Legionella sp. occurrence in urban and rural water systems in South Africa

A Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

Background

Legionella species are intracellular bacteria that thrive in various water systems, posing significant health risks such as Legionnaires' disease. The bacterium survives in natural and man-made water environments, including biofilms, water tanks, and premise plumbing. The occurrence of *Legionella* is particularly concerning in South Africa, where infrastructure challenges, urbanisation, and a high prevalence of immunocompromised individuals exacerbate its public health risk. These bacteria thrive in temperatures ranging from 25°C to 55°C, often multiplying within biofilms and protozoa, which provide nutrients and protection from environmental stresses. *Legionella pneumophila*, the most pathogenic species, is responsible for most human infections, including Legionnaires' disease and Pontiac fever. The bacteria are transmitted through aerosols from contaminated water sources, such as showers, faucets, and air-conditioning systems. Outbreaks of Legionnaires' disease have been reported in hospitals and large buildings due to poorly maintained water systems. Water stagnation, biofilms, and suboptimal temperature control are major factors contributing to bacterial proliferation. Immunocompromised individuals, including those with HIV and tuberculosis, are at increased risk of infection. In South Africa, the incidence of Legionnaires' disease is underreported due to diagnostic limitations and misdiagnosis, with many cases possibly obscured by overlapping symptoms with other respiratory illnesses such as pneumonia. With increasing urbanization, aging infrastructure, and climate change, the public health threat posed by Legionella is expected to rise. Addressing this issue requires enhanced monitoring, improved water management practices, and regular maintenance of water systems. This study explores the prevalence of *Legionella* in water systems across urban and rural areas of South Africa to address the critical need for improved water quality monitoring.

Aims

The project aimed to:

1. Assess the presence and diversity of *Legionella* species in selected urban and rural buildings.
2. Analyse genetic variations in *Legionella* strains using molecular techniques.
3. Provide recommendations for water quality monitoring and safety management in buildings.

Study Design

The study design used in this research is a cross-sectional observational study. It involved the collection of water and swab samples from multiple urban and rural sites in the Gauteng and Limpopo provinces in South Africa during October 2022 to July 2024. The areas included Hillbrow, Vhembe, Melusi, Zandspruit, and Atteridgeville, to assess the presence of *Legionella pneumophila* contamination. The study focused on analysing environmental factors, infrastructure, and water quality characteristics across different socio-economic settings. It did not intervene in the conditions but rather observed and measured the prevalence and concentration of *Legionella* bacteria at a specific point in time across the various locations, making it cross-sectional in nature.

Methodology

The study employed a descriptive approach to assess the prevalence of Legionella species in water distribution systems and biofilms across urban (Hillbrow, Atteridgeville, Zandspruit) and rural (Melusi, Vhembe) areas of South Africa. Ethical clearance was obtained from relevant institutions, and access was secured through the Gauteng Research Triangle and community engagement with local stakeholders. Convenience sampling was used to collect water and biofilm samples from different types of buildings, including high-rise apartments, informal settlements, and household buildings. Samples were collected from multiple points, such as water inlets, showerheads, faucets, storage tanks, and geysers. A combination of microbiological methods was used, including culturing, amoebal enrichment, IDEXX Legiolert™ quantification, and molecular analysis (PCR and qPCR) to identify *Legionella* species. The study also included a risk assessment of *Legionella* exposure based

on collected data and a Quantitative Microbial Risk Assessment (QMRA) framework to estimate health risks associated with aerosolization of contaminated water. Data were analysed using SPSS v28 software for statistical significance and visualized using GraphPad Prism and BioRender® software. The study's findings will be widely disseminated. An information booklet on water safety and *Legionella* prevention will be distributed to stakeholders, including building managers and municipal authorities. Results have been shared at national and international conferences. Ongoing engagement with property managers and households will ensure local communities are informed about water quality. The project also supported academic development, with three Master's students having completed their dissertations.

Results and Discussion

The results of the study demonstrated significant variability in *Legionella* contamination by Legiolert™ across different regions and building types, with notable distinctions between urban and rural areas. In Hillbrow, *Legionella* was highly prevalent, particularly in cold tap water and geysers. In Hillbrow, cold tap water exhibited moderate contamination, with 14 samples in the 11–100 MPN/100mℓ range, whilst one building sample exceeded 1000 MPN/100 mℓ, indicating severe contamination. Geyser water showed lower contamination, with 10 samples <1 MPN/100mℓ and 13 samples in the 11–100 MPN/100mℓ range. No samples exceeded 2272.6 MPN/100mℓ. Biofilm samples from faucets and showerheads also showed levels of contamination, pointing to potential challenges associated with maintaining safe water distribution systems in dense urban environments. In Vhembe, a rural area, the study found considerable *Legionella* contamination, primarily in stored water and geysers, with several samples exceeding acceptable safety limits. In Vhembe, cold tap water contamination was minimal, with most samples <1 MPN/100mℓ, but 2 samples fell into the 11–100 MPN/100mℓ range, and one sample exceeded 2272.6 MPN/100mℓ. Geyser water showed a higher microbial load, with 40 samples <1 MPN/100mℓ, 11 samples in the 11–100 MPN/100mℓ range, and one sample exceeding 101–1000 MPN/100mℓ. Stored water results varied, with most samples <1 MPN/100mℓ, but some reaching up to 101–1000 MPN/100mℓ. The contamination was especially prominent in informal housing structures where storage tanks were not regularly maintained, allowing *Legionella* to thrive. In Atteridgeville, contamination levels were moderate, while some samples from storage tanks and taps did show the presence of *Legionella* at levels generally below those levels found in Hillbrow and Vhembe. In Atteridgeville, 12 cold tap samples were <1 MPN/100mℓ and 3 samples in the 11–100 MPN/100mℓ range; however, one sample exceeded 2272.6 MPN/100mℓ. Geyser water was mostly uncontaminated, with 12 samples <1 MPN/100mℓ and 4 samples in the 11–100 MPN/100mℓ range. Stored water followed similar trends, with most samples showing low microbial counts. However, biofilm samples from infrequently used taps and showerheads still presented contamination risks. The informal settlements of Zandspruit and Melusi had relatively lower levels of contamination. Although most of the samples from these areas fell within acceptable limits, a few exceeded the threshold, particularly in older storage systems and shared community taps. In Zandspruit, microbial contamination was more pronounced, with 18 cold tap water samples in the 11–100 MPN/100mℓ range and one sample in the 101–1000 MPN/100mℓ range. Stored water also showed moderate contamination, with 5 samples <1 MPN/100mℓ and 8 samples in the 11–100 MPN/100mℓ range. In Melusi, microbial contamination was lower but still present, with 7 cold tap water samples in the 11–100 MPN/100mℓ range and one sample <1 MPN/100mℓ. Stored water showed a similar trend, with most samples falling within the 11–100 MPN/100mℓ range. These results indicate that even in areas with lower population density, inadequate infrastructure and sporadic maintenance poses significant risks for *Legionella* growth.

Colilert testing results further identified some contamination with *E. coli*, in Vhembe, indicating faecal contamination in some water systems. This highlights the multifaceted challenges in maintaining water safety, where microbial risks are not limited to *Legionella* but extend to other pathogenic bacteria. In Zandspruit and Melusi, *E. coli*-contamination was less prevalent, suggesting fewer issues with faecal contamination compared to the rural Vhembe region.

Risk assessments suggest that contamination in these areas is driven by a combination of factors, including older infrastructure, biofilm formation, inadequate maintenance, and environmental conditions that promote *Legionella* growth. This underscores the need for regular monitoring, system upgrades, and community-level education to mitigate the public health risks associated with *Legionella*-contaminated water.

General

The project achieved its aims by demonstrating the widespread presence of *Legionella* in diverse water systems across Gauteng and Limpopo Provinces in South Africa. The research highlighted the need for improved water management practices and the importance of regular water monitoring to mitigate the risks of *Legionella* contamination in municipal water and water supply infrastructure. Details of conference presentations and the MSc dissertation resulting from this research are presented below.

Conclusions

The discussion highlights the impact of socio-economic and environmental factors on *Legionella pneumophila* contamination across different regions. Temperature played a critical role, with the bacterium thriving in temperatures between 20°C and 50°C, though it persisted in both lower and higher temperature ranges, particularly in regions with poor water supply infrastructure. Rural areas like Vhembe and informal settlements such as Zandspruit and Melusi demonstrated higher risks of contamination due to inadequate water systems and inconsistent maintenance practices. In contrast, low-income urban areas such as Atteridgeville and Hillbrow exhibited contamination linked to aging water supply infrastructure and biofilm development in complex water systems, exacerbated by high population density.

The study concludes that *Legionella* poses a public health risk in both urban and rural settings, particularly where water systems are poorly maintained or where environmental conditions favour bacterial growth. The findings underscore the need for targeted interventions to improve water safety and reduce the incidence of Legionnaires' disease.

Recommendations

Based on the findings, the following is recommended:

1. Implementing regular maintenance and monitoring of water systems, especially in high-risk areas. This includes ensuring consistent temperature and chlorination levels and conducting regular inspections to prevent biofilm formation and water stagnation.
2. Enhancing public health surveillance and diagnostic capabilities for *Legionella,* with a focus on improving *Legionella* detection using molecular techniques and incorporating routine testing in vulnerable facilities.
3. Developing and distributing educational materials to raise awareness about *Legionella* risks and prevention strategies among building managers and the public.

Conference platform presentations emanating from this project:

1. Buthane KE, Barnard TG and Singh A. 2023. Occurrence of *Legionella* in High-rise Buildings in Hillbrow Johannesburg. South African Society for Microbiology (SASM) Biennial Congress, Protea Hotel Stellenbosch 17-20 September 2023.

2. Buthane KE, Barnard TG and Singh A. 2023. Hidden Threats in the Towers: Unveiling the Presence of *Legionella pneumophila* in Johannesburg's High-Rise Buildings. The Postgraduate and PDRF Annual Research Conference, Emperors Palace Convention Centre, Kempton Park, Johannesburg 20 October 2023.

3. Buthane KE, Delair Z, Barnard TG and Singh A. *Legionella pneumophila* in Urban Water Systems: A Case Study from Johannesburg's High-Rise Buildings. WISA 2024 Biennial Conference and Exhibition 27-29 May 2024, Durban.

4. Singh A, Buthane, KE, Mnisi ZF and Barnard TG. *Legionella pneumophila*: Hidden in Plain Water. IWA World Water Congress and Exhibition, August 2024- Canada.

MSc dissertations emanating from this project:

1. Miss Buthane, Keletso Emily. MHSc, Master's in Health Science (Biomedical Sciences, University of Johannesburg). Occurrence of *Legionella pneumophila* in high-rise buildings in Hillbrow, South Africa. 2024.
2. Miss Mnisi, Zandice Faith. MHSc, Master's in Health Science (Biomedical Sciences, University of Johannesburg). *Legionella* circulating in urban and rural water systems in Gauteng, South Africa. 2025.
3. Mr Mulaudzi Bongani Casner. MSc, Master's of Science (Microbiology, University of Venda). Prevalence of *Legionella* spp in residences around Thohoyandou, South Africa. 2025.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

ARB	Amoeba Resistant Bacteria
ATCC	American Type Culture Collection
BCYE	Buffered Charcoal Yeast Agar
CAP	Community Acquired Pneumonia
CT	Threshold cycle
dNTP	Deoxyribonucleotide Triphosphate
DVCFISH	Direct Viable Count Fluorescent In situ Hybridization
ECM	Extracellular Matrix
FLA	Free Living Amoeba
GRT	Gauteng Research Triangle
HB	Hillbrow Building
ISO	International Standard Organization
LD	Legionnaires Disease
LVC	*Legionella* Containing Vacuole
MIF	Mature Intracellular Form
MPN	Most Probable Number
NNA	Non-Nutrient Agar
NTU	Nephelometric Turbidity Unit
OMV	Outer Membrane Vesicle
PAS	Page's Amoeba Saline
PCR	Polymerase Chain Reaction
L. pneumophila	*Legionella pneumophila*
PVC	Polyvinyl Chloride
RF	Replicating Form
RPM	Rotors Per Minute
RTPCR	Real Time Polymerase Chain Reaction
SA	South Africa
SANS	South African National Standard
spp.	Species
TDS	Total Dissolved Solutes
UK	United Kingdom
USA	United States of America
VBNC	Viable But Nonculturable
VF	Virulent Form
WHO	World Health Organization

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Despite standard treatment procedures, *Legionella* species are known to be ubiquitous in nature and can spread and settle in potable water distribution systems of large buildings and other water supply systems (LeChevallier, 2019; Magdzińska et al., 2023). These opportunistic pathogens are intracellular bacteria that rely on free-living protozoa, amoeba, and biofilms as hosts to survive stressful conditions in water treatment systems, thereby reducing water quality. *Legionella pneumophila* can cause Legionellosis (Pontiac fever or Legionnaires' disease), which manifests with a severe headache, fever, chills, chest pains and dry cough, and can be fatal in vulnerable human populations. Large buildings, hospitals and hotels have reported on outbreaks of Legionnaires' disease internationally, with cooling water systems, showerheads, faucets, and ventilators being the source of water-to-air transmission of *Legionella pneumophila* in droplets (Szwetkowski and Falkinham, 2020). Human infection most commonly occurs as a consequence of inhaling *Legionella*-containing aerosols generated by contaminated manmade water sources, such as showers, hot tubs, plumbing networks, and air-conditioning systems. Other research has shown that aspiration of contaminated water has been suggested as another route of transmission. Water stagnation significantly contributes to *Legionella* proliferation, especially through biofilms. Stagnant water, especially in man-made environments and stored water systems, provides an ideal breeding ground for Opportunistic Premise Plumbing Pathogens (OPPPs), which include *Legionella* bacteria due to water aging and a decrease in disinfectant concentrations, which may lead to an increase in growth. Studies in Europe have emphasized the need for regular water system maintenance to prevent stagnation and subsequent proliferation (Falkinham et al., 2015a; 2015b; Leslie et al., 2021; LeChevallier, 2019). Direct and indirect exposure to dispersed water droplets from stagnant water should also be considered as a risk factor for *Legionella* exposure and an indicator to screen for these bacteria, particularly as these bacteria have also been found to flourish in cold water environments and stagnant sections of indoor plumbing systems (Cassell et al., 2021). Immunocompromised individuals are mainly affected following exposure to *Legionella*, as only 5% of healthy individuals who are exposed develop the disease (Muchesa et al., 2018; Trnková et al., 2018). South Africa is dominated by low-income and immunocompromised people affected by tuberculosis, pneumonia, HIV and AIDS, and these disease burdens create diagnostic biases that obscure potential legionellosis outbreaks. In addition, the threat of Legionnaires' disease is compounded as its victims tend to display the same symptoms as respiratory disease patients, including cough, chills, and fever, making misdiagnosis possible. The reported cases of Legionnaires' disease in South Africa are relatively low compared to other waterborne diseases such as cholera and typhoid fever (Stone et al., 2019). This low incidence may contribute to the perception that *Legionella pneumophila* is not a significant public health threat. It is plausible that cases of Legionnaires' disease are underreported due to misdiagnosis or lack of diagnostic facilities, particularly in rural areas. This could potentially skew the perception of its threat level.

Legionellosis incidence and its associated health risks are on the rise due to global challenges such as urbanization, ageing populations, ageing infrastructure, climatic changes, and circular economy approaches (Graham et al., 2020; Parr et al., 2015). Although field trials are an integral part of studying *Legionella*, these trials often ignore the role of protozoa and biofilms as ecological niches for *Legionella* growth, infectivity, and persistence in water systems.

The screening and control of *Legionella* in water systems is complex, and it is impractical to assume that *Legionella* could be completely removed from treated drinking water systems before use; thus, regular detection is of great importance for risk assessment and disease prevention. The particular focus of this research project is to improve, implement and build capacity for the study of *Legionella* bacteria important to human health using new, innovative, and appropriate detection methods to achieve the global goals set by the World Health Organization for microbial water quality. To stimulate this research agenda, an integrated approach, encompassing traditional and next-generation detection for studying *Legionella* in field-based

systems, is planned. This approach is based on combining the study of complementary sets of information on the water resources of rural and urban settlements and aims to fill the knowledge gap on the circulation of *Legionella* sp in these water systems. The occurrence and distribution of *Legionella* in water sources is still poorly understood in South Africa; therefore, the present study was designed to assess the occurrence of *Legionella* bacteria using different methods of isolation and identification. Sharing knowledge about water resources is essential to ensure water security for all and promote development.

1.2 PROJECT AIM AND OBJECTIVES

The study aimed to determine the occurrence of Legionella spp. In urban and rural water systems in South Africa. In addressing this aim, the following study objectives were developed:

Study objective 1:
To conduct a scoping study on determining the presence and diversity of *Legionella* spp. in selected buildings, in both urban and rural areas

Study objective 2:
To determine any genetic variations in *Legionella* sp. using next-generation molecular techniques and subsequently estimate which water samples posed the greatest health risk of exposure to *Legionella* strains

Study objective 3:
To provide recommendations for water quality monitoring and safety management in buildings

1.3 SCOPE AND LIMITATIONS

This project was designed to provide information on the occurrence of *Legionella* bacteria and Amoeba-associated *Legionella* bacteria within treated and stored water distribution systems in urban and rural areas in the Gauteng and Limpopo provinces in South Africa, respectively. The potential implications for human health risk will also be highlighted from Quantitative Microbial Risk Assessment (QMRA). In addition, a comprehensive pamphlet and or information booklet will be assembled for the safety management of *Legionella* in buildings and stored water systems and distributed to relevant stakeholders at each site. There has been difficulty in accessing the Atteridgeville and Melusi sites due to the rezoning of settlements, and the research team was only allowed to sample in July 2024. Similarly, the research team in Venda could not access sites until permission was granted, which subsequently led to the delay in applying for research ethics clearance and the sample collection began in February 2024. A new informal site Zandspruit, was included in the data.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Legionella species are gram-negative, non-sporing, rod-shaped or filamentous fastidious bacilli, which are ubiquitous in natural aquatic and soil environments, as well as in engineered systems (WHO, 2018; Palazzolo et al., 2020). Water distribution systems such as water treatment works, cooling towers, building plumbing and storage water tanks have been reported to be ideal breeding grounds for *Legionella,* leading to their multiplication at levels that can endanger human life (Buse et al., 2012). The ecology of *Legionella* spp. in natural ecosystems is complex as they can survive in temperatures ranging between 0^{o}C and 68^{o}C and at pH 7-9. In addition, *Legionella* spp. can survive in various nutritional conditions and habitats where they exhibit rod-shaped, coccoid, and filamentous forms. However, in a particular environment, they are usually outcompeted by other bacteria as they require specific nutritional conditions for growth (Diederen, 2008; Kowalczyk et al., 2021). Engineered systems offer favourable conditions for the proliferation of *Legionella* spp. at temperatures between 25^{o}C and 55^{o}C (where optimum growth is at 37^{o}C) and the presence of biofilms and protozoa, which offer nutrients and protection from environmental stresses. To date, the genus has at least 60 species, with more than 70 serogroups, with *Legionella pneumophila* being responsible for most infections (Gattuso et al., 2022). *Legionella pneumophila* was the first species to be isolated in 1976 at an American Legion convention (Brenner et al., 1979) and it consists of 15 serogroups (SG), with serogroup 1 being responsible for at least 90% of infections, mainly due to ecological and physiological features such as the O-antigen proteins. Other *L. pneumophila* serogroups, SG4 and SG6, have also been associated with clinical cases. Non-*L. pneumophila* community acquired infections have also been reported involving infection with *L. bozemanii, L. micdadei, L. dumoffii, L. anisa, L. feelei, L. micdadei and L. longbeachae* (Bartram, 2007; Caicedo et al., 2019). In particular, outbreaks caused by *L. micdadei, L. bozemanii and L. anisa* were associated with building water systems and have only been reported in the USA (Logan-Jackson and Rose, 2021).

2.2 CLINICAL FEATURES OF LEGIONELLA

The outer membrane structure of *Legionella* is highly hydrophilic due to the lipopolysaccharide side chains that enable it to enter host cells, where it forms *Legionella*-containing vacuoles (LCV). The LCV are formed by utilising the host cell mitochondria, ribosomes and endoplasmic reticulum (ER) vesicles, which are involved in the endocytic pathway of the host cells. Formation of LCV will therefore allow the survival of *Legionella* cells by avoiding endocytic maturation and preventing phagolysosome formation which is involved in the degradation of cells. *Legionella* utilises two protein effectors, Type II secretion system (T2SS) and a Type IV secretion system (T4SS), as virulence factors to survive, multiply and cause disease in host cells. *Legionella* spp. is usually transmitted from the environment to humans, mainly through inhalation of contaminated aerosols such as mist or water droplets containing the bacteria (Burillo et al., 2017). When the bacterium is inhaled through aerosols, it enters the lungs and invades alveolar macrophages, which allows it to evade host defences and cause pneumonia (Falkinham, 2020). Individuals can also get infected through less common modes such as aspiration and micro-aspiration of *Legionella*-containing aerosols or direct contact with surgical wounds (Johnson et al., 1985; Marrie et al., 1991). In engineered water systems, contaminated water sources include hot water systems, hot tubs, respiratory equipment, cooling towers, water fountains, shower heads and other water supply infrastructure that can produce the aerosol that contains *Legionella* bacteria (Edelstein, 2007). Waterborne *L. pneumophila* is the leading cause of legionellosis infection, which can present as two different clinical forms: a mild, flu-like illness called Pontiac fever or a severe pneumonia that can lead to permanent lung damage or death known as Legionnaires' disease (LD) (Burillo et al., 2017; Muchesa et al., 2018). The exact incidence of legionellosis worldwide is unknown because countries differ greatly in the methods they use to establish whether someone has the infection and in their reporting of cases (WHO, 2007). According to the United Nations (2014), the number of people diagnosed with legionellosis will rise to around

2.5 billion by 2050 in urbanised areas, emphasising the need for more climatisation solutions (Graham et al., 2020). The number of cases of legionellosis reported in 2017 shows an incidence rate of 1.8 and 2.2 per 100,000 inhabitants in Europe (ECDPC, 2020) and in the US (Barskey et al. 2020), respectively. The symptoms of Legionnaires' disease (LD), which are respiratory in nature, usually appear within 2 to 14 days of exposure to the pathogen, while Pontiac fever symptoms have an incubation time of 3-7 days after exposure to the bacteria. The most common clinical symptoms of Legionellosis are severe headaches, fever, chills, chest pains, shortness of breath and dry cough, with symptoms such as lower respiratory tract infections being absent in Pontiac fever. Risk factors associated with LD include older age, male sex, smoking, chronic lung diseases, cardiovascular diseases, renal disorders, pulmonary, liver dysfunction (Cunha, 2010; Phin et al., 2014; Yakunin et al., 2020; Falkinham, 2020). Some of the sources and risk factors associated with *Legionella* are highlighted in Table 2.1.

Table 2.1. *Legionella*: Transmission, sources, and risk factors by category*

	Community-acquired	Healthcare-acquired (Nosocomial)	Travel-associated
Transmission	Contaminated water: aerosol inhalation	Contaminated water: aerosol inhalation or aspiration. Wound infection	Contaminated water: aerosol inhalation
Sources	Cooling towers, hot and cold water systems, spa pools, thermal pools, storage tanks, springs, humidifiers, domestic plumbing, potting mixes and compost	Cooling towers, hot and cold water systems, spa pools, natural pools, thermal springs, respiratory therapy equipment; medical treatment	Cooling towers, hot and cold water systems, spa pools, thermal springs and pools, humidifiers
Reservoir	Industrial sites, shopping centres, restaurants, leisure centres, sports clubs, private residences	Hospitals, medical equipment	Hotels, cruise ships, campsites, and shopping centres, restaurants, clubs, sports clubs
Personal risk factors	Age >40 years; male; diabetes, chronic heart disease, haematological malignancy, structural pulmonary comorbidity, chronic renal failure; smoking; chronic illness; travel; iron overload; other immunosuppression	Age >25 years; transplant patient; surgery, especially head and neck; cancer, including leukaemia's/ lymphomas; diabetes; treatment with respiratory devices; chronic heart/lung disease; smoking, alcohol abuse	Age >40 years; male; heavy smoking, alcohol abuse; change in lifestyle; underlying disease such as diabetes or chronic heart disease; immunosuppression
Environmental risk factors	Proximity to sources of transmission, poor design or poor maintenance of cooling water systems, inadequate staff training	Stay in accommodation designed for short stays and seasonal use; intermittent water supply and fluctuating water temperature control; complex water systems; lack of trained staff to manage water systems	Complex water distribution system, long pipe runs, poor water temperature control, low water flow rates

* Adapted from WHO (2018), Fields et al. (2002), Joseph (2002, 2004), Ricketts and Joseph (2004), and Marston et al. (1994).

