Evaluation of antibiotic-resistant bacteria and genes associated with tuberculosis treatment regimens from wastewater treatment plants in South Africa

Submitted in fulfilment of the requirements for the degree of Master of Health Sciences in Environmental Health

By

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DECLARATION

I hereby declare that the work reported in this dissertation and submitted at the Department of Community Health Studies at the Durban University of Technology for a Master's degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

23/08/2021

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DEDICATION

This work is dedicated to my family and friends for their love and support but most importantly to my better self, may this motivate you to never limit yourself. To my mother, my biggest cheerleader and supporter, you will forever live in my heart.

LIST OF PUBLICATIONS

a) Review paper

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LIST OF CONFERENCES

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Mtetwa, H.N., Amoah, I.D., Kumari, S., Bux, F and Reddy, P. (2020). Molecular detection of antibiotic-resistant genes in *Mycobacterium tuberculosis* from wastewater. **Oral presentation** at the *Water Institute of South Africa Conference and Exhibition 2020*, Virtual conference. 04 - 11 December 2020.

b) **International conference proceedings**

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ABSTRACT

Essential components of a strong public health system include an efficient surveillance system which helps in early detection and prevention of infectious diseases. This is particularly important for tuberculosis (TB) and multidrug-resistant tuberculosis (MDR-TB), due to increasing globally infections and the associated economic burdens. TB and MDR-TB infections are high in several countries, with South Africa contributing almost 3% of total infections globally. This advocates for improved surveillance systems to help health authorities respond effectively in developing effective policies for managing and controlling diseases. The reliance on clinical case reports, hospital admissions and clinical surveys, as surveillance methods, has proven to be a challenge in developing countries like South Africa, where there are other competing interests for scarce resources. The development and implementation of alternative surveillance tools for identifying disease severity, the emergence of novel strain and resistance patterns is, therefore, a top priority. One such strategy is the use of sewage or wastewater-based analysis, commonly referred to as wastewater-based epidemiology (WBE), which has received attention lately due to its role in developing early warning and surveillance of SARS-CoV-2 (COVID-19) infections. This study evaluates, method development for utilizing WBE approach for monitoring TB and MDR-TB infections via the detection and quantification of tuberculosis-causing mycobacteria and genes (ARGs) associated with resistance to TB treatment in untreated wastewater. Furthermore, the study contributes towards the understanding potential TB transmission through wastewater. To achieve these, conventional and advanced polymerase chain reaction (droplet digital PCR) assays were optimized for the detection and quantification of total mycobacteria, members of the *Mycobacterium tuberculosis* complex (MTBC) and ARGs associated with resistance to first and second-line TB drugs. The mycobacteria targeted in this study were total mycobacteria, *M. tuberculosis* complex, *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. caprae*. The ARGs (and the antibiotic they encode resistance to, in parenthesis) targeted in this study were; *katG* (isoniazid), *rpoB* (rifampicin), *embB* (ethambutol), *pncA* (pyrazinamide), *rrs* (streptomycin), *gyrA* (ofloxacin), *gryB* (moxifloxacin), *atpE* (bedaquiline), *ethR* (ethionamide), *eis* (kanamycin/amikacin). Untreated and treated (post-chlorination) wastewater samples from three wastewater treatments plants (WWTPs) in the city of Durban, South Africa were used for this study. All wastewater samples (untreated and treated) analyzed in this study contained total mycobacteria and MTBC at varying percentages per WWTP studied. The human and animal MTBC pathogens such as *M. tuberculosis*, *M. bovis* and *M. caprae* showed a similar prevalence, except for *M. africanum*, which was less common compared to the others. The highest median concentration detected in untreated wastewater was 4.9(±0.2) Log10 copies/ml for total mycobacteria, 4.0(±0.85) Log10 copies/ml for MTBC, 3.9(±0.54) Log10 copies/ml for *M. tuberculosis*, 2.7(±0.42) Log10 copies/ml for *M. africanum*, 4.0(±0.29) Log10 copies/ml for *M. bovis* and 4.5(±0.52) Log10 copies/ml for *M. caprae*. A statistically significant difference (p-value ≤ 0.05) in concentrations of each organism was observed between the plants. A significant reduction in copy numbers from untreated to treated samples were observed. However, the log reduction in each WWTP did not show any statistically significant differences when compared between the three WWTPs, irrespective of the organism or group of organisms (p-value ≥ 0.05). Furthermore, all targeted ARGS were detected in all samples analyzed at varying concentrations. The most abundant ARG in the untreated wastewater was *rrs*, associated with resistance to the aminoglycosides, specifically streptomycin. In contrast, *pncA* gene associated with resistance to the TB drug pyrazinamide was the least detected. Furthermore, the resistant gene associated with bedaquiline (*aptE*) was also detected in all samples, albeit at low concentrations. This antibiotic is a new addition to the TB treatment regimen in South Africa and it is concerning that resistance has already been detected. The

occurrence and concentration of these ARGs were lower in the treated wastewater in most instances, ranging from 1 log copy/ml to over 4 log copies/ml except for selected genes at few instances. The study makes novel major contributions, firstly, the detection of *M. tuberculosis* complex members in the untreated wastewater at high concentrations signifies a potentially high prevalence of TB in the study area. Secondly, the detection of *M. africanum* in South African wastewater also signifies that some of the TB infections in the communities could be caused by this pathogen. *M. africanum* is the main causative agent of TB in West Africa but is not frequently reported clinically in South Africa. Finally, the presence of diverse ARGs associated with TB drugs also points towards an association between the drug use and resistance profile in the area. These results further support the potential application of WBE to gather data on MDR-TB within communities with limited or no clinical data. The detection of the *aptE* gene also shows that resistance to the new drug, bedaquiline, could already be developing in the communities. The study also observed that the wastewater treatment plant configuration did not significantly influence the removal of these mycobacteria. Furthermore, selective conditions in the WWTPs may contribute to increased concentrations of ARGs during the treatment processes as indicated by increased concentrations for certain ARGs detected in the treated wastewater. This warrants further studies to determine whether the genes detected in the effluent are extracellular or carried in viable microorganisms, to assess the viability and infectivity of the microorganisms carrying these genes in the effluent samples and therefore the potential public health risks associated with the exposure to wastewater. In conclusion, this study establishes the potential of molecular surveillance of wastewater for monitoring TB and MDR-TB infections in communities and supports the use of WBE as a public health strategy to combat infectious diseases.

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LIST OF ABBREVIATIONS

ACOS	Asthma-COPD overlap syndrome
AIC	Akaike Information Criterion
AMB	Amphotericin B
AMK	Amikacin
AMR	Antimicrobial resistance
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic-resistant genes
BCG	Bacillus Calmette-Guerin
BDQ	Bedaquiline
CAP	Capreomycin
COVID-19	Disease caused by SARS-CoV-2
COPD	Chronic obstructive pulmonary disease
CPC	Cetylpyridinium chloride
CFU	Colony-forming units
DDPCR	Droplet digital PCR
DOH	Department of Health
DNA	Deoxyribonucleic acid
DR-TB	Drug-resistant tuberculosis
E	Ethambutol
Eto	Ethionamide
FLQ	Fluoroquinolones
FP	Forward primer
GI	Gastrointestinal
GITB	Gastrointestinal Tuberculosis
HAI	Hospital Acquired Infection
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HIV/AIDS	Human immunodeficiency virus/ acquired immunodeficiency syndrome
INH	Isoniazid
KAN	Kanamycin
Km	Kanamycin
LAM	Lipoarabinomannan
LOD	Limit of detection
LTBI	Latent TB infection
MDR-TB	Multidrug-resistant tuberculosis
MFX	Moxifloxacin
MGEs	Mobile genetic elements
mPTB	Microbiologically confirmed pulmonary tuberculosis
MTBC	*Mycobacterium tuberculosis* complex
MTCT	Mother-To-Child-Transmission
NAL	Nalidixic acid
NDOH	National Department of Health
OFX	Ofloxacin
NICD	National Institute for Communicable Diseases
NTM	Nontuberculous mycobacteria
PACT	Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim
PANTA	Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, Azilocillin
PCR	Polymerase chain reaction

PE	Proline–glutamic acid
PE_PGRS	Proline–glutamic acid (PE) and polymorphic GC-rich sequence
qPCR	qualitative PCR
RDs	Regions of difference
RIF	Rifampicin
RNA	Ribonucleic acid
RP	Reverse primers
RT	Reverse transcriptase
S	Streptomycin
SAMRC	South African Medical Research Council
SANDOH	South African National Department of Health
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
TB	Tuberculosis
TBE	Tris-Borate EDTA
VAN	Vancomycin
VBNC	Viable but non-culturable
WGS	Whole-genome sequencing
WHO	World Health Organization
WHO-AFRO	World Health Organization, African region
WWTPs	Wastewater treatment plants
Z	Pyrazinamide

CHAPTER ONE

1. INTRODUCTION

Tuberculosis (TB) is one of the world's largest public health challenges, mainly caused by the bacterium *Mycobacterium tuberculosis.* Approximately one-third of the world's population is infected with TB and it is the ninth leading cause of death worldwide (WHO-AFRO, 2021). The World Health Organization-African Region (WHO-AFRO) estimates that over 25% of TB deaths globally occur in Africa. This translates to over 417 000 deaths in 2016, against the 1.7 million deaths globally. The WHO also reports that only six countries account for 60% of all TB infections globally. These are Indonesia, Pakistan, India, Nigeria, China and South Africa. The First National TB Prevalence Survey of South Africa reports that the country contributes about 3% of the total TB cases globally (NDOH, 2018). It currently costs about R2500 to treat a common cause of TB and about R115000 to treat a case of MDR-TB (Copenhagen Consensus Centre, 2021). Spending on TB increased from R2.5 billion in 2014/2015 to R2.9 billion in 2016/2017, signifying an annual average increase of 8% (Ndlovu *et al*., 2019). However, there are estimates that to reach the target of reducing TB infections by 90%, additional annual spending of over R5 billion is required (Copenhagen Consensus Centre, 2021). These figures show the serious impact TB infections have on the South African economy. In addition to TB, South Africa is faced with three deadly public health threats; human immunodeficiency virus (HIV), antibiotic-resistant bacteria and drug-resistant tuberculosis (Nnadozie *et al*., 2017).

The rise in resistant infections is one factor contributing to the high costs of TB treatment. Multidrug-resistant tuberculosis infections (MDR-TB) have reached epidemic proportions around the world (Calligaro and Dheda, 2013). MDR-TB is defined as TB that does not respond to at least rifampicin (RIF) and isoniazid (INH), while extensively drug-resistant TB (XDR-TB) is defined as TB resistant to INH and RIF in addition to resistance to any of the fluoroquinolones (FLQ) and at least one of the three second-line injectable drugs: amikacin (AMK), capreomycin (CAP) or kanamycin (KAN) (Zhao *et al*., 2014). The prevalence of MDR-TB is of particular concern on the African continent, given its common co-morbidity with HIV (WHO, 2018a). South Africa has the 3rd highest prevalence of drug-resistant tuberculosis (DR-TB) patients globally (Cox *et al*., 2017), with an estimated 322,000 cases of active TB and HIV in 2017 and 9.6% of these cases were multi-Drug-resistant (MDR TB) (WHO, 2018a). Increased mortality in patients with MDR-TB, especially in HIV-positive patients, is particularly high in the KwaZulu-Natal province (Maharaj *et al*., 2016). Factors

contributing to increased prevalence of MDR-TB include difficulty in accessing laboratory results, late updating of records, lack of unique patient identifiers, HIV co-infection, lack of access to second-line treatment provision in primary care, poverty, high early mortality particularly among HIV-positive patients, poor treatment compliance and under-reporting (Cox *et al*., 2017). According to the WHO Global Tuberculosis report released in 2018, a relatively small proportion (5–10%) of the estimated 1.7 billion people infected with *M. tuberculosis* will develop TB disease during their lifetime. Still, the likelihood of developing TB disease is much higher among people infected with HIV and much higher among people affected by risk factors such as smoking, undernutrition, alcohol consumption and diabetes (WHO, 2018a).

Although MDR-TB represents only 7% of incident TB in South Africa, high drug prices, lengthy treatment and hospitalization lead to exorbitant costs. For instance, in 2014, approximately 65% of the National Tuberculosis Program budget was spent on treating and controlling MDR-TB (Loveday *et al*., 2018). This causes a significant diversion of valuable healthcare resources that could be well spent within the health system. AMR, particularly within the context of TB treatment, is, therefore, a priority. South African TB treatment guidelines have been updated in response to the WHO recommendation of standardized shorter MDR-TB regimens with seven drugs (kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, isoniazid and ethambutol) and a treatment duration of 9-12 months for countries with a great burden of MDR-TB (Caminero *et al*., 2017). These figures on general TB infections and MDR-TB show the serious public health concerns associated with TB.

Surveillance of TB infections has generally relied on clinical-based surveillance, hospital admission data, questionnaires, surveys, motility and morbidity rates and sentinel surveillance (WHO, 2020). However, there are several challenges associated with these approaches, which may be a factor in the continuous health challenges posed by the infections. Some of these challenges include resource constraints, high costs involved in maintaining these surveillance tools and potential reporting biases. This calls for alternative means of monitoring TB infections within communities. One such alternative approach is the detection of the infectious agents in untreated sewage/wastewater from a community, which will indicate the circulation of such agents in the community. This approach referred to as wastewater-based epidemiology (WBE), has gained prominence lately especially during the COVID-19 pandemic. However, before COVID-19, WBE has been applied for monitoring pharmaceutical consumption (Baz-Lomba *et al*., 2016; He, 2020), viral infections (Hou *et al*., 2020), cocaine consumption (Zuccato *et al*., 2005; Mao *et al*., 2020; Tang *et al*., 2020) and for polio surveillance (Nakamura

et al., 2015). Therefore, a similar approach could be adopted for the surveillance of TB infections in connected communities. Furthermore, WBE was also used for antibiotic resistance surveillance (Hutinel *et al*., 2019; Castrignanò *et al*., 2020). Therefore, this approach can be further developed for monitoring MDR-TB infections.

Additionally, the detection of the causative agent for TB in treated wastewater could provide an insight into the possible risks of TB infections through the environmental route. The environmental occurrence of pathogenic mycobacteria has received less attention in comparison to its occurrence in clinical settings. Nevertheless, there is an increasing body of evidence to indicate that water could be an important vehicle for the transmission of these organisms (Dufour, 2004; WHO, 2011; Sims and Kasprzyk-Hordern, 2020). Previous studies revealed that environmental contamination, from faecal shedding, provided the potential and indirect routes for transmission of *M. bovis* infection (Travis *et al*., 2019; Wu *et al*., 2020). The shedding of *M. bovis* cells has already been demonstrated in many animals via oro- nasal mucus, sputum, urine, feaces and wound discharges (Corner *et al*., 2012; Barasona *et al*., 2015; Barbier *et al*., 2017; Vayr *et al*., 2018). The challenge in investigating this type of indirect transmission results from the following important factors i.e., i) shedding of the cells from infected animals into the environment, ii) the persistence of viable microorganisms in various environmental matrices and finally iii) the contact between a new vulnerable host with the contaminated matrices (Barbier *et al*., 2017). This route of transmission has been implicated most frequently in zoonotic infections than human-to-human infections (Travis *et al*., 2019). Velayati *et al*. (2015) isolated *Mycobacteria* from 568 of 1,500 soil and water samples (37.8%) from the Tehran metropolitan area, with *M. tuberculosis* isolated from only 82 soil and water samples (5%). Three of the isolated *M. tuberculosis* strains (3.6%) were MDR-TB. Isolation of *M. tuberculosis* from water and soil raises the possible risk of infection from the environment, especially where soil and water samples remained culturable for at least 6 months after sampling. This highlights the potential risks from exposure to *M. tuberculosis* in the environment, potentially leading to TB and MDR-TB infections.

Wastewater serves as a link between environment and human activities and could be the first medium that may be contaminated with MTBC via faecal shedding. However, studies on the occurrence of MTBC in different environmental matrices has not received priority, therefore there's a lack of proper detection techniques for MTBC in the environment. The study of *M. tuberculosis* in wastewater could potentially help address the challenges with TB and MDR-

TB surveillance and contribute towards understanding the possible role of the environment, specifically wastewater, in the transmission of TB and MDR-TB.

1.1. Rationale

The purpose of this study was therefore to determine the presence and quantity of tuberculosis-causing *Mycobacterium* spp. and associated antibiotic-resistant genes (ARGs) from selected wastewater treatment plants in South Africa. This is an experimental study involving wastewater sample collection (both untreated and treated), screening and quantitative identification of *M. tuberculosis* complex species and associated ARGs through molecular-based methods. Data generated from this project will serve two main purposes. Firstly, it will help to further develop sewage or wastewater-based surveillance tools for monitoring TB infections via detection and quantification of tuberculosis-causing *Mycobacterium* spp. in untreated sewage. The prevalence of these tuberculosis-causing microorganisms in the untreated sewage may provide vital information in estimating not only the occurrence but also resistance in the associated population without clinical data on TB and its antibiotic resistance pattern. This information will also help in the development of sewage analysis as a surveillance tool to ascertain antibiotic use and prevalence of drug resistance TB in settings where data is lacking and for use as an early warning system for detection of the emergence of antibiotic resistance related to *M. tuberculosis.* The study, therefore, contributes to the expansion of wastewater-based epidemiology to cover TB and antibiotic-resistant TB infections.

Secondly, the prevalence of these bacteria and genes in the final treated effluents will provide an understanding of the removal efficacy of these organisms by different wastewater treatment processes and contribute to risk reduction measures associated with the exposure to wastewater and surface water contaminated with wastewater. The inefficient removal by wastewater treatment processes may significantly increase antibiotic-resistant bacteria and antibiotic resistance genes associated with TB in the aquatic environment.

1.2. Aims

Aim: To determine the occurrence and concentration of tuberculosis-causing *Mycobacterium* spp. and antibiotic-resistant genes (ARGs) associated with tuberculosis (TB) treatment regimens in WWTPs in South Africa

1.3. Objectives

1. To determine the prevalence and concentration of *Mycobacterium tuberculosis* complex (MTBC) in untreated and treated wastewater.

This objective has been split into two sub-objectives based, these are;

a) Detection of *Mycobacterium tuberculosis* complex species in wastewater through conventional PCR
b) Quantification of *Mycobacterium tuberculosis* complex species in wastewater through droplet digital PCR

2. To determine the prevalence and concentration of tuberculosis-drug-resistant genes (ARGs) in untreated and treated wastewater.

Objective two was also further split into two sub-objectives for the purposes of clarity. These sub-objectives are;

a) Detection of ARGs associated with tuberculosis resistance in wastewater through conventional PCR

b) Quantification of ARGs associated with tuberculosis resistance in wastewater through droplet digital PCR

1.4. Thesis structure

The thesis is presented in five (5) main chapters. **Chapter one** presented the background, rationale and objectives of the study. The literature review is captured in **Chapter two**, part of this sections presented in this chapter were submitted as a review paper and is under peer review at the time of drafting this thesis. Details of the paper and sections covered are captured in the relevant section of the thesis. **Chapter three** addresses objective 1, which focused on the detection and quantification of tuberculosis-causing *Mycobacterium* spp. in wastewater. It has a brief introduction, a detailed methodology, results and discussion. This chapter has also been submitted as a paper and details of the submission are provided at the beginning of the chapter. **Chapter four** has a similar format to Chapter three, however, it focused on objective 2. Therefore, a brief introduction, detailed methodology, results and discussion is presented for the detection and quantification of antibiotic-resistant genes associated with TB. This chapter has also been submitted as a paper for publication and the details of the submission are presented at the beginning of the chapter. A summary of the main discussion points from each objective is presented in **Chapter five**. This chapter also provides the main conclusions drawn from each objective in relation to the main aim of the study.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Tuberculosis (TB)

Tuberculosis (TB) is a communicable disease that is one of the leading causes of death globally (ranking above HIV/AIDS) (WHO, 2019). It is an airborne disease caused by a group of closely linked, slowly growing mycobacteria collectively termed the *Mycobacterium tuberculosis* complex (MTBC), which infect a large range of mammals, including humans (Forbes *et al*., 2018). The group consists of *Mycobacterium tuberculosis* and other seven very interconnected mycobacterial species, *M. africanum, M. caprae, M. microti, M. canetti, M. bovis, M. pinnipedii* and *M. mungi* (Wanger *et al*., 2018). TB transmission occurs when infected people expel the bacteria into the air; for example, through coughing. Infection happens when an individual (or animal) inhales 1- to 5-m droplet nuclei containing tubercle bacilli that spread to the alveoli of the lungs (Nardell, 2016).

Subsequent to the exposure, either the *M. tuberculosis* complex can be destroyed by the host's immune system or active TB disease arises in different areas of the body such as lungs, brain, regional lymph nodes, larynx, bone and kidneys; or a latent TB infection (LTBI) may be established (Forbes *et al*., 2018). It typically affects the lungs (pulmonary TB) but can also affect other sites (extrapulmonary TB) (WHO, 2016). Latency refers to the state of chronic infection without symptoms of pulmonary TB and clinical signs (Lin and Flynn, 2010). LTBI is noteworthy because these patients could serve as the main reservoir of the *M. tuberculosis* complex in the population for a longer period without showing symptoms and later these patients can develop active pulmonary TB if they become immunosuppressed (Forbes *et al*., 2018). Individuals infected with *M. tuberculosis* have a 10% lifetime risk of developing active TB (WHO, 2019). However, people with compromised immune systems, such as people living with diabetes, malnutrition, or HIV, have a greater risk of progression from LTBI to active disease (Narasimhan *et al*., 2013). For example, individuals living with HIV and infected with *M. tuberculosis* have a 7 to 10% annual risk of developing active TB disease (Holmes *et al*., 2017). Tuberculosis also affects animals where the transmission can either be from human to animal and vice versa and this type of tuberculosis common in animals is known as bovine tuberculosis (Gormley and Corner, 2018).

2.1.1. Taxonomy and cell morphology of the genus *Mycobacterium*

The genus *Mycobacterium* comprises over 190 species and belongs to the family of *Mycobacteriaceae*, class *Corynobacteriales*, type *Actinobacteria*, and kingdom *Bacteria*. *Mycobacterium* spp. have mycolic acids in their cell wall and share this characteristic with bacteria of other genera such as *Tsukamurella, Gordonia*, *Rhodococcus*, and *Nocardia* (Tortoli, 2019). The genus *Mycobacterium* consists of a group of species of which, *M. tuberculosis* and *M. leprae* and *M. africanum* are considered to be obligate human pathogens (Agoro and Mura, 2019), whereas most of the others are opportunistic organisms that cause disease in humans and animal receptors when conditions are optimal. This genus is generally classified into two distinctive groups, related genetically, *M. tuberculosis* complex (MTBC) organisms and nontuberculous mycobacteria (NTM) (Figure 2.1). The latter, also known as environmental mycobacteria because of their ubiquitous presence in soil and water (Tortoli, 2014). In the laboratory, NTM is categorized into slow and fast growers, mainly as a result of their growth rate which usually takes 7–10 days and >14 days to reach maturity for rapid and slow growers, respectively (Gharbi *et al*., 2019; Gao *et al*., 2018).

This characteristic allows these mycobacteria to be distinguished from other bacteria based on staining techniques since the high mycolic acid content in the cell wall makes organisms resistant to decolourization with acid alcohol (i.e., “acid fast”), they are considered as gram neutral due to their difficulty when stained (Pfyffer, 2015; Safaei *et al*., 2018; Forbes *et al*., 2018). *Mycobacterium* spp. are aerobic, acid-fast bacilli (AFB) and non-spore-forming. They are non-motile, and most of them are straight or slightly curved rods, with only a small number of species exhibiting some branching (Pfyffer and Vincent, 2010; Forbes *et al*., 2018).

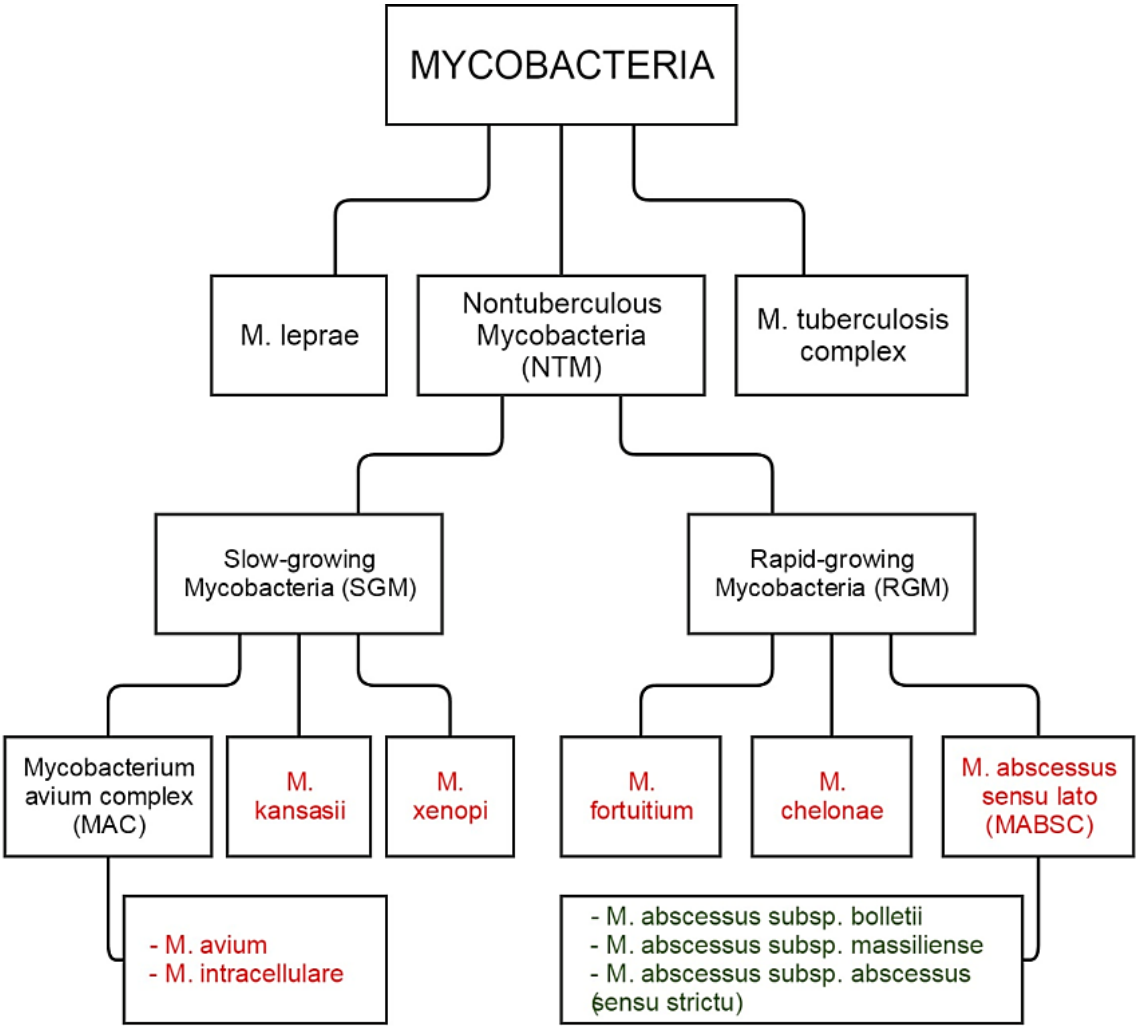

Figure 2.1: Classification of mycobacteria genus (Cauchie, 2016).

Mycobacteria have a polysaccharide cell wall that is similar to that of gram-positive bacteria. The mycobacterial peptidoglycan, on the other hand, is made up of lipids rather than proteins and polysaccharides (Macedo, 2019) (Figure 2.2). Furthermore, except for the inclusion of lipoarabinomannan (LAM), lipomannan, and phosphatidylinositol mannosides, the mycobacterial envelope comprises a plasma membrane that is structurally and functionally comparable to that of other bacteria (Figure 2.2). Overall, the cell wall component of the envelope confers size, shape, osmotic pressure resistance, and likely protects the plasma membrane from detrimental molecules in the environment (Alderwick *et al*., 2015; van Ingen, 2017). When cultivated, certain species develop a yellow or orange pigment that may be constitutive (i.e., scotochromogenic) or triggered exclusively by light (i.e., photochromogenic), while others never create pigment (i.e., non-photochromogenic) (Saviola and Felton, 2011). Mycobacteria grow slowly in comparison to most other bacteria, requiring at least 5 days of i ncubation, with many requiring one or more weeks (Forbes *et al*., 2018).

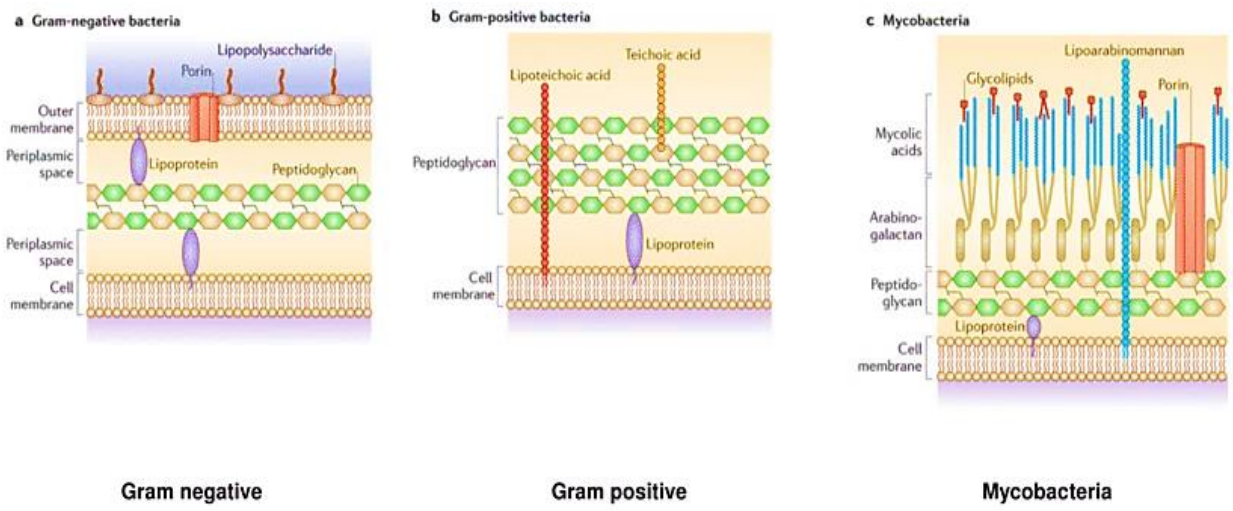

Figure 2.2: Typical bacterial cell walls showings the cell wall of Gram-negative bacteria (a), Gram-positive bacteria (b) and Cell walls of mycobacteria (c) (Alderwick *et al*., 2015).

The *Mycobacterium* genus includes strict pathogens, potential or opportunistic pathogens, and non-pathogenic, saprophytic species (Rahman *et al*., 2014). The similarities of the gene sequences within the genus and the phylogenetic reconstructions confirm the natural division between slowly and rapidly growing mycobacteria, which has been achieved using linked series of sequences of housekeeping genes. This demonstrated that all slowly growing mycobacteria belong to a single evolutionary branch that emerged from the rapidly growing mycobacteria (Somoskovi and Salfinger, 2014; Forbes *et al*., 2018). This feature is essentially linked to their pathogenic ability to infect humans, and therefore, all strict pathogens and most opportunistic pathogens belong to the evolutionary branch of slowly growing mycobacteria (Fedrizzi *et al*., 2017; Somoskovi and Salfinger, 2014).

M. africanum lineages are considered phylogenetically older than *M. tuberculosis* current lineages within the *M. tuberculosis* complex lineages that primarily infect humans (Euro-American, East African Indian, and East Asian) (Sharma *et al*., 2016). *M. africanum* is thought to be indigenous to equatorial Africa, with specimens found in Nigeria, Côte d'Ivoire, Benin, Senegal, Cameroon, Burkina Faso, Gambia, Sierra Leone, and Uganda (Gehre *et al*., 2016; Sharma *et al*., 2016). Patients with tuberculosis in Europe, Brazil, and the United States have also been found to have *M. africanum* (Sharma *et al*., 2016). Human migration from disease-endemic areas in equatorial Africa is likely to attribute to *M. africanum*-caused tuberculosis in non-African countries.

It has been proposed that MTBC members developed from a common ancestor by a series of deoxyribonucleic acid (DNA) deletions and insertions, resulting in the current *Mycobacterium*

speciation and pathogenicity differences (Forrellad *et al*., 2013; Tientcheu *et al*., 2017). These studies relied heavily on genomic analysis, which helped to identify 14 distinct regions (designated as RD1–14) detailed in Figure 2.3. These regions, which are present in the reference laboratory strain *M. tuberculosis* H37Rv, are lacking from the vaccine strain *M. bovis* variant bacille Calmette-Guerin (BCG), allowing researchers to discover pathogenicity-related chromosomal genes. In contrast to other members, the *M. tuberculosis* H37Rv genome lacks six areas designated as H37Rv deletion 1 to 5 (RvD1–5) and *M. tuberculosis* specific deletion 1 (TbD1). *M. canettii*, on the contrary, has all of the RD, RvD, and TbD1 sections and is considered to be the most closely linked genome to the bacilli's ancestor. *M. africanum* strains isolated primarily from West Africa lack the RD9 region, whereas those isolated from East Africa have it but lack the RD3. *M. microti* lacks a distinct region known as RDmic, as well as the regions RD7, RD8, RD9, and RD10. Some vole-isolated strains also omitted a portion of the RD5 region. The most common *M. bovis* strains, known as "classical *M. bovis*," were isolated from bovines in the Netherlands, Spain, the United Kingdom and Argentina, as well as from humans, and had the most RD deletions, lacking regions RD4, RD5, RD6, RD7, RD8, RD9, RD10, RD12, and RD13. *M. caprae* is fairly related to *M. bovis*, however, it has a few nucleotide changes in the gyrB gene that are not found in other MTBC members. Furthermore, during and after the attenuation process, *M. bovis* var BCG lacked the areas RD1, RD2, and RD14 (Forrellad *et al*., 2013).

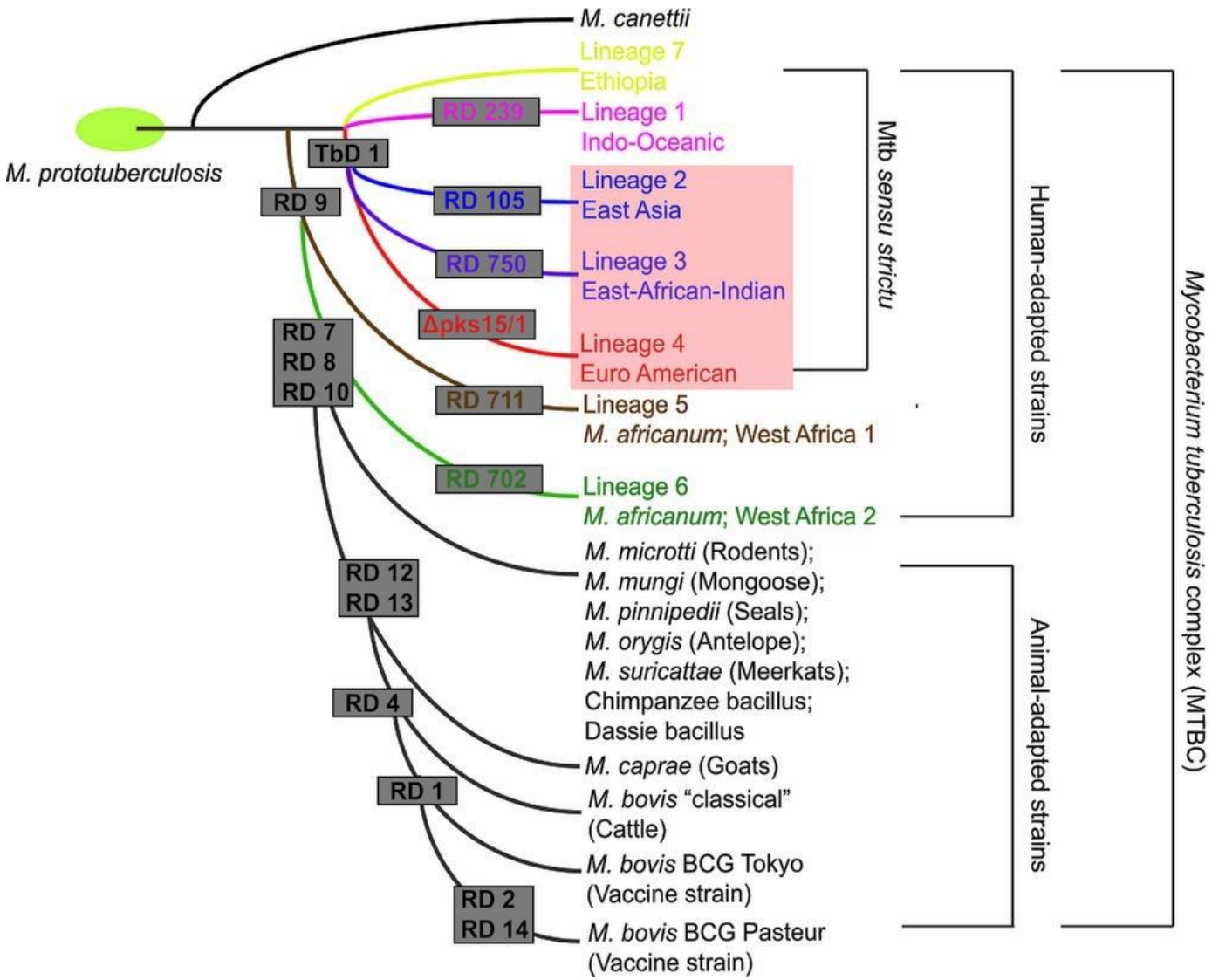

Figure 2.3: Phylogeny of the MTBC and distribution of the 7 main *M. tuberculosis* complex lineages according to the region of differences used to identify members of *M. tuberculosis* complex via molecular identification techniques (Tientcheu *et al*., 2017)

2.1.2. Transmission and pathogenesis of *Mycobacterium tuberculosis* complex

The pathogenicity of *M. tuberculosis* is primarily based on (i) the bacilli's ability to reprogramme host macrophages after primary infection, preventing its elimination; (ii) the formation of granulomas, in which the pathogen survives in equilibrium with the host defence; and (iii) the slowing control of bacterial central metabolism and replication, characterizing the so-called dormant state (Miggiano *et al*., 2020). Figure 2.4 shows the pathogenesis/life cycle of the *M. tuberculosis* of infection in a human host.

