatory tests should be carried out on presumptive colonies and these confirmatory tests include microscopy (e.g. Gram stain), biochemical detection of expression of metabolic enzymes and gas production (Pachepsky et al., 2011). This can be useful in detecting multiple antibiotic resistant microorganisms of clinical concern thus obtaining their multidrug resistant profiles.

According to Boehme et al., (2010) approximately 99% of environmental bacteria cannot be cultured using standard methods and this would be a big drawback of these methods, so it is important that in addition to culture-based, molecular methods be carried out to determining the occurrence, diversity and abundance of bacteria in environmental samples. It is worth noting that culture-based and molecular methods do not provide interchangeable results as the two approaches measure different parameters (Luby et al., 2016). Polymerase chain reaction (PCR) has become a routine method for detecting ARGs in environmental samples (Zhang & Fang 2006). It is less time consuming, highly sensitive and more accurate; it is widely used for obtaining information on DNA sequence of interest. PCR has been successfully used to detect ARGs in agricultural environments (Chee-Sanford et al., 2001). However, there are downfalls to using conventional PCR, because it depends on sample DNA, efficacy of DNA extraction varies across sample matrices and low DNA yielding samples may compromise the PCR results (Goyer et al., 2012). Moreover, conventional PCR provides information only on presence or absence of the target gene and does not indicate expression levels of the gene. However, detection of the gene is still important because extracellular DNA can be taken up and expressed as another bacterium (Chen & Dubnau 2004). PCR products should then be sequenced to confirm the amplified target gene. In Australia the impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks was investigated using high-throughput quantitative PCR and terminal restriction fragment length polymorphism techniques. In this study, diversity, abundance and composition were compared and the results showed that irrigation with wastewater effluent significantly increased the abundance and diversity of ARGs in the soil (Han et al., 2016).

Metagenomic sequencing method has gained popularity in molecular characterization of environmental samples (Streit & Daniel 2017). This is because metagenomic sequencing circumvent PCR and community DNA can be sequenced in a single step. Also, ARGs can be identified by comparison against online databases such as MG-RAST, Integrated Microbial Genome database (IMG) and Comprehensive Antibiotic Resistance Database project (CARD) (Meyer et al., 2008; Kearse et al., 2012; McArthur et al., 2013). Shotgun metagenomic sequencing allows for evaluation of bacterial diversity and abundance in different environments. Moreover, shotgun metagenomics provides higher resolution in studying the majority of unculturable microbial communities that would be otherwise difficult to analyze using PCR methods (Luby et al., 2016). Soil metagenomics analysis has shown that soil contains diverse ARGs (Nesme & Simonet 2015). Metagenomic analysis has also been used to

identify markers of horizontal gene transfer (HGT) such as plasmids, this also give insights on dissemination of ARGs in different environments (Nesme & Simonet 2015). Metagenomic analysis has been used previously to compare plasmid encoded ARGs in manure and agricultural soils to determine the dissemination of ARGs from manure to soil (Udikovic-Kolic et al., 2014).

16S rRNA gene sequencing is a common method used to identify and compare phylogeny of bacteria within a complex and uncultured sample. The principle of 16S rRNA is based on the fact that the prokaryotic 16S rRNA has multiple variable regions interspersed between conserved region, the various regions of the 16S rRNA gene are the ones that are used for high resolution phylogenetic classification in microbial populations (Luby et al., 2016). 16S rRNA has been used previously to successfully determine diversity and abundance of bacterial communities in soil and vegetables (Shen et al., 2019, Fogler et al., 2019).

Integrating culture-based, molecular PCR methods and high throughput metagenomics for surveillance and monitoring of ARGs in agricultural soils allows the ability to detect ARGs, determine the functionality of the genes, identification of microbial taxa harboring individual resistance genes and determination of ARG dissemination in different environments

CHAPTER 3: METHODOLOGY

3.1. Description of study areas

3.1.1. Gaborone (Field surveillance)

Gaborone wastewater treatment plant (24°36’57.5” S; 25°57’94.9” E) is the largest treatment plant in Botswana with influent receiving capacity of 40 000m^3 daily, servicing a population of approximately 230 000 people (Emongor et al., 2005). GWWTP consists of pre-screening, primary and secondary wastewater treatment, following secondary treatment the effluent is discharged into several maturation ponds for biological degradation. The final effluent from the maturation ponds is then discharged into the Notwane River. In dry seasons there is no inflow of water in the river from upstream, therefore wastewater effluent from GWWTP is then used for irrigation of fresh produce by agricultural farms along the Notwane River as illustrated in Figure 3.1.1.

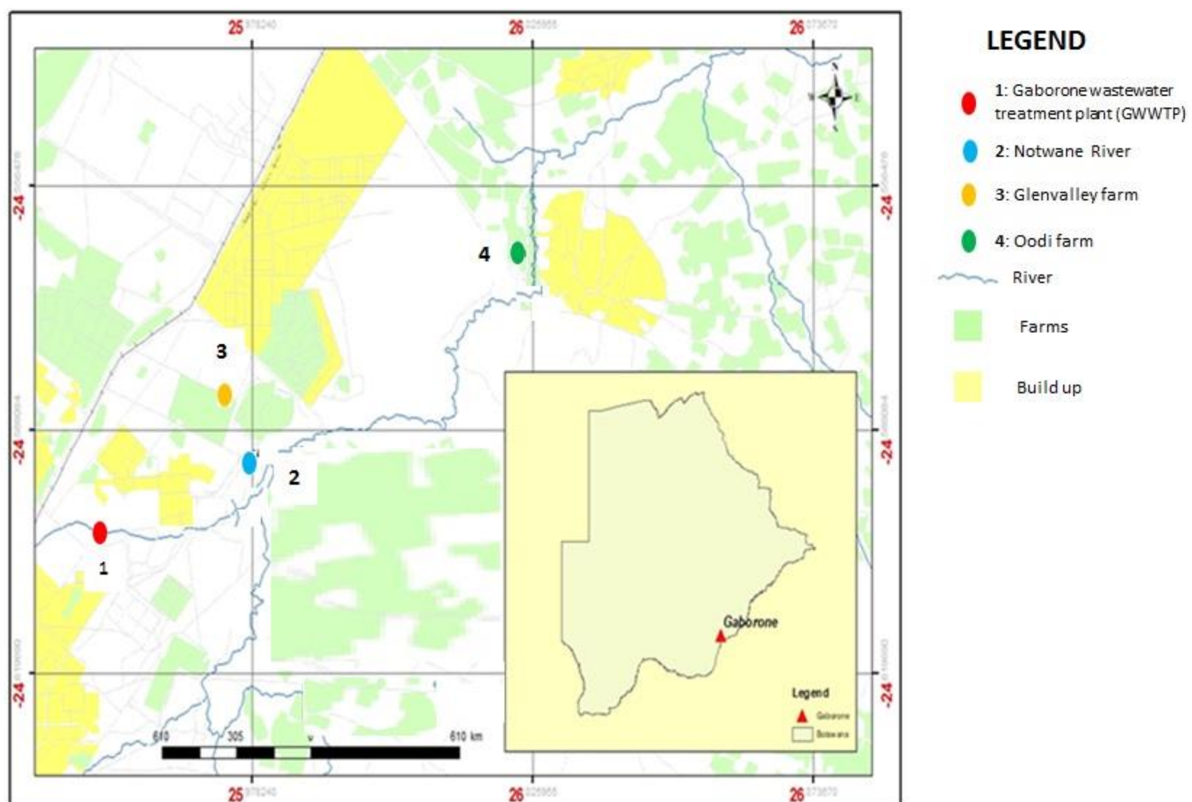

Figure 3.1.1. Map showing field surveillance sampling sites; GWWTP, Notwane River, GWWTP effluent irrigated soil (Glenvalley farm) and Notwane River irrigated soil (Oodi farm) (Onthatile & Rahube 2019).

3.1.2. Palapye (Microcosm experiment)

The irrigation scheme in Gaborone was shown to be a success in terms of youth empowerment and contribution to food security as the vegetables produced are being supplied to local supermarkets in Gaborone and other regions across Botswana. In addition, the wastewater irrigation scheme is also proposed in other parts of the country such as Palapye (population of approximately 37 000 people). The central regions of Botswana are experiencing rapid population increase due to developments such as opening of Botswana International University of Science & Technology (BIUST) and expansion of Morupule coal mine. Some parts of the villages have more prominent rural lifestyles where livestock and humans stay in the same vicinity and pit latrine ablutions are still used. However, some parts of villages are more developed with modern infrastructures. Since Palapye is a combination of both rural and urban lifestyles and it makes it a conducive area for rapid dissemination of antibiotic resistance determinants in the environment. Palapye wastewater treatment plant (PWWTP) is located on the outskirts of Palapye (22° 32'24.0" S; 27° 10'23.2" E), it has a relatively smaller influent receiving capacity of 14000 m^3 per day as compared to GWWTP and uses the pond enhancement treatment operation where anaerobic digestion occurs in a series of ponds followed by degradation with biofilters (Shipin et al., 1998). Final effluent from PWWTP is chlorinated before being discharged into a man-made pond where it is used by the public for various purposes including crop irrigation in backyard gardens.

3.1.3. Microcosm design

Soil was collected around Mahibitswana agricultural field, a proposed irrigation scheme field (approximately 1km from PWWTP) in Palapye into sterile 5L black planting bags filling up to 70% of the bag and transported to BIUST for the microcosm experiment. Wastewater effluent from PWWTP was filled into a 2500L water storage tank and used as a source of water for irrigation of the microcosm garden. Two microcosm experiments were set comprising microplots of spinach (*Spinacia oleracea*), beetroots (*Beta vulgaris*) and carrots (*Daucus carota subsp. Sativus*) that were sown directly in the soil, one set of the microcosm (A) was irrigated with wastewater effluent (experimental), another set (B) was irrigated with tap water (control) and three planting bags (C) remained untreated (not sown, not irrigated) throughout the course of 90 days experiment (Figure 3.1.3). The sets were kept 2 meters apart to prevent cross contamination with the effluent wastewater. In order to mimic the local backyard gardening commonly practiced in local communities, the soil was kept moist with crops irrigated every 2 days (with approximately 1.5 litres of water going into each planting bag),

weeds were removed aseptically by hand, other external environmental factors such as wind, temperature and humidity were not controlled. The experiment was conducted in spring season, which was mostly sunny with temperatures ranging from 17^0 C to 39^0 C. Due to the extreme temperature carrots did not grow therefore only spinach and beetroots were harvested for analysis.

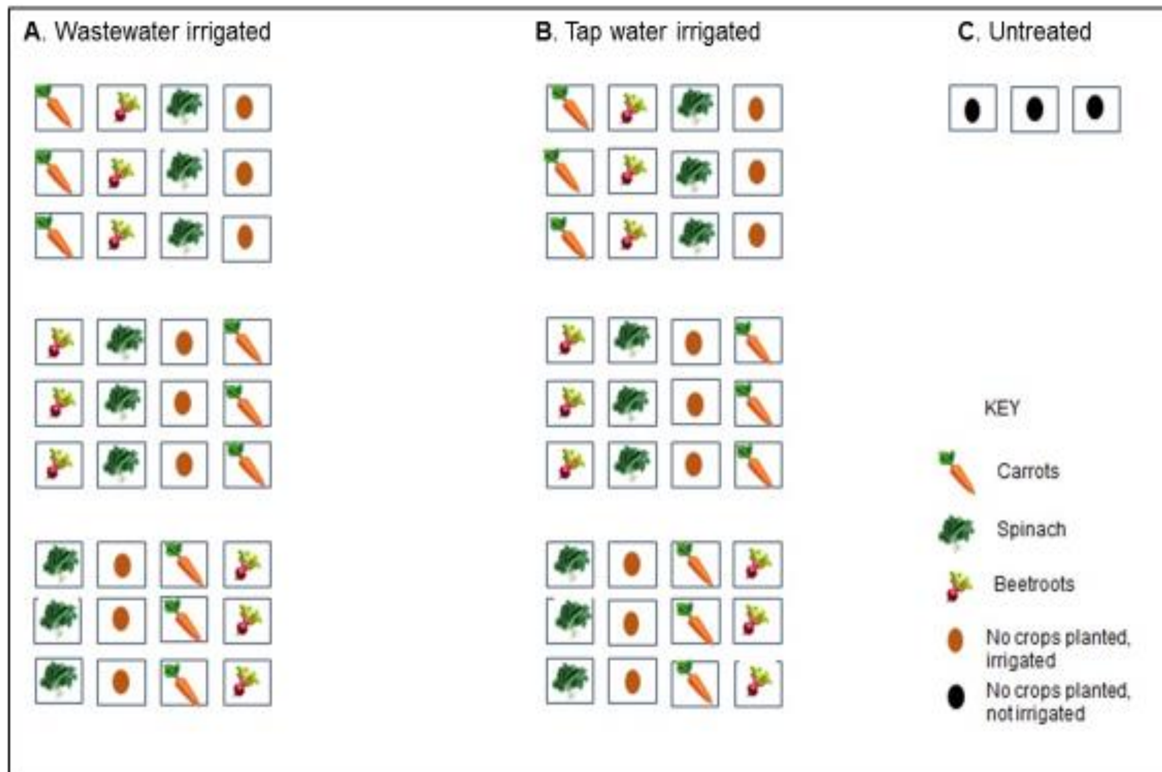

Figure 3.1.2: Microcosm experimental design diagram - A; Experimental microplots showing vegetables that were sown and irrigated using PWWTP effluent B; Control microplots showing vegetables that were sown and irrigated using tap water C; Untreated microplots

3.2. Sample collection

3.2.1. Gaborone field sampling

Water samples (1L from each sampling point) from Gaborone were collected from the GWWTP effluent maturation pond (24°36'57.5" S; 25°57'94.9" E) and Notwane River (24°35'34.3" S; 25°58'39.6" E) into sterile polystyrene bottles. Soil samples (5g from 5 different points in the farm) were collected into sterile zip lock bags from Oodi farm (24°33'58.4" S; 26° 01'25.9" E, 7km from Notwane River sampling site), where water from

the Notwane River is used for irrigation) and from Glenvalley farm (24° 33'58.4" S; 26° 01'25.9" E, 3km from GWWTP), where GWWTP effluent is derived directly from the maturation ponds for irrigation. The samples were then transported in a cooler box with ice packs and analyzed within 4 hours of collection.