2.3 INCIDENCE OF *LEGIONELLA* IN SOUTH AFRICA

Early studies of Legionnaires' disease in South Africa reported 12 cases between 1985 and 1986 at a Johannesburg teaching hospital (Strebel et al., 1988). More recently in South Africa, legionellosis has been classified as a category 2 notifiable disease according to the Notifiable Medical Conditions (NMC) surveillance system of the National Institute for Communicable Diseases (NICD) (Wolter et al., 2020). The NMC reports on outbreaks of *Legionella* within South Africa, and between 2012-2014, over 1800 sputum samples collected from patients diagnosed with HIV or tuberculosis infections at two South African hospitals were screened and reported *Legionella* in 1.2% (n=22) of patients (Wolter et al., 2016). More recently, between 2018 and 2020, 93 cases of legionellosis were reported to the NMC, with most cases (77.4%; n=72) being reported from hospitals within the Western Cape in older male adults presenting with underlying illnesses (Wolter et al., 2020). In South Africa, as Legionellosis infection occurs through inhalation of small water droplets (aerosols) and/or aspiration of contaminated water, cases are still underreported due to the lack of awareness, lack of robust and reliable surveillance mechanisms, and lack of accurate diagnosis (Buse et al., 2012, 2020; Stone et al., 2019). As shown in Figure 2.1, more recent data from the NICD have shown that the Western Cape reported the most cases of Legionellosis (20 cases) from March to August 2023, with the highest number of cases (7) reported in June. This is followed by the Gauteng province with five cases of legionellosis reported in May (1 case), July (2 cases), and August (2 cases) 2023 (NICD, 2023).

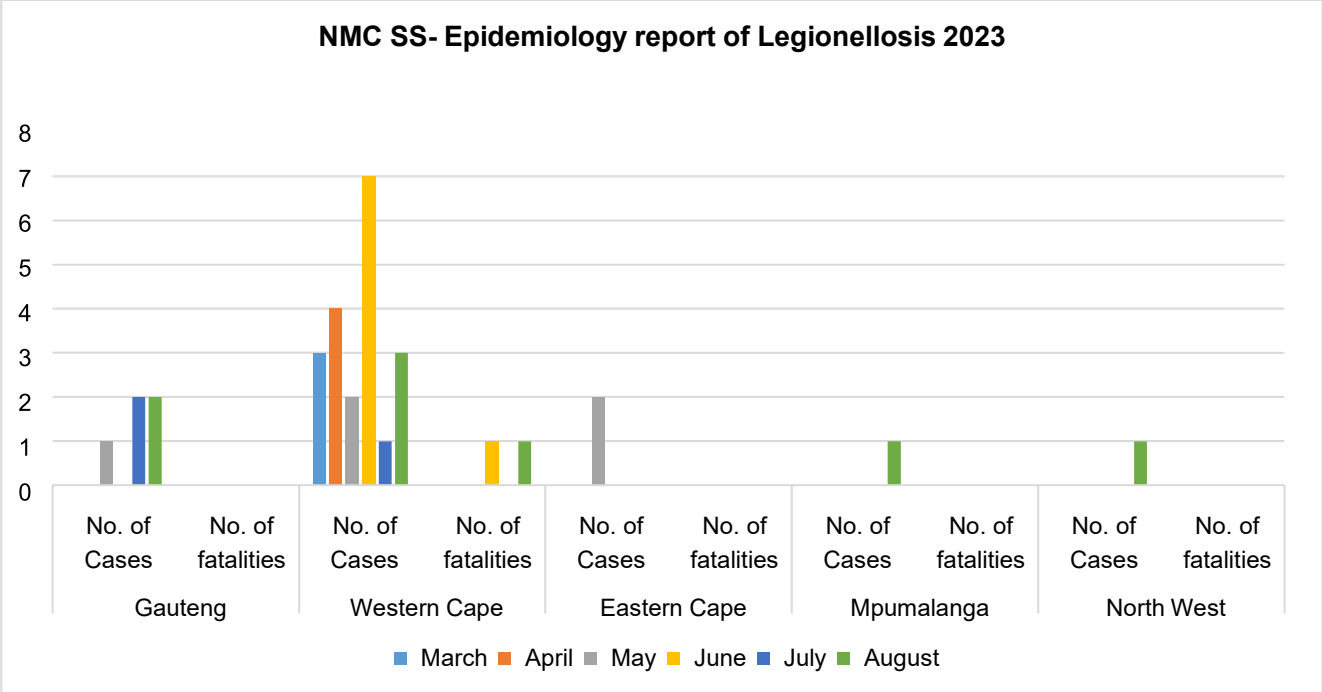


Figure 2-1. Reported incidences of Legionellosis in South Africa from March 2023 to August 2023 (NICD, 2023)

2.4 COVID-19 AND LEGIONNAIRES' DISEASE

Globally, the threat from Legionnaires' disease is compounded because its victims tend to display the same symptoms as patients infected with coronavirus, including cough, chills, and fever, making misdiagnosis a possibility (Cassell et al., 2021). During the ongoing COVID-19 crisis, exposure to aerosolised water from recently reopened buildings should be considered as an epidemiologic risk factor for *Legionella* exposure and an indication to test for these bacteria that proliferate in warm water environments and stagnant sections of indoor plumbing and cooling systems (Cassell et al., 2021). Pneumonia is the most common manifestation of both Legionnaires' disease and COVID-19, and initial presentation for both may include fever, headache, confusion, dyspnoea, nausea, and diarrhoea (Cassell et al., 2021). Individual risk factors for both Legionnaires'

disease and severe COVID-19 include older age, diabetes, and chronic lung disease. Similar to COVID-19 infection, the incubation period for Legionnaires' disease is approximately 5 to 6 days, but may range from 2 to 14 days.

2.5 FACTORS THAT REGULATE *LEGIONELLA* GROWTH AND SURVIVAL

Legionella is ubiquitous in both natural and man-made water systems. *Legionella longbeachae* is isolated from non-aquatic habitats such as potting soil and compost. The correlation between *Legionella* colonization of a water system and the risk of acquiring the disease has been well established (Sciuto et al., 2021). When colonization occurs within a water system, abatement of *Legionella* is difficult and generally eradication is not possible because the bacterium has not only located optimal environmental conditions but also because it can activate survival strategies. Favourable conditions for the multiplication of *Legionella* in water systems include water temperature, water stagnation, and the presence of free-living protozoa, which protect intracellular bacteria from adverse environmental conditions, including water disinfection procedures (Nisar et al., 2020). Scale and organic sediment also provide nutrients for the formation of biofilms in which *Legionella* cells can persist for a long time, sometimes for decades (Filice et al., 2022; Sciuto et al., 2021).

2.5.1 Water temperature

Water temperature plays an important role in *Legionella* survival and growth. *Legionella pneumophila* multiplies at temperatures between 25°C and 42°C, with an optimal growth temperature of 35°C (Caicedo et al., 2019; Falkinham, 2015, 2020). Thus, as indicated by several guidelines and technical reports, a key control measure to reduce colonization of water distribution systems is to maintain elevated temperatures (≥51°C) for the hot water and low temperatures (<20°C) for the cold water (Sciuto et al., 2021). The NICD suggests extending the hot water treatment by storing above 60°C and delivering it to taps above 50°C, and the cold water being stored below 20°C (NICD, 2019; Sciuto et al., 2021). Studies have shown that it is difficult to reach this temperature in large buildings with complex plumbing systems, while, in addition, an elevated water temperature accelerates disinfectant decay (e.g., chloramines and chlorine) and predisposes hot water systems to deteriorating microbial water quality (Sciuto et al., 2021). Laboratory studies have shown that the bacterium can survive for 80–120 min at 50°C and 2 min at 60°C. In real settings, some species, including *L. pneumophila*, were found able to grow at temperatures above 50°C, and to survive up to 63°C (Lesnik et al., 2016). Moreover, in some circumstances, the temperature range between 50–59 °C was identified as the optimal condition for facilitating the emergence of different *Legionella* species (Digler et al., 2018). Adaptations, spontaneous mutations, or horizontal gene transfer from thermophilic *Legionella* species could support this microbial adaptation (Sciuto et al., 2021).

2.5.2 Protozoa and biofilm

Biofilms, as well as protozoa, can help *Legionella* to survive and persist in environments by providing nutrients, stability, and protection from potentially adverse conditions. Biofilms provide an extracellular matrix where *Legionella* can colonise and persist both as free-living species and as intracellular endosymbionts of protozoa. In addition, biofilms are more resistant to disinfectants and extreme heat, allowing *Legionella* species to survive and multiply in water distribution systems where susceptible individuals can be exposed (Taylor et al. 2009; Shaheen et al., 2019)

Legionella can enter free-living amoebae by phagocytosis, where they are generally killed inside lysosomes, where an acidic pH and lysosomal enzymes digest the bacteria. However, *Legionella* has evolved temperature-dependent strategies to prevent lysosome-mediated destruction and can persist in a vacuole within the protozoan hosts. At temperatures >25°C, *L. pneumophila* replicates in amoebae and is released into the water while the protozoan host is killed. At temperatures below 20°C, amoebae infected by *L. pneumophila* eliminate the bacterium to the extracellular environment through a process of encystation (Ohno et al., 2008; Sciuto et al., 2021). The need to avoid protozoan colonization of a water system is important not only to prevent the transmission of protozoa themselves but also to decrease the risk of legionellosis because *Legionella*e inside

protozoan cysts are protected against many harsh conditions. Thus, one of the key issues for controlling the growth of *Legionella*e within protozoa is to recommend an effective disinfection method (Sciuto et al., 2021). When exposed to high temperatures or to chemicals, or in the absence of nutrients or oxygen, free-living protozoa transform to the cyst form to survive. It has been observed that the cysts can remain viable for more than 20 years under dry conditions and 24 years at 4°C in water, because they are metabolically inactive (Sciuto et al., 2021). The minimal temperature to kill the cysts is 65°C for most *Acanthamoeba* spp. isolates, but the cysts of some thermotolerant isolates can resist exposure to 80°C for 10 minutes. Amoebal cysts are also highly resistant to some high-level disinfectants such as biguanides, quaternary ammonium compounds, chlorine, and hydrogen peroxide, as well as to UV, X-ray, and gamma irradiation (Sciuto et al., 2021; Thomas et al., 2010).

Biofilms play an important role in providing favourable conditions in which *Legionella* can grow because they provide protection from environmental stresses or disinfectants, access to higher levels of nutrients, and opportunities for symbiotic interactions with other microbes or protozoa. *Klebsiella pneumoniae, Flavobacterium* spp., *Empedobacter breve, Pseudomonas putida,* and *Pseudomonas fluorescens* can enhance the long-term persistence of *L. pneumophila* in biofilms through the synthesis of capsular and extracellular matrix materials, which support the adherence, or because they provide growth factors for the bacterium (Pécastaings et al., 2010; Stewart et al., 2012). In contrast, other bacterial species such as *Pseudomonas aeruginosa, Aeromonas hydrophila, Burkholderia cepacia, Acidovorax* spp.*, and Sphingomonas* spp. reduce the presence of *L. pneumophila* within the biofilm through the production of homoserine lactone quorum sensing (QS) molecules, or the production of bacteriocins. It has been demonstrated that *Legionella*e inside the biofilm matrix express phenotypes that differ from those of their planktonic counterparts and display an increased resistance to biocide treatments. Thus, disinfectant type as well as substratum play an important role in the survival of *L. pneumophila* in biofilm within drinking water systems (Sciuto et al., 2021).

2.5.3 Stagnation

Water stagnation is a major factor in the control and management of microorganisms, including *Legionella,* in water distribution systems. Studies have shown that overnight stagnation of household drinking water systems can result in changes in bacterial community composition and an increase of up to 3-fold increase in microbial loads (Soderberg et al., 2004; Pepper et al., 2021). Thus, periods of stagnation in a premise's plumbing can increase the number of *Legionella* spp. Stagnation and infrequent water use may concentrate and enhance the release of organic matter in water in plastic pipes and metals in metallic pipes. Over time, when water is not in use in places such as hospitals, schools and buildings that are not occupied for a long period of time, stagnation of the water with no or little sterilisation allows microorganisms to grow and thrive within such systems. Stagnation is not only limited to flow through the system but is also present within specific components of the water system. Studies conducted on hot water tanks showed that water stagnates at the bottom of the tank, where temperatures are lower and promote the growth of *Legionella,* even with operational recirculation of the water through the tank (Armstrong, 2003). Water stagnation is also an important factor in biofilm formation, thereby providing a platform for survival and growth of *Legionella* species and other opportunistic pathogens such as *Mycobacterium avium* (Falkinham et al., 2020). Scale, sediment and other inorganic and organic materials may build up in these tanks and act as a potential food source. Sediments such as these could also furnish the bacteria with protection from high temperatures and or chemical disinfection (Armstrong, 2003).

2.5.4 Organic and inorganic sediment accumulation

Organic and inorganic compounds in water usually enhance colonisation of *Legionella* in plumbing, particularly in the presence of ideal environmental factors such as water temperature or pH, low water pressure or stagnation, loss of disinfectant, or discontinuous disinfection. Small amounts of inorganic nutrients such as iron, zinc, and potassium also enhance the growth of *L. pneumophila* (Sciuto et al., 2021).

2.6 OCCURRENCE OF *LEGIONELLA* SPP. IN ENVIRONMENTS

2.6.1 Community settings

Legionella pneumophila is persistent in the environment, particularly in soil and water, where the bacteria are generally present in low levels and do not cause disease. Given that *Legionella* bacteria are common in aquatic habitats, they can easily contaminate man-made water systems such as faucets, showerheads, decorative fountains, ice machines, hot and cold-water systems, and cooling towers (Cullom et al., 2020; Lombardi et al., 2023), medical equipment, including aerosols from respiratory devices, whirlpools, baths and mist systems (Cullom et al., 2020), hot tubs and air conditioning systems (Lombardi et al., 2023; Muchesa et al., 2018) (Figure 2.2). This microorganism is known to evolve and use methods to survive harsh environments, such as resistance to disinfectants, formation of a biofilm, ability to grow in low-oxygen and low-carbon environments, and evolving pathways to evade lysosome fusion when engulfed by a phagocyte protozoan. This allows it not only to survive phagocytosis and bypass antimicrobial control agents, but to use the protozoa as a replication aid, packed with nutrients to encourage its growth and reproduction in aquatic systems. Several studies have linked outbreaks of waterborne disease to *Legionella* growth in large plumbing systems, and there is a possibility of *Legionella* growth and infection in single households (Schoen and Ashbolt, 2011; Shaheen and Ashbolt, 2018; Stone et al., 2019). When attempting to study *Legionella*, management procedures require a focus on the overall ecology of the system and broader convergence between microbiological approaches to microbial detection.

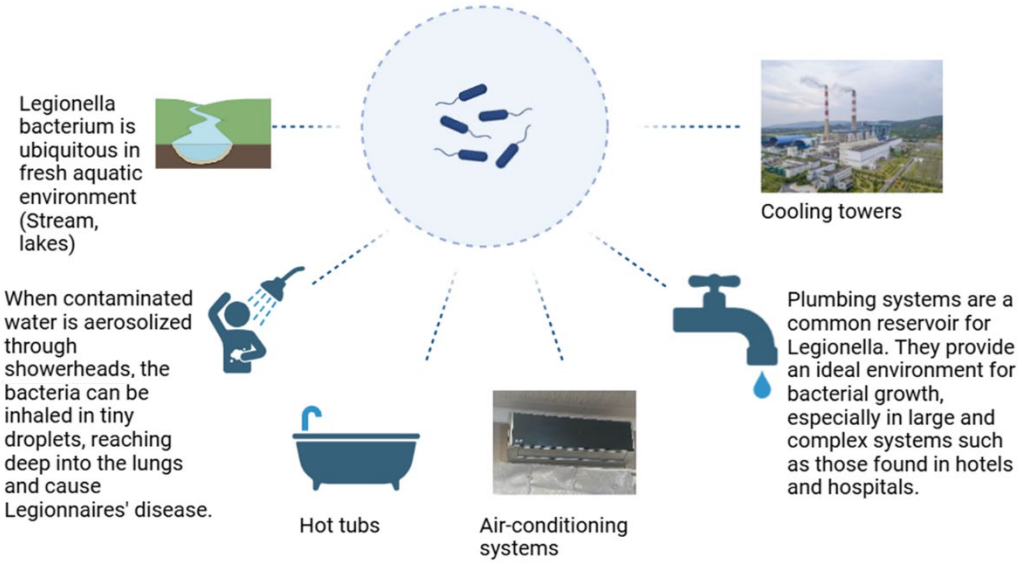


Figure 2-2. Common sources where *Legionella* bacteria thrive, including plumbing systems, showerheads, cooling towers, hot tubs, and air-conditioning systems. Created with BioRender.com

In high-rise urban buildings, the complexity of water systems creates ideal conditions for *the* proliferation of *Legionella*. Understanding the mechanism of infection and disease progression is crucial for developing effective prevention and control strategies. *Legionella* enters building water systems through various sources, including municipal water supplies or natural water bodies. Once in the system, the bacteria find favourable conditions for growth and proliferation supported by various environmental factors, including water temperature, stagnation, and the presence of biofilms (Parsek and Singh, 2003). High-risk areas within building water systems include cooling towers, as they are prone to *Legionella* colonization due to their large water reservoirs and the process of aerosol generation during cooling. Shower heads and faucets are direct points of contact for individuals with water. The warm and moist environment in shower heads is conducive to *Legionella* growth, and the process of showering can aerosolize contaminated water. In rural communities, the

predominant practice involves storing water supplied by municipalities and extracted from boreholes in large storage tanks. Lombardi et al. (2023) highlighted an escalating concern regarding the presence of *Legionella* in these tanks, attributing their proliferation to conditions conducive to *Legionella* growth, such as optimal water temperature and stagnation (Consonni et al., 2021). Notably, *Legionella pneumophila* thrives in temperatures ranging from 25-42°C, a range commonly found in these storage environments (Sciuto et al., 2021).

2.6.2 Hospital settings

The ability of such bacteria to survive in a wide range of environmental conditions, coupled with their presence in engineered water systems, underscores the importance of regular monitoring and control measures to prevent outbreaks. This is particularly crucial in healthcare settings and places with large, complex water systems, where vulnerable populations may be exposed to such bacteria. Investigations of *L. pneumophila* span from cooling towers, hospitals, soil, drinking water, systems, to waste water treatment plants. *Legionella* bacteria in hospital drinking water systems are a common occurrence. A lack of temperature control across a hospital wing has been shown to yield consistent *Legionella* contamination (Bédard et al., 2019). The co-occurrence of *Legionella* with amoeba (*V. vermiformis*) in a South African hospital water system was found to be statistically significant (Muchesa et al., 2018). Gavaldà et al. (2019) established that maintaining a 55°C minimum temperature in a hospital water distribution system was more effective in controlling *Legionella* than a temperature of 50°C. In contrast, another study found *L. pneumophila* serogroups 1 and 4 and 10 (41% and 91% positive, respectively) to be tolerant to 55°C in hot water sampled from a hospital setting (Bédard et al., 2019). The frequency of *Legionella* spp. found in healthcare facilities reflects a threat of LD on immunocompromised patients (Trnková et al., 2018)

2.7 *LEGIONELLA:* THE OPPORTUNISTIC PREMISE PLUMBING PATHOGEN

Opportunistic premise plumbing pathogens (OPPPs) are waterborne pathogens that have adapted to grow in water from man-made drinking water systems. These OPPPs include *Legionella pneumophila, Pseudomonas aeruginosa, Mycobacterium avium complex, Mycobacterium abscessus, Stenotrophomonas maltophilia, Acinetobacter baumannii,* and *Acanthamoeba* spp. (Falkinham, 2020; Rhoads et al., 2016). The presence of these bacteria is not due to contamination as they are naturally occurring in aquatic ecosystems and can therefore grow and survive under a wide range of conditions, including those characteristic of water systems in the “built environment” (Falkinham, 2020). These pathogens have different characteristics (summarised in Table 2.1) from the typical diarrheal pathogens that are a cause of concern in water distribution systems. The mechanisms by which premise plumbing influences *L. pneumophila* and other OPPPs, as well as the broader premise plumbing microbiome, are varied and complex (Cullom et al., 2020). Premise plumbing includes water pipes in houses, hospitals, apartments, and office buildings and is a key conduit for human exposure via showering, handwashing, and other applications that create airborne aerosols.

Table 2.1 Characteristics of Opportunistic Premise Plumbing Pathogens *

	Common features of OPPPs	Resistant features
1	Resistant to chlorine, forms biofilms, can survive low organic carbon environments. Can survive, grow, and persist in natural and drinking water	Can survive in low oxygen, grows within biofilms, resistant to disinfection
2	Not faecal in origin and do not correlate with occurrence of total coliforms/*E.coli,* which results in their not being routinely monitored except in retrospective outbreak investigations	Slow growing, difficult to detect using standard methods
3	Do not rely on human or warm-blooded host to survive and reproduce	Can survive water treatment processes and can grow at low oxygen concentrations and at low levels of organic carbon
4	Adapted for survival, permanence, and growth in the built environment, particularly in hospital and building plumbing installations	Multi-drug resistant, biofilm formation, thrives in hospital water systems; highly adaptable to hospital settings, forms biofilms on respiratory equipment and survives on dry surfaces for long periods
5	Tend to attach to surfaces where they grow to form biofilms with survival and growth occurring with phagocytic free-living amoebae	Can form symbiotic relationships with amoebae, VBNC state
6	Transmission via aerosolization, and inhaling water droplets	Opportunistic pathogen in immunocompromised hosts, survives harsh conditions

*Adapted from Cullom et al. (2020), Falkinham (2015a, 2015b, 2020) and Rhoads et al. (2020).

2.7.1 *Legionella* survival in premise plumbing

Legionella pneumophila is the most widely known waterborne opportunistic premise plumbing pathogen (OPPP). The commensal microflora (protozoa and biofilms) present in water distribution systems greatly impact *Legionella* survival and proliferation (Pereira et al., 2021). Proliferation is reliant on their symbiotic relationships with other microorganisms. The bacterium has evolved a bi-phasic survival mechanism that helps it survive extreme and unfavourable environmental conditions. This mechanism allows the bacteria to alternate between a metabolically active and replicating form (RF) to a motile and stress-resistant virulent form (VF) when faced with environmental conditions that do not favour it (CDC, 2022; CDC and NCIRD, 2021). This cycle is controlled by the bacterial nutrients, metabolism, and environmental stress that induce the transmission between the two forms (Barbosa et al., 2024). The bacteria's growth and egress mechanism inside a protozoan is the same as in human macrophages (Chauhan and Shames, 2021; Mraz and Weir, 2022).

Released strains have been shown to be more virulent and more adapted to invasion of human macrophages (Bartie et al., 2016; Pereira et al., 2021). This interaction leads to multiple physiological changes in the host and the bacteria. The life cycle begins when free-floating *Legionella* are engulfed by phagocytic alveolar macrophages (in humans) or amoebae, where they establish vacuoles that aid them in invading lysosomal digestion (Figure 2.3). When *Legionella* becomes phagocytosed, a host cell defence program is initiated, including antimicrobial metabolic responses aimed at breaking down the pathogen, while the bacteria attempt to sequester nutrients from the host cell and counteract the antimicrobial responses. When favourable conditions are met, the bacteria inhibit transmission and initiate replication. New progeny are formed, and the replication process only stops after the conditions deteriorate and traits are expressed that will help the progeny thrive in the environment. The progeny develops into a mature intracellular form (MIF) that exhibits stress resistance and high virulence. Lysing of the phagosome occurs and the microbes in their MIF are released to the environment, where they exist as free forms until they encounter another phagosome and the cycle begins anew (Bartie et al., 2016; Ji et al., 2014). The ability to proliferate within these symbiont hosts provides *Legionella* with protection from otherwise harmful environmental conditions such as a higher temperature range, water treatment with chlorine, biocides, and other disinfectants (Bartie et al., 2016). Biofilms provide the bacteria with nutrients

for growth and offer protection from adverse environmental conditions (including during water disinfection). Because biofilms colonize drinking water distribution systems, they provide a habitat suitable for *Legionella* growth in potable water, which can lead to human exposure.