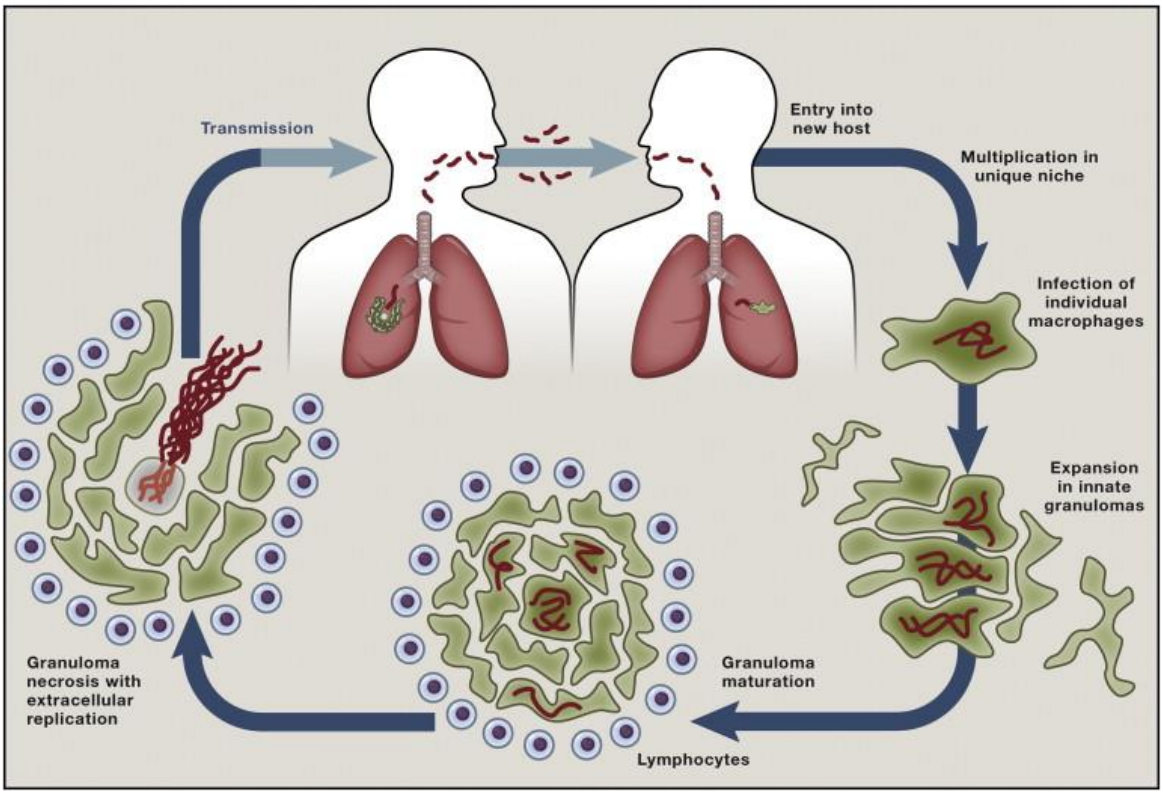

Figure 2.4: Diagram showing the pathogenesis of tuberculosis (Cambier *et al*., 2014)

M. tuberculosis is transmitted in 1–5 micron-sized airborne particles known as droplet nuclei. When people with pulmonary or laryngeal tuberculosis cough, shout, sneeze or sing, infectious droplet nuclei are released (CDC, 2013; Patterson *et al*., 2017). Depending on the environment, these tiny particles can remain suspended in the air for several hours (Sgaragli and Frosini, 2016). Four factors determine the probability of transmission of *M. tuberculosis*, these are; (1) susceptibility (immune status) of the exposed individual, (2) number of infectious bacilli expelled in the air, infectiousness is directly related to the number of bacilli exposed to, (3) environmental factors that affect the concentration of *M. tuberculosis* organisms and (4) exposure (proximity, frequency, and duration of exposure) (Turner *et al*., 2017).

Previous studies revealed that environmental contamination, from faecal shedding, provided the potential and indirect routes for transmission of *M. bovis / M. tuberculosis* infection (Figure 2.5) (Travis *et al*., 2019; Wu *et al*., 2020). *M. bovis* cell shedding has previously been demonstrated in many animals via oro-nasal mucus, sputum, urine, feces, and wound discharges (Barasona *et al*., 2015).

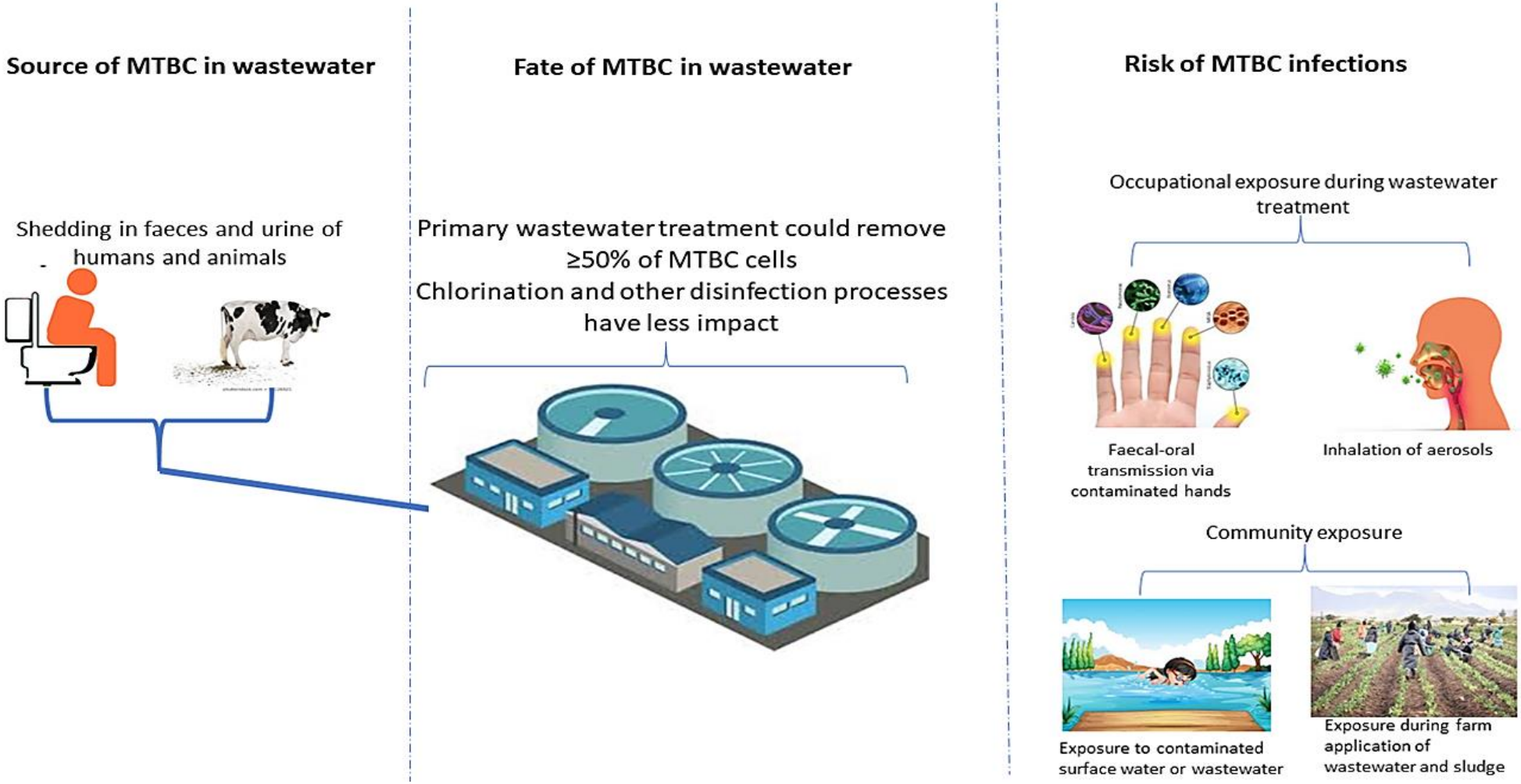

Figure 2.5: The potential sources, fate and possible risks of MTBC transmission in wastewater

2.1.3. Virulence factors of *Mycobacterium tuberculosis* complex

Mycobacteria's ability to cause disease is dependent on a variety of mechanisms that enable colonization, replication, and survival in their host; thus, mycobacterial virulence factors are typically defined as bacterial genes or cellular components that enable their overall survival in the host (Ly and Liu, 2020). In most cases, the infection is contained in the lungs by the formation of granulomas, which are formed when activated macrophages and other immune cells surround the infection site to limit tissue damage and mycobacterial spread. MTBC species that are particularly virulent have developed strategies to avoid or modulate the immune response in their favour. Some bacteria in the granuloma can remain dormant for decades without causing any symptoms (latent tuberculosis). Nonetheless, the dormant bacteria can become active, replicate, and spread into the lungs and other tissues in any immune-depressing condition (Forrellad *et al*., 2013).

Based on their function, molecular features, or cellular localization, the virulence determinants have been divided into the following categories: (1) Lipid and fatty acid metabolism, including cholesterol catabolism, (2) cell envelope proteins, such as cell wall proteins, lipoproteins, and secretion systems, (3) proteins inhibiting macrophage antimicrobial effectors, such as those involved in oxidative and nitrosative stress responses, phagosome arrest, and apoptosis inhibition, (4) protein kinases, (5) proteases, including metalloproteases, (6) metal-transporter proteins (importer and exporter proteins), (7) gene expression regulators, such as two-component systems, sigma factors, and other transcriptional regulators, (8) proteins with unknown functions, such as the PE and PE PGRS families, and (9) other virulence proteins (Forrellad *et al*., 2013; Yu *et al*., 2019). These are primarily involved in MTBC species' interactions with host macrophages. In pathogenic mycobacteria, more than a hundred potential virulence genes have been identified (Forrellad *et al*., 2013).

2.1.4. Treatment protocols

The treatment of drug-susceptible and drug-resistant tuberculosis has been classified according to the first and second-line treatment drugs classified in detail in Table S3 shown in appendices. The first-line treatment drugs are used mainly for drug susceptible TB while the second-line treatment drugs are used for drug-resistant TB (MDR-TB, RR-TB, and XDR-TB). In SA, TB treatment regimens are based on the WHO recommendations for the management of patients with TB disease. Therefore, the management of TB is largely dictated by guidelines as opposed to individualized treatment based on susceptibility patterns. Current recommendations for the

treatment of drug-susceptible TB include a 6-month course of rifampicin, isoniazid, pyrazinamide, and ethambutol (Cohen *et al*., 2019).

2.1.4.1. National guidance for drug-resistant TB treatment in South Africa

Based on the patient's previous TB treatment history, resistance can be classified into three categories: a) Primary resistance: a patient has been infected with an already resistant strain of *Mycobacterium tuberculosis* and has not received any TB treatment (Moodley, 2013; Zhang and Yew, 2015). b) De Novo/Acquired resistance: a patient is taking anti-TB medication or has had one or more previous TB treatment episodes lasting longer than one month. The resistant isolate is thought to have developed resistance as a result of mutations and had a selective advantage as a result of the treatment episodes (Moodley, 2013; Zhang and Yew, 2015). c) Initial resistance: because the patient's history of previous anti-TB treatment is unknown, the patient may have a combination of primary and undisclosed acquired resistance (Moodley, 2013; Zhang and Yew, 2015).

MTB resistance may be classified based on drug susceptibility patterns.

a. Mono-resistant TB: TB caused by *M. tuberculosis* that is resistant to one of the FLDs (Table 2.1) (WHO, 2013)

b. Multi-drug-resistant TB (MDR-TB): TB caused by MTB that is resistant to the two most powerful FLDs, INH and RIF (Moodley, 2013; WHO, 2013).

c. Pre-extensively drug-resistant TB (pre-XDR-TB): TB caused by an MDR MTB strain with additional resistance to either fluoroquinolones or a second-line injectable drug (i.e. CAP, KAN or AMIK), but not both (Moodley, 2013; SANDOH, 2011).

d. Extensively-drug-resistant TB (XDR-TB): TB caused by an MDR MTB strain with additional resistance to one of the fluoroquinolones and any of the second-line injectable drugs (CAP, KAN, AMIK) (Moodley, 2013; WHO, 2013).

e. Totally drug-resistant TB (TDR-TB): TB caused by an XDR-TB strain with additional resistance to 5 other drugs. TDR-TB strains are therefore resistant to a total of 9 different drugs (Moodley, 2013).

Drug susceptible and drug resistance tuberculosis regimens are all designed around the anti-TB drugs in both first-line and second-line drugs (Table 2.1). These regimens differ with time and also the doses varies.

Table 2.1: Classification of TB/MDR-TB drugs and the genes coding for tuberculosis resistance.

Treatment regimen	Drug	Chemical Class	Genes	Reference
First-line drugs	Isoniazid	Pyridine	*katG, inhA*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Rifampicin	Rifamycin	*rpoB*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Ethambutol	Ethylenediamine	*embB, ubiA*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Pyrazinamide	Pyrazine	*pncA, rpsA, panD*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
Second-line drugs	Streptomycin	Aminoglycoside	*rps, rrs, gidB*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Bedaquiline	Diarylquinoline	*atpE, Rv0678*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Delamanid	Nitroimidazole	*fdg1, ddn*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Kanamycin	Aminoglycoside	*rrs, eis, whiB7*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Ethionamide	Pyridine (thionamide)	*etaA /ethaA, ethR, inhA*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Cycloserine	D-Alanine analogue	*alr, dd1, cycA*	Koch *et al*. (2018), Zhang and Yew, 2015
	Amikacin	Aminoglycoside	*rrs, eis, whiB7*	Koch *et al*. (2018), Zhang and Yew, 2015, Lakshminarayana *et al*. (2014)
	Ofloxacin	Fluoroquinolone	*gyrA*	Farhat, Sultana, Lartchouk *et al*. (2016), Lakshminarayana *et al*. (2014)
	p-aminosalicyclic acid	Salicylate	*thyA, dfrA, folC, ribD*	Koch *et al*. (2018), Lakshminarayana *et al*, 2014
	Moxifloxacin	Fluoroquinolone	*gyrA, gyrB*	Koch *et al*. (2018), Lakshminarayana *et al*, 2014
	Terizidone	D-Alanine analogue	*alr, dd1*	Koch *et al*. (2018)

The standard tuberculosis treatment in South Africa includes the use of rifampicin, isoniazid, ethambutol, pyrazinamide and streptomycin taking for a six-month course, however, the regimens vary (Dookie *et al*., 2016). All newly diagnosed MDR- or XDR-TB patients are advised to follow a standardized MDR-TB treatment protocol. The standardized MDR-TB regimen consists of a six-month intensive phase (also known as the "injectable phase") with five drugs, followed by an 18-month (or less) continuation phase with four drugs. During the injectable phase, kanamycin or amikacin, moxifloxacin, ethionamide, terizidone or cycloserine, and pyrazinamide are used. During the continuation phase, moxifloxacin,

ethionamide, terizidone or cycloserine, and pyrazinamide are prescribed (Falzon *et al*., 2013). TB drugs used in SA to treat resistant infections include bedaquiline, delamanid and pretomanid (Conradie *et al*., 2020). These antibiotics (used for both first-line and second-line treatment regimens) in many ways end up in the aquatic environment (Table 2.2 and 2.3).

Table 2.2: Dosage of first-line anti-tubercular drugs in adult patients in South Africa and possible amounts excreted into the aquatic environment (Magwira *et al*., 2019)

Name of Drug	daily dosage[a] (mg/kg body weight)	daily amount for 80 kg adult in mg	annual consumption (Gauteng province)[b]	% of drug excreted and unmetabolised[c]	amount entering environment[d]
Isoniazid	5	400	11,680 kg	20%	2,336 kg
Rifampicin	10	800	23,360 kg	24%	5,606 kg
Ethambutol	15	1200	35,040 kg	50%	17,520 kg
Pyrazinamide	25	2000	58,400 kg	34%	19,856 kg
Streptomycin	15	1200	35,040 kg	29%	10,161 kg

[a] WHO, 2002. Operational Guide for National Tuberculosis Control Programmes on the Introduction and Use of Fixed-Dose Combination Drugs.
[b] Approx. 80,000 patients on TB treatment at any given time, Gauteng DOH, 2016.
[c] Based on urine excretions only.
[d] Amount entering the environment is % of annual consumed compound excreted via urine unmetabolised.

Table 2.3: Dosage of some second-line anti-tubercular drugs and co-trimoxazole in adult patients in South Africa and possible amount excreted into the aquatic environment (Magwira *et al*., 2019)

Name of Drug	daily dosage (mg/kg body weight)	daily amount for 80 kg adult in mg	annual consumption (Gauteng province)	% of drug excreted and unmetabolised	amount entering environment
Kanamycin	15	1200	35,040 kg	90	31,536 kg
Amikacin	15	1200	35,040 kg	94	32,937 kg
Ofloxacin	400[a]	800	23,360 kg	90	21,024 kg
Sulfamethoxazole	400[a]	800	23,360 kg	20	4,672 kg
Trimethoprin	160[b]	320	9,344 kg	80	7,475 kg

[a] 400 mg tablet given to an adult patient twice a day.

[b] 160 mg tablet given to the adult patient twice a day.

2.1.5. Antimicrobial resistance in *Mycobacterium tuberculosis* complex species

Mycobacterium tuberculosis is now considered one of the most successful pathogens among those causing infectious diseases responsible for one-third of the world population's infections. In addition to its innate ability to survive host defence mechanisms, *M. tuberculosis* also has the ability to resist most antimicrobial agents currently available (Nguyen, 2016b). The resistance phenomenon in *M. tuberculosis* has been highly related to mutations in specific genes, and this association has been the base for the implementation of rapid diagnostics kits (Lawn and Nicol, 2011; Juarez-Eusebio *et al*., 2017). Unfortunately, mutations do not completely explain the resistance in all cases, suggesting that other mechanisms could be

involved (Peñuelas-Urquides *et al*., 2018). Drug resistance mechanisms in *M. tuberculosis* can be classified as intrinsic or acquired. The mycobacterial cell wall and drug penetration are both involved in intrinsic drug resistance, a) the unusual composition and structure of the mycobacterial cell envelope have attributed to mycobacteria's inherent resistance to a variety of antibiotics (Nasiri *et al*., 2017; Gygli, 2018), b) antibiotics may be cleaved enzymatically after penetrating the cell wall as an initial defence layer, rendering them ineffective, c) drug target modification by enzymes and d) several efflux systems have been identified in *M. tuberculosis*, but their significance in conferring clinically relevant levels of drug resistance is a point of contention.

Because efflux systems are expressed in a variety of conditions, they could be used as a stepp ingstone to high-level drug resistance (Gygli *et al*., 2017; da Silva *et al*., 2011). Mutations or horizontal gene transfer mediated by phages, plasmids, or transposon elements can cause acquired antibiotic resistance in bacteria. However, horizontal drug resistance gene transfer has not been observed in *M. tuberculosis*, but resistance is mostly caused by chromosomal mutations under the selective pressure of antibiotic use (Nguyen, 2016). *M. tuberculosis* genes to which mutations confer TB drug resistance are listed in Table S2 (in the appendices). Acquired drug resistance involves; a) in *M. tuberculosis*, drug target mutations, non-synonymous mutations in drug target encoding gene(s), and nucleotide substitutions in the op eron encoding ribosomal RNA are all common ways to confer drug resistance (Gygli *et al*., 2017; Hameed *et al*., 2018), b) the abrogation of prodrug activation mechanisms leads to resistance to several antimycobacterial drugs, including isoniazid and pyrazinamide, ethionamide, para-aminosalicylic acid, as well as the two new nitroimidazole drug candidates delamanid and pretomanid (Gygli *et al*., 2017), c) overexpression of drug targets may overcome the inhibitory effect of the drug in question due to the target's abundance. Overexpression of the drug target can be caused by mutations in transcriptional repressors or the promoter of the drug target, as seen with isoniazid, ethambutol, and cycloserine (Gygli, 2018).

2.2. TB epidemiology

Globally, 7.0 million new cases of tuberculosis were reported in 2018, slightly higher compared to 6.4 million in 2017 and significantly higher than the 5.7–5.8 million reported annually between 2009 and 2012 (DTE Staff, 2019; WHO, 2019). The majority of the increase in global tuberculosis cases since 2013 could be attributed to increases in India, South Africa, and Indonesia, the world's first and third largest countries in terms of annual estimated incident cases (DTE Staff, 2019; Glaziou *et al*., 2015). In India, new cases rose from 1.2 million to 2.0

million between 2013 and 2018 (+60%) (WHO, 2019). Meanwhile, in Indonesia, it rose from 331 703 in 2015 to 563 879 in 2018 (+70%), including an increase of 121 707 (+28%) between 2017 and 2018 (WHO, 2019). Despite an increase in tuberculosis infections, there is still a significant gap between the number of new cases reported in 2018 (7.0 million) and the projected 10.0 million (range, 9.0–11.1 million) incident cases for the mentioned year (WHO, 2019). This discrepancy could be due to a combination of underreporting and underdiagnosis, in which people with tuberculosis do not seek medical attention or does not have access to it or are not diagnosed when they do (Naidoo *et al*., 2017).

2.2.1. Tuberculosis statistics in South Africa

According to a report by Statistics South Africa, tuberculosis is the leading cause of death in South Africa, and it continues to be a major public health threat (Nanoo *et al*., 2015). It is listed by the WHO in the three high-burden country lists of TB, TB/HIV and MDR-TB for the period 2016-2020. The provinces with the highest incidence rates in South Africa in 2015 were the Eastern Cape, KwaZulu-Natal, and the Western Cape, with rates of 692, 685, and 681 per 100,000, respectively (Kanabus, 2020). The National Institute for Communicable Diseases of South Africa (NICD) has also reported a higher incidence of DR-TB between 2012-2014 (Figure 2.6). KwaZulu-Natal had the highest annual number of new cases between 2004 and 2012, accounting for 31% of all microbiologically confirmed pulmonary tuberculosis (mPTB) cases in South Africa in 2011 (Nanoo *et al*., 2015).

Resistance to key tuberculosis drugs is said to have emerged in the 1980s (Cox *et al*., 2017). The majority of TB resistance data prior to 1994 came from the South African Medical Research Council's surveillance (SAMRC) (McIntosh *et al*., 2018). According to a retrospective analysis of surveillance data from hospitals in four provinces, between 1965 and 1970, 29% of patients tested had isoniazid-resistant tuberculosis, 34 % had streptomycin-resistant tuberculosis, and 6% had rifampicin-resistant tuberculosis (Barron and Padarath, 2017; Cox *et al*., 2017). Multi-drug-resistant tuberculosis (MDR-TB) incidence was reported to be less than 2% among *M. tuberculosis* isolates in the period 1980–1988 (Charles *et al*., 2011; Cox *et al*., 2017). Resistance to isoniazid decreased dramatically over the three time periods studied, from 29 % in 1965–1970 to 14% in 1980–1988. These findings suggest that if adequate first-line treatment is maintained, TB drug resistance will decline (Barron and Padarath, 2017; Cox *et al*., 2017).

Despite the fact that the annual number of TB patients has decreased in recent years, South Africa's TB epidemic has remained among the worst in the world for the past two decades (Churchyard *et al*., 2014). The World Health Organisation (WHO) statistics estimated an incidence of 360,000 active TB cases in 2019. This is a rate of 615 per 100,000 population (WHO, 2020).

South Africa has the 9th highest incidence of rifampicin-resistant tuberculosis (RR-TB) in the world, with 14,000 cases estimated in 2017 (Director and Kruger, 2018). While South Africa treats the third-largest number of RR-TB patients in the world, there is still a significant gap between the number of cases reported as diagnosed and those started on second-line treatment; treatment delays are also common (Barron and Padarath, 2017; Cox *et al*., 2017). In summary, treatment is successful for about half of the patients who begin treatment, which is comparable to the global treatment success rate (Barron and Padarath, 2017; Naidoo *et al*., 2017). In 2001, South Africa became one of the first high-burden MDR-TB countries to implement a national rollout of second-line MDR-TB treatment, and the country has since implemented innovative strategies to improve case detection and patient outcomes (Barron and Padarath, 2017).

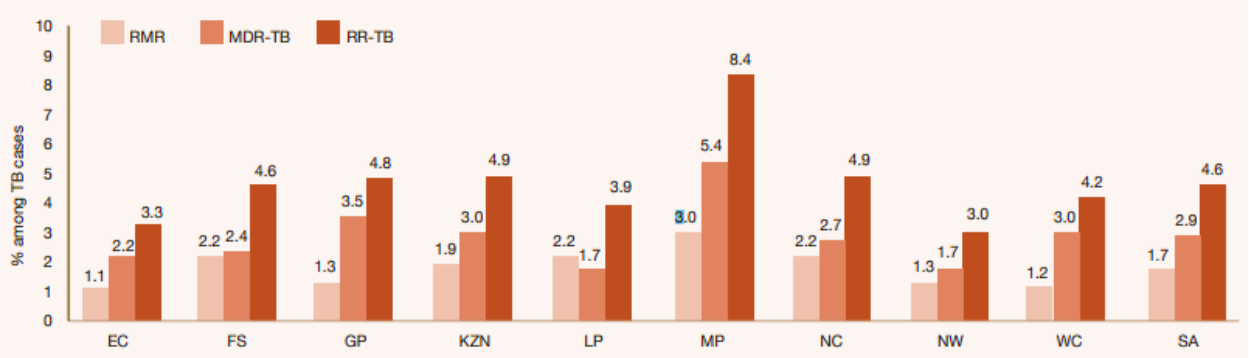

Figure 2.6: Percentage of TB cases found to have rifampin monoresistance tuberculosis (RMR-TB), rifampicin-resistant tuberculosis (RR-TB) and multidrug-resistant tuberculosis (MDR-TB) across provinces in South Africa through the 2012-2014 (NICD, 2016).

While the prevalent view in South Africa, and indeed globally, was that resistance was primarily caused by acquired resistance during poor first-line TB treatment, such as poor patient adherence, inadequate treatment regimens, or lower drug quality, this was not the case (Barron and Padarath, 2017; Cox *et al*., 2017), several studies suggest that since the mid-1990s, there has been significant community transmission of DR-TB strains in South African settings (Barron and Padarath, 2017; Pillay and Sturm, 2007; Cox *et al*., 2017). According to the Departments of Health and Agriculture, Forestry and Fisheries for South Africa, the drivers of antibiotic resistance include the total volumes of antibiotics that are used by humans and in

animals, the lack of veterinary health professionals, the national reliance on broad-spectrum antibiotics and weak regulations and enforcement mechanisms, poor practices for infection control leading to the acquisition and spread of hospital-acquired infection (HAI) (DOH, 2014).

2.3. TB surveillance

Tuberculosis surveillance involves monitoring and analysing tuberculosis disease data. This data could include, demographics, diagnostic methods used and geographic information. Globally, this data is recognized as a vital data source for assessing the burden of disease and epidemiological trends. Therefore, the WHO emphasizes the importance of having quality TB surveillance data. Currently, TB surveillance is achieved through several methods, and these include clinical-based surveillance, hospital admission data, questionnaires, surveys, motility and morbidity rates and sentinel surveillance (Figure 2.7) (WHO, 2020). However, the most common TB surveillance method used globally is the case notifications, with expert opinion or standard adjustment (Figure 2.7). Despite implementing such a variety of surveillance tools, the WHO still estimates that about 3.5 out of 10 active TB cases are missed globally (Ajudua, and Mash, 2020). This is even higher in sub-Saharan Africa, with an estimated 5 out of 10 cases missed (Ajudua, and Mash, 2020). This could be due to the reliance on case notifications for TB data acquisition.

Case notifications for TB surveillance in the regulated diagnostic practices, rational use of TB medication and the availability of diagnostic and treatment facilities for drug-resistant TB infections may pose additional challenges (Nagpal, and Chawla, 2013). These challenges, mentioned above, may be less of a challenge in developed countries with proper regulatory systems in place. For instance, in Germany, an estimated 90% of all TB cases were captured in the German TB surveillance program (Domaszewska *et al*., 2020). This indicates that case notifications could be a good surveillance system if the right policies are implemented. However, a recent review of the German surveillance program highlighted an increase in underreporting (Domaszewska *et al*., 2020). In South Africa, a National TB Program (NTP) was developed to address this issue of "missing TB patients" (Podewils *et al*., 2015). However, a WHO-led review of this NTP identified several challenges with the surveillance system. This review noted variability in the completeness and quality of the TB records, a backlog of data entry and an incomplete understanding of the TB indicators (Podewils *et al*., 2015). These challenges could be due to several reasons, for instance, the TB pandemic is prevalent in poor, vulnerable and often overcrowded communities, where health-seeking behaviours may differ between individuals (Ajudua, and Mash, 2020). Furthermore, the NTP program focused on

facility-based surveillance for the detection and treatment of TB (Ajudua, and Mash, 2020). However, the failures identified with this approach shows the need for a community-based initiative aimed at early detection of TB. Therefore, new monitoring and management approaches are necessary for the early detection and prevention of tuberculosis and associated resistance in the population.

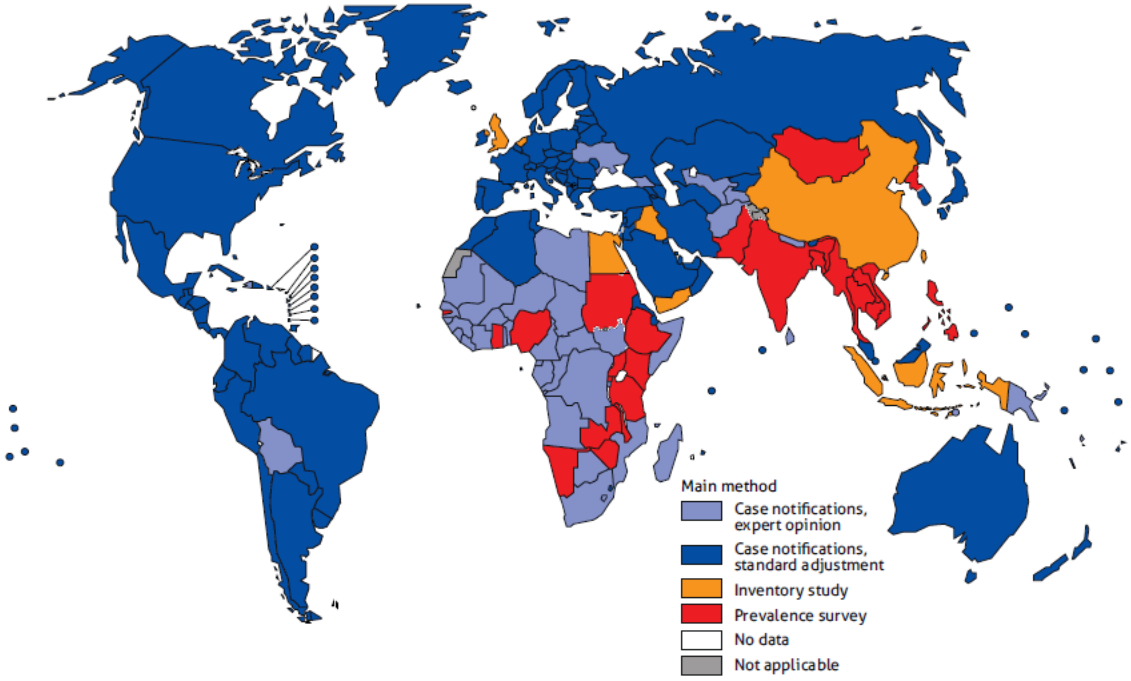

Figure 2.7: Surveillance methods used to estimate TB incidence (WHO, 2020)

2.4. MTBC in wastewater: a possible indirect route of transmission?

The main infection route for TB has been reported to be through exposure to aerosols from infectious patients (Escombe *et al*., 2009; Escombe *et al*., 2008; Escombe *et al*., 2007; Dharmadhikari *et al*., 2012; Dharmadhikari *et al*., 2014; Churchyard *et al*., 2017). This fundamentally shows the airborne transmission of pulmonary TB and is currently widely accepted as the primary mechanistic transmission route (Yates *et al*., 2016; Martinez *et al*., 2019). Although airborne transmission is the main route for TB, other routes have been reported. For example, in 1905, Calmette and Guérin postulated that TB could be transmitted through contaminated food (Barberis *et al*., 2017; Martinez *et al*., 2019). Gao *et al*. (2018) also provided evidence that guinea pigs could be infected by drinking MTBC contaminated water. Clinical and pathological findings in infected animals were similar to those observed in guinea pigs infected via respiratory or subcutaneous routes. These observations show the possible oral transmission of TB in exposed individuals.

