

ASSESSMENT OF AIRBORNE BACTERIAL DIVERSITY AND ANTIBIOTIC
RESISTANCE PATTERNS IN HOSPITALS, PUBLIC TRANSPORT AND
WASTEWATER TREATMENT PLANTS IN BOTSWANA

by

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The undersigned certifies that he/she has read and hereby recommends for acceptance by the College of Science a thesis titled: Assessment of Airborne Bacterial Diversity and Antibiotic Resistance Patterns in Hospitals, Public Transport and Wastewater Treatment Plants, in fulfillment of the requirements for the degree of Master of Science in Biotechnology of the BIUST.



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DEDICATION

I am indebted to my loving and caring parents, and husband who words can never express my heartfelt gratitude. They trusted and believed I will make it despite all the odds; their encouraging words helped me face the world each day more especially when I was on the verge of distrusting myself and to them I dedicate this work.

TABLE OF CONTENTS

DECLARATION AND COPYRIGHT	ii
CERTIFICATION	iii
ACKNOWLEDGEMENT	iv
DEDICATION.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	x
1.0 INTRODUCTION.....	1
1.1. Statement of the problem	9
1.2. Objectives.....	10
1.3. Hypothesis.....	10
1.4. Significance of study.....	10
2.0. LITERATURE REVIEW.....	12
2.1. Airborne bacteria in clinical environment.....	12
2.2. Airborne bacteria in non-clinical environment	13
2.3. Antibiotic resistance in airborne bacteria.....	14
2.4. Effects of temporal variations on airborne microorganisms	16
3.0. MATERIALS AND METHODS	18
3.1. Experimental setting/ study sites.....	18
3.2. Identification and quantification of airborne bacteria.....	25
3.3. Antibiotic susceptibility test.....	26
3.4. Detection of antibiotic resistance genes	26
3.5. Statistical analysis	29
4.0. RESULTS AND DISCUSSION	30
4.1. Occurrence and abundance of airborne bacteria in hospitals.....	30
4.2. Diversity and abundance of airborne bacteria in hospitals	33
4.3. Occurrence, abundance and diversity of airborne bacteria in wastewater treatment plants	36
4.4. Occurrence, abundance and diversity of airborne bacteria in public transport systems	42
4.5. Occurrence and diversity of antibiotic resistance in airborne bacteria	45
4.5.1. Detection of antibiotic resistance genes	51
4.6. Temporal variation of airborne bacterial communities.....	54

5.0. CONCLUSIONS AND RECOMMENDATIONS.....	61
Conclusions.....	61
Policy recommendations	62
Recommendations for further research	62
REFERENCES	64
APPENDICES	78
Appendix A: WWTP Sampling points.....	78
Appendix B: Agarose Gel Electrophoresis results of the <i>mph</i> (A) Resistant gene	79

LIST OF TABLES

	Page
1.1. Airborne Nosocomial Pathogens, health effects upon infection, infection exposure.....	4
1.2. Antibiotic classes, mechanism of actions and targeted bacteria for each antibiotic class.....	6
3.1. Summary of sampling points in hospitals, public transport and WWTP	23
3.2. List of primer pairs used to amplify antibiotic resistance genes in this study.....	27

LIST OF FIGURES

	Page
3.1. The various wastewater treatment plants under study.....	21
4.1. Abundance of airborne bacteria in different hospitals' operating theatre and paediatric ward.....	30
4.2. Bacterial diversity of Isolates in (a) operating theatres and (b) paediatric wards.....	35
4.3. Bacterial concentrations in the air surrounding the wastewater treatment plants.....	37
4.4. Bacterial abundance and diversity in (a) upwind and (b) downwind air.....	41
4.5. Airborne bacterial concentrations in public transport; bus and mini bus..	43
4.6. Bacterial diversity of Isolates in a bus as compared to the mini bus....	44
4.7. The average percentages of antibiotic resistant and sensitive bacterial isolates from public transport, hospitals and wastewater treatment plants....	46
4.8. Multidrug resistance to various clinical antibiotics used.....	48
4.9. Percentage of antibiotic resistance against clinically relevant antibiotics	50
4.10. Percentage of antibiotic resistance genes detected in airborne bacterial isolates from clinical and non-clinical environments.....	52
4.11. Temporal distribution of bacterial concentration isolated from hospitals' operating theatres, versus temperature and humidity.....	52
4.12. Temporal distribution of bacterial concentration isolated from WWTP downwind versus temperature and humidity.....	55
4.13. Temporal distribution of bacterial concentration isolated from bus and mini bus versus temperature and humidity	57

ABSTRACT

There is presently insufficient information on the atmospheric microbial level in Botswana, the occurrence and diversity of airborne microbes in Botswana is not well understood. In addition, there is also growing concern in the global spread of antimicrobial resistant bacterial pathogens that continue to emerge and pose a huge challenge to human health. This study, being the first of its kind in the country was aimed at understanding the occurrence, distribution and relative diversity of bacteria in the atmosphere surrounding wastewater treatment plants, in hospitals and public transport. The focus was also to further understand the effects of atmospheric conditions, temperature and humidity on the concentration of airborne bacteria. Air impaction was applied for the collection of airborne bacterial community samples using Microbial Air Sampler MAS-100 NT® device at a flow rate of 100 L/min. Air sampling was done in three different months representing 3 seasons between 2016 and 2017. Antibiotic susceptibility was assessed qualitatively. Antibiotic resistance genes were detected through Polymerase Chain Reaction assays. The highest level of culturable bacteria was detected in aerosols (up to 4.4×10^2 CFU/m³) wastewater treatment plant (Palapye) downwind.. The assessment of airborne bacteria in clinical environment revealed abundance of bacteria in paediatric wards than in the operating theatres. The highest bacterial concentration was observed at LMH paediatric ward (5.7×10^2 CFU/m³). At all the wastewater treatment plants, highest bacterial concentrations in the air were isolated from the downwind of the WWTP. The downwind air of PWWTP had the highest average bacterial concentrations (4.4×10^2 CFU/m³). The atmospheric bacterial population was directly affected by temperature and relative humidity; the highest airborne bacterial concentration was recorded during autumn followed by spring while the lowest was observed during winter season. In all the environments (hospitals, public transport and wastewater treatment plants), *Pseudomonas* species was the most frequently detected bacterium (27.1%) followed by *Brucella* (15.3 %), *Listeria* (10.7 %) and *Staphylococcus* (8.9 %) species. Diversity of genes encoding resistance to various antibiotics was also detected in air samples collected in various environments. This study remains important to better understand and monitor the atmospheric basal microbial levels in Botswana.

Keywords: Airborne, bacterial diversity, antibiotic resistance, wastewater treatment plant, hospital, public transport

1.0 INTRODUCTION

Although air is not a suitable growth medium for microorganisms, it is considered as an inanimate transmitting vehicle by carrying biological agents from one host to another. Thereby the atmosphere acts as a dissemination medium for airborne diseases. Besides, humans, animals and plants rely on the atmosphere for continued survival, and exposure to airborne pathogenic microorganisms put their lives at risk. Microorganisms in the atmosphere exist in various forms that vary according to the environmental conditions and locations; these microorganisms have an effect on the climate, human, animal and ecosystem health. In recent years, scientists began to be aware of the diversity of environmental and human health problems potentially caused by airborne microscopic entities. Griffin *et al.*, (2003), indicated that airborne microorganisms in the atmosphere exist either as free-floating particles or may be bound to organic or inorganic material; that is they may adhere to dust particles. The airborne bacteria, fungi, bacterial and fungal spores, toxins, yeast, viruses, and other microbial entities may also be associated with liquid droplets such as splash drops from sewage processing or food processing, resulting in the formation of aerosols of biological origin (bioaerosols) (Huffman *et al.*, 2013).

The major sources of airborne bacteria among others include soil, water, human beings, animals, and plants (Soto, *et al.*, 2009; Jeon *et al.*, 2011). The survival and distribution of airborne bacteria is highly dependent on the bacteria's survival capabilities and the meteorological conditions (Lighthart, 1997). Burge and Rogers, (2000) and Kumar *et al.*, (2011) indicated that the atmospheric microbial concentration depends on abundance of source and factors controlling the release of airborne bacteria and dispersal from the surface boundary layer. Bioaerosols are rapidly dispersed in the atmosphere due to their lightweight and microscopic sizes. Bacteria have a diameter ranging from 0.25 to 8 μm , fungal spores are 1–30 μm in diameter while the diameter for viruses are typically not more than 0.3 μm (Thompson, 1981; Jones and Harrison, 2004; Korzeniewska, 2011). Variations in quantity and concentration of bacteria in the atmosphere are temporal as well as spatial, meaning the concentration not only vary among areas but also over various time-scales, including seasons, months, days and hours (Jensen *et al.*, 1994; Chen *et al.*, 2012).

The rapid increase in migration of people from rural areas to urban areas largely contributes to

the abundance of airborne microbial concentration. The migration of people to urban areas leads to high levels of anthropogenic activities such as constructions, consequently the increase in traffic (Fang *et al.*, 2008; 2015; Chen *et al.*, 2013). The increase in traffic thereby causes disturbances in soil layers on roadsides, increasing the abundance of airborne bacteria as more bacteria are released into the atmosphere from the soil. Moreover, production processes or industrial operations that usually take place in urban areas or developing regions, such as food processing plants, fermentations, animal rendering and sewage treatment emit viable microorganisms into the air (Cullinan *et al.*, 2001; Mouli *et al.*, 2005). Exposure to bioaerosol pollution is almost an inescapable feature of urban living throughout the world; this type of pollution has been associated with serious economic losses stemming from the food and water contamination (Ho *et al.*, 2005). The release of bioaerosols, comprising of pathogenic bacteria and their toxins (such as endotoxins) act as biological weapons in the atmosphere leading to healthcare-associated (HA) infections and community-acquired (CA) infections, therefore it is particularly critical to be aware of these bioaerosols and study their dynamics (Stetzenbach *et al.*, 2004).

Urban areas are usually overpopulated as more people migrate to cities and towns in search for employment opportunities, therefore may be regarded as potential targets for bioterrorism acts that have recently been a major concern in the many countries. Thousands of people lost their lives as a result of bioterrorism, such as the “9/11” terrorism attack in the United States, where spores with anthrax-causing microorganisms were sent to unsuspecting people through postal system (Wells, 2006; Ruggiero and Vos, 2013). Concerns of the increase of bioterrorism threats have therefore increased the importance of studying bioaerosols. The releases of these biological threat agents are usually a result of deliberate action or an unintentional release from anthropogenic sources. Majority of biological threat agents induce minimal immediate effects in humans that are unnoticeable even after fatal exposures. And without proper monitoring or surveillance system, the public would not be aware of the presence of biological threat agents until affected individuals seek medical support which could be days after exposure and probably after damage to some body organs (Dybwad *et al.*, 2012). There are currently no previously reported cases of deliberate dispersion of biological threat agents in Botswana but such actions cannot be ruled out in the future. An early warning, detection, and response scheme could

contribute to minimizing the consequences of such incidents.

Pathogenic aerosol-associated microorganisms cause a wide range of diseases in human beings and animals (Lee *et al.*, 2003; Chen *et al.*, 2012). Nosocomial infections that involve airborne transmission routes may include pathogens that are inhaled, or that settle from the air. In humans, persistent exposure to pathogenic airborne microorganisms leads to respiratory disorders and some adverse infections such as allergic reactions and hypersensitivity pneumonitis and sick building syndromes (Gorny *et al.*, 2002; Fracchia *et al.*, 2006; Griffin, 2003; Yassin and Almouqatea, 2010). According to World Health Organization, WHO (2010), the indoor air pollution contributes 2.7% of the global burden of diseases such as those listed in Table 1.1, with pneumonia being the leading cause of death, followed by chronic obstructive pulmonary disease. The most susceptible people to these airborne nosocomial infections are children (paediatric infections), elderly and immunocompromised patients. Among airborne nosocomial pathogens; *Staphylococcus aureus* is a leading cause of healthcare-associated infections and is increasingly responsible for life-threatening community-acquired infections in otherwise healthy persons (Lindsay and Holden, 2004; 2006; Garcia- Navarro *et al.*, 2008). *Clostridium difficile* is also a major health care-associated pathogen responsible for a wide spectrum of disease (*Clostridium difficile* infections, CDIs), such as diarrhea, abdominal pains and pseudomembranous colitis (Tenover *et al.*, 2012). Patients with *C. difficile* infection shed large amounts of spores, which are transmitted among patients through air (Weinstein and Hota, 2004; Roberts *et al.*, 2008). *C. difficile* has developed multi-drug resistance to a number of antibiotics (Wieczorkiewicz *et al.*, 2016) making CDIs difficult to treat.

Table 1.1. Airborne Nosocomial Pathogens, health effects upon infection, infection exposure

Organism	Health effect	Exposure
----------	---------------	----------

<i>Staphylococcus aureus</i>	Skin infections	Contaminated building
<i>Aspergillus fumigatus</i>	Infection, allergy	Mold-contaminated building, compost
<i>Aspergillus versicolor</i>	Allergy,	Mold-contaminated building
<i>Bacillus anthracis</i>	Anthrax	Bioterrorism, animal handlers, veterinarians
<i>Francisella tularensis</i>	Tularemia	Potential WMD, infected rodents
<i>Legionella pneumophila</i>	Pneumonia	Aerosols from water spray
<i>Mycobacterium tuberculosis</i>	Tuberculosis	Person-to-person

(Adapted from: Stetzenbach *et al.*, 2004)

Not only does the presence of harmful airborne bacteria affect human, animals and plant health, but also they are also major determinants of weather and climate (Griffin, 2003). Several airborne bacteria serve as effective cloud condensation nuclei, which explain the role of bioaerosols on weather and climate (Bauer *et al.*, 2003; Andreae, 2007). Furthermore, they influence the earth's energy budget by scattering and absorbing radiation (Hamilton and Lenton, 1998). Bioaerosols absorb or reflect sunlight or thermal radiation emitted onto the earth's surface changing the amount of sun's energy reaching the earth through the "direct effect" (Möhler *et al.*, 2007; Bowers *et al.*, 2012). The most dominant bacteria isolated from the atmosphere belong to *Micrococcus* species, *Staphylococcus* species, *Streptococcus* species (Soto *et al.*, 2009) as well as *Bacillus* species (Stetzenbach *et al.*, 2004; Dybwad *et al.*, 2011). Certain infectious bacterial species that easily spread through the air include *S. aureus*, *Bordetella pertussis*, *Clostridium diphtheriae*, *Mycobacterium tuberculosis*, *Neisseria meningitidis* and *Yersinia pestis* (Lighthart and Stetzenbach, 1994; Buemi *et al.*, 1999; Griffin *et al.*, 2003; Oren, 2004; Brodie *et al.*, 2007; Rao *et al.*, 2007). *Staphylococcus* and *Streptococcus* species, which are linked with most skin infections, are relatively resistant to environmental influences such as humidity and temperatures and may remain viable in the atmosphere for longer periods (Luciano, 2012). Most of these infectious bacteria such as *Staphylococcus* have developed resistance to multiple antibiotics in the last decades and have been labeled as "superbugs". *S. aureus* and *M. tuberculosis* has gained substantial public attention due to increasing mortality associated with multidrug resistance.

Antibiotic resistance is widely distributed within various environments. Antibiotic resistance development occurs naturally over time, commonly through genetic mutations and gene exchange mechanisms (Baquero *et al.*, 2008). In addition, the introduction of some antibiotics to bacteria may not be effective, as some bacterial strains may have already developed efficient mechanisms to resist them. Thereby increasingly hindering treatment of life-threatening illnesses resulting in prolonged hospitalization and subsequently death. The misuse (such as taking antibiotics for viral infections) and overuse (such as using antibiotics as growth promoters in livestock) of antibiotics has been acknowledged as major factors accelerating the development of antibiotic resistance (World Health Organization, WHO, 2016). Furthermore, in the absence of effective antibiotics for treatment of infections, the success of major surgery and cancer chemotherapy would be compromised. WHO (2016) reported that 480 000 people develop multidrug resistant TB each year globally, and this complicates the fight against HIV/AIDS. Botswana being one of the sub-Saharan countries largely affected by HIV/AIDS has been faced with the escalating population of people dying as a result of the disease, and this is worsened by the increase in multidrug resistant *M. tuberculosis* among this HIV/AIDS infected population.

Moreover, the intensified use of antibiotics for medical and agricultural purposes has become a major cause of propagation of antibiotic resistance genes (ARGs), and can be considered a major anthropogenic environmental threat affecting the natural environment, where they are distributed into the surrounding environment by water and atmospheric circulation (Batt *et al.*, 2006; Brown *et al.*, 2006; Cabello, 2006; Hawkey, 2008; Rizzo, 2013; Segawa^[1]_{SEP} *et al.*, 2013). The ARGs are transmitted from one bacterium to the other more especially in environments where dense bacterial communities are frequently exposed to intensive uses of antibiotics such as agricultural fields and clinical settings (Segawa^[1]_{SEP} *et al.*, 2013). The dense bacterial communities form biofilms in aquatic environments such as drinking water distribution systems and sewage treatment plants, ARGs become a threat in such environments because gene transfers can occur through both vertical and horizontal gene transfer mechanisms. The main concern for the release of antibiotics into the environment (such as the release of antibiotics by the urban wastewater treatment plants) is related to the development of antibiotic resistant

bacteria and antibiotic resistant genes that lessen the therapeutic potential against bacterial pathogens (Kemper, 2008; Zhang *et al.*, 2009b; Rizzo, 2013). The development of antibiotic resistance by bacteria is highly owed to the bacteria's remarkable array of mechanisms that they develop to exhibit resistance to antibiotics that include drug inactivation or modification, alteration of the target site, alteration in the metabolic pathway, and reduced drug accumulation (Katzung 2004; Gatica *et al.*, 2015). Antibiotic classes and their mechanisms of action are summarized in Table 1.2.

Table 1.2. Antibiotic classes, mechanism of actions and targeted bacteria for each antibiotic class

Antibiotic class	Antibiotic	Mechanism of action and Targeted bacteria	Antibiotic resistance genes examples
Penicillins (β -lactams)	Penicillin Ampicillin	Inhibition of cell wall synthesis, Efflux pump, Broad spectrum bacteria	Penicillin resistance genes, repressor (<i>blaI</i>), antirepressor (<i>blaR1</i>), and structural (<i>blaZ</i>) genes
Cephalosporin (β -lactams)	Erythromycin Cephalosporin	Inhibition of cell wall synthesis, Altered target Effective against Gram positive bacteria	β -lactamase genes such as <i>blaCTM-X</i> , <i>blaTEM</i>
Sulfonamide	Trimethoprim Sulfonamide	Folic acid metabolism Broad spectrum bacteria	Sulfonamide resistance genes, <i>sul1</i> , <i>sul2</i> , and <i>sul3</i>
Macrolide	Erythromycin	Protein synthesis (50S inhibitors), Target modification due to enzymatic degradation or modification, Efflux Broad spectrum bacteria	Efflux pump genes such as <i>mef(A)</i> genes. <i>ermA</i> , <i>ermB</i> , <i>ermC</i> , <i>msrA/msrB</i> , <i>ereA</i> and <i>ereB</i>
Fluoroquinolone	Ciprofloxacin	Inhibit nucleic acid synthesis; bacteria resistant to fluoroquinolones are often multi-resistant.	<i>qnr</i> genes (<i>qnrA</i> , <i>qnrB</i> , <i>qnrS</i>), <i>aac(6')-Ib-cr</i> , <i>qepA</i> and <i>oqxAB</i> genes

		Broad spectrum bacteria	
Tetracycline	Tetracycline	Efflux pump Broad spectrum effective against Gram positive and negative bacteria and also Mycoplasma	Tetracycline resistance genes <i>tet(A)</i> , <i>tet(B)</i> , <i>tet(M)</i> , <i>tet(O)</i> , <i>tet(S)</i> and <i>tet(W)</i>
Carbapenem	Meropenem	Inhibition of cell wall synthesis, Cell death by covalently binding to penicillin-binding proteins (PBPs) Broad spectrum	<i>bla</i> _{KPC-13} , <i>bla</i> _{IMP-14a} , <i>bla</i> _{IMP-1} , <i>bla</i> _{VIM-11}
Aminoglycosides	Streptomycin	Protein synthesis (30S inhibitors) Broad spectrum	Aminoglycoside resistance genes <i>aac(6')-Ie-aph(2'')-Ia</i> , <i>aph(2'')-Ib</i> , <i>aph(2'')-Ic</i> , <i>aph(2'')-Id</i> , <i>aph(3')-IIIa</i> , and <i>ant(4')-Ia</i>

In general, resistance is acquired by mutational change (vertical evolution; such as point mutations, deletions and insertions in bacterial genome) or by the acquisition of resistance-encoding genetic material located on various types of mobile DNA elements through lateral or horizontal gene transfers (Dzidic and Bedekovic, 2003). The horizontal gene transfer is the most common bacterial mechanism of acquiring antibiotic resistance genes. Horizontal gene transfer involves transduction, (the transfer of DNA between bacterial cells through bacteriophages or bacteria-specific viruses), conjugation (the transfer of plasmids between bacterial cells which happens when there is direct cell-cell contact between two cells), transformation (involves the uptake of bacterial DNA from external environment, due to the lysis or death of another bacterium).