3.2.2. Palapye microcosm sampling

From the effluent wastewater in the storage tank, 1L was collected into sterile polystyrene bottle at day 1 (immediately after filling the tank) and every 30 days for 3 months for quantitative analysis. Soil samples (5g) were collected into sterile zip lock bags from each plating bag at the beginning of the experiment before irrigation and every 30 days post irrigation for 3 months. The samples were immediately analyzed in the laboratory. All spinach leaves and beetroots were aseptically harvested (40 days post sowing for spinach and 60 days for beetroots) into sterile zip lock bags and immediately taken to the lab for analysis.

3.3. Bacteria isolation and enumeration

Non selective (nutrient agar) and various selective media such as Mannitol Salt agar, Listeria mono differential agar, Harlequin Salmonella agar, Campylobacter selective agar, Harlequin Pseudomonas agar and MacConkey agar were used for primary isolation, presumptive identification and quantification of bacterial isolates from wastewater effluent, river water, soil samples, spinach leaves and beetroots surface. To calculate viable bacterial cells from water and soil samples, the samples were serially diluted using phosphate buffered saline (PBS), plated on nutrient agar and incubated at 37^{0} C. Excess soil was removed from spinach leaves and beetroots with paper towel to achieve visual cleanliness. Beetroots (50g) and spinach leaves (10g) were washed in 100ml of buffered peptone water in a sterile Ziploc bag, the wash water was serially diluted and spread plated as previously described by (Obinna & Destiny 2016). Bacteria enumeration was carried using a routine method of counting colonies (between 30-300) on culture plates and calculating cfu/ml (water samples) and cfu/g (soil and vegetable samples) using the formula; cfu/ml or cfu/g = [number of colonies on plate (cfu)] / [(dilution factor x volume plated)].

Colonies from selective media were then sub-cultured to obtain pure culture, which were then stored at -80^{0} C in LB broth containing 50% glycerol at a ratio of 1:1 for further analysis.

3.4. Antibiotic susceptibility testing

Pure culture isolates were plated into LB agar which was supplemented with different antibiotics at clinical break-point concentrations (Table 3.4.1) using a sterile toothpick, the plates were then incubated at appropriate temperatures and conditions as previously described by Tapela & Rahube (2019).

Table 3.4.1: Classes of antibiotics used and their clinical break point concentration

Class	Antibiotic	Clinical breakpoint concentration (µg/ml)
Beta- lactams	Penicillin (Pen)	16
	Ampicillin (Amp)	32
Quinolones	Ciprofloxacin (Cip)	4
Tetracyclines	Tetracycline (Tet)	16
Aminoglycosides	Streptomycin (Str)	64
Macrolides	Erythromycin (Ery)	8
Carbapenem	Meropenem (Mer)	4
Cephalosporins	Cephalosporin (Cep)	32

3.5. DNA extraction

DNA was extracted from the samples within 4 hours of collection. Briefly 500ml of wastewater effluent from GWWTP, PWWTP, Notwane River water and vegetable wash water were filtered through a 0.45µm filter paper and DNA was extracted using the ZR microbe DNA extraction kit (Zymo Research USA) following manufacturer's instructions. Effluent irrigated soil samples, tap water irrigated samples and untreated soil samples from random sampling points were respectively bulked together and homogenized. DNA was extracted in triplicates following DNA extraction protocol using the ZR microbe DNA extraction kit (Zymo Research USA) following manufacturer's instructions. The yield of the extracted DNA was quantified and checked for purity using a nano drop spectrophotometer (Lasec, Jenway Genova nano) at an absorbance of 260nm. All the DNA samples obtained were stored at -20^0 C for further analysis.

3.6. Polymerase Chain Reaction (PCR) assay

Conventional PCR was used to detect several clinically relevant ARGs; *bla*$_{TEM}$, *sul*1, *dfr*A, *tet*A and *aad*A conferring resistance to beta-lactams, sulfonamides, trimethoprim, tetracyclines, aminoglycosides respectively in the effluent wastewater, soil and vegetable DNA samples. PCR assays with positive and negative controls consisted of a total reaction volume of 25µl which constituted of 12.5µl Emerald Amp® GT PCR Master Mix, 1.5 µl each primer, 7.5µl nuclease free water and 2µl DNA template. Target genes were amplified in a conventional PCR machine (ProFlex PCR system), the temperature profile entailed initial denaturation of 95°C for 5 minutes, followed by 35 cycles of 98°C for 10 seconds, 1-minute annealing at specific primer temperatures, 72°C for 1 minute with a final extension at 72°C for 1 minute. The annealing temperatures are specified in table 3.6.1. PCR products were analyzed by 1% (w/v) agarose gel electrophoresis stained in 4 µl /g ethidium bromide for 90 minutes in 1× TAE buffer and viewed using UV light (Gel doc-IT® imager UVP, Cambridge, UK). The sizes of the PCR products were confirmed against Quick-Load 1 Kb DNA ladder (BiLabs inc, England).

Table 3.6.1: List of primers and their annealing temperatures used in this study

Gene	DNA sequence 5'-3'	Annealing temperature	Product (bp)	Reference
***bla*TEM**	**F**- TCCGCTCATGAGACAATAACC **R-** TTGGTCTGACAGTTACCAATGC	58.0ºC	431	Asir et al., 2015
***sul*1**	**F**-GTGACGGTGTTCGGCATTCT **R**-TCCGAGAAGG TGATTGCGCT	54.7ºC	921	Lanz et al., 2003
***dfr*A**	**F**-CCCAACCGAAAGTATGCGGTCG **R**-GTATCTACTTGAT CGAT CAGG	45.6ºC	171	Sunde, 2005
***tet*A**	**F**CATATAATCATCACCAATGGCA **R**-GGCGGTCTTCTTCATCATGC	46.2ºC	500	Kozak et al., 2005
***aad*A**	**F-** GTGGATGGCGGCCTGAAGCC **R**- AATGCCCAGTCGGCAGCG	68.0 ºC	525	Titilawo et al., 2015

3.7. Metagenomics analysis

3.7.1. 16S rRNA gene sequencing and bioinformatics analysis

Uncultured and community DNA from wastewater effluent and wastewater effluent irrigated soil were analyzed by 16S rRNA gene sequencing on the Illumina MiSeq system following a bacterial metagenomics workflow by Klindworth et al., (2013) at Inqaba Biotec™. The protocol included genomic DNA being PCR amplified using a universal primer pair 341F and 785R - targeting the V3 and V4 region of the 16S rRNA gene. Amplicons were then purified by gel, end repaired and illumina specific adapter sequence were ligated to each amplicon. The samples were quantified and individually indexed followed by another purification step. Amplicons were then sequenced on illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit. 20Mb of data (2x300bp long paired end reads) were produced for each sample. Reads were processed through usearch (https://drive5.com/usearch) and taxonomic information was determined based on the Ribosomal Database Project's (http://rdp.cme.msu.edu/index.jsp) 16S database v16 or in the case of ITS1F, the RDP ITS V2 database.

3.7.2. Shotgun metagenomics and bioinformatics analysis

Uncultured and community DNA from wastewater effluent and wastewater effluent irrigated soil samples were fragmented using an enzyme-based approach following part of the protocol from New England BioLab's Next Ultra II kit™. Resulting fragments were purified (size selected), end-repaired and an Illumina specific adapter sequence was ligated to all fragments. The samples were quantified, individually indexed followed by a second size selection step using AMPure XP Beads. The libraries were quality controlled on a DNA chip (Agilent 2100 Bioanalyzer) and then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit according to the manufacturer's protocol.

SPAdes was used through PATRIC (https://www.patricbrc.org/) to assemble reads obtained from illumina shotgun metagenomics sequencing. Annotation of contigs was carried out with Rapid Annotation using Subsystem Technology (RAST) tool kit (http://rast.theseed.org/FIG/rast.cgi), PATRIC k-mer based tool (https://www.patricbrc.org/) was used to assign ARG functional annotation and broad antibiotic resistance mechanisms. ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) and CARD (https://card.mcmaster.ca/) were used to identify acquired ARGs and PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) to identify plasmids in the DNA sequence.

3.8. Software and statistical analysis

Sample site mapping was carried out using ArcGIS mapping v10.6. The means and standard deviations of bacterial counts were calculated from the replicate numbers obtained from the individual plate counts which were imported into Sigma Plot 12.0 Systat Software (Addilink Software Scientific, Barcelona, Spain). To determine if there is a significant difference in the bacterial counts at different sampling sites, one-way analysis of variance (ANOVA) was carried out with a critical p-value of 95% confidence ($p < 0.05$). Diversity of bacterial species were presented in krona charts designed using PATRIC where assembly parameters of the contigs were set to default settings (https://patricbrc.org/).

CHAPTER 4: RESULTS

4.1. Total viable bacterial quantitative analysis

Wastewater effluent is derived directly from the GWWTP maturation ponds and used for irrigation in Glenvalley farm. Viable bacterial count from non-selective media was found to be 1.6×10^5 cfu/ml and 4.6×10^5 cfu/g from GWWTP effluent and effluent irrigated soil in Glenvalley farm respectively. Oodi farm uses effluent discharged into the Notwane River for irrigation of their produce. Quantitative analysis of total viable bacteria from non-selective media showed 1.1×10^5 cfu/ml viable bacteria from Notwane River which was significantly lower than 1.1×10^6 cfu/g ($p = 0.0016$) viable bacteria enumerated from the river irrigated soil in Oodi farm (Figure 4.1.1).

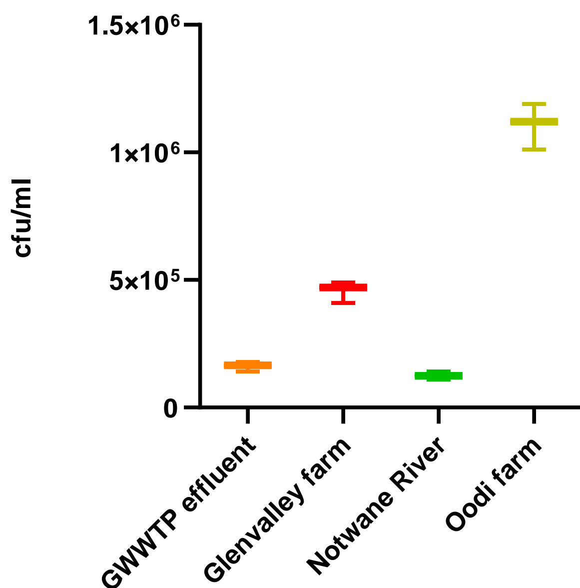

Figure 4.1.1: Viable bacterial quantification from GWWTP effluent, Glenvalley farm, Notwane River and Oodi farm.

From the microcosm experiment, viable bacterial counts of PWWTP effluent stored in the tank were monitored for 3 months compared to tap water. Immediately after filling the storage tank (Day 1), bacterial quantitative analysis recorded 8.7×10^5 cfu/ml viable bacteria from PWWTP effluent, which was at least three orders of magnitude higher than 3.0×10^2 cfu/ml recorded for tap water. After 1 month (day 30) of filling the storage tank with effluent, bacterial quantity significantly ($p = 0.0032$) declined to 7.9×10^4 cfu/ml. The bacterial quantity in the effluent storage tank continued to decline for 3 months, with 6.6×10^3 cfu/ml and 3.7×10^3 cfu/ml viable bacteria recorded on the second (day 60) and third month (day 90) respectively. Bacterial

quantity in tap water however remained relatively constant throughout the three months (Figure 4.1.2).

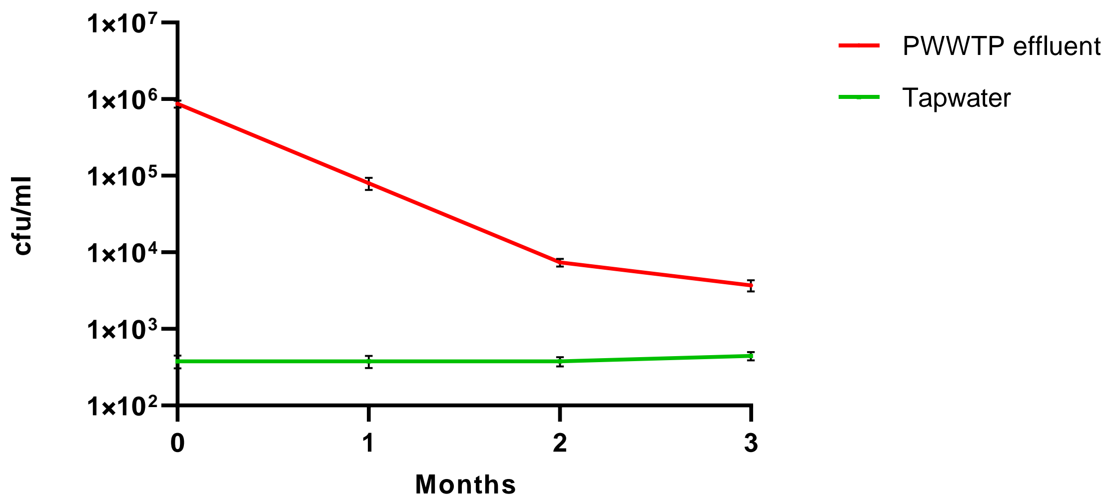

Figure 4.1.2: Bacterial quantification in PWWTP effluent filled storage tank in comparison to BIUST tap water.

From the vegetable surfaces, PWWTP effluent irrigated spinach had 3.4×10^4 cfu/ml of viable bacteria compared to 2.5×10^3 cfu/ml from tap water irrigated spinach. Wastewater irrigated beetroots had a significantly (p= 0.0061) higher bacteria quantity of 5.5×10^6 cfu/ml in comparison to 7.0×10^5 cfu/ml from tap water irrigated beetroots (Figure 4.1.3).