The abundance in water systems of free-living protozoa, such as *Acanthamoeba* species, has led to an ineffectiveness of antimicrobial control agents to control *Legionella*, as these amoebae species provide a nutrient-rich refuge that allows the *Legionella* to proliferate and grow freely (Cervero-Aragó et al., 2015; Dobrowsky et al., 2017). This endosymbiotic relationship between *Legionella* and amoebae has resulted in an increase in the pathogenicity of *Legionella* and plays a role in their increased ability to invade and manifest disease in humans.

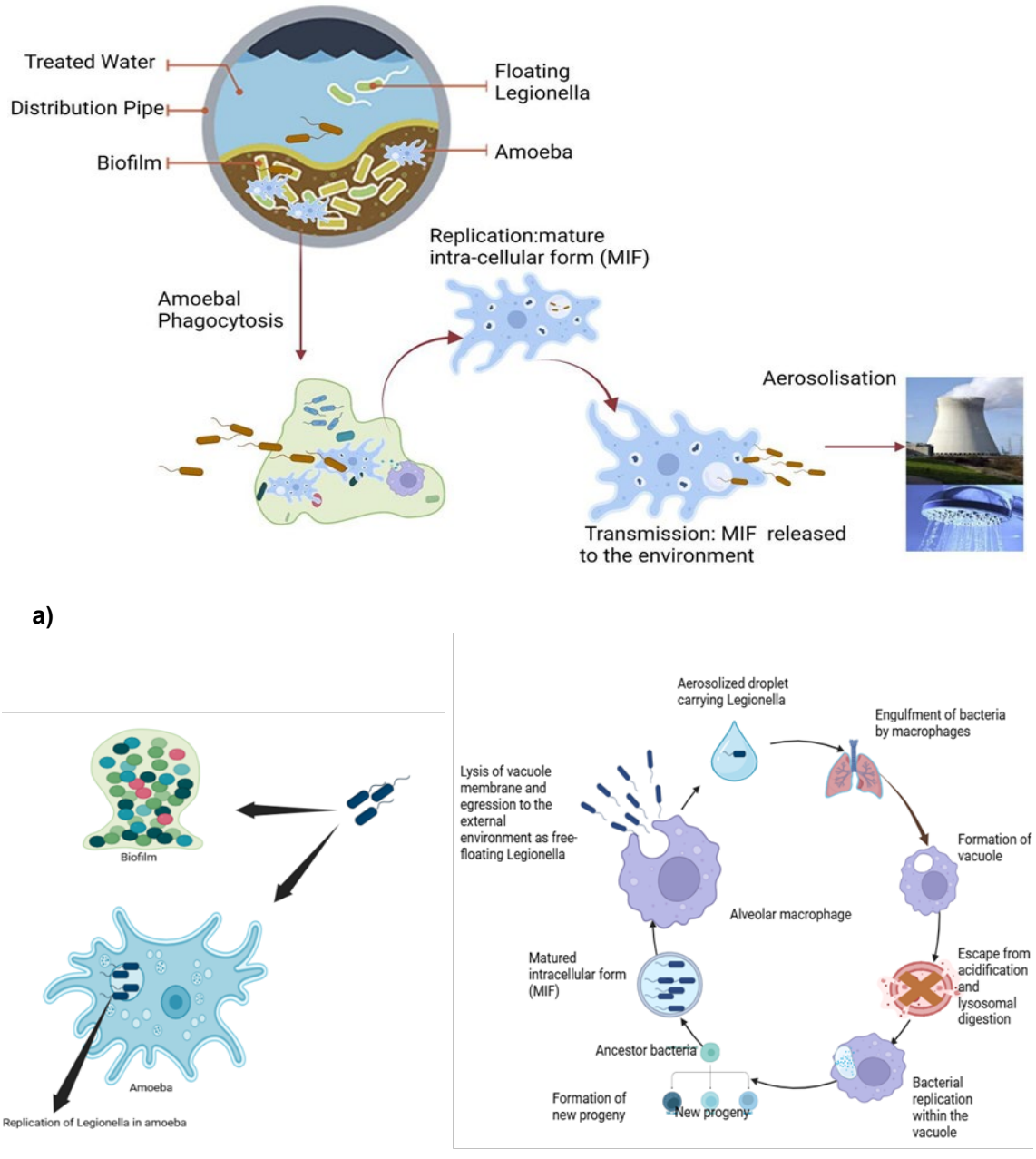


Figure 2-3. Lifecycle of *Legionella* in water systems (a) and human macrophages (b). Generated with BioRender.com.

2.7.2 *Legionella* risk assessment

In South Africa, *Legionella* falls under the Occupational Health and Safety Act, No 85 of 1993 – Regulations for Hazardous Biological Agents (HBAs). *Legionella* is identified in hazardous biological agent regulations as "Group 2 HBA" and requires the control of exposure of individuals to *Legionella*. The HBA regulations highlight the need for risk assessment, control program, training and auditing. While *Legionella* is legislated in South Africa, the legislation is poorly policed, and no actual guidelines currently exist that are specific to *Legionella*. Although legionellosis is a notifiable disease, it is rarely reported in South Africa (Stone et al., 2019). *Legionella* outbreaks in hospitals and other public areas are the only investigated incidences of *Legionella* in South Africa. In a study to identify methods for *Legionella* detection in South Africa, before the SANS standard method was introduced, it was found that 92.9% (26) of 28 samples collected from different environments (industrial representing water, mine water, and biofilms) were positive for *Legionella pneumophilia* (Bartie et al., 2003). South Africa is faced with the dual epidemics of HIV (a prevalence of 12.7% in 2016) and TB (781 cases per 100,000 population in 2016), as well as resource and medical care limitations (Stone et al., 2019). This heavy burden of disease creates both a diagnosis bias, hiding many other diseases, and an immune-compromised population susceptible to many other diseases. This might explain the lower rate of reported Legionellosis cases in South Africa in comparison to developed countries. South Africa has benchmarked rainwater harvesting as one of the supplementary sources of water, but high concentrations of both *Legionella* and *Acanthamoeba* have been found in harvested rainwater in water tanks in South Africa, thus indicating a high risk of Legionellosis to the public targeted by the initiative (Dobrowsky et al., 2017).

Water distribution systems should be assessed in isolation for a comprehensive *Legionella* risk assessment, taking into consideration the susceptibility of the building inhabitants, vulnerability to infection the possible transmission routes and the characteristics of the building. Other factors include the design and maintenance of the water distribution system, the presence or absence of *Legionella* colonies from the system, the presence or absence of protozoa which promote *Legionella* proliferation and the population and profile of people who might be exposed the waterborne infectious agents. In addition, water stagnation, disinfection of the water distribution system, water temperature, oxygen levels, biofilm formation and nutrient concentration also play a critical role in the growth and presence of OPPPs such as *Legionella* in drinking water systems and premise plumbing (Falkinham et al., 2015a, 2015b).

2.8 DETECTING *LEGIONELLA* THROUGH CULTURE-BASED METHODS

2.8.1 ISO 11731: 2017

Environmental surveillance of *Legionella* has been done mainly by culture-based methods as guided by the Water Quality and Enumeration of *Legionella* standard (ISO 11731: 2017). However, the plate culture of *Legionella* is labour intensive in terms of processing the samples, and some of the process steps can decrease the concentration of the viable organisms. The methodology also requires experience in terms of knowing which media to use, and when you eventually get colonies on a plate, you have to know what you are looking at; it is thus much more difficult to look at *Legionella* than many of the other gram-negative or gram-positive bacteria that are routinely screened. Briefly, culture occurs on buffered charcoal yeast extract (BCYE) as the bacteria do not grow on standard media and require L-cysteine and iron salts (Cunha, 2010). The optimal pH for growth is 6.7 to 6.9, and colonies appear to be greyish-white after 7-14 days incubation at 36°C, with examination of the BCYE plates every two days. Significant problems with the cultivation methods can arise: the more rapid generation times of other microbes present in the sample might obscure slow-growing *Legionella* on plates and the presence of *Legionella* in the viable but non-culturable (VBNC) state is a problem. *Legionella* grows optimally at temperatures between 25°C and 45°C (optimum: 25°C - 35°C); therefore, water supplies with temperatures in this range support the highest levels of growth of this organism (NAS, 2019). They are also thermotolerant, capable of surviving at temperatures between 55°C and 70°C (Cervero-Aragó et al., 2015).

2.8.2 Amoeba-associated *Legionella* detection

The widespread survival of *Legionella* species in nature has been attributed to their interactions with other microorganisms, such as protozoa, in the environment where they thrive in biofilms (Declerck, 2010). Survival of *Legionella* in protozoan cysts following exposure to 80°C has been demonstrated (NAS, 2019). As reservoirs of *Legionella* in the environment, amoebae can therefore be used as tools to isolate *Legionella* from water distribution systems using the amoeba enrichment technique and amoeba co-culture.

Amoebae enrichment involves: (I) Isolation of indigenous amoebae from the environment that potentially contain *Legionella* (Kebbi-Beghdadi and Greub, 2014) and (2) culturing lysed amoebae cells. The environmental sample is inoculated (directly or after concentration) onto a non-nutrient agar (NNA) medium seeded with Gram-negative bacteria such as *Escherichia coli*, which will act as a food source after incubation. Non-nutritive agar plates are incubated in a humidified atmosphere and microscopically examined daily for the morphological appearance of amoebae trophozoites and/or cysts. Culture plates positive for amoebae are sub-cultured on fresh NNA plates seeded with viable or heat-killed gram-negative bacteria. The amoebae are then lysed to release potential *Legionella* spp. Amoebae co-culture is a cell culture method in which an axenic isolate of amoebae is used as an eukaryotic host to sustain the growth of *Legionella* (Greub and Raoult, 2004; Lamoth and Greub, 2010). Environmental samples that potentially contain amoeba-resistant bacteria (ARB) are concentrated and inoculated onto axenic amoebae and incubated to facilitate the intracellular *Legionella* present in the sample to infect and multiply in the amoebal host. The amoeba co-cultures are sub-cultured onto fresh axenic amoebae after one week of incubation. After that, the amoebal cocultures are observed daily for intracellular bacteria and/or amoebal lysis (Greub and Raoult, 2004). After amoebal lysis is observed, the culture is screened for the presence of ARB by differential stains and molecular characterizing methods such as Gram staining, Gimenez staining, neutral red staining and fluorescent in situ hybridization (FISH), polymerase chain reaction (PCR), rRNA sequencing and transmission electron microscopy (TEM) depending on the type of ARB (Lienard and Greub, 2011; Tosetti et al., 2014)

2.8.3 Most Probable Number Method

The Legiolert™ ® most probable number (MPN) quantification is a liquid culture method for the enumeration and quantification of *L. pneumophila* from potable and non-potable water samples. The method uses a powdered growth medium to which a measured aliquot of the sample is added. The substrate present in the Legiolert™ ® reagent is utilized by an *L. pneumophila*-based enzyme. This MPN is not labour intensive and doesn't encourage the growth of non-target *Legionella* species. The IDEXX Legiolert™ ® method for *Legionella* testing is a more robust procedure that can handle the analysis of larger samples and is simpler to perform, with easy-to-read end-point reactions with a rapid processing time. Launched by IDEXX in Europe in 2017, Legiolert™ ® can accurately and sensitively quantify *Legionella pneumophila* in water and provide a confirmed result within 7 days, compared to up to 14 days with smear culture methods. In addition, Legiolert™ ® is less burdensome to the organism than some of the culture methods and the specificity averages a higher number for *Legionella pneumophila* than plate methods (Scaturro et al., 2020).

2.8.4 Microscopy

Electron microscopy provides maximum resolution for the investigation of the morphology and ultrastructure of *Legionella*. The bacterium displays varied morphology, demonstrating coccoid, bacillary (~0.3 µm to 0.6 µm by ~3 µm), and/or long filamentous (~8 µm to 50 µm) forms that are influenced by temperature, available nutrients or metabolites, growth environment (e.g., inside amoebae), and medium type (Greub and Raoult, 2003). Other microscopy detection methods described by Abu Khweek and Amer (2018), Fields et al. (2002) and Steinert et al. (2002) include:

a) Transmission Electron Microscopy (TEM): The TEM method offers high-resolution images of *Legionella*, enabling the study of intracellular structures and bacterial interaction with host cells. This

technique is instrumental in understanding the pathogen's invasion and replication mechanisms within host cells.

b) Scanning Electron Microscopy (SEM): The SEM method provides detailed surface morphology of *Legionella*, crucial for studying its environmental survival and biofilm formation. This technique aids in visualizing the structural adaptations that facilitate bacterial persistence in various environments.

c) Fluorescence Microscopy: This technique, especially when combined with specific staining methods, allows for the visualization of *Legionella* in environmental samples and within host cells. It is particularly useful in studying the distribution and dynamics of the bacteria in different settings.

2.8.5 Alternative detection methods for environmental samples

Detection and monitoring of *Legionella* in water systems is necessary for risk assessment and prevention of legionellosis. Although *L. pneumophila* detection in water is characteristically performed by culture isolation on selective media, it has several limitations, including long incubation times and the inability to detect viable but non-culturable bacteria (VBNC) that may represent a public health hazard, especially when a reclamation treatment is performed (Bonetta et al., 2018; Bonetta and Bonetta, 2020). Cells in the VBNC state are characterized by their inability to grow on standard medium and in contrast to dead cells, VBNC cells are metabolically active and have intact cell walls (Li et al., 2014). For this reason, alternative methods for the fast, sensitive, and precise detection of *Legionella* in water samples have been proposed. To identify potential sources of contamination/infection, high-resolution genotyping of new isolates (eg, sequence-based typing, multilocus variable number of tandem repeats) is required to correlate environmental with clinical isolates (Bonetta and Bonetta, 2020).

2.8.6 Polymerase Chain Reaction (PCR)

Yin et al. (2022) highlighted in their research that PCR is easier, rapid and more accurate in detecting *L. pneumophila* in environmental water samples, which is also used for large-scale detections. The 16S rRNA gene is commonly used for genus-level identification of *Legionella* spp. Farhat et al. (2018) applied PCR systems targeting the 16S rRNA gene to detect *Legionella* spp. in environmental water samples, demonstrating its broad applicability in monitoring water systems. Similarly, Eble et al. (2021) highlighted the utility of the Macrophage Infectivity Potentiator (*mip*) gene, which is specific to *L. pneumophila*, for species-level identification and differentiation of strains. The mip gene, encoding the macrophage infectivity potentiator protein, is a highly conserved marker used to detect virulent strains of *L. pneumophila*.

2.8.7 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) can overcome many of the disadvantages of traditional culture methods. However, qPCR does not distinguish between viable cells and dead cells. A promising approach to detecting viable cells involves using qPCR together with photoactivatable DNA intercalators such as propidium or ethidium monoazide (PMA or EMA), which can penetrate the membranes of compromised cells and block PCR amplification (Delgado-Viscogliosi et al., 2009; Mansi et al., 2014; Scaturro et al., 2016), with PMA having been shown to be more specific for dead cells compared to EMA (Scaturro et al., 2016). Some studies using the PMA-qPCR method to detect *Legionella* in water samples have already been published (Ditommaso et al., 2014; 2015; 2016), all of which have dealt with water reclaimed with traditional disinfection strategies.

2.8.8 Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) is an advanced sequencing technology known for its remarkably high throughput, scalability, and rapid results (Yadav et al., 2023). This technique is employed to identify the order of nucleotides in entire genomes or targeted regions of DNA or RNA (Danakumara et al., 2024). Additionally,

studies have utilized NGS technique to detect *L. pneumophila* using CRISPR/Cas9-based targeted enrichment and FLASH-NGS to sequence selected genes directly from clinical samples (Domazetovska et al., 2022)

2.8.9 Flow Cytometry

A rapid flow cytometry detection method was developed to count bacterial species, including *Legionella,* in recreational water samples, and this was found to be a quicker technique for real-time monitoring of bacterial dormancy/ activity after exposure to disinfectants (Taguri et al., 2011). Flow cytometry can differentiate between the viability of bacterial cells based on cell permeability, and this can be determined by using a combination of dyes that differ in their abilities to penetrate bacterial cells (Buchrieser and Hilbi, 2019). Syto® 9, a green, fluorescent nucleic acid stain, penetrates bacteria in all states, and propidium iodide, a red fluorescent nucleic acid stain, stains bacteria with damaged cell membranes, are the most-used dyes for determining bacterial cell viability, coupled with microscopy or flow cytometry (Buchrieser and Hilbi, 2019).

2.8.10 Diagnostic methods for clinical *Legionella* detection

The diagnosis of *Legionella* infection necessitates laboratory confirmation, employing various specimens like sputum, respiratory secretions, tissue, blood, serum, or urine (Reller et al., 2003; Murdoch et al., 2012). The primary diagnostic methods include:

a) Serological and antibody-based assays: Initially, the preferred method, serology, has seen a decline in use due to the advent of more rapid and user-friendly techniques. However, it retains importance in retrospective epidemiological studies and cases where the infectious agent is elusive (Avni et al., 2016).

b) Urinary Antigen Tests: These tests, detecting *Legionella* cell wall components in urine, are favoured for their simplicity, low cost, and rapid results. However, their limitation lies in detecting only *L. pneumophila* SG1, necessitating the development of assays for other Legionella serogroups and species.

c) Immunomagnetic Separation (IMS) methods: Legipid® *Legionella* Fast Detection Test (Biótica, Spain) is a rapid and culture-independent assay for detecting *Legionella* bacteria. It works by adding magnetic particles that bind to *Legionella* cells, forming complexes that can be separated by a magnet. These complexes are then labelled with an enzyme and detected calorimetrically (Ortí-Lucas and Luciano, 2022). The test offers fast results, typically within 24 hours, and is useful for onsite detection, though its effectiveness may be reduced by low bacterial numbers or sample complexity, especially in potable water (Walker and McDermott, 2021).

2.8.11 Treatment and antimicrobial resistance of *Legionella* infections

Legionella species are generally susceptible to antibiotics, particularly macrolides (e.g., erythromycin, azithromycin) and fluoroquinolones (e.g., levofloxacin). However, antimicrobial resistance (AMR) has emerged as a critical global health threat (Murray et al., 2022), and *Legionella* is increasingly affected by this trend. While erythromycin was the treatment of choice following the initial LD outbreak in 1976, *Legionella* resistance has developed over time, necessitating alternative therapies (Pappa et al., 2020). Epidemiological studies report resistance rates of 50-100% for *L. pneumophila* strains against erythromycin, azithromycin, and levofloxacin (De Giglio et al., 2015; Rahimi and Vesal, 2017). This growing resistance is attributed to the inappropriate and excessive use of antibiotics and the emergence of resistance-conferring genetic mutations (Agyeman et al., 2022; Shi et al., 2022). Projections suggest that by 2050, AMR could lead to 300 million premature deaths globally, including 4.5 million in Africa, positioning it as one of the leading causes of mortality (Taviani et al., 2022).

2.9 CONTROL OF LEGIONELLA PNEUMOPHILA

2.9.1 Regulations for *Legionella* control

Risks that are posed by *Legionella* in community and hospital settings cannot be eliminated but appropriate control measures can be taken to reduce *Legionella* risk. The South African drinking water standard, South African National Standard (SANS) 241, does not explicitly mention *Legionella,* but it sets out comprehensive guidelines for microbiological, physical, aesthetic, and chemical determinants in drinking water, ensuring overall water safety. The South African Water Quality Guidelines for Domestic Water Use (Volume 2, 1993) also provides an overview of factors that can be used to determine the quality of water, namely pH, total coliforms available and presence of faecal coliforms, as well as other organic and inorganic components. The hygienic quality of water is determined by the presence of total coliforms in the water, which should normally not be detectable in drinking water, and if present, it is seen as a sign of ineffective water treatment, contamination of water, and unchecked growth of nutrients which may increase the growth of pathogenic microorganisms. The World Health Organisation (WHO) has provided specific guidelines to protect the public from *Legionella* infection in three principal documents: Guidelines for Drinking-water Quality (WHO, 2004), Guidelines for Safe Recreational Water Environments (WHO, 2006), and Guide to Ship Sanitation (WHO, 2007).

2.9.2 Control measures for *Legionella*

Several fundamental factors are important to *Legionella* proliferation in water distribution systems. These factors help to describe key strategies for *Legionella* control in building water systems. These factors include temperature, disinfectants, the presence of nutrients and stagnation. Control measures should not only focus on limiting or minimizing the growth of *Legionella* but also on reducing the risk of exposure for humans. *Legionella* colonization and growth are encouraged at warmer temperatures. All these control strategies have interactive effects and don't occur in isolation. Minimizing *Legionella* concentrations in the impacted system and preventing the spread of the bacteria to susceptible individuals is required to reduce or eliminate the risk of legionellosis. Various methods are utilized to manage *Legionella* in domestic plumbing systems, and research has demonstrated that they are successful, while presenting minimal drawbacks. These measures involve temperature regulation, chlorine treatment, copper-silver ionization, UV disinfection, and regular maintenance of the plumbing system.

2.9.3 Pasteurization

Pasteurization (also known as super-heat and flush) has been identified as a potential disinfection method for remediating engineering water systems. This process involves raising the hot water temperature to 71°C – 77°C so that it reaches at least 65°C at the outlets. At this temperature, outlets should be flushed for between 10 and 30 minutes (Bentham and Whiley, 2018; Whiley and Taylor, 2016).

2.9.4 Temperature

Various methods have been used to control *Legionella* in domestic sanitary water systems, although each method has its own level of effectiveness and there are also disadvantages. Thermal pasteurization (also known as heat treatment) is one of the simplest methods. To control and manage *Legionella* in buildings, hot and cold-water systems should have temperature conditions that do not allow *Legionella* colonization and growth. Therefore, temperatures outside 25°C to 45°C should be able to control the proliferation of *Legionella*. Elevating temperatures for *Legionella* control to greater than 55°C in hot water systems and less than 25°C in cold-water systems has been shown to be effective in reducing *Legionella* samples, cases and/or outbreaks (Arvand et al., 2011). Curative strategies may be applied once or many times, for an hour to a day, and over a range of temperatures (60°C to 70°C) to destroy *L. pneumophila* strains in water distribution systems. This method needs to be repeated every few weeks as it is unreliable in the long term and bacteria can recolonize the system if not repeated (Springston and Yocavitch, 2017). Moreover, frequent heat shocks can promote the emergence of heat-resistant *L. pneumophila* strains, as observed in hospital water systems subjected to

periodic extreme temperature (24 hours at 65°C a few times a year), while no such resistance was observed for strains isolated from the system where heat shock treatments (70°C for 30 minutes) were sparingly applied (Allegra et al., 2011).

2.9.5 Disinfectants

Chlorine is widely used for its strong oxidizing power for primary disinfection treatment of potable water. It reacts with a variety of bacterial cellular components and can permeabilize the cytoplasmic membranes, causing leakage of proteins and DNA damage. However, *L. pneumophila* has shown resistance to high levels of chlorine by the formation of biofilms, and pipe corrosion can be expected when the disinfectant is dosed in a water distribution system (Sciuto et al., 2021). The World Health Organization recommends maintaining a free chlorine concentration of 0.5 mg/L to inactivate microbial contaminants and prevent regrowth in water distribution systems (WHO, 2022). As *L. pneumophila* can resist high chlorine levels by forming biofilms, and high residual chlorine can corrode copper and iron pipes, maintaining a pH above 8 and using poly- and orthophosphates can mitigate this. Chlorine also forms potentially toxic and carcinogenic disinfection by-products (DBPs) such as trihalomethanes (THMs), which are difficult to remove and require resource-intensive processes (Cooper and Hanlon, 2010; Assaidi et al., 2020). Although chlorine dioxide and monochloramine as a secondary disinfectant have been found to be successful against biofilms and waterborne pathogens (Springston and Yocavitch., 2017; Sciuto, 2021), other studies suggested that to mitigate the risk of *Legionella* there should be a combination of two or more different disinfectants used together (Sciuto, 2021). Chlorine dioxide, a water-soluble gas, is superior to chlorine in penetrating biofilms and inactivating free-living protozoa, making it effective as a secondary disinfectant in hospital settings. It is less corrosive than chlorine but can damage polyethylene pipes and requires careful monitoring to control DBP formation (Yee et al., 2020; Sciuto et al., 2021; Scotmas, 2023). Monochloramine, formed by reacting ammonia with chlorine, is used for primary disinfection with recommended concentrations varying by organization, such as 3 mg/L by the WHO and 4 mg/L by the EPA. It is effective as a secondary disinfectant with fewer DBPs than chlorine, making it a viable alternative in certain water systems (Marchesi et al., 2020; CDC, 2020; Waterline, 2024). These disinfectants are effective for *Legionella* spp. in biofilms and planktonic form. Factors that affect the efficacy of disinfectants include temperature, pH, organic carbon, water hardness, presence of protozoa, culture condition of *Legionella* spp. and concentration. The concentration and exposure time (CT) are determined by the type of disinfectant used. Choice of a disinfectant also needs to consider corrosion impacts on pipe materials, reliability, and safety. Because *Legionella* spp. can use protozoa and their cysts as a protective shield against disinfectants, it is imperative to consider the efficacy of the disinfectants. Chlorine is the most widely used disinfectant, which is usually applied as chlorine (chlorine gas), sodium hypochlorite solution, or dry calcium hypochlorite. Chlorine disrupts the cell membrane, nucleic acids, respiration, and enzymatic activity of *Legionella* spp., leading to their inactivation. Generally, maintenance of a free chlorine residual in potable water systems is effective for control of *Legionella* spp. (Springston and Yocavitch, 2017). However, in outbreaks, hyperchlorination with 4 to 6 mg/L decreased *L. pneumophila* in plumbing systems by 5 to 6 logs over six hours. A higher dose of chlorine was required at 43°C to overcome thermal decomposition and maintain a chlorine residual of 4 to 6 mg/L (Kim et al., 2002). Copper-silver ionization is a cost-effective and low-maintenance method for controlling *Legionella* in water systems. Copper and silver ions, produced through electrolysis, work together to disrupt bacterial cell walls and inhibit nutrient uptake (LeChevallier, 2023). Copper ions create openings for silver ions, binding to cellular components, immobilizing the bacteria, and preventing cell division. This combination effectively controls bacterial growth in various environments (NASEM et al., 2019).