Plasmids, which are extra-chromosomal materials, carry accessory or functional genes that encode for toxins, virulence factors, specific metabolic pathways, and protective mechanisms including resistance to heavy metals and antimicrobials (Gatica *et al.*, 2015). They allow the transfer of genes encoding for antibiotic resistance not only between bacteria of the same

population, but between bacterial species and genera. Plasmids may be large self-transmissible plasmids or smaller plasmids that can be mobilized by self-transmissible plasmids and are frequently part of transposons or conjugative transposons (Dzidic and Bedekovic', 2003). In addition, the plasmids may carry resistance genes for single or multiple antimicrobial agents. An example is the resistance of *Staphylococci* species to various antibiotics like penicillin, rifampicin, streptomycin, fusidic acid and novobiocin and fluoroquinolones (Ombui *et al.*, 2000; Akinjogunla and Enabulele, 2010).

Moreover, other mobile genetic elements such as integrons and transposons also facilitate the transfer of genetic material from one bacterium to another or within an individual cell (Bennet, 2008; Gatica *et al.*, 2015). Integrons, which are exclusively found in the Gram-negative bacteria, recognize and capture mobile gene cassettes that confine antibiotic resistance genes (van Belkum *et al.*, 2013), which provides for horizontal transfer by several mechanisms. Integrons are classified as resistance integrons and super-integrons. Resistance integrons carry mostly gene cassettes that encode resistance against antibiotics, disinfectants and heavy metals, and can be located either on chromosome or on plasmids and can move between bacteria by horizontal gene transfer (Hall and Stokes, 2004). A variety of integron classes have been described, such as class 1, 2 and 3. Most resistance integrons belong to class 1, linked to many clinically relevant bacteria (Weldhagen, 2004) such as *Pseudomonas* species (Fluit and Schmitz, 2004).

There are several ways that microbial communities in the atmosphere affect the ecosystem functioning, and this can be either negative or positive. Airborne microorganisms have been linked with severe commercial losses as a result of deterioration of buildings and medicines (Lee *et al.*, 2003). Bioaerosols may attach and grow on surfaces causing adverse health effects, material damages and unpleasant odors (Yassin and Almouqatea, 2010). Biodeterioration of stored collections in archive repositories have been reported to be worsened by various microorganisms such as fungal and bacterial contamination, some of the archived materials are made up of organic compounds like cellulose, keratin, collagen and elastin that provide excellent substrates for the fungi and bacteria (Nunes *et al.*, 2013). Furthermore, airborne bacteria have been highly associated with food contamination that occurs mainly during food

processing due to the exposure of processing systems (machines, equipment and food products) to contaminated aerosols. The microorganisms associated with food contamination are vegetative bacteria such as *Micrococcus*, *Staphylococcus* and *Salmonella* species. The content of microorganisms in the atmosphere, which can reflect the quality of air condition, is an important parameter of atmospheric pollution and thus it needs regular monitoring. Bioaerosols are monitored by measuring both viable (culturable and nonculturable) microorganisms that have the ability to reproduce and are metabolically active, and nonviable microorganisms (Jensen and Schafer 1998).

1.1. Statement of the problem

There is information on the atmospheric microbial levels in Botswana and as such when incidences of airborne diseases do occur, little will be known of the origin/occurrence of the diseases. Botswana is therefore insufficiently prepared to protect its citizens from potential bioterrorism acts and disease outbreaks like Foot and mouth and influenza leaving animals and people vulnerable to these threats. There is also a growing concern in the global spread of antimicrobial resistant bacterial pathogens such as multi-drug resistant *S. aureus* and extensively drug resistant *M. tuberculosis*. In addition, airborne bacteria producing the Extended-Spectrum Beta-Lactamase (ESBL) enzyme that confer resistance to most beta-lactam antibiotics continue to emerge and pose a huge challenge to human health. Despite all the negative influence and the public health implications of airborne microorganisms, the microbial content of air in Botswana, particularly in public places such as hospitals and public transport is yet to be investigated. The research on the diversity of airborne bacteria and their antibiotic resistance patterns in Botswana is unknown. Therefore, the assessment of airborne bacterial concentrations is vital to better understand the atmospheric basal microbial levels in public places like hospitals, public transport and wastewater treatment plants in Botswana. The assessment would also help understand the occurrence, abundance and diversity of airborne and antibiotic resistant bacteria.

1.2.Objectives

This study is aimed at determining the occurrence/presence, abundance, and relative diversity of bacteria in the atmosphere of selected areas; hospitals (paediatric wards and operating wards), wastewater treatment plants (upwind and downwind air), and public transport (bus and mini bus). The study is also aimed at identifying the antibiotic resistance patterns of the bacterial isolates by determining the antibiotic resistance phenotypes and genotypes in bacteria captured from hospitals, public transport and wastewater treatment plants. Furthermore, the study aims to find correlation between the different meteorological conditions (temperature and relative humidity) and the bacterial concentrations in the air.

1.3. Hypothesis

High human population density coupled with high level of anthropogenic activities lead to high occurrence of diverse bacterial populations in the environments. Higher bacterial concentrations that are more diverse are expected from WWTPs environments as a major source. This study hypothesizes that higher occurrence and diversity of multi-drug resistant bacterial strains and resistance genes will be observed from hospital and WWTPs environments compared to public transport.

1.4. Significance of study

Findings from this study will provide information on the atmospheric bacterial concentration from different clinical and non-clinical environments. Knowledge of the occurrence, abundance and diversity of airborne and antibiotic resistant bacteria particularly in areas that are supposed to be free of bacterial contamination such as operating theatre rooms can lead to major key alerts to potential life threatening situations that need immediate intervention. Hence responsible personnel will find the need to regularly monitor the inflow of bacterial contaminated air in such areas. The public will also be sensitized of possible airborne spreading of potentially pathogenic and antibiotic resistant bacteria so as to avoid overcrowding in buses

and other modes of transport. The study will also be informative enough to urge health ministry to develop airborne bacterial surveillance systems and criteria for setting the recommended maximum limits of bacterial concentrations that could be used in risk assessments.

2.0.LITERATURE REVIEW

2.1. Airborne bacteria in clinical environment

The atmospheric microbial contamination in hospital environment, mainly in operating theatre wards continue to increase prevalence of nosocomial infections which lead to high mortality rates among hospitalized patients going through post-operative surgery (Weigelt *et al.*, 2010; Hailemariam *et al.*, 2016). A study conducted by Hailemariam and colleagues (2016) on bacterial concentration and antibiotic susceptibility pattern of isolates in operating rooms and the immediate zone (changing rooms) of a hospital, revealed that the female changing room of the operating room had the highest bacterial counts (6.5×10^2 CFU/dm²) whereas the operating room had the lowest bacterial count (3.2×10^1 CFU/dm²). These results indicate that the surrounding areas furnish the air microbial contamination of the operating theatre. The results are supported by Spagnolo *et al.*, (2013), whose findings indicated that pathogenic microorganisms in operating theatres are acquired from exogenous sources such as the operating theatre surrounding environment, surgical personnel and equipment brought to the sterile field during operation. In previous studies, the predominant bacterial species isolated from operating theatre wards were *Staphylococcus* species (the leading cause of surgical site infections, SSI), *Enterococcus* species, *Escherichia coli* and *Pseudomonas aeruginosa* (Giacometti, 2000; Wolcott *et al.*, 2009; Hailemariam *et al.*, 2016). Matthias and colleagues (2000) assessed the indoor and outdoor air contamination at a secondary care hospital and reported high percentages of *Staphylococcus epidermidis* followed by *P. aeruginosa*. Additionally, they discovered that various hospital environments act as reservoirs of multi-drug resistant nosocomial pathogens, with the most contaminated site being the labor room followed by the dressing room and the operation theatre.

In addition to the operating theatre, the paediatric wards have been widely studied for their harboring of airborne bacteria. In a study conducted by Okten and Asan (2012) Edirne Government Hospital's paediatric unit in Turkey, 2.4×10^3 bacterial colonies were isolated from 13 sampling points; the most prevalent bacterial genera were *Staphylococcus*, *Bacillus*,

Corynebacterium and *Micrococcus*. Similarly, *S. aureus* was found to be the most common microorganism isolated from a paediatric ward, which was 16.2% of the total bacterial isolates (Qudiesat *et al.*, 2009). Another similar study conducted in a paediatric ward was by Coetzee *et al.* (2013), which indicated the highest isolation of *P. aeruginosa* and these results are similar to those of Behzadnia *et al.* (2014) in a similar ward, paediatric ward. In addition, *S. aureus*, *Serratia marcescens*, *Acinetobacter* species and *Klebsiella* species were also among isolated bacteria (Behzadnia *et al.*, 2014).

2.2. Airborne bacteria in non-clinical environment

Wastewater treatments plants (WWTPs) are customarily located outside urban settlements, however with the high rates of migration to towns and cities resulting in the growth of the urban areas WWTPs are increasingly found very close to urbanized areas (Korzeniewska, 2011; Gotkowska-Płachta *et al.*, 2013). The size, treatment procedures as well as varying techniques involved during wastewater treatment (Sánchez-Monedero *et al.*, 2008) affect the environment varyingly. Bioaerosols are generated and emitted into the air in large numbers at different wastewater treatment stages, mostly at the primary and secondary stages (Filipkowska *et al.*, 2002; Sánchez-Monedero *et al.*, 2008). The primary treatment stage involves treating raw wastewater by removal of solid inorganic constituents through physical means. Whereas the secondary stage involves sludge activation, where pre-treated sewage is aerated to promote the growth of bacteria (cells) that gradually degrade the wastewater's biodegradable organic materials into tiny particles and additional biomass. The wastewater mixture of suspended cells or solids (sludge forming materials) and water is then separated in a settling tank or clarifier.

Wastewater aeration by dipper wheels, sprinklers, diffusers and aerators usually increases the bacterial concentration in the atmosphere (Korzeniewska, 2011). These techniques are also likely to facilitate the transfer of microorganisms from the wastewater at WWTPs to the air nearby. The bioaerosols dispersed in the atmosphere are transferred over considerable distances depending on the meteorological and physicochemical air conditions, topographies, season, times of day, and the techniques involved during wastewater treatment (Filipkowska *et al.*, 2000; Bauer *et al.*, 2003; Karra and Katsivella, 2007; Sanchez-Monedero *et al.*, 2008). Due to

the small sizes of the airborne microorganisms, they are easily carried by the wind to other places over long distances thereby posing a potential biological hazard not only to site workers but also to residents living far away from WWTPs (Korzeniewska, 2011). *Enterobacter*, *Citobacter*, *Serratia*, *Klebsiella* and *Pantoea* species were prevalent near biological and mechanical treatment sites of WWTPs. *Escherichia*, *Salmonella* or *Shigella* species were rarely isolated from such environments (Filipkowska *et al.*, 2002; Korzeniewska *et al.*, 2008; Korzeniewska, 2011).

Very few studies have highlighted the airborne microbial diversity and the ecology of bioaerosols in public transport systems such as buses. Instead, studies tend to focus more on bus stations and metro/subway stations than public transport systems. Bioaerosol concentrations study in various public restroom environments (lecture building, student center, library, shopping center and subway station) conducted by Lee *et al.*, (2012), indicated higher concentrations of bioaerosols in the subway station than most of the public restrooms. The abundance in the airborne bacteria in subway stations could be attributed by the capacity of the building, and the anthropogenic activities, usually in other public restrooms people are relaxed and with less movements compared to subway stations. A study by Mouli *et al.*, (2005) on the outdoor airborne bacteria at institutional, health care, commercial, traffic, industrial and agricultural areas of Tirupati (India), indicated that a central bus station located at the center of the town was among the sampling areas that showed a greater level of airborne bacteria. In addition, a review on the evidence for airborne infectious disease transmission in public ground transport by Mohr and colleagues (2012), noted that passengers using public transport may be at risk of infectious disease such as tuberculosis when they are seated closeby infected passengers during bus and train trips. More information concerning the bioaerosol concentration not only in wastewater treatment plants and health-related facilities, but also in many public enclosed indoor environment such as public transport systems, shopping centers and schools, where numerous people are exposed to indoor air every day is should be generated.

2.3. Antibiotic resistance in airborne bacteria

Antibiotic-resistant airborne bacteria are more common in hospitals, where the antibiotic drugs

are mostly used. Thus the emergence of bacteria resistant to antibiotics is common. Antibiotic resistant airborne bacteria has also increasingly emerged in aquatic environments such as wastewater treatment plants. Centers for Disease Control and Prevention (CDC, 1999) have documented increases in the number of nosocomial infections caused by antibiotic-resistant bacteria. In particular, antibiotic resistance has caused concern in the paediatric population where bacterial infections are the most frequent cause of childhood diseases. The use of antimicrobial agents such as antibiotics has become a routine practice for the treatment of paediatric illnesses such as gastroenteritis. However, very few studies have highlighted the airborne microbial diversity and the antibiotic resistance in this type of environment. A study by Gilbert and colleagues (2010) on airborne bacteria and antibiotic resistance genes in hospital rooms revealed that erythromycin resistance genes were abundant in paediatric ward air whereas tetracycline resistance genes were sporadically detected. A study by Joshi and colleagues (2011) indicated high percentages of antibiotic resistant *P. aeruginosa* isolated in a hospital were resistant to various antibiotics: trimethoprim (100%), amoxicillin (100%), ampicillin (100%), tetracycline (100%), ticarcillin (100%), carbenicillin (90%), ceftazidime (80%), clavulanic acid (69.2%), imipenem (60%), cefepime (52%), gentamicin (50%), and ciprofloxacin (40%). Hailemariam *et al*, (2015) studied the bacterial concentration and antibiotic susceptibility pattern of isolates in operating rooms at Hawassa University Referral Hospital (southern Ethiopia) and revealed that *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa* and *Pseudomonas mirabilis* and *S. aureus* were the predominant species and highly resistant to Ampicillin, Gentamicin, Chloramphenicol, Cloxacillin and Ceftriaxone. The findings indicated that *S. aureus* was highly resistant to penicillin, tetracycline and trimethoprim at 83.3%, 81.5% and 63%, respectively.

Antibiotic-resistant bacteria and antibiotic resistance genes are also emitted into the atmosphere by urban wastewater treatment plants (Schiller *et al.*, 2015). These antibiotic resistant bacteria emitted into the atmosphere from wastewater treatment plants may include the resistant strains to various antimicrobial compounds. Diverse classes of genes encoding resistance to tetracycline: *tet(32)* *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(H)*, *tet(J)*, *tet(K)*, and *tet(L)*, *tet(M)*, *tet(O)*, *tetB(P)*, *tet(Q)*, *tet(S)*, *tet(T)*, *tet(W)*, *tet(Y)*, *tet(Z)*, *tet(30)*, have been revealed to be present in air surrounding wastewater treatment plants by Villedieu *et al.*, (2003).

2.4. Effects of temporal variations on airborne microorganisms

The diversity, survival and distribution of airborne microorganisms is controlled by daily and seasonal variations; temperature, wind velocity and relative humidity (Harrison *et al.*, 2005; Mouli *et al.*, 2005; Soto *et al.*, 2009; Kumar *et al.*, 2011). Temperature and humidity controls the rate of change of water vapor and the rate of change of heat between the surface and environment. These changes ultimately affect the viability of airborne bacteria through the evaporation of their cellular water (Mouli *et al.*, 2005). Several studies have in the past investigated the effect of meteorological conditions (such as temperature, humidity and sunlight/radiation) on the survival of airborne infectious agents (Tong and Lighthart; 2000; Jones and Harrison, 2004; Mouli *et al.*, 2005; Burrows *et al.*, 2009; Bowers *et al.*, 2012) contributed to the lifespan of airborne microbes. In addition, the dispersal and sedimentation of microorganisms in the atmosphere is determined by wind velocity (Soto *et al.*, 2009). Solar irradiance, air ions, oxygen and open-air factors also largely influence the growth of microorganisms. According to studies by Lighthart (2000), and Raisi and colleagues (2010), the variation of the quantity and quality of airborne bacteria at different times of the day and year justifies the effects of solar heating on the survival of bacteria cells in suspension. This however, depends on the activities at a particular area. Due to some activities such as wastewater treatment, the bacterial contamination of the air surrounding the wastewater treatment plant increases throughout the day despite the changes in solar heating. Karra and Katsivela (2007) discovered the highest bacterial concentrations around the wastewater treatment plants in the late afternoon and evening during summer.

Mouli *et al.*, (2005), through a multiple linear regression modelling approach showed that temperature, relative humidity and wind velocity have significant influence on airborne bacterial concentration. The models obtained justified that in terms of wind velocity, temperature and relative humidity for each sampling location, the bacterial concentrations varied widely according to varying wind velocity, temperature and humidity. In another study to find the correlation between relative humidity and the survival of bacteria, Peccia *et al.*, (2001) revealed that aerosolized bacterial cells showed increased water sorption from the

atmosphere (when atmospheric relative humidity is between 20 and 95%) protecting them from UV-induced inactivation. Annually, Korzeniewska *et al.*, (2008) and Gotkowska-Płachta *et al.*, (2013) observed the maximum number of airborne bacteria at the wastewater treatment plant in winter and early spring respectively. Whereas the study of seasonal variability in airborne bacterial communities at a high-elevation site by Bowers and colleagues (2012) indicated that bacterial abundances varied by season. The highest bacterial concentrations were observed during the fall and spring seasons while lower bacterial concentrations were recorded during summer and winter. However, in contrary to some previous studies, no strong correlations were discovered between airborne bacterial concentrations and the basic meteorological indices.

3.0.MATERIALS AND METHODS

3.1. Experimental setting/ study sites

3.1.1 Clinical environment

Hospitals

Bacteria suspended in the indoor air of operating theatre wards and paediatric wards of Palapye Primary Hospital (Palapye), Nyangabwe Referral Hospital (Francistown) and Letsholathebe II Memorial Hospital (Maun) were assessed. An operating theater is one of the hospital's supposedly sterile facilities without windows and feature controlled temperature and humidity, where surgical operations are performed. It is usually separated from other hospital facilities, and accessed only by authorized personnel. The paediatric ward is the hospital's facility assigned to provide health care for children under the age of 12. The operating theater and paediatric wards were both selected in determination of the risk and possible transmission of opportunistic bacterial pathogens in highly immunocompromised surgery patients and children. Palapye Primary Hospital is located in Palapye, Central district, built in the 1970s and it caters for the surrounding villages as it receives referrals from local and nearby clinics as well as health posts. Nyangabwe Referral Hospital is in Francistown, North East district, built around 1989 is by far the largest hospital in the northeastern Botswana, and thereby receives most referrals from the region. Letsholathebe II Memorial Hospital is one of the recently constructed hospitals in the northern region of Botswana (Ngamiland district). The hospital was opened in 2008 and is located along the southern edges of the Okavango Delta in the region that has been previously hit by major southern African epidemics and pandemics; influenza, smallpox, bubonic plague, sleeping sickness, malaria, and bilharzia (Molefi 2001, 2003; Mosothwane, 2015).