However, the environmental occurrence of pathogenic mycobacteria has received less attention in comparison to its occurrence in clinical settings. Nevertheless, there is a growing body of evidence to show that water could be a significant vehicle for the transmission of these organisms (Dufour, 2004; WHO, 2011; Sims and Kasprzyk-Hordern, 2020). Previous studies revealed that environmental contamination, from faecal shedding, provided the potential and indirect routes for transmission of *M. bovis* infection (Travis *et al*., 2019; Wu *et al*., 2020). The shedding of *M. bovis* cells has already been demonstrated in many animals via oro- nasal mucus, sputum, urine, feaces and wound discharges (Corner *et al*., 2012; Barasona *et al*., 2015; Barbier *et al*., 2017; Vayr *et al*., 2018). Investigating this type of indirect transmission is challenging as discussed previously. This transmission route has been implicated most frequently in zoonotic infections than human-to-human infections (Travis *et al*., 2019). Wastewater serves as a link between human activities and the environment and could be the first medium that may be contaminated with MTBC via faecal shedding. However, studies on the occurrence of MTBC in different environmental matrices has not received priority, therefore there's a lack of proper detection techniques for MTBC in the environment

2.4.1. Source of *Mycobacterium* spp in wastewater

The occurrence of MTBC in wastewater (Table 2.4) could be from various sources including domestic, industrial and agricultural activities.

i) Domestic wastewater: This could be primarily due to gastrointestinal infections with MTBC, resulting in the shedding of MTBC cells in human excreta. The human sewage microbiome is referred to as the collective microbes in sewage from human domestic waste such as faeces, urine, sweat, washing, bathing, etc. (Vinnerås *et al*., 2013; Orumwense *et al*., 2013; Cai *et al*., 2014). This is mainly derived from the human body including the skin, respiratory tract, oral cavity, gastrointestinal tract, and urogenital tract which ends up in wastewater treatment plants (Cai and Zhang, 2013; Cai *et al*., 2014). Pathogens are abundant in wastewater from hospitals and facilities that receive patients infected with contagious microorganisms such as *Mycobacterium* spp. (Petrovich *et al*., 2020; Nguyen *et al*., 2016; Jia and Zhang, 2020). Jensen (1954) demonstrated the occurrence of tubercle bacilli in considerable numbers in the wastewater systems of several towns containing tuberculosis clinics. Therefore, it is important to note that sewage systems from communities with high TB infections, facilities and institutions receiving pathogen carriers are at risk of contamination due to the presence of these organisms at high concentrations in the community (Francy *et al*., 2011; Nguyen *et al*., 2016).

ii) Industrial wastewater: This includes wastewater from slaughterhouses and may constitute the largest source of the contamination of the environment in some regions (Franke-Whittle and Insam, 2013; Kundu *et al*., 2013; Bustillo-Lecompte and Mehrvar, 2017). Improper management of slaughterhouse wastes and subsequent disposal either directly or indirectly into river bodies portends serious environmental and health hazards (possibly, infection from *M. bovis*) both to aquatic life and humans (Malama *et al*., 2014; Pokam *et al*., 2019). Irshad *et al*. (2015) reported that improper disposal of wastes from slaughterhouses could lead to the transmission of pathogens to humans and cause zoonotic diseases such as bacillosis, salmonellosis, brucellosis, and helminths. Pokam *et al*. (2019) reported that *M. bovis* could be transmitted by aerosol and ingestion of infected carcasses.

iii) Wastewater from agricultural fields: This includes animal excrement, manure and other components: Agricultural fields using manure as a soil amendment could potentially contribute significantly to the pathogen, such as MTBC, in wastewater. Different pathogens in manure have been reported extensively (Manyi-Loh *et al*., 2016; Burch *et al*., 2018; Manyi-Loh *et al*., 2018; Zhang *et al*., 2020). MTBC cells, most especially *M. bovis* have been detected commonly in manure (Donat *et al*., 2016; Hahn *et al*., 2017; Avilez *et al*., 2019), this could therefore significantly result in the contamination of water sources with these pathogens. Additionally, the occurrence of these pathogens in manure could potentially result in the infection of both humans and animals. In addition to the manure, the reports of shedding of MTBC cells in excreta from animals could be a significant source of these in wastewater or runoffs from agricultural fields.

Table 2.4: Occurrence of MTBC in wastewater

Specific MTBC organism	Sample matrix	Study location	Detection method	Reference
M. tuberculosis	Raw sewage, sewage effluent	Poland	Culture-based	Ogielski and Zawadzki, 1961
M. tuberculosis	Sanatorium sewage: inlet, settling tank and outlet	India	Culture-based	Saldanha *et al*. (1964)
M. bovis, M. tuberculosis	Sewage from cattle farm used for pastures	Poland	Culture-based	Skurski *et al*. (1965), Szulga *et al*. (1965)
M. bovis, M. tuberculosis	Sewage from tuberculous sanatorium and hospitals, towns and sewage purification plants	Poland	Culture-based (Sewer swabs)	Buczowska, 1965, Buraczewski and Osinski, 1966
M. tuberculosis	Sewage sediment	Poland	Culture-based	Bedryńska-Dobek, 1966
M. bovis, M. tuberculosis	Sewage water around tuberculous sanatoria	Kazakhstan	Culture-based	Blagodarnyi and Vaksov, 1972
M. tuberculosis	Wash-off water from wearing apparel, crockery, household utensils, etc	Russia	Culture-based	Poptsova,1974
M. tuberculosis, M. bovis	River sediment (wastewater present)	Romania, Portugal,	PCR-based	Kazda, 2010; Santos *et al*. (2015)
M. tuberculosis	Fresh sewage used for pastures and fields	Germany	Culture-based	Kazda, 2010
M. tuberculosis	Activated sludge and effluent	Hong Kong	PCR-based	Cai and Zhang, 2014
M.microti/tuberculosis/africanum/pinnipedii	River (sediment/ water)	Portugal	PCR-based	Santos *et al*. (2015)
M. tuberculosis	soil and water	Tehran, Iran	Culture, biochemical and PCR-based	Velayati *et al*. (2015)
M. tuberculosis	Drinking water and sewage water	Pakistan	Culture, biochemical and PCR-based	Suliman *et al*. (2017)
Mycobacterium tuberculosis complex	water	South Africa	PCR-based	Ntloko *et al*. (2019)

2.4.2. Fate of MTBC in wastewater

MTBC in wastewater could be affected by several processes, such as natural die-off and removal during wastewater treatment. This section addresses the impact of these processes on MTBC in the wastewater environment.

2.4.2.1. Factors affecting the survival of MTBC in wastewater

The survival of MTBC in different matrices could be influenced by several factors, such as temperature, moisture, pH, inhibitors and protection against solar radiation (ultra-violet) (Barbier *et al*., 2017). Mycobacterial cells are known to be hydrophobic (Hruska and Kaevska, 2012), which may result in their attachment to solid particles in the water environment. This could also play a role in the extensively reported biofilm formation by mycobacterial cells (Hegde, 2020; Esteban and García-Coca, 2018; Aboagye and Rowe, 2018; Trivedi *et al*., 2016). Biofilm formation is a process that represents the most successful adaptation of bacteria against several environmental factors. It has become increasingly evident that biofilms in drinking water supply systems provide a transient or long-lasting habitat for many microbes, including human pathogens (Ramamurthy *et al*., 2014). Biofilms provide protection against environmental stresses, e.g., desiccation, starvation and the presence of toxics (Flemming *et al*., 2016, Xue *et al*., 2012).

Additionally, microorganisms including *M. tuberculosis* have been reported to be amoeba-resistant which may enhance their survival in wastewater. *M. tuberculosis* (Hagedorn *et al*., 2009) and *M. bovis* (Taylor *et al*., 2003) could survive for hours to days in the amoebal trophozoites. The observation that *M. tuberculosis* and *M. bovis* organisms were engulfed by *Acanthamoeba polyphaga* trophozoites agreed with previous observations made when co-culturing *M. tuberculosis* organisms with the free-living amoeba *Dictyostelium discodium* (Medie *et al*., 2011; Butler *et al*., 2020). Mycobacteria survived in the cysts for up to 18 days and cysts protected *M. tuberculosis* organisms against mycobactericidals (5 mg/mL streptomycin and 2.5% glutaraldehyde). This data indicates that MTBC organisms are amoeba-resistant organisms, as previously demonstrated for non-tuberculous, environmental mycobacteria (Medie *et al*., 2011; Bartie *et al*., 2016; Delafont *et al*., 2017). Inter-cystic survival of tuberculous mycobacteria, except for *M. canettii*, could therefore protect them against biocides and play a role in their survival (Medie *et al*., 2011; Ghodbane *et al*., 2014).

There is evidence to suggest that under starvation caused by nutrient limitations, low pH and lack of oxygen, a non-replicating state is induced in some mycobacterial cells caused by the

metabolic state of the pathogen (Archuleta *et al*., 2005). Some MTBC organisms, like *M. avium,* can survive rapid shifts in oxygen content for prolonged periods by altering their metabolism from aerobic to anaerobic and vice versa (Lewis and Falkinham, 2015). Intrinsically, MTBC cells can withstand desiccation due to the presence of a dense external cell wall composed of a large number of fatty acids (Rodríguez-Hernández *et al*., 2016). For instance, *M. tuberculosis* was found to still be viable after exposure to high temperatures for several months (Russell *et al*., 2012). Although the mechanisms responsible for this feature are not well-known, reports have indicated a possible role of endogenous synthesis of trehalose (McIntyre *et al*., 2007; Rodríguez-Hernández *et al*., 2016). Coupled with their natural ability to withstand desiccation, the wastewater environment with high suspended solids could provide an additional layer of protection for MTBC cells, enhancing their survival. Therefore, it is plausible that MTBC may survive in wastewater, through both intrinsic (cell wall) and extrinsic factors (biofilms). However, the lack of information on the survival of MTBC in wastewater, as mentioned before, makes it difficult to conclusively determine the impact wastewater conditions may have on this group of organisms.

2.4.2.2. Removal of *Mycobacterium tuberculosis* complex during wastewater treatment

Wastewater treatment plants (WWTPs) serve as the guts of the population, receiving and digesting various human pathogens (Cai and Zhang, 2013). Several studies demonstrate that human pathogenic or opportunistic bacteria may survive treatment processes (Al-Gheethi *et al*., 2018; Chahal *et al*., 2016; Talan and Tyagi, 2020). Radomski *et al*. (2011) reported *Mycobacterium* concentrations of 5.5×10^5 ($\pm3.9\times10^5$) copies/L in untreated wastewater and $0.74\times10^4 \pm 1.40\times10^4$ copies/L (in 7 positive samples among 13) detected in the final treated wastewater after decantation and biofiltration, and $1.04\times10^6 \pm 1.75\times10^6$ copies/g (in 3 positive samples among 6) in sludge. They reported that the most removal of mycobacteria (98.6 ± 2.7%, i.e. 2.4 ± 0.7 $\log_{10}$) was achieved by physical-chemical decantation (primary treatment) and the remaining mycobacteria were removed by biofiltration (secondary treatment). Chandra and Arora (2018) also reported a 50% removal of mycobacterial load during primary sewage treatment processes.

In some other findings, *M. tuberculosis* was detected more frequently in both the activated sludge and effluent, than the influent (Cydzik-Kwiatkowska and Zielińska, 2016; Guo *et al*., 2019). Additionally, pathogenic *Mycobacterium* spp. have been reported in treated wastewater effluents from a WWTP treating salty wastewater (Ye and Zhang, 2013; Cydzik-Kwiatkowska

and Zielińska, 2016). Da Silva *et al*. (2015) investigated the microbial communities present in effluent samples from two independent field-scale swine WWTPs. They concluded that *Mycobacteria* were abundantly observed in the final effluent. This is corroborated by Cai and Zhang (2013) through metagenomic analysis, where a low abundance of the genus *Mycobacterium* was observed in the influent as compared to both the activated sludge and effluent. These reports indicate that MTBC cells may be removed mainly through settling, however, the final effluent may still contain high loads of these pathogens.

2.4.2.3. Impact of wastewater disinfection processes on MTBC

Tertiary treatment of wastewater usually involves the use of disinfection processes aimed at inactivating microbial organisms before discharge. These processes include; chlorination, ozonation, and UV treatment (Carra *et al*., 2018; Tong *et al*., 2019; Burch *et al*., 2019; Arzate *et al*., 2019). Previous researchers have reported that several strains of mycobacteria are 100−330 times more resistant to chlorine than *E. coli* (Amha *et al*., 2017; Wang *et al*., 2019), which is usually used as an indicator for wastewater treatment efficiency. Slow-growing mycobacteria are unaffected at the higher chlorine disinfection, confirming past reports of their high resistance to chlorination (Amha *et al*., 2017; Guo *et al*., 2019). Several other studies have observed resistance of some mycobacteria to the normal chlorination process used either in drinking water or wastewater treatment plants (Dubrou *et al*., 2013; Moghim *et al*., 2012). The unusual structure of the mycobacterial cell wall skeleton explains mycobacteria's high resistance to chlorination (Le Dantec *et al*., 2002). The peptidoglycan is covalently linked to mycolic acids, which are long fatty acids with up to 90 carbon atoms, in mycobacteria via an arabinogalactan bridge. Mycobacteria are acid-fast due to mycolic acids, which form a thick, hydrophobic barrier that prevents diffusion and lowers permeability (Donohue *et al*., 2019; Le Dantec *et al*., 2002). Chen *et al*. (2012) showed that the resistance of *Mycobacteria* to free chlorine was attributed to the cell membrane composition. They observed that the richness of the long-chain saturated fatty acid or rareness of unsaturated fatty acid in the cell membrane might partly explain the higher chlorine resistance of *Mycobacteria* over other bacteria. The high concentration of mycolic acid and slow growth, adherence to surface and hydrophobicity of mycobacteria have been reported to be primarily responsible for the high resistance of mycobacteria to chemical disinfection (Lee *et al*., 2010; Edirisinghe *et al*., 2017). Comparatively, UV irradiation was more effective in eliminating *Mycobacterium*. However, Lee *et al*. (2010) reported that mycobacteria are 2–10 times more resistant to UV than *E. coli*. Nevertheless, the absence of residual disinfection and low penetrability in water containing

suspended solids are the major disadvantages of UV irradiation on a mass scale (Edirisinghe *et al*., 2017), especially in wastewater treatment.

The reported MTBC in treated and untreated wastewater could result in infections for different populations that may be exposed either directly or indirectly (Ogundeji *et al*., 2015). Direct exposure to wastewater could be a major route mainly for WWTP workers, farmers using the treated wastewater for irrigation and the general public exposed to either untreated wastewater within the community or effluent discharge from WWTPs. Despite this potential risk, there is a scarcity of studies in this regard. The following section, therefore, discusses the potential of infection using information from related fields but not specifically for MTBC.

2.4.3. Potential risks of infection for wastewater operators/workers

Most MTBC infections are usually through inhalation of aerosols or droplets, produced either through the coughing or sneezing of infected individuals (Tellier *et al*., 2019). Therefore, inhalation of water aerosols may represent the major route of exposure to MTBC in wastewater. Exposure through this pathway may expose three main groups of people: (1) individuals that shed viable pathogens into the toilet and are then exposed to these pathogens during the flush of the toilet, (2) individuals that come into contact with wastewater containing viable pathogens during the collection and treatment process, and (3) individuals that contact untreated wastewater containing pathogens during a spill or release of wastewater from the piping and collection system (Chattopadhyay and Taft, 2018). Liquid (droplet) aerosols notably are generated during wastewater aeration and also during the spray application of wastewater including sludge suspensions onto land. Aerosols generated during wastewater treatment might serve as a source of disease in wastewater treatment workers (Hurst, 2018).

It is well known that exposure of wastewater treatment workers to bioaerosols carries a risk of negative health outcomes (Gaviria-Figueroa *et al*., 2019; Choi *et al*., 2020). This is based on the fact that sewage is known to contain a range of potential pathogens (Al-Gheethi *et al*., 2018) and that some studies have suggested a correlation between exposure to WWTP bioaerosols and a range of respiratory and gastrointestinal symptoms (Mbareche *et al*., 2019; Osunmakinde *et al*., 2020). Occupation has never been considered as a factor in contracting tuberculosis and its associated morbidity. Sewage workers are responsible for entering manholes and closed channels, as well as maintaining sewage treatment facilities. They work in confined spaces, closed channels, and sewage treatment plants, where they use technologies such as up-flow anaerobic sludge blankets, activated sludge processes, fluidized aerobic bioreactors,

sedimentation, trickling filters, and a series of waste stabilization ponds, which emit noxious fumes and bioaerosols (Chandra and Arora, 2018; Bressani-Ribeiro *et al*., 2018).

Chandra and Arora (2019) conducted a study consisting of 104 sewage workers with average occupational exposure to sewage work of 21.28 (±10.54) years. Approximately, 21% of the sewage workers had tuberculosis and 92.31% had at least one of the chronic respiratory diseases (COPD, Asthma or ACOS). They concluded that sewage workers have an adverse chronic morbidity profile for tuberculosis. Therefore, there is an urgent need for epidemiological research and targeted screening and public health intervention for tuberculosis in sewage workers as an occupational group.

2.4.4. Community infection risks from exposure to wastewater

Particles are easily carried by wind and dispersed over long distances due to their small size and light weight (Wang *et al*., 2018), which may cause infection in on-site workers as well as downwind residents. Temperature, wind velocity, smog, and specific humidity are factors that influence aerosol spread and microorganism survival in the air (Vtzová *et al*., 2013; Pamionka, 2019). Microbes are dehydrated at very low humidity and high temperatures, whereas high humidity may protect cells from solar radiation (Wang *et al*., 2018; Vantarakis *et al*., 2016; Pierce and Scott, 2019). The maximum distance for droplet transmission is currently unknown, though pathogens transmitted via the droplet route have not been transmitted over long distances through the air (Capolongo *et al*., 2017). The distance droplets travel is likely determined by their velocity and mechanism of propulsion, the density of the secretions, environmental factors such as temperature and humidity, and the pathogen's ability to maintain infectivity over that distance (D'Alessandro and Fara, 2017; Capolongo *et al*., 2017). Air microbiological analyses have commonly been conducted close to sewage treatment plants (Vantarakis *et al*., 2016). Concentrations of airborne bacteria varied in a wide range of 23–4878 CFU/m^3 (Yang *et al*., 2019). Brenner *et al*. (1988) recorded concentrations of 86–7143 bacterial CFU/m^3 air at a distance of 25m from the surface of an aeration basin well (Vantarakis *et al*., 2016). High microbial numbers were also reported in locations close to the WWTP (Vantarakis *et al*., 2016).

In addition to aerosols generated during wastewater treatment, the reuse of wastewater for irrigation could also lead to the generation of aerosols (Miller-Robbie *et al*., 2017; Ungureanu *et al*., 2018). Aerosols generated during wastewater treatment and reuse are affected by the same factors as aerosols from the WWTPs. These processes could therefore be a significant

route through which the general public may be exposed to MTBC in wastewater leading to infections. However, despite these potential risks, a few studies to date have focused on measuring the risks of infection with TB as a result of aerosols (de Sousa *et al*., 2020; Wood *et al*., 2016) but no study has focussed on measuring the risks of infection with TB as result of aerosols containing MTBC from WWTPs. This is therefore a research niche that requires further studies.

The detection of pathogenic mycobacteria in treated wastewater (Ye and Zhang, 2013; Cydzik-Kwiatkowska and Zielińska, 2016) could potentially result in surface water contamination. Therefore, exposure to this contaminated surface water may result in infections. However, it is worth noting that the main route of transmission of TB is through aerosols, therefore the risks of infection from exposure to surface water may be low unless the exposure involves the generation and inhalation of these aerosols. The presence of MTBC in high abundance in wastewater especially their detection in sludge or the final effluent presents possibilities of respiratory infections especially tuberculosis but the worse outcome is having the resistant version of these group of organisms from the environment and the resistant genes being shared among other cohabitants in the wastewater or surface water.

2.5. Occurrence of TB associated resistance genes in water and wastewater and possible risks associated with exposure

The relationship between the concentration of antibiotics in the environment and the development of bacterial pathogen resistance has remained largely unknown. However, the prevalence and persistence of antibiotic resistance in bacterial pathogens have emerged as a serious threat to public health, causing widespread concern (Gao *et al*., 2012). Multiple genes encoding for antibiotic resistance have been frequently detected both in liquid (wastewater, surface water, groundwater and even drinking water) and solid (sludge, soil and sediment) environmental media (Gao *et al*., 2012). The spread of ARGs and resistance bacteria could pose health risks to humans. Resistant microorganisms may enter human bodies directly or indirectly, and resistance genes are distributed in various environmental media and widely disseminated in the environment via the horizontal gene transfer (HGT) mechanism (Figure 2.8). Antibiotic resistance genes may also be taken up by plants in the agro-ecosystem via wastewater reuse, posing a risk of human and animal exposure. The majority of antibiotics in the environment are derived from sewage, which is partially eliminated in wastewater treatment processes and is present in effluents before reaching ambient surface waters (Xu *et al*., 2015).

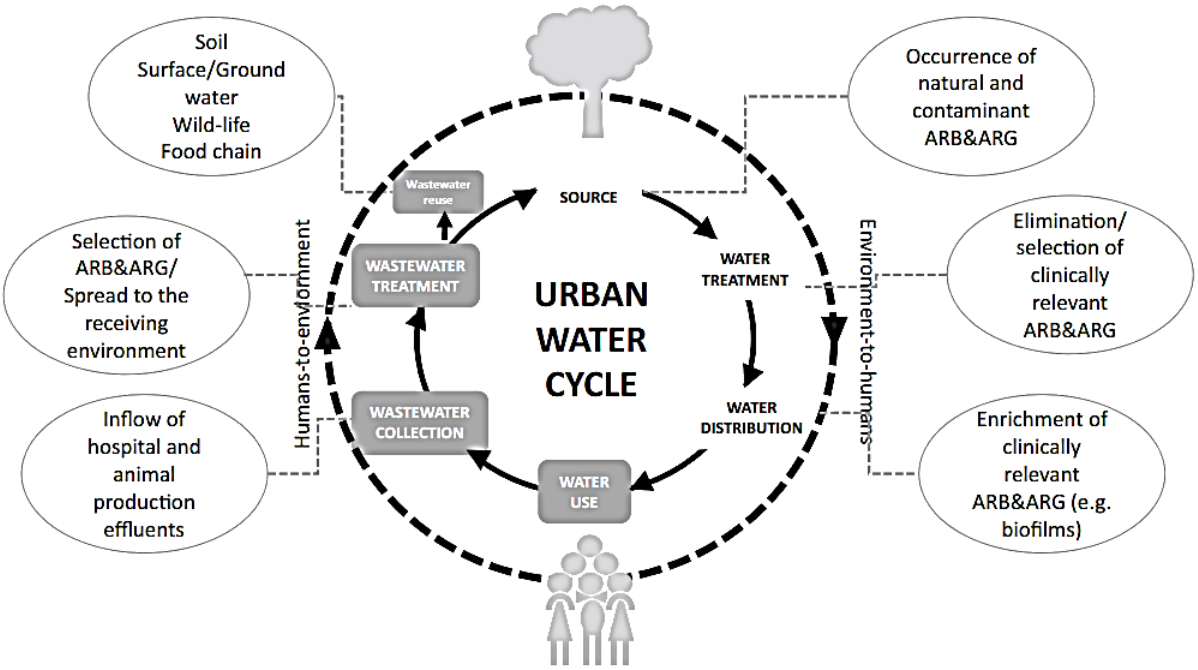

Figure 2.8: Pathways for the distribution of antibiotic-resistant microorganisms and their related genes in the environment (Manaia *et al*., 2016)

Despite the multiple shreds of evidence that wastewater treatment plants may be responsible for the discharge of antibiotic-resistant bacteria or resistance genes to the environment (Lupo *et al*., 2012; Manaia *et al*., 2016; Rizzo *et al*., 2013), there is still much uncertainty regarding the risks of transmission back to human pathogenic or commensal bacteria. Indeed, although water and soil are regarded as potential antibiotic resistance reservoirs, either naturally or due to environmental contamination by humans (Bush *et al*., 2011; Forsberg *et al*., 2012), only in a few cases (e.g. qnr and blaCTX-M, Poirel *et al*., 2002; Poirel *et al*., 2005) that it is possible to demonstrate the passage of the resistance genes from the environment to clinically relevant bacteria or clarify the mechanisms that make such a gene transfer possible.

The relationship between the environmental contamination profile and the antibiotic-resistance type is rather complex and difficult as a result of these effects. As a result, antibiotic-resistance determinants discovered in the environment do not always correspond to the most persistent and commonly found antibiotic residues (e.g. ofloxacin, sulfamethoxazole, tetracyclines) (Novo *et al*., 2013; Oberlé *et al*., 2012).

2.5.3. The role of wastewater in the dissemination of tuberculosis resistance

Antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) resistomes, also known as AR hotspots, have been discovered in the aquatic environment (Ekwanzala *et al*., 2018). Surface water bodies (rivers, lakes, and streams), groundwater, hospital effluents, and municipal wastewater are examples of these aquatic environments (Ekwanzala *et al.*, 2018). Wastewater from hospitals and facilities that receive patients infected with contagious microorganisms may have dense concentrations of pathogens including *Mycobacterium* spp., which may represent a threat to public health (Nguyen *et al*., 2016). Antibiotic resistance is spreading rapidly in wastewater treatment plants, which are considered important hotspots. This idea is frequently supported by three major arguments. The first is that antibiotic residues and other substances that may exert selective pressure, as well as antibiotic-resistant bacteria and resistance genes, are routinely discharged into municipal sewage systems. Antibiotics' persistence in wastewater is largely determined by their water degradability, which varies between antibiotics (Magwira *et al*., 2019). The second is that conditions during the wastewater treatment process may favour antibiotic resistance determinant selection or horizontal gene transfer. The third point is that, regardless of efficiency or operational conditions, wastewater treatment results in the production of final effluents containing antibiotic-resistant bacteria, sometimes in higher percentages than in the raw inflow. In general, three major categories of factors are thought to influence the fate of antibiotic-resistant bacteria during wastewater treatment: abiotic conditions, bacterial community composition and structure, and the presence of possible selective pressure factors (Novo *et al*., 2013). As a result, wastewater treatment plants are potential reservoirs for antibiotic resistance to evolve and spread (Lood *et al*., 2017; Cacace *et al*., 2019). It is widely believed that the presence of antibiotic-resistant determinants is due to selection pressure from antibiotics and that reducing or eliminating antibiotic use will result in a reduction in resistant bacterial strains (Davies and Davies, 2010).

2.6. Methods used for the detection and quantification of *Mycobacterium* spp in wastewater

The lack of data on the occurrence of MTBC in environmental matrices could be mainly due to the lack of sensitive and mass-scalable techniques to detect these organisms in environmental samples (Santos *et al*., 2015; Barbier *et al*., 2016; Martinez *et al*., 2019). Methods for the detection of MTBC in the environment can be categorised into two; culture-based and molecular techniques, these are discussed below and presented in Figure 2.9.

2.6.1. Culture-based methods for the detection of MTBC in wastewater

Isolation and culturing of MTBC from wastewater require two key steps, disinfection/decontamination of the samples to remove other microorganisms capable of interfering with their (MTBC) growth and concentration of the samples.

Disinfection/decontamination is usually achieved using 1-4% NaOH, 1% Oxalic acid, 1% HCL (Brooks *et al*., 1984a), or 1% Cetylpyridinium chloride (CPC) and 12% sulfuric acid (H_2SO_4) (du Moulin and Stottmeier, 1978; Radomski *et al*., 2011; Fine *et al*., 2011; Velayati *et al*., 2015; Sattar *et al*., 2018). All these chemicals exert adverse effects on the growth of other microflora that may be in the sample. Numerous studies have previously recommended CPC as the most suitable chemical for decontamination (Corner *et al*., 2012; Radomski *et al*., 2010; Sattar *et al*., 2018). This is because the low toxicity of CPC to mycobacteria enables a fast recovery rate of mycobacteria (Radomski *et al*., 2010; Sattar *et al*., 2018).

However, the elimination of non-target microorganisms by chemical decontamination is insufficient (Whittington, 2009). The incorporation of antimicrobials in the decontamination procedure will remove most of the contaminant bacteria and provide the opportunity for bacilli to grow, which results in highly positive cultures (Moravkova *et al*., 2011; Sattar *et al*., 2018). The use of antibiotics, such as nalidixic acid (NAL), vancomycin (VAN) and amphotericin B (AMB), in previous studies, has shown desired effects by reducing contamination rate and improving culture sensitivity (Sattar *et al*., 2018). In addition to inactivating other microorganisms, the disinfection/decontamination agents may also inactivate some of the mycobacteria but to a lesser extent (Brooks *et al*., 1984; Corner *et al*., 1995; Sattar *et al*., 2018). The lesser impact of these decontamination chemicals could be due to the tough cell wall of these mycobacteria. Therefore, it is recommended that the chemical effects should be balanced to support mycobacterial growth and eliminate contaminating microorganisms (Sattar *et al*., 2018). Minor inhibitory effects can be ignored because of the significant improvement in the sensitivity of culture due to the use of antibiotics (Whittington, 2009; Sattar *et al*., 2018).

The next step after decontamination/disinfection is the concentration of the MTBC cells. The most common methods of MTBC concentration from wastewater are filtration (0.2-0.5 um) and centrifugation (Martin *et al*., 2018; Kshirsagar *et al*., 2017). After cell concentration, the mycobacterial cells are isolated using specific culture media. Middlebrook 7H9 broth (mostly used for enrichment or recovery of MTBC), 7H10 agar, 7H11 agar or Lowenstein Jensen (L-J) slants are the most commonly used isolation media for *Mycobacterium* spp, with

recommended incubation temperature of 35°C-37°C for 6-12 weeks for slow-growing mycobacteria (Velayati *et al*., 2015; Suliman *et al*., 2017; David *et al*., 2018). Solid media may also at times be supplemented with a group of antibiotics such as Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim (PACT) or Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, Azilocillin (PANTA) (Kang *et al*., 2020; Srivastava *et al*., 2020). Furthermore, malachite green, which is the selective antifungal agent in L–J, shows inhibitory effects on the growth of different mycobacterial species (Sattar *et al*., 2018).

MTBC has been successfully cultured from environmental samples (Velayati *et al*., 2015; Barbier *et al*., 2016), using the approaches mentioned above. However, limited sensitivity has been observed due to bacterial overgrowth and the presence of "differentially culturable" (or "viable but nonculturable") MTBC organisms (Barbier *et al*., 2016; Chengalroyen *et al*., 2016; Mukamolova *et al*., 2010). Many bacteria, including a variety of important human pathogens, are known to respond to various environmental stresses by entry into a novel physiological state, where the cells remain viable but are no longer culturable on standard laboratory media (Ramamurthy *et al*., 2014; Batyrshina and Schwartz, 2019 Trutneva *et al*., 2020). On resuscitation from this 'viable but nonculturable' (VBNC) state, the cells regain culturability and the renewed ability to cause infection. In the case of wastewater, some members of MTBC have also been reported as amoeba-resistant (Medie *et al*., 2011). Additionally, MTBC in wastewater may enter into the VBNC state in response to stresses such as lack of oxygen, nutrient scarcity, predation (e.g amoeba), chemical stress (chlorine). This could be one of many ways through which they may be able to survive most wastewater treatment processes in addition to their intrinsic abilities to survive extreme environmental conditions. The VBNC state is likely a survival strategy, although several interesting alternative explanations have been suggested. For example, it appears that the 'latent' or the 'dormant' phase of *M. tuberculosis* infections represents the VBNC state in this pathogen (Shleeva *et al*., 2004; Young *et al*., 2009; Batyrshina and Schwartz, 2019) and that the recurrence of tuberculosis years after a person was thought to be tuberculosis free is due to resuscitation of this pathogen from the VBNC state (Pai *et al*., 2000; Ramamurthy *et al*., 2014). As cells in the VBNC state are no longer culturable, alternate nonculture methods must be used to demonstrate that cells in this state are alive. Commonly used are reagents (e.g. the BacLights Live/Dead assay) designed to demonstrate, through direct microscopic examination, the presence of an intact cytoplasmic membrane (e.g. the BacLights Live/Dead assay) (Oliver *et al*., 2010). Despite the application

of these methods for the detection of these MTBC cells using culture-based techniques, there is no consensus on the method yielding the highest number of mycobacteria.

Suliman *et al*. (2017) reported on the detection of MTBC organisms from hospital sewage water and drinking water by conventional culturing techniques, followed by biochemical analysis. They were successful in detecting *M. tuberculosis* from 80% of hospital wastewater samples from different locations. Velayati *et al*. (2015) also reported a higher recovery of *M. tuberculosis* from water (86.5%) than soil (13.4%). The majority of *M. tuberculosis* isolates were recovered from raceway systems (56 of 500, 11.2%) or dump water (15 of 200, 7.5%). Three multidrug-resistant *M tuberculosis* (MDR-TB) (3.6%), four mono drug-resistant strains (three isoniazid and one rifampicin, 4.8%), and 58 pan susceptible strains (70%) were also detected among the water and soil isolates.

2.6.2. Molecular methods for the detection of MTBC in wastewater

The development of molecular methods has assisted in addressing some of the challenges associated with the detection of MTBC cells in environmental matrices. While conventional molecular methods (e.g. polymerase chain reaction (PCR)) do not distinguish viable from non-viable organisms, several molecular methods have been developed to do so, including detection of mRNA or selective detection of intracellular markers (Cangelosi and Meschke, 2014; Li *et al*., 2010; Weigel *et al*., 2013). An increasingly popular molecular method that can be used to detect MTBC cells in the VBNC state is reverse transcriptase (RT)-PCR, which detects RNA. Because the half-life of bacterial mRNA is typically only 3–5 min (Conway and Schoolnik, 2003), continued gene transcription by non-culturable cells is considered an excellent indicator of bacterial cell viability. Molecular detection of MTBC has been demonstrated in filtered air samples (Wood *et al*., 2016), however, no study to date has applied these molecular techniques to detect MTBC in wastewater samples. This is despite an increasingly robust literature on the detection of various pathogens in natural and built environments (Afshinnekoo *et al*., 2015; Martinez *et al*., 2019). Therefore, there is a knowledge gap in relation to molecular detection of MTBC in wastewater using PCR techniques.

2.6.3. High-throughput sequencing for the detection of MTBC in wastewater

Other genomic or molecular methods such as sequencing have been applied to successfully identify pathogens (Table 2.5), study population structure and pathogen evolution among other outcomes (Gilchrist *et al*., 2015; Schürch *et al*., 2018). For instance, whole-genome sequencing (WGS) has become the preferential technique for infectious disease epidemiology such as

tuberculosis, support for public health and veterinary health professionals in decision making (Orloski *et al*., 2018; Meehan *et al*., 2019; Crisan *et al*., 2019). WGS approaches use DNA sequencing platforms to reconstruct DNA sequences of the genome of an organism (Meehan *et al*., 2019). MTBC strains have a single-chromosome genome, which makes these organisms well suited for WGS (Gordon and Parish, 2018). The use of WGS for the design and implementation of direct patient treatment and improvement of surveillance systems has been reported in certain countries in relation to *M. tuberculosis (*Meehan *et al*., 2019; Tagliani *et al*., 2018).

Irrespective of the sequencing platform, there is a common pathway or workflow, these are; (1) nucleic acid extraction (either DNA or RNA) is first extracted from the samples or isolates; (2) enzymatic processing of extracted nucleic acid; (3) sequencing of multiple fragments of nucleic material in parallel; and (4) finally bioinformatic analyses of data generated from the sequencing (Guimaraes, and Zimpel, 2020).

The use of sequencing approaches such as sanger sequencing for the detection of MTBC organisms in wastewater samples has seen an increased interest in recent years. Some of these reports do not provide species identification, with identification down to only the genus (Fang *et al*., 2018; Oluseyi Osunmakinde *et al*., 2019; Ng *et al*., 2019; Balcom *et al*., 2016; Bedoya *et al*., 2019; Leddy *et al*., 2017). However, others have identified known human pathogens, such as *M. tuberculosis* (Rosso *et al*., 2018) and animal pathogens, *M. bovis* (Li *et al*., 2015). Additionally, other lesser-known species have been identified through this sequencing approach (Li *et al*., 2015; Giwa *et al*., 2019). Table 2.5 presents some of the publications on the use of different sequencing approaches for the detection of mycobacteria in wastewater and sludge. Therefore, advanced molecular sequencing methods/techniques such as shotgun sequencing could potentially play a significant role in the detection of diverse MTBC in wastewater (Table 2.5).