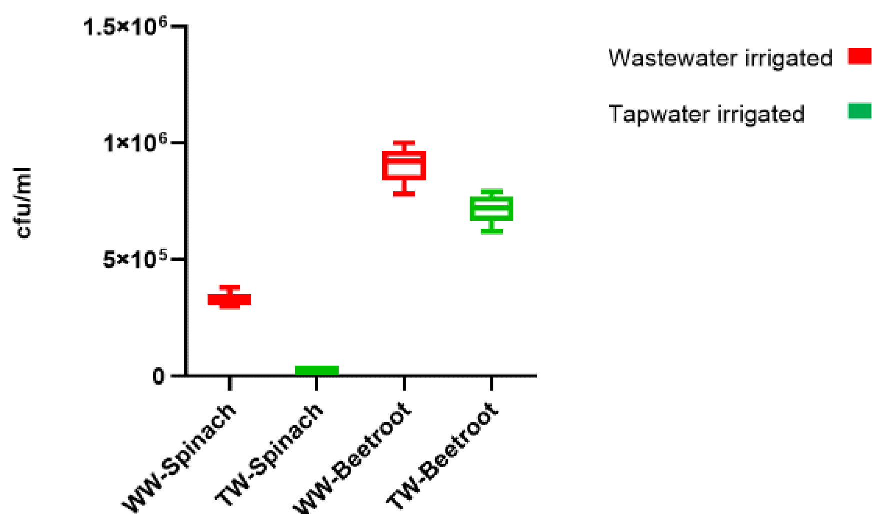

Figure 4.1.3: Bacterial quantification in vegetables from the microcosm experiment (WW- wastewater irrigated, TW- tap water irrigated).

4.2. Bacterial species presumptive isolation and quantification

From the field surveillance study, presumptive bacterial species *Listeria* (4.1×10^4 cfu/ml), *Enterobacter* (3.7×10^4 cfu/ml), *Staphylococcus* (2.7×10^4 cfu/ml), *Campylobacter* (8.4×10^3 cfu/ml), *Pseudomonas* (1.1×10^3 cfu/ml), *Shigella* (4.0×10^2 cfu/ml) and *E.coli* (1.0×10^2 cfu/ml) were identified in GWWTP effluent. A similar pattern except *Enterobacter* was observed in Glenvalley farm soil which is irrigated with GWWTP effluent; *Campylobacter* species (2.8×10^4 cfu/ml), *Staphylococcus* species (2.2×10^4 cfu/ml), *E.coli* (1.9×10^4 cfu/ml), *Pseudomonas* species (1.8×10^4 cfu/ml), *Listeria* species (1.7×10^4 cfu/ml) and *Shigella* species (1.2×10^4 cfu/ml) were also identified.

GWWTP effluent going through the Notwane River is used for irrigation of produce in Oodi farm. From the Notwane River, *Listeria* species (1.0×10^3 cfu/ml), *E.coli* (8.4×10^2 cfu/ml), *Campylobacter* (8.4×10^2 cfu/ml), *Staphylococcus* species (5.3×10^2 cfu/ml), and *Shigella* (4.2×10^2 cfu/m) were isolated. *Staphylococcus* species (5.9×10^3 cfu/ml) and *Listeria* species (3.9×10^3 cfu/ml), were also isolated from the soil irrigated with Notwane River water in Oodi farm (Figure 4.2.1).

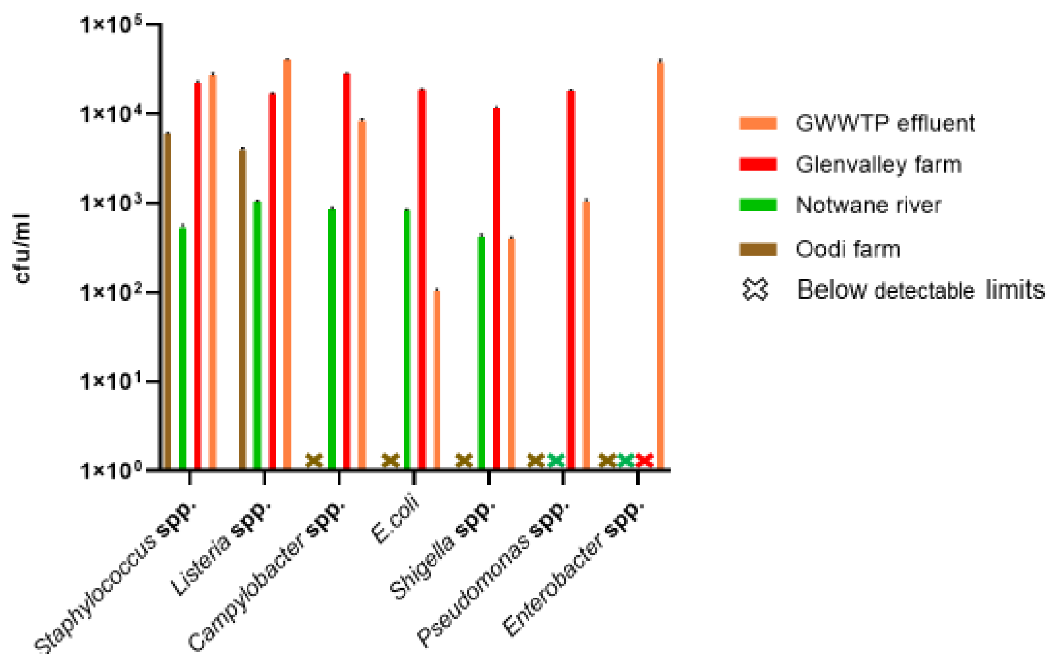

Figure 4.2.1: Quantities of presumptive viable bacterial spp. in GWWTP effluent, Glenvalley farm soil, Notwane River and Oodi farm soil.

From the PWWTP effluent filled tank, different presumptive bacterial species were isolated with *Campylobacter* species being the highest with a bacterial load of 9.1×10^2 cfu/ml

followed by *Pseudomonas* species with 9.0×10^2 cfu/ml, *Shigella* (8.3×10^2 cfu/ml), *Enterobacter* (6.3×10^2 cfu/ml) and *Listeria* species (1.4×10^2 cfu/ml). One set of the vegetables was irrigated with PWWTP effluent, the other with tap water while 3 planting bags remained untreated/unirrigated to serve as controls in the microcosm experiment. *Staphylococcus* species (1.5×10^3 cfu/ml), *Campylobacter* species (3.3×10^2 cfu/ml) and *Listeria* species (2.3×10^2 cfu/ml) were isolated in PWWTP effluent irrigated soil. *Staphylococcus* species were also isolated in tap water irrigated soil (3.1×10^2 cfu/ml) and in untreated soil (2.2×10^2 cfu/ml) as shown in Figure 4.2.2. From the vegetables harvested, *Staphylococcus* species were isolated in wastewater irrigated beetroots surface (1.3×10^3 cfu/ml), tap water irrigated beetroots surface (3.2×10^2 cfu/ml), wastewater irrigated spinach leaves (2.8×10^2 cfu/ml) and tap water irrigated spinach leaves (2.0×10^2 cfu/ml). *Listeria* species were isolated from wastewater irrigated beetroots (8.3×10^2 cfu/ml) and wastewater irrigated spinach (3.6×10^2 cfu/ml) while *Campylobacter* species was identified in wastewater irrigated beetroots (2.1×10^3 cfu/ml), tap water irrigated beetroots (3.2×10^2 cfu/ml) and wastewater irrigated spinach (3.6×10^2 cfu/ml). *Enterobacter* (1.1×10^3 cfu/ml), *Pseudomonas* (4.8×10^2 cfu/ml) and *Shigella* (3.0×10^2 cfu/ml) were isolated from wastewater irrigated beetroots (Figure 4.2.3).

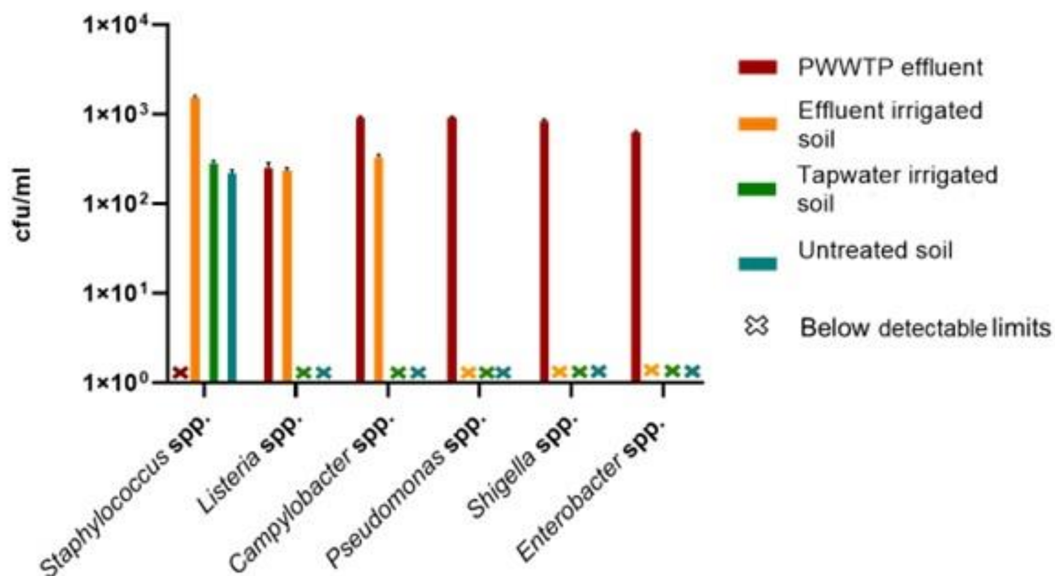

Figure 4.2.2: Quantities of presumptive viable bacterial spp. in PWWTP effluent (at day 1), effluent irrigated soil (at day 90), tap water irrigated soil (at day 90) and untreated soil (at day 90).

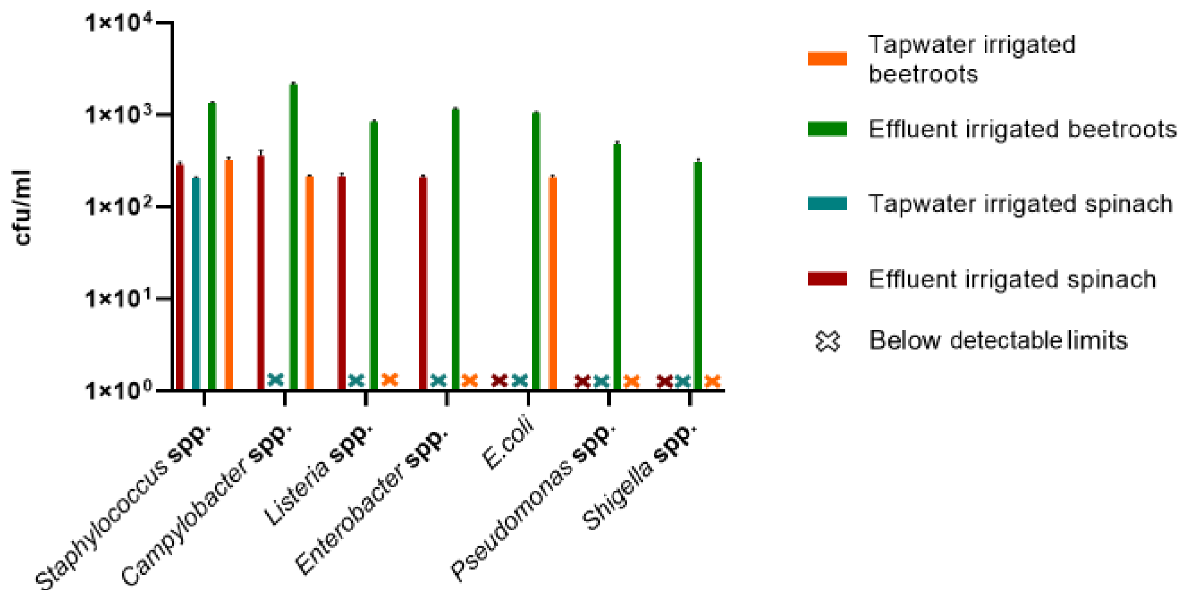

Figure 4.2.3: Quantities of presumptive viable bacterial spp. in PWWTP effluent irrigated vegetables and tapwater irrigated vegetables (day 40 for spinach, day 60 for beetroots) from the microcosm experiment.

4.3. Antibiotic resistance profiles of total bacterial isolates from effluent, soil and vegetables

A total of 110 isolates from GWWTP effluent were tested against eight different antibiotics at clinical breakpoint concentration. 96% of the isolates were resistant to Penicillin, 84% to Ampicillin and Cephalosporin, 71% to Erythromycin, 60% to Tetracycline and 25% to Streptomycin, the isolates were all susceptible to Meropenem and Ciprofloxacin. From the 90 GWWTP effluent irrigated soil (Glenvalley farm) isolates, 98% were resistant to Penicillin, Ampicillin, Cephalosporin and Erythromycin. Resistance to Streptomycin (39%) and Tetracycline (39%) was also observed. Isolates from the Notwane River (n=90) showed 98% resistance to Penicillin, Ampicillin and Cephalosporin. 84% of the isolates were resistant to Erythromycin, 57% to meropenem and 24% to Streptomycin and Tetracycline. From the 90 isolates from the Notwane river irrigated soil (Oodi farm), 95% were resistant to Ampicillin and Cephalosporin, 50% to Penicillin and 24% to Erythromycin (Table 4.3.1).

Bacterial isolates from the microcosm experiment were tested against eight antibiotics at clinical breakpoint concentrations. From the 110 isolates from PWWTP effluent, 98% were resistant to Penicillin, Meropenem, Ampicillin and Erythromycin, 45% to Streptomycin and

28% to Cephalosporin. All isolates from the PWWTP effluent were susceptible to Tetracycline and Ciprofloxacin at clinical breakpoint concentrations. Of the 90 isolates from PWWTP effluent irrigated soil, 98% of the isolates showed resistance to Penicillin, while 14% were resistant to Ampicillin, Erythromycin and Meropenem. Tap water irrigated soil (n=90) isolates showed 81% resistance to Penicillin however they were all susceptible to the rest of the antibiotics tested. From the soil that did not receive any water treatment, 82% and 75% of the 90 isolates were resistant to Penicillin and Ampicillin respectively (Table 4.3.1).