CHAPTER 3: CHARACTERISATION OF STUDY SITES AND BUILDINGS

3.1 INTRODUCTION

The study focused on diverse settings such as the densely populated urban environment of Hillbrow, the low-income urban area of Atteridgeville, the rural district of Vhembe, and informal settlements like Zandspruit and Melusi (Figure 3.1). This chapter provides an understanding of the possible risk of *Legionella* infection across the different settings, regions, infrastructure conditions, and population densities in Gauteng and Limpopo provinces, South Africa. The results offer insights into the varying urban and rural contexts, and how these may contribute to the spread of waterborne diseases.

Figure 3-1. Study sites and sampling areas in Vhembe: High-rise building (a) Household building (b), Melusi (c), Zandspruit (d, e); Atteridgeville (f) and high-rise buildings in Hillbrow (g).

3.2 DESCRIPTION OF STUDY SITES

Permission to conduct this study was obtained from the relevant stakeholders (GRT-INSPIRED), building managers and households in Gauteng (Appendix A). The Gauteng Research Triangle (GRT) is a partnership between the Universities of Johannesburg, Pretoria, and the Witwatersrand (Wits). The GRT oversees a range of areas, but one of its main projects is a new health and demographic surveillance site (HDSS) with a split node located in Hillbrow, Atteridgeville West, and Melusi, an informal settlement in Pretoria (Figure 3.2). Ethical clearance was obtained for the collection and processing of samples for the Hillbrow area from the Faculty of Health Sciences Research Ethics Committee at UJ (REC-1656-2022 and amended REC-1656-2022). Ethical clearance was obtained for Atteridgeville, Melusi and Zandspruit from the University of Johannesburg in October 2023 (REC-2418-2023) and for the Vhembe site (FSEA/23/BMY/29) from the University of Venda in December 2023.

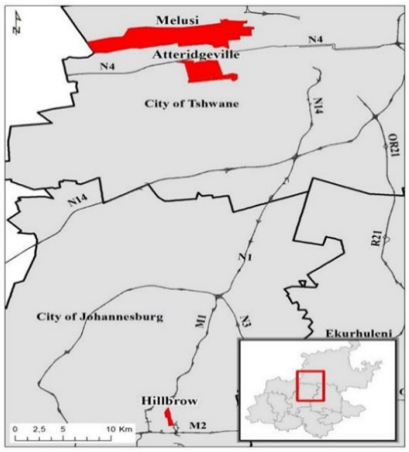


Figure 3-2. Map showing Hillbrow, Atteridgeville and Melusi sites from the GRT, in the Gauteng Province, South Africa (GRT-SAPRIN, unpublished).

3.2.1 Hillbrow Johannesburg

Hillbrow, situated in Johannesburg within the Gauteng Province of South Africa, serves as an inner-city residential neighbourhood (Figures 3.1, 3.2 and 3.3). Geographically, it is located at a latitude of -26.1867 and a longitude of 28.0428 (GRT-SAPRIN, unpublished). The area is a focal point of urban density, characterized by a high concentration of residential buildings, including both high-rise apartments and smaller housing units.

Figure 3-3. Map of Johannesburg inner city showing the high-rise buildings (represented as dots) in the Hillbrow location. The South 1 (green) and South 2 (purple) regions were sampled during this study (GRT-SAPRIN, unpublished).

The research team has received access to more than 100 residential apartment buildings in the south of Hillbrow, with varying floor levels and residential apartments. From these buildings, we were able to identify 32 buildings that were safe to access (used as rental residential accommodation) with > 4 floors. To de-identify building names, the buildings were coded numerically from HB1 onwards. Before sampling could begin, an email was sent out to the building property managing agencies and building managers to ask for permission to access the sites and an appointment was requested to assess the buildings for this study through the UJ GRT Community Engagement Officer. All methods and techniques were optimised with the samples obtained from the Hillbrow area.

3.2.2 Atteridgeville

Atteridgeville is a housing township located to the west of Pretoria, in the Gauteng Province of South Africa (Figures 3.1 and 3.2). The GPS coordinates for Atteridgeville are approximately -25.7879 latitude and 28.1011 longitude (GRT-SAPRIN, unpublished). The area features a diverse range of building infrastructures, including formal low-rise houses, apartment complexes, and informal settlements. The area reflects a diverse socio-economic landscape, with varying levels of income and educational attainment. Atteridgeville, which was established in 1939, presents a distinct contrast to Hillbrow. Instead of vertical apartment buildings, Atteridgeville features matchbox “township” houses and sprawling backyard dwellings. In the 2011 Census, the Atteridgeville sub-place covered an area of 7.25 km², with a population of approximately 43,000 people residing in 11,000 households.

3.2.3 Melusi

The Melusi informal settlement did not exist according to Census 2011, but now houses approximately 40 000 people. Melusi shares an almost seamless border with the suburb of Daspoort Melusi (Figure 3.2), now consisting of three sections and expanding, and is situated around old but highly polluted dams, positioned between formal suburban areas and the nearby hillside(GRT-SAPRIN, unpublished). Melusi has more freestanding informal housing structures with open spaces between and around them, eliminating the need for consolidating individual structures into larger collective compounds. Access to this site was put on hold for 2023 due to unplanned expansion, which posed a significant challenge in terms of accessibility and resource allocation. Accessibility was provided in July 2024.

3.2.4 Vhembe (Thohoyandou)

This is a region in the Vhembe District, situated in the northern part of the Limpopo Province, South Africa (Figure 3.4). The GPS coordinates for Thohoyandou are approximately -22.9456 latitude and 30.4850 longitude. Thohoyandou features a mix of modern and traditional housing infrastructure that varies from formal housing developments to informal settlements. Thohoyandou is the administrative centre of Vhembe District Municipality and Thulamela Local Municipality. It is also known for being the former capital of the bantustan of Venda. Thohoyandou is a town that falls under the Thulamela municipality of Vhembe District. Thulamela Local Municipality has an estimated population of 575,929 (Statistics SA, 2022). The area is surrounded by small rural townships such as Maungani, Makwarela, Manini, Duthuni, Vondwe, Ngovhela, Muledane, Phiphidi, Shayandima and Tshisahulu. Today, Thohoyandou is one of the fastest-growing towns in Limpopo. It is also home to the University of Venda.

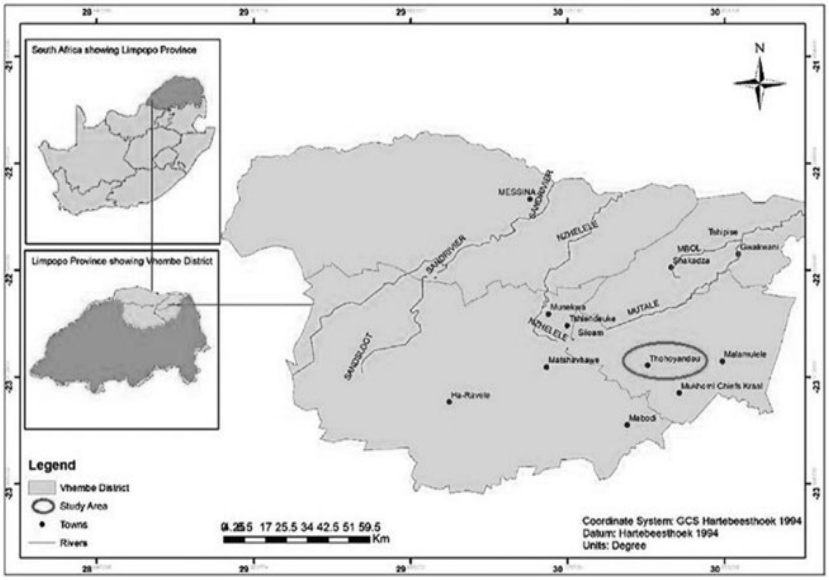


Figure 3-4. Map of Vhembe District showing Thohoyandou town and other towns, Limpopo Province, South Africa.

3.2.5 Zandspruit informal settlement (Johannesburg)

Zandspruit informal settlement is located in Johannesburg, in the Gauteng Province of South Africa (Figure 3.5). It has an area of approximately 4 km² in Region C City of Johannesburg Metropolitan Municipality. Zandspruit is an invasion site on privately owned farmland. Established in 1994, the 2011 census reported a population of 31,716 residents (Stats SA, 2011), but by 2013, Dawson et al. (2013) estimated that the population had grown to around 80,000 with an estimated 14,500 freestanding informal housing structures. However, current population statistics do not actually exist. The settlement is comprised of formalized (developed) and non-formalized (underdeveloped) areas.

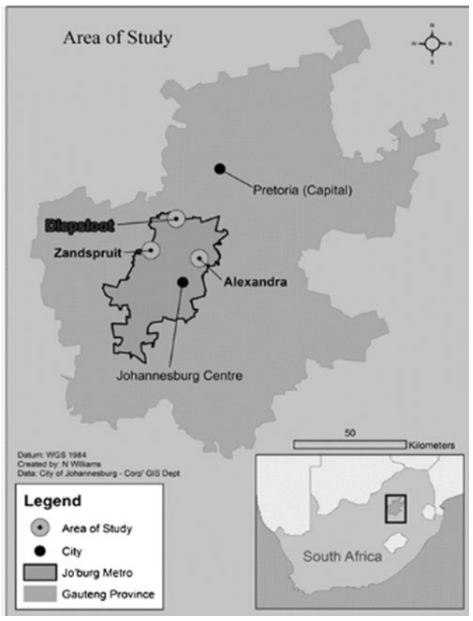


Figure 3-5. Map of Gauteng showing the Zandspruit informal settlement, South Africa.

3.3 BUILDING CHARACTERISATION

Access to the Hillbrow and Atteridgeville study sites was approved through the SAMRC-Gauteng Research Triangle Initiative for the Study of Population, Infrastructure and Regional Economic Development (GRT-INSPIRED) (Appendix B). Access to the Vhembe sites was facilitated by Ethics Approval from the University of Venda, whilst access to Zandspruit was achieved through Mr Wihann van Reenen (IIE MSA, Ruimsig). Building classes according to SANS 10400 A:2022 was considered for the Hillbrow, Atteridgeville and Vhembe sites (Table 3.1). According to the SANS 10400 A:2022 Edition 4 standard, informal housing structures such as those in the rural Vhembe and Zandspruit informal settlement region generally do not meet the formal requirements outlined for building construction and design in South Africa. However, it is important to note that the standard does recognize the existence of such structures and the need for their improvement.

Table 3.1. SANS 10400A:2022 Occupancy or Building Classification

Building Class of Occupancy	Occupancy and Population
H1	**Hotel:** Where a person rents furnished rooms, not being dwelling units. The total number of persons per bedroom is 2.
H2	**Dormitory:** Where groups of people are accommodated in 1 room. The number of person/s per m^2 is 1.
H3	**Domestic Residence:** Consisting of two or more dwelling units on a single site. The total number of persons per bedroom is 2.
H4	**Dwelling House:** Consisting of a dwelling unit on its own site, including a garage and other domestic outbuilding, if any. The total number of persons per bedroom is 2.
H5	**Hospitality:** Where unrelated persons rent furnished rooms on a transient basis within a dwelling house or domestic residence with sleeping accommodation for not more than 16 persons within a dwelling unit. The total number of persons per room is not more than 4.
"dwelling house": is a single dwelling unit and any garage and other domestic outbuildings, situated on its own site "dwelling unit": is a unit containing one or more habitable rooms and provided with adequate sanitary and cooking facilities	

3.3.1 Building Classification

The SANS 10400A:2022 Occupancy or Building Classification was used to categorise the study buildings sampled in Hillbrow, Vhembe, Atteridgeville, Melusi and Zandspruit. Hillbrow and Vhembe (high-rise Buildings) were classified as Building Class H5: Hospitality, where unrelated persons rent furnished rooms on a transient basis within a dwelling house or domestic residence with sleeping accommodation for not more than 16 persons within a dwelling unit. Atteridgeville was classified as Building Class H4 Dwelling House: Consisting of a dwelling unit on its own site, including a garage and other domestic outbuilding, if any. Vhembe households comprised Building Class H3: Domestic Residence: Consisting of two or more dwelling units on a single site. The household buildings in Zandspruit and Melusi could not be classified, and the occupancy could not be determined, due to the areas being informal settlements.

Urban and rural areas offer ideal environments for investigating the multifaceted effects of urbanization on public health, particularly in the context of disease proliferation and management. As one of the most densely populated metropolitan areas in Southern Africa, Hillbrow provides a unique case study. With an estimated population of 75,000 individuals residing in approximately 200 structures—ranging from residential apartments to freestanding houses, some exceeding 15 stories- this neighbourhood exhibits a population density of about 68,418 people per square kilometre, spread over nearly one square kilometre (Stadler and Dugmore, 2017). The area accommodates around 24,857 households within roughly 10,000 flats, along with a few motels and other housing structures (Turok and Borel-Saladin, 2016).

The study extends beyond Hillbrow to include other significant sites, each with distinct socio-economic and environmental characteristics that influence the spread and impact of pathogens such as *Legionella pneumophila*. Atteridgeville, another urban low-income area, contrasts with Hillbrow in its infrastructure and population density, offering insight into how different urban environments affect health risks (Coovadia et al., 2009). Vhembe, a rural area in Limpopo, provides a rural perspective, highlighting the challenges in water quality management outside urban settings (Bessong et al., 2009). The informal settlements of Zandspruit and Melusi present another layer of complexity, where inadequate infrastructure and high population density create conditions ripe for the spread of waterborne diseases (Naicker et al., 2015). These sites collectively contribute to a comprehensive understanding of the diverse factors influencing public health in both urban and rural settings across South Africa.

3.3.2 Building description

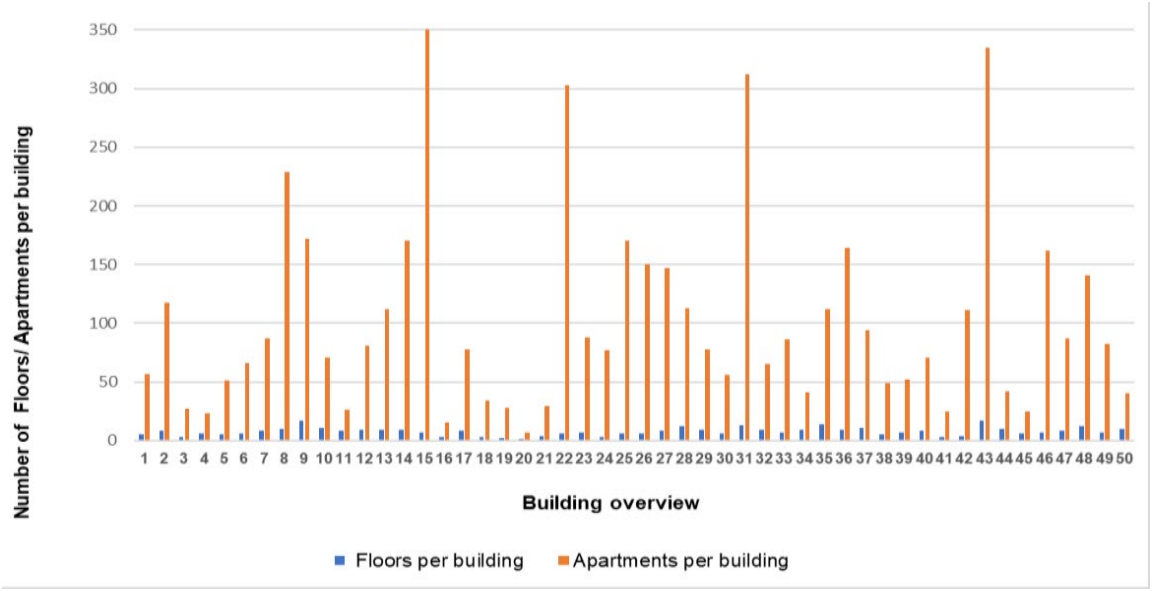
The GRT-SAPRIN team received access to 122 residential apartment buildings in the south of Hillbrow. After screening the buildings from the GRT study, it was noted that the managing agencies and building managers refused access to 19 buildings, while 34 buildings were unsafe to access. The researchers were granted full access to only 69 buildings, and these had varying floor levels, and the number of residential apartments ranged from 40 to 140 (Figure 3.6). The 69 buildings were categorised according to safety and access (at the level of the building manager) (Appendix D, Table 6), and it was found that 50 of these buildings were safe to access, whilst access was lost to eight buildings due to unforeseen circumstances.

Access was denied by some property management agents as they feared tenants might think there is an issue with the building's water, and others didn’t see how this project would be of benefit to them. During this study sampling period, a total of 15 buildings were visited for risk assessment and sampling once permission and consent were received. Buildings were selected based on safety and accessibility first, thereafter, the number of floors per building was taken into consideration (Figure 3.6). Building occupancy ranged from 60-100% across the buildings.

3.3.3 Building Occupancy

In Hillbrow, 87% (13/15) of the buildings were domestic residences and fell into Class H3, whilst one building was a student residential apartment (HB40) and the remaining building was a residence that was also being used as a hotel and fell into Class H5 (Figure 3.7). The Class H3 dwellings are family homes where activities related to home life, such as showering, sleeping and cooking, occur with the number of occupants ranging from 2 to approximately 6 people per dwelling (GRT-SAPRIN).

(a)



(b)

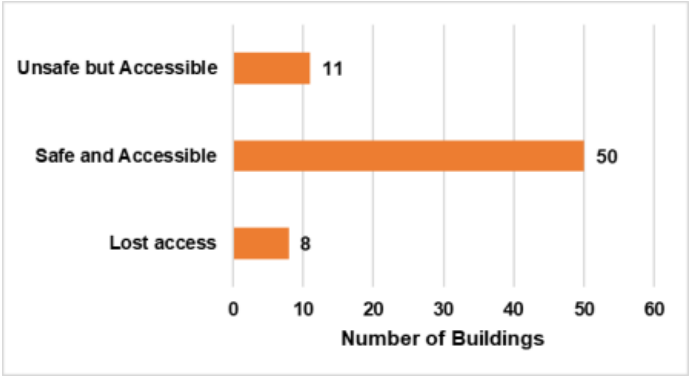


Figure 3-6. Summary of the building characteristics (a), and their accessibility and safety (b) in the south of Hillbrow (n=69) between October 2022 and May 2023.

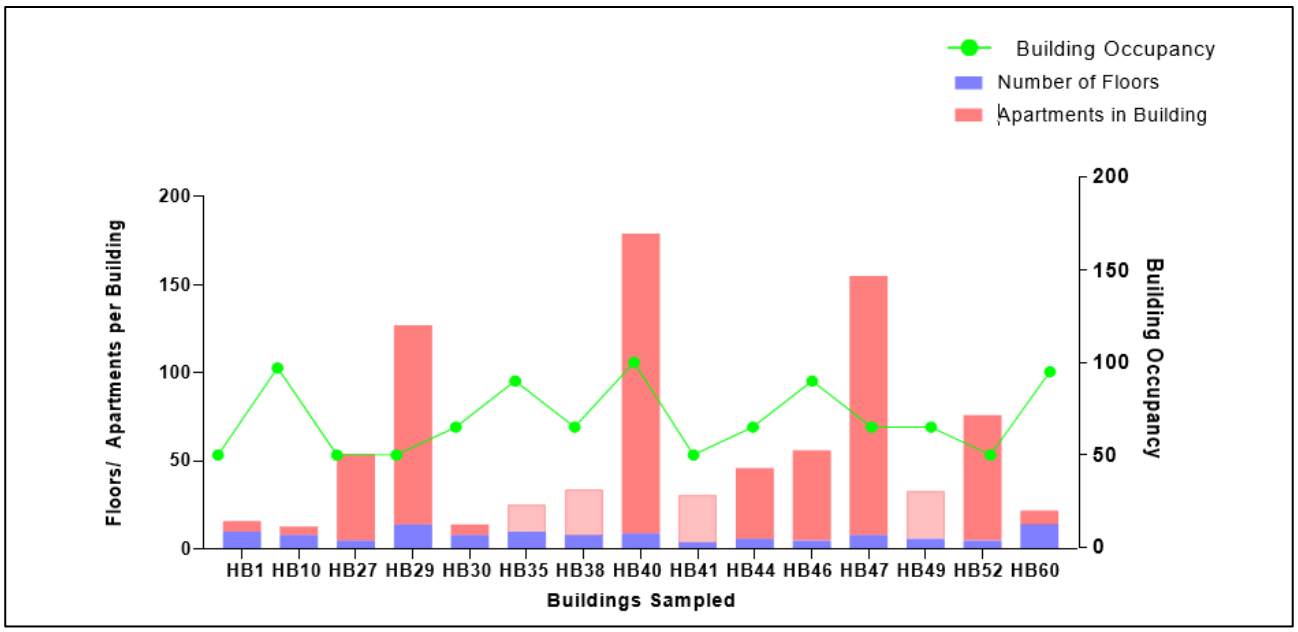


Figure 3-7. Overview of the number of floors, apartments and occupancy per building (n=15) accessed in Hillbrow.

In the Vhembe region, a comprehensive assessment of 14 buildings, including high-rise structures and households, revealed a range of aspects related to building infrastructure, water sources, and water quality. The building assessment checklist for the Vhembe region was categorized into general building information, water distribution, water use, and risk classification. A total of 14 buildings were sampled in Vhembe, with 28.57% (4/14) being high-rise residences and the remainder (71.43%; n=10) being domestic residences that fell into Class H3 (Table 3.2). Among the buildings, most (71.43%; n=10) were fully occupied, with the remainder (28.57%; n=4) being partially occupied. Household occupancies varied, with the largest household (V10) having 14 rooms, and others ranging from 5 to 12 rooms.

Table 3.2. Building occupancy and general information of buildings in the Vhembe region.

Building Type:	Rooms	Building Occupancy
High-Rise Building V1	324	Fully Occupied
High-Rise Building V2	324	Fully Occupied
High-Rise Building V3	904	Fully Occupied
High-Rise Building V4	880	Fully Occupied
Household V5	12	Partially Occupied
Household V6	9	Fully Occupied
Household V7	11	Fully Occupied
Household V8	5	Partially Occupied
Household V9	9	Fully Occupied
Household V10	14	Partially Occupied
Household V11	7	Fully Occupied
Household V12	5	Fully Occupied
Household V13	10	Partially Occupied
Household V14	8	Fully Occupied

3.4 WALKTHROUGH ASSESSMENT OF BUILDINGS

Walkthrough site assessments were conducted for Hillbrow, Melusi, Atteridgeville, Zandspruit and Vhembe prior to sampling to assess the potential risk of exposure to *Legionella* spp. The water distribution systems in the high-rise buildings were mapped with the assistance of the maintenance/building managers who had a working knowledge of the plumbing and air conditioning systems in the building, where applicable. Building assessment checklists (Appendix B, C) was done to characterize the sampling sites based on general information on the building: the number of floors, occupancy, water source, water quality, water distribution throughout the building, water aerosolization points, suitable water sampling sites, water storage, including hot water availability and usage. The assessments were conducted to determine the areas that may be considered a risk for infection by aerosolization and inhalation of *Legionella* to people living in these areas. The building assessments provide information on:
a) Identifying areas in the water distribution systems and buildings where water may stagnate, such as air conditioners, water fountains, storage tanks and/frequently used taps and showers
b) The number of at-risk water sources, i.e., taps and showers, geysers, and water boilers, as well as open toilet flushing systems and toilets that have no lids.