Table 2.5: Detection of *Mycobacterium* spp organisms using sequencing approaches

Specific MTBC organism	Sample matrix	Study location	Detection method	Reference
Mycobacterium avium, Mycobacterium abscessus, Mycobacterium bovis, Mycobacterium kansasii, Mycobacterium marinum	Wastewater	Hong Kong	Illumina HTS	Li *et al*. (2015)
Mycobacterium sp., *Mycobacterium fortuitum*	Wastewater and sludge	China	Illumina HiSeq	Giwa *et al*. (2019)
Mycobacterium sp		China	Illumina Hiseq	Fang *et al*. (2018)
Mycobacterium sp	Wastewater	South Africa	16S-rRNA-Based Amplicon Sequencing	Oluseyi Osunmakinde *et al*. (2019)
Mycobacterium sp	wastewater	Singapore	Illumina HiSeq2500	Ng *et al*. (2019)
Mycobacterium sp	wastewater	Vietnam	Illumina TruSeq	Balcom *et al*. (2016)
Mycobacterium sp	Biosolids	Colombia	Illumina MiSeq	Bedoya *et al*. (2019)
Mycobacterium tuberculosis, Mycobacterium sp	wastewater	USA	Illumina MiSeq	Rosso *et al*. (2018)
Mycobacterium sp	wastewater	Taiwan	Illumina HiSeq PE150	Liu *et al*. (2019)
Mycobacterium sp	wastewater	USA	NGS—next-generation sequencing	Leddy *et al*. (2017)

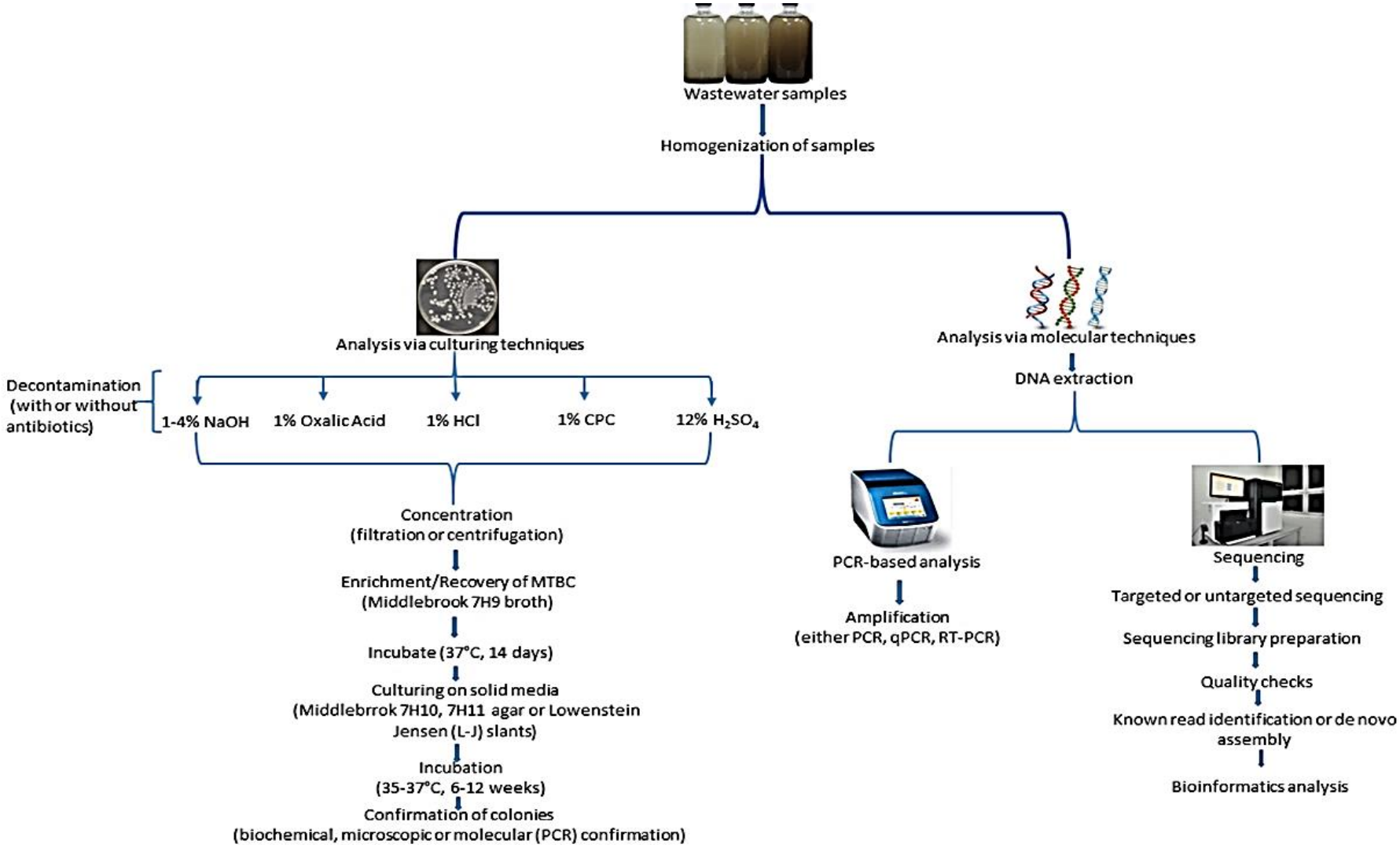

Figure 2.9: Representation of the common sample-processing framework for the detection of MTBC in wastewater samples

2.7. One health approach to AMR

The One Health and Global Health approaches have been used to address problems associated with infectious disease in general, and AMR in particular (Figure 2.10) (Hernando-Amado *et al*., 2019). The concept of One Health and Global health both integrate the knowledge of the biological elements necessary for understanding the evolution of AMR, including the microorganisms and vectors involved in its emergence and dissemination, the host organisms (human or animals) and the environments involved, and the socioeconomic and cultural features that facilitate its spread (Hernando-Amado *et al*., 2019). The One Health approach is "an integrative effort of multiple disciplines and multiple government sectors and partners working locally, nationally, and globally to attain optimal health for people, animals, and the environment" (Mackenzie *et al*., 2014). Together, the three make up the One Health triad; the health of each being inextricably connected to that of the others (DOH, 2017). In its most basic form, a description of One Health recognizes the relationships between human, animal and environmental health, and applies inter-and multi-disciplinary tools to solve complex public health problems (DOH, 2017). AMR occurs at the local level across the borders between different ecosystems, such as farms, hospitals, wastewater treatment plants and natural environments. This is a One Health problem, where the health of any of these ecosystems may affect the health of the others, including human health. Therefore, one health can be understood as a local version of Global health, which addresses communication among local ecosystems and the global conditions that facilitate the worldwide spread of AMR. This may occur through the worldwide interchange of goods by human travellers, migrating animals and even through the help of natural phenomena such as El Nino, which can expand the area for the interchange among geographical areas. Corridors and bridges, therefore, exist that promote the globalization of gene spread, encouraging the appearance of similar microbial communities whenever the same process occurs (Hernando-Amado *et al*., 2019).

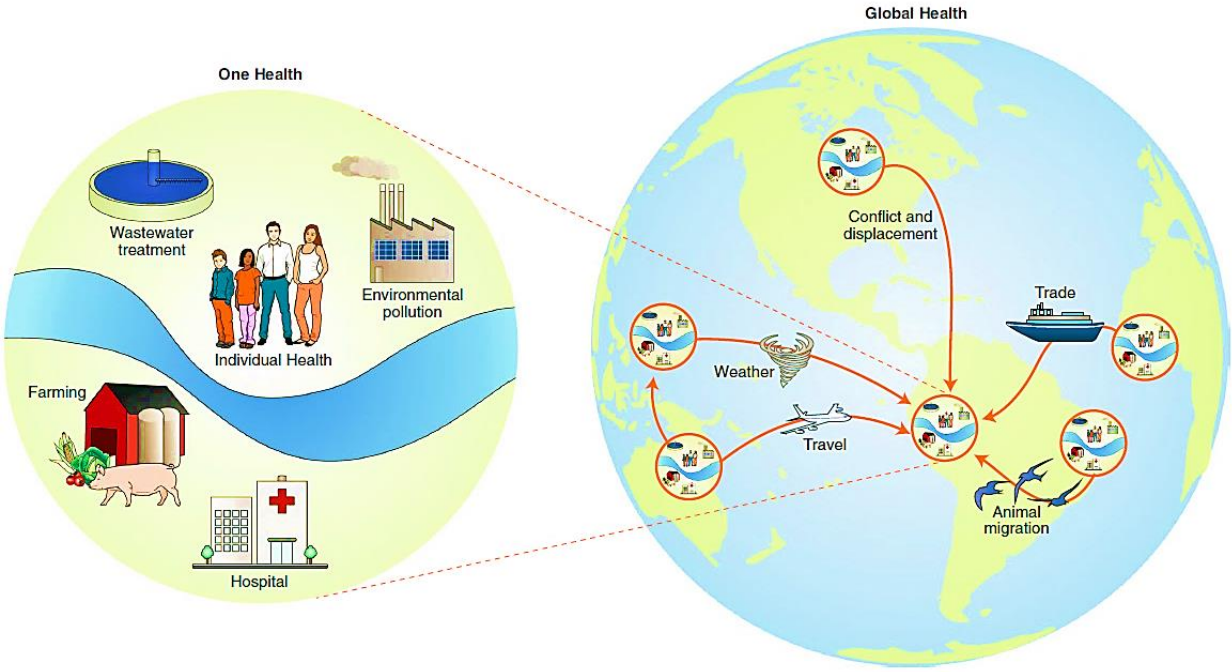

Figure 2.10: The One Health and global Health axes of antibiotic resistance (Hernando-Amado *et al*., 2019)

AMR emerges as the result of local confluences between bacteria colonizing different hosts (including humans and animals) and their shared environments where ARGs can be transferred from their original hosts to bacterial pathogens (Hernando-Amado *et al*., 2019). While resistomes across habitats are linked to the phylogeny of microbial populations along ecological gradients, clinically important resistance genes associated with mobile genetic elements (MGEs) can cross habitat boundaries (Pehrsson *et al*., 2016). Besides, microorganisms that form part of non-clinical ecosystems are, on many occasions, the original hosts of clinically important ARGs that have been transferred from environmental microorganisms to human pathogens (Hernando-Amado *et al*., 2019).

2.8. Application of Wastewater-based epidemiology approach

Wastewater-based epidemiology is an approach based on the human health biomarkers excreted via faeces and urine that end up in sewage (Figure 2.11). The untreated sewage/wastewater is collected either at a wastewater treatment plant or at the building level and analysed for these markers. This allows researchers affords researchers to ascertain the presence of the biomarkers of interest from a community perspective. Most studies have investigated this approach as an important tool to estimate the population habits such as substance consumption (Feng *et al*., 2018; Archer *et al*., 2018), human health (Daughton, 2012; Vitale *et al*., 2021) and nutritional status (Bowes and Halden, 2019).

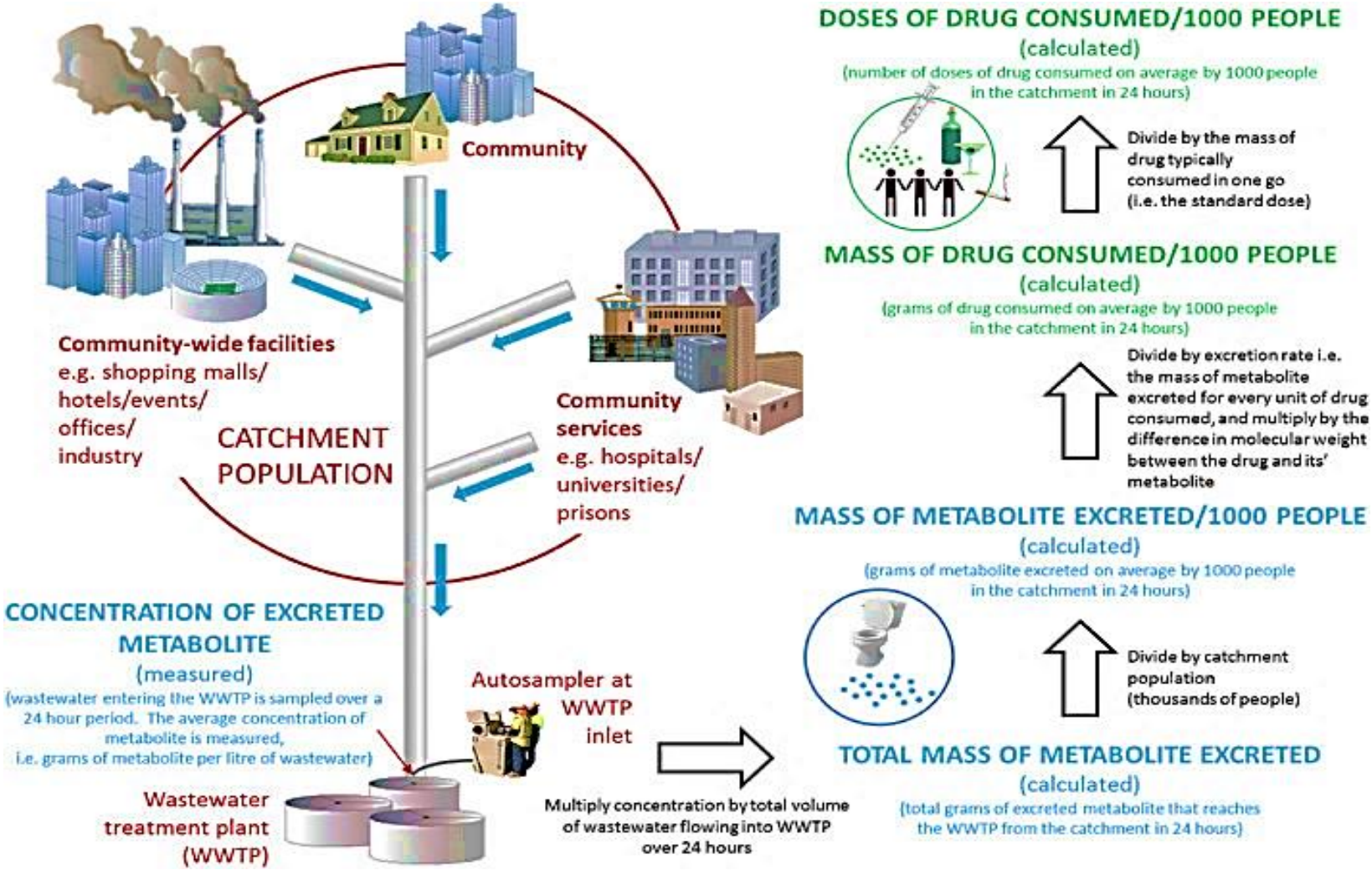

Figure 2.11: Detailed WBE approach (Choi *et al*., 2018b)

Recent studies also report this approach as a promising tool for the real-time collection of exposure/effects data that reflects the overall average health of entire communities (Devault *et al*., 2018). Some studies have applied WBE for antibiotic consumption (Choi *et al*., 2018b; Feng *et al*., 2018) and monitoring of diseases such as polio (Mao *et al*., 2020). The current COVID-19 pandemic has highlighted the usefulness of WBE in disease monitoring. This approach has been adopted and used to monitor COVI-19 infections in several countries, including South Africa (Ahmed *et al*., 2020; Medema *et al*., 2020; Wu *et al*., 2020; Kumar *et al*., 2020; Randazzo *et al*., 2020; La Rosa *et al*., 2020; Wurtzer *et al*., 2020; Pillay *et al*., 2021). O'Brien and Xagoraraki. (2019) proposed a water-focused one-health approach for the early detection and prevention of viral outbreaks, this could potentially be applied to other infectious diseases outbreaks such as drug-susceptible and drug-resistant tuberculosis. The use of biomarkers for infectious disease in analysing untreated wastewater could assist in broadly monitoring these infectious diseases in near-real-time (Choi *et al*., 2018). Analyses of the untreated wastewater could therefore provide an insight into the ARGs circulating in the connected population. Therefore, this approach contributes to the development of alternate AMR surveillance systems to complement the existing clinical-based surveillance, hospital admission data, questionnaires, surveys, motility and morbidity rates and sentinel surveillance systems (Mao *et al*., 2020).

CHAPTER THREE

3. Molecular surveillance of tuberculosis-causing mycobacteria in wastewater

(This paper is under review at *Heliyon*)

3.1. Introduction

Tuberculosis (TB) is a communicable disease caused by a group of closely related, slow-growing mycobacteria collectively named *Mycobacterium tuberculosis* complex (MTBC) (Forbes *et al*., 2018). Most TB infections in humans are caused by *M. tuberculosis,* however, there are other members of the MTBC that cause TB in both humans and animals. These include *M. bovis*, the causative agent of tuberculosis in both animals and humans (via zoonotic infections) (Walter *et al*., 2012; Howell *et al*., 2019; Nugent *et al*., 2017; Inlamea *et al*., 2020), *Mycobacterium africanum*, the causative agent of human tuberculosis (mainly in Western Africa) (Gehre *et al*., 2016; Tientcheu *et al*., 2016) and *Mycobacterium canetti* isolated in the Horn of Africa (Loukil *et al*., 2019) and reported in human infections however, its natural reservoir, host range, and mode of transmission still remains debatable. Lesser-known members of this group are *M. microti*, *M. caprae, M. pinnipedii* and *M. mungi,* usually associated with animal infections with possible transmission to humans.

Globally, an estimated 10.0 million (range, 8.9–11.0 million) people were infected with TB in 2019, with a mortality of 1.5 million deaths annually (Visca *et al*., 2021; WHO TB report, 2020). Human immunodeficiency virus (HIV) infection is considered an important risk factor for contracting TB in most African countries, especially in South Africa, with co-infection associated with increased morbidity and mortality (Mesfin *et al*., 2014; Travis *et al*., 2019). Over 70% (6 million) of humans co-infected with TB and HIV/AIDS live in sub-Saharan Africa where bovine TB represents a potential health hazard to humans (Ayele *et al*., 2004; Ntloko *et al*., 2019). Estimates of the burden of disease caused by TB and measured in terms of incidence, prevalence and mortality are produced annually by WHO using information gathered through surveillance systems (case notifications and death registrations), special studies (including surveys of the prevalence of disease), mortality surveys, "inventory studies" of under-reporting of detected TB, in-depth analysis of surveillance and other data, expert opinion and consultations with countries (Glaziou *et al*., 2016). However, in resource-poor countries, monitoring of tuberculosis/ drug-resistant tuberculosis (DR-TB) is a major challenge because assays are costly and time-consuming, and laboratories are ill-equipped. This has led

to underestimation/reporting of TB cases in such countries (Oga-Omenka *et al*., 2020; Zarowsky *et al*., 2020; WHO, 2020). Therefore, alternative means of estimating to complement the existing surveillance systems would be beneficial. There is evidence to support intestinal infections associated with extra-pulmonary TB due to the detection of tuberculosis causing organisms in human faeces (Bosch, 2018; Abaye *et al*., 2017; Walters *et al*., 2018). Few studies from the 1960s-70s reported on the isolation of *M. tuberculosis* from the environment, such as hospital sewage (Buczowska, 1965; Buraczewski and Osinski, 1966), households (Buczowska, 1965, Buraczewski and Osinski, 1966; Poptsova,1974) and farms (Kazda, 2010; Skurski *et al*., 1965, Szulga *et al*., 1965). Therefore, the concept of wastewater-based epidemiology (WBE) could be adopted to provide additional information on the TB burden. WBE is based on the assumption that any stable substance that is excreted by humans and in sewage/wastewater can be used to estimate the original concentration excreted by the serviced population. The same concept can be used for the analysis of pathogen circulation in sanitary sewers in a given population, when excreted in the faeces/urine of infected people (Mao *et al*., 2020; Polo *et al*., 2020). Such an approach is a useful tool, especially where resources for clinical diagnosis are limited and when reporting systems are unavailable or inefficient (Kitajima *et al*., 2020; Hart and Halden, 2020; Thompson *et al*., 2020; Prado *et al*., 2021). This approach has seen increased interest during the COVID-19 pandemic (Ahmed *et al*., 2020; Gonzalez *et al*., 2020; Hart and Halden, 2020). WBE studies could potentially help reconstruct spatially explicit transmission chains: not only "who infected who", but where they were infected, which may give an idea of how they were infected (Sims and Kasprzyk-Hordern, 2020; Martinez *et al*., 2019).

TB primarily spreads from person-to-person by aerosolized infective tubercle particles (Shiloh, 2016). However, there are observations that TB could be transmitted through other means, such as through faecal-oral transmission (Santos *et al*., 2015). Despite the potential role of the environment in TB transmission, there is limited information on the occurrence of the causative organisms in the environment. This could be attributed to the lack of sensitive and scalable techniques to detect MTBC in environmental samples (Santos *et al*., 2015; Barbier *et al*., 2016; Martinez *et al*., 2019). *M. tuberculosis* can be cultured from soil and other materials, but sensitivity may be limited due to bacterial overgrowth and the presence of “differentially culturable” (or “viable but non-culturable”) organisms (Chengalroyen *et al*., 2016; Mukamolova *et al*., 2010). A better understanding of *M. tuberculosis* complex viability in various environmental matrices requires methods for optimal promotion of their growth following recovery from the environment. This challenge could be addressed with the use of

molecular techniques, such as the polymerase chain reaction (PCR). Molecular detection of *M. tuberculosis* complex has been demonstrated in filtered air samples (Wood *et al*., 2014), but few studies are investigating its detection in water or wastewater samples (Fine *et al*., 2011; Li *et al*., 2015; Rosso *et al*., 2018; Guo *et al*., 2019).

The use of more sensitive and accurate molecular methods for the detection of tuberculosis-causing mycobacteria in wastewater could play a significant role in developing the WBE approach for estimating TB burden. This will complement or be used as an alternative to the current surveillance methods in place. Additionally, these methods could theoretically be used to ascertain the potential risk of TB infection in community settings due to exposure to wastewater. The paper aims to evaluate a molecular surveillance strategy for the detection of tuberculosis-causing mycobacteria in both untreated and treated (post-chlorination) wastewater in KwaZulu Natal (KZN), South Africa.

3.2. Methodology

3.2.1. Study site

Municipal wastewater was sampled from three wastewater treatment plants (WWTPs) in the city of Durban, South Africa referred, to as WWTP A, WWTP B and WWTP C. The selection of the WWTPs was based on plants serving at least a population of 10 000 individuals, and those receiving hospital sewage. These WWTPs also had different treatment configurations and capacities, details of which are presented in Table 3.1. Wastewater samples were taken on four separate occasions.

Table 3.1: Details of the wastewater treatment plants used for this study.

Treatment works	Design Capacity (Mℓ/d)	Primary Settling	Activated Sludge	Secondary clarification	Bio-filters	Sludge Digestion	Tertiary treatment	Remarks
WWTP A	18.8	Yes	No	No	Yes	Yes	Yes	Receives from **Hospital A**, which offers health services to the community at regional and district levels and has 17 clinics attached. The hospital is one of the sites for Mother-To-Child-Transmission (MTCT) of HIV and has the largest crisis centre - now called the 'Place of Comfort'.
WWTP B	4.90	N/A	Extended Aeration	Yes	No	No	Yes	Receives from **Hospital B** which serves the population of Chatsworth and the surrounding area, Inner and Outer West, the boundaries commence from Yellowwood Park to Richmond. This is also a referral hospital for another hospital and clinic
WWTP C	70.0	Yes	Conventional	Yes	No	Yes	Yes	Receives from **Hospital C** complex which offers specialised services for **multi-drug-resistant (MDR) and complicated TB**

Information sourced from Cross and Buckley (2016).

3.2.2. Sample collection and processing

One litre composite sample from all the WWTP was taken from the influent (raw/untreated wastewater) and effluent (treated wastewater) each. Composite samples comprising of many subsamples were taken, i.e. approximately 100 ml water samples were additively taken at 30 second intervals until a total of 1 L was achieved. Samples were transported to the laboratory in a cooler box with ice, stored at 4°C and analysed within 48 hours. Two samples (Influent and effluent (post-chlorination)) were taken per WWTP. Before analysis, samples were homogenised, and 50 mL subsamples were taken and centrifuged at 3000 rpm for 20 min. The supernatant was discarded, and the pellet was used for DNA extraction. DNA was extracted using a DNeasy Powersoil DNA extraction kit (QIAGEN), following the extraction procedure provided by the manufacturer with no modification. The quantity and quality of the extracted DNA were determined using IMPLEN NanoPhotometer NP80 – All-in-One Spectrophotometer. All analyses were done in triplicate.

3.2.3. Optimization of Polymerase Chain Reaction (PCR) conditions for detection of target organisms in wastewater

Method optimization was done using published primers targeting total mycobacteria, *M. tuberculosis* complex, *M. tuberculosis*, *M. africanum, M. bovis* and *M. caprae*. In this study, regions of differences (RDs) in these various organisms were targeted based on their uniqueness. However, it must be noted that some of these regions of differences are shared among the species within the *M. tuberculosis* complex and different studies report them differently. For instance, the total mycobacteria were targeted using the 16s rRNA gene (Chae *et al*., 2017), Rv0577 for *M. tuberculosis* complex (Chae *et al*., 2017), RD9 for *M. tuberculosis* (Perez-Osorio *et al*., 2012; Chae *et al*., 2017), RD8 present- 150 bp (Asante-Poku *et al*., 2015), RD1 present for *M. bovis* (Kim *et al*., 2013), RD4 present for *M. caprae* (Domogalla *et al*., 2013).

The limit of detection (LOD) of the PCR protocol was determined using positive control DNA of *M. tuberculosis* H37Rv strain. The concentration of the target was diluted (10^{-1}- 10^{-4}) to the following copies/µl: 59.2, 18.6, 13.75 and 7.06 respectively for conventional PCR to determine the lowest concentration of *M. tuberculosis* detectable.

The PCR mixture for all targeted organisms contained 12.5 ul of OneTaq 2X Master Mix with Standard Buffer (New England BioLabs inc), 2 µl of primer mix containing 1 µl of each primer set (final concentration of 0.2-0.4 µM), 1 µl (<60 ng/µl) of DNA template, and 9.5 µl of

molecular grade water in a final volume of 25 µl in a reaction tube. PCR amplification was performed in Veriti™ 96-Well Thermal Cycler. Optimised thermocycling conditions were initial denaturation step at 95°C for 10 min and followed by 30 cycles of 96°C for 45 s. The annealing temperatures varied for each primer (organisms), total mycobacterial species (16s rRNA gene- 500bp) at 61.5°C for 45 s, *M. tuberculosis* complex (Rv0577-700bp) at 54°C for 60 s, *M. tuberculosis* (RD9 present- 369 bp) at 59°C for 60s, *M. africanum* (RD8 present- 150 bp) at 68°C for 60 s, *M. bovis* (RD1 present-264bp) at 57°C for 60s and *M. caprae* (RD4 present) at 58°C for 60s and extension at 72°C for 40 s. The final extension step was performed at 72°C for 10 min.

PCR products were analyzed by 2% agarose gel electrophoresis performed at 70 V in 1 X Tris-Borate EDTA (TBE) buffer. The agarose gel was pre-stained with ethidium bromide (final concentration of 02.-0.5 µg/mL. The PCR products were visualized using a Bio-Rad Gel Doc™ XR (Biorad).

3.2.4. Determination of the presence of total mycobacteria, MTBC, *M. tuberculosis, M. africanum, M. bovis and M. caprae* in treated and untreated wastewater by conventional PCR

The optimized conventional PCR protocol (was used to determine the presence of the selected organisms or group of organisms in wastewater from Durban, South Africa. Wastewater samples were collected and processed using the methodology described above (Section 3.2.2).

3.2.5. Determination of the concentration of total mycobacteria, MTBC, *M. tuberculosis, M. africanum, M. bovis and M. caprae* in treated and untreated wastewater

The Rv0577 primer for *M. tuberculosis* complex mentioned in section 3.2.3 was used to determine the limit of detection for *M. tuberculosis* using a measured DNA template (1.5 ng/µl of *M. tuberculosis* H37Rv strain). The DNA template was serially diluted from 10^{-1} to 10^{-9}.

The concentration of these organisms was determined using the droplet digital PCR (ddPCR). The same set of primers presented in Table S1 (appendices) were used. The ddPCR analysis was performed in a 20 µL reaction volume, containing, 10 µL of 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad), 1–20 ng/µL of template DNA quantified by the IMPLEN NanoPhotometer NP80 – All-in-One Spectrophotometer, forward primers (FP) and reverse primers (RP), each at a final concentration of 250 nM and RNase/DNase free water.

Droplets were generated using the automated droplet generator and the following amplification protocol was followed: Optimised thermocycling conditions included an initial denaturation step at 95°C for 10 min and followed by 30 cycles of 96°C for 45 s, the annealing temperatures varied for each primer (organisms), total mycobacterial species (16s rRNA gene- 500bp) at 61.5°C for 45 s, *M. tuberculosis* complex (Rv0577-700bp) at 54°C for 60 s, *M. tuberculosis* (RD9 present- 369 bp) at 59°C for 60s, *M. africanum* (RD8 present- 150 bp) at 68°C for 60 s, *M. bovis* (RD1 present-264bp) at 57°C for 60s and *M. caprae* (RD4 present) at 58°C for 60s and final incubation step was performed at 98°C for 10 min (ramp rate 2.2°C/s). These conditions were applied to the wastewater samples. After thermal cycling, the ddPCR plates were read using the QX200 droplet reader (Bio-Rad). Droplet counts and amplitudes were analysed with QuantaSoft™ analysis Pro software (Bio-Rad).

3.2.6. Statistical analysis

Descriptive statistics were performed with Microsoft Excel, and a test of normality was performed based on the Akaike Information Criterion (AIC) score measured with @Risk (Palisade Inc. USA). Based on the normality tests, a comparison of the concentration of the different tuberculosis-causing mycobacteria was achieved using the Kruskal-Wallis tests followed by Dunn's Multiple Comparison tests. The difference in the concentration of the mycobacteria between untreated and treated wastewater was compared using the Mann-Whitney tests. All statistical tests were performed at a 95% confidence interval and a p-value ≤ 0.05 was considered statistically significant. All statistical analyses were done using GraphPad Prism (Version 7.0, GraphPad Software, USA).

3.3. Results

3.3.1. Determination of the presence of total mycobacteria, MTBC, *M. tuberculosis, M. africanum, M. bovis and M. caprae* in treated and untreated wastewater by conventional PCR

Conventional PCR was optimized with the limit of detection determined to be 18.6 copies/µl using Rv0577 for the total *M. tuberculosis* complex primer. The detected mycobacterial organisms varied between the three WWTPs. Total mycobacteria was detected in all (treated and untreated) wastewater samples analysed (Figure 3.1). Similarly, *M. tuberculosis* complex (MTBC) was present in all (100%) untreated and the majority (75%) of treated wastewater samples from all three WWTPs (Figure 3.1). *M. bovis* and *M. caprae* were detected in 100% of all untreated wastewater and *M. bovis* was present in 50%, 75% and 100% of the treated wastewater in WWTP A, WWTP B and WWTP C respectively. *M. tuberculosis*, the main

causative agent for human tuberculosis, was detected in 75% of untreated samples from both WWTP A and WWTP C and 100% of untreated wastewater samples from WWTP B.

The least prevalent was *M. africanum*, which was detected in 25% of untreated samples in WWTP A, 50% in WWTP B, and not detected in WWTP C. However, their presence in the treated wastewater was lower, with 25% at WWTP A and no detection in both WWTP B and WWTP C.

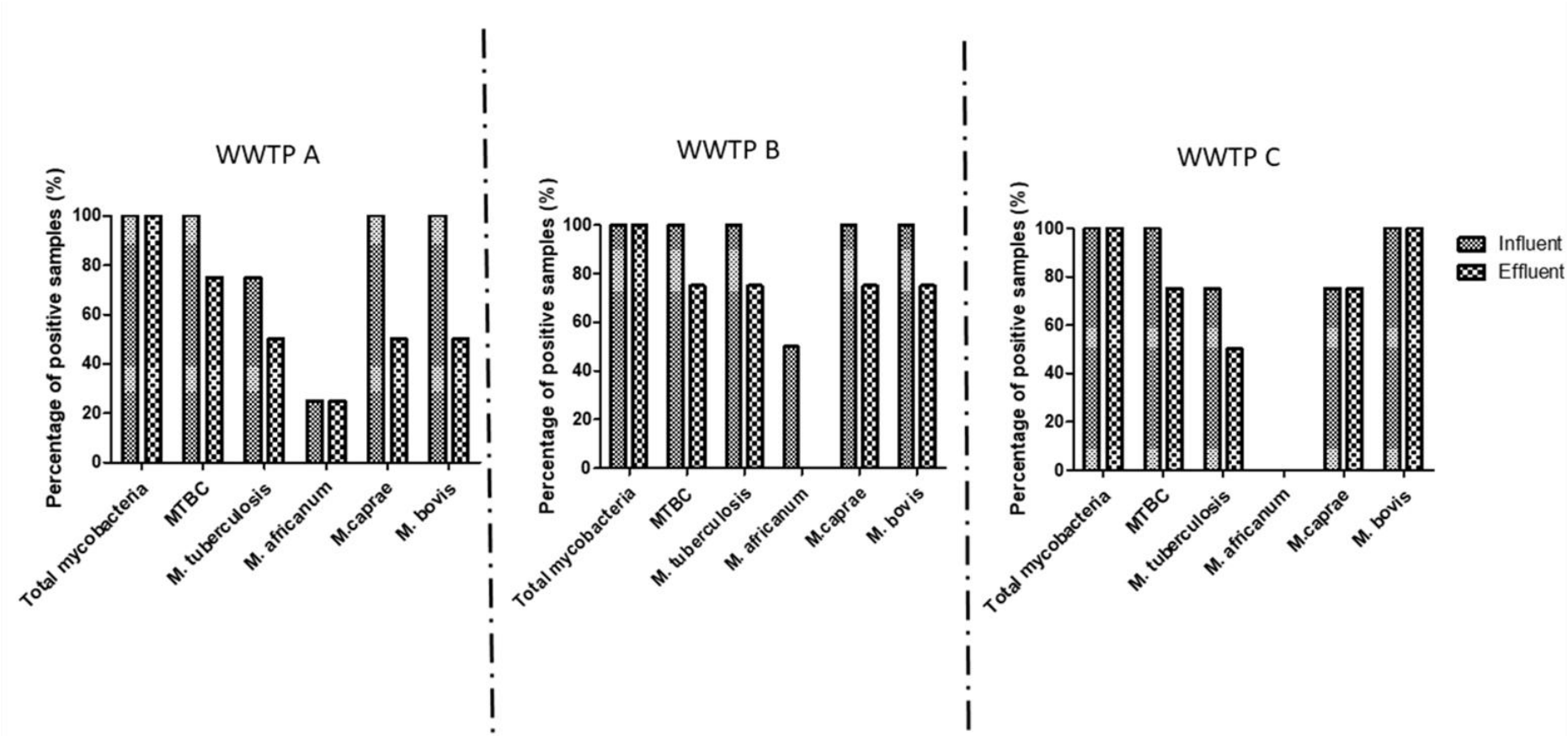

Figure 3.1: Percentage of influent and effluent wastewater samples showed positive for total mycobacteria, *M. tuberculosis Complex, M. tuberculosis, M. africanum, M. bovis and M. caprae* ([1]N=12).

[1] Wastewater samples were taken on four separate occasions for both influent and effluent. All analysis were in triplicate.

3.3.2. *M. tuberculosis* H37Rv strain limit of detection for the ddPCR assay

The standard/reference *M. tuberculosis* (H37Rv strain) DNA was determined to have an average of 9226(±642.1) copies/mL. The LOD after ten-fold serial dilutions was determined to be 3.0 (±0.06) gc/ml (Figure 3.2) with an average of 18,892 droplets generated per well.

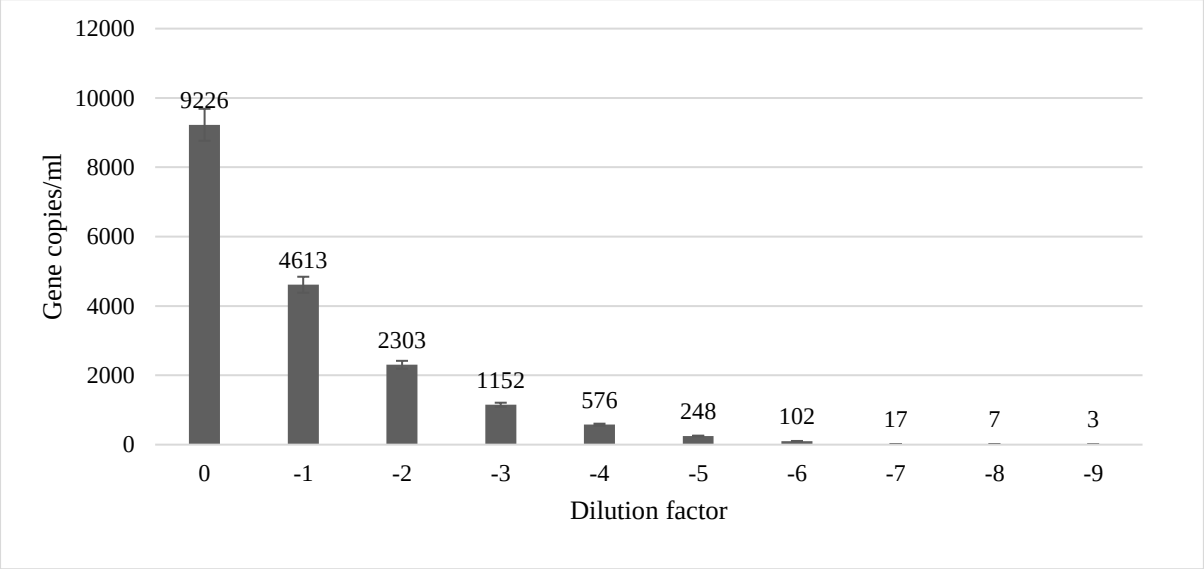

Figure 3.2: Limit of detection for *M. tuberculosis* using the droplet digital PCR.