Antibiotic resistance was not observed in tap water irrigated spinach isolates, however of the 90 isolates from wastewater irrigated spinach 11% were resistant to Penicillin and Ampicillin. 98% resistance to Penicillin and Ampicillin was observed in effluent irrigated beetroots, 84% to Erythromycin and Cephalosporin, and 29% resistance to Streptomycin and Tetracycline (n=90). Isolates from tap water irrigated beetroots (n=90) showed 89% resistance to Penicillin, 66% resistance to Ampicillin and 18% to Erythromycin (Table 4.3.1).

Table 4.3.1: Prevalence of antibiotic resistance phenotypes from effluent, soil and vegetables

Source	High Resistance (%)	Low Resistance (%)	No Resistance
GWWTP effluent (n=110)	Pen (96), Amp (84),Cep (84), Ery (71),Tet (60)	Str (25)	Mer, Cip
Glenvalley farm (n=90)	Pen (98), Amp (98), Cep (98), Ery (98)	Str (39), Tet (39)	Mer, Cip
Notwane River (n=90)	Pen (98),Amp (98),Cep (98),Ery (84),Mer (57)	Str (24), Tet (24)	Cip
Oodi farm (n=90)	Amp (95), Cep (95), Pen (50)	Ery (24)	Str, Tet, Mer, Cip
PWWTP effluent (n=110)	Pen (98), Amp (98), Ery (98), Mer (98)	Str (45), Cep (28)	Tet, Cip
PWWTP effluent irrigated soil (n=90)	Pen (98)	Amp (14), Ery (14), Mer (14)	Str, Cep, Tet, Cip
Tap water irrigated soil (n=90)	Pen (81)		Amp, Ery, Mer, Str, Cep, Tet, Cip
Untreated soil (n=90)	**Pen (82), Amp (75)**		
Effluent irrigated beetroots (n=90)	Pen (98), Amp (98), Ery (84), Cep (84)	Str (29), Tet (29)	Mer, Cip
Tap water irrigated beetroots (n=90)	Pen (89), Amp (66)	Ery (18)	Cep, Str, Tet, Mer, Cip
Effluent irrigated spinach (n=90)		Pen (11), Amp (11)	Ery, Cep, Str, Tet, Mer, Cip
Tap water irrigated spinach (n=90)			Pen, Amp, Ery, Cep, Str, Tet, Mer, Cip

% Resistance of ≥ 50% considered high resistance whereas <50% considered low resistance. (Pen– Penicillin, Amp – Ampicillin, Ery – Erythromycin, Str – Streptomycin, Mer – Meropenem, Tet – Tetracycline, Cep – Cephalosporin)

4.4. Antibiotic resistance profiles of different bacterial species

From the total *Staphylococcus* species isolated from field surveillance, 40% were resistant to four antibiotics tested 33% were resistant to a total of five antibiotics, and 27% of the *Staphylococcus* species isolated were sensitive to all eight antibiotics tested. (Figure 4.4.1). *Staphylococcus* species isolated from GWWTP effluent were resistant to Cephalosporin (91%), Penicillin (51%), Tetracycline (47%), Ampicillin (39%) and Streptomycin (6%). *Staphylococcus* species from the effluent irrigated soil in Glenvalley farm also showed resistance to Cephalosporin (62%), Penicillin (61%), Ampicillin (53%) and Streptomycin (10%). From the Notwane River, *Staphylococcus* isolates showed low resistance to Ampicillin (31%), Penicillin (23%), Cephalosporin (19%) and Streptomycin (8%). No resistance was detected in *Staphylococcus* species isolated from Notwane River irrigated soil in Oodi farm (Figure 4.4.1). From the microcosm experiment, 10% of the total *Staphylococcus* species isolated were resistant to only one antibiotic tested, 34% to two antibiotics and 39% were resistant to a total of three antibiotics bringing a total of multiple resistant *Staphylococcus* species from the microcosm experiment to 83%. *Staphylococcus* species isolated from PWWTP effluent irrigated soil showed 61% resistance to Penicillin, 33% to Ampicillin and 5% Cephalosporin. High resistance to Penicillin (81%) was observed in tap water irrigated soil isolates. *Staphylococcus* isolates from untreated soil showed high resistance to Penicillin (83%) and Ampicillin (73%). From both effluent irrigated and tap water irrigated beetroots *Staphylococcus* isolates were resistant to Penicillin (57% and 44%) and Ampicillin (38% and 19%) respectively. 22% of the *Staphylococcus* species isolated from effluent irrigated beetroots were also resistant to Cephalosporin. *Staphylococcus* from both effluent and tap water irrigated spinach were not resistant to any of the antibiotics tested at clinical breakpoint concentration (Figure 4.4.2).

Campylobacter species from the field surveillance also exhibited multi-drug resistance, 63% of the total *Campylobacter* species were resistant to a total of six antibiotics tested and 37% of the total *Campylobacter* species from field surveillance were susceptible to all the eight antibiotics tested. *Campylobacter* species were identified in GWWTP effluent, the isolates showed resistance to Tetracycline (91%), Penicillin (91%), Ampicillin (89%), Cephalosporin (81%), Erythromycin (69%) and Streptomycin (11%). Resistance against Ampicillin (95%), Penicillin (89%), Cephalosporin (65%), Erythromycin (51%), Tetracycline (46%) and Streptomycin (5%) was also observed in *Campylobacter* species isolated from GWWTP effluent irrigated soil (Glenvalley farm). *Campylobacter* isolated from Notwane River also

showed multi-drug resistance against Penicillin (96%), Ampicillin (96%), Erythromycin (66%), Cephalosporin (61%), Tetracycline (26%) and Streptomycin (13%) at clinical breakpoint concentrations (Figure 4.4.1). From the microcosm experiment, 98% of *Campylobacter* species exhibited diverse multi-drug resistant patterns, 11% of the total *Campylobacter* species were resistant to three antibiotics, 6% to four antibiotics, 34% to five and 47% of *Campylobacter* species were resistant to a total of 6 antibiotics tested. *Campylobacter* species isolated from PWWTP effluent showed high resistance against to Cephalosporin (81%), Penicillin (81%), Ampicillin (78%), Meropenem (73%) and Erythromycin (67%), lower resistance against Streptomycin (23%) was also observed. Effluent irrigated soil *Campylobacter* isolates were resistant to Penicillin (63%), Ampicillin (57%), Erythromycin (21%), Cephalosporin (19%) and Meropenem (10%). From the PWWTP effluent irrigated beetroots, *Campylobacter* species were resistant to Ampicillin (63%), Penicillin (51%), Cephalosporin (51%) and Erythromycin (33%). *Campylobacter* isolated from tap water irrigated beetroots showed resistance against Erythromycin (18%), Penicillin (8%) and Ampicillin (8%). In effluent irrigated spinach, 18% of the *Campylobacter* isolates were resistant to Penicillin and Ampicillin.

Multi-drug resistant *Pseudomonas* species were also observed from the field surveillance, 64% of the total *Pseudomonas* isolates were resistant to five antibiotics tested and 36% were susceptible to all eight antibiotics tested GWWTP effluent *Pseudomonas* isolates showed high resistance to Cephalosporin (97%), Penicillin (81%), Ampicillin (79%), Tetracycline (59%) and low resistance to Streptomycin (9%). High resistance against Ampicillin (89%), Penicillin (86%), and Cephalosporin (81%) was also observed in *Pseudomonas* species isolated from GWWTP effluent irrigated soil (Glenvalley farm) and lower resistance was observed against Tetracycline (55%) and Streptomycin (41%) as illustrated on (Figure 4.4.1). *Pseudomonas* species were also isolated in the microcosm experiment, 13% of the total *Pseudomonas* isolates were resistant to four antibiotics and 16% to five antibiotics tested (Figure 4.4.2). PWWTP effluent irrigated isolates showed low resistance to Ampicillin (32%), Penicillin (21%), Cephalosporin (13%), Meropenem (10%) and Streptomycin (6%) whereas *Pseudomonas* from effluent irrigated beetroots was resistant to Ampicillin (21%), Penicillin (13%), Streptomycin (10%) and Cephalosporin (6%).

E. coli isolates from the field surveillance had expressed multi-drug resistance to antibiotics tested, 14% of the isolates were resistant to four antibiotics and 44% were resistant to a total of 6 antibiotics tested (Figure 4.4.1). High resistance against Ampicillin (81%), Penicillin

(72%), Cephalosporin (61%), Tetracycline (59%) and low resistance against Streptomycin (22%) and Erythromycin (12%) was observed in *E. coli* isolated from GWWTP effluent irrigated soil in Glenvalley farm. Low resistance against Cephalosporin (23%), Erythromycin (13%), Penicillin (11%) and Ampicillin (7%) was observed in *E. coli* isolated from the Notwane River. Multi-drug resistant *E. coli* was also isolated in PWWTP effluent irrigated beetroots from the microcosm experiment, 25% of the isolates were resistant to six antibiotics tested. From the resistant isolates 39% were resistant to Cephalosporin, 33% to Ampicillin, 31% to Penicillin, 23% to Erythromycin, 16% to Meropenem and 12% to Tetracycline. None of the *E. coli* isolated from tap water irrigated beetroots showed resistance against antibiotics tested (Figure 4.4.2).

Enterobacter from the field surveillance exhibited 66% resistance to a total of 6 antibiotics tested . *Enterobacter* was isolated only in GWWTP effluent, the isolates were resistant to Penicillin (91%), Cephalosporin (80%), Erythromycin (73%), Ampicillin (66%), Tetracycline (66%) and Streptomycin (19%) (Figure 4.4.1). Multi-drug resistant *Enterobacter* was also isolated from the microcosm experiment with 16% of the isolates resistant to a total of 6 antibiotics and 84% were susceptible to all eight antibiotics tested. *Enterobacter* isolated from PWWTP effluent showed low resistance against to Penicillin (31%), Ampicillin (23%), Erythromycin (21%), Cephalosporin (16%), Meropenem (11%) and Tetracycline (9%). *Enterobacter* isolated from PWWTP effluent irrigated beetroots also showed low resistance against Penicillin (21%), Ampicillin (19%), Erythromycin (15%), Cephalosporin (13%), Meropenem (9%) and Tetracycline (5%). *Enterobacter* isolates from effluent irrigated spinach were susceptible to all antibiotics tested (Figure 4.4.2).

From the microcosm experiment, 16% of *Shigella* species isolated showed resistance to a total of four antibiotics and 19% showed resistance to five antibiotics. *Shigella* species isolated from PWWTP effluent showed low resistance to Erythromycin (26%), Penicillin (24%), Ampicillin (21%), Meropenem (17%) and Cephalosporin (6%). Low resistance against Penicillin (21%), Erythromycin (21%), Ampicillin (16%), and Cephalosporin (4%) was also observed in *Shigella* species isolated from effluent irrigated beetroots (Figure 4.4.2).

Multi-drug resistant *Listeria* was observed from the field surveillance with44% of the *Listeria* isolates expressing resistance to a total of four antibiotics tested (Figure 4.4.1). *Listeria* species isolated from GWWTP effluent were resistant to Penicillin (91%), Ampicillin (81%), Erythromycin (60%) and Tetracycline (42%). The same resistance pattern was observed in

GWWTP effluent irrigated soil (Glenvalley farm) with 91% resistance to Ampicillin, 86% resistance to Penicillin, 53% resistance to Erythromycin and 23% resistance to Tetracycline. Low resistance against Penicillin (41%), Ampicillin (34%), Streptomycin (26%) and Erythromycin (20%) was observed in *Listeria* species isolated from Notwane River. From Notwane River irrigated soil in Oodi farm, *Listeria* species were resistant to Ampicillin (26%), Penicillin (13%), Erythromycin (11%) and Streptomycin (11%). *Listeria* species isolated from the microcosm experiment exhibited 16% of the total isolates resistant to four antibiotics, 53% resistant to a total of six antibiotics tested, and 31% of the total *Listeria* species were susceptible to all eight antibiotics tested. *Listeria* species from PWWTP effluent in the storage tank were resistant to Erythromycin (92%), Meropenem (78%), Penicillin (74%), Ampicillin (70%), Streptomycin (43%) and Cephalosporin (28%). From the effluent irrigated soil in the microcosm experiment *Listeria* species showed resistance to Penicillin (92%), Ampicillin (22%), Erythromycin (18%), and Meropenem (14%). High resistance was observed against Ampicillin (81%), Penicillin (75%), Cephalosporin (68%), Erythromycin (64%), and low resistance against Streptomycin (23%) and Tetracycline (18%) in *Listeria* isolated from effluent irrigated beetroots. *Listeria* species isolated from effluent irrigated spinach was susceptible to all antibiotics tested at clinical breakpoint concentration as seen on Figure 4.4.2