A walkthrough site assessment of the 15 Hillbrow and 14 Vhembe buildings was conducted on separate occasions, and each walkthrough was aided by the building manager and caretaker. Site assessments were also performed for Melusi, Atteridgeville and Zandspruit. These assessments were done to determine occupancy, water quality, water distribution throughout the building, water aerosolization points, to locate a suitable sampling site, hot water availability and usage.

3.4.1 Hillbrow buildings

For the Hillbrow buildings, the residents within the apartments were contacted in advance to ensure their availability.

3.4.1.1 Plumbing maintenance

The age of the building could not be provided. There was no official water distribution plan, and the plumbing materials used included iron, PVC, or copper pipes (Figure 3.8). Most buildings (93%, 14/15) had plastic storage tanks, while one (7%) had a metal tank. Plumbing materials were not specified in 8 of the 15 buildings; 4 used iron pipes, 1 used copper, and 2 combined copper and PVC.

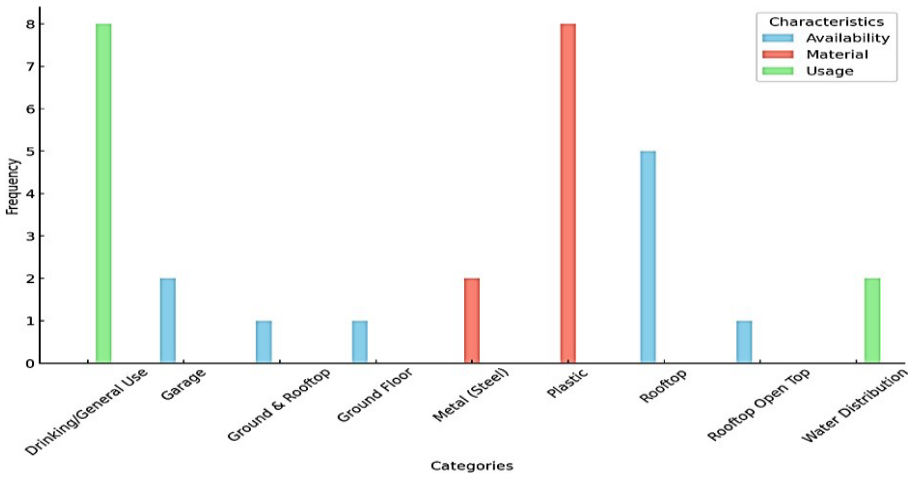


Figure 3-8. Storage tank characteristics in Hillbrow showing availability, locations, material types, and usage patterns.

Table 3.3 shows the water quality management practices employed in the study buildings. Four (n=4) of the buildings had an assigned water quality manager, unlike the remainder (n=11) that did not employ a water quality manager. Monthly water quality monitoring was done in six (n=6) of the 15 buildings, but nine (n=9) buildings did not monitor water quality. All buildings had toilet seat covers in all their apartments. No points of aerosolization were observed.

Table 3.3. Summary of water quality management practices in the Hillbrow buildings.

Management aspect	Not present (0)	Present (1)	Other (2)
Water treatment	9	5	**1**
Water quality monitoring	9	6	**0**
Water quality manager	**11**	**4**	**0**

As shown in Figure 3.9, building plumbing maintenance was only done, when necessary, in eight (n=8) of the buildings. Monthly maintenance occurred in three (n=3) of the buildings, and daily maintenance was done in two (n=2) of the buildings, one annually and one weekly.

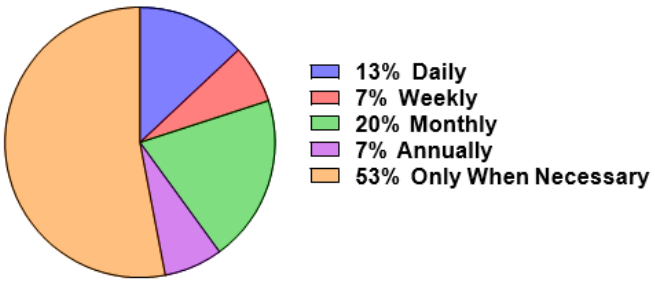


Figure 3-9. Pie chart showing the frequency of building plumbing maintenance in Hillbrow.

3.4.1.2 Water Distribution System in Hillbrow Buildings

All 15 buildings employed a storage tank in their drinking water distribution systems. The location of the storage tanks for 60% of the buildings was on the roof, where water was pumped from the source up to the roof, then down to the residences. Alternatively, the storage tank was located at the bottom level of the building, from which water was pumped up to the residences. Most buildings (60%; n=9) had the following water distribution network in place, whereby:

> "*Water is piped from the ground floor of the building to a water tank on the top of the building and then this 'tank water' is piped back down to the apartment*" (Figure 3.10a).

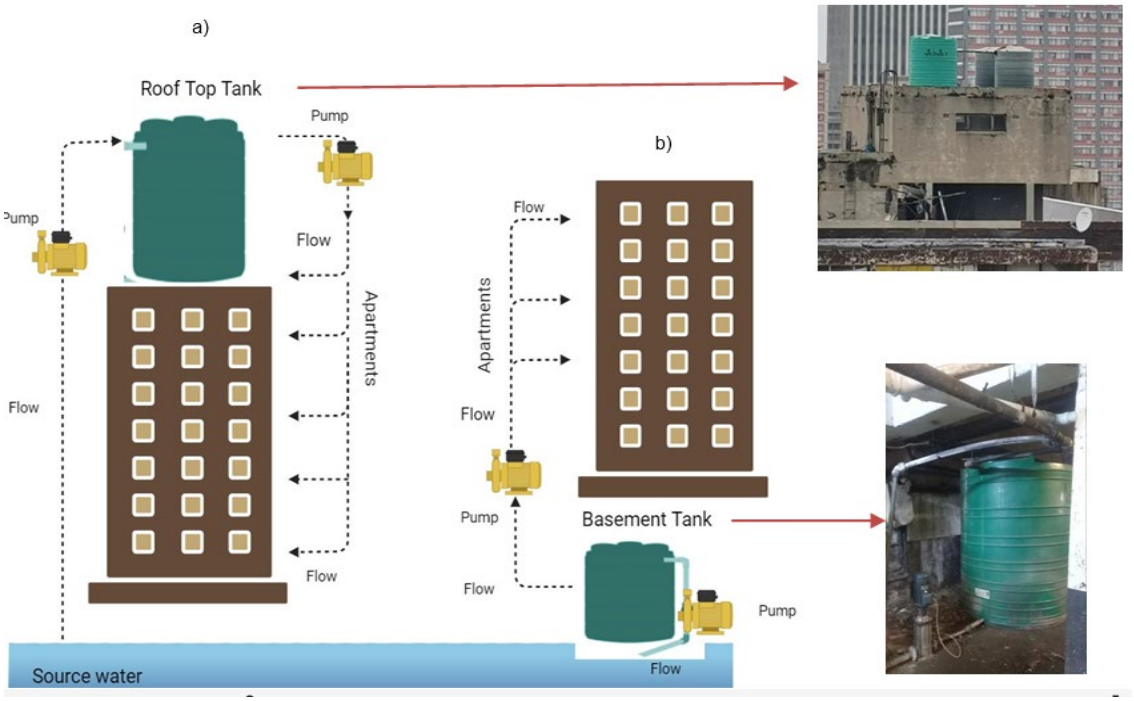


Figure 3-10. Water distribution systems in the Hillbrow high-rise buildings. a) Unidirectional flow of water from the source of water to the rooftop tank and back down to the apartments, b) Directional flow of source water from the basement floor to a tank in the basement and back up to the apartments.

Visual inspection showed that these 'Jo-Jo' tanks were placed on the rooftop of the building, and in one building, this was not fully covered. Only two of the 15 buildings (HB40 and HB46) had water distribution that was piped from the ground floor of the building to apartments at the top of the building (Figure 3.10b). Water heating varied throughout the day, with some buildings having geysers on each floor (continuously heated), and others having geysers or water boilers in each apartment (heating at the discretion of the resident). Buildings HB10 and HB29 had a borehole onsite, and this water was treated and circulated as treated potable water throughout the building. Buildings HB30, HB35, HB41 and HB60 had their water treated onsite.

3.4.1.3 Water Availability (Hot and Cold), distribution and use in Hillbrow

The quality of water was primarily assessed through taste, smell and colour. All 15 Hillbrow buildings stated that the water had no unusual taste or smell, and that the colour was clear. In contrast, one building had an open storage tank, 13 buildings relied on a municipal water supply, and two buildings had a man-made borehole as a drinking water source. Of the 15 buildings, five had secondary water treatment on-site, one building only employed on-site water treatment when deemed necessary, and nine buildings did neither. None of the buildings had a water feature or a drinking water fountain, and a plumbing distribution plan was unavailable for all 15 buildings. As shown in Figure 3.11, the availability of hot water differed per building and was subject to the hot water distribution system that each building employed. Thus, hot water availability included geysers per floor, geysers in each apartment, shared water heaters, tenant-installed geysers, water heating per floor, water boilers in each apartment, and non-working heat pumps. The water heating schedule ranged from no hot water in the building to individual preferences and continuous heating throughout the day in 4 out of the 15 buildings. This continuous heating was due to a central water heater/boiler. Although 9 of the 15 buildings had geysers per apartment, water heating was at the discretion of the tenant. Two of the 15 buildings had no water heating equipment in place. The building risk assessment checklist recorded hot water usage, availability, and patterns. The majority of Hillbrow tenants in the present study had a hot water system in place, but opted not to use it. Instead, 27% of tenants used a bath dish, 16% of tenants preferred to boil

their own water, 3% used an electric kettle to boil water and 4% had to install their own geyser. Only 7% of tenants used their geysers. Figure 3.12 highlights the comments made by the tenants based on their outlook towards water distribution and use per building.

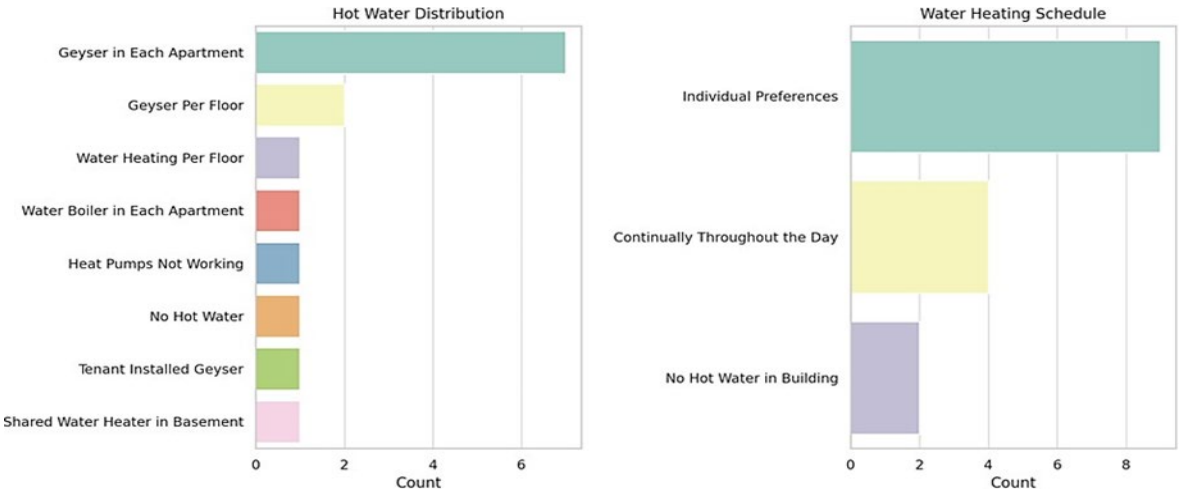


Figure 3-11. Hot water distribution (left): Frequency for each method of hot water availability. Water heating schedule (right): Frequency of water heating across the buildings during the day.

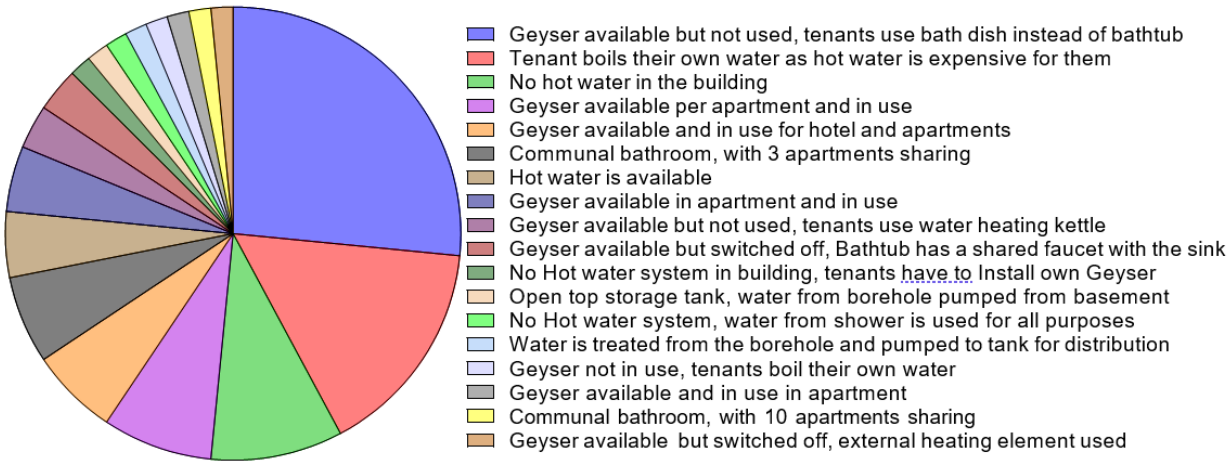


Figure 3-12. Comments from study participants as to hot water availability and use in Hillbrow buildings.

3.4.2 Vhembe building

A general walk-through assessment was done for the high-rise residences and households. The age of student residences ranged from 2 to 4 years, while household buildings were between 15 and 40 years old. Of all the buildings, 42.86% (6/14) had undergone plumbing renovations, while 8 had no record of such renovations. Plumbing maintenance was conducted weekly in four (n=4) buildings, annually in nine (n=9) buildings, and one (n=1) building performed maintenance only when necessary.

3.4.2.1 Water storage and distribution

In the 14 Vhembe buildings (high-rise buildings n=4 and one level rural households n=10) included in the present study, 14% (n=2) used boreholes as their water source, 36% (n=5) relied on the municipality for water

provision, and 50% (n=7) had access to both borehole and municipal water (Table 3.4). All buildings had continuous access to water throughout the day. None of the four high-rise buildings had storage tanks, as water was supplied directly from water reservoirs. In contrast, all 10 households had plastic JoJo storage tanks, with most tanks installed on stainless steel stands, except for one household where the tank had no stand. Four (n=4) buildings used copper exclusively for their plumbing, one (n=1) used iron only, five (n=5) used a combination of copper and PVC, one (n=1) used iron and PVC, and three (n=3) buildings utilized a combination of copper, iron, and PVC.

Table 3.4. Assessment of plumbing in Vhembe buildings.

Category	Details	Count
With Leaks		5
Without Leaks		9
Plumbing Renovation	Yes	6
	No	8
Maintenance Frequency	Daily	0
	Weekly	4
	Monthly	0
	Annually	9
	Other (When Necessary)	1
Unused Water Fixture (Household)	Have	4
	Don't Have	6
Source of Water	Borehole	2
	Municipal	7
	Both	5
Availability of Storage	Yes	10
	No	4
Cleaning Frequency	Weekly	1
	Monthly	1
	Annually	5
	Other	3
Temperature Monitoring	Yes	3
	No	9
	Other	2
Type of Plumbing	Copper	11
	Iron	6
	PEX	0
	PVC	8
	Other	0
Hot Water Distribution	Central Water Heating	14
	Water Heating/Boiling on Each Floor	0
	Geyser in Each Apartment	0
Heating Times	Continually Throughout the Day	7
	Only at Peak Hours	2
	Other	5
Taste and Color of Water	Good	11
	Poor	3
Smell of Water	No Smell	13
	Don't Know	0
	Other	1

3.4.2.2 Plumbing and maintenance

In terms of plumbing assessment (Table 3.4), over a third (35.71%; n=5) of the buildings had leaks while 42.86% (n=6) of the buildings had undergone plumbing renovations. Plumbing maintenance frequency varied,

with 28.57% (n=4) of the buildings conducting weekly maintenance, most (64.29%; n=9) conducting annual maintenance, and the remaining building (7.14%; n=1) performing maintenance only when necessary.

3.4.2.3 Water source

Among the households (Table 3.4), nearly half (40%; n=4) had unused water fixtures, while the remaining households (60%; n=6) used their water fixtures. Regarding water sources, 14.29% (2/14) of the buildings used boreholes, 50% (7/14) relied on municipal water, and 35.71% (5/14) had access to both borehole and municipal water. Storage tanks were present in 71.43% (10/14) of the buildings, with the remaining 28.57% (4/14) lacking storage facilities. The cleaning frequency of these storage tanks varied: 7.14% (1/14) were cleaned weekly, 7.14% (1/14) were cleaned monthly, 35.71% (5/14) were cleaned annually, and 21.43% (3/14) were cleaned using other schedules. For temperature monitoring, only 21.43% (3/14) of the buildings had temperature monitoring in place, while 64.29% (9/14) did not.

3.4.2.4 Hot water distribution and heating times

All high-rise buildings (100%) had centralized water heating systems (Table 3.4). Hot water was available continuously throughout the day in 50% (7/14) of the buildings, only during peak hours in 14.29% (2/14), and under other schedules in the remaining 35.71% (n=5) of the buildings. Of the buildings surveyed, 78.57% (11/14) reported good taste and colour of water, while 21.43% (3/14) indicated poor taste and colour. Most (92.86%; n=13) reported no smell to the water, and 7.14% (1/14) were uncertain about the smell of the water.

Figure 3.13 provides a summary of water system conditions and water awareness from Vhembe residents. In general, among the surveyed buildings, two student residences reported that students were not aware of the risks associated with *Legionella* bacteria. In two cases, geysers were available but were not used due to high electricity costs. One residence did not have a geyser, with only cold-water showers available. Another household reported a storage tank pipe distribution system showing signs of rust and dirt accumulation. In one instance, a geyser was available, but this only boiled water during peak hours. Additionally, biofilm formation was observed in one building's shower system, indicating potential water quality issues.

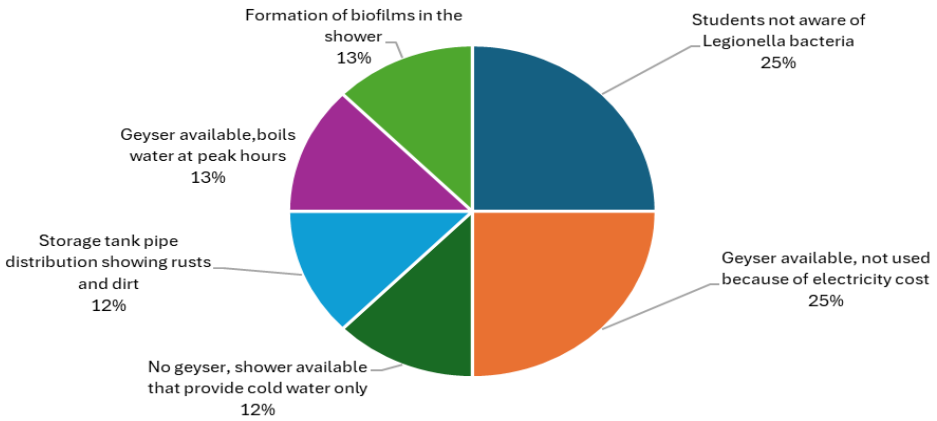


Figure 3-13. General comments on the buildings and Legionella from participants in Vhembe.

3.4.3 Melusi Household Building

Melusi is a rural settlement outside of Pretoria with little to no known plumbing renovations or maintenance done on the water supply. Water taps are located outside houses and have irregular water flow. Thus, 54% (n=20) of households stored their water. Plastic buckets were used by all 20 households who stored their water, and used it for everyday use such as drinking, cooking, bathing, laundry and cleaning. As indicated in

Figure 3.14, the water in the storage buckets was changed once a day by 45% (9/20) of the households, 30% (6/20) households fetched water every second day, whilst 15% (3/20) fetched water more than once a day. and 5% (1/20) collected water every three days and only 1 household changed their water once a. The general water quality was noted as good by 94% (34/36) of the households, but the remaining 6% (2/36) of the households noted a discolouration and a metallic taste in the water. There were no individual sanitation facilities in Melusi; consequently, all households had access to pit toilets.

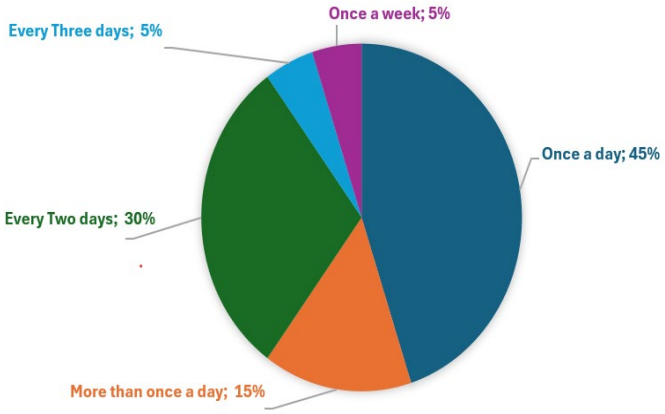


Figure 3-14. Frequency of refilling water for storage by households in Melusi.

3.4.4 Atteridgeville buildings

Atteridgeville is a township outside Pretoria, with fully installed water and sanitation facilities. Plumbing renovations were done recently for two of the households and no record of renovations exists for 94% (34/36) of the households. Only 8% (3/36) of households arrange annual plumbing maintenance, while 92% (33/36) do not employ any plumbing maintenance. The plumbing material used to transport water was unspecified/unknown for most (58%; n=21) of the households, PVC was used in 17% (6/36), while 14% (5/36) used copper, 8% (3/36) used iron and 3% (1/36) used PEX. Water treatment was noted by 47% (17/36) of the households and the other 53% (19) knew of no water treatment in their households/area. Some of the households (39%; n=14) opted to store their water in plastic buckets and use it instead of using the water straight from the tap, as most (22 of the 36) households did. The stored water was replaced daily by 79% (11/24) of the households, while 21% (3/14) changed water more than once a day. Most of the households 53% (19/36) used a geyser, 28% (10/36) used an electric kettle to boil their water and 19% (7/36) used a boiler. All 36 households had a flushing toilet, but only 58% (21/36) of the households had a lid for the toilet (Figure 3.15).

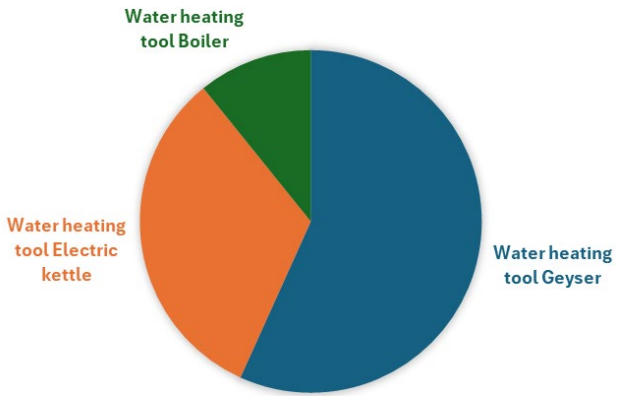


Figure 3-15. Water heating in Atteridgeville.

3.4.5 Zandspruit buildings

Zandspruit informal settlement has water taps (standpipes) located near a few houses per street, which are shared by households on that street. Most of the sampled households 87% (26/30), have a tap and 13% (4/30) store their water in plastic containers. This stored water was used for household purposes and collected daily. The general water quality, assessed through taste, smell, and colour, was reported as good by all the households. There were no known plumbing renovations, maintenance, or water treatment done, nor was the plumbing material specified in the sampled households. All 30 households used a kettle to boil their water. The toilets in all 30 households were flushing toilets; only 13% (4/30) had a toilet lid, while the majority, 87% (26/30), had no toilet lid.