3.1.2 Non-clinical environment

Public transport

The airborne bacteria of public transport, shared passengers transport service available for commuting by the general public: bus (75 seater) and mini bus (25 seater) were assessed. Public transport bus services that were selected for this study are those confined to the country's main road (A1) plying between Francistown and Palapye. The occurrence, abundance and diversity of airborne bacteria were compared between the two public transport systems due to their differences in passenger carrying capacity. Other factors taken into account include inside air volume capacity and the frequency of the bus plying between origins of departure and destinations. Buses have larger carrying capacity that cater for both seated and standing passengers compared to mini buses.. The differences in the size and carrying capacity of the bus and mini bus may influence the volume of air inside them, with buses having more volume of air than mini buses. The other difference between the two public transport systems is the frequency each travels. Buses generally travel longer distances and make on average less trips in a day compared to mini buses. There are also striking differences in inhalable particles depending on the different route used by the bus and mini bus, ranging from dusty roads in mostly rural areas, tarred roads in main highways and through towns/cities.

Wastewater treatment plants

Wastewater treatments plants in Palapye, Gaborone and Maun were selected as the main reservoirs for airborne bacteria. Palapye wastewater treatment plant (PWWTP) (22°32'26" S 27°10'23" E) is a medium-size sewage treatment plant with a capacity of 14000 m³ per day. Palapye WWTP collects wastewater from Palapye town (Central district) with a population of 36 211 people (Botswana Population and Housing Census, 2011). Palapye is one of the fastest growing towns in the country, with a new educational institution, Botswana International University of Science and Technology and the recently expanded coal power station (Morupule power station) leading to the gradual increase in residential and industrial properties. This rapid growth of the town puts pressure on the wastewater treatment plants as the volume of the wastewater increases. Gaborone wastewater treatment plant (GWWTP) (24°36'45" S 25°58'5"

E) lies on the southeastern of Gaborone City, the capital city of Botswana. The city has experienced substantial population growth, thereby considered the economic capital harboring most of the institutional, industrial and many other organizational headquarters. The city's wastewater generated by the massive population depends on Gaborone WWTP also known as Glen Valley WWTP; a municipal wastewater treatment plant with a receiving capacity of 45,000 m³ of sewage daily (Nkegbe *et al.*, 2005). While Maun wastewater treatment plant (MWWTP) (19°57'36" S 23°24'47" E) receives water from Maun town, in the northwestern part of Botswana. Maun is considered the tourist capital of the country, and its population has substantially increased over the years. The increase in population, coupled with intensive infrastructure development has led to the generation and accumulation of toxic wastewater, high release of aerosol and potential spread of airborne bacteria into the atmosphere from the wastewater treatment plants.

All three wastewater treatment plants (Gaborone, Palapye and Maun) are characterized by various sewage treatment stages as shown in Figure 3.1. The Gaborone WWTP consists of primary and secondary wastewater treatments. The primary treatment is firstly done by screening to remove coarse, inorganic and large objects in grit chambers, followed by sedimentation to facilitate the removal of organic and inorganic solids. At this stage, total suspended solids, oils and grease are removed. The GWWTP involves round primary sedimentation tanks; in which settled solids (primary sludge) are removed through sludge raking. The primary sludge is then processed biologically by anaerobic digestion performed in covered tanks (anaerobic digesters). The effluent is then subjected to the secondary treatment, as an activated sludge process. This stage involves aerobic microorganisms particularly bacteria to metabolize the organic matter in the wastewater. As a result, more microorganisms and gases such as CO₂ and NH₄ are emitted into the atmosphere. The secondary stage involves activation of sludge that occurs in aeration basin. A suspension of the wastewater and microorganisms are mixed vigorously by aeration devices thus emitting bioaerosols into the surrounding air. The effluent from the secondary treatment is then discharged to maturation ponds near the GWWTP where the water then flows into Notwane river. The Palapye wastewater treatment plant is almost similar to GWWTP, but the PWWTP further treats the wastewater aerobically through the Pond Enhanced Treatment and Operation (PETRO) system. The PETRO system increases

the efficiency of the secondary treatment stage by employing trickling filter, whereby a tank is embedded with stones and settled wastewater is continuously sprayed on this stones and trickles to the bottom, followed by drainage into the secondary clarifiers. At this stage, more bioaerosols are generated hence contribute to the downwind air pollution. Maun wastewater treatment plant is characterized by primary and secondary treatments, however there is minimal treatment during the secondary stage compared to GWWTP, as there are no aeration tanks and few primary sedimentation basins and the effluent from the MWWTP is much more of primary effluent. The effluent then flows into Thamalakane river (upstream).

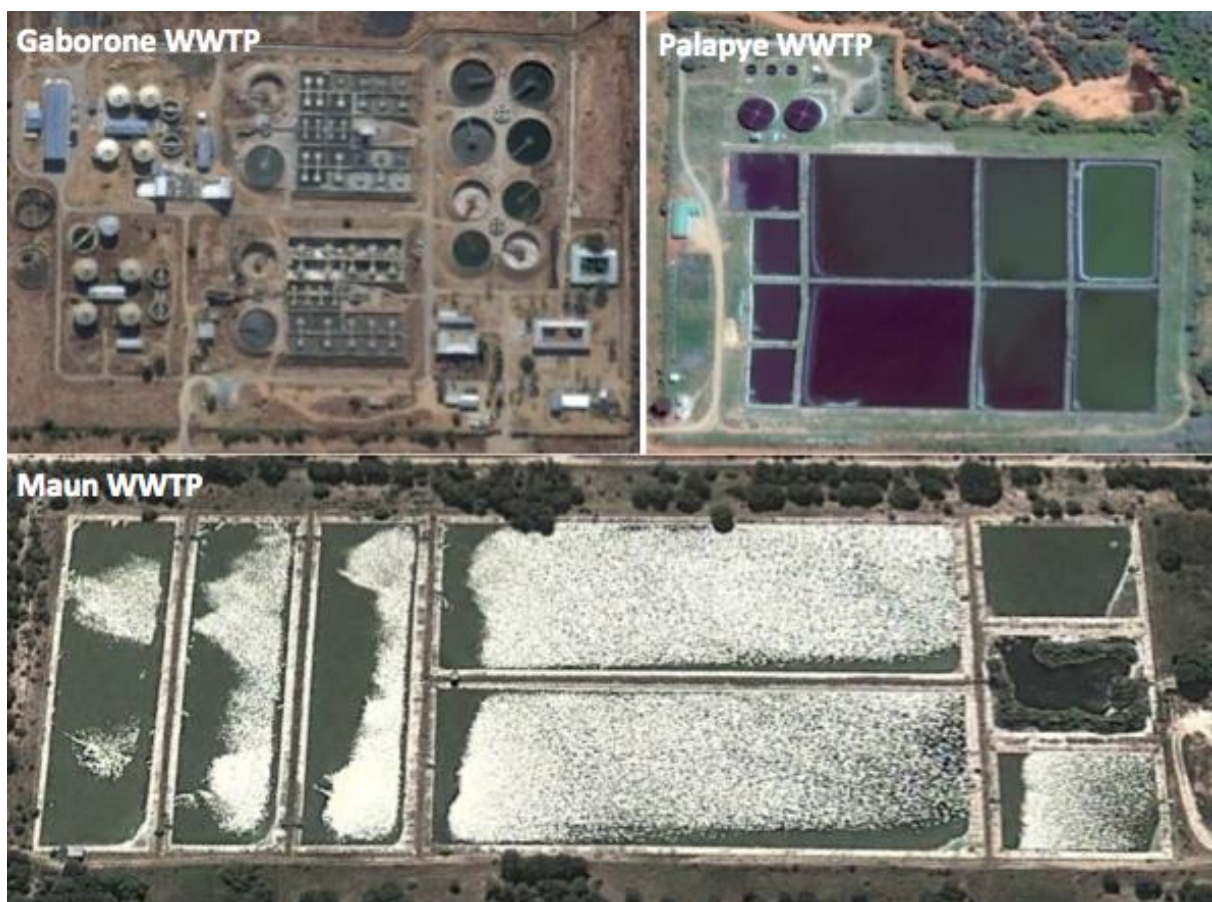


Figure 3.1. The various wastewater treatment plants under study

Sampling points

There were 16 sampling sites under study as presented in Table 3.1, and in all the sampling sites, samples were collected in 3 different seasons between March 2016 and April 2017. Botswana's climate is characterized by four seasons; hot summers (November, December and January), wet autumns (February, March and April), cold and dry winters (May, June and July) and arid windy springs (August, September and October). In 2016, samples were collected in 2 seasons (summer and winter), and 1 season in 2017 (autumn). Samples were collected in mornings between 0530 and 1100 hours when there are minimal activities at the wastewater treatment plant and hospitals. The same sampling seasons and sampling times were maintained for public transport, where a total of 3 buses and 3 mini buses were involved in the study.

Table 3.1. Summary of sampling points in hospitals, public transport and WWTP

Sampling Point	<u>Denomination</u>		
Clinical environment	Hospitals	Palapye Primary Hospital (PPH) – Palapye	- Operating theater ward - Paediatric ward (Medical + surgical)
		Nyangabwe Referral Hospital (NRH) – Francistown	- Operating theater ward - Paediatric ward (Medical)
		Letsholathebe II Memorial Hospital (LMH) – Maun	- Operating theater ward - Paediatric ward (Medical)
Non-clinical environment	Transport	Bus	- 72 seater
		Mini Bus	- 25 seater
	Wastewater Treatment Plant	Palapye (PWWTP)	-Upwind (90 m from the wwtp) -Downwind (within wwtp) - Extension 1 (downstream of wwtp- 440 meters from downwind air sampling point)
		Gaborone (GWWTP)	-Upwind (94 m from the wwtp) -Downwind (within wwtp) - Tsholofelo Extension (downstream of wwtp- 510 meters from downwind air sampling point)
		Maun (MWWTP)	-Upwind (91 m from the wwtp) -Downwind (within wwtp)

Meteorological data

Temperature (T_m) and relative humidity (RH) were recorded at each sampling site with a handheld Thermocron iButtons (Dallas Semiconductors, Model DS1920). T_m and RH were then reported as an average of 1 hour 30 minutes sampling period at each sampling location.

Air sampling method

The principle of air impaction was applied for the collection of airborne bacterial community samples. Air was directed against the media plates using a portable Microbial Air Sampler MAS-100 NT® device (Merckmillipore, Merck KGaA Darmstadt, Germany), which is considered the gold standard among air samplers, at a flow rate of 100 L/min. The air sampler was placed 1.5 m above ground level during the wastewater treatment plants and hospital wards sampling, whereas in public transport the air sampler was placed at heights that mimic the average height of seated passengers (about 0.91 m). Airborne bacteria were collected by impaction onto various selective and differential agar media; Mannitol Salt agar (selective isolation and enumeration of staphylococci; Coagulase-positive staphylococci and coagulase negative staphylococci, and *Micrococcus*), Brucella agar (*Brucella* species), Pseudomonas Agar F (*Pseudomonas* species), Campylobacter agar (*Campylobacter* species), Listeria agar (*Listeria* species), Salmonella agar (*Salmonella* species), Chromocult agar (*E. coli* and coliforms) and *Escherichia coli* agar (*E. coli*). Cycloheximide (1 µg/µL, Sigma-Aldrich Co., St. Louis, MO, USA), previously shown not to affect bacterial counts (Dybwad *et al.*, 2012) was added to each media to inhibit growth of fungi (particularly saprophytic molds). Aerosolized bacteria were collected for 10 minutes on each of the 8-selective/differential media (in duplicates) and nutrient agar media as the control.

3.2. Identification and quantification of airborne bacteria

After each sampling session, the culture media plates were incubated for 24 hours at 37°C for enumeration of viable airborne bacteria. To estimate airborne bacterial concentrations, the number of colony forming units growing in selective/differential media were counted and related to the volume of the air sampled. The total viable count of each site bacteria was calculated by adding all the bacterial counts from the three months (2 months representing 2 seasons in 2017 and 1 month representing 1 season in 2017). Bacterial concentrations were expressed as the average values of colony-forming units per cubic meter (CFU/ m³). The volume of air collected by the air sampler per sampling done was 1000 L hence expressed as cubic meter. With an inflow rate of 100 liter/minute, the volume of air collected by the MAS-100 NT® air sampler per plate was 1000 liters (as the sampling time for each plate was 10 minutes), expressed as cubic meter. The actual microbial count per m³ of air was corrected and calculated using the integrated Feller conversion table (or FELLER's statistical correction table) provided by MAS-100 NT® air sampler operating manual. The Feller conversion table is based on the principle that as the number of organisms per sampling rise, the chance that several organisms will enter the same hole in the perforated lid also increases. To determine abundance and diversity of airborne bacterial species, bacteria growing on selective/differential medium was counted and expressed in CFU/m³ as previously described. Prior to characterization, the colonies were purified. Bacteria were then identified by morphological characterization and Gram staining procedures according to Bergeys Manual of Determinative Bacteriology (Holt *et al.*, 1994). Morphological characterization of the airborne bacteria was carried out by examining colony size, shape, and cellular arrangement. Few colonies were also picked and further confirmed by biochemical tests. Of all the bacterial isolates counted, 1200 isolates were selected (75 bacterial isolates from each of the 16 sampling sites that best represented each selective medium plate) and stored at -80°C in nutrient broth with 50% glycerol solution prior to antibiotic resistance typing and DNA extraction.

3.3. Antibiotic susceptibility test

Antibiotic susceptibility was assessed qualitatively by designating isolates (isolates that were previously stored in 50% glycerol and then thawed), as being resistant or sensitive based on the presence or absence of colony growth after incubation at 30°C for 48 h. All isolates were assayed for resistance against various antibiotics by cultivation on nutrient agar plates supplemented with various concentrations of different antibiotics. Isolates were picked using sterile toothpicks or sterile disposable inoculation loops. The following antibiotics were included in the determination of resistance patterns; Trimethoprim (16 µg/ml), Ampicillin (32 µg/ml), Cephalosporin (32 µg/ml), Penicillin (16 µg/ml), Erythromycin (8 µg/ml), Sulfonamide (512 µg/ml), Meropenem (4 µg/ml), Tetracycline (16 µg/ml), Ciprofloxacin (4 µg/ml) and Streptomycin (30 µg/ml). The selective culture media were supplemented with antibiotics at clinical breakpoint concentrations, similar to those reported as minimum inhibitory for target bacteria. This method was adapted from Watkinson *et al.*, (2007), Novo and Manaia, (2010), and Dybwad *et al.*, (2012), the method allows the rapid characterization of a large number of samples as in this study. It is ideal for the definition of resistance profiles (Rizzo *et al.*, 2013).

3.4. Detection of antibiotic resistance genes

A total of 30 antibiotic resistant strains were selected for detection of various antibiotic resistance genes. The isolates were selected based on their phenotypic expression of multi-drug resistance. These were bacteria isolates that showed the highest antibiotic drug resistance, they were resistant to more than 9 antibiotics that they were subjected to. Different bacterial species isolated from each sampling site were randomly selected for the screening of antibiotic resistance genes that made up the total of 30 bacterial isolates; all those resistant to the ten antibiotics (n=18) and some resistant to nine antibiotics (n=12).

Genomic DNA extraction from isolates

DNA extraction was carried out using the method described by Neela *et al.*, (2015) with slight modification. Pure culture isolates were inoculated on nutrient broth and incubated for 24 hours

at 37 °C. The overnight-cultured bacterial cells were harvested by centrifugation at 13000 rpm for 10 minutes; the pellet was resuspended in 600 µL of lysis buffer (50mM Tris hydrochloride - 50mM EDTA, pH 8.0, 50 mM NaCl and 5% SDS). To re-suspend the pellet, and avoiding formation of foam, the tube was inverted 25 times and gentle pipetting was done. Followed by incubation at 37 °C for 30 minutes. Nucleic acid purification was done by adding 200 µL of phenol and chloroform/isoamyl alcohol, followed by addition of 200 µL of chloroform and vortexing for 20s. The sample was then centrifuged at 13000 rpm for 3 minutes to pellet the protein, and the supernatant was transferred into a new tube for nucleic acid precipitation. This was followed by adding 600 µL of isopropanol and precipitation of the DNA by centrifugation at 13000 rpm for 30 minutes to obtain the DNA pellet. The extracted DNA was washed in 500 µL ice-cold ethanol (70%) and inverting the tube gently to precipitate the DNA. Another centrifugation was done at 13000 rpm for 5 minutes, the supernatant was discarded and the pellet allowed drying for 5 minutes. The DNA was resuspended TE buffer and stored at -20 °C before PCR assays.

Polymerase Chain Reaction (PCR) assays

The antibiotic resistance genes were detected by PCR method. Specific primers for the following resistance genes; Sulfonamide (*sul1* and *sul2*), tetracycline (*tetA* and *tetB*), erythromycin (*ermA*, *ermB* and *ermC*), streptomycin (*strA* and *strB*), quinolone (*qnrA*), dihydrofolate reductase (*dfr1*) and class 1 integron gene cassette (*intI1*) were used to amplify the respective antibiotic resistance genes. The primers used are listed in Table 3.2 below.

Table 3.2. List of primer pairs used to amplify antibiotic resistance genes in this study.

Target gene	Resistance phenotype	Primer pairs*	Primer sequence	Ann. Temp. (°C)	Product (bp)	Reference
<i>tetA</i>	Tetracycline	tetA-L	GGCGGTCTTCTTCATCATGC	46.2	500	Boerlin <i>et al.</i> ,2005
		tetA-R	CGGCAGGCAGAGCAAGTAGA			
<i>tetB</i>	Tetracycline	tetB-F	CATTAATAGGCGCATCGCTG	50.5	930	Memon <i>et al.</i> ,2016
		tetB-R	CATTAATAGGCGCATCGCTG			
<i>mph(A)</i>	Macrolide	<i>mph(A)</i> -F	GCCGATACCTCCCAACTGTA	52.8	403	Liang <i>et al.</i> ,2017
		<i>mph(A)</i> -R	AGGCGATTCTTGAGCATTGC			

ermA	Erythromycin	ermA-F ermA-R	GTTCAAGAACAATCAATACAG AG GGATCAGGAAAAGGACATTTT AC	58.0	421	Castro-Alarcó'n <i>et al.</i> , 2011
ermB	Erythromycin	ermB-F ermB-R	AAAACCTTACCCGCCATACCA TTTGCCGTGTTTCATTGCTT	43.4	359	Castro-Alarcó'n <i>et al.</i> , 2011
ermC	Erythromycin	ermC-F ermC-R	GAAATCGGCTCAGGAAAAGG TAGCAAACCCGTATTCCACG	50.7	572	Castro-Alarcó'n <i>et al.</i> , 2011
qnrA	Quinolones	qnrA-F qnrA-R	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	49.4	516	Robicsek <i>et al.</i> , 2006
dfr	Trimethoprim	dfr-F dfr-R	CCCAACCGAAAGTATGCGGTC G GTATCTACTTGATCGATCAGG	45.6	171	Sunde, 2005
int1	Class1 Integrase	int1-F int1-R	GCATCCTCGGTTTTCTGG GGTGTGGCGGGCTTCGTG	50.4	457	Yousefi <i>et al.</i> , 2010
strA	Streptomycin	strA-F strA-R	CCTGGTGATAACGGCAATTC CCAATCGCAGATAGAAGGC	50.0	546	Boerlin <i>et al.</i> , 2005
strB	Streptomycin	strB-F strB-R	ATCGTCAAGGGATTGAAACC GGATCGTAGAACATATTGGC	46.9	500	Madsen <i>et al.</i> , 2000
sul1	Sulfamethoxazole	sul1-L sul1-R	GTGACGGTGTTCCGGCATTCT TCCGAGAAGGTGATTGCGCT	54.7	921	Lanz <i>et al.</i> , 2003
sul2	Sulfamethoxazole	sul2-L sul2-R	CGGCATCGTCAACATAACCT TGTGCGGATGAAGTCAGCTC	51.5	721	Boerlin <i>et al.</i> , 2005

* F; forward primer, R; reverse primer, Ann. Temp.; annealing temperature

The PCR amplification was performed with a PCR Thermal Cycler (Proflex PCR system, Applied Biosystems). The PCR mixture contained: 2 µL of template DNA, 12.5 µL premix (EmeraldAmp® PCR Master Mix, TAKARA BIO INC), 1.5 µL of each of the primers (forward and reverse) and 7.5 µL deionised water. The following thermocycler parameters were used: initial denaturation at 95 °C for 5 min followed by 30 cycles at 98 °C, annealing for 40 seconds to 1 minute (the annealing temperature varied among the primers, as indicated on Table 3.2), this was followed by extension at 72 °C for 1 minute with a final elongation at 72 °C for 1 minute.