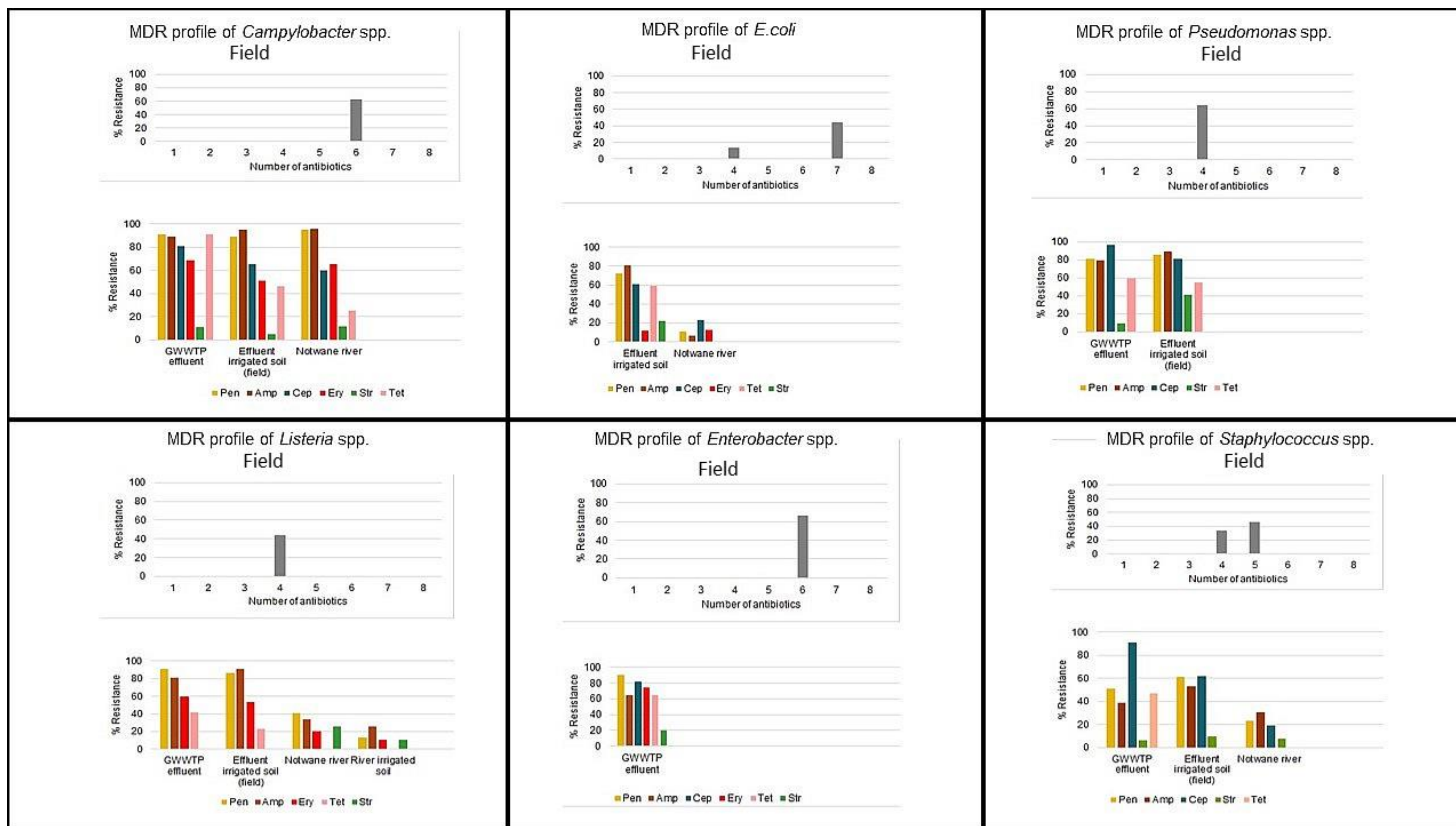

Figure 4.4.1: Multi-drug resistance profiles of field surveillance bacterial isolates . Top (grey) graph shows percentage (%) resistance exhibited by the total bacterial species against number of tested antibiotics. Bottom (coloured) graph shows antibiotic resistance perecentage (%) of the bacterial isolates from different sampling sites

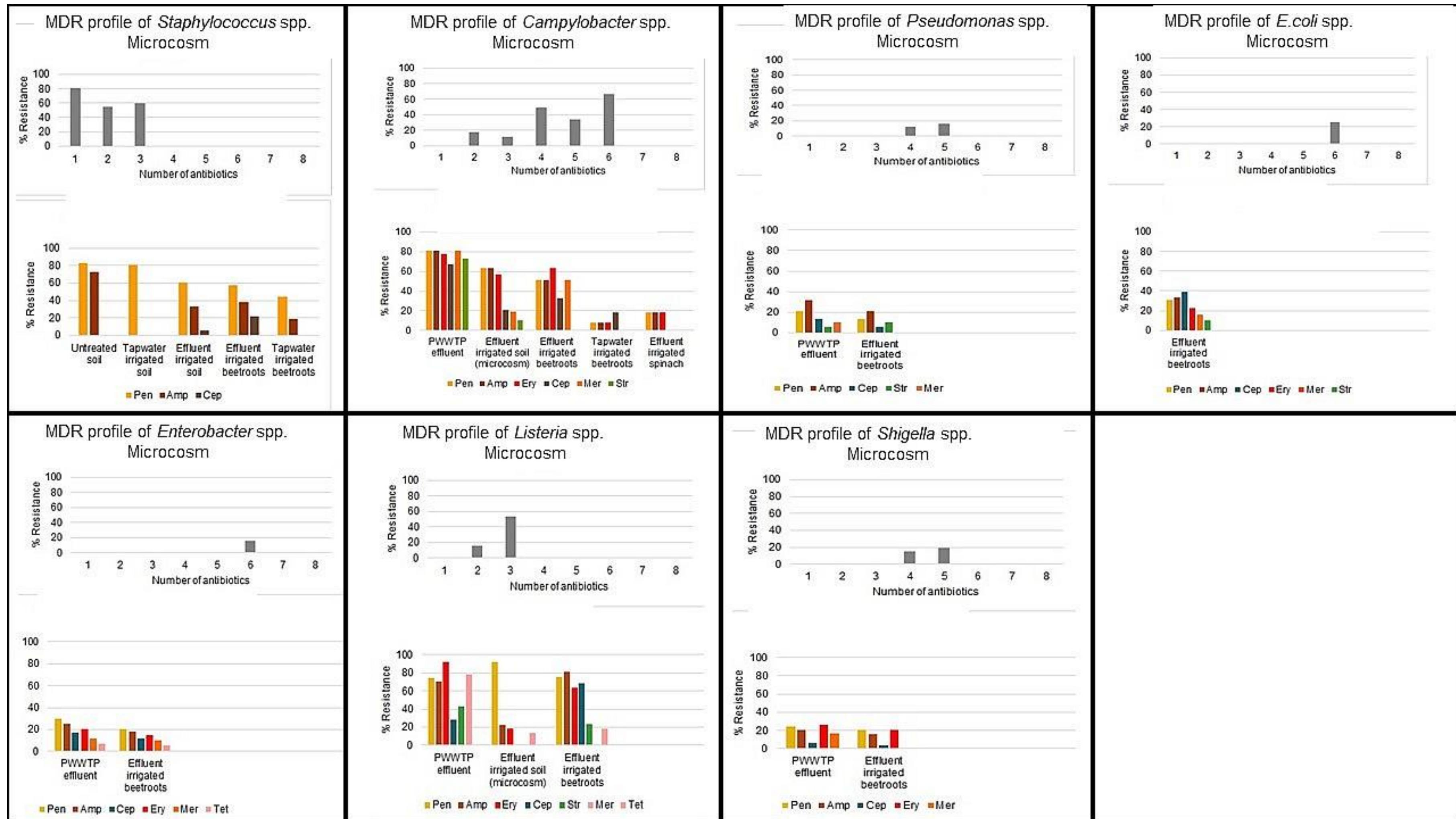

Figure 4.4.2: Multi-drug resistance profiles of microcosm experiment bacterial isolates. Top (grey) graph shows percentage (%) resistance exhibited by the total bacterial species against number of tested antibiotics. Bottom (coloured) graph shows antibiotic resistance perecentage (%) of the bacterial isolates from different sampling sites

4.5. Detection of antibiotic resistance genes in effluent irrigated soil and vegetables

The occurrence of ARGs from the field surveillance; GWWTP, GWWTP irrigated soil (Glenvalley farm), Notwane River and Notwane River irrigated soil (Oodi farm), and microcosm experiment; PWWTP effluent irrigated soil and vegetables, was determined using conventional PCR. From the five targeted ARGs, *bla*$_{TEM}$ and *dfr*A were detected in GWWTP effluent, GWWTP effluent irrigated soil, Notwane River and river irrigated soil. ARGs *tet*A and *sul*1 were only detected in GWWTP effluent and *aad*A in GWWTP effluent and GWWTP effluent irrigated soil. From the microcosm experiment *bla*$_{TEM}$ was detected in effluent irrigated spinach and beetroots. Although the gene was not detected in untreated soil, it was detected in soil irrigated with effluent for 30, 60 and 90 days. (Table 4.5.1).

Table 4.5.1: Antibiotic resistance genes detected in both the field and microcosm experiments using PCR

	Field surveillance (Gaborone)				**Microcosm experiment (Palapye)**				
Gene Target	GWWTP Effluent	Glenvalley farm	Notwane River	Oodi farm	Untreated soil	Soil 30 days post WW irrigation	Soil 60 days post WW irrigation	Wastewater irrigated spinach	Wastewater irrigated beetroot
***bla*$_{TEM}$**	+	+	+	+	-	+	+	+	+
***dfr*A**	+	+	+	+	-	-	-	-	-
***aad*A**	+	+	-	-	-	-	-	-	-
***tet*A**	+	-	-	-	-	-	-	-	-
***sul*1**	+	-	-	-	-	-	-	-	-

KEY: + Presence

-Absence

4.6. 16s rRNA gene metagenomics analysis

Metagenomics sequencing of the16S rRNA gene was carried out to further determine the diversity of bacterial phylogenetic groups in PWWTP effluent, untreated soil and PWWTP effluent irrigated soil. From the PWWTP effluent, Cyanobacteria phylum was over-represented with 48% followed by Firmicutes (21%), Proteobacteria (17%), Actinobacteria (13%) and Bacteroidetes (1%). At class level Oscillatoriophycideae showed a high percentage of 43%, Bacilli (20%), Gammaproteobacteria (14%), Actinobacteria (13%), Betaproteobacteria (2%),

Alphaproteobacteria (1%), and Bacteroidia was least represented with 1% in the total bacterial population of PWWTP effluent sample. Bacteria identified were classified into different genus, *Streptococcus* (18%), *Pasteurella* (8%), *Rothia* (8%), *Enterobacter* (5%), *Pseudomonas* (3%), *Escherichia* (2%), *Actinomyces* (2%), *Neisseria* (2%), *Stella* (1%) and *Salmonella* (1%) (Figure 4.6.1).

Five phyla were identified in untreated soil sample, these include Proteobacteria (88%), Firmicutes (5%), Actinobacteria (4%), Planctomycetes (2%) and Bacteroidetes (2%). From these phylum Gammaproteobacteria was shown to be the highest class with 79% representation, with Comamonas aquatica representing 9% of the total bacteria. At genus level, 25% of the bacteria classified were *Pseudomonas, Comamonas* and *Provencia* had 9% representation each genus, *Escherichia*, *Klebsiella* and *Citrobacter* each had 7% representation. *Streptococcus* was represented with 5% and *Enterobacter* and *Acinetobacter* by 2% of the total bacteria from untreated soil sample (Figure 4.6.2).

From the PWWTP effluent irrigated soil sample, Actinobacteria was the most abundant phylum with 42% representation, followed by Proteobacteria with 22%. Firmicutes (14%), Plantomycetes (10%), Acidobacteria (2%), Chloroflexi (3%), Bacteroidetes (2%) and Gemmatimonadetes (2%) were also identified. At class level, Actinobacteria was over-represented with 34% followed by Bacilli (11%), Alphaproteobacteria (12%), Gammaproteobacteria (5%), Planctomycetia (10%), Betaproteobacteria (4%). Ktedonobacteria accounted for 3%, Clostridia 2% and Deltaproteobacteria 1%. Out of all the identified bacterial species, 15% were classified as *Streptococcus*, 11% as *Bacillus*, *Conexibacter* 7% and *Solurobacter* 6%. *Streptomyces* and *Methylobacterium* accounted for 5% of the species identified, *Escherichia* had 3% representation. *Salmonella* and *Neisseria* were the least represented genus with 1% each genus (Figure 4.6.3).

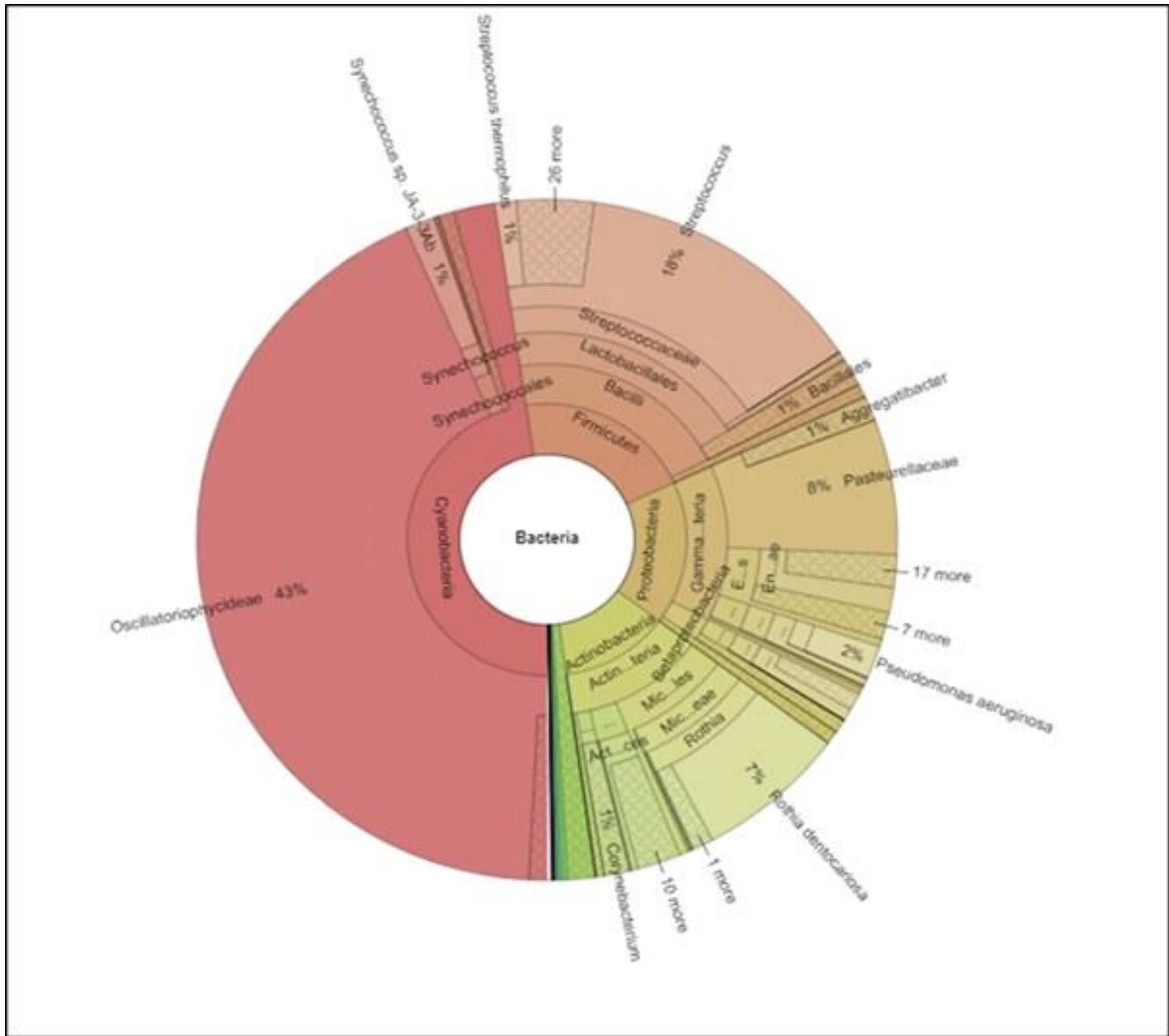

Figure 4.6.1: Bacterial diversity in PWWTP effluent showing bacterial up to species level.