**Key: 0= no dilution (standard/reference strain); -1= 10^{-1} dilution; -2 =10^{-2} dilution; -3 = 10^{-3} dilution; -4=10^{-4} dilution; -5= 10^{-5} dilution; -6=10^{-6} dilution; -7=10^{-7} dilution; -8=10^{-8}; -9= 10^{-9} dilution.*

3.3.3. Determination of the concentration of total mycobacteria, MTBC, *M. tuberculosis* spp., *M. africanum* spp., *M. bovis* spp., *M. caprae* spp. in treated and untreated wastewater

Comparing the concentration of the six organisms or group of organisms measured in the untreated wastewater across the three WWTPs showed differences in their concentrations. Between the WWTPs, a statistically significant difference in concentrations of each organism was observed. The highest median concentration for total mycobacteria in the untreated wastewater was recorded in WWTP A (4.9(±0.2) Log10 copies/ml), with the lowest median concentration of 4.8(±0.06) and 4.8(±0.47) Log10 copies/ml in WWTP B and WWTP C, respectively. The members of *M. tuberculosis* complex were more abundant in WWTP A, with a concentration of 4.0(±0.85) Log10 copies/ml as compared to both WWTP B (3.8(±0.33) Log10 copies/ml) and WWTP C (3.3(±0.52) Log10 copies/ml).

WWTP A and WWTP B influent had similar median concentrations of *M. tuberculosis* (Table 3.2) and a lower concentration of 2.8(±0.75) Log10 copies/ml was recorded in WWTP C. The organism with the lowest concentrations recorded in all untreated wastewater, irrespective of WWTP was *M. africanum,* this correlated with the percentage of samples with positive detection of this organism described in section 3.3.1 above (Figure 3.1). As shown in Table 3.2, the other two rarely occurring mycobacteria (*M. bovis* and *M. caprae*) were also recorded in low concentrations. However, comparing with *M. bovis* and *M. caprae,* the concentrations of *M. africanum* in the untreated wastewater samples were significantly lower (P-value ≤0.05).

Furthermore, within each WWTP, the concentration of these organisms was lower in the treated wastewater compared to the untreated wastewater concentrations described above. For instance, the concentration of total Mycobacteria in the treated and untreated wastewater were statistically significant (P-value ≤ 0.05) in all the WWTPs, except WWTP C. However, the difference in MTBC, *M. tuberculosis, M. africanum, M. bovis* and *M. caprae* concentrations in the treated and untreated wastewater in all the WWTPs was not statistically significant (P-value ≥ 0.05). Despite the reductions observed in all the WWTPs, concentrations up to 4log10 for these mycobacteria organisms are released into receiving water environments (Table 3.2).

Table 3.2: Median (standard deviation) concentration and range of the concentration (Log10 copies/mL) of total Mycobacteria, *M. tuberculosis* Complex, *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. caprae* in influent and effluent wastewater at the three WWTPs

	WWTP A				**WWTP B**				**WWTP C**			
	Influent		**Effluent**		**Influent**		**Effluent**		**Influent**		**Effluent**	
	Median(±SD*)	**Range**	**Median(±SD)**	**Range**	**Median(±SD)**	**Range**	**Median(±SD)**	**Range**	**Median(±SD)**	**Range**	**Median(±SD)**	**Range**
Total mycobacteria	4.9(0.2)	4.8-5.2	4.4(0.03)	4.3-4.4	4.8(0.06)	4.8-4.9	3.9(0.15)	3.8-4.2	4.8(0.47)	4.4-5.2	4.0(0.8)	3.2-4.7
MTBC[#]	4.0(0.85)	2.3-4.2	2.9(0.94)	1.8-3.9	3.8(0.33)	3.2-4.0	3.4(0.52)	2.8-4.0	3.3(0.52)	2.5-3.8	3.0(0.44)	2.5-3.4
M. tuberculosis	3.9(0.31)	3.3-4.0	3.5(0.34)	3.3-4.1	3.9(0.54)	2.9-4.1	3.7(0.44)	3.2-4.1	2.8(0.75)	2.8-4.3	2.7(0.62)	2.5-3.8
M. africanum	2.3(0.29)	2.0-2.7	2.5(0.31)	2.0-2.7	2.5(0.29)	2.2-2.8	2.3(0.18)	2.2-2.6	2.7(0.42)	2.1-3.1	2.5(0.16)	2.3-2.6
M. bovis	4.0(0.29)	3.5-4.2	2.8(0.97)	2.0-4.1	3.8(0.43)	3.4-4.2	3.8(0.74)	2.5-4.1	3.6(0.44)	3.2-4.2	3.9(0.68)	3.2-4.5
M. caprae	4.0(0.36)	3.7-4.5	3.7(0.36)	3.2-4.1	4.5(0.52)	3.5-4.7	4.3(0.87)	2.7-4.6	4.1(0.81)	2.5-4.3	3.8(0.84)	2.7-4.6

*Means Standard deviation, [#] refers to *M. tuberculosis* complex, n=12

3.3.4. Reduction in the concentration of tuberculosis-causing mycobacteria during wastewater treatment

The observed log reduction in each WWTP as presented in Figure 3.3-3.5 did not show any statistically significant differences when compared between the three WWTPs, irrespective of the organism or group of organisms (P-value ≥ 0.05). The highest median log reduction of 0.91 (±0.20) for total Mycobacteria was achieved by WWTP B. Similarly, WWTP A had the highest reduction in *M. tuberculosis* complex members of 0.71 (±0.65). Despite these differences in log reduction as can be seen in Figure 3.3, the Kruskal-Wallis test did not show any statistically significant differences.

Specifically, looking at tuberculosis causing mycobacteria, the highest median reduction in *M. tuberculosis* was observed at WWTP B (0.36 (±0.41)) and the lowest at WWTP A (0.21 (±0.29)). In respect of *M. africanum*, the highest reduction was recorded at WWTP C (0.22 (±0.28)) and the lowest at WWTP A (see Figure 3.4). The highest log reduction of *M. bovis* and *M. caprae* was observed in WWTP A (Figure 3.5). This study could not identify any single WWTP to have the most efficient log reduction for all mycobacteria tested.

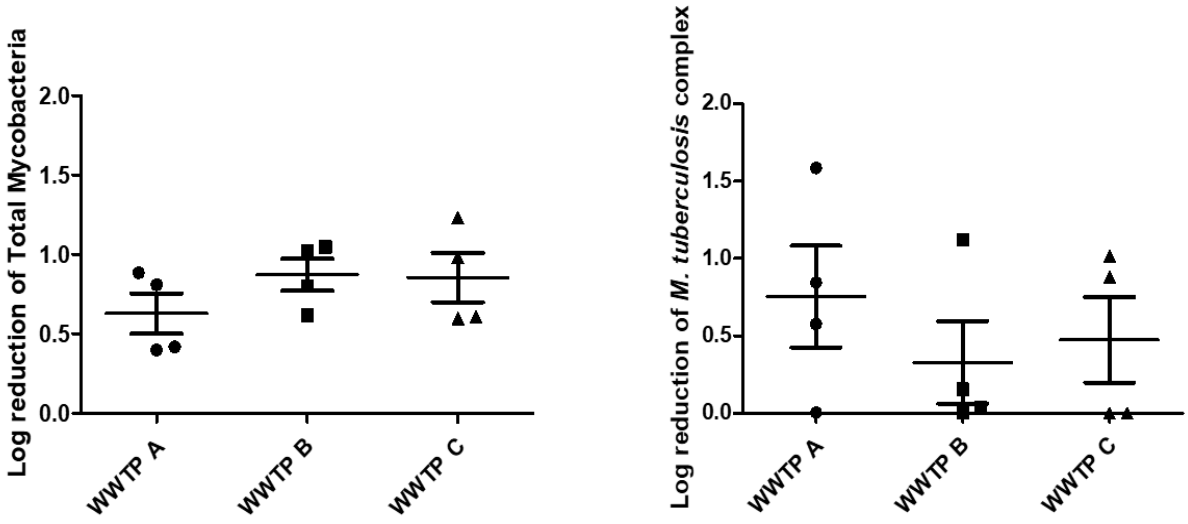

Figure 3.3: Log reduction of total mycobacteria and *M. tuberculosis* complex achieved by each WWTP.

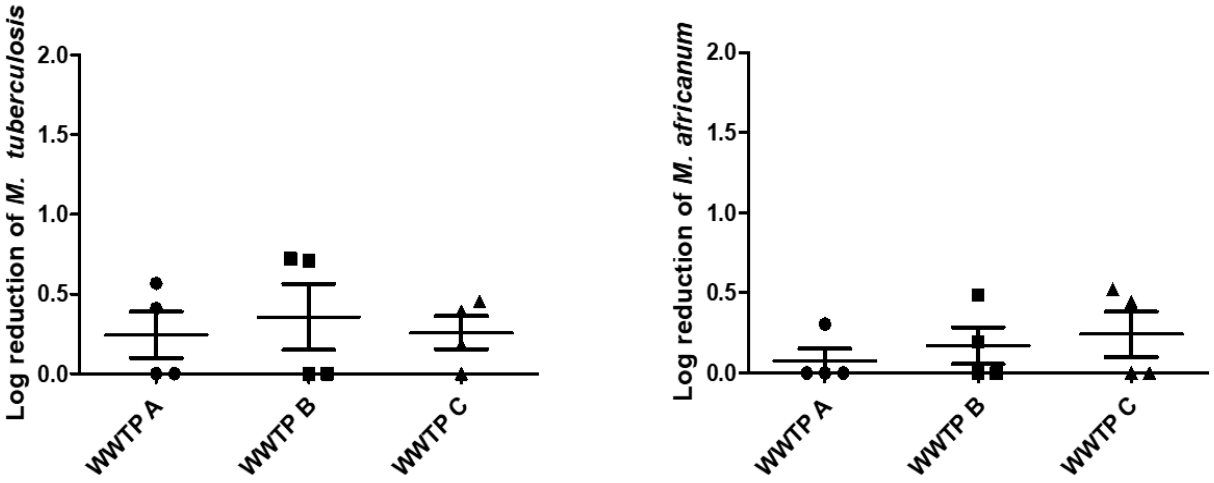

Figure 3.4: Log reduction of *M. tuberculosis* and *M. africanum* achieved by each WWTP.

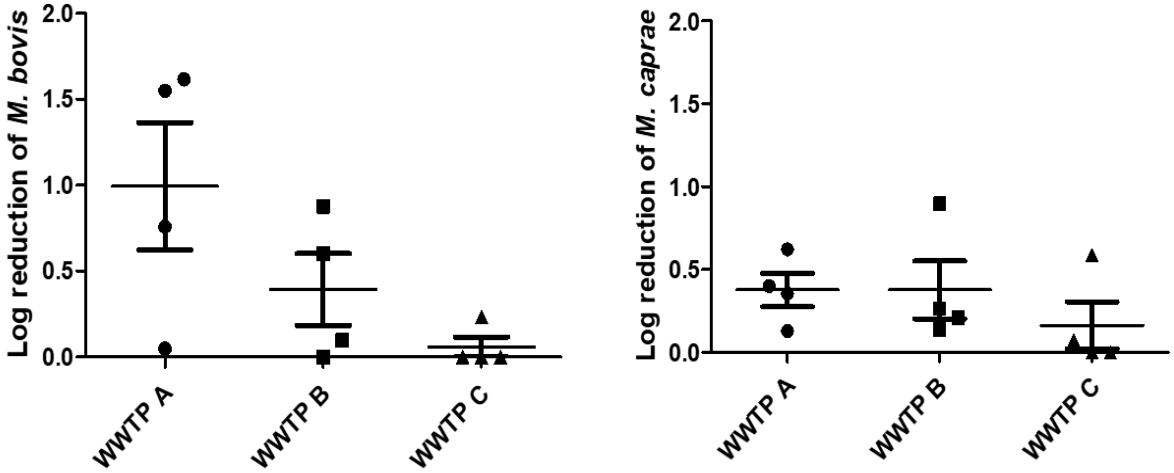

Figure 3.5: Log reduction of *M. bovis* and *M. caprae* achieved by each WWTP.

3.4. Discussion

Wastewater contamination with total mycobacteria, members of the *M. tuberculosis* complex and the other tuberculosis-causing mycobacteria could be attributed to several factors, including shedding of these organisms in human and animal faeces which end up in wastewater treatment plants from hospital sewage or domestic sewage. This study has shown the presence of different species of *Mycobacterium* in SA wastewater in varying abundance. The presence of mycobacteria known to cause tuberculosis infections in humans was considerably lower than the total mycobacteria, which is expected as it is comprising of all species of *Mycobacterium.* The prevalence of *M. tuberculosis* in untreated wastewater was between 75%-100% and *M.*

africanum ranged from 25%-50% (Figure 3.1). Comparing the two species, the prevalence of *M. tuberculosis* in this study correlates with the high prevalence reported in clinical studies conducted in KZN (Mzembe, 2020; Naidoo *et al*., 2018; Brown *et al*., 2019). *M. tuberculosis* was detected in wastewater using high-throughput shotgun sequencing techniques (Cai and Zhang, 2013) this study advocates for the presence of these human pathogens in wastewater. *M. caprae* and *M. bovis* were prevalent in 100% of the untreated wastewater from WWTP A and WWTP B and 75% of untreated wastewater for WWTP C, for *M. bovis*. Although *M. bovis* and *M. caprae* are mainly known as causative agents of TB in animals, reports of human TB caused by these mycobacteria have been published (Prodinger *et al*., 2014; Lan *et al*., 2016). Therefore, their higher prevalence compared to the other two known human pathogenic mycobacteria could result from their shedding in both human and animal faeces/urine from the domestic sewage (Cadmus *et al*., 2019). One of the significant findings is the detection of *M. africanum* in wastewater within South Africa. This organism consists of two phylogenetically distinct lineages, the *M. africanum* West African 1 (MAF1) and *M. africanum* West African 2 (MAF2) (Gagneux *et al*., 2006). *M. africanum* is endemic to West Africa and is known to cause up to half of the human TB in that region (De Jong *et al*., 2010). Therefore, the detection of *M. africanum* in wastewater from South Africa potentially indicates that some of the TB infections within the country could be caused by *M. africanum*. A study in the mid-2000s reported *M. africanum* as not the cause of tuberculosis in Cape Town, South Africa (Demers *et al*., 2010). However, besides this study being over a decade ago, it was specific to one province. The detection of these organisms could be due to the migration of people from West African countries into South Africa, thereby leading to the potential spread of the mycobacteria within the South African population. The detection of the various tuberculosis-causing mycobacteria, most especially *M. africanum*, in the untreated wastewater shows the potential for molecular surveillance of these organisms in wastewater contributing to WBE. Lorenzo and Picó (2019) proposed that this approach can become an early warning system for outbreaks of disease and a unique tool for the identification of hotspots for pandemics. The usefulness of WBE has already been exhibited with the current studies in relation to COVID-19 infections, where several countries, such as Australia (Ahmed *et al*., 2020), the Netherlands (Naughton *et al*., 2021), USA (Gonzalez *et al*., 2020), France (Barcelo, 2020) and South Africa (Pillay *et al*., 2021) have established national WBE systems. Therefore, the results obtained in this study further advocates this approach in complementing the existing surveillance systems for TB infections.

The concentration of the mycobacteria analyzed varied both by WWTP and by the organism. However, it was observed that total mycobacteria and MTBC concentrations were largely within the 4-$\log_{10}$ concentrations per ml of wastewater. Comparatively, the concentrations recorded in our study for total mycobacteria or MTBC were higher than the results published by Radomski *et al*. (2011). In the referenced study, mycobacteria concentrations of up to 5.5×10^5 ($\pm3.9\times10^5$) copies/L in untreated wastewater were reported. However, it is worth mentioning that Radomski *et al*. (2011) focused on non-tuberculosis mycobacteria (NTM). These are also known as environmental mycobacteria, consisting of more than 150 species, and are globally ubiquitous in both natural and man-made environments (Nishiuchi *et al*., 2017; Tortoli, 2014; Cai and Zhang, 2013; Cai and Zhang, 2014). The higher concentrations determined in this study could potentially be attributed to higher infection numbers in connected populations and their ability to survive in the environment for a long time (Hruska and Kaevska, 2012; Donohue *et al*., 2015). The high concentrations observed in WWTP A for all species could be attributed to the hospital sewage that the WWTP receives, WWTP A receives from 17 clinics which could represent highly concentrated sewage compared to the other two plants, regardless of the volume received daily.

The high concentration of the mycobacteria in the treated wastewater (up to 4 log10) could be due to the resistant nature of these organisms to environmental conditions and predators. For example, tuberculosis-causing bacteria have been reported to be amoeba-resistant which may enhance their survival in the environment, especially wastewater (Ghodbane and Drancourt, 2013). *M. tuberculosis* and *M. bovis* could survive for hours to days in the amoebal trophozoites (Hagedorn *et al*., 2009; Mardare *et al*., 2013). The observation that *M. tuberculosis* and *M. bovis* organisms were engulfed by *Acanthamoeba polyphaga* trophozoites agreed with previous observations made when co-culturing *M. tuberculosis* organisms with the free-living amoeba *Dictyostelium discodium* (Medie *et al*., 2011; Butler *et al*., 2020). Additionally, the higher concentrations observed in this study could be due to the use of the ddPCR platform for quantification of these microbes as against the qualitative PCR (qPCR) technique used by Radomski *et al*. (2011). The ddPCR platform has been reported to be more sensitive, accurate and less affected by PCR inhibitors, compared to qPCR (Rački *et al*., 2014; Jahne *et al*., 2020).

The percentage of treated wastewater samples with these organisms was lower than the untreated samples (Figure 3.2). This could be attributed to the reduction achieved by the wastewater treatment processes. The log reductions of the mycobacteria achieved by the three WWTPs varied, perhaps due to differences in treatment configuration or performance.

However, it was observed that each WWTP achieved the highest removal for at least one member of MTBC. For instance, the highest log removal for total mycobacteria was WWTP C, WWTP A achieved the highest removal of MTBC and WWTP B had the highest removal of *M. tuberculosis*. This indicates that despite differences in the treatment configuration for these WWTPs, there was no difference in their effectiveness in removing these mycobacteria. Therefore, the removal of mycobacteria could potentially be due to other factors apart from the WWTP configuration. These factors could include the ability of mycobacteria to resist some disinfection processes (Loret and Dumoutier, 2019) and the attachment of the mycobacteria cells to solids in the wastewater and the capacity of these cells to form biofilms due to the hydrophobic nature of these organisms (Radomski *et al*., 2011; Loret and Dumoutier, 2019; Cao *et al*., 2019; Jing *et al*., 2018). Hruska and Kaevska (2012) observed that as of 2012, there were no internationally accepted legal directives on how to control the public health risk associated with environmental mycobacteria. Additionally, the operational conditions of the WWTPs could have influenced the reduction achieved. For instance, the sub-optimal performance of the WWTPs could potentially result in a less efficient treatment process, thereby resulting in the detection of the bacteria in the treated wastewater. Therefore, the detection of potentially human pathogenic mycobacteria, like *M. tuberculosis* and *M. africanum* and potentially zoonotic species like *M. bovis* and *M. caprae* in the treated wastewater could potentially cause public health issues. However, it must be noted that detection of these organisms via DNA-based PCR does not necessarily indicate the presence of infectious pathogens as the assessment of the viability of these detected microorganisms was outside the scope of this study.

3.4.1. Limitations of the study and remarks on further work.

Although this study detected members of MTBC in both untreated and treated wastewater, the presence of these organisms may not necessarily translate to tuberculosis infections. Studies on the viability and infectivity of these organisms isolated from wastewater are essential. Also, longitudinal studies to assess the presence of these organisms over a period of time is warranted. The detection of these organisms in higher concentrations in both untreated and treated wastewater does highlight the need for further studies on the possibilities of health implications from the exposure to untreated wastewater and surface water that may be contaminated with wastewater.

3.5. Conclusion and recommendations

This study was successful in the application of molecular techniques for the detection of total mycobacteria, members of *M. tuberculosis* complex in total including *M. tuberculosis*, *M. africanum*, *M. bovis* and *M. caprae* in untreated and treated wastewater. Detection of these tuberculosis-causing mycobacteria in wastewater could potentially provide insight into infection epidemiology in the connected sewershed, provide information on potential infection risks and help in assessing the efficiency of wastewater treatment plants in removing these organisms. The detection of *M. africanum* in wastewater within South Africa shows the likelihood that some of the TB infections reported in the region could be caused by this bacterium, which is largely reported to be endemic in Western African countries. The findings, therefore, make a significant contribution towards the adoption of wastewater-based epidemiology as a cost-effective tool for TB surveillance.

It was also observed that the reduction in mycobacteria concentrations in wastewater could be due to other factors apart from the WWTP configuration. This is based on the observation that the reduction achieved by the different WWTPs were not statistically significant. For instance, the WWTP operational parameters could have potentially impacted the log reductions observed. Additionally, each WWTP reported the highest log reductions for at least one mycobacteria. The detection of potentially human pathogenic species of mycobacteria, such as *M. tuberculosis* and *M. africanum* highlights the potential health risks for populations that may be exposed to either the treated or the untreated wastewater. However, due to the inability of the molecular data obtained in this study to provide information on the infectivity of these bacteria, it is recommended that there should be further studies that focus on the determination of viability and infectivity. This will provide additional information necessary for decision-making with respect to risk reduction strategies.

CHAPTER FOUR

4. Wastewater-based surveillance of antibiotic resistance genes associated with tuberculosis treatment regimen in KwaZulu Natal, South Africa

(This paper is published at *Antibiotics*)

4.1. Introduction

Antimicrobial resistance (AMR) is a major threat to global health and a growing concern worldwide (WHO, 2016; Kairigo *et al*., 2020), and is mainly attributed to the excessive use and misuse of antibiotics (Varela *et al*., 2014). AMR related to tuberculosis (TB) has been extensively reported in clinical studies (Suthar *et al*., 2018; Fletcher, 2015; WHO, 2018). South Africa has one of the highest recorded prevalence of drug-resistant tuberculosis (DR-TB) patients globally (Cox *et al*., 2017; WHO, 2020), and particularly with the increasing burden of human immunodeficiency virus (HIV) coinfection (Nnadozie *et al*., 2017). Drug susceptible TB and DR-TB treatment regimens are classified into two groups; first-line and second-line TB drugs (Nguyen, 2016a; Koch *et al*., 2018). The TB/DR-TB treatment regimen in South Africa includes first-line TB drugs such as isoniazid, rifampicin, ethambutol and pyrazinamide, while the second-line TB drugs used for rifampicin-resistant (RR-TB), multidrug-resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), includes ofloxacin, moxifloxacin, bedaquiline, ethionamide, kanamycin, amikacin. Other drugs such as streptomycin have been reported in both first-line and second-line treatment regimens (Pitso *et al*., 2019). The One Health approach to combating AMR argues that human, animal and environmental health are interconnected and that all these sectors should be considered in the fight against AMR (Kahn, 2017). AMR in environmental matrices such as wastewater, surface water and treated water has been reported in various studies (Sobsey *et al*., 2014; Prestinaci *et al*., 2015; Sabri *et al*., 2018). However, none of these studies focused specifically on tuberculosis resistance or related genes in wastewater. The presence of these genes in wastewater may be attributed to secretions in the faeces and urine of infected individuals or animals. In urban areas with centralized sewage systems, the wastewater discharged from households, hospitals and pharmaceutical industries are collected and treated together in wastewater treatment facilities (Butkovskyi *et al*., 2015; Nanninga *et al*., 2012) and this wastewater reflects the health and habits of the community served by that treatment plant (Mao *et al*., 2020). Analyses of untreated wastewater (influent) could therefore provide an insight into the prevalence of drug-resistant TB in the served population. The analysis of raw

wastewater for surveillance of infections in connected populations has been proposed by several researchers (Daughton, 2020; Sims and Kasprzyk-Hordern, 2020; Mao *et al*., 2020; Pillay *et al*., 2021). This approach, referred to as wastewater-based epidemiology (WBE), could therefore contribute to the development of alternate AMR surveillance systems for TB to complement the existing clinical-based surveillance, hospital admission data, questionnaires, surveys, motility and morbidity rates and sentinel surveillance systems (Mao *et al*., 2020). Linking WBE and the One Health approach in monitoring and management of the occurrence and spread of drug-resistant TB in the population could contribute to early detection and public health mitigation strategies.

Additionally, AMR development in wastewater could be due to the presence of antimicrobials in this environment. It is reported that only 30% of the antibiotics consumed are metabolized in the human body whilst the major percentage is released to the environment through faeces and urine, either in their original form or as residues and conjugates (Nnadozie *et al*., 2017). Therefore, the extensive use of antibiotics used in TB/DR-TB treatment regimens could result in the excess release of antibiotics in wastewater and related environments, especially where there is a high prevalence of TB in the population (Tehrani and Gilbride, 2018). The presence of these antibiotics/antimicrobials, together with the high bacterial density, high nutrient and high oxygen conditions within wastewater treatment plants (WWTP) provides conducive conditions for the transfer of antibiotic resistance genes (ARGs), development of new antibiotic-resistant bacteria (ARB) and for creating hotspots for the spread of resistant bacteria and genes into the environment (Nnadozie *et al*., 2017; Rizzo *et al*., 2013). Therefore, the detection of ARGs coding for resistance to tuberculosis drugs in untreated and treated wastewater can be used to estimate the contribution of WWTPs in the emergence and dissemination of ARGs.

This study presents the molecular surveillance of ARGs associated with drug resistance in tuberculosis infections in both treated and untreated wastewater from Kwa-Zulu Natal, South Africa. This surveillance, through the use of both conventional and digital droplet polymerase chain reaction (ddPCR) techniques, may advocate the use of WBE for drug-resistant TB surveillance.

4.2. Methodology

4.2.1. Study site

Three WWTPs, receiving municipal wastewater in the city of Durban, South Africa referred to as WWTP A, WWTP B and WWTP C were investigated in this study. The selection of the WWTPs was based on plants serving at least a population of 10 000 individuals, and those receiving hospital sewage. These WWTPs also had different treatment configurations and capacities, details of which are presented in Table 4.1. Wastewater samples were taken on three separate occasions.

Table 4.1: Details of the wastewater treatment plants used for this study.

Treatment works	Design Capacity (Mℓ/d)	Primary Settling	Activated Sludge	Secondary clarification	Bio-filters	Sludge Digestion	Tertiary treatment	Remarks
WWTP A	18.8	Y	N	N	Y	Y	Y	Receives from **Hospital A**, which offers health services to the community at regional and district levels and has 17 clinics attached. The hospital is one of the sites for Mother-To-Child-Transmission (MTCT) of HIV and has the largest crisis centre
WWTP B	4.90	N/A	Extended Aeration	Y	N	N	Y	Receives from **Hospital B** which serves predominantly residential areas. This is also a referral hospital for another hospital and clinic
WWTP C	70.0	Y	Conventional	Y	N	Y	Y	Receives from **Hospital C** complex which offers specialized services for **multi-drug-resistant (MDR) and complicated TB**

Availability of the treatment process (Y=Yes/N=No) Information sourced from Cross and Buckley. (2016).

4.2.2. Sample collection and processing

One litre composite sample was taken from the influent (raw/untreated wastewater) and final effluent (post chlorinated effluent) at each WWTP. Thus, two one-litre samples (influent and effluent) were taken per WWTP. Composing of many subsamples (100 ml) taken every 30 minutes interval until a final volume of 1 litre was achieved.

Samples were transported to the laboratory in a cooler box with ice, stored at 4°C and analysed within 48 hours. Before analysis, samples were homogenised, and 50 mL subsamples were taken and centrifuged at 3000 rpm for 20 min, the supernatant discarded, and the total DNA was extracted from the pellet using a DNeasy Powersoil DNA extraction kit (QIAGEN), following the extraction procedure provided by the manufacturer, with no modification. The quantity and quality of the extracted DNA were determined using the IMPLEN NanoPhotometer NP80 – All-in-One Spectrophotometer. All analyses were done in triplicate.

4.2.3. Selection of antibiotic resistance genes

The genes coding for resistance to a variety of antibiotics used in drug-susceptible and drug-resistant tuberculosis (TB/DR-TB) treatment regimens were selected for the study (Table 4.2), based on the availability of information and common usage in South Africa (DoH, 2015). Those antibiotics used exclusively for treating TB infections are categorized into the first and second-line treatment regimen. The first-line treatment regimen drugs are isoniazid, rifampicin, ethambutol and pyrazinamide (Tiberi *et al*., 2017; Farah Aldour *et al*., 2018; Bea *et al*., 2021; Pérez-Osorio *et al*., 2012). The second-line treatment regimen are kanamycin, amikacin, delamanid, bedaquiline and ethionamide (Tiberi *et al*., 2017; Ektefaie *et al*., 2021). Additionally, other genes coding for resistance to drugs that may be used to treat other infections apart from TB were included, as they are sometimes used as part of the TB treatment regimen. These are streptomycin, cycloserine, ofloxacin, moxifloxacin (Tiberi *et al*., 2017; Kadigi *et al*., 2020).

Table 4.2: Antibiotic resistance genes selected for this study and the antibiotics that they code resistance to.

	Chemical class	Drug name	Gene	References
First-line drugs	Pyridine	Isoniazid (H)	*katG*	Koch *et al*. (2018); Zhang and Yew, 2015; Rodwell *et al*. (2014); Lakshminarayana *et al*. (2015); Cuevas-Córdoba *et al*. (2013a); Cuevas-Córdoba *et al*. (2013b)
	Rifamycin	Rifampicin (R)	*rpoB*	
	Ethylenediamine	Ethambutol (E)	*embB*	
	Pyrazine	Pyrazinamide (Z)	*pncA*	
Add-on drug	Aminoglycoside	Streptomycin (S)	*rrs*	
Second-line drugs	Fluoroquinolone	Ofloxacin (Ofx)	*gyrA*	Koch *et al*. (2018); Lakshminarayana *et al*. (2015); Farhat *et al*. (2016)
		Moxifloxacin (Mfx)	*gyrB*	
	Diarylquinoline	Bedaquiline (Bdq)	*atpE*	
	Pyridine	Ethionamide (Eto)	*ethR*	Koch *et al*. (2018); Zhang and Yew, 2015; Lakshminarayana *et al*. (2015)
Injectable drugs	Aminoglycoside	Kanamycin (Km)	*eis*	Koch *et al*. (2018); Zhang and Yew, 2015; Lakshminarayana *et al*. (2015)
		Amikacin (Amk)	*eis*	

4.2.4. Optimization of Polymerase Chain Reaction conditions

Method optimization was done using published primers targeting the genes presented in Table S1 (in the appendices). The PCR mixture for all targeted genes contained 12.5 ul of OneTaq 2X Master Mix with Standard Buffer (New England BioLabs inc), 2 µl of primer mix containing 1 µl of each primer set, (final concentration of 0.2-0.4 µM), 1 µl (20ng/µl) of DNA template, and 9.5 µl of molecular grade water in a final volume of 25 µl in a reaction tube. PCR amplification was performed in Veriti™ 96-Well Thermal Cycler. Optimised thermocycling

conditions were initial denaturation step at 95°C for 10 min and followed by 30 cycles of denaturation at 96°C for 45 s, annealing temperatures varied for each primer, *eis* (50°C for 60 s), *gyrB*, *rrs*, *ethR* (52°C for 60 s), *pncA*, *gyrA*, *katG*, *aptE* (54°C for 60 s), *rpoB*, *embB* (60°C for 60 s) and extension at 72°C for 40 s. The final extension step was performed at 72°C for 10 min.

PCR products were analyzed by 2% agarose gel electrophoresis performed at 70 V in 1 X Tris-Borate EDTA (TBE) buffer. The agarose gel was pre-stained with ethidium bromide (final concentration of 0.2-0.5 µg/mL). The electrophoresed PCR products were visualized using a Bio-Rad Gel Doc™ XR, a gel documentation system.

The above optimized PCR protocol was used to determine the presence of the selected genes coding for tuberculosis resistance in wastewater from Durban, South Africa. Wastewater samples were taken and processed using the protocol described in Section 4.2.2.

4.2.5. Optimization of ddPCR for detection and quantification of tuberculosis resistance genes in wastewater

The concentration of the selected ARGs in the wastewater samples was determined using ddPCR analysis. The ddPCR was performed in a 22 µL reaction volume, containing 10 µL of 2X QX200 ddPCR EvaGreen Supermix (Bio-Rad), 1 µL (20 ng/µL) of template DNA, 1.25 µL each of the forward primers (FP) and reverse primers (RP), each at a final concentration of 250 nM and RNase/DNase free water.

Droplets were generated using the automated droplet generator (Bio-Rad) and were amplified using a C1000 Touch™ Thermal Cycler (Bio-Rad). The following thermal cycling conditions were followed: initial denaturation step at 95°C for 10 min followed by 45 cycles of denaturation at 96°C for 45 s, the annealing temperatures varied for each primer (genes), *eis* (50°C for 60s), *gyrB*, *rrs*, *ethR* (52°C for 60s), *pncA*, *gyrA*, *katG*, *aptE* (54°C for 60s), *rpoB*, *embB* (60°C for 60s), incubation step was performed at 98°C for 10 min (ramp rate 2.2°C /s) and held at 4°C for 30 min before reading the plate. After thermal cycling, the ddPCR plates were read using the QX200 droplet reader (Bio-Rad). Droplet counts and amplitudes were exported to and analysed with the QuantaSoft™ analysis Pro software (Bio-Rad).

4.2.6. Statistical analysis

Descriptive statistics were performed with Microsoft Excel, and a test of normality was performed based on the Akaike Information Criterion (AIC) score measured with @Risk (Palisade Inc. USA). Based on the normality tests, a comparison of the concentration of the

different genes coding for tuberculosis resistance and the three WWTPs was achieved using the Kruskal-Wallis tests followed by Dunn's Multiple Comparison tests. The difference in the concentration of the genes between untreated and treated wastewater was compared using the Mann-Whitney tests. All data were log-transformed (log10 copies/ml) before analysis. All statistical tests were performed at a 95% confidence interval and a p-value ≤ 0.05 was considered statistically significant. All statistical analyses were done using GraphPad Prism (Version 7.0, GraphPad Software, USA).

4.3. Results

4.3.1. Detection of genes associated with resistance to drugs used in TB treatment regimen using conventional PCR.

The genes associated with resistance to TB/DR-TB drugs used in this study were found in the majority of the samples tested from all the three WWTPS investigated. The genes coding resistance to the second-line TB drugs such as moxifloxacin, bedaquiline, ethionamide and kanamycin/amikacin (*gyrB, atpE, ethR* and *eis*) were the most frequently detected from both influent and effluent samples (Table 4.3). However, *gyrA* gene associated with fluoroquinolone resistance, specifically ofloxacin, used in the second-line treatment regimen was only detected from one of the treatment plants (WWTP C) investigated.

The prevalence of the genes coding for resistance to first-line TB drugs varied depending on the gene, WWTP and the type of PCR analysis used. All the wastewater samples analysed were positive for all the genes investigated using ddPCR for the detection, however, there were some genes not detected via conventional PCR. The *rrs* gene (conferring resistance to streptomycin) was found in all wastewater samples, both influent and effluent. In contrast, the gene associated with the resistance to pyrazinamide (*pncA*) was not detected in any of the samples via conventional PCR. However, *pncA* gene was detected in all wastewater samples and in low concentrations (Figures 4.1-4.3) using the ddPCR. Other less frequently detected ARGs included the *rpoB* gene associated with rifampicin resistance. Table 4.3 presents the prevalence of the various ARGs in wastewater from all the WWTPs.

Table 4.3: Detection of antimicrobial resistance genes associated with drug-resistant TB

			Percentage (%) of samples positive (*n= 9 per sampling point)											
			WWTP A				WWTP B				WWTP C			
Category of treatment	Gene detected	Antibiotic/s	Conventional PCR		ddPCR		Conventional PCR		ddPCR		Conventional PCR		ddPCR	
			Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
First-Line TB treatment	*katG*	H	100	100	100	100	100	100	100	100	0	100	100	100
	rpoB	R	100	0	100	100	0	0	100	100	100	100	100	100
	embB	E	100	100	100	100	100	0	100	100	100	100	100	100
	pncA	Z	0	0	100	100	0	0	100	100	0	0	100	100
	rrs	S	100	100	100	100	100	100	100	100	100	100	100	100
Second-line TB treatment	*gyrA*	Ofx	0	0	100	100	0	0	100	100	100	100	100	100
	gyrB	Mfx	100	100	100	100	100	100	100	100	100	100	100	100
	atpE	Bdq	100	100	100	100	100	100	100	100	100	100	100	100
Other drugs	*ethR*	Eto	100	100	100	100	100	100	100	100	100	100	100	100
Injectables	*eis*	Km/ Amk	100	100	100	100	100	100	100	100	100	100	100	100

* Samples were taken on three separate occasions for each WWTP, and analysis was done in triplicates for each organism.