The PCR products were analyzed using gel electrophoresis on a 1.0% (w/v) agarose gel stained with ethidium bromide. Electrophoresis was run at a constant voltage of 60V for about 1 hour 30 minutes in a horizontal tank with 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. After electrophoresis, the gels were visualized on a UV transilluminator.

3.5. Statistical analysis

The variations in total bacteria in clinical and non-clinical environment were assessed using an analysis of variance (ANOVA) test. The one-way ANOVA was used to evaluate the difference between the bacterial concentrations in hospitals, WWTPs and public transport. Results with a p-value less than or equal to 0.05 ($p \leq 0.05$) were considered to be statistically significant whereas those with greater than 0.05 p-value were considered statistically not significant. The tests were performed with the software Graphpad Prism 7.0 (Zhou and Wang, 2013).

4.0.RESULTS AND DISCUSSION

4.1.Occurrence and abundance of airborne bacteria in hospitals

The assessment of airborne bacteria in clinical environment revealed abundance of bacteria in paediatric wards than in the operating theatres. The highest bacterial concentration was observed at PPH paediatric ward; 7.9×10^2 CFU/m³, followed by LMH paediatric ward (6.4×10^2 CFU/m³), followed by PPH paediatric ward (5.8×10^2 CFU/m³). The highest bacterial concentration in operating theater was observed at PPH (3.7×10^2 CFU/m³), followed by NRH (2.7×10^2 CFU/m³) and the least at LMH (2.6×10^2 CFU/m³) as shown by Figure 4.1.

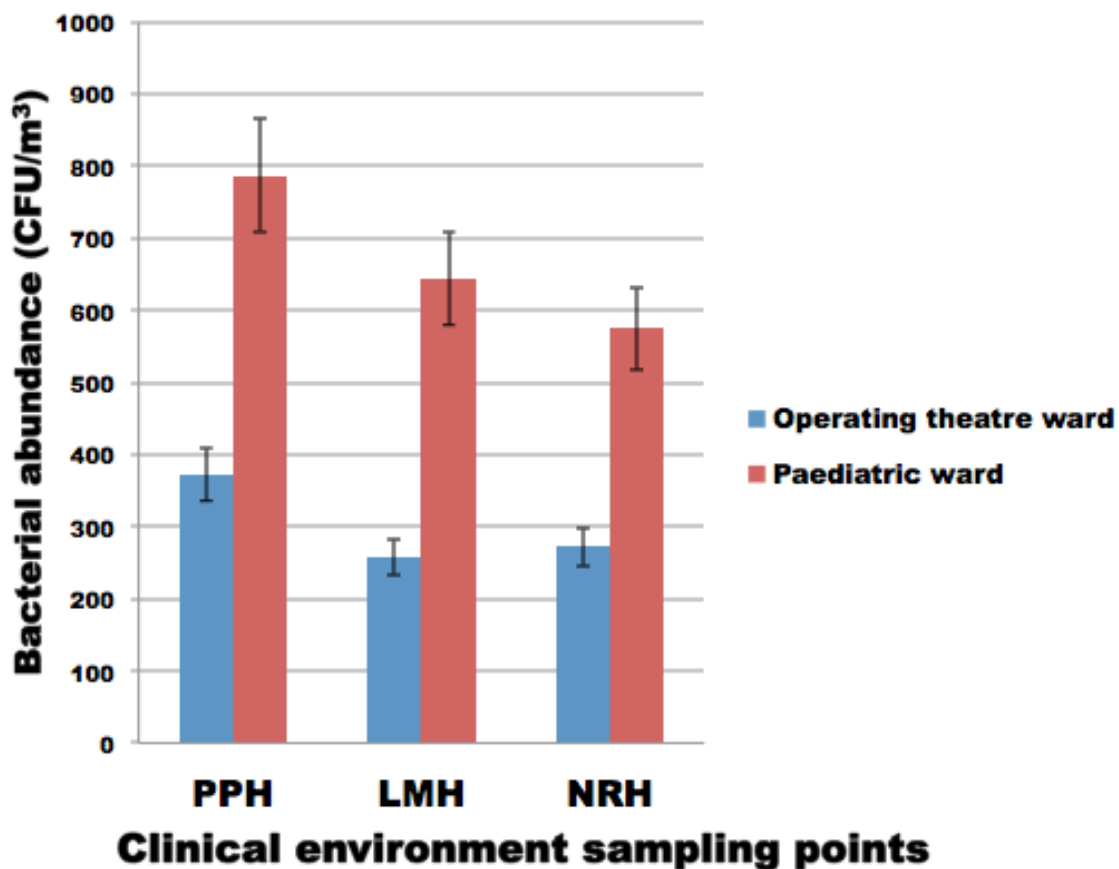


Figure 4.1. Abundance of airborne bacteria in different hospitals' operating theatre and paediatric ward.

(PPH – Palapye Primary Hospital, LMH – Letsholathebe II Memorial Hospital, NRH – Nyangabwe Referral Hospital).

Comparing between the paediatric and operating theater wards, the operating theatre ward had the lowest airborne bacterial concentrations and the paediatric wards showed the highest level of bacterial contamination. Figure 4.1 clearly indicate there is a consistent difference in bacterial concentrations between operating theatre and paediatric wards among all the hospitals. The results are expected, and the higher bacterial concentrations in paediatric wards could have been greatly influenced by the nature and usage, including higher population of patients at the ward compared to those of the operating rooms. Several other factors such as lack of ventilation systems and depending on the outdoor air may have attributed to the vast difference in bacterial concentration between the two different hospital units. Qudiesat *et al.*, (2009), reported that microbial concentrations in the operation rooms were dependent on the hospital setup; operating theatre wards nearer to other hospital units had higher bacterial concentrations compared to those located away from the rest of the hospital units. The level of hygiene and practice of aseptic techniques, with the dirty areas usually separate from the operating theater rooms, the patient flow, from arrival to discharge may also contribute to airborne bacterial concentrations. The high bacterial concentrations in PPH operating theatre ward could be attributed by the washrooms located adjacent to the entrance of the operating room as well as uncontrolled traffic and activities by health care workers around the areas in close proximity to the operating theater. The PPH operating theater room had the highest airborne bacterial concentration compared to other operating theaters, PPH operating theater is the hospital's only major operating room, and therefore it experiences large volumes of activities daily. The PPH hospital is very old, more than 30 years old, the design, layouts, furnishings, fittings, floor coverings and ventilation systems may have a significant impact on the cleaning of the unit consequently giving rise to the airborne bacterial contamination. In addition, PPH autoclave room that receives dirty materials and equipment from the operating room and the rest of the hospital is closer to the operating room thereby increasing the transfer of airborne bacteria to the operating room. Some studies have also reported that the sink drains are frequently colonized by large numbers of bacteria therefore serve as potential reservoir for aerosolized pathogens or opportunistic microorganisms (Moore *et al.*, 2002; McBain *et al.*, 2003).

NRH operating theatre wards also had concerning abundance of airborne bacteria, similar to PPH. NRH operating theatre ward is also poorly located; the main entrance to the ward is along one of the busy corridors of the hospital and also opposite the Intensive Care Unit (ICU). Compared to PPH, NRH is newer and a relatively better condition, however, the ventilation system of most of the hospital units at NRH are malfunctioning, there have been reports from the NRH operating theatre wards that the air conditioning systems were for a long time in bad conditions resulting in poor air ventilation and unstable indoor temperatures which were experienced during sampling. The findings at LMH operating theatre ward were as expected, the bacterial concentration was at the lowest compared to the other hospitals' operating theatre wards indicating a much cleaner indoor air, which could have been attributed to the cleanliness of the ward, whereas those of operating theatre wards of NRH and PPH were of much concern as the bacterial load was about 2-folds and more than 3-folds higher respectively. Similar studies on the assessment of bacterial concentrations in hospital operating rooms such as at State Railway Hospital (Lublin, Poland) revealed that the airborne bacterial concentrations varied from 10 to 10^2 CFU/m³ (Augustowska and Dutkiewicz, 2006), which is in agreement with the findings of this study. The results are also supported by other findings (McCarthy *et al.*, 2000; Li and Hou 2003) where microbial concentrations in operating rooms were lower compared to other hospital units.

Although the paediatric wards care for children less than 12 years, those under the age of 7 years are usually accompanied by parents during hospitalization hence the higher population of the occupants, consequently this setup leads to increased inflow of airborne bacteria. In addition, the products such as food, fruits and inanimate objects such as toys from the external environment are frequently brought in by visitors into the paediatric wards and may contribute to the increase in airborne bacteria; these were documented as important source of hospital contamination (Jaffal *et al.*, 1997; Qudiesat *et al.*, 2009). Similar to operating theater wards, poor and deficient hygienic conditions due to minimal disinfection procedures throughout the paediatric wards might have given rise to the concentration of observed airborne bacteria. The large numbers of airborne bacteria within the PPH paediatric ward might also be attributed to the age of the building. PPH paediatric ward is dimensionally too small to accommodate the inpatients, the ward is made up of a single room that is usually congested resulting in poor air

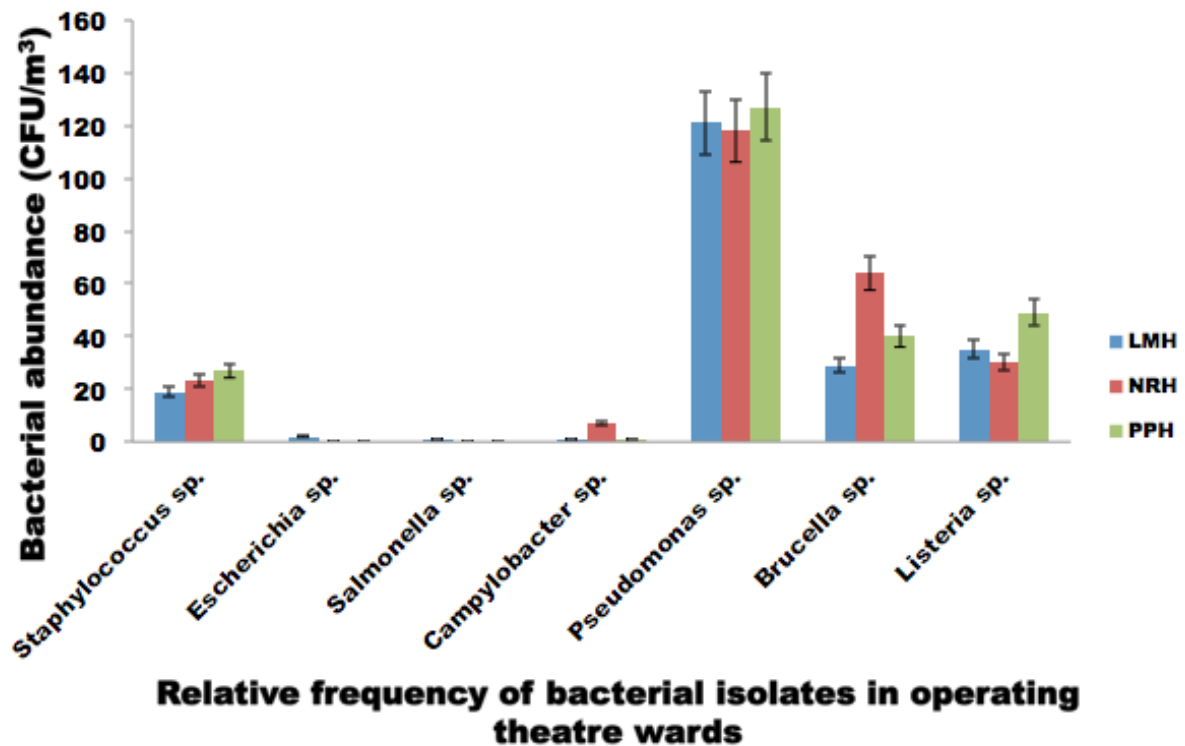
circulation and also this makes it hard for cleaning the ward. NRH paediatric ward also had high concentration of airborne bacteria, the ward has multiple rooms that are incompletely partitioned and separated from each other, hence allowing for free airflow between the rooms. There are no air conditioning systems at the NRH paediatric wards, therefore the ward depends on windows and doors for ventilation and this could lead to more inflow of airborne bacteria from the surrounding outdoor air. The paediatric ward of the LMH is ideally constructed, the rooms are well spaced making it easy for appropriate cleaning, the air conditioning system is in a good conditions, and also the ward is not congested. All this could have attributed to the minimal airborne bacterial abundance compared to other paediatric wards. A study on airborne transmitted infections in hospitals revealed mean bacterial contamination values of $3.0 - 7.0 \times 10^2$ CFU/m³ during hours with less human activities and 6.0×10^3 CFU/m³ during bed making hours (Woldu *et al.*, 2013). Roberts *et al.*, (2006) ascertains that influence of anthropogenic activities have an effect on the rise of airborne microbial concentrations in hospital rooms.

4.2. Diversity and abundance of airborne bacteria in hospitals

The occurrence of diverse airborne bacterial species was observed in all the hospitals' operating theatre and paediatric wards. The prevalence of *Pseudomonas*, *Listeria*, *Brucella*, and *Staphylococcus* species in those hospitals' units was the highest, whereas the least isolated bacteria were *Escherichia coli* and *Campylobacter* species. The bacterial species isolated from the operating theatre wards were as follows; *Pseudomonas* (1.2×10^2 CFU/m³, 1.2×10^2 CFU/m³ and 1.3×10^2 CFU/m³ isolated from LMH, NRH and PPH respectively), *Listeria* (3.4×10^1 CFU/m³, 2.0×10^1 CFU/m³ and 5.0×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively), *Brucella* (2.8×10^1 CFU/m³, 7.5×10^1 CFU/m³ and 4.5×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively), *Staphylococcus* (2.5×10^1 CFU/m³, 1.9×10^1 CFU/m³ and 3.6×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively), *Escherichia coli* (0.2×10^1 CFU/m³, 0 CFU/m³ and 0.1×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively) and *Campylobacter* (0.1×10^1 CFU/m³, 0.2×10^2 CFU/m³ and 0.1×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively). There were no *Salmonella* species found in any of the operating theatre wards. The bacterial species isolated from paediatric wards were as follows; *Pseudomonas* (2.8×10^2 CFU/m³, 1.8×10^2 CFU/m³ and 2.7×10^2 CFU/m³ isolated from LMH,

NRH and PPH respectively), *Listeria* (1.8×10^2 CFU/m³, 1.7×10^1 CFU/m³ and 1.0×10^2 CFU/m³ isolated from LMH, NRH and PPH respectively), *Brucella* (1.8×10^2 CFU/m³, 3.0×10^1 CFU/m³ and 9.0×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively), *Staphylococcus* (5.0×10^1 CFU/m³, 1.2×10^2 CFU/m³ and 6.0×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively), *Escherichia coli* (0.1×10^1 CFU/m³ isolated from each hospital) and *Campylobacter* (0.6×10^1 CFU/m³, 2.3×10^1 CFU/m³ and 2.6×10^1 CFU/m³ isolated from LMH, NRH and PPH respectively) and *Salmonella* (0.1×10^1 CFU/m³ isolated from each hospital).

(a)



(b)

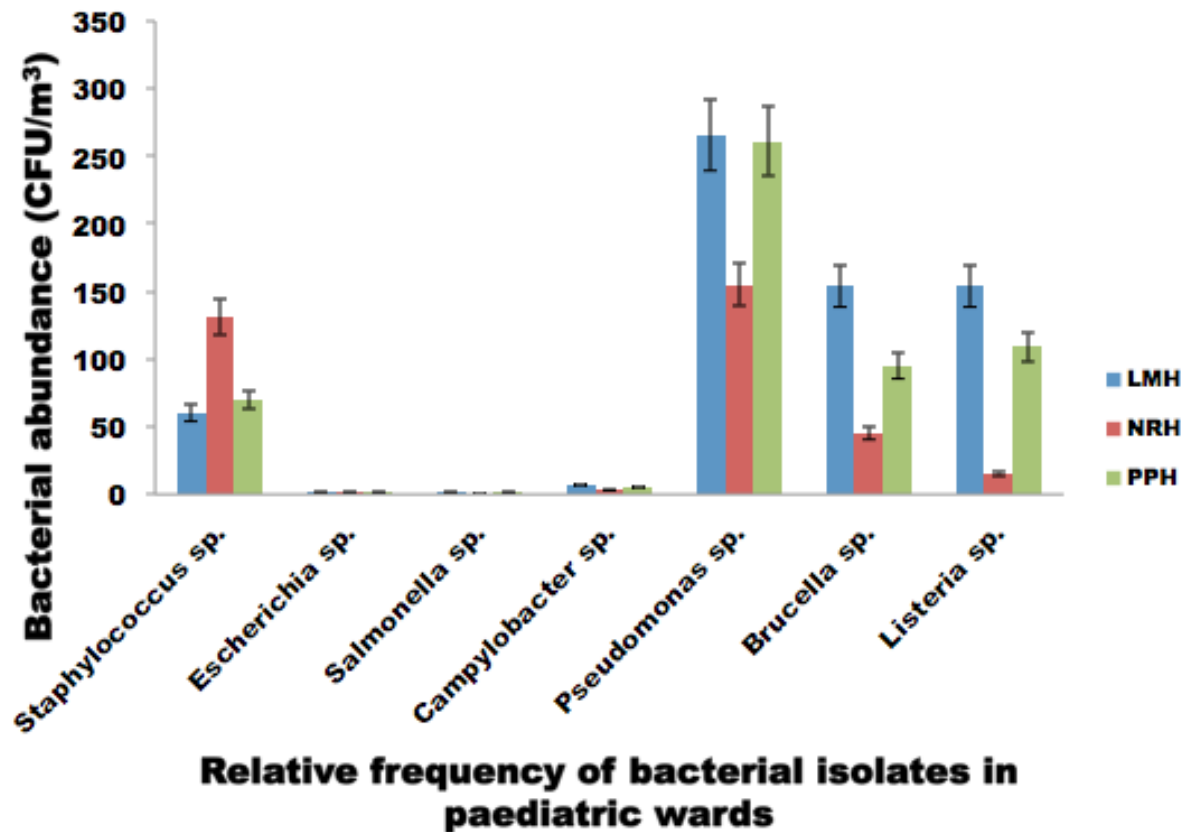


Figure 4.2. Bacterial diversity of Isolates in (a) operating theatres and (b) paediatric wards. (LMH-Letsholathebe II Memorial Hospital, NRH-Nyangabwe Referral Hospital, PPH-Palapye Primary Hospital)

Pseudomonas species were the most abundant bacterial isolates from all the operating rooms and the paediatric wards, *Brucella*, *Listeria* and *Staphylococcus* species were also predominant. Similarly, findings from other studies where airborne bacteria were isolated in hospitals are in accordance with our results (Borrego *et al.*, 2010; Qudiesat *et al.*, 2009; Gilbert *et al.*, 2010), these studies highlighted the high presence of potentially pathogenic bacteria in the hospital air such as *Staphylococcus* species, *Pseudomonas* species, and *Streptococcus* species. *Pseudomonas* species, especially *P. aeruginosa* was found to be prolific in the hospital atmosphere. *P. aeruginosa* is of particular importance due to its immense association with fatal nosocomial outbreaks (Trautmann *et al.*, 2005; Hota *et al.*, 2009; Gilbert *et al.*, 2010). It is amongst the leading causes of nosocomial pneumonia associated with high mortality rates in

healthcare settings (Krzowska-Firych *et al.*, 2014). In hospitals it has been previously isolated from various environments and surfaces especially around water sources such as sink drains (Trautmann *et al.*, 2005). Hota and colleagues (2009) supports these findings and further mentions that the *P. aeruginosa* is then aerosolized during hand washing thereby easily transmitted throughout the atmosphere. The abundance of airborne *P. aeruginosa* in our study is a serious health threat and its aerial transmission should be lessened. Other bacteria genera that were isolated in this study were *Listeria*, these were also observed in similar studies conducted in hospitals such as Sarica *et al.*, (2002), where airborne *Listeria* among others were reported to be predominant in various areas of the hospital under study. *Listeria* species such as *Listeria monocytogenes* have been reported to be a health concern leading to fatality rates of 20–30% among hospitalized patients (King *et al.*, 2003). These bacteria although they are foodborne, they are likely to enter the atmosphere from the hospitalized patients and/or their food, this is supported by the concentration of the *Listeria* species in paediatric wards than operating theatre wards where food is allowed into the hospital units.