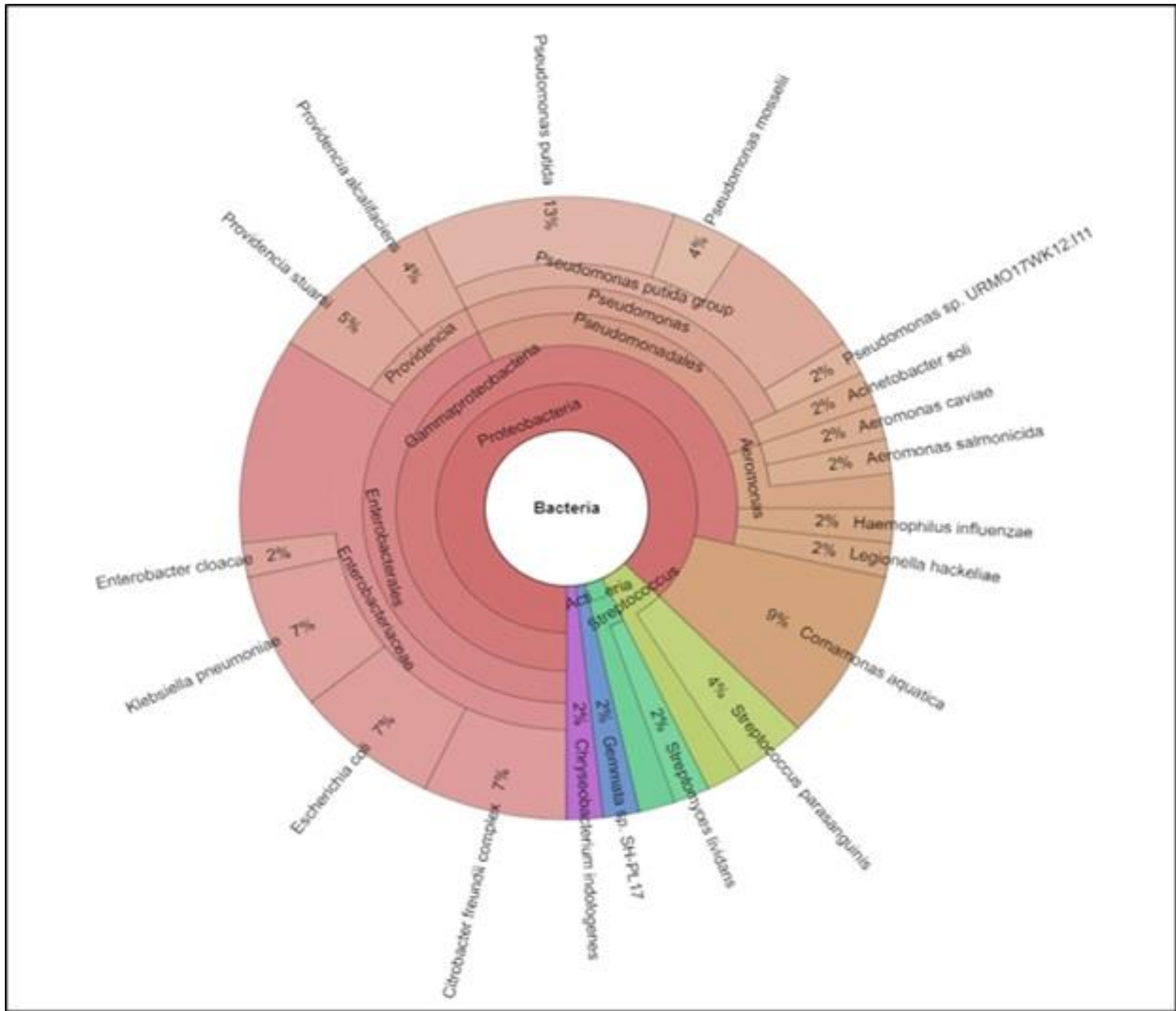

Figure 4.6.2: Bacterial diversity in untreated soil sample from the microcosm experiment showing bacterial phylogeny at up to species level.

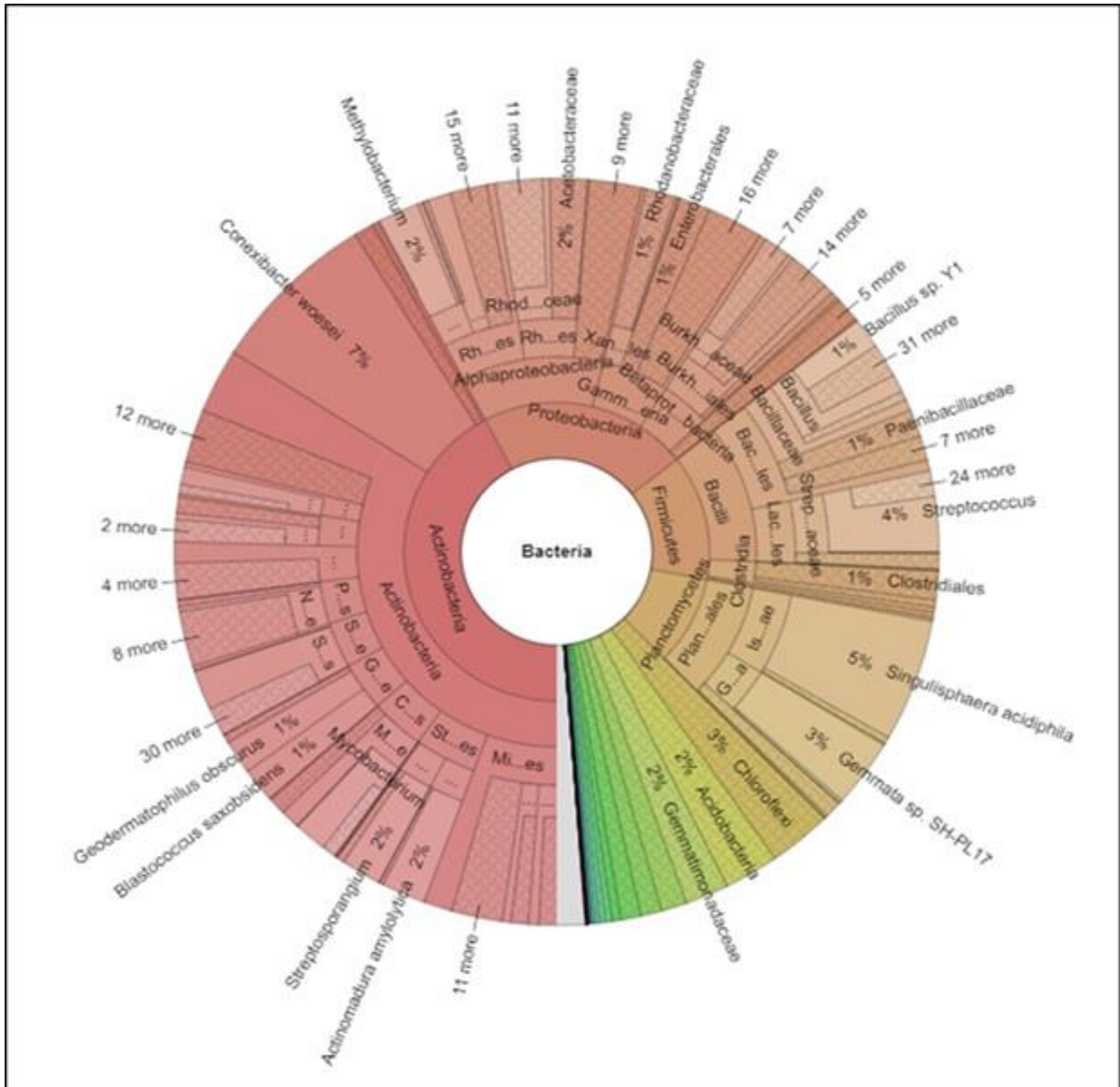

Figure 4.6.3: Bacterial diversity in PWWTP effluent irrigated soil showing bacterial phylogeny up to species level.

Proteobacteria and Firmicutes phyla were compared between PWWTP effluent, untreated soil and 90 days effluent irrigated soil to determine the effects of effluent wastewater on the bacterial communities in the soil before and after irrigation.

Proteobacteria was identified in PWWTP effluent, untreated soil and 90 days effluent irrigated soil (Figure 4.6.4). At phyla level a reduction in Proteobacteria was observed from 88% (in untreated soil) to 22% following irrigation with wastewater effluent that comprised of only 17% Proteobacteria population. A notable reduction in Proteobacteria is further observed at class level with 90% gammaproteobacteria in untreated soil and 25% in effluent irrigated soil where 84% gammaproteobacteria was observed in PWWTP effluent. Other bacteria classes such as betaproteobacteria, alphaproteobacteria and deltaproteobacteria are seen to be introduced into the soil where they were not identified before irrigation with PWWTP. At order level Enterobacteriales and Pseudomonadales also reduced from 55% and 34% to 25% and 8% respectively. At family level Enterobacteriacea slightly reduced from 79% in untreated soil to 72% in effluent irrigated soil where 62% Enterobacteriacea were observed in PWWTP effluent. Yersiniaceae and Erwiniaceae also appear to be introduced from effluent into soil after irrigation, since these bacterial families were not observed in untreated soil.

Firmicutes were identified in PWWTP effluent, untreated soil and 90 days effluent irrigated soil. An increase in firmicutes phyla was observed in soil as 5% was identified in untreated soil and 14% in PWWTP effluent irrigated soil with 21% firmicutes observed in PWWTP effluent wastewater. At class level, only bacilli (100%) was observed in untreated soil whereas 84% was observed in effluent irrigated soil and 97% in PWWTP effluent wastewater. Negativicutes and Clostridia were identified in both PWWTP effluent and effluent irrigated soil. At order level only Lactobacillales were identified in untreated soil, a notable reduction was observed in effluent irrigated soil with 37% Lactobacillales and 75% Bacilli of which PWWTP effluent had 7% Bacilli. Lactobacillaceae (8%), Leuconostacaceae (3%) and Enterococcaceae (3%) were observed in effluent irrigated soil but not in PWWTP effluent and untreated soil.

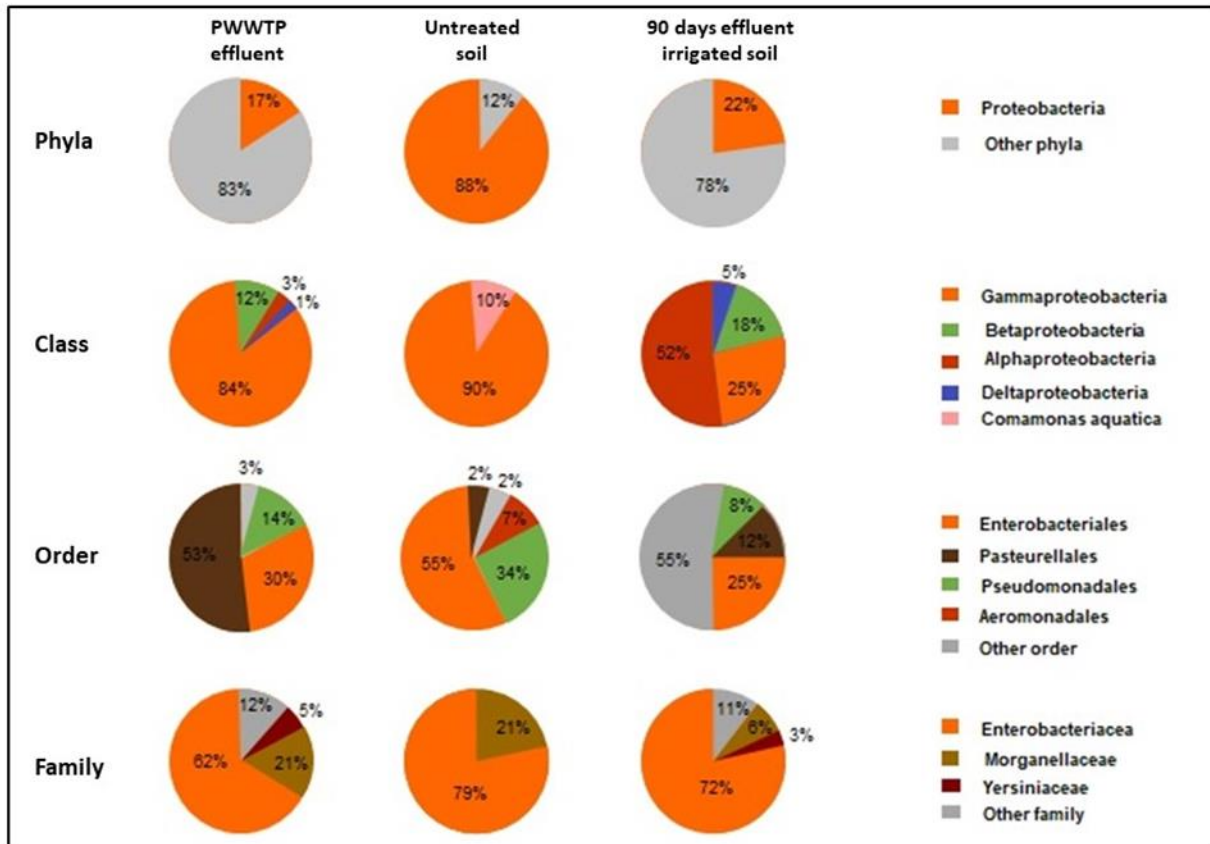

Figure 4.6.4: Taxonomic classification comparison based on Proteobacteria found in PWWTP effluent, untreated soil and effluent irrigated soil