CHAPTER 4: STUDY DESIGN AND METHODS

4.1 INTRODUCTION

The sampling plan and optimised methods are based on the study done in the Hillbrow buildings. Before any assessment or sampling could start, property agents and building managers were contacted for an appointment. Once approval was obtained, walkthrough assessments were done onsite during day 2, and residents were contacted for permission based on the convenience sampling method, whereby residents (from apartments) who were present on the day 2 site assessments were asked to participate in the study on day 3. The sampling procedure followed for all sites in the study is delineated in Figure 4.1.

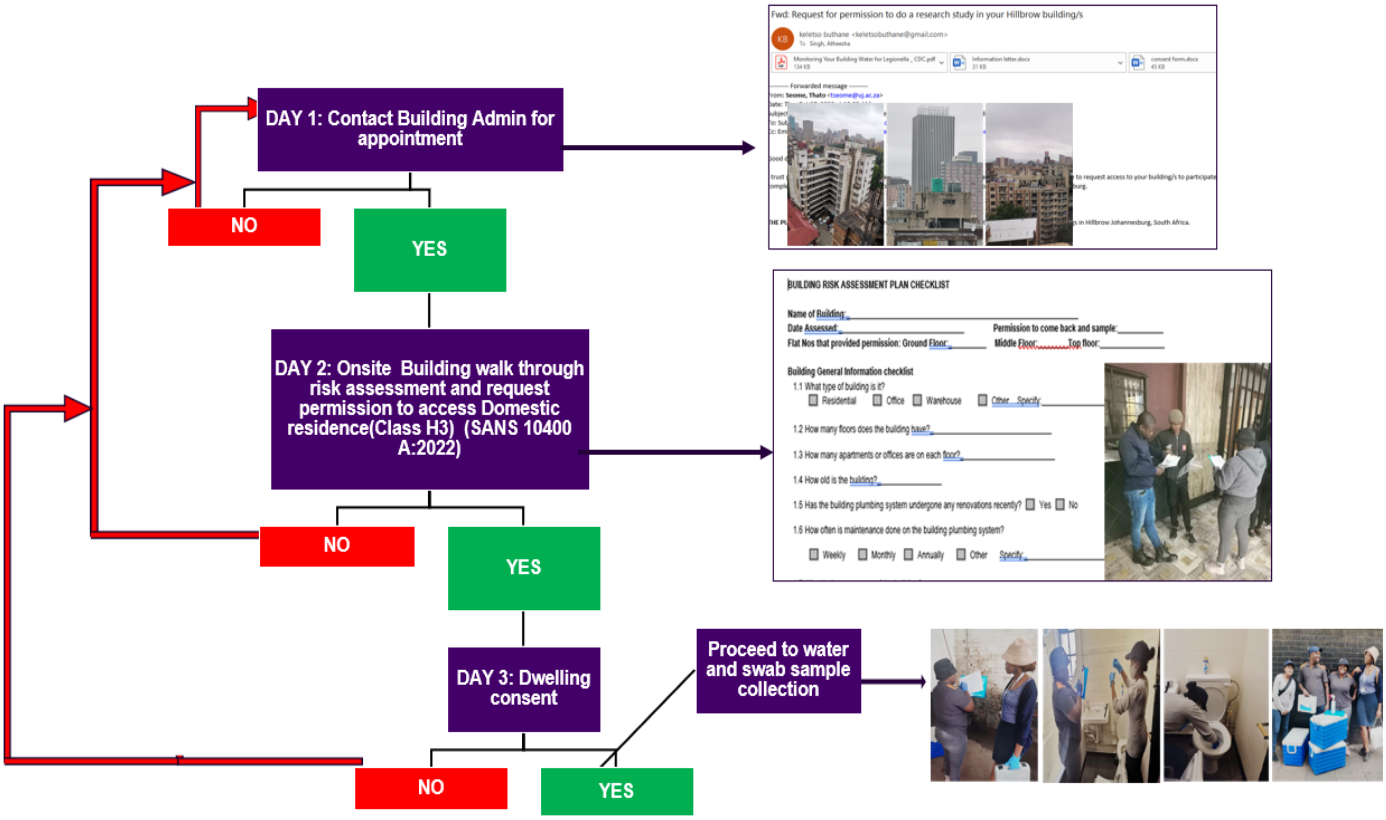


Figure 4-1. Building assessment and sampling strategy

4.2 SAMPLE COLLECTION AND INFIELD ANALYSIS

At least six sampling sessions were done in Hillbrow during October 2022 - April 2023, and two sampling sessions were conducted in Zandspruit, Melusi and Atteridgeville. In total, 15 high-rise buildings were accessed in Hillbrow, 30 household buildings were accessed in Zandspruit, Melusi and Atteridgeville and four high-rise buildings and 10 household buildings were accessed from the Vhembe region. Samples were collected from the source of water, from the top, middle, and bottom (ground) floors of apartments in each high-rise building and from main water and stored water sources in single-floor households. Samples of hot and cold water were collected (2 L) from building inlets, showerheads, faucets, geysers, boilers and storage water tanks. Biofilm samples were collected using sterile swabs in transport media using Copan's Fecal Swab Cary-Blair Collection and Transport System (Copan Diagnostics, Inc., Murrieta, CA, USA) by swabbing the inside surfaces of the tap (faucet), showerhead, and toilet bowl. For the collection of biofilm samples, the (boiler/ geyser) water faucet was opened for a few seconds to moisten the pipe, and then closed and a

sampling swab was inserted deep into the faucet/pipe beyond the bend to swab the inside surface. The swab was placed back into the transport medium and stored on ice. After collecting the biofilm swab, the water faucet was turned on until the water was warm but not hot to obtain water currently in the piping behind the fixture, along with any material shed from the “biofilm”. Water (2L) from the faucet was then collected into pre-sterilized 5L sampling bottles containing a volume of 1 mL of 0.1N sodium thiosulfate solution to neutralize residual chlorine.

A total of 131 households were selected using convenience sampling: Hillbrow ((Buildings (n=15), water samples (n=27), Vhembe ((Buildings (n=14), water samples (n=40) biofilm samples (n=54)), Zandspruit ((households (n=30), water samples (n=31), biofilm samples (n=62)), Melusi ((households (n=36), water samples (n=36), biofilm samples (n=72)), and Atteridgeville ((households (n=36), water samples (n=40), biofilm samples (n=72)). Samples were processed immediately upon arrival in the laboratory for Legiolert™ (IDEXX) and Colilert (IDEXX) quantitrays and within 24- 48 h after arrival for the other methods, with storage at 4°C until analysis.

Figure 4-2 shows the sample collection and processing flow diagram. The outlined sampling approach was followed for all samples collected from Hillbrow, Vhembe, Zandspruit, Atteridgeville and Melusi from October 2022 to July 2024. In-field water testing analysis according to LeChevallier (2019) included testing the temperature, pH, total dissolved solids (TDS) and conductivity of the water using a portable Combo Tester® (Hanna, SA). Residual chlorine was also measured onsite using a chlorine photometer (Hanna, SA), according to the manufacturer's instructions. Turbidity measurements were taken at the laboratory using the TN100 EUTECH turbidimeter (Oakton DW-35635-00).

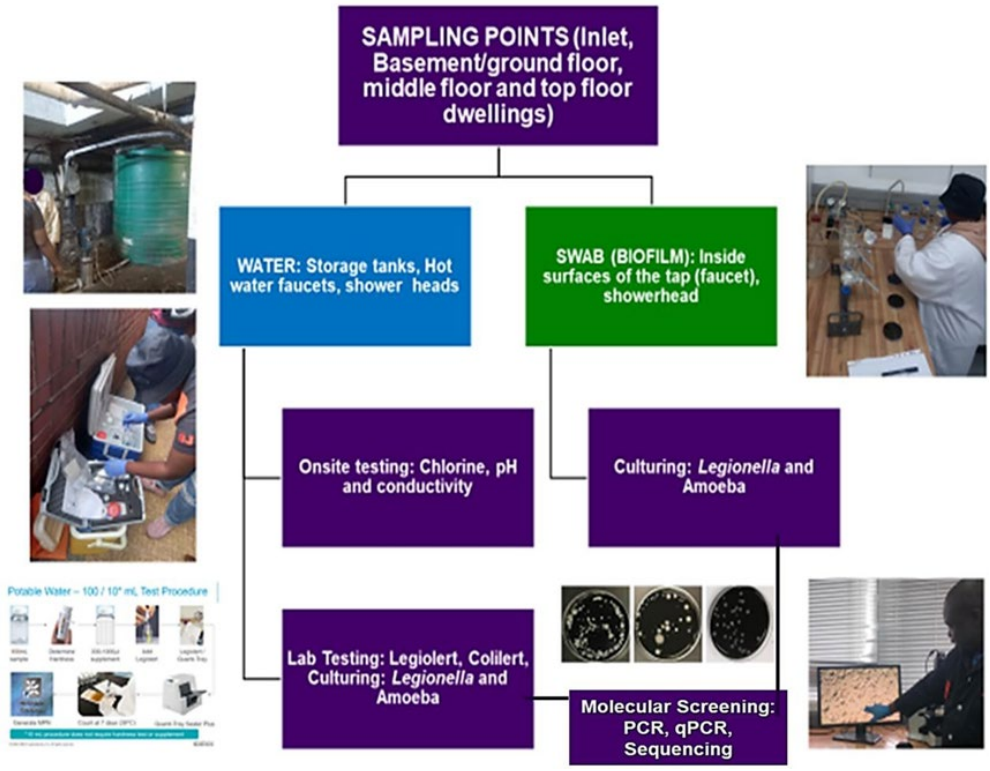


Figure 4-2. Sample collection and processing flow diagram

4.3 MICROBIOLOGICAL ANALYSIS

4.3.1 Amoeba-associated *Legionella* isolation

The amoebal enrichment technique for the isolation of intracellular *Legionella* was performed on the collected swab biofilms and 500 mL water samples according to Bartie et al. (2016) from Thomas et al. (2006) and Lamoth and Greub (2010). The enrichment techniques include seeding experiments and microscopy. Briefly, for each water sample, two filtrations of 500 mL were poured through cellulose nitrate membranes. The two membranes were placed on NNA plates (Non-Nutrient Agar) (Oxoid, UK) and overlaid with a suspension of heat-killed *Escherichia coli (E. coli*). Swabs were vortexed at maximum speed for 30 s in 10 mL Page's amoebal saline (PAS) buffer (Oxoid, UK) in individual sterile tubes. For each swab sample, two filtrations of 5 mL were performed and these suspensions were concentrated and inoculated on NNA plates as per the water samples. The plates were incubated at 32°C and examined for the appearance of amoeba, trophozoites and/or cysts every 3 days for 2 weeks using a light microscope (Olympus, Japan) with a 10x and 40x objective lens. Samples were considered negative if no amoebal development was observed after 3 weeks. Plates positive for amoebae were sub-cultured by cutting small agar plugs, placing them upside down onto fresh NNA spread *E. coli* plates and incubating as before. This was done three to four times to purify amoebae cells. Once purified, the amoebae cells were harvested by gently scraping the agar surface and resuspending in Page's amoebal saline buffer. The presumptive intracellular bacteria were released from the amoeba by passing the suspension through a 25-gauge needle syringe three times. A volume of 100 µL was inoculated onto Buffered Charcoal Yeast Extract agar (BCYE)(Oxoid, UK) culture media for *Legionella* and incubated aerobically at 37°C for up to 14 days. A water tray was placed beside the samples to ensure a humid growth environment. Typical *Legionella* colonies were tested for cysteine dependency (CD), by inoculation and incubation of BCYE and nutrient agar plates until growth was observed on the BCYE agar. Colonies growing on BCYE, but not on nutrient agar, were regarded as cysteine dependent (CD+). The CD+ colonies were reported as presumptive *Legionella*. After incubation, the presumptive *Legionella* cells were inoculated into Lenoxx broth (10g/L Tryptone, 5g/L Yeast extract, 5g/L sodium chloride, autoclaved at 121°C for 15 minutes, BCYE supplement) and incubated for 48 hours at 37°C. The broth was used for DNA extraction.

4.3.2 Isolation of pathogenic bacteria associated with Amoeba

The amoebal lysate was also cultured onto selective media specific for the detection of presumptive pathogens and incubated at 37°C for up to 24 hours as follows: *Escherichia coli* was cultured on Brilliance *E. coli* selective agar (Oxoid, UK), *Enterobacter* spp. were cultured on MacConkey agar (Oxoid, UK), *Staphylococcus aureus* was cultured on Mannitol salt agar with 7.5% NaCl (MSA agar) (Oxoid, UK) *Klebsiella* spp. were cultured on *Klebsiella* media + Klebsiella Selective Supplement (Oxoid, UK), *Acinetobacter baumannii* on Campylobacter media (Oxoid, UK), *Pseudomonas aeruginosa* on Cetrimide agar (Oxoid, UK), and *Enterococcus* spp. on Slanets and Bartley media (Oxoid, UK). After isolation, each presumptive isolate was sub-cultured onto nutrient agar and incubated at 37°C for up to 24 hours. The presumptive pure culture was then inoculated onto LB broth and incubated at 37°C. The broth was used for identification and antibiotic susceptibility testing using the VITEK® 2 compact system. The broth was then preserved in 50% (v/v) glycerol and stored for long term at -80°C.

4.3.3 Detecting *Legionella pneumophila* according to SANS 11731:2017

Testing for culturable *Legionella pneumophila* was done using the South African National Standard (SANS) 11731:2017 method. From the samples collected, 100 mL of water sample was filtered onto a 0.45 µM nitrocellulose membrane, after which 30 mL of acid buffer was added directly onto the filtration membrane and left to stand for 5 minutes, before being filtered out. Washing out the acid buffer was done with 20 mL Page's saline and filtered out. The filter membrane was then placed onto Buffered Charcoal Yeast Extract agar (BCYE)(Oxoid, UK) (grids facing up) and incubated at 37°C for up to 10 days. A water tray was placed beside the samples to ensure a humid growth environment. Presumptive colonies were sub-cultured onto a clean BCYE agar plate and on Nutrient agar to check for cysteine dependency. Colonies growing on BCYE agar but

not on nutrient agar were considered positive for *Legionella*. After incubation, the presumptive *Legionella* were inoculated into Lenoxx broth and incubated for 48 hours at 37°C. The broth was used for DNA extraction.

4.3.4 Detecting *Legionella* with IDEXX Legiolert™

Legionella was detected using the Legiolert™ Quanti-tray system according to the manufacturer's instructions (IDEXX, bioMérieux, Inc.). Discrete sampling was performed wherein 100 mL of each 1L collected potable water sample was aliquoted into a sterile anti-foam sampling bottle and brought to room temperature (20°C). The hardness of the water sample was first tested using a dip-strip (Aquadur® hardness test strip) supplied with a Legiolert™ system (IDEXX). Water hardness was detected as follows: if the reading was 0-2 pads, the sample was deemed low (positive), and if it was 2-3 pads, then the sample was marked as having a high hardness. Supplements were added to the water sample according to the hardness reading determined and shaken until everything had dissolved for 2-3 min: for samples with low hardness, 0.33 mL of supplement was added and for samples with high hardness, 1.0 mL of supplement was added. The contents of the Legiolert™ ® blister pack were added to the IDEXX Quanti-Tray and sealed using IDEXX Quanti-Tray sealer plus, which can accommodate the Legiolert™ tray. The Quanti-trays were incubated at 39°C for 7-10 days in a sterile plastic bag to prevent moisture loss.

Positive and negative controls were included for all the sample sets. The positive control was made up with the *Legionella pneumophila* control strain (ATTC 33152). The negative control was made up of autoclaved distilled water and *Enterococcus faecium*. To create a humid environment and prevent trays from drying, cotton wool soaked in distilled water was placed in the plastic containing the IDEXX Quanti-Trays, and a beaker of distilled water was placed in the incubator. Positive results assumed that actively growing strains of *Legionella pneumophila* will use the added substrate to produce a brown colour and/or microbial growth (as evidenced by turbidity) relative to a negative control. The number of tray wells that were positive was counted for using the most probable number (MPN) count, which was then read from the appropriate MPN table supplied with the system.

4.3.4.1 Legionella validation experiment for Legiolert™

A frozen microbank of *Legionella pneumophila* ATCC 33152 control strain was sub-cultured onto Buffered Charcoal Yeast Extract (BCYE) agar and incubated at 37°C for two to four days. The colonies were suspended in 0.85% Phosphate Buffered Saline (PBS) as an initial suspension, with 0.1 optical density at 600 nm, which equates to 1.0×10^8 colony forming units (cfu)/mL. A series of dilutions was prepared with the PBS in triplicate. Three (3) sets of experiments were performed, where serial dilutions were prepared from 1.0×10^7 cfu/mL to 1.0×10^2 cfu/mL. This involved adding 10 mL of the initial suspension (1.0×10^8 cfu/mL) to 90 mL of PBS to prepare a 1.0×10^7 cfu/mL. From this, 10mL of the 1.0×10^7 cfu/mL solution was added to 90 mL of PBS to dilute this to 1.0×10^6 cfu/mL. This dilution process was repeated until a dilution of 1.0×10^2 cfu/mL was prepared. The full volume for each dilution was added to a 100 mL anti-foam bottle and Legiolert™ blister pack was added, after mixing, the contents were added onto Legiolert™ Quanti-Tray and sealed with a Quanti-Tray sealer. The Legiolert™ trays were incubated at 39°C for 7 days and results were read and recorded. The repeatability (closeness of agreement between test results by the same method) of the IDEXX Legiolert™ method was determined by calculating the standard deviation (variation from the mean) of the three sets done. The following equation was used:

$$\mathrm{SD} = \sqrt{\frac{\sum(X - \dot{x})^2}{N}}$$

where, $\sum$= the sum of, X= each value in the sample set (MPN/100mL), $\dot{x}$= mean of the sample set, N= the total number of values. Repeatability is expressed as a percentage coefficient of variance (SD/mean × 100) and the method is verified as repeatable when the %CV is ≤10 (SANAS TR 28-03).

4.3.5 Serological determination

The serogroup of each presumptive *Legionella* colony isolated from Legiolert™ and amoeba was tested using the Oxoid *Legionella* Latex Test kit according to the manufacturer's instructions. The Oxoid Legionella Latex Test kit is an immunological assay used for the rapid identification of *Legionella pneumophila* from cultured isolates, particularly *L. pneumophila serogroup* 1 and sometimes other serogroups. It operates based on latex agglutination, where latex particles are coated with antibodies specific to *Legionella* antigens. When mixed with a bacterial culture containing *L. pneumophila*, the particles clump together (agglutinate) if the corresponding antigen is present. The positive control was made up with the *Legionella pneumophila* control strain (ATTC 33152). The negative control was made up of autoclaved distilled water and *Enterococcus faecium*. A positive result was recorded if agglutination was observed.

4.3.6 Microbial controls

The type strains *Acanthamoeba castellanii* ATCC 30010, *Legionella pneumophila* ATCC 33152 (Serogroup 1), *Escherichia coli* ATCC 25922 and *Saccharomyces cerevisiae* ATCC 9763 were obtained from the American Type Culture Collection and used as positive controls for experimental analysis (Bartie et al. 2016).

4.3.7 Detection of *Escherichia coli* and total coliforms by IDEXX Colilert® Quanti-Tray™

The IDEXX Colilert® Quanti-Tray™ most probable method was used on the same water samples tested on Legiolert™. The samples were inoculated and enumerated in an IDEXX Colilert® Quanti-Tray®/2000 according to the manufacturer's instructions. All sample lots included positive and negative controls to confirm sterility and product performance. These included a coliform positive control (*Klebsiella pneumonia* ATCC No. 31488), a coliform negative control (*Pseudomonas aeruginosa* ATCC No. 27853), an *E. coli* positive control (*E. coli* ATCC No. 25922), and autoclaved distilled water as a product control. The yellow wells were identified as positive for total coliforms and all fluorescent yellow (and white) wells under UV light were identified as positive for the presence of *E. coli.*

4.4 MOLECULAR DETECTION OF *LEGIONELLA* SPECIES

4.4.1 DNA isolation from amoeba and *Legionella*-positive samples

Both *Legionella* (from Legiolert™ and colonies from SANS culturing) and Amoebal DNA was isolated from lysed amoeba-positive cells and *Legionella*-positive samples, respectively, using the Guanidium Thiocyanate method as described by Omar and Barnard (2014). Briefly, 2 mL of the broth was centrifuged for 2 min at 13000 rpm (Vacutec Neofuge 15R) to concentrate the bacterial cells. The supernatant was discarded. To the pellet, 700 µL of lysis buffer was added and the reaction was incubated at 70°C for 10 min. Then, 250 µL of 100% ethanol was added to each lysate and incubated at 56°C for 10 min. To each preparation, 50 µL of celite was added and incubated at room temperature for 10 min. The mixture was transferred (in 2 steps) to a spin column (prepared in-house) and centrifuged. The column was washed with 800 µL of wash buffer in 2 steps, followed by 800 µL of 70% (v/v) ethanol in 2 steps. Thereafter, 100 µL of PCR grade water was added directly to the column filter and incubated at 56°C for 2 min. The columns were centrifuged as described by Omar and Barnard (2014) using the Vacutec Neofuge 15R, and the DNA was eluted, and concentration was measured using the Nanodrop instrument (Jenway Genova Nano, USA). The DNA was stored at -20°C for downstream molecular analysis.

4.4.2 Polymerase chain reaction (PCR) analysis for *Legionella*

Legionella spp. and *L. pneumophila* were detected by amplification of the extracted DNA using primer pairs targeting the specific regions of the 16S rDNA and the *mip* gene for *L. pneumophila*. Details of these primers are supplied in Table 4.1. All samples were tested for the presence of *Legionella* spp and *L. pneumophila*. Each PCR amplification was performed in a 20 µL reaction mixture containing 2 µL of 10 x PCR Buffer

(QIAGEN® Hotstart *Taq* DNA polymerase and 15 mM $MgCl_2$), 0.6 µL (20 mM each) dNTP mix, 2 µL (10 µM each forward and reverse primer mix), 2 µL of template DNA, 0.1 µL HotStar *Taq* DNA polymerase, 2 µL $MgCl_2$ and 11.3 µL PCR grade water. The PCR was performed in a BIORAD® T100™ thermal Mycycler with the following conditions for *Legionella* species: an initial enzyme activation of 95°C for 15 minutes, followed by 30 cycles of denaturation at 95°C for 1 minutes, annealing at 55°C for 90 sec and elongation at 72°C for 1 min with final extension of 72°C for 10 minutes. The cycling conditions for *L. pneumophila* were as follows: 95°C for 15 minutes, followed by 34 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 45 sec and elongation at 72°C for 5 minutes with a final extension of 72°C for 5 min. All amplicons were separated on a 2% (w/v) agarose gel.

Table 4.1. Primers used for the detection of *Legionella* spp. and *L. pneumpohila*

Primer	Sequence	Size (bp)	Reference
Leg 225 (F)	5'- AAG ATT AGC CTG CGT CCG AT -3'	654	**Rafiee *et al*. (2014)**
Leg 858 (R)	5'- GTC AAC TTA TCG CGT TTG CT -3'		
***L. pneumophila* (F)**	5′- CCGATGCCACATCATTAGC -3′	150	**Schwake *et al*. (2015)**
***L. pneumophila* (R)**	5′- CCAATTGAGCGCCACTCATAG -3′		

4.4.3 Polymerase chain reaction (PCR) analysis for Amoeba

Acanthamoeba PCR involved amplification of the extracted DNA using primer sequences CFLA(F) CAGGTTAAGGTCTCGTTCGTTAAC and CFLA(R) CAGGTTAAGGTCTCGTTCGTTAAC (Coskun et al., 2013). The reaction mixture was as follows: Each PCR reaction was performed in a 20 µL reaction mixture volume containing 2 µL of the 10 x PCR Buffer, 0.1 µL QAGEN® Hotstart *Taq* DNA polymerase, 1 µL 5 x Q-solution, 0.6 µL (10 mM each) dNTP mix, 1 µL (10 µM) each forward and reverse primer, 4 µL of template DNA, 2 µL (25 mM) $MgCl_2$, and 8.3 µL PCR grade water.

4.4.3.1 Confirming PCR through Spiking Experiments

From the PCR results above, if the samples were found to be negative for *Legionella*, the samples were then spiked with a positive control strain DNA (*Legionella pneumophila* ATTC:33152) to determine the presence or absence of an inhibitor and to test the accuracy of the results.