The gastrointestinal pathogen, which is also a foodborne bacterium, *Salmonella* species was isolated only in paediatric wards in LMH and PPH at low concentrations. Similar to *Listeria*, *Salmonella* species isolated may also have entered the air from the contaminated food from the hospital kitchen or the food brought in by visitors, or aerosolized upon release during baby nappy changing of an infected child. The *Salmonella* species is likely to proliferate and/or settle on uncovered drinking water and food thereby contaminating it leading to new infections within the ward hence prolonged hospitalization for some of the patients. *Salmonella* outbreaks have been reported in hospitals (mostly paediatric wards and nurseries) and nursing homes (Villarino *et al.*, 1992; CDC, 1999).

4.3. Occurrence, abundance and diversity of airborne bacteria in wastewater treatment plants

At all the wastewater treatment plants, highest bacterial concentrations in the air were isolated from the downwind of the WWTP. The downwind air of Palapye WWTP had the highest

average bacterial concentrations (5.2×10^2 CFU/m³), followed by Gaborone WWTP downwind air (4.7×10^2 CFU/m³) and Maun WWTP Downwind air (2.4×10^2 CFU/m³). Whereas the upwind air of Gaborone had the highest bacterial concentration (2.6×10^2 CFU/m³) followed by that of Palapye (1.2×10^2 CFU/m³) and lastly of Maun (9.2×10^1 CFU/m³). Settlements near the WWTPs had lower levels of airborne bacterial concentrations, in Palapye Extension 1 had slightly lower (1.4×10^2 CFU/m³) total airborne bacteria than Gaborone's Tsholofelo extension (1.7×10^2 CFU/m³) whereas Maun WWTP remains far away from any settlement, (Figure 4.3).

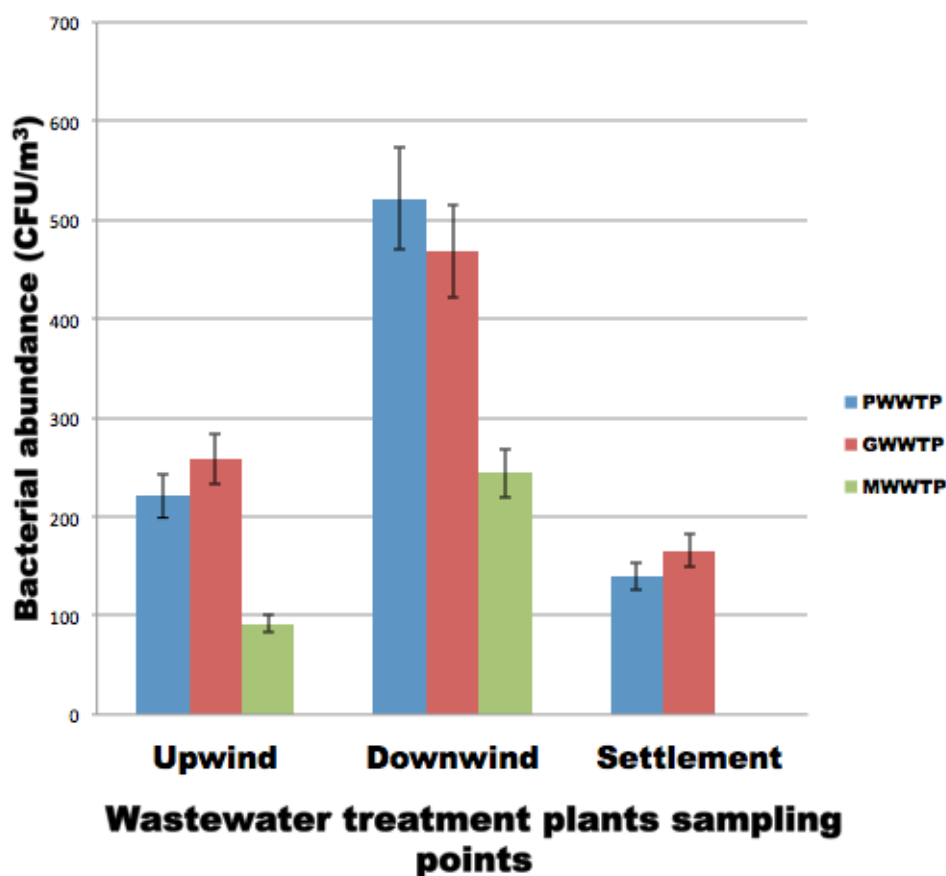


Figure 4.3. Bacterial concentrations in the air surrounding the wastewater treatment plants. The higher numbers of bacteria in the downwind air samples which were collected within the premises of the WWTPs near the mechanical sewage treatment devices (grit chamber Palapye and Gaborone, and sedimentation pond in Maun) of the plant could have been caused by the various treatment stages of the wastewater at each plant. The abundance of airborne bacteria in downwind air of PWWTP was the highest of all the wastewater treatment plants, which could be a result of constant inflow of raw wastewater through the aerated grit chamber and the

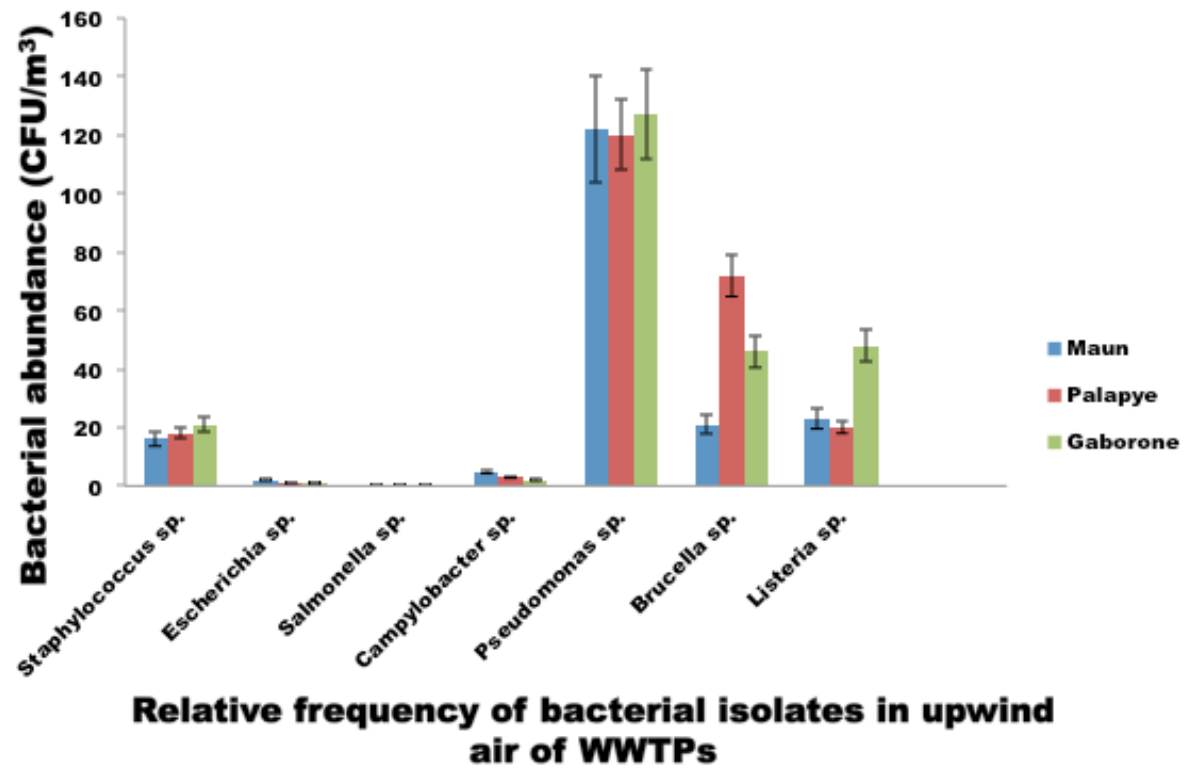
aeration of effluent during the trickling stage. The trickling effect at the trickling tank of the secondary treatment elevated the concentration of airborne bacteria as small droplets of bioaerosols were produced and dispersed to the surrounding air by wind. In Gaborone, the aeration chamber of the treatment plant is equipped with a fine bubble deep aeration system that does not produce large turbulence, and consequently generate few amounts of bioaerosols compared to the use of the trickling filter in Palapye. Maun WWTP generated lower bioaerosol contaminations due to the simplicity of the operating system, which does not involve any aerating chambers; hence few bioaerosols are emitted into the atmosphere. The results show that differences in the sources of sewage water and the population that each WWTP serves may also have had an effect on the airborne bacterial concentration in each of the city/towns. The findings of the occurrence and abundance of airborne bacteria were as expected based on the differences of the stages of wastewater treatment at each facility. These results are in agreement with similar studies on the effect of operational conditions on airborne bacteria done by Prażmo *et al.*, (2003) and Sa´nchez-Monedero *et al.*, (2008), where the bacterial contamination in the atmospheres were highly affected by the emission of the bioaerosols from the WWTPs. These results are however contrary to findings by Gotkowska-Płachta *et al.*, (2013) that it is not easy to delineate whether differences in bioaerosol emissions are related to the type and amount of treated wastewater, or to the processes utilized by the plants

The abundance of airborne bacteria in GWWTP upwind air was high compared to the other wastewater treatments plants. Upwind air is regarded as clean air before contamination by the wastewater treatments activities. However, GWWTP upwind air is not so clean, and this could be as a result of series of maturation ponds surrounding the wastewater treatment plant. The abundance of bacteria in upwind air of the PWWTP was contributed by the soil, plants and animals (cattle and small livestock) around the upwind sampling areas. Animals as well as plants shed bacteria, and the movement of these animals loosens the soils resulting in the bacteria from the soil entering the surrounding air thus elevating the bacterial concentration in the air. In addition, the wind direction during sampling could have largely contributed to the abundance of airborne bacteria in the upwind air, during sampling the air would rarely blow in a single direction and the abundance of airborne bacteria may be associated with unstable wind. Results indicated that the settlements nearby the wastewater treatment plants are affected by

the WWTP. Bioaerosols may travel over long distances, and affect various environments. Palapye and Gaborone residents use the treated wastewater from the WWTPs for irrigation purposes and construction works among others. Thereby, wastewater-carrying vehicles frequent the WWTPs effluent sites and are likely to pollute the immediate air as the wastewater gets aerosolized during transportation to its destination. This is likely to raise levels of bacterial concentration in upwind air of WWTPs as well.

Bacterial diversity and abundance of isolates detected in WWTPs upwind and downwind air are shown in Figure 4.4. The bacterial species and concentrations detected in the upwind air of WWTPs were as follows; *Pseudomonas* (1.2×10^2 CFU/m³, 1.2×10^2 CFU/m³ and 1.3×10^2 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Listeria* (2.0×10^1 CFU/m³, 1.5×10^1 CFU/m³ and 4.9×10^1 CFU/m³ were detected in Maun, Palapye and Gaborone respectively), *Brucella* (1.5×10^1 CFU/m³, 6.5×10^1 CFU/m³ and 4.0×10^1 CFU/m³ detected in from Maun, Palapye and Gaborone respectively), *Staphylococcus* (1.0×10^1 CFU/m³, 1.2×10^1 CFU/m³ and 1.4×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Escherichia coli* (0.2×10^1 CFU/m³ detected in Maun, and 0.1×10^1 CFU/m³ from Palapye and Gaborone) and *Campylobacter* (0.4×10^1 CFU/m³, 0.6×10^1 CFU/m³ and 0.3×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively). There were no *Salmonella* species isolated from the upwind air of all the wastewater treatment plants. Whereas the bacterial species and concentrations detected in the downwind air were as follows; *Pseudomonas* (1.9×10^2 CFU/m³, 2.8×10^2 CFU/m³ and 1.8×10^2 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Listeria* (1.3×10^2 CFU/m³, 1.2×10^2 CFU/m³ and 1.2×10^2 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Brucella* (1.8×10^2 CFU/m³, 2.8×10^1 CFU/m³ and 9.0×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Staphylococcus* (1.8×10^1 CFU/m³, 8.5×10^1 CFU/m³ and 2.0×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively), *Escherichia coli* (0.1×10^1 CFU/m³, 0.3×10^1 CFU/m³ and 0.2×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively) and *Campylobacter* (0.9×10^1 CFU/m³, 0.5×10^1 CFU/m³ and 0.3×10^1 CFU/m³ detected in Maun, Palapye and Gaborone respectively) and *Salmonella* (0.3×10^1 CFU/m³ detected in Palapye).

(a)



(b)

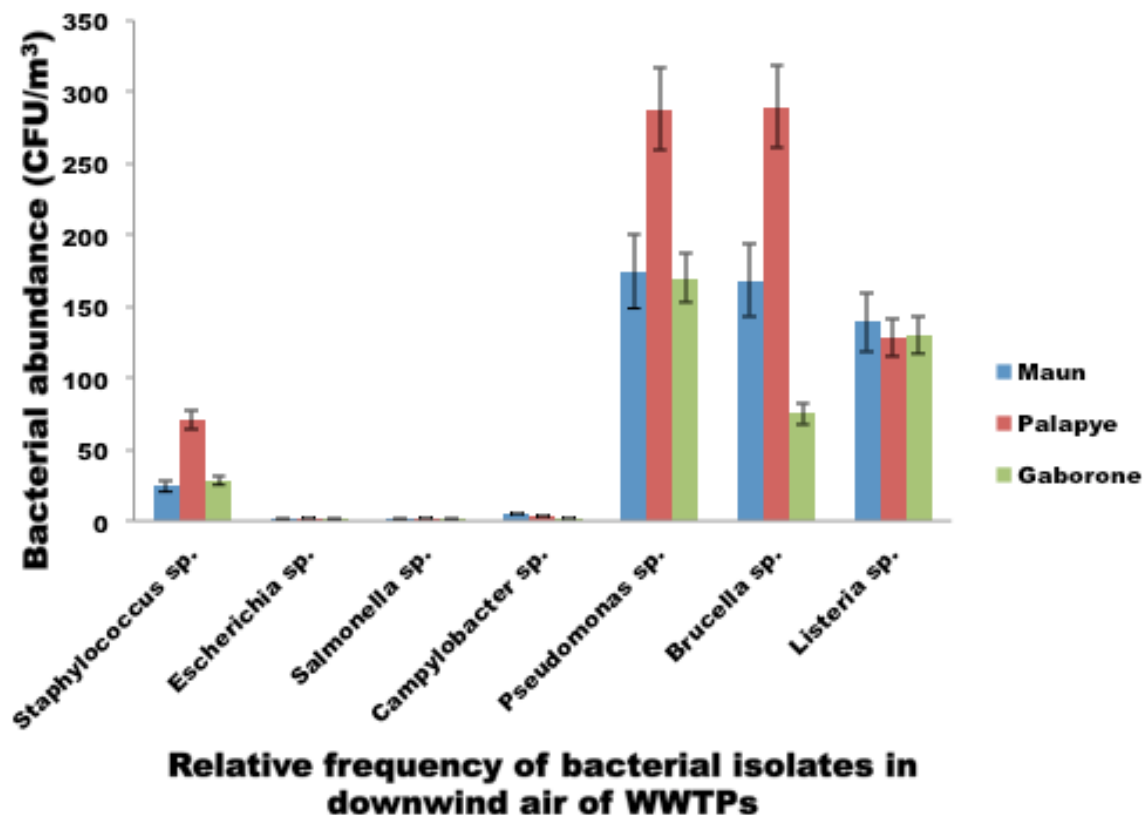


Figure 4.4. Bacterial abundance and diversity in WWTPs (a) upwind and (b) downwind air.

The results reveal that the dominant bacteria in atmospheric air within the WWTPs and the immediate areas were *Pseudomonas*, *Brucella*, *Listeria* and *Staphylococcus* species. The highest diversity of bacteria (7 bacteria genera) was isolated from the air sampled within the premises of the WWTPs (downwind air). The results indicate that the qualitative composition of bacteria in the atmosphere is affected by aerosolization of wastewater from the wastewater treatment plants. According to several studies, most diverse airborne bacteria are found around domestic sewage. These include bacteria such as *Pseudomonas* species, *Brucella* species, *Escherichia* species, *Campylobacter* species, *Salmonella* species, *Listeria* species, and *Staphylococcus* species, isolated from various municipal wastewater treatment plants (Mulloy, 2000; Korzeniewska *et al.*, 2008; Sa' nchez- Monedero *et al.*, 2008). The results of the current study were surprising, as fecal coliforms such as *E.coli* were expected in higher abundance in the downwind air of wastewater treatment plants. However, they were isolated in low concentrations. The Gram-negative bacteria such as *Pseudomonas* species and *Brucella* species

were found in high concentrations, these bacteria prefer moist environments, and have increased survival time in aerosolized state in the atmosphere surrounding WWTPs. *Pseudomonas* species is the most abundant airborne bacteria near WWTPs, mostly *P. aeruginosa* have been widely reported in wastewater from urban sewerage systems (containing hospital and commercial wastewater), in the effluents and the water bodies downstream from these plants (Schwartz *et al.*, 2003; Slekovec *et al.*, 2012). These bacteria then enter the atmosphere where they contribute to an increase in the number of community-acquired infections. The number of *Staphylococci* species isolated from the downwind of each WWTP was in minimal concentrations, between $2.4 - 3.3 \times 10^3$ CFU/m³. These results are supported by various studies that reported that *Staphylococci* species were of minor concern in the sewage and in the air in the WWTP's area (Filipkowska, 2003; Korzeniewska *et al.*, 2008). *Staphylococcus* species have pigments that enable them to withstand harsh conditions in the atmosphere hence survive longer; an example is the carotene pigments in species that enable them to survive exposure to radiation (Velez-Quinones, 2013). Wastewater treatment is one of the main sources of airborne bacteria among human activities, largely affecting bacterial concentrations and populations in the outdoor environment (Recer *et al.*, 2001; Bauer *et al.*, 2003; Prazmo *et al.*, 2003).

4.4. Occurrence, abundance and diversity of airborne bacteria in public transport systems

The highest bacterial concentration in the air within public transport system was detected in the public bus, which accommodates a larger number of passengers compared to those isolated from the mini bus that accommodates lesser passengers. Figure 4.5 shows the average concentrations of cultivable airborne bacteria in each public transport system over a period of 3 seasons, 5.6×10^2 CFU/m³ and 4.3×10^2 CFU/m³ were observed in a bus and mini bus respectively. The data obtained from this study indicate the existence of a significant level of bacterial contamination in the public transport in Botswana.

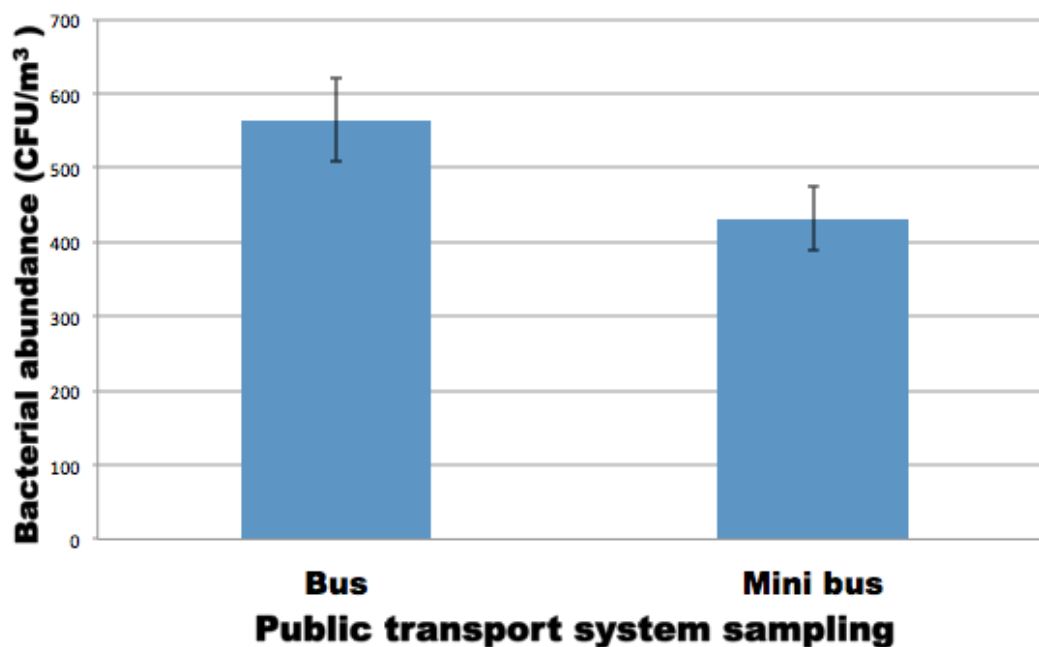


Figure 4.5. Airborne bacterial concentrations in public transport; bus and mini bus.