H: isoniazid, R: rifampicin, E: ethambutol, Z: pyranzinamide, S: streptomycin, Ofx: ofloxacin, Mfx: moxifloxacin, Bdq: Bedaquiline, Eto: ethionamide, Km/Amk: kanamycin/amikacin

4.3.2. Abundance of antimicrobial resistance genes in untreated and treated wastewater.

4.3.2.1. Concentration of ARGs in the WWTPs

The highest concentration of ARGs in both influent and effluent in WWTP A was *rrs* gene, conferring resistance to streptomycin, with concentrations ranging from 4.35-5.19 log copies/ml. The other most abundant ARGs in both influent and effluent wastewater were *gyrB*, *eis*, *aptE*, *embB* and *ethR* genes associated with resistance to fluoroquinolones, (specifically moxifloxacin), aminoglycosides (kanamycin/amikacin), bedaquiline, ethambutol and ethionamide, respectively, with median concentrations ranging from 3.05(±0.08)-4.51(±0.04) log copies/ml. As shown in Figure 4.1, the least abundant ARG among the genes was *pncA*, with measured concentrations ranging from 2.10-2.20 log copies/ml in the influent and 1.80-1.96 log copies/ml in the effluent. However, no statistically significant difference was observed when the influent and effluent concentrations were compared using the Mann-Whitney test at a 95% confidence interval.

A similar trend was observed in WWTP B, the highest concentration of ARGs in both influent and effluent was *rrs* gene, with concentrations ranging from 4.56-4.78 log copies/ml. The other most abundant ARGs were *eis*, *ethR*, *atpE* and *embB* genes as shown in Figure 4.2. The least abundant ARG among the genes was *pncA*, with measured concentrations ranging from 1.90-2.27 log copies/ml in the influent and 1.77-2.14 log copies/ml in the effluent.

Similarly, the ARG with the highest concentration in both influent and effluent at WWTP C was *rrs* gene, with concentrations ranging from 4.62-4.74 log copies/ml. The highest concentrations of *eis* gene were most abundant in the effluent (4.07(±0.09) log copies/ml compared to the influent (3.88(±0.07) log copies/ml. Again, as shown in Figure 4.3, the least abundant ARG was *pncA*, with median concentrations of 1.90(±0.26) log copies/ml in the influent and 2.09(±0.01) log copies/ml in the effluent.

These results show that the ARG with the highest concentration amongst all the WWTPs was *rrs* gene, however, there was no statistically significant difference ($p \geq 0.05$) in the concentrations of this gene in the influent samples of all WWTPs when compared. In contrast, a significant difference in their concentration was observed in the effluent samples when all the WWTPs were compared ($p \leq 0.05$). In addition to *rrs*, the other ARGs found abundantly across all the WWTPs were *eis*, *ethR* and *atpE* genes with median concentrations ranging from 3.27(±0.03)-4.30(±0.09) log copies/ml for both influent and effluent samples. Irrespective of

the WWTP, the least concentrations of ARGs detected were that of *pncA* gene with no statistically significant difference in the concentrations when all WWTPs were compared. ($p \geq 0.05$). Furthermore, no statistically significant difference ($p \geq 0.05$) was achieved when comparing the influent to effluent concentrations of all the ARGs within each WWTP and across all the WWTPs using the Mann-Whitney test at 95% confidence interval.

4.3.2.2. Variation in the concentration of ARGs responsible for resistance to first- and second-line TB treatment drugs and other related drugs

Comparatively, the difference in concentration of the ARGs associated with resistance to first- and second-line TB treatment drugs in all the WWTPs was statistically significant (p-value $\leq$ 0.05), with the exception of the concentrations of *gyrA*. The statistical difference in *eis* and *ethR* concentration was driven by differences between WWTP A and WWTP C, and the difference in *atpE* and *gyrB* concentrations by differences between WWTP A and WWTP B. However, both the Kruskal-Wallis Test and Dunn's multiple comparison tests did not show any statistically significant differences in the concentration of *gyrA* between the various WWTPs (p-value $\geq$ 0.05).

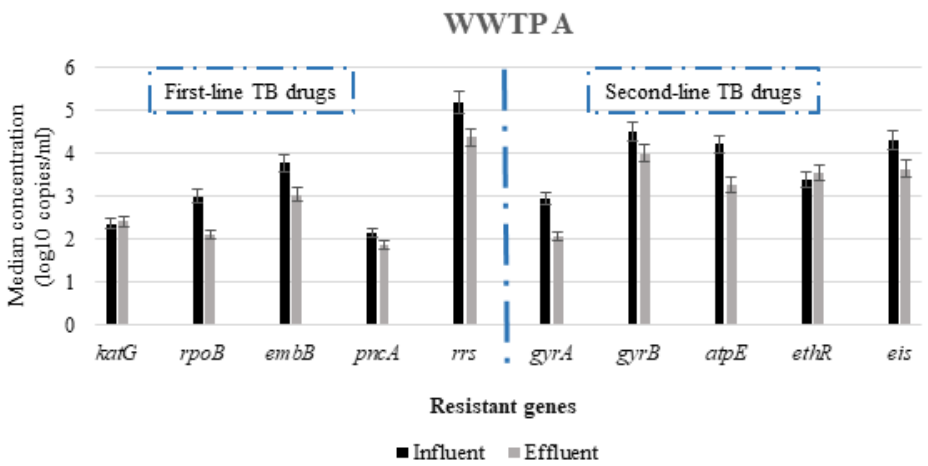

Figure 4.1: Median log copies/ml concentration of selected antimicrobial resistance in WWTP A

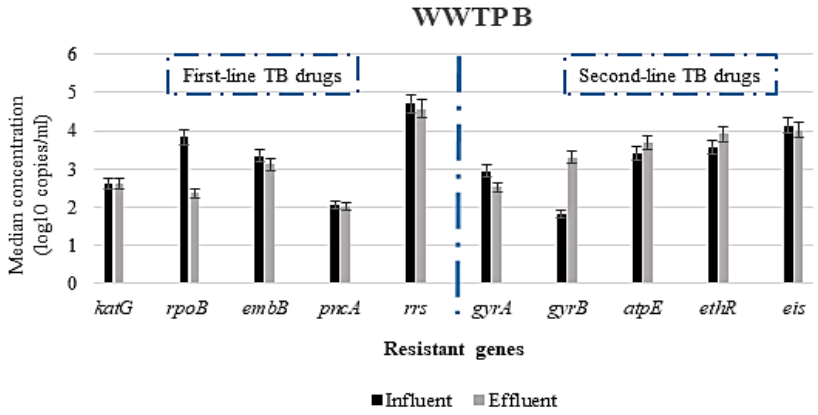

Figure 4.2: Median log copies/ml concentration of selected antimicrobial resistance in WWTP B

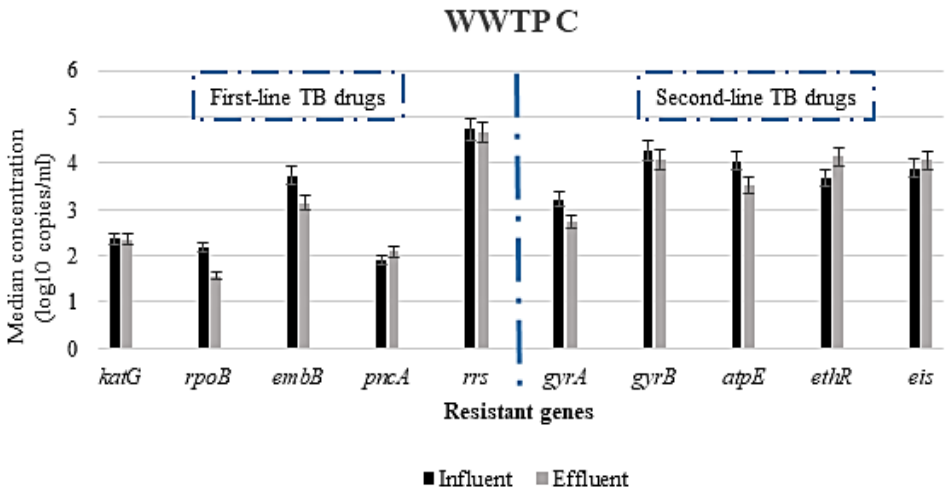

Figure 4.3: Median log copies/ml concentration of selected antimicrobial resistance in WWTP C

4.3.3. Reduction in antimicrobial resistance genes during wastewater treatment

The concentration of the various ARGs detected differed between the untreated and treated wastewater samples (Figures 4.1-4.3). In most instances, the concentrations in the treated wastewater (effluent) were higher than in the untreated wastewater. For instance, the highest log reduction in these ARGs was observed for *rpoB*, *gyrA* and *embB*, with median log reduction ranges of 0.62-0.89, 0.41-0. 87 and 0.23-0.73 respectively (Figure 4.4). In contrast, for some of the ARGs, the concentrations measured in the treated wastewater were higher than in the untreated wastewater. This was observed for *ethR* in all the WWTPs, with median log reductions ranging from -0.16 to -0.47. In WWTP B, an increase of effluent concentrations for *gyrB* and *atpE* was observed where *gyrB* median log reduction was -1.49 and -0.27 for *atpE* (Figure 4.4). These results show that the concentration of these ARGs in the treated wastewater was still high, ranging from 1 log copy/ml to over 4 log copies/ml as shown in Figures 4.1-4.3.

The log reduction of ARGs associated with first-line TB treatment did not show any statistically significant differences between all three WWTPs (p-value ≥ 0.05). In contrast, all ARGs associated with second-line TB treatment (except *gyrA*), as well as all the other drugs used in TB treatment, showed statistically significant differences in the log reduction between all WWTPs (p-value $\leq$ 0.05). The statistically significant differences observed in log reductions achieved for *atpE* and *gyrB* genes was due to the differences between WWTP A and WWTP B. The difference in the log reductions between WWTP A and WWTP C was responsible for the statistically significant reduction reported for *eis* and *ethR* genes. When comparing log reductions between first-line and second-line ARGs, no statistically significant difference was observed at 95% confidence interval using the Mann-Whitney test.

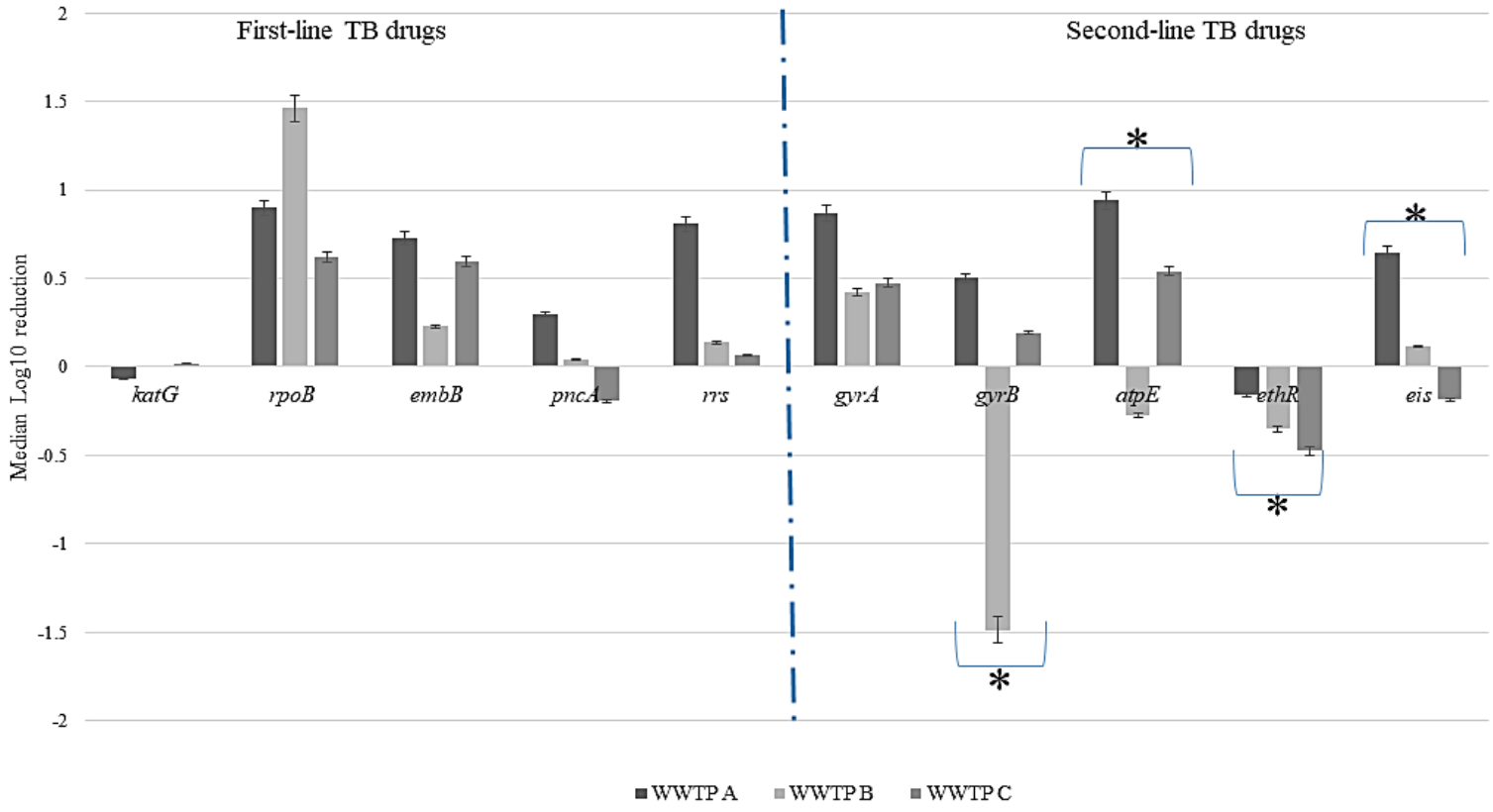

Figure 4.4: Median (SD) Log reduction achieved for the various antimicrobial resistance genes in the three-wastewater treatment plants.

*****_The difference in median log reduction for the ARGs marked were statistically significant using the Kruskal-Wallis tests (p-value≤0.05)._

4.4. Discussion

Antibiotic-resistant bacteria (ARB) and genes (ARGs) are an emerging concern (Zhang *et al*., 2015) to public health worldwide and DR-TB is a global health concern that threatens the significant progress made in TB care and prevention. South Africa is one of the countries with the highest prevalence of drug-resistant tuberculosis (DR-TB) globally (Cox *et al*., 2017; WHO, 2020). Therefore, there is a need for alternative surveillance systems to augment the fight against DR-TB. This study successfully optimized both conventional and droplet digital PCR assays for the detection of ARGs associated with TB treatment. Therefore, showing the potential of using wastewater as a snapshot of ARG circulation in the connected population. However, the utility of this approach is largely dependent on the sensitivity of the methods used. The use of two PCR assays with different sensitivity in this study highlighted the importance of these sensitive assays in wastewater-based surveillance for ARGs. Using conventional PCR, ARGs responsible for resistance to the second-line TB treatments were present in all samples, in contrast, the prevalence of ARGs responsible for resistance to first-line TB drugs was lower. Based on conventional PCR analysis, *pncA* was not detected in any of the samples, and low frequency of detection for *rpoB*. However, all the genes were detected using ddPCR. This could be due to the sensitivity of the ddPCR platform to detect even the lowest concentrations when compared to other PCR platforms (Demeke and Dobnik, 2018; Li *et al*., 2018; Cao *et al*., 2020). The concentrations of these ARGs as determined with ddPCR provided a better estimation of occurrence in wastewater, indicating the prevalence of these ARGs in the populations served by the different WWTPs.

The most abundant ARG was *rrs*, responsible for streptomycin resistance (Cuevas-Córdoba *et al*., 2013a; Ghosh *et al*., 2020). The high concentration of this ARG could be due to the common use of streptomycin, not just for both drug-susceptible and drug-resistant TB infections but also for the treatment of other infections caused by other *Mycobacterium* related species (Dal Molin *et al*., 2018) and other bacteria such as *Xanthomonas* spp (Xu *et al*., 2010), *Salmonella typhimurium*, *Yersinia pestis* (Dai *et al*., 2021). Detection of the *rrs* gene responsible for streptomycin resistance in clinical *M. tuberculosis* isolates has been reported in South Africa (Cohen *et al*., 2020), China (Wang *et al*., 2019), Iran (Khosravi *et al*., 2017) and Myanmar (Oo *et al*., 2018). Additionally, the *rrs* gene has been implicated in cross-resistance to other aminoglycosides (amikacin/kanamycin), also used in TB treatment (Sirgel *et al*., 2012). Therefore, the high prevalence and concentration

of this gene could be attributed to the high prevalence of resistance to streptomycin or the aminoglycosides in the connected populations.

In contrast, *pncA* gene concentrations were very low in the wastewater across all WWTPs, which could explain the non-detection of this gene by the conventional PCR. This gene (*pncA*) confers resistance to pyrazinamide, which is used exclusively for the treatment of active TB (not latent TB) (Cuevas, 2013). This drug is mostly used in combination with rifampicin, isoniazid, and either streptomycin or ethambutol (Florou *et al*., 2021; Padda and Reddy, 2020). This show that DR-TB may be driven by resistance to rifampicin or isoniazid and streptomycin or ethambutol while pyrazinamide remains relatively effective (Koch *et al*., 2018). However, Allana *et al*. (2017) reported that 70% of multidrug-resistant and 96% of extensively drug-resistant *M. tuberculosis* isolates in South Africa and Georgia were positive for the *pncA* gene. Detection of this gene in clinical *M. tuberculosis* isolates have been published from other countries such as Pakistan (Malik *et al*., 2019) and Uganda (Naluyange *et al*., 2020). The data obtained in our study, therefore, shows that the environmental abundance of this gene could be driven by its prevalence within the local population/communities.

A major finding worth highlighting in this study is the abundance of the ARG, *aptE,* responsible for resistance to bedaquiline. Bedaquiline is one of the newer drugs added to the MDR-TB treatment regimen in South Africa which has been associated with a reduced DR-TB mortality rate (Schnippel *et al*., 2018). This antibiotic is used as either add on to existing treatment regimens or used in cases of resistance, to certain core TB/MDR-TB drugs. The abundance of this ARG may allude to a high prevalence of MDR-TB which may contribute to the epidemiological profile of the population served by these WWTPs.

The relatively high concentration of these ARGs may not only be associated with TB treatment or even mycobacteria infections. For instance, *rpoB* gene, responsible for resistance to rifampicin, has been reported in environmental mycobacteria such as *Mycobacterium abscessus* (Goldstein, 2014; Rominski *et al*., 2017) and other bacteria such as *Staphylococcus aureus* (Goldstein, 2014; Wang *et al*., 2019), *Streptococcus* spp. (Ferrandiz-Avellano *et al*., 2005), *E. coli* (Goldstein, 2014) and other species (Goldstein, 2014). Additionally, fluoroquinolone resistance genes (*gyrA*/*gyrB*) have also been reported in *Legionella pneumophila* (Hennebique *et al*., 2017). Detection of these ARGs may be attributed to several other factors, such as high concentrations in wastewater (due

to improper waste management) of unused/expired drugs and overuse of TB/MDR-TB medication. The presence of the antibiotics together with the high bacterial density, high nutrient and high oxygen conditions within WWTPs provides conducive conditions for the transfer of ARGs, development of new ARB and for creating hotspots for the spread of resistant bacteria and genes into the environment (Nnadozie *et al*., 2017; Rizzo *et al*., 2013). This may not necessarily relate to the presence of antibiotics in higher concentrations in the wastewater, as ARBs and ARGs have been reported in the environment without the actual presence of antibiotics relating to resistance (Sabri *et al*., 2018; Barancheshme and Munir, 2019). Horizontal gene transfer (HGT) is also reported as the most common route for which the ARGs are transferred among different bacterial communities in the WWTPs, which could also account for the higher concentrations in the effluent samples found in our results. Similar findings have been reported by other authors (Aali *et al*., 2014; Kraemer *et al*., 2019; Barancheshme and Munir, 2019).

The reduction in the concentration of the ARGs associated with first-line TB treatment did not show any statistically significant differences (p-value ≥ 0.05). This could be a result of numerous reasons, including the ineffectiveness of the treatment plants in removing these ARBs and ARGs or the ability of these ARBs to survive and attach to sludge due to their hydrophobic nature (Loret *et al*., 2019; Ibekwe and Murinda, 2019; Cydzik-Kwiatkowska and Zielińska, 2016; Barancheshme and Munir, 2018) or genes being carried by more than one type of bacteria (Claeys and Robinson, 2018). The negative log reductions (or increased concentrations in the effluent) observed for the genes responsible for resistance to second-line drugs raises concerns due to the release of high concentrations (Figures 4.1-4.3) of these ARGs into surface water, which could be attributed to the presence of *Mycobacterium* spp. in high concentrations in treated wastewater (Cui and Liang, 2019) or cross-resistance in the bacterial communities in wastewater. The high concentrations in the treated wastewater, sometimes higher than the untreated wastewater, could be due to selective pressures that increase the concentrations of ARB by inhibiting antibiotic susceptible bacteria or contributes to the selection of mutations (Zhang *et al*., 2015). Additionally, cell lysis/disruption from different chemical and physical treatment processes used in the wastewater treatment (Samer, 2015; Jäger *et al*., 2018) leading to the release of the nucleic material in the wastewater could also account for the high concentration observed in the treated wastewater. Some of the treatment processes, such as biofiltration, conventional activated sludge and biological nutrient removal have been reported to promote the growth of slow-growing microbial populations in wastewater due to

higher solids retention time or sludge recirculation (Manaia *et al*., 2016; Krzeminski *et al*., 2019). This could also explain the high concentrations of *rrs*, *ethR*, and *atpE* gene found in the effluent of especially WWTP B for the ARGs when all WWTPs were compared. This WWTP is the only one with an activated sludge process incorporating an extended aeration treatment. Therefore, the increase in the concentration of the ARGs during treatment in this WWTP could potentially be mediated by this treatment process. Additionally, protozoans have been reported as environmental hosts for *Mycobacterium* spp. They have known to survive within protozoa (Wang *et al*., 2012; Samba-Louaka *et al*., 2018). This could be one means of survival for this bacterium within the treatment plants and also could bypass the disinfection stage as protozoans are resistant to chlorination. However, these results are inconclusive to categorically state the impact of the treatment process of removal of the ARGs. These ARGs could either be present in dead bacterial cells, live/viable bacteria or even extracellular. Therefore, there is the need for further studies in this regard, especially taking into consideration the potential health implication and risk associated with the release of ARBs and ARGs in treated wastewater.

4.5. Conclusion and recommendations

Wastewater based surveillance of antibiotic resistance associated with TB treatment using advanced molecular techniques provides a unique opportunity to complement surveillance systems aimed at monitoring TB resistance. This study was able to show that resistance to drugs used in both first- and second-line TB treatment are common in untreated wastewater, potentially indicating their widespread circulation in the populations. Furthermore, this study shows that the concentration of these ARGs is associated with the extent of use of the drugs in TB treatment due to the observation that antibiotics used in both TB treatment as well as other infections, such as streptomycin, had a higher prevalence and concentrations compared to lesser used drugs such pyrazinamide. This, therefore, shows the potential of wastewater-based surveillance systems in complementing the existing TB resistance surveillance systems.

This study also established that wastewater treatment plants have varying efficiencies in removing these ARGs. However, there was no distinct WWTP configuration that was observed to have higher efficiency in removing these ARGs. Concentrations of up to 4 log copies/ml of these ARGs were still detected in the treated wastewater, which could potentially result in the spread of these resistance genes in the aquatic environment. These genes could be extracellular or carried in the

mycobacterial cell or other bacterial species. Therefore, further studies are recommended to ascertain whether the genes detected in the treated wastewater are either extracellular or carried in viable microorganisms and to understand the diversity of these carrier bacteria and on identifying the potential bacterial communities carrying these genes through advanced microbial community analysis.

CHAPTER FIVE

5. Summary, conclusion and recommendations

5.1. Summary

Tuberculosis infection continuous to be a major health challenge globally. The increasing impact of antimicrobial resistance, has led to increased mortalities, and high costs of treatment. Improved surveillance systems for monitoring TB and MDR-TB infections are therefore important. The focus of this study was to determine the occurrence and concentration of tuberculosis-causing *Mycobacterium* spp. and antibiotic-resistant genes (ARGs) associated with (TB) infections in wastewater from Durban, South Africa. The rationale was to contribute towards the development of wastewater-based epidemiology techniques for the detection of these pathogens. Durban, in the KwaZulu-Natal (KZN) province, as the study location was deliberate to the high prevalence of TB and MDR-TB in the province as a result of the co-infection with HIV. Since KZN has the highest burden of both HIV and TB co-infections in the country, an alternative surveillance tool for monitoring TB and MDR-TB infections will contribute towards South Africa's fight against this public health burden. Furthermore, analysis of both treated and untreated wastewater could provide an insight into the possible role of wastewater in the transmission of TB infections. Sanitation coverage is a major challenge in the communities with high TB disease burden, therefore exposure to untreated wastewater or partially treated wastewater could be a common phenomenon. This exposure could lead to increased risk of TB infections.

Different species of mycobacteria were detected in the tested wastewater samples, both treated and untreated. Mycobacteria species known to be associated with animal infections were most common in all the samples. For instance, *M. caprae* and *M. bovis* were present in 100% of the untreated wastewater from two of the WWTPs and 75% in the third WWTP. In contrast, *M. tuberculosis* presence in untreated wastewater was between 75%-100% and *M. africanum* ranged from 25%-50%. The concentration of these organisms also followed a similar trend. For instance, *M. caprae* and *M. bovis* concentrations ranged from 4.0(±0.36) to 4.5(±0.52) Log10 copies/mL and 3.6 (±0.44) to 4.0 (±0.29) Log10 copies/mL respectively. *M. tuberculosis* and *M. africanum* concentrations ranged from 2.8 (±0.75) to 3.9 (±0.54) Log10 copies/mL and 2.3 (±0.29) to 2.7 (±0.42) Log10 copies/mL respectively. These results indicate the possible circulation of these species in the connected populations. The animal pathogenic species are expected to be common

due to the contribution from agricultural industries, potential runoffs from farmlands and even pets faecal latter in the wastewater. However, the significant result of this phase of the study was the detection of *M. africanum* in South African wastewater. This species of mycobacteria is primarily responsible for TB infections within West Africa, where it is reported to cause up to 50% of all TB cases (De Jong *et al*., 2010) and is responsible for reduced sensitivity of rapid identification tests kits used for TB detection (Ofori-Anyinam *et al*., 2016). Therefore, its detection in South African wastewater could imply that some of the TB infections in the country, or at least within the study area, could be caused by *M. africanum*. This has implications for TB diagnostics and treatment due to the reported reduction in diagnostic sensitivity. These results indicate that wastewater analysis could potentially provide community-based surveillance data for TB infections.

Beyond surveillance of TB infections via wastewater analysis, this study also aimed at evaluating WBE with respect to MDR-TB surveillance as well. The choice of antibiotic-resistant genes associated with TB was specific to target drugs used for treating both drug-susceptible and drug-resistant TB infections. These ARGs included *katG* (isoniazid), *rpoB* (rifampicin), *embB* (ethambutol), *pncA* (pyrazinamide), *rrs* (streptomycin), *gyrA* (ofloxacin), *gryB* (moxifloxacin), *atpE* (bedaquiline), *ethR* (ethionamide), *eis* (kanamycin/amikacin). Based on the results obtained with the ddPCR assay, the *rrs* gene was the most abundant. This is expected due to the widespread use of streptomycin to treat several other infections, in addition to TB. The widespread detection and high concentration of this ARG can therefore be related to its widespread use. Several studies around the globe and in South Africa have reported the occurrence of this ARG in clinical isolates of *M. tuberculosis* (Khosravi *et al*., 2017; Oo *et al*., 2018; Wang *et al*., 2019; Cohen *et al*., 2020). Therefore, its presence may indicate streptomycin-resistant TB infections in the community. The occurrence of MDR-TB within the communities was also corroborated via the wastewater analysis. This is due to the detection of the ARG *pncA,* albeit in low concentrations compared to the others. This gene is responsible for resistance to pyrazinamide, which is used exclusively to treat TB infections. In instances of drug-resistant TB, this drug is usually administered in combination with rifampicin, isoniazid, and either streptomycin or ethambutol (Florou *et al*., 2021; Padda and Reddy, 2020). Clinical studies have also shown that over 96% of extensively-drug-resistant TB in South Africa contains this gene. As discussed and mentioned in Chapter five, similar findings from clinical isolates have been reported in other countries. Therefore, the results obtained in this study

shows that MDR-TB could be common in the study area when evaluated by WBE. This corroborates clinical data and national estimates on MDR-TB stratified by province (NICD, 2016; Ismail *et al*., 2018). An interesting finding in this study is the detection of the ARG responsible for resistance to bedaquiline (*aptE*). This is a new drug added to the South African MDR-TB treatment regime. It is mainly prescribed as an add-on to existing medications or prescribed when resistance to the main MDR-TB drugs is reported. The detection of the genes responsible for resistance to this drug indicates the potential circulation of *M. tuberculosis* resistant to this drug in the population already. This could have serious consequences for TB treatment in the region and requires urgent attention from the health authorities.

The treated wastewater (effluent) also contained high concentrations of mycobacteria, in some instances up to 4 log10 units per mL. This implies the possible passage of these microorganisms through the treatment processes without any impact. This could be explained by their ability to survive in harsh environmental conditions like wastewater. As discussed in Chapter 4, several studies have reported the ability of these bacteria to withstand even phagocytic activity of amoeba (Hagedorn *et al*., 2009; Mardare *et al*., 2013). However, it is worth noting that molecular methods such as the ddPCR platform used in this study do not necessarily differentiate between living or dead cells. Therefore, the extracted DNA could have been from dead mycobacterial cells or could be extracellular DNA. In contrast to the concentration of the mycobacteria in the treated wastewater, where concentrations were generally lower than the untreated wastewater, some ARG concentrations were higher in the treated wastewater compared to the untreated. This could be attributed to several reasons or factors. Firstly, it could be due to the inability of the WWTPs to remove these ARBs and ARGs or the ability of these ARBs to survive and attach to sludge due to their hydrophobic nature. Furthermore, horizontal gene transfer (HGT) could account for the increasing concentrations of the ARGs during wastewater treatment. The wastewater environment has been reported to be ideal for HGT (Aali *et al*., 2014; Kraemer *et al*., 2019; Barancheshme and Munir, 2019). Therefore, it could have played a role in these increasing concentrations, which was mostly observed for the second-line TB drug related ARGs. It is worth noting that the detection of the mycobacteria and ARGs in the treated wastewater does not necessarily mean these bacteria are viable or the associated ARGs are contained in viable cells. Therefore, further studies are required to ascertain the viability of mycobacteria in treated wastewater, which could provide an insight into the possible risks of infection from exposure to treated wastewater.

5.2. Conclusions

This study was successful in determining the prevalence of tuberculosis-causing *Mycobacterium* in wastewater from all the three WWTPs studied. The first objective was achieved via optimization of both conventional and advanced PCR to detect and quantify tuberculosis-causing mycobacteria and the genes associated with tuberculosis resistance from untreated and treated wastewater. The detection of these microorganisms in the untreated wastewater from all the three WWTPs indicates the potential prevalence of these organisms in the connected populations. The findings also show the potential for the adaptation of WBE for the determination of TB infections in the served communities. The most significant finding was the detection of *M. africanum* in wastewater from South Africa. This shows that it is possible that some of the TB infections in the community could be caused by this species of *Mycobacterium*, widely reported to be endemic in West Africa and report to be most genetically similar to *M. tuberculosis*. In addition, the detection of these microorganisms in the treated wastewater, show the potential risks of infections to exposed populations, which may be attributed to wastewater reuse schemes as well as exposure to wastewater contaminated surface water bodies. Furthermore, this supports the One Health perspective in relation to the risks associated with the exposure to either treated and untreated wastewater or surface water contaminated by wastewater. This study was able to show that resistance to drugs used in both first- and second-line TB treatment are common in untreated wastewater, potentially indicating their widespread circulation in the served populations. Furthermore, this study shows that the concentration of these ARGs is associated with the extent of use of the drugs in TB treatment due to the observation that antibiotics used in both TB treatment, as well as other infections, occur in higher concentrations. These findings support the potential of WBE in providing an additional surveillance tool for monitoring TB infections and drug-resistant TB infections in communities.

5.3. Recommendations

The following recommendations are made based on the findings of this study;

1. The adoption of WBE as an additional surveillance method for the prevalence of tuberculosis and its resistance in the population especially in low-income countries.
2. Although this study was able to detect the members of MTBC and the genes associated with tuberculosis resistance, further studies on the viability and infectivity are warranted.

This is very relevant because the detection of MTBC via ddPCR or conventional PCR does not necessarily indicate the presence of viable/infectious organisms. Therefore, to further understand the potential risks there is the need to determine if the MTBC detected in the wastewater are viable and infectious.

3. There is a need for studies on the survival of MTBC in wastewater and factors influencing their survival. This will help in the adoption of technologies aimed at enhancing the removal or inactivation of MTBC in wastewater.
4. The detection of MTBC in the treated and untreated wastewater could potentially increase the risks of TB infections for the worker in these WWTPs, therefore the implementation of risk reduction measures such as the use of personal protective equipment is recommended.
5. Further studies on the risk assessment for infection for nearby communities is warranted.
6. Evaluation of whether the genes detected in treated wastewater are either carried by viable microorganisms or are extracellular.
7. Further studies on the correlation of TB/MDR-TB drug consumption in the population and ARGs profile in wastewater are recommended.

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Appendix I

This appendix section entails some of the information not included in the methodology section in chapter three and four. The information contained below is the map indicating the sampling site for WWTPs in KwaZulu Natal, South Africa selected for this study, the sequences for the primers used in this study to identify the selected microorganisms and genes coding for TB resistance for this study.

Method Optimization

Figure S1 shows the location of the sampling points within Durban, KwaZulu-Natal Province. Primer sequences and genes target for total mycobacteria and tuberculosis-causing mycobacteria and associated ARGs mentioned in Chapters three and four are presented in Table S1 and S2. Table S3 contains additional information on the classification of drugs used in TB treatment and their mechanism of action.