4.5 REAL-TIME QUANTITATIVE PCR (QPCR)

Real-Time quantitative PCR (qPCR) was carried out to detect *Legionella pneumophila* from all the Legiolert™ and Amoeba-associated *Legionella*-positive samples using the primers in Table 4.2. The amplification was performed in 20 µL reaction mixture containing 2 µL of the 10 x PCR Buffer (QIAGEN® Hotstart *Taq* DNA polymerase and 15 mM $MgCl_2$), 0.6 µL (20 mM each) dNTP mix, 2 µL (10 µM Lpneu F, Lpneu R, LpneuP probe mix), 2 µL of template DNA, 0.1 µL HotStar *Taq* DNA polymerase, 2 µL $MgCl_2$ and 11.3 µL PCR grade water. The reaction mixture was amplified in a Rotor-Gene Q (QIAGEN) with the following cycling conditions: *Taq* polymerase activation at 95°C for 15 minutes, followed by 43 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 60 sec.

Table 4.2. Strain and primer sequence for *L. pneumophila* detection used in qPCR

Strain	Primer	Sequence
L. pneumophila	LpneuF	**F- 5′-CCGATGCCACATCATTAGC-3′**
	Lpneu R	**R- 5′-CCAATTGAGCGCCACTCATAG -3′**
	LpneuP (Probe)	**5'-6-carboxyfluorescein[FAM]-TGCCTTTAGCCATTGCTTCCG-BHQ1–3'.**

4.6 GEL ELECTROPHORESIS

The PCR products were separated on a 2% (w/v) horizontal agarose gel slab (Celtic Molecular, UK) containing ethidium bromide (0.5 µg/mL) using TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.3). Electrophoresis was conducted at 80 volts for 45-60 minutes. All gels were viewed using a UV transilluminator and digitally photographed using the Omega Fluor™ Gel Documentation System. The relevant sizes of the DNA fragments were estimated by comparing their electrophoretic mobility to that of a standard 1 kbp marker (Fermentas®, USA) that was run with the samples on each gel.

4.7 VITEK®2 PATHOGENIC BACTERIAL IDENTIFICATION ANALYSIS AND ANTIMICROBIAL PROFILING

The VITEK®2 (bioMérieux Inc.) compact system was used to confirm and identify positive pathogenic bacterial colonies isolated from the amoebal lysates. Gram staining using standard Gram staining reagents (crystal violet, Gram's iodine, 95% ethyl alcohol and safranin) (Diagnostic Media Products, NHLS, South Africa) was used to confirm the Gram reaction of each isolate before identification with the VITEK® 2 System. After the Gram reaction of the isolates was confirmed, the isolates were inoculated onto the VITEK® 2 identification cards. Briefly, the isolates were suspended in 0.45% saline solution (bioMérieux Inc.) to obtain the specified McFarland standard of 0.5 to 0.63, according to the manufacturer's instructions. Gram-positive (GP) cocci and Gram-negative (GN) bacilli were then identified using the specific VITEK® 2 identification cards (GP and GN). Antimicrobial Susceptibility Tests (AST) of the isolated pathogens were done using the consumables prescribed by the manufacturer. Antimicrobial susceptibility testing (AST) cards were used as follows: AST-N256 for Gram-negative bacilli (bioMérieux Inc.) and AST-P645 for Gram-positive cocci (bioMérieux Inc.). The AES (Advanced Expert System) software automatically analysed the data, correlating identification with sensitivity and determining the presence of resistance mechanisms, which is based on Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (CLSI, 2021; EUCAST, 2021). Appropriate positive and negative controls were integrated into the AST cards to ensure the reliability of the test results.

4.8 SEQUENCING OF *LEGIONELLA* SPECIES

Sequencing of PCR-confirmed *Legionella pneumophila* isolates was done for all sites (outsourced to Inqaba Biotech). Only two single isolates were obtained for *Legionella pneumophila* after culturing on BCYE, however, these bacteria did not have consistent growth, which prevented whole genome sequencing from being conducted. It was then decided that one sample from each site with the highest Legiolert™ MPN/100mL value would be sent for Next Generation sequencing (NGS). These samples were sent to Inqaba Biotech for sequencing using the PacBio's Sequel platform (long read chemistry), allowing for gapless genome assembly using 500 MB of data, so that it gives a good coverage. Results for the NGS will be published in research articles and are not part of this report.

4.9 RISK ASSESSMENT

Potential health risks associated with exposure to *Legionella* from water samples were determined using a conceptual model for risk analysis. This assists in identifying which water samples pose a greater health risk following exposure to aerosolized *Legionella pneumophila*. The method of Quantitative Microbial Risk Assessment (QMRA) evaluation is based on the average *Legionella* concentrations in water and data found in the literature (Denissen et al., 2023). To assess the potential risk of *Legionella* aerosolization in Hillbrow and Vhembe, a structured four-step approach was followed, which included:

Hazard Identification: Water samples from both regions were collected and analysed for the presence of *Legionella pneumophila,* which formed the empirical basis for further risk analysis.

Exposure Assessment: Exposure scenarios, such as showering/ bathing by pouring water and toilet flushing, were modelled to estimate the dose of *Legionella* inhaled by individuals. Inhalation and ingestion rates were derived from existing literature for each activity.

Dose-Response Assessment: The beta-Poisson model was applied to estimate the probability of infection based on pathogen doses in the water.

Risk Characterization: The probability of infection for each exposure scenario was calculated using Monte Carlo simulations in RStudio, with 500,000 iterations per scenario. This approach accounted for uncertainties in dose, exposure frequency, and individual variability, producing robust risk estimates.

Key variables considered in the analysis included *Legionella* concentrations (measured in MPN/100 mL), inhalation rates, exposure duration, and the number of exposure events per year.

4.10 DATA MANAGEMENT PLAN

The data acquired from this study is stored in a secure open-access (OA) data repository courtesy of the University of Johannesburg, taking into consideration confidentiality and ethics. This repository provides a confidential data cloud storage and collects raw data for observation, generation, and creation, while still preserving the data. This system benefits researchers since it ensures safe and protected electronic cloud data storage, with a transparent accreditation to raw data publication quality. This OA collection in the repository forms a complete record of the research output from the study, which is openly accessible to grant funders, the public domain, and publishers.

4.11 VALIDITY AND RELIABILITY

This study follows the SANS 241 guidelines for drinking water quality as well as the SANS 11731:2017 - Water quality — Enumeration of *Legionella* standard for water quality-detection and enumeration of *Legionella*, in conjunction with the IDEXX Legiolert™ method. All products, laboratory consumables and chemicals purchased for this study were procured from commercial laboratory and pharmaceutical suppliers, who supply proficient high-quality certificates to ensure results repeatability and validity. All appropriate controls are included in this study to ensure the reliability and trustworthiness of the results reported.

4.12 DATA ANALYSIS

The descriptive statistics of the overall sample will be determined using SPSS v28. To analyse the differences in *Legionella* contamination, a one-way analysis of variance (ANOVA) is performed (results are considered statistically significant when $p < 0.05$). Data are graphed and imaged using GraphPad Prism v10 and Biorender® software, respectively.

CHAPTER 5: ISOLATION AND DETECTION OF LEGIONELLA PNEUMOPHILA IN SAMPLES

5.1 INTRODUCTION

This chapter presents the results of the study conducted across multiple sites to assess the prevalence of *Legionella pneumophila* in the samples. For the Hillbrow study, water samples were collected from a single apartment on three different floors in each building: the top floor, the middle floor, and the ground or basement level (Figure 5.1). This approach ensured a representative assessment of water quality variations across vertical distribution within each building. Additionally, where the water inlet supply was available from the storage tank, a water sample was also collected. Water samples were mainly collected from water faucets (taps) in the bathroom, either from the showerhead or the basin. In the Vhembe region overall, 85 water samples were collected from 14 buildings, which include 4 student residences and 10 households. A summary of the collected water sample types is shown in Table 5.1.

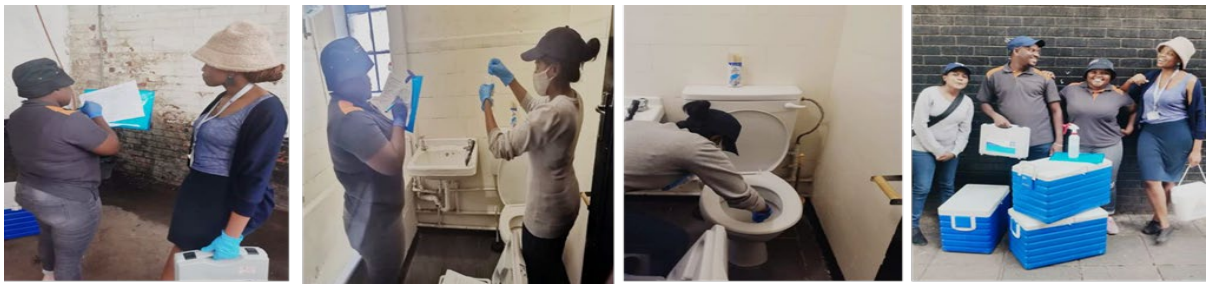


Figure 5-1. Research team sampling in a residential apartment

Table 5.1. Water Samples Collected per Site

	Cold Tap	Geyser/Boiler	Stored Water
Vhembe	45	27	13
Hillbrow	30	36	4
Atteridgeville	22	18	0
Zandspruit	26	0	5
Melusi	15	0	20

5.2 GENERAL WATER QUALITY PARAMETERS

5.2.1 Physicochemical parameters

The general quality of the water samples was determined by the odour, taste and any observed discoloration. All the water samples (100%) were odourless and presented no discernible discoloration or taste. The temperature, pH, total dissolved solids, electrical conductivity and turbidity of each sample were tested, and the results were recorded. All collected samples were then stored in cooler boxes with ice. As shown in Figure 5.2, the water quality across the different study sites of Vhembe, Hillbrow, Atteridgeville, Zandspruit, and Melusi was assessed using various parameters, including pH, temperature, conductivity, turbidity, and total dissolved solids (TDS). Each site presented distinct characteristics that reflect the environmental conditions and potential water quality issues unique to those locations. For all sites, the total chlorine levels in the water samples ranged from 0 to 3.22 mg/L, while free chlorine levels ranged from 0 to 3.17 mg/L.

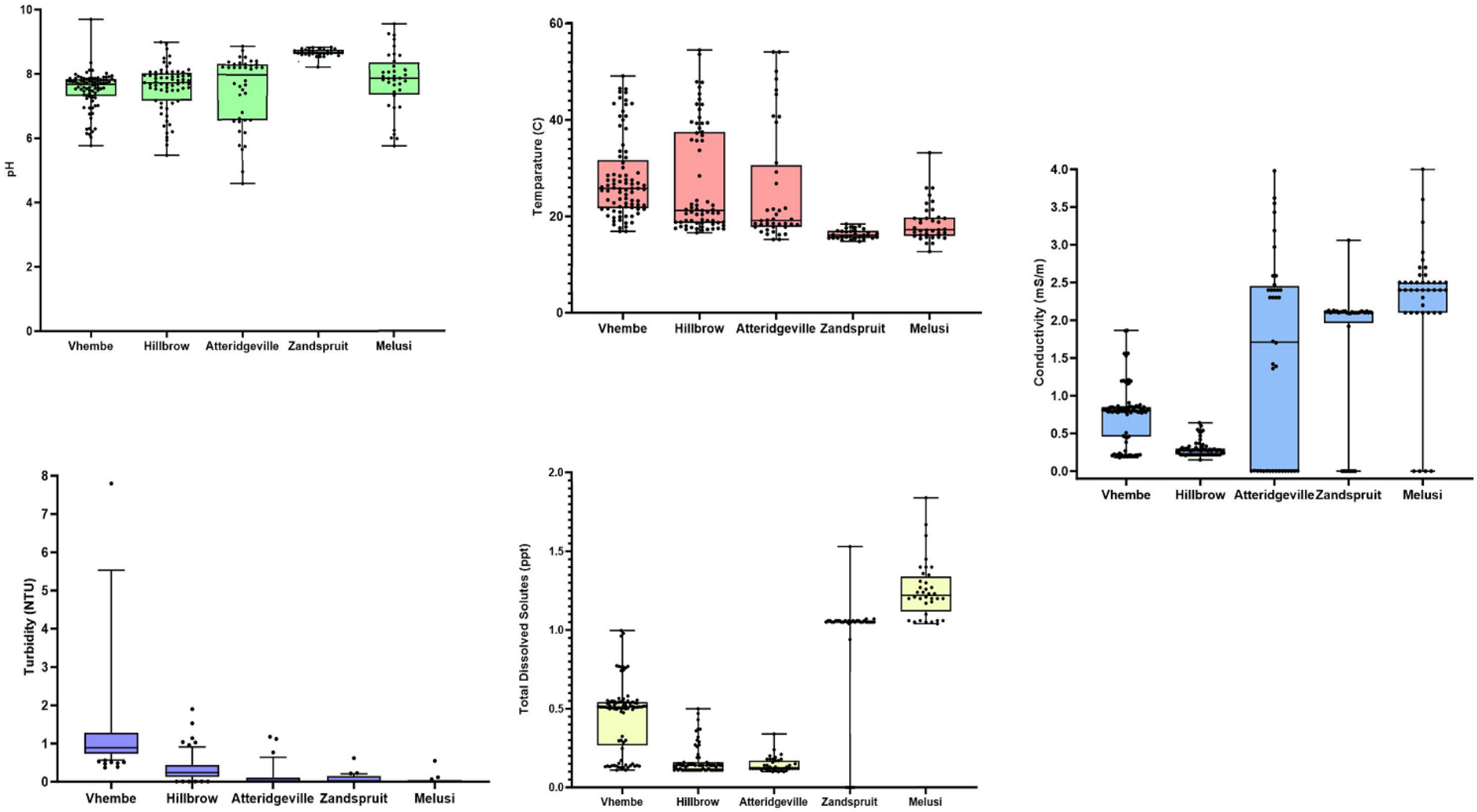


Figure 5-2. Summary of all physicochemical parameters (pH, conductivity, total dissolved solutes, temperature and turbidity) tested for each sample from all buildings and sites sampled.

At Vhembe, the pH values ranged from 6.03 to 9.70, indicating that the water varied from slightly acidic to moderately alkaline. Such variability in pH suggests fluctuations in the chemical compounds dissolved in the water, which could be due to natural factors or pollution. The water temperature at Vhembe also varied widely, from as low as 16.9°C to as high as 49.1°C, which might reflect changes in water heating. Conductivity readings fluctuated, with values ranging from 0.18 to 1.861 µS/cm, suggesting varying levels of dissolved ions. Additionally, turbidity at Vhembe was highly variable, with some periods reaching as high as 28.9 NTU, indicating potential episodes of contamination. The TDS levels ranged from 0.206 to 1.861 ppt, further highlighting the site's fluctuating water quality.

In contrast, Hillbrow showed a more stable pH range, mostly between 7.0 and 8.5, although there were instances of more acidic conditions with pH as low as 5.47. The temperature also ranged from 16.6°C to 54.5°C. Conductivity at Hillbrow was consistently low, generally around 0.22 µS/cm, indicating low levels of dissolved ions. The turbidity ranged from 0.01 to 1.9 NTU, showing mostly low turbidity. TDS values were similarly low, with minor fluctuations, indicating stable but relatively low levels of dissolved solids.

At Atteridgeville, the pH ranged from 4.59 to 8.86, showing occasional acidic conditions that could be of concern. Temperature readings varied, with values ranging from 15.2°C to 54.1°C. Conductivity data presented some periods showing very low values and others higher, up to 4.34 µS/cm, indicating fluctuating ion concentrations. Turbidity was generally low, with most readings at or near 0 NTU, except for a few outliers. The TDS values at this site were also low, ranging from 0.1 to 0.24 ppt, suggesting minimal dissolved solids.

Zandspruit presented water that had pH levels consistently in the alkaline range, between 6.0 and 8.78. Temperature readings were lower, ranging from 14.8°C to 18.4°C. Conductivity remained consistent, with values of 2.09 to 3.06 µS/cm, indicating moderate ion concentrations. Turbidity was low, with most readings below 0.62 NTU, reflecting clear water conditions.

TDS values were higher at Zandspruit compared to other sites, ranging from 1.05 to 1.53 ppt, indicating a higher presence of dissolved solids. Melusi showed a pH range from 5.76 to 9.56, reflecting varying water chemistry from acidic to highly alkaline conditions. The temperature varied from 12.7°C to 33.2°C, with some periods of moderate warmth. Water conductivity at Melusi was moderate, with values ranging from 2.1 to 4.0 µS/cm, suggesting a consistent presence of dissolved ions. Turbidity was generally low, similar to Zandspruit, with readings mostly at or near 0 NTU. The TDS levels were among the highest observed, ranging from 1.04 to 1.84 ppt, indicating a notable concentration of dissolved solids.

In summary, each site displayed unique water quality characteristics. Vhembe showed the most variability across all parameters, indicating potential water quality concerns. Hillbrow, while generally stable, had instances of low pH and high temperatures that could impact water quality. Atteridgeville's occasionally acidic pH and variable conductivity are points of interest, while Zandspruit and Melusi had more stable conditions, with Zandspruit showing higher TDS levels. These findings highlight the diverse environmental conditions and potential influences on water quality.

5.2.2 Microbiological indicator parameters

The occurrence of pollution indicator bacteria (total and faecal coliform) was used as a sanitary parameter for evaluating the quality of drinking water. It is known that these indicators are associated with disease-causing organisms, which are of great concern to public health (Pal, 2014; Aram et al., 2021). The Colilert Quanti-tray method was adopted to determine the presence of total coliform and *E. coli,* as is recommended by the SANS 241 document for water quality. As indicated in Figure 5.3, results for the present study are presented as the Log_{10} Most Probable Number (MPN) of *Escherichia coli* (*E. coli)* or Total Coliforms per 100 mL of water. This logarithmic scale provides a normalized way to express the bacterial concentration, enhancing comparability across different samples by reducing variability and emphasizing order-of-magnitude differences.

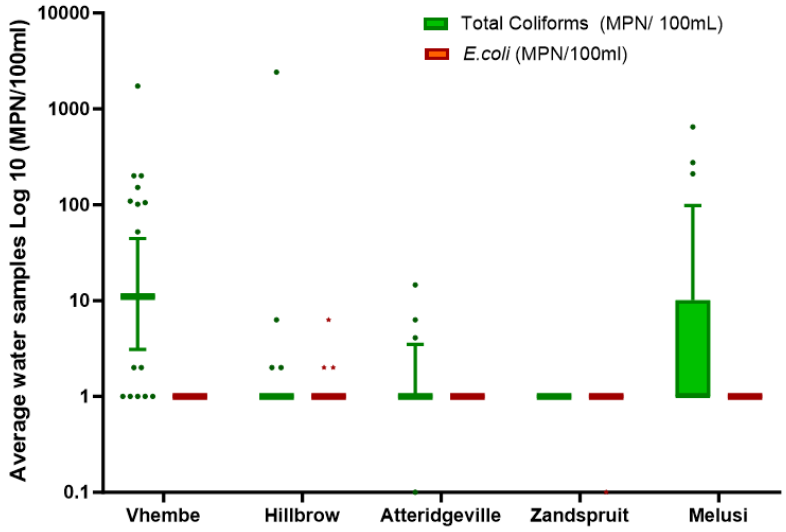


Figure 5-3. Log_{10} Most Probable Number (MPN) of total coliforms and *E. coli* per 100 mL of water

5.2.2.1 Hillbrow

From the samples tested, nearly all (87%; n=13) of the buildings had no coliform growth in their water systems, and only two (n=2) of the buildings were positive for total coliforms, while one building (n=1; 7%) was positive for faecal coliform *E. coli* in 3 of the 5 floors (60%) sampled. The level of detection was low for all samples for both coliforms and *E. coli*, except for sample PBWT01 from building HB60, which had a high level of coliforms at 2419.6 MPN/ 100mL. The general quality of the water was determined by the taste, odour and any observed discoloration in the water at tap level. All the samples (100%) were deemed odourless, having no discernible discoloration and no taste to their water.

5.2.2.2 Zandspruit

From the 30 Colilert samples tested, only two (n=2) water samples were positive for total coliforms and none were positive for *E.coli*.

5.2.2.3 Melusi

From the 36 water samples tested using the Colilert® assay, 11 water samples were positive for total coliforms and none were positive for *E. coli*.

5.2.2.4 Atteridgeville

Of the 36 Colilert samples tested, no water samples were positive for either total coliforms or *E.coli*.

5.2.2.5 Venda

For the Vhembe region, 32,94% (28/85) of the water samples had total coliforms localised to 9 of 14 buildings inspected. Of these, 28 water samples showed the presence of total coliforms, 50% (14/28) of the samples were below the standard limit (≤10 CFU/100 mL) according to SANS 241 guidelines for drinking water quality. While total coliforms themselves are not harmful, their presence suggests that the integrity of the water distribution system might be compromised. However, this result also highlights the concern about the potential

for other pathogenic organisms to enter the water supply. *E. coli* was observed in 1,17% (1/85) of samples collected from one building. However, the presence of this suggests that the water is not suitable for human consumption since they do not fall within the standard limit according to SANS 241 document of zero CFU/100mℓ.

5.3 *LEGIONELLA* ISOLATION WITH IDEXX LEGIOLERT™

The Legiolert™ test is an innovative approach to detecting *Legionella pneumophila* bacteria in water samples. This assay employs a microbial enzyme substrate process, where the reagent in the test specifically reacts with an enzyme produced by *Legionella* , leading to a colorimetric change (brown colour) that can be visually assessed. This is indicated in Figure 5.4. The result is expressed in Most Probable Number (MPN) units, which provide an estimate of the concentration of *Legionella pneumophila* bacteria per 100 mL of water.

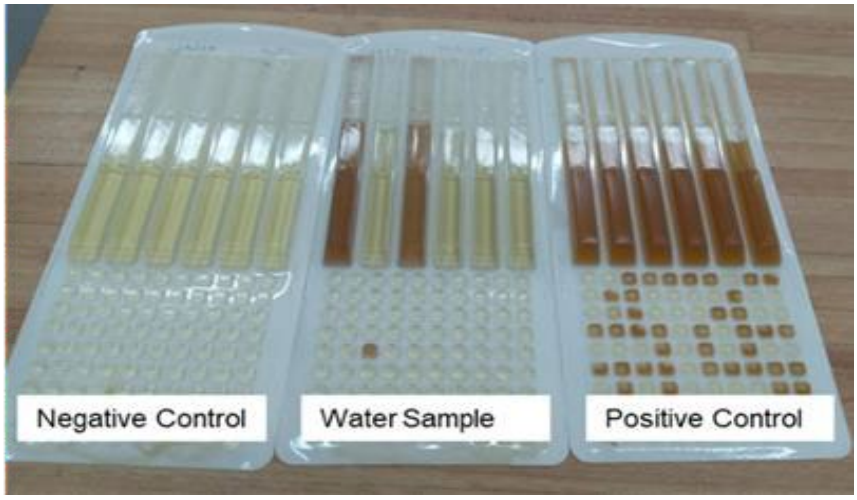


Figure 5-4. Legiolert™ Quantitrays showing positive results in comparison to a control strain in water samples.

5.3.1 Method validation

The repeatability (closeness of agreement between test results by the same method) of the IDEXX Legiolert™ method was done by calculating the standard deviation (variation from the mean) of the three (n=3) sets done

$$\mathrm{SD} = \sqrt{\frac{\sum(X-\dot{x})^2}{N}}$$

where, $\sum$= the sum of, X= each value in the sample set (MPN/100mL), $\dot{x}$= mean of the sample set, N= the total number of values.

Repeatability is expressed as percentage coefficient of variance (SD/mean × 100) and a method is verified as repeatable when the %CV is ≤10 (SANAS TR 28-03). This resulting %CV is within the range (≤10) which verifies this method as repeatable.

5.3.2 Legiolert™ results from all sites

The Legiolert™ results obtained from all sites – Hillbrow, Atteridgeville, Vhembe, Zandspruit, and Melusi – indicated the presence and distribution of *Legionella* spp. across various water sources, including cold taps, geysers/boilers, and stored water. The *Legionella* contamination levels varied significantly across the different locations and types of water sources. Zandspruit's cold tap water and Vhembe's geyser/boiler water showed higher contamination levels, with some samples reaching up to the 101-1000 MPN range. However, the majority of samples across all locations fell below the 100 MPN threshold, indicating low to moderate contamination overall. The presence of samples exceeding 2272.6 MPN in Atteridgeville cold taps and Vhembe cold taps is concerning and warrants further investigation. A summary of these results is presented in Figure 5.5.