Public buses usually carry 60 to 75 passengers, and during rush hours or “busy” days such as weekends and month ends, buses allow for standing of the extra passengers. Whereas mini buses carry between 15 – 25 passengers and no extra standing passengers are allowed on board. The differences in the number of passengers and consequently the volume of air (buses have more air capacity than mini buses due its bigger size) could have a greater effect on the bacterial concentrations in the air within various public transport systems. The higher the number of occupants confined in any enclosed space, the higher the microbial concentration as beings occupying the space are considered as essential sources of indoor atmospheric contamination. Observations that support this study were made by Soto *et al.*, (2009), where occupants in various public places were found to directly affect the microbial concentration. The high bacterial concentrations in buses and mini buses may also be as a result of the build-up of airborne bacteria released into the indoor air from the luggage and the food brought in by the passengers. Considering that the human’s daily intake of air (more than 10.000 liters) (Soto *et al.*, 2009) and that a mean value of $4.1 - 5.2 \times 10^2$ CFU/m³ was obtained as the average values of bacteria concentration from bus and mini bus, this study reveals that one public transport passenger may inhale a total estimate of no less than about 4.0×10^2 CFU/m³ microorganisms

from the air per hour on each trip made and individuals are thereby likely to be exposed and get infected with airborne nosocomial infections.

The bacterial species isolated from the bus were as follows; *Pseudomonas* species (3.8×10^2 CFU/m³), *Listeria* species (9.5×10^1 CFU/m³), *Brucella* species (7.9×10^1 CFU/m³), *Staphylococcus* (8.5×10^1 CFU/m³), *Escherichia coli* (1.9×10^1 CFU/m³), *Campylobacter* species (1.0×10^1 CFU/m³) and *Salmonella* species (0.4×10^1 CFU/m³). Whereas the bacterial species isolated from the mini bus were as follows; *Pseudomonas* species (1.5×10^2 CFU/m³), *Listeria* species (1.9×10^2 CFU/m³), *Brucella* species (8.3×10^1 CFU/m³), *Staphylococcus* species (8.0×10^1 CFU/m³), *Escherichia coli* (1.7×10^1 CFU/m³), *Campylobacter* species (0.2×10^1 CFU/m³) and *Salmonella* species (0.3×10^1 CFU/m³) as indicated by the figure below (Figure 4.6).

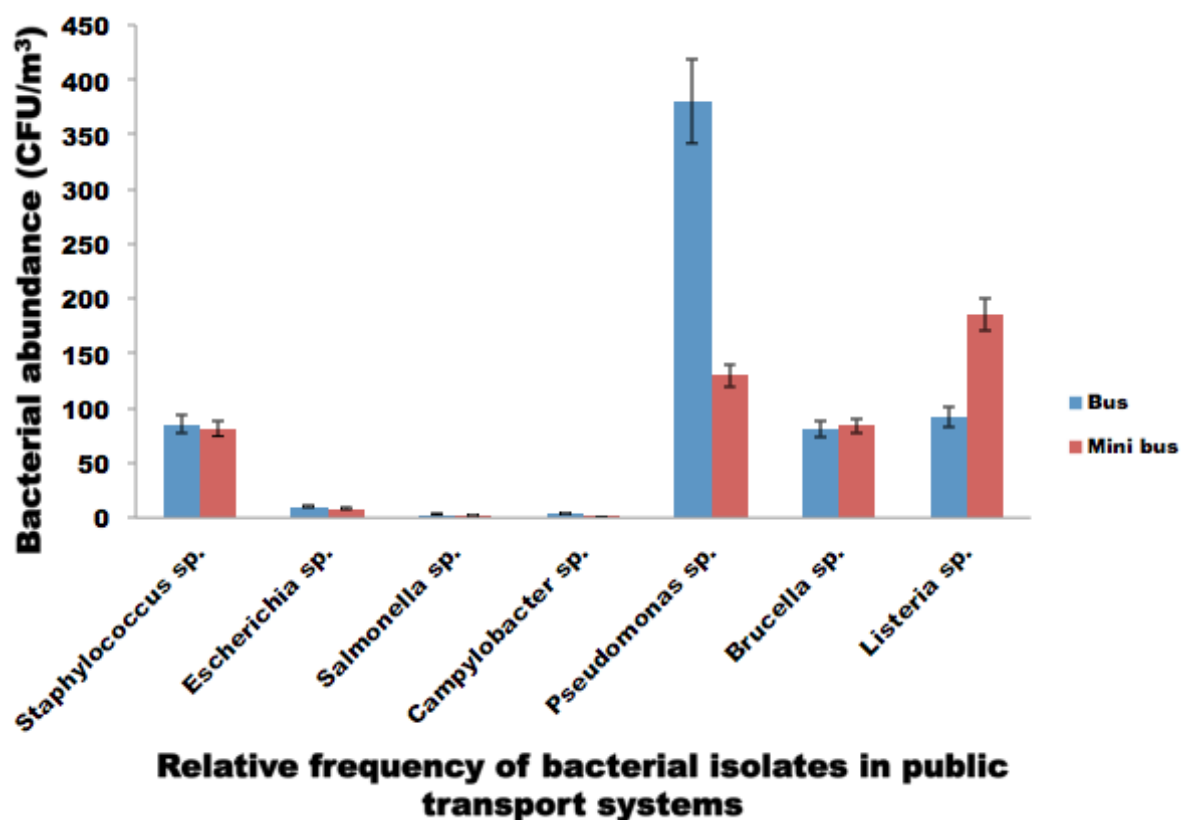


Figure 4.6. Bacterial diversity of isolates in a bus as compared to the mini bus.

The isolation of *Pseudomonas* species in public transport system as well as the other environments that were studied (hospital units indoor air and air surrounding wastewater treatment plants) is a cause of a serious concern. These pathogens are highly associated with hospital-acquired infections as they are linked with airborne transmission of cystic fibrosis (Sudharsanam *et al.*, 2015; Wood *et al.*, 2016). *Pseudomonas* species such as *P. aeruginosa* are usually released into the air by individuals with cystic fibrosis and could travel for up to 4 meters where they survive for almost 45 minutes in air upon release by cystic fibrosis patients (Wood *et al.*, 2016). These findings suggested that *Pseudomonas* species, which usually colonize moist environments, remain viable long enough in air to cause an airborne infection risk as they are resistant to rapid biological decay hence their abundance in diverse environments. *Listeria* species isolated from the buses were also abundant; this could be as a result of the accumulation of dust particles within the buses or their transfer into the transportation system through the dusty roads as soil is one of the major sources of *Listeria*, where it can survive for extended periods. In the entire clinical and non-clinical environment involved in this study, public transportation systems had the highest prevalence of *Staphylococcus* species. The findings are as expected because human beings are one of the major sources of *Staphylococcus* species. For example *S. aureus* is a known commensal of the nasal cavity (Loeto *et al.*, 2007). These pathogenic bacteria survive for several months in dust particles (Qudiesat *et al.*, 2009) and may be transmitted within the public transport systems by air. The minimal cleaning and poor ventilation (windows are usually closed throughout the trip) of the public transport may result in the accumulation of these airborne bacteria. Passengers using public transport are thereby at high risk of acquiring infectious diseases caused by bacteria. The isolation of bacterial species that are indicator organisms of foodborne disease outbreaks such as *E. coli* and *Salmonella* species in public transportation system denoted possible fecal contamination. This is a cause of concern, as these bacterial species are likely to settle on commuters' food resulting in bacterial gastroenteritis upon consumption of the contaminated food.

4.5. Occurrence and diversity of antibiotic resistance in airborne bacteria

In this study, a total of 1201 bacterial isolates were tested against ten antibiotics; Trimethoprim,

Ampicillin, Cephalosporin, Penicillin, Erythromycin, Sulfonamide, Meropenem, Tetracycline, Ciprofloxacin and Streptomycin. Of all the 1201 bacterial isolates, 79% (951) were resistant to at least one antibiotic while only 21% (250) were sensitive to all the ten antibiotics. The mean abundance of antibiotic resistant bacteria from the public transport: bus and mini bus was 82% and 85% respectively. While the mean abundance of antibiotic resistant bacteria in hospitals ranged between 76% and 97%; PTW- 84%, PPW- 76%, NTW- 95%, NPW-97%, LTW- 87% and LPW- 79%. Lastly, antibiotic resistant bacteria from Gaborone, Maun and Palapye wastewater treatments plants (this included bacterial isolates from upwind, downwind and a nearby settlement) had a mean abundance of 76%, 77% and 67% respectively, (Figure 4.7).

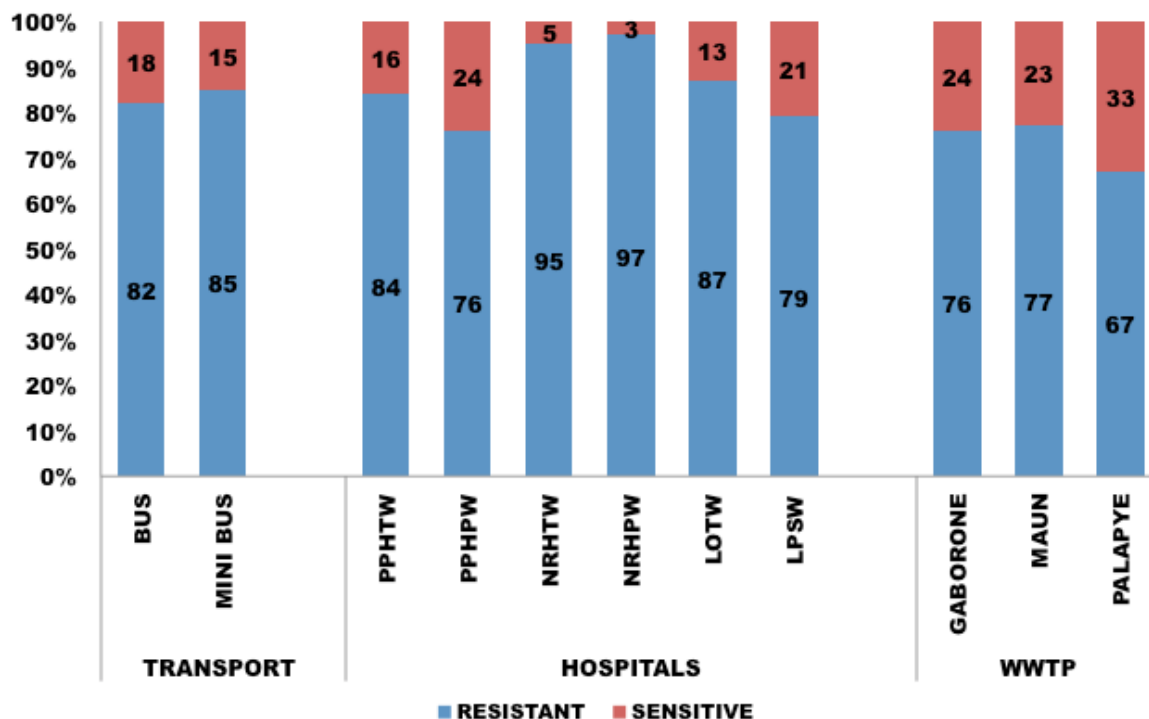


Figure 4.7. The average percentages of antibiotic resistant and sensitive bacterial isolates from public transport, hospitals and wastewater treatment plants.

(PTW- Palapye Primary Hospital Operating Theatre ward, PPW- Palapye Primary Hospital Paediatric ward, NTW- Nyangabwe Referral Hospital Operating Theatre ward, NPW- Nyangabwe Referral Hospital Paediatric ward, LTW- Letsholathebe II Memorial Hospital Operating Theatre ward, LPW- Letsholathebe II Memorial Hospital Paediatric ward)

The results from this study indicate that the highest antibiotic resistant bacteria were isolated from the hospitals (87 – 97%), followed by public transport (82 – 85%) and lastly the wastewater treatments plants (67 – 77%). The results are not as expected, as it was hypothesized that the wastewater treatment plants would have the highest antibiotic resistant bacterial population followed by hospitals and lastly the public transportation. Among the three hospitals under study, Nyangabwe Referral Hospital had the highest antibiotic resistant bacteria, both in paediatric ward (NPW) and operating theatre ward (NTW) with antibiotic resistance percentage of 97 and 95 respectively. Similarly, other hospitals' wards had bacteria with high antibiotic resistance, > 75%. The majority of hospitalized patients receive antibiotics for therapy or prophylaxis during their inpatient stay, and this is one of the factors driving antibiotic resistance in hospitals. Also, patients who enter hospitals for the treatment of resistant bacterial infections are a source of resistant bacteria. In Botswana, most of the broad-spectrum antibiotics such as ampicillin are used indiscriminately and narrow-spectrum antibiotics like erythromycin are also used inappropriately. In a study that compared the usage and prescription of one or more antibiotics in Botswana's hospitals, lowest percentages of antibiotic prescriptions were recorded (Fisher *et al.*, (2009). And a similar study by Woldu *et al.*, (2013), reported the highest usage and prescription of one or more antibiotics in Ethiopia as compared to Botswana.

Public transportation has alarming antibiotic resistant bacterial populations, and it is not very clear of the source contributing to higher numbers of airborne antibiotic resistance bacteria in buses. Antibiotic resistant bacterial strains released into the environment through the aerial route may contribute to acquisition and spread of antibiotic resistance to wild-type strains. In wastewater treatment plants the source of antibiotic resistance could be mostly associated with hospitals and other clinical settings (Baquero *et al.*, 2008). Antibiotic residues and resistant bacteria from hospital discharges enter the wastewater treatment plant and may ultimately enter into the atmospheric air where they are carried over long distances to various environments. The reported multiple antibiotic resistant bacterial concentrations in hospitals, public transport and the wastewater treatment plants pose a serious threat to the inpatients and health workers, commuters, workers and the public in nearby inhabitants.

The majority of bacterial isolates from public transport, hospitals and wastewater treatments

(n=1208), showed resistance to various antibiotics used as indicated in Figure 4.8 below (none=21% (250), one=11% (131), two=12% (137), three=14% (158), four=9% (114), five=10% (128), six=7% (87), seven=7% (85), eight=5% (59), nine=3% (34) whereas 1% (18) were resistant to all (ten) antibiotics.

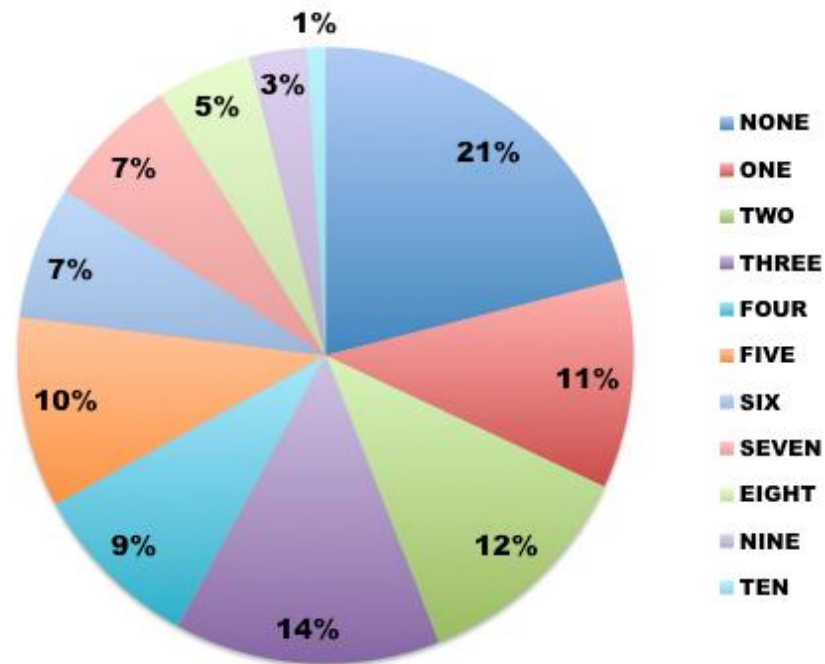


Figure 4.8. Multidrug resistance to various clinical antibiotics used.

Results of this study indicate that most of the airborne bacterial population were resistant to many of the clinical antibiotics tested; only 21 % of the bacterial isolates were all susceptible to all the ten antibiotics. Multidrug resistance (resistance to more than two antibiotics) was observed in the majority (68.1%) of the bacterial isolates. Bacterial isolates that showed resistance to all (ten; Trimethoprim (16 µg/ml), Ampicillin (32 µg/ml), Cephalosporin (32 µg/ml), Penicillin (16 µg/ml), Erythromycin (8 µg/ml), Sulfonamide (512 µg/ml), Meropenem (4 µg/ml), Tetracycline (16 µg/ml), Ciprofloxacin (4 µg/ml) and Streptomycin (30 µg/ml).) antibiotics were only 1%. These findings were as expected; the majority of the isolated bacterial species were the *Pseudomonas* species *Pseudomonas* species, particularly *P. aeruginosa* has intrinsic resistance to multiple antimicrobial agents and is amongst the list of the ‘superbugs’.

These multi-drug resistant bacterial species are associated with hospital-acquired infections, causing infections such as surgical site infections, nosocomial pneumonia (chronic lung infection) and urinary tract infection (Richards *et al.*, 2000; Cortes *et al.*, 2009). The treatment of these infections caused by *Pseudomonas* species is commonly a major challenge due to the constant emergence of antibiotic-resistant variants, resulting in the increases in the prevalence and mortality. In hospital settings, patients with *Pseudomonas* species infections are a source of antibiotic resistant bacterial species, whereas the wastewater (such as that from hospital sewer containing urine of patient infected with *Pseudomonas* species) environments are a source of these antibiotic resistant *Pseudomonas* species in air surrounding wastewater treatment plant.

Compared to other Gram-negative bacteria whose outer membrane act as selective barrier to uptake of antibiotics, *Pseudomonas* species have demonstrated increased resistance to a wide variety of antibiotics, (including beta-lactams (such as Penicillin), aminoglycosides (Streptomycin) and fluoroquinolones (Ciprofloxacin)) due to the low permeability of their outer membrane (Breidenstein *et al.*, 2011). The mechanism limits the rate of penetration of antibiotic molecules into the cells due to the inefficient porins accelerating developing antibiotic resistance by *Pseudomonas* species. Plasmids, transposons and bacteriophages containing multiple resistance cassettes are easily transferred between *Pseudomonas* species (Lambert, 2002). In addition, bacterial exposure to a single antibiotic drug has been reported to increase antibiotic resistance to a majority of antibiotics, a phenomenon known as cross protection (Lambert, 2002; Mesaros *et al.*, 2007). Chopra and Roberts, (2001) and Huang *et al.*, (2012) reported that in air samples collected in their study *Pseudomonas* species represented the highest frequently detected and abundant bacteria which are intrinsically resistant towards many antibiotics.

Figure 4.9 shows the percentage of bacteria with antibiotic resistance against clinically relevant antibiotics; Trimethoprim (11%), Ampicillin (38%), Cephalosporin (55%), Penicillin (59%), Erythromycin (83%), Sulfonamide (55%), Meropenem (19%), Tetracycline (46%), Ciprofloxacin (12%) and Streptomycin (11%). Bacterial isolates showed high levels of resistance towards antibiotics that have been available therapeutically for a long time including

erythromycin, penicillin and sulfonamide, whereas resistance towards meropenem, ciprofloxacin and trimethoprim were lower.