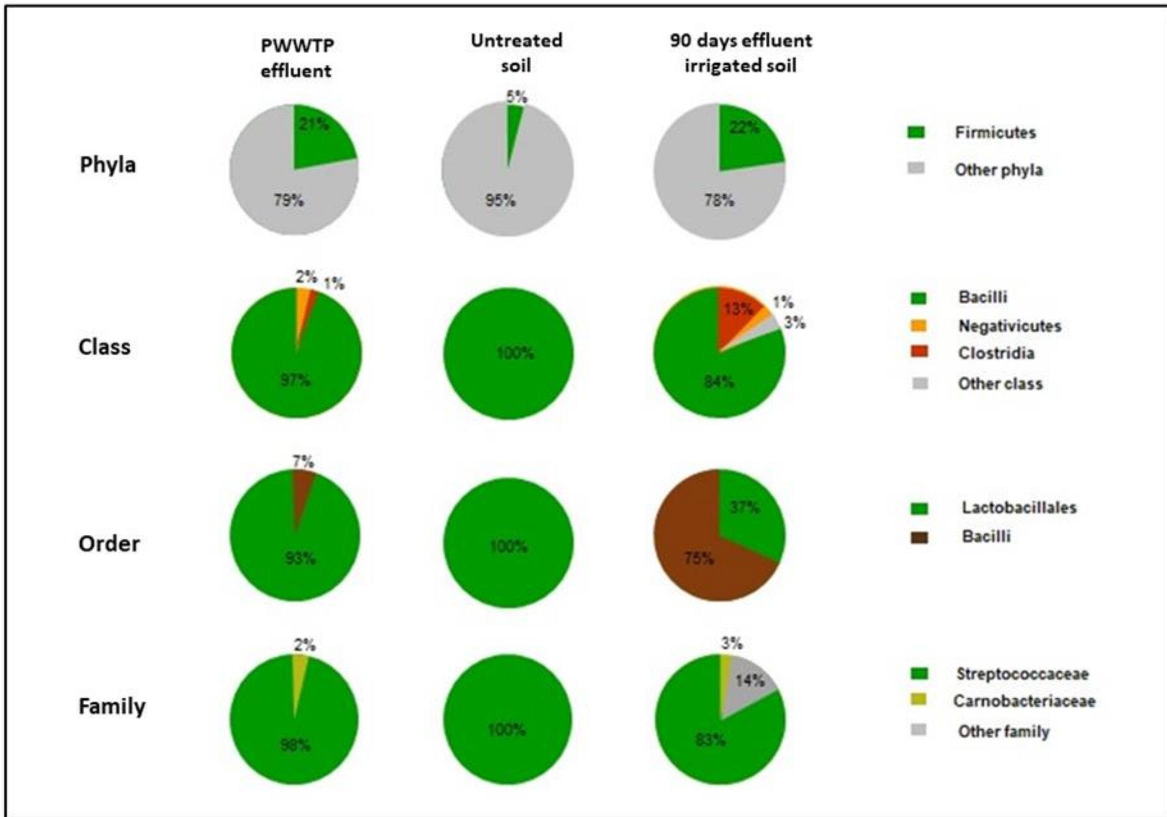

Figure 4.6.5: Taxonomic classification comparison based on Firmicutes found in PWWTP effluent, untreated soil and effluent irrigated soil

4.7. Shotgun metagenomic analysis

Using ResFinder and CARD, diverse acquired ARGs were identified in PWWTP effluent and grouped under several clinically important classes of antibiotics; aminoglycosides, beta-lactamase, trimethoprim, macrolide, glycopeptide, tetracycline, sulfonamides, quinolones and oxazolidinone. Specific antibiotic inactivation mechanisms were identified associated with aminoglycosides (*aad*A5, *aac(2)*-la*, aph(6)*-id, *aph (3)*-ib), beta-lactamase (*bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{TEM-122}$, *bla*$_{SHV-163}$, *bla*$_{OXA-663}$, *ampC*) and macrolide (*mph*A) genes. Beta-lactamase *ompk35* gene was associated with conferring resistance through a different mechanism that reduces permeability to antibiotics. Trimethoprim (*dfr*A1*, dfr*A14, *dfr*A17), sulfonamides (*sul*1, *sul*2, *sul*3) and some quinolones (*qnr*B5*, qnr*B10*, qnr*S1*, qnr*D1*, qnr*D2) ARGs were identified and these triggers resistance through modification of antibiotic targets. The glycopeptides (*tol*C, *acr*A, *acr*B, *acr*D, *acr*F, *cpx*A), tetracycline (*gad*W, *gad*X*, tet*(A), *tet*(B), *tet*(C), *tet*(D)*, tet*(R)*, tet*(39)*, evg*S), some quinolones (*emr*A, *emr*B, *emr*K, *emr*Y) and oxazolidinome (*mdt*B, *mdt*F, *mdt*K, *mdt*H, *mdt*O, *mdt*P) genes were identified and confer resistance through the antibiotic efflux mechanism (Table 4.6.1).

Aminoglycoside (*aad*A6) gene associated with the antibiotic inactivation resistance mechanism was identified in effluent irrigated soil . Only *bla*$_{TEM}$ (Beta-lactam) acquired resistant gene was observed in both effluent and effluent irrigated soil in the microcosm experiment (Table 4.6.2).

Using PlasmidFinder, at least three classes of plasmids were identified from PWWTP wastewater effluent namely 1) Col plasmids (Col BS512, Col KPHS6, Col MG828, Col 156, Col 3M, Col 440I, Col 440II, Col pVC, Col IRGK), 2) IncF (Inc FIA, Inc FIB, Inc FIB (K)) and 3) Inc R. The plasmids identified from the shotgun sequenced PWWTP effluent showed high percentage identity (95% to 100%) to the known plasmids variants in the PlasmidFinder database (Table 4.6.3). However, none of the classes were identified in effluent irrigated soil.

Table 4.6.1: Acquired ARGs detected in PWWTP effluent

Antibiotic class	ARGs	% identity	% Length of Reference sequence	Resistance Mechanism
Aminoglycosides	*aad*A5	100	19.85	Antibiotic inactivation
	aac(2)-la	99.08	61.24	Antibiotic inactivation
	aph(6)-id	99.64	100	Antibiotic inactivation
	aph(3)-ib	100	13.45	Antibiotic inactivation
Beta-lactamases	bla_{TEM}	100	94.06	Antibiotic inactivation
	bla_{CTX-M}	100	64.21	Antibiotic inactivation
	$bla_{TEM-122}$	100	94.06	Antibiotic inactivation
	$bla_{SHV-163}$	97.18	36.36	Antibiotic inactivation
	$bla_{OXA-663}$	100	84.21	Antibiotic inactivation
	ampC	100	8.29	Antibiotic inactivation
	*omp*k35	100	21.93	Reduced permeability to antibiotic
Macrolides	*mph*A	100	100	Antibiotic inactivation
Trimethoprim	*dfr*A1	99.36	100	Antibiotic target replacement
	*dfr*A14	100	100	Antibiotic target replacement
	*dfr*A17	99.07	58.15	Antibiotic target replacement
Glycopeptides	*tol*C	99.51	4.41	Antibiotic efflux
	*acr*A	98.64	17.21	Antibiotic efflux
	*acr*B	96.18	12.58	Antibiotic efflux
	*acr*D	95.52	5.45	Antibiotic efflux
	*acr*F	100	4.45	Antibiotic efflux
	*cpx*A	100	24.29	Antibiotic efflux
Tetracycline	*gad*W	100	10.27	Antibiotic efflux
	*gad*X	94.21	17.19	Antibiotic efflux
	tet(A)	100	97.88	Antibiotic efflux
	tet(B)	100	5.99	Antibiotic efflux
	tet(C)	99.51	22.47	Antibiotic efflux
	tet(D)	100	40.86	Antibiotic efflux
	tet(R)	100	28.85	Antibiotic efflux
	tet(39)	100	22.28	Antibiotic efflux
	*evg*S	99.68	56.22	Antibiotic efflux
Sulfonamides	*sul*1	100	100	Antibiotic target replacement
	*sul*2	100	100	Antibiotic target replacement
	*sul*3	100	100	Antibiotic target replacement
Quinolones	*qnr*B5	100	100	Antibiotic target protection
	*qnr*B10	99.12	100	Antibiotic target protection
	*qnr*S1	100	100	Antibiotic target protection
	*qnr*D1	100	100	Antibiotic target protection
	*qnr*D2	100	13.55	Antibiotic target protection
	*emr*A	100	7.42	Antibiotic efflux
	*emr*B	100	4.3	Antibiotic efflux
	*emr*K	97.67	12.82	Antibiotic efflux
	*emr*Y	100	18.34	Antibiotic efflux
Oxazolidinome	*mdt*B	100	2.50	Antibiotic efflux
	*mdt*F	96.77	4.34	Antibiotic efflux
	*mdt*K	96.3	5.70	Antibiotic efflux
	*mdt*H	99.21	31.59	Antibiotic efflux
	*mdt*O	98.55	20.20	Antibiotic efflux
	*mdt*P	100	2.90	Antibiotic efflux

Table 4.6.2: Acquired ARGs detected in PWWTP effluent irrigated soil

Antibiotic class	ARGs	% identity	% Length of Reference sequence	Resistance Mechanism
Aminoglycosides	*aad*A6	100	97.92	Antibiotic inactivation
Beta-lactamase	bla_{TEM}	100	100	Antibiotic inactivation

Table 4.6.3: Plasmids identified in PWWTP effluent

Plasmids	% identity	Query/Template length	Contig	Accession Number
Col (BS512)	100	233/233	Contig 2439_cov_358.383151	NC010656
Col (KPHS6)	100	178/178	Contig 5437 _cov_41.035933	NC016841
Col (MG828)	95.43	219/262	Contig 3398_cov_7.37533	NC008486
Col 156	96.94	98/154	Contig 532_cov_5.39207	NC009781
Col 3M	97.45	157/157	Contig 3058_cov_215.31997	JX514065
Col 440I	95.5	111/114	Contig 2268_cov_66.745214	CP023920.1
Col 440II	97.87	282/282	Contig 518_cov_47.534427	CP023921.1
Col pvC	97.41	193/193	Contig 3268_cov_95.587640	JX133088
Col IRGK	98.38	185/184	Contig 1980_cov_7.551446	AY543071
IncFIA	96.91	388/388	Contig 25260_cov_2.788584	AF250878
IncFIB	97.3	629/682	Contig 7042_cov_2.618613	AP001918
IncFIB (K)	99.21	379/560	Contig 11524_cov_3.819899	JN233704
IncR	99.6	251/251	Contig 6778_cov_5.781333	DQ449578

CHAPTER 5: DISCUSSION

Wastewater effluent remains an important source of irrigation water in many developing countries. However due to the poor infrastructure of wastewater treatment plants and lack of regulations on safe use of effluent, this water source potentially spread antibiotic resistance determinants in agricultural soil and vegetables posing a serious public health concern. This study was carried out to determine the impact of wastewater effluent irrigation on the abundance and diversity of bacterial communities in soil and the dynamics of antibiotic resistance genes in soil and vegetable produce.

5.1. Bacterial abundance in a storage tank

Storing effluent in tanks and reservoirs is a common practice in water-stressed countries to provide consistent supply of water for irrigation. In this study we predicted that the storage tank will act as a secondary reservoir to enhance the microbial load, it is plausible to assume since wastewater effluent contain high concentrations of nutrients (Muamar et al., 2014). However, the results showed a significant decline in bacterial populations by at least 2 orders after 3 months. A previous study on the effect of wastewater storage in a tank on *E.coli* concentrations that was carried out for 3 months revealed a 100-fold increase in the growth of *E.coli* (Appling et al., 2013). It has however been established that some water storage vessels encourage the growth of microorganisms more than others. Duru et al., (2013) suggests that calabash and clay pot vessels enhance the growth of microorganisms the most compared to other materials with plastic and glass storage tanks being the least materials to encourage microbial growth. This is mainly due to the high dissolved oxygen in calabash and clay pots which promotes the growth of microorganisms. This study showed that storing wastewater effluent in a plastic tank for an extended period reduces the number of viable microorganisms in the effluent. In this study, bacterial abundance continued to decline for the three months that the effluent was stored in the tank. A plastic tank is affected by heat and pressure, bacteria in wastewater effluent may therefore decline due to hot weather temperatures and pressure as well as low dissolved oxygen (Duru et al., 2013). In the storage tank, nutrients also get used up and sediment at the bottom of the tank therefore becoming unavailable to the bacteria in the tank resulting in declined bacteria numbers (Al-Gheethi et al., 2018).