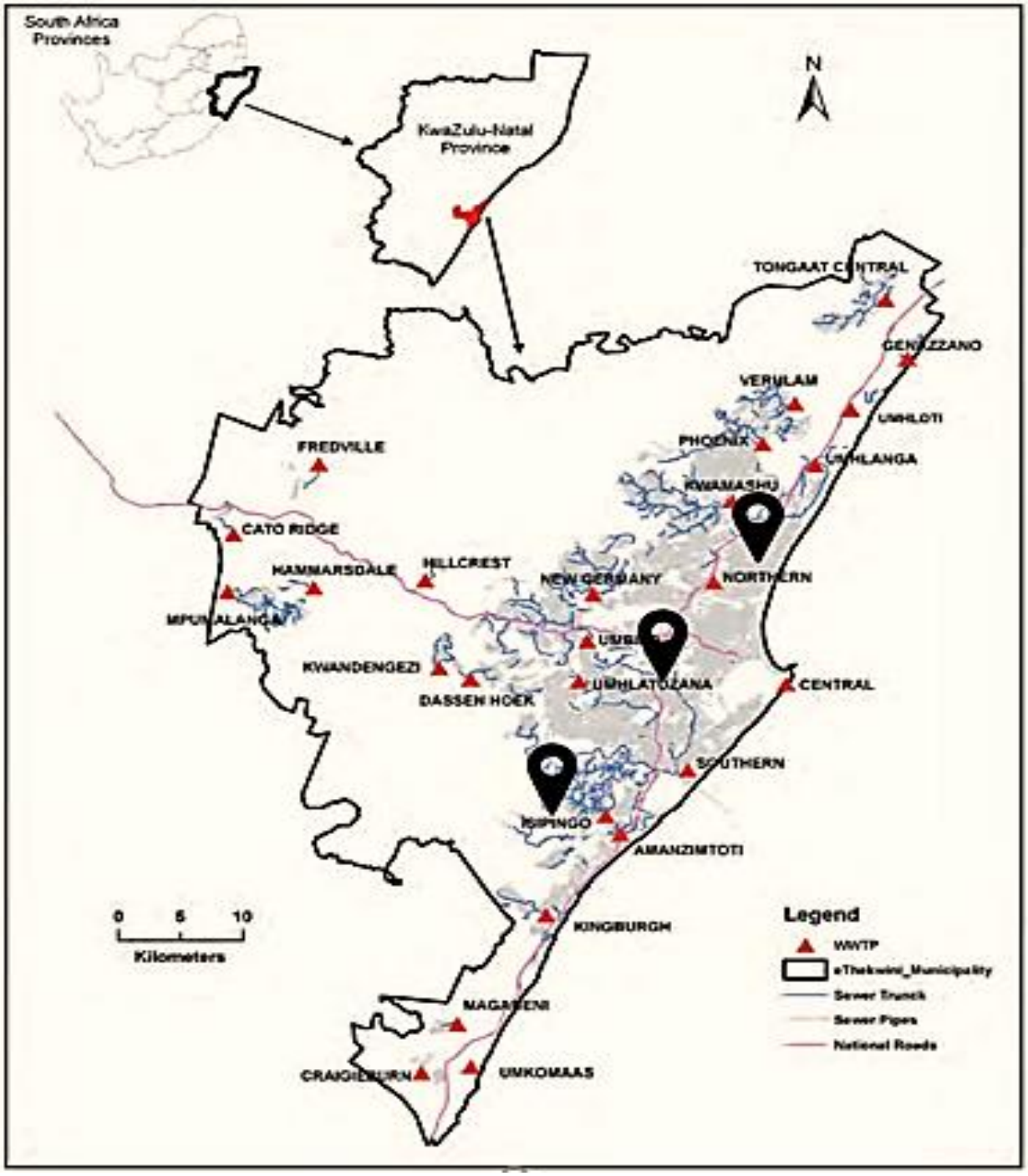


Figure S1: Sampling site for WWTPs in KwaZulu Natal, South Africa

Table S1: Primers and targeted genes used for the detection of total mycobacteria and MTBC members

Gene	Drug/ organism name	Primer sequence	Reference
16S rRNA gene	All mycobacterial species	F:5'-GAGATACTCGAGTGGCGAAC-3' R:5'CAACGCGACAAACCACCTAC-3'	Chae *et al*. (2017)
Rv0577	*M. tuberculosis* complex	F:5'ATGCCCAAGAGAAGCGAATACA-3' R:5' AATGTCAGCCGGTTCCGCAA-3'	Chae *et al*. (2017)
IS6110		F; 5' GGATCCTGCGAGCGTAGGCGTCGG-3' R; 5' CCTGTCCGGGACCACCCGCGGCAA-3'	
RD8 present	*M. africanum*	F:5'- GTCGAAGCGGGGCGCTCT -3' R:5'- GCGCAACGGATTTCCATCGT -3'	Asante-Poku *et al*. (2015)
RD9	*M. tuberculosis*	F:5'-GTGTAGGTCAGCCCCATCC-3' R:5'-GTAAGCGCGTGGTGTGGA -3'	Chae *et al*. (2017); Perez-Osorio *et al*. (2012)
RD 4		forward = 5′-ATGTGCGAGCTGAGCGATG-3′	Hlokwe *et al*. (2017)
		internal = 5′-TGTACTATGCTGACCCATGCG-3′	
		reverse = 5′-AAAGGAGCACCATCGTCCAC-3	
RD9		forward: 5′-CAAGTTGCCGTTTCGAGCC-3′	Hlokwe *et al*. (2017)
		internal: 5′-CAATGTTTGTTGCGCTGC-3′	
		reverse: 5'GCTACCCTCGACCAAGTGTT-3′	
RD12		forward: 5′-GGGAGCCCAGCATTTACCTC-3′	Hlokwe *et al*. (2017)
		internal: 5'GTGTTGCGGGAATTACTCGG-3′	
		reverse: 5′-AGCAGGAGCGGTTGGATATTC-3	
RD1	*M. bovis*	External forward 5′AAGCGGTTGCCGCCGACCGACC	El-Tawab *et al*. (2016)
		Internal forward 5′CTGGCTATATTCCTGGGCCCGG	
		External reverse 5′GAGGCGATCTGGCGGTTTGGGG	
RD9	*M. Africanum*	F; 5' ACT CCC AGC GCT CGG CGG TGA CGG TAT CGT 3'	Vasconcellos *et al*. (2010)
		R; 5' ATT CCG TGG GCG CTG CGG CCA ATG TTT GTT 3'	
RD8 deleted		F-GTCGAAGCGGGGCGCTCT	Kim *et al*. (2013)
		R-GGTTCTTGGCGTCTTGGAAGG	
RD1		F-CGAGGGGAAGCAGTCCCTGA	Kim *et al*. (2013)

	R-AGGTCGAACTCGCCCGATCC

Table S2: Target resistant genes and their PCR primer sequences

Target gene-locus	**Encoded protein**	**Drug name**	**Primer sequence**	**References**
rpoB	β-Subunit of RNA polymerase	Rifampicin	F:5'-CGAGGTGCCGGTGGAAAC-3'	Farah Aldour *et al*. (2018); Pérez-Osorio *et al*. (2012); Rodwell *et al*. (2014); Nguyen, 2016a
			R:5'-GTCGTCGTGCTCCAGGAAGG-3'	
KatG	catalase-peroxidase	Isonizaid	F:5'-GAGCCCGATGAGGTCTATTG-3'	Farah Aldour *et al*. (2018); Pérez-Osorio *et al*. (2012); Rodwell *et al*. (2014); Nguyen, 2016a
			R:5'-GTCCTTGGCGGTGTATTGC-3'	
inhA	Enoyl ACP reductase		F:5'-GAGCGTAACCCCAGTGCGAA-3'	Rodwell *et al*. (2014); Nguyen, 2016a
			R:5'-TCCGGTAACCAGGACTGAAC-3'	
embB	arabinosyltransferase	Ethambutol	F:5'-CATGTCATCGGCGCGAATTCG-3'	Nguyen, 2016a
			R:5'-TGGCAGGCGCATCCACAGACT-3'	
PncA	pyrazinamidase	Pyrazinamide	F:5'-GACGTATGCGGGCGTTGA-3'	Farah Aldour *et al*. (2018); Pérez-Osorio *et al*. (2012)
			R:5'-CCATCAGGAGCTGCAAACCA-3'	
gyrA	DNA gyrase subunit A	Ofloxacin, Moxifloxacin	F:5'-GGTGCTCTATGAAATGTTCG-3'	Rodwell *et al*. (2014)
			R:5'-GCTTCGGTGTACCTCATCG-3'	
gyrB	DNA gyrase subunit B		F:5'-CGATGTTCCAGGCGATACTT-3'	Rodwell *et al*. (2014)
			R:5'-ATCTTGTGGTAGCGCAGCTT-3'	

rrs	16S ribosomal RNA	Kanamycin, Amikacin	F:5'-GTAATCGCAGATCAGCAACG-3'	Rodwell *et al*. (2014); Nguyen, 2016a
			R:5'-TTTTCGTGGTGCTCCTTAGAA-3'	
eis	Aminoglycoside acetyltransferase	Amikacin, Kanamycin	F:5'-AAATTCGTCGCTGATTCTCG-3'	Rodwell *et al*. (2014)
			R:5'-CGCGACGAAACTGAGACC-3'	
rpsL or *rrs*	30S ribosomal protein S12/16S ribosomal RNA	Streptomycin	F:5'-GCGCCCAAGATAGAAAG-3'	Nguyen, 2016a
			R:5'-CAACTGCGATCCGTAGA-3'	
ddn	deazaflavin-dependent nitroreductase	Delamanid	F:5'-CGAGCGCACCGACCAGAGC-3'	Yang *et al*. (2018)
			R:5'-GCATGGCCCGCAGGTGGACAA-3'	
fbiA	2-phospho-L-lactate transferase		F:5'-GCGGTTCTGTTGTGGTTGGG-3'	Yang *et al*. (2018)
			R:5'-CCGATGACGGGCAGGATCTCGATGG-3'	
fgd1	F420-dependent glucose-6-phosphate		F:5'-CGTGGCCGCGAGCGAGGTGAA-3'	
			R:5'-CGCCCGAACCGTCAACAACACTGG-3'	
Rv0678	hypothetical protein	Bedaquiline	F:5'-GTATCCAGGCACGCTTGA-3'	Yang *et al*. (2018)
			R:5'-CCCCACAATCGATAACC-3'	
atpE	ATP synthase subunit C		F:5'-GTACTTCAGCCAAGCGATGG-3'	Yang *et al*. (2018)

			R:5’-CCGTTGGGAATGAGGAAGTTG-3’	
ethA	monooxygenase EthA	Ethionamide	F:5′-CCTGGCAGCTTACTACGTGTC-3	Tan *et al*. (2017)
			R:5′-CGGCATCATCGTCGTCTG-3′	
ethR	HTH-type transcriptional repressor		F:5′-TTTTCCAGGATGGCGTAGC-3′	Tan *et al*. (2017)
			R:5′-CCGACCGGATCGTCAACA-3′	
alr	alanine racemase	Cycloserine	F:5’-GAAAATAAAAGACACGCCTACTTTCGCTCCA-3’	Chen *et al*. (2017)
			R:5’-GACATCCATCGCCATGGCAATACCCTT-3’	

Table S3: Classification of TB/ MDR-TB drugs

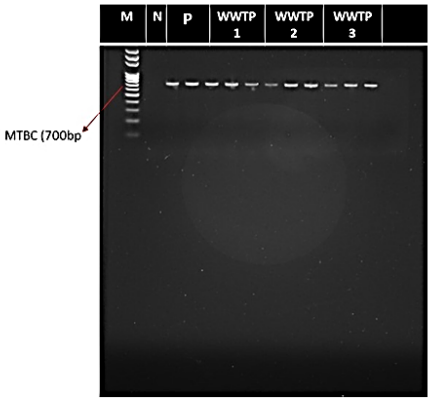
WHO category	Drug or drug class	Resistance Genes	Gene function	Mechanism of drug resistance	Reference(s)
First-line agents	Rifamycins (for example, rifampicin)	*rpoB*	RNA polymerase	Target modification	Telenti *et al*. (1993)
		ponA1	Probable bifunctional penicillin-binding protein	Unknown	Farhat *et al*. (2013)
	Isoniazid	*katG*	Catalase-peroxidase enzyme	Decreased drug activation	Heym *et al*. (1995)
		inhA	NADH-dependent enoyl-acyl carrier protein	Target amplification or modification	Rozwarski *et al*. (1998); Banerjee *et al*. (1994)
	Pyrazinamide[b]	*pncA*	Pyrazinamidase	Decreased drug activation	Sreevatsan *et al*. (1997); Scorpio *et al*. (1997)
		panD	Aspartate decarboxylase	Unknown	Zhang *et al*. (2013)
		rpsA	Ribosomal protein S1	Target modification	Shi *et al*. (2011)
	Ethambutol[b]	*embCAB operon*	Arabinosyltransferase	Target modification	Safi *et al*. (2013)
		ubiA	Arabinogalactan synthesis	Gain-of-function	Alcaide *et al*. (1997)
Second-line drugs					
Group A	Levofloxacin	*gyrA*	DNA gyrase A	Target modification	Maruri *et al*. (2012); Takiff *et al*. (1994)
	Moxifloxacin	*gyrB*	DNA gyrase B	Target modification	Takiff *et al*. (1994)
	Bedaquiline	*atpE*	ATP synthase	Target modification	Andries *et al*. (2005)

		pepQ	Putative Xaa-Pro aminopeptidase	Unknown	Almeida *et al*. (2016)
		Rv0678	Transcriptional regulator of mmpL5	Drug efflux	Hartkoorn *et al*. (2014); Andries *et al*. (2014)
	Linezolid	*Rrl*	23S rRNA	Target modification	Hillemann *et al*. (2008)
		rplC	50S ribosomal protein L3	Target modification	Beckert *et al*. (2012)
Group B	Clofazimine	*pepQ*	Putative Xaa-Pro aminopeptidase	Drug efflux	
		Rv0678	Transcriptional regulator of mmpL5	Drug efflux	
	Cycloserine	*Ald*	L-alanine dehydrogenase	Substrate shunting	Desjardins *et al*. (2016)
	Terizidone	*alr*	Alanine racemase	Target modification	
		ddl	D-alanine-D-alanine ligase	Target modification	
		cycA	Bacterial D-serine/L-and D-alanine/glycine/Dcycloserine proton symporter	Mechanism not confirmed	
Group C	Delamanid	*ddn*	Oxidative stress	Decreased drug activation	
	Pretomanid	*fgd1*	Glucose-6-phosphate oxidation	Decreased drug activation	
	Imipenem/cilastatin	*crfA*	Unknown	Drug inactivation	
	Amikacin, Capreomycin, Kanamycin[c]	*Rrs*	16S rRNA	Target modification	
	Streptomycin	*rpsL, rrs*	12S ribosomal protein	Target modification	

		rrs	16S rRNA	Target modification
		gidB	7-Methylguanosine methyltransferase	Target modification
	Ethionamide	*ethA*	Mono-oxygenase	Decreased drug activation
	Prothionamide	*ethR*	Transcriptional regulatory repressor protein (TetR)	Decreased drug activation
	Para-aminosalicylic acid (PAS)	*folC*	Folate pathway	Decreased drug activation
		dfrA	Dihydrofolate reductase	Target amplification
		thyA	Thymidylate synthase	Target modification
		thyX	Catalyzes dTMP and tetrahydrofolate	Mitigating target inhibition
		ribD	Enzyme in riboflavin biosynthesis	Mitigating target inhibition
Other medicines[c]	Kanamycin	*Eis*	Aminoglycoside acetyltransferase	Inactivating mutation
	Capreomycin	*tlyA*	rRNA methyltransferase	Target modification

Appendix II

This section of the appendix presents the results from the initial screening which was done after optimization of the primers to screen the presence of the genes for the desired organisms and resistant genes of interest for this study. This preliminary test was done initially on the influent samples from all the three WWTPs selected for this study. These preliminary results assisted in selecting optimized primers for this study mentioned in chapter three and four.



Detection of *Mycobacterium tuberculosis* complex from three wastewater treatment plant's influent in Durban; Isipingo (**WWTP1**), Shallcross (**WWTP2**) &Northern works (NW)

Table 1: Detection of Total *Mycobacteria* & MTBC in influent samples from three wastewater treatment plants in Durban, South Africa

Organism	Primer name	WWTP 1	WWTP 2	WWTP 3
Total *Mycobacterium*	16s rRNA	+	+	+
Mycobacterium tuberculosis complex	Rv0577	+	+	+

M-Marker, **N**-Negative control, **P**-Positive control, **WWTP1**-ISIPINGO, **WWTP2**-SHALLCROSS, **WWPT3**-NORTHERN WORKS

Figure S2: Preliminary results of total mycobacteria and MTBC species on influent wastewater samples by conventional PCR

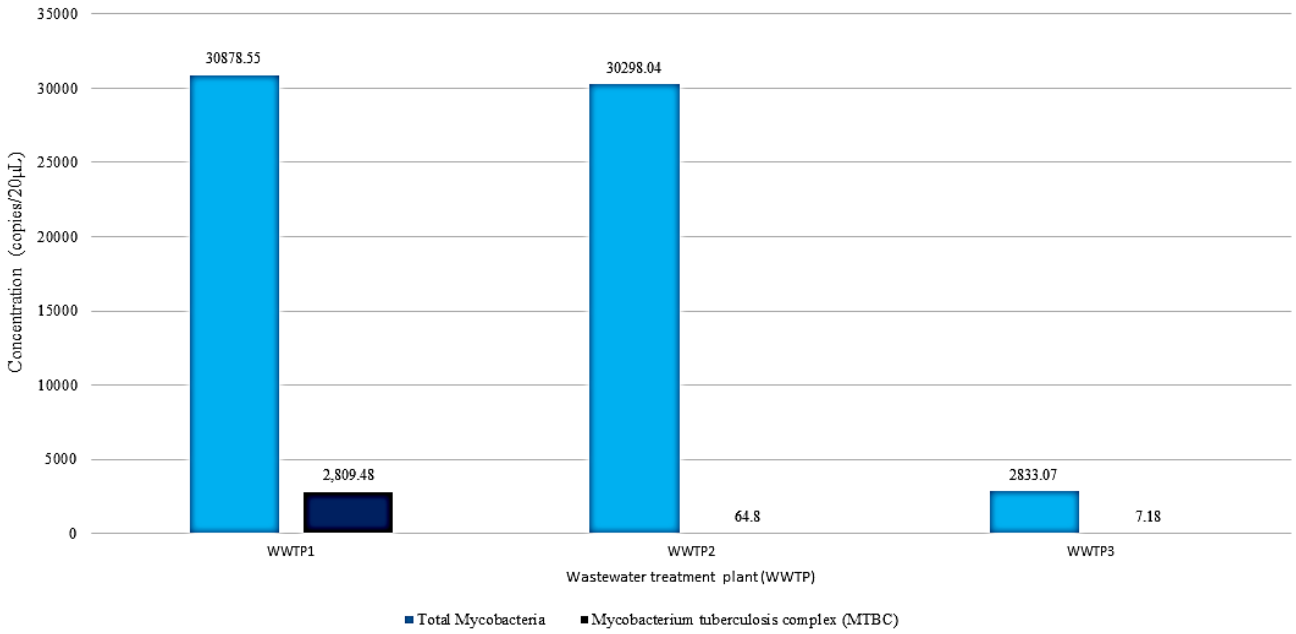
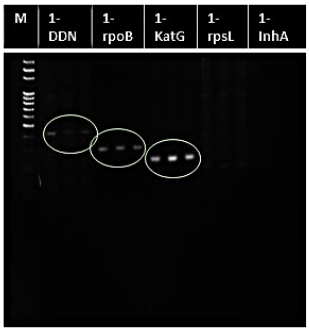


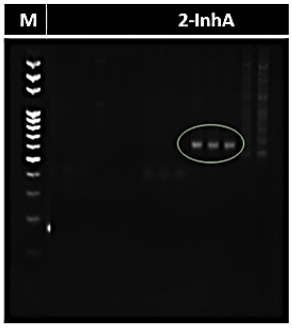
Figure S3: Preliminary results on the quantification of total mycobacteria and MTBC in untreated wastewater samples via ddPCR

Preliminary Screening of ARGs associated with tuberculosis resistance on wastewater samples

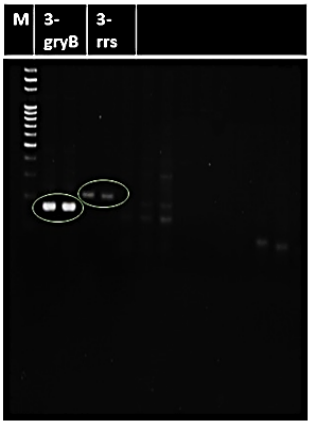
- Detection by PCR



Detection of genes coding for genes in delamanid (ddn),rifampicin (rpoB) & isoniazid (katG) from Isipingo WWTP's influent



Detection of genes coding for genes in isoniazid (inhA) from Shallcross WWTP's influent



Detection of genes coding for genes in delamanid (ddn),rifampicin (rpoB) & isoniazid (katG) from Isipingo WWTP

Figure S4: Preliminary results of genes coding for TB resistance in untreated wastewater samples using conventional PCR

Table 4: Prevalence of genes associated with tuberculosis resistance from three WWTPs in Durban, KwaZulu Natal.

Drug	Gene	WWTP 1	WWTP 2	WWTP 3
Rifampicin	*rpoB*	+	+	+
Isoniazid	*KatG*	+	+	+
	inhA	+	+	+
Ethambutol	*embB*	+	+	-
Pyrazinamide	*PncA*	+	+	-
Ofloxacin, Moxifloxacin	*gyrA*	+	+	+
	gryB	+	+	+
Kanamycin, Amikacin	*Rrs*	+	+	+
Amikacin, Kanamycin	*Eis*	+	+	-
Streptomycin	*rpsL*	-	+	+
Delamanid	*Ddn*	+	+	-
	fbiA	+	+	+
	fgd1	+	+	+
Bedaquiline	*Rv0678*	+	+	+
	atpE	+	+	+
Ethionamide	*ethA*	+	+	+
	ethR	+	+	+
Cycloserine	*Alr*	+	+	+

WWTP1- ISIPINGO, **WWTP2**- SHALLCROSS & **WWTP3**-NORTHERN WORKS

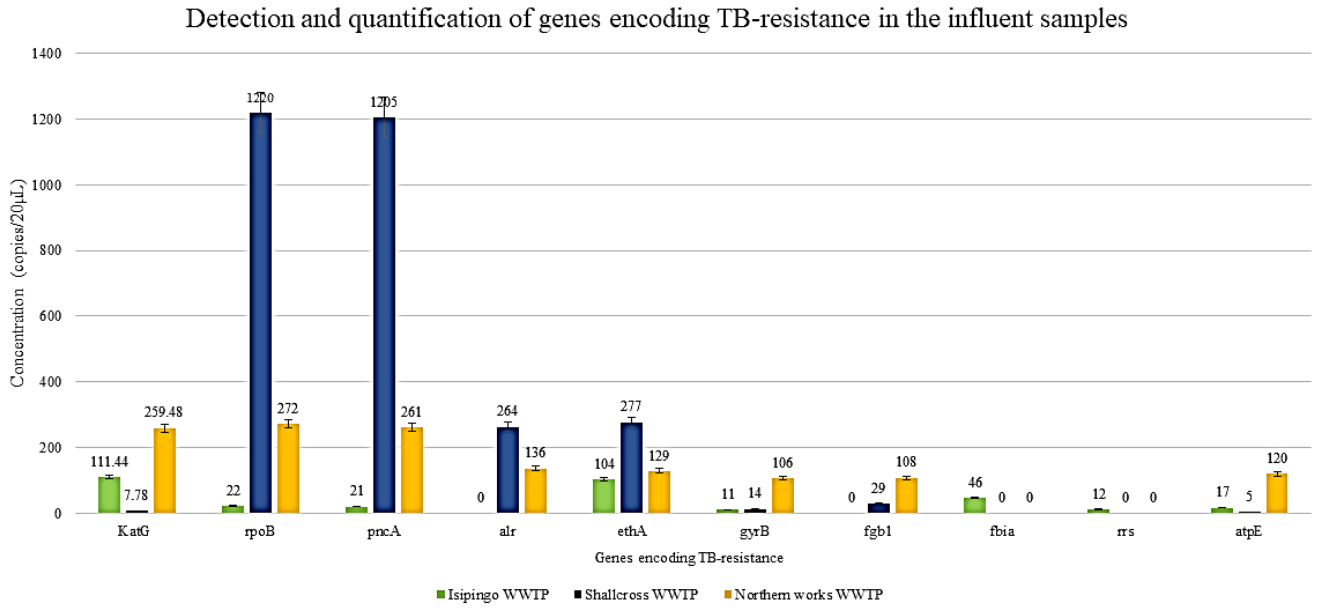


Figure S5: Preliminary results of genes coding for TB resistance in untreated wastewater samples from three WWTPs using ddPCR

Appendix III

The following is part of the literature review which has been extended into a full manuscript that has been submitted as a review paper to *BMC Public health* as well as the materials used in this study alongside the suppliers. Please note that prices are subjected to change, always request for a quotation from the supplier.

The source and fate of *Mycobacterium tuberculosis* Complex in wastewater and possible routes of transmission

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Abstract

Background: The *Mycobacterium tuberculosis* complex (MTBC) consists of causative agents of both human and animal tuberculosis and is responsible for over 10 million annual infections globally. Infections occur mainly through airborne transmission, however, there are possible indirect transmissions through a faecal-oral route which is poorly reported. This faecal-oral transmission could be through the occurrence of the microbe in environments such as wastewater. This manuscript, therefore, reviews the source and fate of MTBC in the wastewater environment, including the current methods in use and the possible risks of infections.

Results: The reviewed literature indicates that about 20% of patients with pulmonary TB may have extra-pulmonary manifestations such as GITB, resulting in shedding in feaces and urine. This could potentially be the reason for the detection of MTBC in wastewater. MTBC concentrations of up to 5.5×10^5 ($\pm3.9\times10^5$) copies/L of untreated wastewater have been reported. Studies have indicated that wastewater may provide these bacteria with the required nutrients for their growth and could potentially result in environmental transmission. However, 98.6 (± 2.7) %, removal during wastewater treatment, through physical-chemical decantation (primary treatment) and biofiltration (secondary treatment) has been reported. Despite these reports, several studies observed the presence of MTBC in treated wastewater via both culture-dependent and molecular techniques.

Conclusion: The detection of viable MTBC cells in either treated or untreated wastewater, highlights the potential risks of infection for wastewater workers and communities close to these wastewater treatment plants. The generation of aerosols during wastewater treatment could be the main route of transmission. Additionally, direct exposure to the wastewater containing MTBC could potentially contribute to indirect transmissions which may lead to

pulmonary or extra-pulmonary infections. This calls for the implementation of risk reduction measures aimed at protecting the exposed populations.

KEYWORDS: *Mycobacterium tuberculosis* complex (MTBC), Wastewater, Sewage, Environment

1. Background

Tuberculosis (TB) is a communicable disease and one of the top ten causes of death globally, ranking above human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) (1, 2). It is caused by a group of closely related slowly growing mycobacteria, collectively named *Mycobacterium tuberculosis* complex (MTBC), which infect a large spectrum of mammals, including humans (3, 4). This includes *M. bovis*, the causative agent of tuberculosis in both animals and humans (5, 6, 7, 8) and *M. africanum*, the causative agent of human tuberculosis (mainly in Western Africa (9, 10). Lesser-known members of this group are *M. microti*, *M. caprae*, *M. pinnipedii*, *M. canetti* and *M. mungi*, usually associated with infections animals with possible transmission to humans. According to the World Health Organisation (WHO), an estimated 10 million people (as of 2018) were infected with TB worldwide (11). Geographically, Africa accounted for 24% of the reported TB cases in 2018 (12). Human immunodeficiency virus (HIV) is considered an important risk factor for contracting TB in most African countries especially South Africa, with co-infection associated with increased morbidity and mortality (13, 14, 15). Over 70% (6 million) of humans co-infected with TB and HIV/AIDS live in sub-Saharan Africa where bovine TB represents a potential health hazard to humans as well (16, 17).

The main infection route for TB has been reported to be through exposure to aerosols from infectious patients (18, 19, 20, 21, 22, 23). This fundamentally shows the airborne transmission of pulmonary TB and is currently widely accepted as the primary mechanistic transmission route (24, 25). Although airborne transmission is the main route for TB, other routes have been reported. For example, in 1905, Calmette and Guérin postulated that TB could be transmitted through contaminated food (26, 25). Gao et al. (27) also provided evidence that guinea pigs could be infected by drinking MTBC contaminated water. The clinical and pathological observations in the infected animals were similar to those found in guinea pigs infected via the

respiratory or subcutaneous routes. This shows the possible oral transmission of TB in exposed individuals.

The environmental occurrence of pathogenic mycobacteria has received less attention in comparison to its occurrence in clinical settings. Nevertheless, there is a growing body of evidence to show that water could be a significant vehicle for the transmission of these organisms (28, 29). Previous studies revealed that environmental contamination, from faecal shedding, provided the potential and indirect routes for transmission of *M. bovis* infection (15, 30). The shedding of *M. bovis* cells has already been demonstrated in many animals via oro-nasal mucus, sputum, urine, feaces and wound discharges (31, 32, 26, 33). Investigating this type of indirect transmission is challenging because it results at least from the combination of three essential factors i.e., i) the environmental contamination by shedding from infected animals, ii) the persistence of the bacteria under a viable state in environmental matrices and finally iii) the interaction between a new susceptible host with the contaminated matrices (26). This route of transmission has been implicated most frequently in zoonotic infections than human-to-human infections (15).

Wastewater serves as a link between human activities and the environment and could be the first medium that may be contaminated with MTBC via faecal shedding. However, studies on the occurrence of MTBC in different environmental matrices has not received priority, therefore there's a lack of proper detection techniques for MTBC in the environment. The study of *M. tuberculosis* in wastewater could potentially address limitations in our understanding of transmission, which is currently achieved almost exclusively through studying clinical samples. Therefore, this review systematically summarized the current knowledge on the occurrence of MTBC in wastewater. The current methods of detection and the risk of infections due to exposure to wastewater contaminated with these pathogens are also discussed.

2. Methodology

2.1 Literature Search Strategy

All the papers reviewed were taken from the sources that are available publicly. Publications of potential interest were retrieved from these databases, Google Scholar, Web of Science, Science Direct, and PubMed. The keywords and word strings used were, tuberculosis OR *Mycobacteria* OR *Mycobacterium tuberculosis* OR *Mycobacterium tuberculosis* complex OR *Mycobacterium bovis* AND Wastewater OR Sewage OR Water. Only papers written in the English language were reviewed with no limitation on the year of publication and geographical location of studies. After searching each database, individual article titles and abstracts were assessed to determine their relevance to the scope of this review. Three categories of empirical studies were included in the review: detection of mycobacteria or *Mycobacterium tuberculosis* complex in wastewater and the environment, public health risks from the exposure to wastewater or water contaminated by wastewater (specifically to sewage workers, health care workers and the nearby community), health risks of wastewater irrigation, indirectly health risks, and studies on contamination of crops used for human consumption. Studies that included soil or wastewater contamination by members of the *Mycobacterium tuberculosis* complex were also included in the review.

2.2 Data Extraction

Relevant data extracted included authorship, year of publication, location of study, isolated bacteria, sample matrix, and finally the results obtained. The results obtained included MTBC species, survival period in the environment, source of the pathogenic bacteria, genetic epidemiology of pathogenic bacteria detected. The retrieved information was reviewed and presented in different sections focusing on, the source of the MTBC in human excreta (Section 3), the occurrence and fate of these MTBC cells in the wastewater environment (Section 4) and

144 an assessment of the potential risks associated with MTBC occurrence in wastewater (Section
145 5).

146 **3. Source of MTBC cells in excreta**

147 The occurrence of MTBC cells in wastewater could be a result of the shedding of these cells in
148 excreta and other bodily fluids from infected individuals. This section reviews the available
149 literature on gastrointestinal infections associated with TB (GITB) that may result in excretion
150 in feaces and reports of the actual detection of these cells in excreta.

151 **3.1 Gastrointestinal (GI) infections with MTBC**

152 The primary site of TB is usually the lungs, from which it can get disseminated into other parts
153 of the body (34). The other routes of spread can be contiguous involvement from adjacent
154 tuberculous lymphadenopathy or primary involvement of extrapulmonary organs (35, 36, 37).
155 It is estimated that close to 20% of patients with pulmonary TB may have extra-pulmonary
156 manifestations such as GITB (38, 39). GITB is usually caused primarily through ingestion of
157 the pathogenic MTBC in water and food (such as non-heat-treated milk, vegetables and meat
158 (40, 41). Animals ingest relatively more vegetable feed, which is often contaminated with
159 mycobacteria, or their surface is contaminated with soil often containing mycobacteria (41,
160 42). The ingestion of water and food contaminated with MTBC is therefore the main route of
161 GITB (43, 44, 45). Other routes include infected sputum, hematogenous spread from distant
162 tubercular focus, contagious spread from infected adjacent foci and through a lymphatic
163 channel (35).

164 The mucosal layer of the gastrointestinal (GI) tract can be infected with the bacilli with the
165 formation of epithelioid tubercles in the lymphoid tissue of the submucosa (35). The most
166 common region within the GI tract affected is the ileocaecal region, due to its richness in
167 lymphoid tissue and increased absorption rate (38). Some of the symptoms of GITB include

168 diarrhoea, nausea and vomiting (46). Diagnosis of gastrointestinal tuberculosis (GITB) is
169 difficult resulting in increased morbidity (47). However, GITB infections have been reported
170 over the years by multiple studies (48, 49, 47, 50, 51, 52, 35, 53).

171 **3.2 *Mycobacterium tuberculosis* complex in excreta**

172 Most mycobacterial pathogens, causing tuberculosis and tuberculosis-like infections in other
173 soft tissues or lymph nodes, are excreted via human urine if the infection is in kidneys or stool
174 for GITB infections (54, 55, 56, 57, 58). The detection of TB through human stool analysis has
175 also been reported (59). Mitchell et al. (60) reported that clinical signs of TB infection are often
176 correlated with high shedding levels (above 50 colony forming units (CFU)/gram of faeces).
177 *M. bovis* has been detected in both goat and cattle faeces (15). Despite being a human pathogen
178 *M. tuberculosis* has been detected in cattle as well (15). This indicates interspecies infection,
179 which can be determined based on the detection of these different species in faecal samples.
180 For instance, the urine of badgers and possums has been reported to aid in the interspecies
181 transmission of bovine tuberculosis to cattle due to the detection of this pathogen in the urine
182 (61, 41). Therefore, these reports of MTBC shedding in urine and stool could result in their
183 occurrence in wastewater (discussed below, Section 4).

184 **4. Occurrence and fate of *Mycobacterium tuberculosis* complex (MTBC) in wastewater**

185 The occurrence of MTBC in wastewater has been reported over the years as shown in Table 1.
186 These studies detected the presence of MTBC in different matrices, such as wastewater or
187 sewage and surface water impacted by wastewater. The literature on MTBC occurrence in
188 wastewater has focused largely on the molecular-based detection of these cells with less to no
189 quantification data presented. This could be attributed to the challenges with the methods for
190 quantification as described in Section 4.1. The most commonly reported MTBC in wastewater
191 are *M. tuberculosis* and *M. bovis* (See Table 1). These bacteria were commonly reported, using

conventional culture-based, biochemical analysis and molecular-based techniques, in raw wastewater (62, 63, 64, 65), treated wastewater (65, 66, 67), activated sludge (66), soil (68) and water (17, 62, 68). This could be attributed to the high number of human infections caused by these pathogens (69). *M. bovis* is the second most commonly reported member of the MTBC based on the number of publications reporting the detection of this bacteria in wastewater. The presence of these pathogens in wastewater could either result in potential infections directly or indirectly through the contamination of drinking water. Suliman et al. (62) reported the occurrence of *M. tuberculosis* in both drinking water and wastewater from a hospital in Pakistan, this could be an example of wastewater contamination of drinking water (70). In some instances, these wastewater samples or wastewater impacted surface water and soil were taken from areas connected to hospitals (71, 72), households (71, 72, 73) and farms (63, 74, 75). The available information on MTBC detection in wastewater and other environmental matrices shows an early interest in this domain. The first available reports in this area were from the early 1960s (65), subsequently, a reduction in output was observed in the 1980s. In the last decade, there has been an increase in the number of publications on the occurrence of MTBC in wastewater possibly due to advancements in molecular-based detection methods and high-throughput sequencing data (See Table 1).

Table 1: Occurrence of MTBC in wastewater

4.1 Methods used for the detection of MTBC in wastewater

The lack of data on the occurrence of MTBC in environmental matrices could be mainly due to the lack of sensitive and mass-scalable techniques to detect these organisms in environmental samples (76, 77, 25). Methods for the detection of MTBC in the environment can be categorised into two, culture-based and molecular techniques, these are discussed below and presented in Figure 1.

Figure 1: Representation of the common sample-processing framework for the detection of MTBC in wastewater samples.

4.1.1 Culture-based methods for the detection of MTBC in wastewater

Isolation and culturing of MTBC from wastewater require two key steps, disinfection/decontamination of the samples to remove other microorganisms capable of interfering with their (MTBC) growth and concentration of the samples.

Disinfection/decontamination is usually achieved using 1-4% NaOH, 1% Oxalic acid, 1% HCL (78), or 1% Cetylpyridinium chloride (CPC) & 12% sulfuric acid (H_2SO_4) (79, 80, 81, 68, 82). All these chemicals exert adverse effects on the growth of other microflora that may be in the sample. Numerous studies have previously recommended CPC as the most suitable chemical for decontamination (31, 80, 82). This is because the low toxicity of CPC to mycobacteria enables a fast recovery rate of mycobacteria (80, 82).

However, the elimination of nontarget microorganisms by chemical decontamination is insufficient (83). The incorporation of antimicrobials in the decontamination procedure will remove most of the contaminant bacteria and provide the opportunity for bacilli to grow, which results in highly positive cultures (84, 82). The use of antibiotics, such as nalidixic acid (NAL), vancomycin (VAN) and amphotericin B (AMB), in previous studies, has shown desired effects by reducing contamination rate and improving culture sensitivity (82). In addition to

inactivating other microorganisms, the disinfection/decontamination agents may also inactivate some of the mycobacteria but to a lesser extent (78, 82, 85). The lesser impact of these decontamination chemicals could be due to the tough cell wall of these mycobacteria. Therefore it is recommended that the chemical effects should be balanced to support mycobacterial growth and eliminate contaminating microorganisms (82). Minor inhibitory effects can be ignored because of the significant improvement in the sensitivity of culture due to the use of antibiotics (82, 83).

The next step after decontamination/disinfection is the concentration of the MTBC cells. The most common methods of MTBC concentration from wastewater are filtration (0.2-0.5 um) and centrifugation (86). After cell concentration, the mycobacterial cells are isolated using specific culture media. Middlebrook 7H9 broth (mostly used for enrichment or recovery of MTBC), 7H10 agar, 7H11 agar or Lowenstein Jensen (L-J) slants are the most commonly used isolation media for mycobacterium, with recommended incubation temperature of 35°C-37°C for 6-12 weeks for slow-growing mycobacteria (62, 68, 87). Solid media may also at times be supplemented with a group of antibiotics such as Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim (PACT) or Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, Azilocillin (PANTA) (88, 89). Furthermore, malachite green, which is the selective antifungal agent in L–J, shows inhibitory effects on the growth of different mycobacterial species (82).