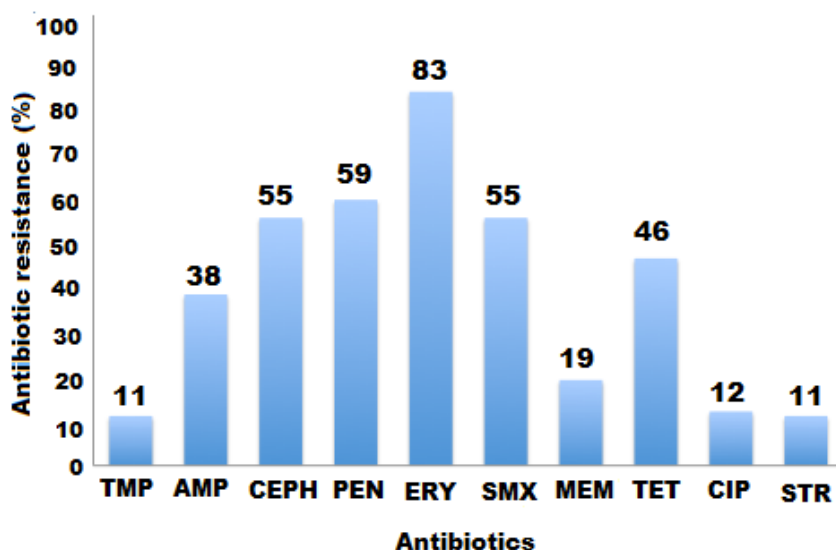


Figure 4.9. Percentage of antibiotic resistance against clinically relevant antibiotics (TRI-Trimethoprim, AMP-Ampicillin, CEP-Cephalosporin, PEN-Penicillin, ERY-Erythromycin, SUL-Sulfonamide, MEM-Meropenem, TET-Tetracycline, CIP-Ciprofloxacin and STR-Streptomycin.)

The isolated bacterial species from the clinical and non-clinical environments had the highest resistance towards erythromycin than any other antibiotic that were included in this study. The majority of airborne bacterial species that were isolated belonged to the Gram-negative bacteria. These bacterial species are naturally resistant to minimal levels of macrolides with erythromycin (Arthur And Courvalin, 1986) thus higher percentage of erythromycin resistance. According to Pratt and Fekety, (1986), intrinsic resistance towards erythromycin may be due to the chromosomal mutation or plasmid mediated, the same could be said about the findings of this study. The synthesis of erythromycin esterase by some bacterial species increases development of erythromycin resistance. The enzyme hydrolyzes the antibiotic's lactone ring whereas the bacterial species' resistance to macrolide may be as a result of decreased permeability of antibiotics through the bacterial cell envelope occurring mostly in Gram-negative and some Gram-positive bacterial species such as *Staphylococcus* (Pratt and Fekety,

1986). Airborne bacterial species isolated during this study were found to be highly resistant to penicillin. These results were as expected as the majority of the bacterial species belonged to the penicillin resistant *Pseudomonas* genera. *Pseudomonas* species have developed resistance mechanisms like pseudomonal outer-membrane impermeability and efflux mechanisms that promotes resistance to penicillins, cephalosporins and ampicillins commonly used to treat pseudomonal infections (Morita *et al.*, 2013). Mostly, bacterial species are susceptible to broad-spectrum antibiotics, Fluoroquinolone (Ciprofloxacin), Tetracycline and Carbapenem (Meropenem). The findings of this study showed low bacteria percentages of resistance to these antibiotic drugs. In Botswana it has been revealed that there is improper prescription of some antibiotics in some health care facilities leading to the rise of antibiotic resistance. According to Truong *et al.*, (2011), patients with STIs likely caused by *S. aureus* in Botswana are routinely treated with clindamycin, erythromycin, tetracycline and penicillin leading to high levels of MRSA resistance to these antibiotics.

4.5.1. Detection of antibiotic resistance genes

Among the 1201 bacterial isolates that were analyzed for the antibiotic susceptibility patterns, a total of 30 (18 bacterial strains that demonstrated resistance to all the ten antibiotics and 12 with resistance to nine antibiotics) were selected and screened for presence of antibiotic resistance genes. Antibiotic resistance genes detected in the isolates were; *dfr1* (40%), *strB* (4%), *ermC* (10%), *mph(A)* (30%). In addition, genes encoding the class 1 integron mobile gene cassette *intI1* (53%) were also detected (Figure 4.12) . Genes encoding for other antibiotic resistance, such as *tetA*, *tetB*, *qnrA*, *strA*, *ermA*, *ermB*, *sul1* and *sul2* were not detected in any of the bacterial isolates tested.

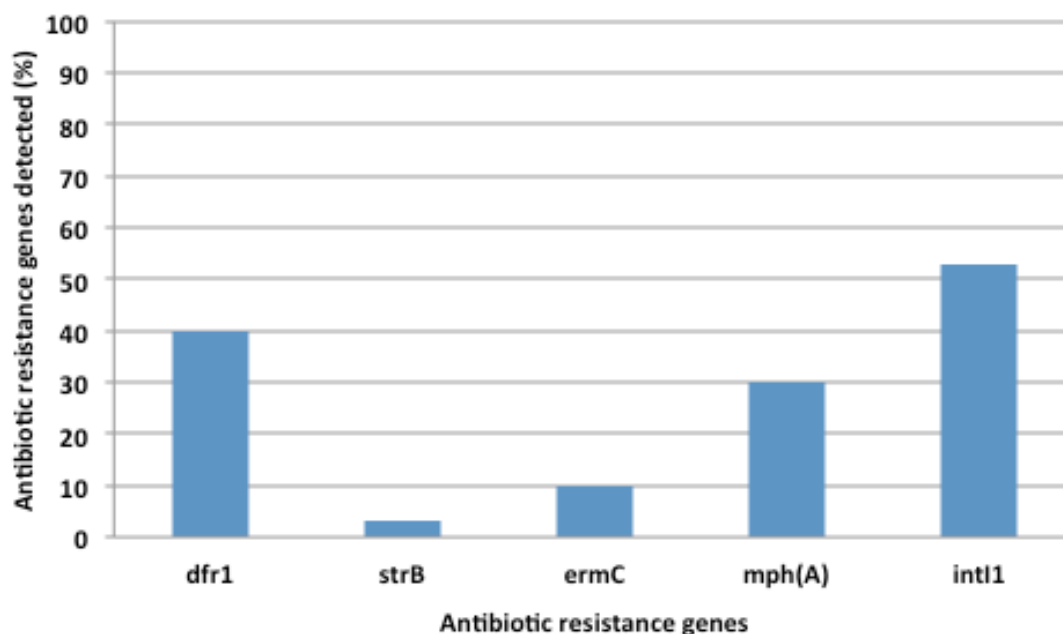


Figure 4.10. Percentage of antibiotic resistance genes detected in airborne bacterial isolates from clinical and non-clinical environments

In the current study, the presence of gene encoding macrolide 2'-phosphotransferase I, *mph(A)* gene were identified in 30% of the airborne bacterial isolates that belonged to the following bacterial species; *Pseudomonas*, *Brucella*, *Listeria* and *Staphylococcus*. The bacterial isolates from which *mph(A)* genes were isolated were from various environmental sources; Palapye WWTP upwind, Maun WWTP upwind and downwind, Gaborone WWTP downwind, Tsholofelo Extension, Nyangabwe Referral Hospital paediatric ward, Letsholathebe II Memorial Hospital operating theatre ward and the paediatric ward. The findings of this study are in agreement with those by Nakamura *et al.*, (2000) where *mph* genes were identified in *P. aeruginosa* clinical isolates. *mph(A)* genes were also detected in clinical isolates belonging to *Enterobacteriaceae* and *Staphylococcaceae* (Sutcliffe *et al.*, 1996).

Other macrolide resistance genes, *ermA* and *ermB*, that confer resistance to erythromycin were not identified in any of the bacterial isolates; however the percentage of the airborne bacterial isolates harboring the *ermC* gene was 10%. The bacterial strains with *ermC* belonged to the *Staphylococcus* and *Pseudomonas* species, isolated from Palapye WWTP upwind, Gaborone

WWTP downwind and LMHPW respectively. Phenotypically, *Pseudomonas* species isolates had high resistance to the antibiotics tested (mostly erythromycin). This study detected genes conferring resistance to macrolides in most of the *Pseudomonas* species. Similar results were found in a study conducted by Türkyilmaz *et al.*, (2010) where genes conferring resistance to macrolides like *ermC* were detected in the majority of *Pseudomonas* species.

In the sulfonamide and trimethoprim resistance gene families, *sul1* and *sul2* resistance genes were not detected from all the bacterial isolates that were screened whereas the genes that confer resistance to trimethoprim, (*dfr1*) dihydrofolate reductase was detected in most of the bacterial isolates (40%). *dfr* genes were widespread amongst bacterial isolates; these were identified from *Staphylococcus* species (isolated from LMHTW, LMHPW, public transport), *Escherichia coli* (isolated from PWWTP downwind), *Brucella* species (isolated from NRHTW, T.TEXT), *Listeria* (isolated from MWWTP downwind) and *Pseudomonas* species (isolated from PPHTW, PPHPW, GWWTP downwind). Most of these genes are detected in isolates that are related to the hospital and wastewater treatment plants downwind atmospheric air. The high prevalence of *dfr* genes in these environments is likely due to the wide application of trimethoprim in the treatment of urinary tract infections that are common in hospitalized patients. According to Jury *et al.*, (2010), *dfr* genes are mostly associated with integrons and use elaborate transfer mechanisms to laterally spread and proliferate within the bacterial community.

Class 1 integrons (*intI1*) were detected in 53.3% of the bacterial isolates, mostly the *Pseudomonas* species. Most of the bacteria species harboring the *intI1* were isolated from the clinical environment and the wastewater treatment plants. The *intI1* gene is highly associated with multiple antibiotic resistances and this gene also regulates the expression of some exogenous resistance genes such as sulfonamide resistance gene (*sul1*) that is located adjacent to class 1 integrons (Gillings *et al.*, 2015). The findings of this study is similar to those by Shin *et al.*, (2015), in which the class 1 integron carrying multigene was highly prevalent among gram-negative bacilli. A similar study by White *et al.*, (2001) revealed that integrons were significantly associated with resistance to certain antibiotics including ampicillin, tetracycline, trimethoprim, chloramphenicol, kanamycin, gentamicin, streptomycin, tobramycin, and sulfafurazole.

In the present study *tetA* and *tetB* resistance genes were not identified in any of the airborne bacterial isolates. Similarly, no *strA* resistance genes were detected in this study; *strB* resistance gene was however identified in only 1 bacterial isolate belonging to *Pseudomonas* species from the GWWTP downwind air. The *qnr* resistance gene was not detected in any of the bacterial isolates that were screened for the antibiotic resistance genes. Other authors (Pereira *et al.*, 2007) described the low frequency of detection of the *qnr* gene. Furthermore, Jacoby *et al.*, (2003) evaluated 91 bacterial isolates and from all those, identified only one isolate harboring the *qnr* gene. The *qnr* gene codes for quinolones, which are also not frequently used compared to macrolides and penicillins.

4.6.Temporal variation of airborne bacterial communities

Indoor air such as in hospital facilities, unlike outdoor air where there are no ventilation systems is not affected by atmospheric temperature and humidity. However, buildings like hospitals have air-conditioning systems that facilitate temperature and humidity regulation thus the temperature and humidity recorded in hospital facilities are independent of the season. Figure 4.11 indicates the findings of the effects of temperature and humidity on airborne bacterial concentration in operating theatre wards of the hospitals under study.

The bacterial concentration at Palapye Primary Hospital was almost similar during May 2016 (4.6×10^2 CFU/m³) and March 2017 (4.2×10^2 CFU/m³) with higher bacterial concentrations and lower in August (2.1×10^2 CFU/m³), while that of Letsholathebe II Memorial Hospital was low in May 2016 (5.5×10 CFU/m³) and April 2017 (8.8×10 CFU/m³) and high in August (6.3×10^2 CFU/m³), additionally, the bacterial concentration at Nyangabwe Referral Hospital was almost similar in June (9.9×10 CFU/m³) and August (1.6×10^2 CFU/m³) and was the highest in April (3.9×10^2 CFU/m³).

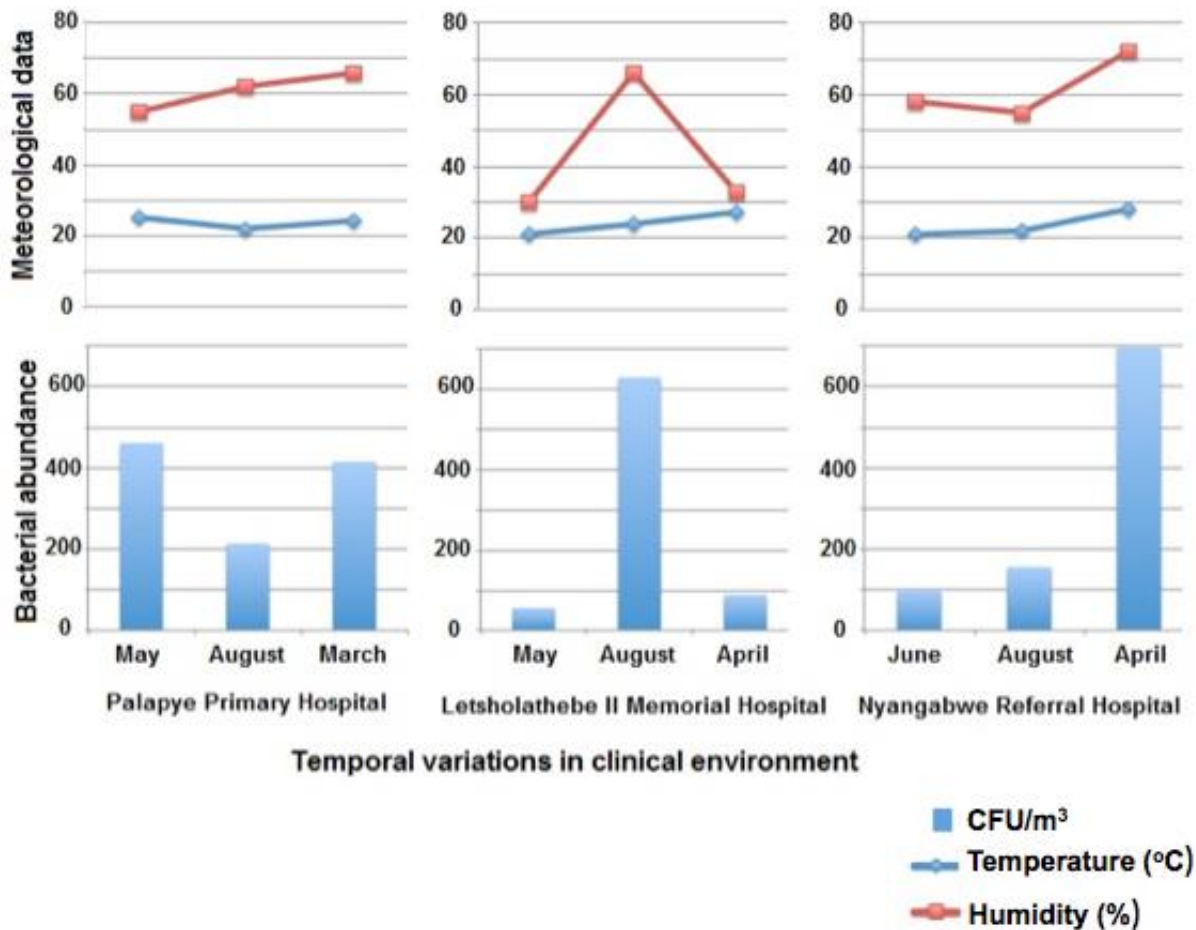


Figure 4.11. Temporal distribution of bacterial concentration isolated from hospitals' operating theatres, versus temperature and humidity. Temperature and relative humidity (RH) are reported as an average of a 1 hour 30 minutes sampling period for each sampling. During the study, temperature ranged between 9 and 29°C, RH ranged between 42 and 72%.

The results indicate that in addition to human activities, the atmospheric bacterial population is directly affected by temperature, when temperatures are low (below 22 degree Celsius) the bacterial concentration in the atmosphere is low then rises with the increase in temperatures (about 24 degree Celsius). According to Mouli *et al.*, (2009) temperature is a significant factor for airborne bacteria, which governs the rate of change of water vapor and the rate of change of heat between the surface and environment. Furthermore, temperature affects the viability of airborne bacteria through the evaporation of their cellular water. Although atmospheric humidity affects the bacterial concentrations in the atmosphere, the findings of this study

indicated that there is no distinct trend between the effects of humidity and microbial concentration.

In hospital facilities, through the use of the air conditioning systems, the temperatures are usually maintained around 24 °C. However, in instances where the air conditioning systems are malfunctional, the temperature and relative humidity in the outdoors may have an impact on the indoor air. In some occasions during this study, some of the hospitals' air conditioning systems were malfunctioning, thus affecting the desired temperature and humidity consequently affecting the microbial abundance. This suggests that the management and control of temperature, ventilation and relative humidity inside the AUC is effective in maintaining low contamination values. Guimet *et al.*, (2011) reported that air movements caused by ventilation could avoid high microbial concentrations in the air. Qudiesat *et al.*, (2009) further supports the effects of ventilation on airborne bacteria by reporting that old and poor ventilation systems might serve as potential source of airborne bacteria in hospital units. Augustowska and Dutkiewicz (2006) studied the diurnal fluctuations of the airborne bacteria levels in pneumonological department ward of a hospital over a period of a year. Their findings showed significant variation, highest airborne microbial concentration was recorded during autumn whereas the lowest was observed during winter.

Outdoor environments such as the WWTPs are highly affected by the atmospheric temperature and humidity all year round, the temperatures and humidity recorded are dependent on seasons. Figure 4.12 shows the total bacterial concentration isolated downwind air of all WWTPs under study. PWWTP downwind air had the highest total bacterial concentration in autumn (March 2017, 7.5×10^2 CFU/m³) and at the beginning of winter season (May 2016, 7.0×10^2 CFU/m³) compared to the end of the winter season (July 2016, 3.0×10^2 CFU/m³). Whereas at GWWTP downwind air, the bacterial concentration in the atmosphere was the highest in spring (August 2016, 5.5×10^2 CFU/m³) followed by autumn (March 2017, 5.2×10^2 CFU/m³) and the lowest in winter (May 2016, 3.4×10^2 CFU/m³), while at MWWTP bacterial concentration was much higher in spring (August 2016, 6.77×10^2 CFU/m³) and autumn (April 2017, 5.3×10^2 CFU/m³) and the lowest in winter (May 2016, 5.6×10 CFU/m³). The results of this study indicate that in all the three seasons, total bacterial concentration was highest during the autumn and spring.

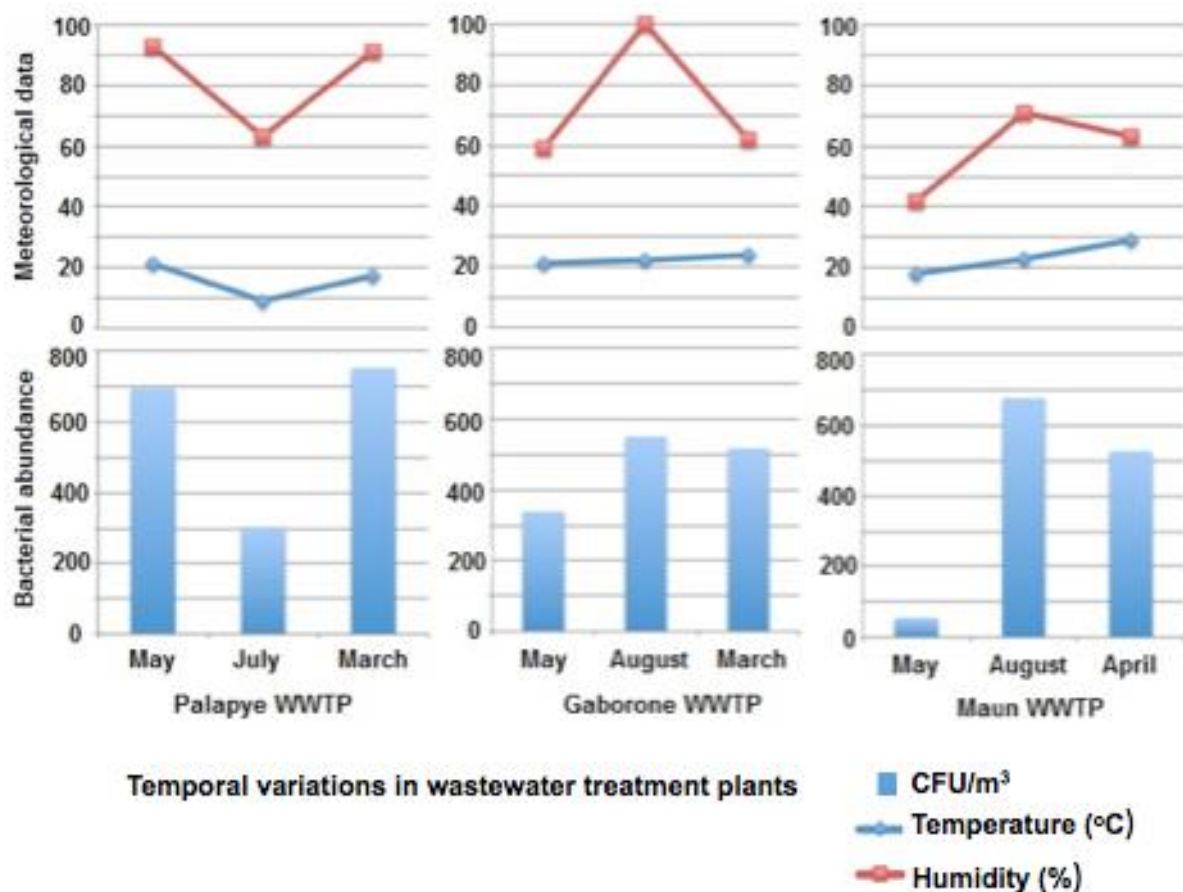


Figure 4.12. Temporal distribution of bacterial concentration isolated from WWTP downwind versus temperature and humidity. Temperature and relative humidity (RH) are reported as an average of a 1 hour 30 minutes sampling period for each sampling. During the study, temperature ranged between 9 and 29°C, RH ranged between 42 and 100%.