5.2. Impact of wastewater irrigation on bacterial abundance and diversity in soil

Although Botswana has wastewater effluent standards, they lack proper monitoring and implementation as previously shown by the high bacterial abundance in wastewater effluent (Tapela & Rahube., 2019). The results of this study suggest that bacteria are disseminated from the effluent into the soil and subsequently to the vegetable surfaces through effluent irrigation as similar bacterial species were isolated in effluent, soil and vegetable surfaces. It is also important to note from the results that bacterial species were more abundant in the field surveillance study compared to the microcosm experiment. Hidri et al., (2010) supports the results of this study as bacterial abundance has been shown to increase after long term periods following irrigation with wastewater effluent. Long-term irrigation with wastewater effluent results in increased pH, carbon and nitrogen sources. Because microbial diversity is influenced by both biotic and non-biotic factors, long-term irrigation with effluent will result in a significant change in microbial communities (Bougnom et al., 2019). The abundance of viable bacterial in GWWTP effluent and downstream Notwane river did not reveal a statistical difference ($p = 0.056$), results are not surprising since there has not been any inflow of water from upstream Notwane river over the years, therefore Notwane river remains dominated by effluent wastewater from GWWTP. Soil is a natural habitat for diverse bacterial species hence it is expected that bacterial abundance in soil is high especially after irrigation with effluent wastewater as this increases the nutritive value of soil hence providing a conducive environment for proliferation of bacteria. This can be seen on the results as Oodi and Glenvalley farm soils had relatively high bacterial abundance compared to GWWTP effluent wastewater and Notwane river water.

Next generation sequencing of the 16s rRNA gene was carried out to determine bacterial diversity in PWWTP effluent and PWWTP effluent irrigated soil. Proteobacteria was found to be the predominant phyla in untreated soil, with gammaproteobacteria being the most abundant class of proteobacteria. Gammaproteobacteria comprises of foodborne pathogens such as *Escherichia, Salmonella and Enterobacteriaceae*, gammaproteobacteria is important in the global cycling of carbon, nitrogen and sulfur hence expected to be found in soil (Mhete et al., 2019). A reduction in gammaproteobacteria was observed in the soil following wastewater effluent irrigation, 90% gammaproteobacteria was observed in untreated soil and 52% in effluent irrigated soil. Previous studied carried out by Broszat et al., (2014) indicate that soil irrigated with wastewater for a period of 100 years in Mexico showed 26.7% increase in the relative abundance of proteobacteria. It has also been reported that the relative abundance of

proteobacteria increases with high carbon availability in soil (Mhete et al., 2019). This however contradicts the results of this study since a reduction in gammaproteobacteria was observed following wastewater irrigation. The microcosm study was carried out for 3 months and the overall trend of proteobacteria may have not been completely captured during the short-term microcosm experiment.

Secondary wastewater treatment provides a conducive environment for growth of cyanobacteria, proliferation of cyanobacteria is then enhanced by increased light and high summer temperatures (Martins et al., 2011). This justifies the high abundance of cyanobacteria in PWWTP effluent. The growth of cyanobacteria in wastewater effluent drastically changes the ecology of the microbial communities. Moreover, the presence of cyanobacteria in wastewater effluent may result in toxin production which presents a serious public health issue when disseminated to downstream environments (Martins et al., 2011). In the microcosm soil irrigated with PWWTP effluent actinobacteria was found to be the most dominant phyla. Actinobacteria consists of many gram-negative bacteria that play an important role in carbon cycling and degrading environmental chemicals. The results of this study are supported by Ouyang et al., (2017) who previously identified the phyla as the most prevalent in activated sludge and wastewater treated soils.

Following cyanobacteria, firmicutes were most abundant in PWWTP effluent. Firmicutes were identified in PWWTP effluent (21%), untreated soil (5%) and 90 days effluent irrigated soil (14%). An increase in firmicutes abundance is observed from untreated soil to wastewater irrigated soil. This is expected because an increase in soil carbon content increases the nutritive value of soil hence increase in bacterial proliferation. An increase in firmicutes abundance in soil presents a public health issue as firmicutes comprise of notable gram-positive bacteria such *Clostridium, Streptococcus* and *Staphylococcus* species associated with causing diseases in humans (Mhete et al., 2019).

5.3. Dynamics of ARB and ARGs in soil and vegetables following wastewater irrigation

The occurrence of antibiotic residues in wastewater treatment plants promote the selection of ARB and ARGs. Palacios et al., (2017) suggests that the presence of multidrug resistant bacteria in the soil is an indicator of wastewater use in agriculture. From this study GWWTP effluent isolates also showed resistance to beta lactams (Penicillin, Ampicillin), Cephalosporin, Erythromycin, Tetracycline and Streptomycin. The same resistant pattern was observed effluent irrigated soil which would suggest the dissemination of antibiotic resistance from

effluent to agricultural soil. Wastewater effluent used to irrigate the microcosm and the investigated field were previously reported to be strong vectors of antibiotic resistance bacteria (Tapela & Rahube 2019). From the PWWTP effluent, a high resistance percentage was observed against beta-lactams (penicillin, ampicillin), Meropenem, Erythromycin and Streptomycin whereas low resistance was observed against cephalosporin. The resistance against these antibiotics was also observed in effluent irrigated soil and vegetables. Soil is a substantial environmental reservoir of antibiotic resistance that accounts for 30% of known antibiotic resistance genes in public repositories (Nesme et al., 2014). Although MDR are isolated from untreated soil, their high abundance and persistence in effluent irrigated soils suggest that the use of wastewater effluent for irrigation enhance proliferation of multi-drug resistant bacteria (Palacios et al., 2017). The shared antibiotic resistance pattern between effluent, soil and vegetables suggests a potential dissemination of ARGs into human microbiome through the food chain (Zhang et al., 2019).

The pathway for transmission of ARGs from effluent and soil to vegetables is still not understood. From the shotgun metagenomic sequencing results of this study, antibiotic resistance genes for most antibiotic classes were identified in PWWTP effluent. However only beta-lactamase and aminoglycoside genes were identified in both effluent and effluent irrigated soil, and only beta-lactamase genes in vegetable surfaces. This may be attributed to the short-term irrigation of the microcosm experiment. bla_{TEM}, *tet*A, *aad*A,*sul*1, , *dfr*A were also identified in GWWTP effluent but only bla_{TEM}, *aad*A and *dfr*A were detected in effluent irrigated soil, Notwane River and river irrigated soil. A study carried out by Zhang et al., (2019) on the dissemination of ARGs from manure treated soils to lettuce showed the ability of the plant tissues to take up ARGs, the rhizosphere of the lettuce harboring the most ARGs compared to the leaf and phyllosphere because of its direct contact with the soil. The presence of multi-drug resistant bacteria and ARGs in soil is a serious public health concern as it has been previously reported that plant tissues are able to uptake ARGs from soil (Zhang et al., 2019). With the increased consumption of raw and minimally processed foods this could result in transfer of ARGs to human commensal and pathogens.

Plasmids have led to rapid dissemination of ARGs in the environment, this is because they can move between bacteria and therefore are considered important vectors in the transfer of ARGs (Ragupathi et al., 2019). From this study three classes of plasmids were identified, Col plasmids, incF and incR plasmids. Col plasmids contain genes that code for bacteriocins and are often associated with *E.coli* (Rozwandowicz et al., 2018). IncF plasmids are conjugative,

their host range is limited to *Enterobacteriacea* and they are reported to harbor genes encoding carbapenemases, aminoglycoside-modifying enzymes and plasmid-mediated quinolone resistance (Ragupathi et al., 2019). Plasmids belonging to Inc R class are considered broad range, they have been shown to carry genes conferring resistance to β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines, chloramphenicol and trimethoprim (Rozwandowicz et al., 2018).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.0. Conclusion

Antibiotic resistance genes are not recognized as environmental contaminants in Botswana, wastewater effluent use remains unregulated which poses serious threat to public health . It is therefore imperative that research on antibiotic resistance and dissemination from wastewater treatment plants to agricultural environments is prioritized. This study shows that irrigation with wastewater effluent significantly changes the bacterial community profile in soil, potentially introduces ARB and ARGs into the soil and subsequently into fresh vegetable produce. This study supports other studies around the world that highlights the potential dissemination of ARB and ARGs from effluent to agricultural soils and vegetable crops. The government of Botswana has implemented an irrigation scheme that aim to use effluent for vegetable crops to empower horticulture farmers and improve food security. However, without any antimicrobial resistance surveillance systems in place, the risk of potentially disseminating antibiotic resistance and pathogenic bacteria through the food chain remains. Considering the overall occurrence, abundance and diversity of antibiotic resistance determinants in agricultural settings and adding to the increase in consumption of raw vegetables, it is critical to put in place mitigation measures to reduce the risk of transmission of microbial infectious diseases. Hence this study has shed crucial findings on the impact of wastewater effluent irrigation in the spread of ARB and ARGs in agro-systems. This study will also be important in making evidence-based decisions that will form parts of the policies aimed at regulation, safe and sustainable use of wastewater effluent in Botswana.

6.1. Study limitations

Due to the budget constraints, next generation sequencing of DNA samples from field surveillance samples (GWWTP effluent, effluent irrigated soil, Notwane River and river irrigated soil) could not be conducted.

6.2. Recommendations

- The results of this study will be shared with the Ministry of Agricultural Development and Food Security so that it guides policies and regulations on safe and sustainable use of effluent. Currently in Botswana, antimicrobial resistance is being considered as a public health issue, it is therefore important that evidence-based research is conducted to guide policies .

- In addition to adapting a national action plan for combating antibiotic resistance, wastewater treatment facilities in Botswana need to be assessed on the effectiveness of operating conditions and environmental factors on development and proliferation of ARB and ARGs to minimize the risks of downstream environmental contamination with antibiotic resistance determinants that are being released with effluent wastewater.
- The use of plastic tanks to store effluent for irrigation could be considered as mitigation strategy to reduce bacterial loads prior to irrigation. This is because a decline in bacterial abundance was observed overtime in wastewater effluent stored in the plastic tank. However, more long-term studies will need to be carried out to monitor the changing dynamics of bacterial populations and antimicrobial resistance on seasonal basis.
- Antibiotic resistance has been shown to be accelerated by anthropogenic activities, such as agricultural wastewater irrigation, therefore it is important to educate the public and all stakeholders on the proper use and disposal of antibiotics remains critical to minimize rapid development and dissemination of antibiotic resistance determinants.

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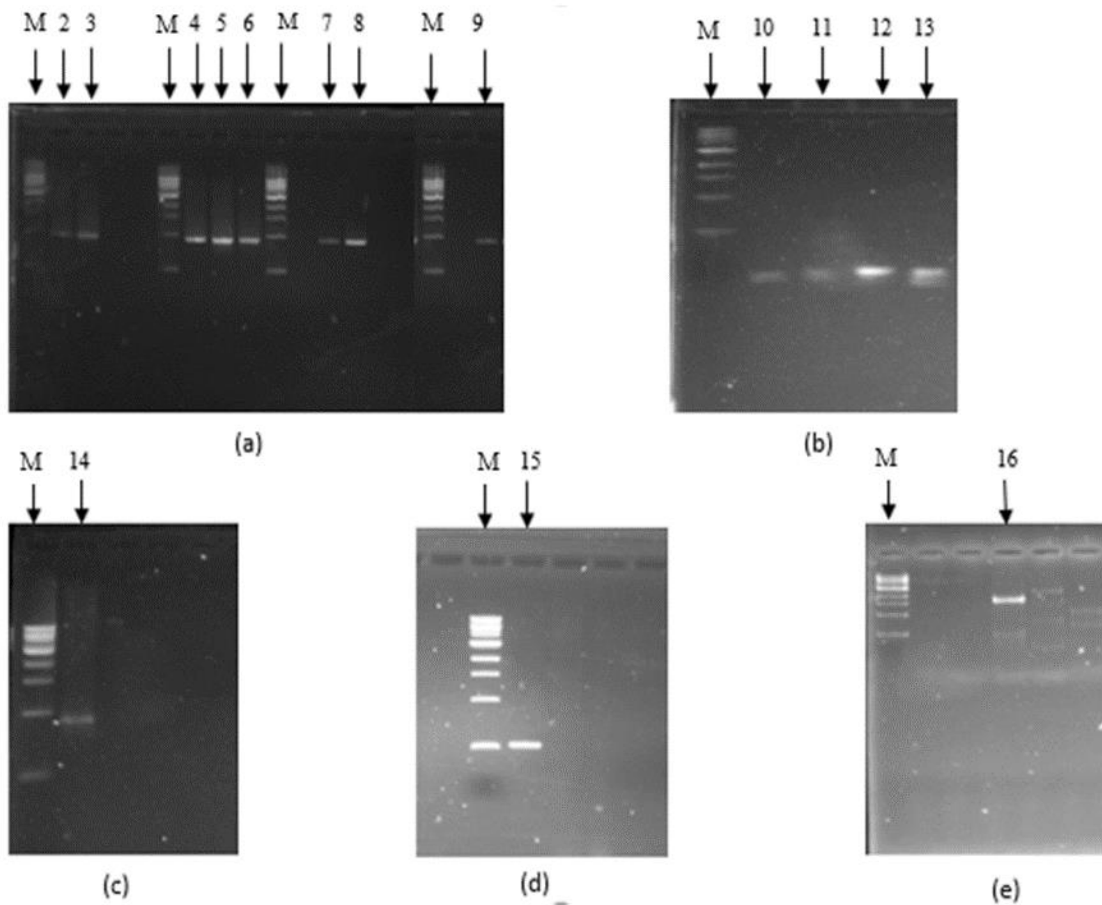
APPENDICES



A: Positive PCR amplification of targeted ARGs (a) *bla*$_{TEM}$ (b) *dfr*A (c) *tet*A (d) *aad*A (e) *sul*1. (Key: M: 1KB DNA ladder, 2: Spinach 3: Beetroot 4: Glenvalley farm, 5: Notwane River 6: Oodi farm, 7: 30 days irrigated soil, 8: 60 days irrigated soil 9: GWWTP effluent 10: GWWTP effluent 11: GWWTP effluent 12: Notwane River 13: Oodi farm 14: GWWTP effluent 15: GWWTP effluent 16: GWWTP effluent)

B. Cabbage (Brassica oleracea var. capitate) in Oodi farm irrigated with water from Notwane River (©O.Onalenna)

C. Bell peppers (Capsicum annuum) and Butternuts (Cucurbita moschata) in Glenvalley farm irrigated with water directly from GWWTP effluent (©O.Onalenna)

D: PWWTP downstream effluent pond

E*:* Microcosm experiment showing positions of experimental and control plants (©O.Onalenna)

F: Microcosm experiment showing position of the water tank outside microcosm experiment (©O.Onalenna)