MTBC has been successfully cultured from environmental samples (68, 77), using the approaches mentioned above. However, limited sensitivity has been observed due to bacterial overgrowth and the presence of “differentially culturable” (or “viable but non-culturable”) MTBC organisms (77, 90, 91). Many bacteria, including a variety of important human pathogens, are known to respond to various environmental stresses by entry into a novel physiological state, where the cells remain viable but are no longer culturable on standard laboratory media (92, 93, 94). On resuscitation from this ‘viable but non-culturable’ (VBNC)

state, the cells regain culturability and the renewed ability to cause infection. In the case of wastewater, some members of MTBC have been reported as amoeba-resistant (95) also detailed in section 4.3.2. Additionally, MTBC in wastewater may enter into the VBNC state in response to stresses such as lack of oxygen, nutrient scarcity, predation (e.g amoeba), chemical stress (chlorine). This could be one of many ways through which these bacteria may be able to survive most wastewater treatment processes in addition to their intrinsic abilities to survive extreme environmental conditions. It is likely that the VBNC state is a survival strategy, although several interesting alternative explanations have been suggested. For example, it appears that the 'latent' or the 'dormant' phase of *M. tuberculosis* infections represents the VBNC state in this pathogen (93, 96) and that the recurrence of tuberculosis years after a person was thought to be tuberculosis free is due to resuscitation of this pathogen from the VBNC state (92, 97). As cells in the VBNC state are no longer culturable, alternate nonculture methods must be used to demonstrate that cells in this state are alive. Commonly used are reagents (e.g. the BacLights Live/Dead assay) designed to demonstrate, through direct microscopic examination, the presence of an intact cytoplasmic membrane (e.g. the BacLights Live/Dead assay) (98). Despite the application of these methods for the detection of these MTBC cells using culture-based techniques, there is no consensus on the method yielding the highest number of mycobacteria.

A study by Suliman et al. (62) reported the detection of MTBC organisms from hospital sewage water and drinking water by conventional culturing techniques, followed by biochemical analysis. Velayati et al. (68) and his colleagues were successful in detecting *M. tuberculosis* from 80% of hospital wastewater samples from different locations. (185) also reported a higher recovery of *M. tuberculosis* from water (86.5%) than soil (13.4%). The majority of *M. tuberculosis* isolates were recovered from raceway systems (56 of 500, 11.2%) or dump water (15 of 200, 7.5%). Three multidrug-resistant *M. tuberculosis* (MDR-TB) (3.6%), four mono

drug-resistant strains (three isoniazid and one rifampin, 4.8%), and 58 pan susceptible strains (70%) were also detected among the water and soil isolates.

Some limitations have been identified with the use of culture-based methods for the detection of MTBC in wastewater, per the published literature the main issues are contamination from fast growing bacteria. This may result in overgrowth of these bacteria on culture media, which could out compete the MTBC. Decontamination/disinfection or the use of antibiotics has been introduced as a means to reduce the impact of fast growing bacteria on culture plates, however, as discussed above these decontamination/disinfection techniques could also have a detrimental effect on some of the mycobacteria. Furthermore, MTBC in wastewater could easily enter into the VBNC state which may lead to their non-detection via culturing. This could potentially result in the underestimation of concentrations in wastewater.

4.1.2 Molecular methods for the detection of MTBC in wastewater

The development of molecular methods has assisted in addressing some of the challenges associated with the detection of MTBC cells in environmental matrices. While conventional molecular methods (e.g. polymerase chain reaction (PCR)) do not distinguish viable from non-viable organisms, several molecular methods have been developed to do so, including detection of mRNA or selective detection of intracellular markers (99, 100, 101). An increasingly popular molecular method that can be used to detect MTBC cells in the VBNC state is reverse transcriptase (RT)-PCR, which detects RNA. Because the half-life of bacterial mRNA is typically only 3–5 min (102), continued gene transcription by non-culturable cells is considered an excellent indicator of bacterial cell viability. Molecular detection of MTBC has been demonstrated in filtered air samples (103), however, no study to date has applied these molecular techniques to detect MTBC in wastewater samples. This is despite an increasingly robust literature on the detection of various pathogens in natural and built environments (104, 25). Therefore, there is a knowledge gap in relation to molecular detection of MTBC in

wastewater using PCR techniques. The biggest limitation associated with the molecular detection of bacteria in wastewater is the inability to differentiate between live or dead cells. This is a major issue especially in the context of wastewater treatment efficiency and risk of infection assessment. The introduction of RT-PCR has the potential to address this challenge however as mentioned above, this has not been applied yet for the study of MTBC in wastewater.

4.1.3 High-throughput sequencing for the detection of MTBC in wastewater

Other genomic or molecular methods such as sequencing have been applied to successfully identify pathogens, study population structure and pathogen evolution among other outcomes (105, 106). For instance, whole-genome sequencing (WGS) has become the preferential technique for infectious disease epidemiology such as tuberculosis, support for public health and veterinary health professionals in decision making (107, 108, 109). WGS approaches make use of DNA sequencing platforms for the reconstruction of DNA sequences of the genome of an organism (108). MTBC strains have a single-chromosome genome, which makes these organisms well suited for WGS (110). The use of WGS for the design and implementation of direct patient treatment and improvement of surveillance systems has been reported in certain countries in relation to *M. tuberculosis* (108, 111).

Irrespective of the sequencing platform, there is a common pathway or workflow, these are, (1) nucleic acid extraction (either DNA or RNA) is first extracted from the samples or isolates, (2) enzymatic processing of extracted nucleic acid, (3) sequencing of multiple fragments of nucleic material in parallel, and (4) finally bioinformatic analyses of data generated from the sequencing (112).

The use of such sequencing approaches for the detection of MTBC organisms in wastewater samples has seen an increased interest in recent years. Some of these reports do not provide

species identification, with identification down to only the genus (113, 114, 115, 116, 117, 118). However, others have identified known human pathogens, such as *M. tuberculosis* (156) and animal pathogens, *M. avium* and *M. bovis* (119). Additionally, other lesser-known species have been identified through this sequencing approach (120, 121). Table 2 presents some of the publications on the use of different sequencing approaches for the detection of mycobacteria in wastewater and sludge. Therefore, molecular sequencing methods/techniques are useful tools that could potentially play a significant role in the detection of MTBC in wastewater. However, it must be noted that several laboratories do not have access to these sequencing platforms and in some instances, access does not address the issue of costs and skills. This has limited the widespread adoption of these methods. Therefore, there is a need to identify and optimize cost-effective alternative sequencing approaches.

Table 2: Detection of MTBC organisms using sequencing approaches

4.2 Source of *Mycobacterium tuberculosis* complex in wastewater

The occurrence of MTBC in wastewater (Table 1) could be from various sources including domestic, industrial and agricultural.

i) *Domestic wastewater:* This could be primarily due to gastrointestinal infections with MTBC (See Section 3.1) which results in the shedding of MTBC cells in human excreta. The human sewage microbiome is referred to as the collective microbes in sewage from human domestic waste such as feaces, urine, sweat, washing, bathing, etc. (66). This is mainly derived from the human body including the skin, respiratory tract, oral cavity, gastrointestinal tract, and urogenital tract which ends up in wastewater treatment plants (122, 66). Wastewater from hospitals and facilities that receive patients infected with contagious microorganisms has dense concentrations of pathogens which include *Mycobacterium* spp. (123). A study by Jensen (124)

357 demonstrated the occurrence of tubercle bacilli in considerable numbers in the wastewater
358 systems of several towns containing tuberculosis clinics. It is therefore important to note that
359 sewage systems from communities with high TB infections, facilities and institutions receiving
360 pathogen carriers are at risk of contamination due to the presence of these organisms at high
361 concentrations (125, 123).

362 ii. *Industrial wastewater:* This includes wastewater from slaughterhouses and may constitute
363 the largest source of contamination of the environment in some regions (126, 127, 128).
364 Improper management of abattoir wastes and subsequent disposal either directly or indirectly
365 into river bodies portends serious environmental and health hazards (possible infection from
366 *M. bovis*) both to aquatic life and humans (129, 130). Irshad et al. (102) reported that improper
367 disposal of wastes from slaughterhouses could lead to the transmission of pathogens to humans
368 and cause zoonotic diseases such as bacillosis, salmonellosis, brucellosis, and helminths.
369 Pokam et al. (149) reported that *M. bovis* can be transmitted by aerosol and ingestion of infected
370 carcasses.

371 iii. *Wastewater from agricultural fields:* This includes animal excrement, manure and other
372 components: Agricultural fields using manure as a soil amendment could potentially contribute
373 significantly to the pathogen, such as MTBC, in wastewater. The presence of different
374 pathogens in manure has been reported extensively (132, 133, 134, 135). MTBC cells, most
375 especially *M. bovis* have been detected commonly in manure (136, 137, 138), this could
376 therefore significantly result in the contamination of water sources with these pathogens.
377 Additionally, the occurrence of these pathogens in manure could potentially result in the
378 infection of both humans and animals. In addition to the manure, the reports of shedding of
379 MTBC cells in excreta from animals (Section 3.2) could be a significant source of these in
380 wastewater or runoffs from agricultural fields.

4.3 Fate of MTBC in wastewater

MTBC in wastewater could be affected by several processes, such as natural die-off and removal during wastewater treatment. This section addresses the impact of these processes on MTBC in the wastewater environment.

4.3.1 Survival of MTBC in excreta and wastewater

The survival of MTBC in wastewater has not been studied, however, several studies have investigated the survival of tubercle bacilli in other environments, such as soil, water, manure, feces and urine (Table 3). It was observed that tubercle bacilli inoculated in rivers at temperatures 8–12°C and 15–20°C can survive for 50 days (25). Survival up to 6 months has also been reported for *M. tuberculosis* in water (68) and up to 41 months for *M. avium*, which is a common environmental mycobacteria (139). There are also reports of the survival of this bacterium in water (140, 141, 68), using biodegradable organic material in the water especially in biofilms as carbon source (142). The presence of these bacteria in urine could also provide further insights into their possible survival in wastewater (143). The survival times of *M. bovis* and *M. tuberculosis* in human urine has been reported to be over 10 days at 4^{o}C and below three days at 22^{o}C (144). In contrast, at 15 ^{o} C, mycobacteria have been reported to survive up to 6 weeks (55). According to Scanlon and Quinn. (143), the survival time of *M. tuberculosis* in sterilized manure kept at room temperature was up to 172 days. There are a few reports of extended survival for a year or more, generally in faeces or soil under optimal laboratory conditions. A study by Singh et al. (146) reported the survival of *M. tuberculosis* in faeces for 8 weeks or longer if protected from light. Table 3 gives examples of these studies on the survival of MTBC in different environments, showing the paucity of data for wastewater. The characteristics or complexity of wastewater differ significantly from water, feaces and urine, therefore the survival in these matrices may be different from wastewater. This warrants further

research in understanding the survivability of MTBC in wastewater, especially considering the potential for GITB infections as a result of the ingestion of contaminated water and food.

Table 3: Reports on the survival of MTBC in different environmental matrix

4.3.2 Factors affecting the survival of MTBC in wastewater

The survival of MTBC in different matrices could be influenced by several factors, such as temperature, moisture, pH, inhibitors and protection against solar radiation (ultra-violet) (77). Intrinsically, MTBC cells can withstand desiccation due to the presence of a dense external cell wall composed of a large number of fatty acids (161). For instance, *M. tuberculosis* was found to still be viable after exposure to high temperatures for several months (162). Although the mechanisms responsible for this feature are not well-known, reports have indicated a possible role of endogenous synthesis of trehalose (163, 161). Additionally, mycobacterial cells are known to be hydrophobic (42), which may result in their attachment to solid particles in the water environment. This could also play a role in the extensively reported biofilm formation by mycobacterial cells (147, 148, 149, 150). Biofilm formation is a process that represents the most successful adaptation of bacteria against several environmental factors. It has become increasingly evident that biofilms in drinking water supply systems provide a transient or long-lasting habitat for many microbes, including human pathogens (95). Biofilms provide protection against environmental stresses, e.g., desiccation, starvation and the presence of toxics (151, 152). Coupled with their natural ability to withstand desiccation, the wastewater environment with high suspended solids enhancing biofilm formation could provide an additional layer of protection for MTBC cells, enhancing their survival. Mycobacteria are known to have a narrow pH range between 6.2 and 7.3 (215). For instance, *M. tuberculosis* is reported to have extreme sensitivity to acid (215), but there have been reports of the intrinsic

ability of some mycobacteria to maintain intra-cellular pH (216). This gives *M. tuberculosis* the ability to survive in acidic wastewater conditions.

Additionally, microorganisms including *M. tuberculosis* have been reported to be amoeba-resistant which may enhance their survival in wastewater. *M. tuberculosis* (153) and *M. bovis* (154) could survive for hours to days in the amoebal trophozoites. The observation that *M. tuberculosis* and *M. bovis* organisms were engulfed by *Acanthamoeba polyphaga* trophozoites agreed with previous observations made when co-culturing *M. tuberculosis* organisms with the free-living amoeba *Dictyostelium discodium* (95, 155). Mycobacteria survived in the cysts for up to 18 days and cysts protected *M. tuberculosis* organisms against mycobactericidals (5 mg/mL streptomycin and 2.5% glutaraldehyde). This data indicates that MTBC organisms are amoeba-resistant organisms, as previously demonstrated for non-tuberculous, environmental mycobacteria (95, 156, 157). Inter-cystic survival of tuberculous mycobacteria, except for *M. canettii*, could therefore protect them against biocides and play a role in their survival (95, 158). There is evidence to suggest that under starvation caused by nutrient limitations, low pH and lack of oxygen, a nonreplicating state is induced in some mycobacterial cells caused by the metabolic state of the pathogen (159). Some MTBC organisms, like *M. avium*, can survive rapid shifts in oxygen content for prolonged periods by altering their metabolism from aerobic to anaerobic and vice versa (160).

Therefore, it is plausible that MTBC may be able to survive in wastewater, through both intrinsic (cell wall) and extrinsic factors (biofilms). However, the lack of information on the survival of MTBC in wastewater, as mentioned before, makes it difficult to conclusively determine the impact wastewater conditions may have on this group of organisms.

4.3.3 Removal of *Mycobacterium tuberculosis* complex during wastewater treatment

Wastewater treatment plants (WWTPs) serve as the guts of the population, receiving and digesting various human pathogens (122). Several studies demonstrate that human pathogenic or opportunistic bacteria may survive treatment processes (164, 165, 166). Radomski et al. (80) reported *Mycobacterium* concentrations of 5.5×10^5 ($\pm3.9\times10^5$) copies/L in untreated wastewater and $0.74\times10^4 \pm 1.40\times10^4$ copies/L (in 7 positive samples among 13) detected in the final treated wastewater after decantation and biofiltration, and $1.04\times10^6 \pm 1.75\times10^6$ copies/g (in 3 positive samples among 6) in sludge. The most removal of mycobacteria (98.6 ± 2.7%, i.e. 2.4 ± 0.7 $\log_{10}$) was achieved by physical-chemical decantation (primary treatment) and the remaining mycobacteria were removed by biofiltration (secondary treatment) in this study. A study by Chandra and Arora, (167) also reported 50% removal of mycobacterial load during primary sewage treatment processes.

Despite these reports of *M. tuberculosis* removal during wastewater treatment, there are contrasting reports where these organisms are reported to be detected more frequently in both the activated sludge and effluent, than the influent (168, 169). Additionally, pathogenic *Mycobacterium* sp have been reported in treated wastewater effluents from a WWTP treating salty wastewater (170, 168). Da Silva et al. (171) investigated the microbial communities present in effluent samples from two independent field-scale swine WWTPs and concluded that *Mycobacteria* were abundantly observed in the final effluent. This is corroborated by Cai and Zhang, (122) through metagenomic analysis, where a low abundance of the genus *Mycobacterium* was observed in the influent as compared to both the activated sludge and effluent. These reports indicate that wastewater treatment plants may have varying efficiencies in the removal of MTBC cells.

4.3.3.1 Impact of wastewater disinfection processes on MTBC

Tertiary treatment of wastewater usually involves the use of disinfection processes aimed at inactivating microbial organisms before discharge. These processes include chlorination, ozonation, and UV treatment (172, 173, 174, 175). Previous researchers have reported that several strains of mycobacteria are 100−330 times more resistant to chlorine than *E. coli* (176, 177), which is usually used as an indicator for wastewater treatment efficiency. Slow-growing mycobacteria are unaffected at the higher chlorine disinfection, confirming past reports of their high resistance to chlorination (176, 169). Several, other studies have observed resistance of some mycobacteria to the normal chlorination process used either in drinking water or wastewater treatment plants (178,179). The peculiar structure of the mycobacterial cell wall skeleton partly explains the high resistance of mycobacteria to chlorination (180). In mycobacteria, the peptidoglycan is covalently linked to mycolic acids, consisting of long fatty acids up to 90 carbon atoms, through an arabinogalactan bridge. Mycolic acids confer acid fastness to bacilli and represent a thick, hydrophobic barrier preventing diffusion and lowering permeability (180, 181). Chen et al. (182) showed that the resistance of *Mycobacteria* to free chlorine was attributed to the cell membrane composition and observed that the richness of the long-chain saturated fatty acid or rareness of unsaturated fatty acid in the cell membrane might partly explain the higher chlorine resistance of *Mycobacteria* over other bacteria. The high concentration of mycolic acid and slow growth, adherence to surface and hydrophobicity of mycobacteria have been reported to be primarily responsible for the high resistance of mycobacteria to chemical disinfection (183, 184). Comparatively, UV irradiation was more effective in eliminating *Mycobacterium*, however, Lee et al. (183) reported that mycobacteria are 2–10 times more resistant to UV than *E. coli*. Nevertheless, the absence of residual disinfection and low penetrability in water containing suspended solids are the major disadvantages of UV irradiation on a mass scale (184), especially in wastewater treatment.

5. The potential risk of infection with *Mycobacterium tuberculosis* complex found in wastewater during wastewater treatment processes

The reported MTBC in treated and untreated wastewater could result in infections for different populations that may be exposed either directly or indirectly (185). Direct exposure to wastewater could be a major route mainly for WWTP workers, farmers using the treated wastewater for irrigation and the general public exposed to either untreated wastewater within the community or effluent discharge from WWTPs. Despite this potential risk, there is a scarcity of studies in this regard. This section, therefore, discusses the potential of infection using information from related fields but not specifically for MTBC.

5.1. Potential risks of infection for wastewater operators/workers

Most MTBC infections are usually through inhalation of aerosols or droplets, produced either through the coughing or sneezing of infected individuals (186). Therefore, inhalation of water aerosols may represent the major route of exposure to MTBC in wastewater. Exposure through this pathway may expose three main groups of people: (1) individuals that shed viable pathogens into the toilet and are then exposed to these pathogens during the flush of the toilet, (2) individuals that come into contact with wastewater containing viable pathogens during the collection and treatment process, and (3) individuals that contact untreated wastewater containing pathogens during a spill or release of wastewater from the piping and collection system (187). Liquid (droplet) aerosols notably are generated during wastewater aeration and also during the spray application of wastewater including sludge suspensions onto land. Aerosols generated during wastewater treatment might serve as a source of disease in wastewater treatment workers (188).

522 It is well known that exposure of wastewater treatment workers to bioaerosols carries a risk of
523 negative health outcomes (189, 190). This is based on the fact that sewage is known to contain
524 a range of potential pathogens (164) and that some studies have suggested a correlation
525 between exposure to WWTP bioaerosols and a range of respiratory and gastrointestinal
526 symptoms (191, 192). Occupation per se has not been considered as a determinant of
527 contracting TB and its consequent morbidity. Sewage workers enter manholes and closed
528 channels as part of their duties and also man the sewage treatment facilities. They work in
529 confined spaces, closed channels and sewage treatment plants which employ technologies like
530 up-flow anaerobic sludge blanket, activated sludge process, fluidized aerobic bioreactor,
531 sedimentation, trickling filters, series of waste stabilization ponds which produce noxious
532 fumes and bioaerosols (167, 193).

533 A study conducted by Chandra and Arora, (194) consisting of 104 sewage workers with
534 average occupational exposure to sewage work of 21.28 (±10.54) years. Approximately, 21%
535 of the sewage workers had tuberculosis and 92.31% had at least one of the chronic respiratory
536 diseases (COPD (Chronic obstructive pulmonary disease), Asthma or ACOS (Astha-COPD
537 overlap syndrome)). It was concluded that sewage workers have an adverse chronic morbidity
538 profile for tuberculosis. Therefore, there is an urgent need for epidemiological research and
539 targeted screening and public health intervention for tuberculosis in sewage workers as an
540 occupational group.

541 **5.2. Community infections from exposure to wastewater**

542 Due to their small size and lightweight, particles are easily carried by wind and dispersed over
543 considerable distances (195), which may cause infection in on-site workers as well as
544 downwind residents. Several atmospheric factors, such as temperature, wind velocity, smog,
545 and specific humidity, influence the aerosol spread as well as the ability of microorganisms to
546 survive in the air (196, 197). At very low humidity and high temperature, microbes face

547 dehydration, whereas high humidity may give cells protection against solar radiation (198,
548 199). The maximum distance for droplet transmission is currently unresolved, although
549 pathogens transmitted by the droplet route have not been transmitted through the air over long
550 distances (200). It is likely that the distance droplets travel depends on the velocity and
551 mechanism by which they are propelled from the source, the density of the secretions,
552 environmental factors such as temperature and humidity, and the ability of the pathogen to
553 maintain infectivity over that distance (201, 200). Air microbiological analyses have commonly
554 been conducted close to sewage treatment plants (198). Concentrations of airborne bacteria
555 varied in a wide range of 23–4878 CFU/m^3 (202). A study by Brenner et al. (203) recorded
556 concentrations of 86–7143 bacterial CFU/m^3 air at a distance of 25m from the surface of an
557 aeration basin well (198). High microbial numbers were also reported in locations close to the
558 WWTP (198).

559 In addition to aerosols generated during wastewater treatment, the reuse of wastewater for
560 irrigation could also lead to the generation of aerosols (204, 205). Aerosols generated during
561 wastewater treatment and reuse are affected by the same factors as aerosols from the WWTPs.
562 These processes could therefore be a significant route through which the general public may
563 be exposed to MTBC in wastewater leading to infections. However, despite these potential
564 risks, a few studies to date have focused on measuring the risks of infection with TB as a result
565 of aerosols (206, 207) but no study has focussed on measuring the risks of infection with TB
566 as a result of aerosols containing MTBC from WWTPs. This is, therefore, a research niche that
567 requires further studies.

568 The detection of pathogenic mycobacteria in treated wastewater (170, 168) could potentially
569 result in the contamination of surface water. Therefore, exposure to this contaminated surface
570 water may result in infections. However, it is worth noting that the main route of transmission

of TB is through aerosols, therefore the risks of infection from exposure to surface water may be low unless the exposure involves the generation and inhalation of these aerosols.

6. Conclusion and Recommendations

The reviewed literature showed that MTBC could potentially survive in wastewater for months, this could be attributed to their cell physiology and ecology. Additionally, although wastewater treatment has been shown to reduce the concentration of several bacteria, including these MTBC members, there are a significant number of reports on their occurrence in treated wastewater.

The possible exposure of WWTP workers to aerosols generated during wastewater treatment raises the potential risks for infection through this route. Several studies have shown the occurrence of pathogens in aerosols from WWTPs. Additionally, risks of infection could exist for the general public due to the transport of these aerosols further away from the WWTPs or to aerosols generated during wastewater reuse.

This review also exposed gaps in our knowledge on the occurrence and fate of MTBC in wastewater. This calls for further studies to address these areas,

1. Survival in wastewater: No study has explicitly looked at the survival of MTBC in wastewater and the factors influencing these. The conclusion drawn in this review on MTBC survival in wastewater was made based on survival data gathered for other environments like water and urine. Therefore, there is a need to determine their survival in wastewater under field conditions.
2. Risk reduction for sewage workers: The potential risks of infection for sewage workers due to exposure to aerosols requires the implementation of protective measures. Personal respiratory protection devices including the use of particulate respirators (N95

respirators or equivalent could potentially reduce or eliminate the risks of infection with MTBC through the inhalation of contaminated aerosols.

3. Risk assessment: There is the need for a further study to ascertain full pathogen (MTBC) occurrence and concentration in aerosols and determine the link with infections within the workers (occupational health study for WWTP workers)
4. Change in technology: It has been suggested that the use of diffused aeration technology results in a drastic reduction in the generation of aerosols. This could potentially eliminate the transmission of pathogens through aerosols. Alternatively, some researchers have theorized that it should be possible theoretically to reduce the size of the bubble for aeration so that eventually the resulting droplets and particles would be too small to carry any microorganisms.
5. Adherence to the distancing of settlements and WWTPs/wastewater reuse cites: Siting WWTPs and wastewater reuse irrigation sites away from residential areas could potentially reduce the exposure of the general public to aerosols generated during these processes.
6. There is also a need for an improvement on the methods of surveillance that are being used to track the prevalence of tuberculosis as it has been reported that there may be other potential sources of TB from the environment. There is a need for understanding the prevalence, and distribution of *Mycobacterium tuberculosis* complex organisms in the environment specifically wastewater. The prevalence of these tuberculosis-causing microorganisms in the untreated sewage may provide vital information in estimating not only the occurrence but also resistance in the associated population without clinical data on TB and its antibiotic resistance pattern.

7. List of abbreviations

AMB	Amphotericin B
COPD	Chronic obstructive pulmonary disease
ACOS	Asthma-COPD overlap syndrome
CPC	Cetylpyridinium chloride
CFU	Colony forming units
GI	Gastrointestinal
GITB	Gastrointestinal Tuberculosis
HIV	Human immunodeficiency virus
HIV/AIDS	Human immunodeficiency virus/ acquired immunodeficiency syndrome
MDR-TB	Multidrug-resistant *M. tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NAL	Nalidixic acid
PACT	Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim

PANTA	Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, Azilocillin
PCR	Polymerase chain reaction
RT	Reverse transcriptase
TB	Tuberculosis
VAN	Vancomycin
VBNC	Viable but nonculturable
WGS	Whole-genome sequencing
WWTPs	Wastewater treatment plants

8. Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All reviewed articles, books or websites are included under the reference section

Competing interests

No competing interests to declare.

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Authors' contributions

All authors were involved in the conceptualization of the manuscript, data collection was performed by H.N. Mtetwa, I.D. Amoah. Writing of the original draft manuscript was done by H.N. Mtetwa under the supervision of P. Reddy, I.D. Amoah, S. Kumari and F. Bux. Initial reviewing and editing of the manuscript was done by I.D. Amoah, P. Reddy, S. Kumari and F. Bux. The final revision of the manuscript and approval was done by all authors.

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1455 **Tables**

1456 **Table 1: Occurrence of MTBC in wastewater**

Specific MTBC organism	Sample matrix	Study location	Detection method	Target	Reference
M. tuberculosis	Raw sewage, sewage effluent	Poland	Culture-based		(65)
M. tuberculosis	Sanatorium sewage: inlet, settling tank and outlet	India	Culture-based		(67)
M. bovis, M. tuberculosis	Sewage from cattle farm used for pastures	Poland	Culture-based		(74,75)
M. bovis, M. tuberculosis	Sewage from tuberculous sanatorium and hospitals, towns and sewage purification plants	Poland	Culture-based (Sewer swabs)		(71,72)
M. tuberculosis	Sewage sediment	Poland	Culture-based		(208)
M. bovis, M. tuberculosis	Sewage water around tuberculous sanatoria	Kazakhstan	Culture-based		(64)
M. tuberculosis	Wash-off water from wearing apparel, crockery, household utensils, etc	Russia	Culture-based		(73)
M. tuberculosis, M. bovis	River sediment (wastewater present)	Romania, Portugal,	PCR-based	16SRNA sequence	(63,76)
M. tuberculosis	Fresh sewage used for pastures and fields	Germany	Culture-based		(63)
M. tuberculosis	Activated sludge and effluent	Hong Kong	PCR-based	16S rRNA gene & IS6110	(64)
M.bovis/caprae/microti/tuberculosis/africanum/pinnipedii	River (sediment/ water)	Portugal	PCR-based	16SRNA sequence	(76)

M. tuberculosis	soil and water	Tehran, Iran	Culture, biochemical and PCR-based	16S-23S RNA gene spacer polymerase chain reaction	(68)
M. tuberculosis	Drinking water and sewage water	Pakistan	Culture, biochemical and PCR-based	RNA converted to cDNA for amplification	(62)
Mycobacterium tuberculosis complex	water	South Africa	PCR-based	Genomic DNA	(17)

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Table 2: Detection of MTBC organisms using sequencing approaches

Specific MTBC organism	Sample matrix	Study location	Detection method	Sequencing method	Reference
Mycobacterium avium, Mycobacterium abscessus, Mycobacterium bovis, Mycobacterium kansasii, Mycobacterium marinum	Wastewater	Hong Kong	Illumina HTS	HTS-based metagenomic analysis	(120)
Mycobacterium sp., *Mycobacterium fortuitum*	Wastewater and sludge	China	Illumina HiSeq	Metagenomic sequencing	(121)
Mycobacterium sp		China	Illumina Hiseq	Paired-end sequencing	(113)
Mycobacterium sp	Wastewater	South Africa	16S-rRNA-Based Amplicon Sequencing	Paired-end sequencing	(114)
Mycobacterium sp	wastewater	Singapore	Illumina HiSeq2500	Metagenomic sequencing	(116)
Mycobacterium sp	wastewater	Vietnam	Illumina TruSeq	Cluster generation and paired-end sequencing	(115)
Mycobacterium sp	Biosolids	Colombia	Illumina MiSeq	Metagenomics and 16S-amplicons sequencing	(117)
Mycobacterium tuberculosis, Mycobacterium sp	wastewater	USA	Illumina MiSeq	Shotgun metagenomic analyses	(119)
Mycobacterium sp	wastewater	Taiwan	Illumina HiSeq PE150	Paired-end sequencing	(209)
Mycobacterium sp	wastewater	USA	NGS—next-generation sequencing	Shotgun whole genome sequencing	(118)

1473 **Table 3: Reports on the survival of MTBC in different environmental matrix**

Specific MTBC organism	Sample matrix	Survival period	References
M. bovis	Soil	150 days	(210)
M. tuberculosis, *M. bovis* and *M. canetti*	Soil	Survival of the distinct mycobacteria in the soil for 12 months	(158)
M. tuberculosis	Soil & water	persisted for 9 months	(68)
M. bovis		88 days in soil, 58 days in water and hay, and 43 days on corn	(81)
M. bovis	Manure	172 days	(145)
M. bovis	River water & distilled water	After 50 days, could still be cultured	(25, 211)
M. bovis	liquid manure	176 days	(143)
M. bovis	vegetables stored at -20^{o}C and 23^{o}C	112 days	(212)
M. bovis	Soil, urine and faeces	6 weeks	(53, 213)
M. bovis	wet soil	21 months	(214)

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QUOTATION

Client name:	Hlengiwe N. Mtetwa	**Created by:**	Bruce Pillay
Customer:	DURBAN UNIVERSITY OF TECHNOLOGY	**Quote no:**	2006-302133024
Address:	Stores , 41/43 M L Sultan Rd, Greyville, Durban, 4001, South Africa	**Date:**	22 July 2020
Floor Level:	1st Floor	**Quote Expires on:**	27 July 2020
Department:	Institute For Waste Water Technology	**Page:**	1 of 2

Note:
Price are valid ONLY for 5 days.

Stock code and description	Pack size	Note	Quantity	Unit Price	Sub-total
BBRDMSB1001 PLATE PCR SEAL ADHESIVE OPTICAL	PK 100	13 Units currently available. Subject to prior sale.	1	3 147.85	3 147.85
BBRD1864120 PIPET TIPS AUTO DG (QX100/200)	PK 20	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	7 865.91	7 865.91
BBRD12001925 DDPCR PLATE 96W SEMI SKIRT PK 25	EA	3 Units currently available. Subject to prior sale.	1	2 745.38	2 745.38
BBRD1864034 QX200 DDPCR EVAGREEN SUPERMIX 500 RXN 5 X 1ML	EA	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	9 136.67	9 136.67
BBRD1814040 PLATE PCR PIERCEABLE FOIL HEAT SEAL	EA	3 Units currently available. Subject to prior sale.	1	1 617.12	1 617.12
BBRD1864108 DG32 CARTRIDG QX100/QX200 DG	PK 30	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	26 834.90	26 834.90
BBRD1864112 AUTO DG OIL EG (QX200)	EA	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	9 821.46	9 821.46
BAMPA608204 5X DNA LOADING BUFFER BLUE 5X1ML	PK5	Price valid ONLY for 4 Units currently available. Subject to prior sale.	1	485.00	485.00
Or alternative below					
BBRD1665111 UVIEW 6X LOADING DYE 200UL	EA	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	815.36	815.36
BAMPA610541 DNA LADDER A610541	EA	Price valid ONLY for 8 Units currently available. Subject to prior sale.	1	650.00	650.00
BAMPA4112416 NUCLEASE FREE WATER PCR GRADE 1L	EA	price valid ONLY for 10 Units currently available. Subject to prior sale.	1	1 200.00	1 200.00
BBRD1610733 BUFFER10X TRIS BORIC EDTA 1L	EA	Currently we have no stock available. Lead time of 6-8 weeks expected.	1	1 997.19	1 997.19

Total excl. VAT	R 66 316.84
VAT 15%	R 9 947.53
Total incl. VAT	R 76 264.37

We trust this meets with your approval and assure you of our continued service

Bruce Pillay

For and on behalf of Lasec SA (Pty) Ltd

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Department:	Institute For Waste Water Technology	**Page:**	2 of 2

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Order Confirmation

Prepared for: Durban University of Technology (DUR001) Hlengiwe Mtetwa Steve Biko Campus S10, Level 1, Room 112 Durban, Kwazulu-Natal South Africa Phone: 031 373 2346	**Quotation Number:** SA2021/104948 **Your Order Reference:** PO190058 **Quotation Date:** 08 February 2021 **Date Ordered:** 17 February 2021 **Your Reference:** PO190058 **Sales Contact:** Pule Shabalala	**Delivery Address:** Durban University of Technology (DUR001) Hlengiwe Mtetwa Steve Biko Campus S10, Level 1, Room 112 Durban, Kwazulu-Natal South Africa Phone: 031 373 2346

Code	Description	Supplier	In Stock	Qty	Price Per Unit	% Disc	VAT	Subtotal excl. VAT
IB OL0001	4 Oligonucleotide, 0.01 umole scale, per mer	inqaba biotec	No (0)	82	R 5.33	0.00	15 %	R 437.06
NEB B7021S	Gel Loading Dye, Blue (6X) - 4,0 ml; Storage Temp: RT/4°C/-20°C; Shipping: RT Product Link (Click to Open)	New England Biolabs	No (0) , Incoming	1	R 800.02	0.00	15 %	R 800.02
NEB M0486S	OneTaq Quick-Load 2X Master Mix with Standard Buffer - 100 rxns; Storage Temp: -20°C; Shipping: Cool Packs Product Link (Click to Open)	New England Biolabs	Yes (12)	2	R 586.68	0.00	15 %	R 1,173.36
NEB N3231S	DNA Ladder 100 bp - 100 gel lanes; Storage Temp: -20°C; Shipping: Cool Packs Product Link (Click to Open)	New England Biolabs	Yes (9)	2	R 944.02	0.00	15 %	R 1,888.04
NEB N3232S	DNA Ladder 1 kb - 200 gel lanes; Storage Temp: -20°C; Shipping: Cool Packs Product Link (Click to Open)	New England Biolabs	No (0) , Incoming	4	R 880.02	0.00	15 %	R 3,520.08

Total Excluding VAT	R 7,818.56
VAT 15%	R 1,172.78
Total Including VAT	R 8,991.34

Specification of Oligonucleotide(s):

Name	Scale	Purification	5' Modification	Sequence	3' Modification	Bases	Comments
16S rRNA F	0.01 (umole)	Desalting	None	GAGATACTCGAGTGGCGAAC	None	20	
16S rRNA R	0.01 (umole)	Desalting	None	CAACGCGACAAACCACCTAC	None	20	
Rv0577 F	0.01 (umole)	Desalting	None	ATGCCCAAGAGAAGCGAATACA	None	22	
Rv0577 R	0.01 (umole)	Desalting	None	AATGTCAGCCGGTTCCGCAA	None	20	

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