The analysis of the correlation of atmospheric temperature and humidity and total microbial concentration revealed that levels of microbiological air pollution depend on climatic conditions, a greater number of bacteria was observed in the months with lower temperatures (autumn, winter and spring), and high temperatures have the potential to significantly reduce bacterial concentrations in the air. This study is consistent with the study by Michałkiewicz *et al.*, (2011) in Kostrzyń WWTP premises, where bacteria isolates were recorded in abundance in autumn (3.0×10^3 CFU/m³), which made the air severely polluted at that time and lesser

airborne bacteria concentrations in spring and winter. They further explain that bacteria emitted at a particular time can cause lighter or heavier pollution, depending on the season which emphasize the effects of climatic conditions such as elevated temperature which is conducive to bioaerosols formation on airborne bacteria concentration. Additionally, a review by Korzeniewska (2011), explains that culturable bioaerosols around wastewater treatment plants have shown to exhibit both diurnal and annual cyclic patterns in relation to the meteorological conditions; maximum number of airborne bacteria at the WWTP were observed in the late afternoon and evening (Karra and Katsivella, 2007). The diurnal variability on airborne bacteria could be due to the greater turbulence as a result of mixing and the aerations during daytime resulting in longer residence time for substances emitted during the day than for those emitted at night.

Whereas annually, Gotkowska-Płachta *et al.*, (2013) and Korzeniewska *et al.*, (2008) found the largest number of airborne microorganisms at the WWTPs area during early spring. The bacterial cells are found to absorb water from the atmosphere when the relative humidity ranges between 20 and 95% thus explaining the abundance of airborne bacteria within that range. Peccia *et al.*, (2001)'s study on aerosolized bacterial species such as *B. subtilis*, *S. marcescens* and *Mycobacterium* confirmed that when they are exposed to a relative humidity exceeding 50% they tend to demonstrate increased water sorption, which protects the cells from UV-induced inactivation. According to Karra and Katsivella (2007), Korzeniewska *et al.*, (2013), Korzeniewska *et al.*, (2009) and Gotkowska-Płachta *et al.*, (2013), the dispersal of bioaerosols outside the facilities of WWTPs, as well as the type, amount and survival rate of airborne microorganisms depend on physicochemical and meteorological air conditions, landscape features, the time of the day, the season, and the type of treatment technology.

Temporal distribution of bacterial concentration isolated from bus and mini bus against temperature and humidity are demonstrated in Figure 4.13. The results reveal that the total bacterial concentration in public transport was highest during the autumn (March 2017, bus- 7.1×10^2 CFU/m³, mini bus- 4.9×10^2 CFU/m³), followed by summer (December 2016 bus- 5.3×10^2 CFU/m³, mini bus- 4.8×10^2 CFU/m³) and was the lowest in winter (June 2016, bus- 4.6×10^2 CFU/m³, mini bus- 3.3×10^2 CFU/m³). The findings of this study clearly indicate that the

abundance of airborne bacteria in public transport systems is not directly influenced by humidity and temperature.

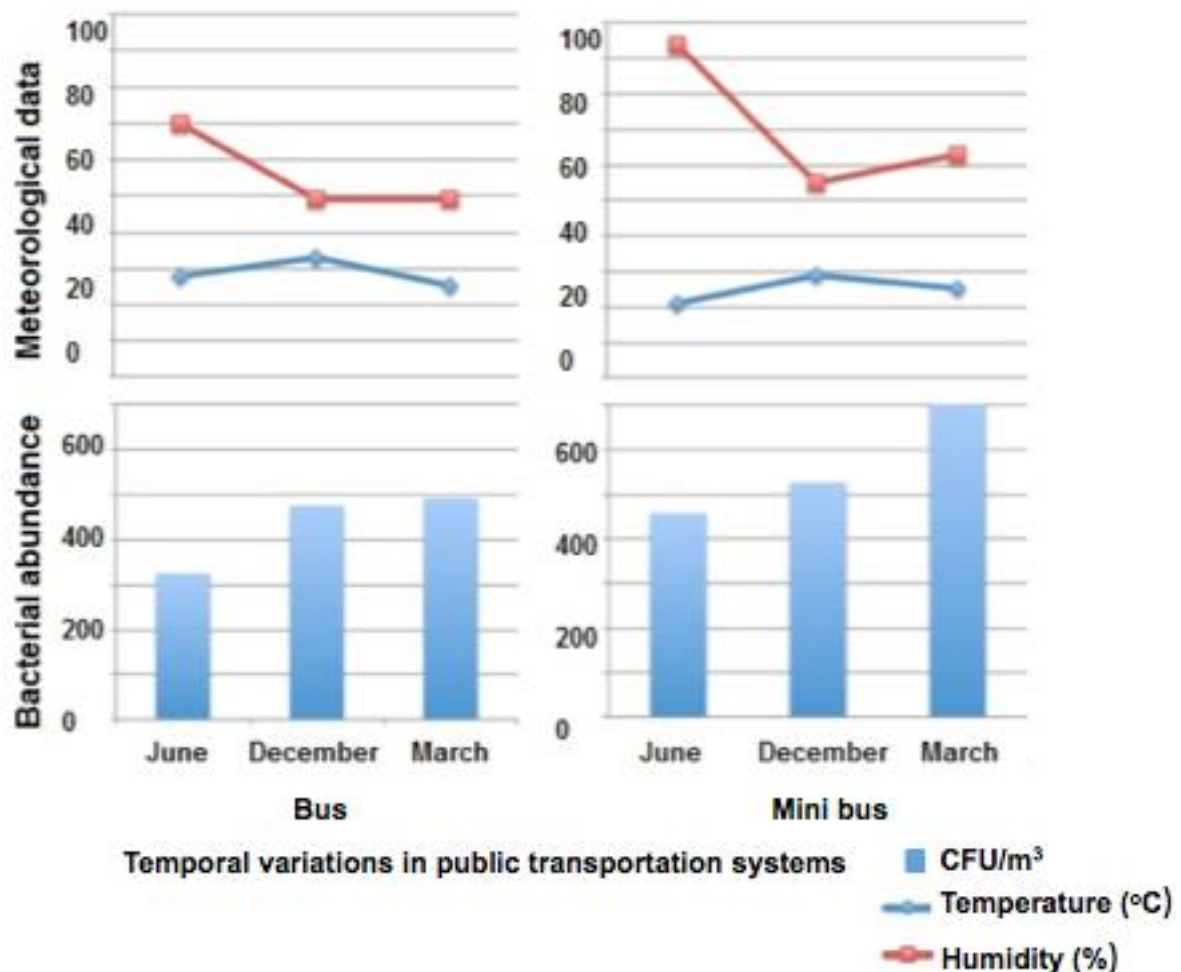


Figure 4.13. Temporal distribution of bacterial concentration isolated from bus and mini bus versus temperature and humidity. Temperature and relative humidity (RH) are reported as an average of a 1 hour 30 minutes sampling period for each sampling. During the study, temperature ranged between 19 and 33°C, RH ranged between 49 and 94%.

Although most of the airborne bacteria in public transport are emitted by indoor sources like humans, some may enter either by means of passive ventilation or by means of ventilation systems from the surrounding areas such as bus stations. However, the majority of local public transports systems such as buses, mini buses and taxis lack ventilation systems such as air

conditioners that could regulate the indoor air temperature and humidity disabling a sweeping out effect. Therefore growth conditions like excessive humidity in the transportation systems is encountered on a more frequent basis, which in most cases is the contributing factor for microbial growth. Our results are in accordance with the studies of Bowers *et al.*, (2012) which demonstrated that bacterial abundances varied by season with the highest concentrations in autumn and spring.

Although Bowers *et al.*, (2012) studied the seasonal variability in airborne bacterial communities at a high-elevation site which differs from the sites of our study, they reported that the total bacterial concentrations across all seasons ranged from 10^3 to 10^5 cells/m³ with spring and fall seasons having the highest bacterial concentrations while summer and winter had lower bacterial concentrations. Similarly, Soto *et al.*, (2009) explained that viable bacterial contamination in the air analyzed appears to reach a maximum peak during autumn and a minimal level during spring and summer, which is in agreement with the deleterious effects of high temperature and sunstroke on most bacteria.

5.0.CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Hospitals' paediatric wards indoor air, downwind air of wastewater treatment plants and settlements closer to wastewater treatment and public transport have high airborne bacterial concentration. Lower concentrations of airborne bacteria were found in operating theatre wards of the hospitals. Although these results were generally not surprising, the bacterial abundance in public transport was much higher than the expected as it almost equaled those of the downwind air of the wastewater treatment plants. In addition, public transport also has high abundance and diversity of antibiotic resistant bacteria. Therefore there is need to regularly monitor airborne bacteria in hospitals, wastewater treatment plants and other environments where human beings are at risk. The results also suggest that human beings are exposed to high levels of airborne bacteria daily. Such prolonged exposure during commuting may promote the acquisition of community-acquired and hospital-acquired infections. The airborne microbial abundance in public transport has been under-studied, not only in Botswana but the entire world. There are also relatively few published reports on the measurement of bioaerosol concentrations at public places such as bus and train stations, airports and malls in Botswana. The most frequently isolated bacterial species were *Pseudomonas*, followed by *Brucella* and *Listeria*, whereas the least isolated bacteria genera was *Salmonella* species in all the sampling sites which was expected due to the nature of most of the sampling sites as they are associated to water bodies (wastewater treatment plants as well as hand wash basins in the hospital units). The leading airborne bacterial species in the atmosphere are mostly the opportunistic human pathogens such as *P. aeruginosa* that cause appalling chronic infections and diseases. The results of this study further provide evidence that hospitals and wastewater treatment plants are hotspots for antibiotic resistance genes, which could be due to recurrent contamination with both antibiotics and resistant bacteria resulting in the increase of multidrug resistant bacteria in the air. There was a correlation between seasons and the airborne bacterial concentration, atmospheric temperature and relative humidity have an effect the airborne bacterial concentrations. The highest bacterial population was observed autumn (mostly March) and lowest in winter. The antibiotic resistant bacteria seem to be present everywhere in different

clinical and non-clinical environments. Robust monitoring system, effective control and prevention measures need to be put in place to reduce mortality and morbidity that may be associated with airborne and antibiotic resistant bacteria.

5.2 Policy recommendations

Most of the wastewater treatment facilities in Botswana are characterized by wastewater treatment techniques involving such as aeration chamber, biofilters and grit chambers that disperse bioaerosols. **Therefore** pathogenic bacteria are usually transmitted along with the wind over long distances. These bioaerosols are much likely to infect individuals through respiration, swallowing or direct contact. Therefore, the release of the bioaerosols could be reduced from WWTPs by covering grit tanks and aeration chambers. With the intensification of urbanization in towns and cities such as Gaborone, residential, industrial and other facilities are situated closer to WWTP, exposing individuals to airborne bacteria emitted by the WWTPs. Therefore policies implemented on the construction of WWTPs away from cities and towns should be considered and more regulations adapted during urbanization when the residential sites are extended to curb the new settlements near WWTPs facilities. In clinical settings, the inflow and outflow of air, which could be as a result of unmonitored movement in and out of hospital units, should be strictly monitored to minimize the pollution of indoor air. The ventilation systems in all hospital facilities should be improved to reduce the airborne bacterial concentrations. Appropriate educational intervention on routine cleaning practices and housekeeping should be provided for hospitals' sanitary personnel. The results of this study indicated that poor ventilation systems had an effect on the concentration of airborne bacteria in operating theatre rooms and paediatric wards. The same recommendation applies to implementation of the monitoring of ventilation systems and cleaning practices should be applied to public transportation systems.

5.3 Recommendations for further research

In addition to using only the culture-based techniques, direct DNA extraction from liquid samples such as that obtained through liquid impingement or filtration may be implemented for

the investigation of the bacterial diversity in air samples, as this would also allow for the assessment of non-culturable bacteria, which are neglected following culturing methods. The form of air sampling that is culture independent would enable the application of the 16S rDNA sequencing to the airborne bacterial isolates, which is highly recommended to enhance the understanding of the diversity of the airborne bacteria. The culture-independent air sampling would also enable the measurements of pro-inflammatory components such as endotoxins that may serve as markers of high concentrations of airborne Gram-negative bacteria or of particular microbial species.

The pathogenicity profiles of airborne bacterial isolates from the clinical and non-clinical environments should be determined, the presence of virulence genes in these isolates would aid in provision of an insight to the public on the potential health risks associated with exposure to virulent airborne bacteria. Moreover, this study was limited only to test few culturable bacterial species whereas there are many other airborne microorganisms including fungi and viruses. Future investigations can be performed to compare the diversity of airborne bacteria in public hospitals and private hospitals in the country, the assessment of the immediate surroundings of the hospitals' facilities under study should also be performed in identifying the source of the wards' bacterial pollution. There is also a need to explore the impacts of the dusty roads of Botswana on the airborne bacterial community.

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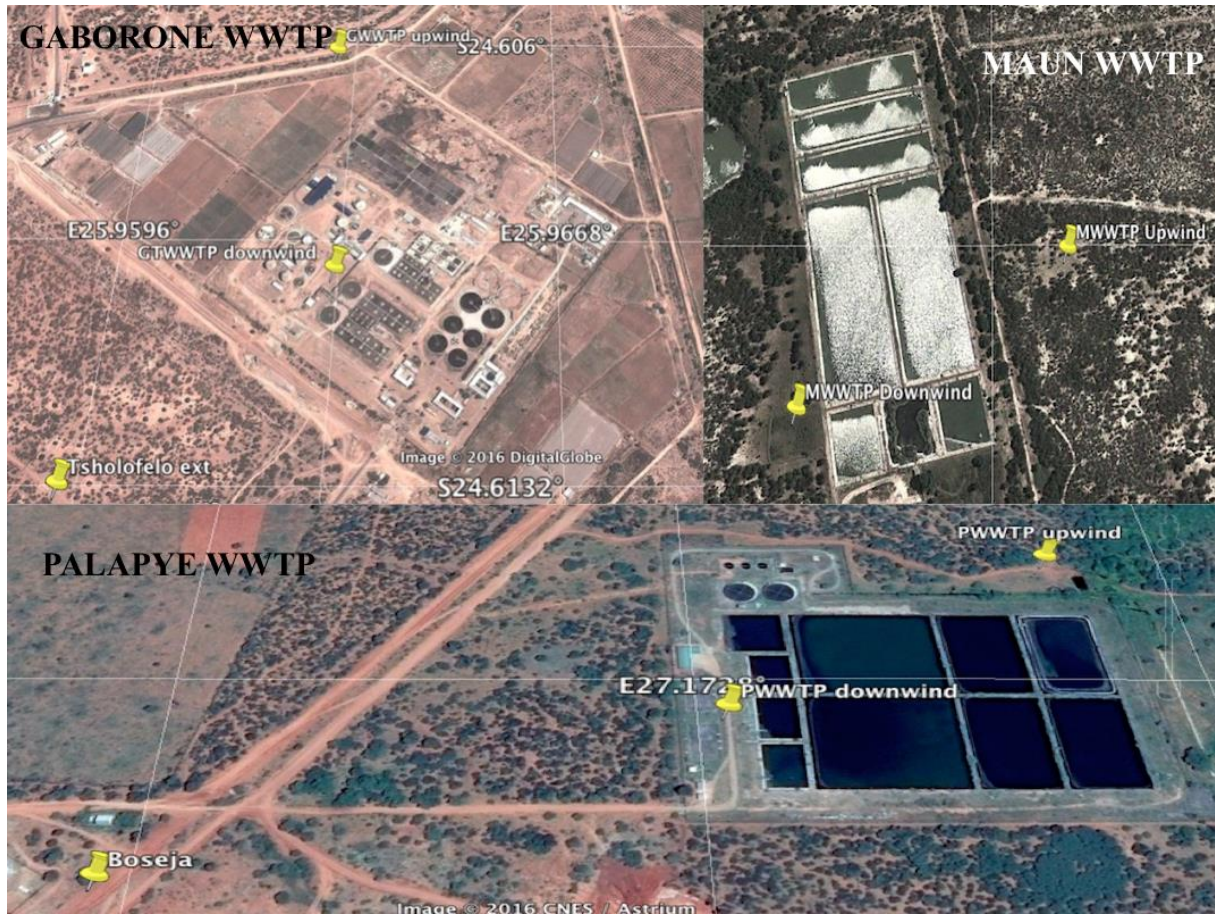
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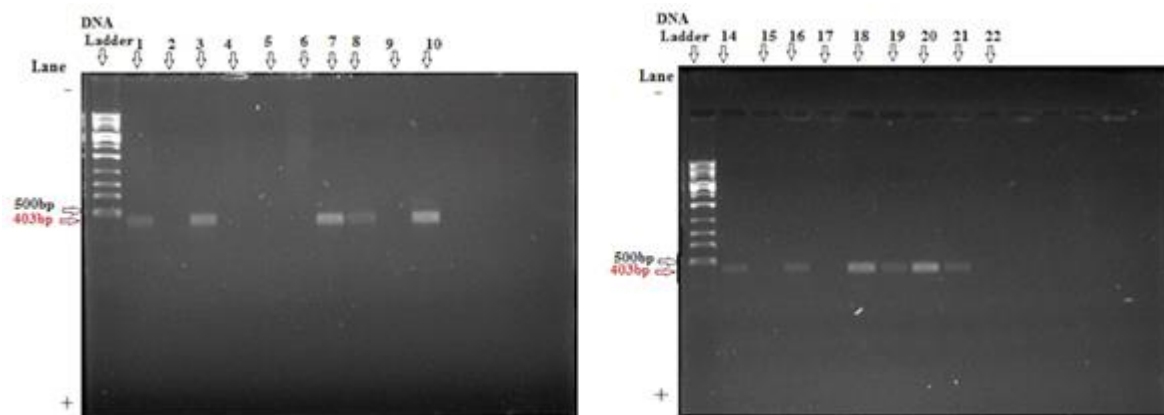
APPENDICES

Appendix A: WWTP sampling points



Gaborone, Maun and Palapye Wastewater Treatment Plants

Appendix B: Agarose Gel Electrophoresis results of the *mph*(A) Resistant gene



Agarose Gel Electrophoresis of the amplified *mph*(A) gene by PCR Assay. Line 1, Molecular weight marker; Line 2, positive control strain Line 3, negative control strain

The presence of Macrolide 2'-phosphotransferase A, *mph*(A) gene was identified in 9 of the 30 airborne bacterial isolates; *Pseudomonas* species (lane 3, 7, 10), *Listeria* species (lane 8,19), *Brucella* species (lane 14) and *Staphylococcus* species (lane 16, 18, 21). All the bacterial isolates screened for *mph* (A) gene were resistant to erythromycin, however the percentage of the airborne bacterial isolates harboring the *mph*(A) gene was 30% (Figure 4.10).