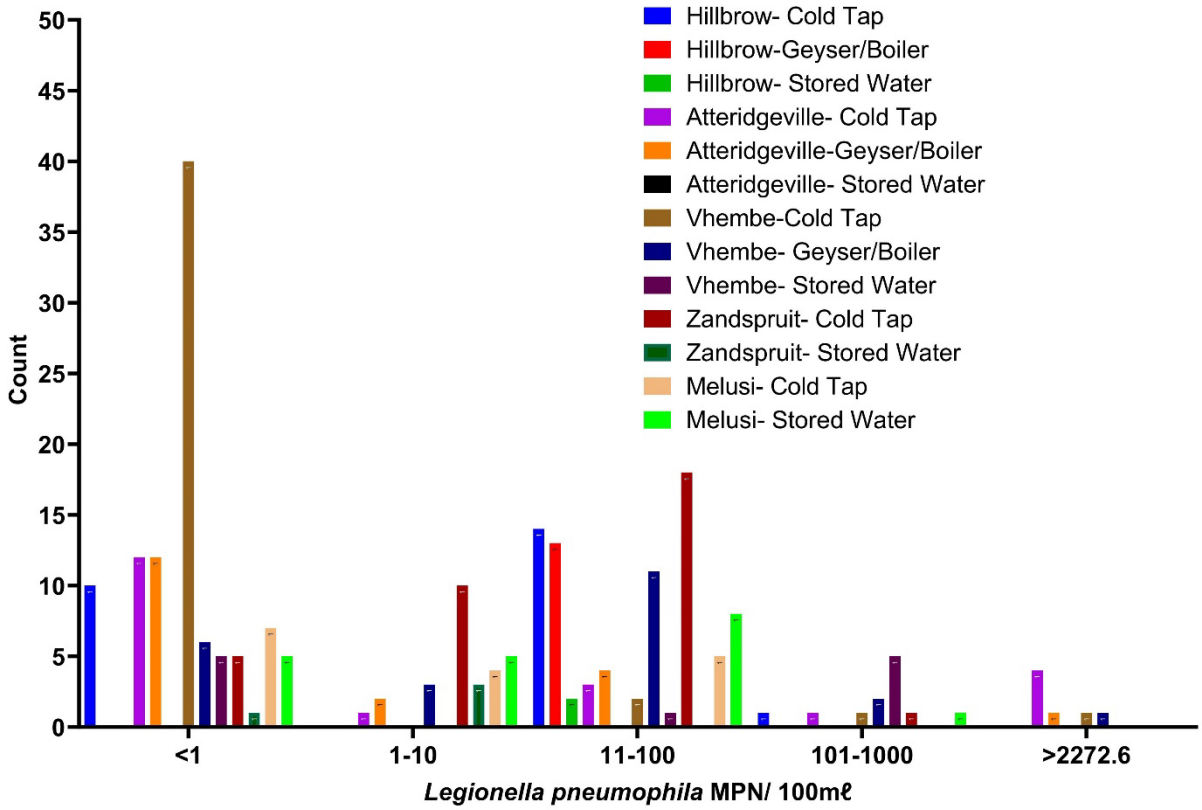


Figure 5-5. Legiolert™ results (in MPN/ 100mL) obtained for water samples from all the study sites (Hillbrow, Atteridgeville, Vhembe, Zandspruit, and Melusi).

5.3.2.1 Hillbrow:

For the cold tap, 14 samples were found within the 11-100 MPN/100mL range, with one sample in the 101-1000 MPN/100mL range. There were no samples exceeding 2272.6 MPN/100mL. For the Geyser/Boiler, 10 samples showed counts <1 MPN/100mL, and 13 samples were in the 11-100 MPN/100mL range. No significant contamination was detected in stored water.

5.3.2.2 Atteridgeville:

Cold Tap: Most samples showed low contamination, with 12 samples <1 MPN/100mL and 3 in the 11-100 MPN/100mL range. However, one sample exceeded 2272.6 MPN/100mL. Geyser/Boiler: The majority of samples (n=12) were <1 MPN/100mL, while four (n=4) samples were within the 11-100 MPN/100mL range. Stored Water: Demonstrated similar trends, with most samples <1 MPN/100mL.

5.3.2.3 Vhembe

Cold Tap: The majority of samples indicated low contamination, with most <1 MPN/100mL, although two (n=2) samples fell in the 11-100 MPN/100mL category, and 1 exceeded 2272.6 MPN/100mL.
Geyser/Boiler: This site showed relatively higher contamination, with 40 samples <1 MPN/100mL, 11 samples between 11-100 MPN/100 mL, and one sample exceeding 101-1000 MPN/100mL.
Stored Water: The results varied, with most samples being <1 MPN/100mL, and 37,03% samples reaching up to 101-1000 MPN/100mL.

5.3.2.4 Zandspruit

Cold Tap: This location showed notable contamination, with 18 samples in the 11-100 MPN/100mL range and one sample in the 101-1000 MPN/100mL range.
Stored Water: 5 samples were <1 MPN/100mL, while eight (n=8) samples were within the 11-100 MPN/100mL range, showing moderate contamination.

5.3.2.5 Melusi

Cold Tap: Seven (n=7) samples were in the 11-100 MPN/100mL range, while one (n=1) sample showed low contamination <1 MPN/100mL.
Stored Water: The results were similar, with most samples in the 11-100 MPN/100mL range, indicating a consistent level of contamination across stored water sources.

5.3.2.6 Comparative Summary

1. Hillbrow exhibited the highest levels of *L. pneumophila* contamination, particularly in cold tap and geyser/boiler water sources, with several samples exceeding 101-1000 MPN/100 mL, and one even crossing the >2272.6 MPN/100 mL threshold.
2. Atteridgeville showed a lower contamination profile, with most samples in the 1-10 and 11-100 MPN/100 mL ranges. However, the presence of samples in the 101-1000 MPN/100 mL range suggests a moderate risk of contamination.
3. Melusi had mixed contamination levels, with most samples in the lower ranges but with a few samples indicating higher contamination, especially involving stored water.
4. Vhembe demonstrated significant contamination, particularly in stored water, where some samples reached the >2272.6 MPN/100 mL range, posing a high health risk.
5. Zandspruit displayed a moderate contamination profile, with most samples in the 11-100 MPN/100 mL range, but with occasional instances of severe contamination.

5.3.3 Legionella pneumophila MPN/100mL ranges

To further analyse the data, *Legionella pneumophila* concentrations (MPN/100mL) were grouped into specific ranges, and then the instances of these concentrations were linked according to each temperature range per site (Figure 5.6), where:

- Low Concentration: 1 - 50 MPN/100mL
- Moderate Concentration: 51 - 500 MPN/100mL
- High Concentration: 501 - 1000 MPN/100mL
- Very High Concentration: >1000 MPN/100mL

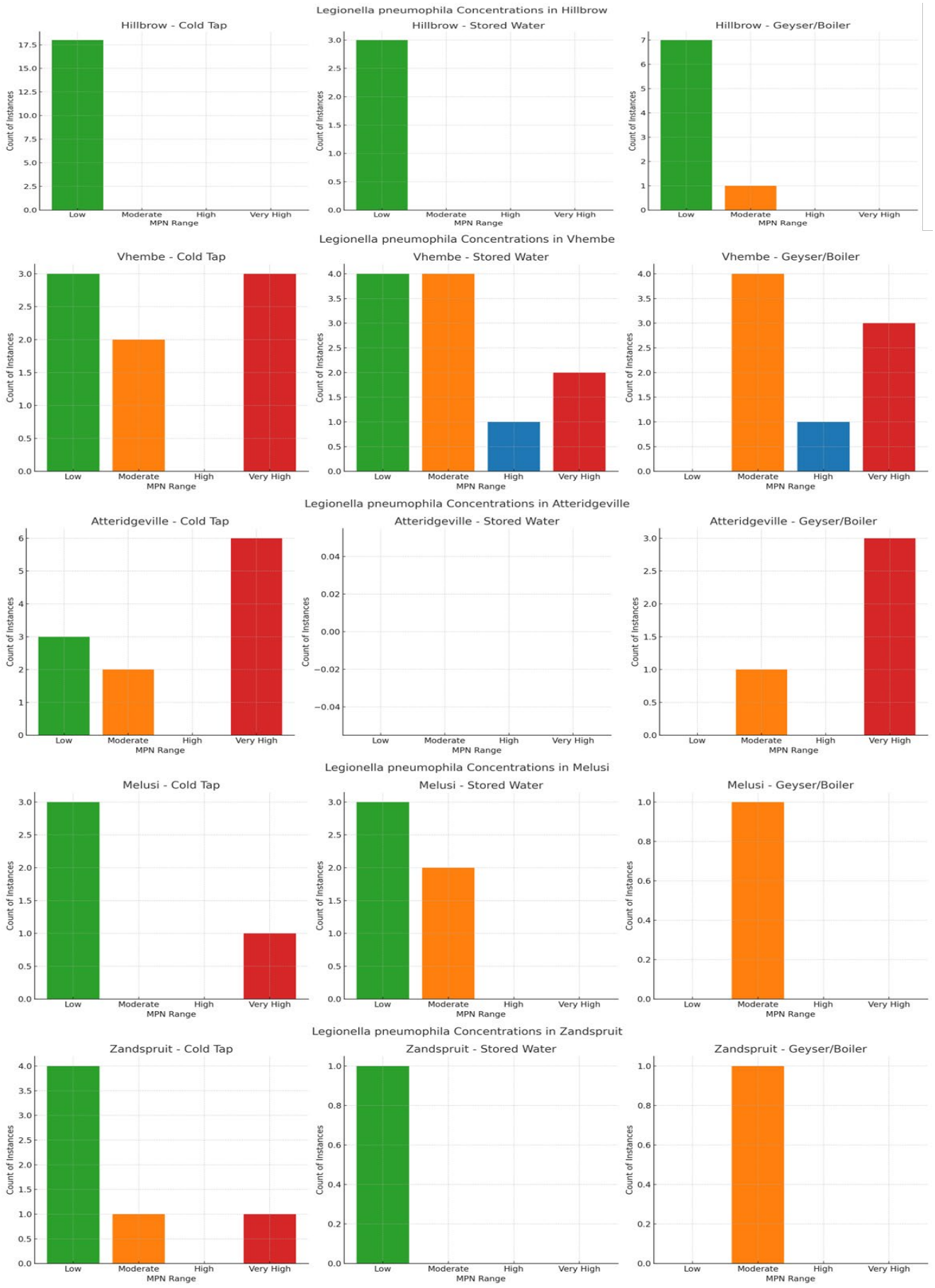


Figure 5-6. Bar graphs displaying the distribution of *Legionella pneumophila* concentrations as ranges across different water sample types for each study area.

Key Observations from Figure 5.6 are as follows:

- **Vhembe:** Stored water and water from the geyser/boiler showed a higher percentage of moderate (51-500 MPN/100mL) and high (501-1000 MPN/100mL) concentrations, with the geyser/boiler also showing a significant proportion (15.79%) in the very high range (>1000 MPN/100mL). Cold tap water has a lower overall percentage in the very high range, but still shows some presence of *L. pneumophila*.
- **Hillbrow:** Cold tap water has a high percentage (36.73%) in the low concentration range (1-50 MPN/100mL), with no significant instances in higher MPN ranges. The geyser/boiler water also showed the presence of *L. pneumophila* in the low range.
- **Atteridgeville:** Cold tap water has a notable percentage (31.58%) in the very high MPN range, indicating a potential area of concern. Other water types in Atteridgeville have fewer occurrences of *L. pneumophila* in higher ranges.
- **Zandspruit:** Most water types show a lower occurrence of *L. pneumophila* in the moderate and very high MPN ranges, with cold tap water having a slightly higher presence.
- **Melusi:** Cold tap and stored water showed some presence in the low range, with a small percentage (5.26%) of cold tap water indicating *L. pneumophila* in the very high range.

5.3.4 *L. pneumophila* MPN Values (Presence) vs Water Temperature

A detailed comparison of positive *L. pneumophila* concentrations in relation to water temperature across different areas is presented in Figure 5.7, highlighting areas and conditions with higher *L. pneumophila* bacterial counts. The pie charts in Figure 6.7 illustrate the percentage distribution of *Legionella pneumophila* positive results, detected using the Legiolert™ ® method, across various temperature and categorised into four ranges: 17-29°C, 30-39°C, 40-49°C, and 50-60°C. in different areas: Vhembe, Hillbrow, Atteridgeville, Zandspruit, and Melusi. These percentages indicate the proportion of positive water samples falling within specific temperature ranges.

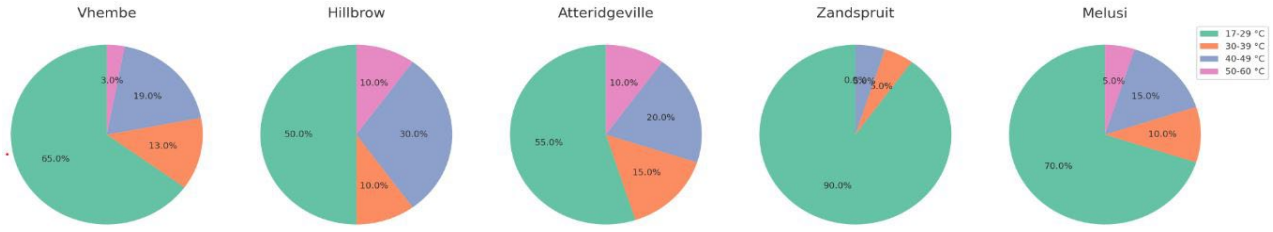


Figure 5-7. Percentage distribution of *Legionella pneumophila* positive results, detected using the Legiolert™ method, across various temperature ranges in different areas: Vhembe, Hillbrow, Atteridgeville, Zandspruit, and Melusi.

5.3.4.1 Vhembe. L. pneumophila MPN values vs. water temperature

- 65% of the positive samples were found in the 17-29°C range. This suggests that a significant number of water sources in Vhembe harbour *Legionella* even at relatively cooler temperatures.
- 19% of positive samples occurred in the 30-39°C range, which is within the optimal growth range for *Legionella*.
- 13% of the positive samples were detected in the 40-49°C range, a temperature range often associated with higher risks for *Legionella* proliferation.
- A small percentage (3%) of positive samples were in the 50-60°C range, indicating that *Legionella* can still be found even at higher temperatures, albeit less frequently.

5.3.4.2 Hillbrow. L. pneumophila MPN values vs. water temperature

- Half of the positive results (50%) were detected in the 17-29°C range, showing a similarity to *L. pneumophila* detected in Vhembe water samples, indicating that *Legionella* can thrive in cooler water sources.
- A significant portion (30%) of positive samples were within the 30-39°C range, consistent with *Legionell"*s preferred growth conditions.
- 10% of positive samples were found in each of the 40-49°C and 50-60°C ranges, suggesting a presence of *Legionella* in hotter water sources, which might be less common but still possible.

5.3.4.3 Atteridgeville. L. pneumophila MPN values vs. water temperature

- The majority of positive results (55%) were in the 17-29°C range, indicating a substantial risk of *Legionella* contamination even at lower temperatures.
- 20% of positive samples were detected in the 30-39°C range, which is optimal for *Legionella* growth.
- 15% of the positive samples occurred in the 40-49°C range, with 10% in the 50-60°C range, highlighting the presence of *Legionella* in hotter environments, potentially from poorly maintained hot water systems.

5.3.4.4 Zandspruit. L. pneumophila MPN values vs. water temperature

- A dominant 90% of positive samples were found in the 17-29°C range, indicating that in Zandspruit, *Legionella* is primarily associated with cooler water sources.
- The remaining 6% and 3.4% of positive results were found in the 30-39°C and 40-49°C ranges, respectively, suggesting fewer issues with *Legionella* in warmer water sources.

5.3.4.5 Melusi L. pneumophila MPN values vs. water temperature

- 70% of positive samples were detected in the 17-29°C range, consistent with other areas, showing a significant risk of *Legionella* in cooler water sources.
- 15% of positive samples occurred in the 30-39°C range, and 10% were in the 40-49°C range, indicating the potential for *Legionella* in warmer temperatures.
- 5% of the positive results were found in the 50-60°C range, similar to findings in other areas, indicating that *Legionella* can persist in higher temperatures, though it is less common.

Across all sampling areas, the majority of *Legionella*-positive results were determined in the cooler water samples within the 17-29°C temperature range, highlighting the need for vigilance even regarding cooler water sources. In addition, water in the 30-39°C range also showed a consistent presence of *Legionella* across all areas, aligning with the bacterium's optimal growth conditions associated with water at lower temperatures. While appearing at a lower frequency, the presence of *Legionella* in the water samples within the 40-49°C and 50-60°C ranges suggests that hot water systems, particularly those that were not properly maintained, can still be sources of *Legionella* contamination.

These findings underscore the importance of monitoring water systems for *Legionella* across a wide temperature range, not just the traditionally recognized temperature "danger zone". In addition, regular maintenance, temperature control, and effective water management practices are crucial in mitigating the risk of *Legionella* maintaining a growth environment, which promotes the chances of *Legionella* infection and possible outbreaks. The study data emphasises the importance of comprehensive monitoring of *Legionella pneumophila* across all temperature ranges. Public health strategies should prioritize the regular assessment of cooler to moderately warm water sources, environmental temperatures where *Legionella* is most prevalent,

to mitigate the risk of bacterial growth maintenance and exposure to individuals using the Legionella-contaminated water systems. This is especially crucial in environments such as residential buildings, hospitals, and public facilities where vulnerable populations are at greater risk of infection.

5.4 CULTURING LEGIONELLA

All samples collected in the study, including stock water samples, storage tank water samples and swabs, were subjected to culturability analysis using the SANS: 11731 methods to detect *Legionella pneumophila* and amoeba-associated *Legionella*. The samples positive for *Legionella* are shown in Figure 5.8 to compare the number of samples tested during each sampling run per sampling area. The graph illustrates the distribution of *Legionella pneumophila*-positive samples (determined using the Legiolert™ ® assay), relative to the total samples analysed, and those successfully cultured by the SANS 11731:2017 method, across the five study regions of Vhembe, Hillbrow, Atteridgeville, Zandspruit, and Melusi.

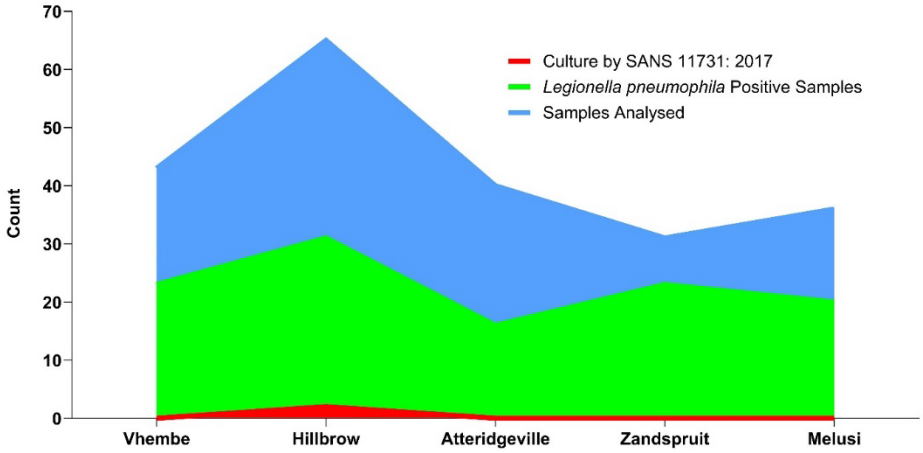


Figure 5-8. Representation of all water samples tested showing positive detection *of L. pneumophila* by Legiolert™ and the SANS 11731:2017 method.

Overall Trends

Samples analysed (Blue Area): The largest category in the graph is the blue area, representing the total number of samples analysed across all regions. Most samples analysed were collected in Hillbrow, closely followed by the number of samples collected in Atteridgeville and Vhembe. This supports the suggestion that there is a willingness on the part of individuals in these areas to allow monitoring of their water quality.

Legionella pneumophila-positive samples (Green Area): The green area indicates the relative number of samples that tested positive for *Legionella pneumophila*. Notably, Hillbrow showed the highest counts of positive samples, implying that this area might have a more significant presence of *Legionella* contamination, while Zandspruit showed a relatively increased frequency of water samples containing *Legionella pneumonia*. Vhembe, Atteridgeville, and Melusi showed relatively lower counts but still indicate the presence of these pathogens.

Culture by SANS 11731:2017 (Red Area): The smallest area, represented in red, reflects those samples cultured according to SANS 11731:2017 standards. The minimal detection of *Legionella* in this category suggests that only a small portion of the total analysed samples tested positive when using this specific culture method. This could indicate a limitation to this culturing assay and so Legionella analysis results obtained when using this protocol run the risk of under-reporting the prevalence of *Legionella pneumonia* in water

samples. This disparity might also suggest that *Legionella* species present in the water samples are in a viable but non-culturable (VBNC) state, meaning they remain undetectable through traditional culturing methods.

Swab samples were also tested for the presence of *Legionella* and amoeba from four of the sites. Of these, *L. pneumophila* was found in one toilet sample from Atteridgeville. However, none of the swab samples obtained from the other sample collection sites tested positive for *Legionella,* but were positive for the presence of amoeba. The summarized results can be seen in Figure 5.9.

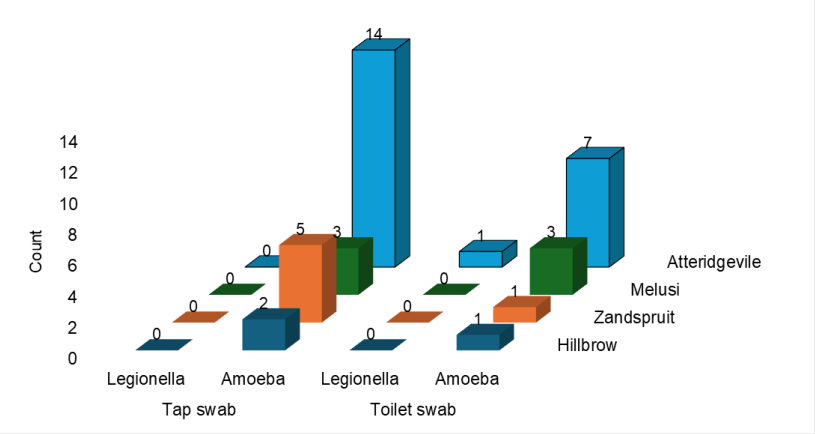


Figure 5-9. Schematic representation of toilet and tap swabs positive for the presence of *Legionella* sp. and amoebae.

5.5 SERO TESTING

All presumptive *Legionella* spp. isolates that were successfully cultured on BCYE agar underwent serological testing. However, none of the isolates showed positive agglutination results. This serological test detects *Legionella pneumophila* serogroups 1-14. However, the *L. pneumophila* isolates identified in this study may not belong to any of these serogroups, which could explain the negative agglutination results. Additionally, it is possible that some of the isolates may belong to *Legionella non-pneumophila* species, which are not detectable by this specific test. This supports the need to use modern, more sensitive detection methods such as those making use of molecular analysis.

5.6 MOLECULAR DETECTION OF *LEGIONELLA* FROM WATER SAMPLES

Bacterial DNA was isolated from Legiolert™ Quanti-trays that were positive for *Legionella pneumophila* as well as presumptive colonies growing on BCYE agar plate.

5.6.1 Legionella genus PCR

A total of 169 *Legionella* spp. PCR tests were carried out, of which 117 were positive for the *Legionella* 16S gene (Table 5.2). An example of the positive PCR is shown in the gel results in Figure 5.10. From the samples that were negative, a few were randomly selected and spiked with *Legionella positive* control DNA (ATTC strain 33152) to determine PCR inhibition. The results were all positive, indicating that there was no inhibition in the PCR.

Table 5.2. PCR results for the detection of *Legionella* spp. and *Legionella pneumophila* across sites: Hillbrow, Zandspruit, Melusi, Vhembe and Atteridgeville.

Site	n	*Legionella spp*	*L. pneumophila*
Hillbrow	27	21	20
	%	78	74
Zandspruit	31	2	25
	%	6	81
Melusi	36	16	29
	%	44	81
Atteridgeville	40	16	34
	%	40	85
Vhembe	35	23	22
	%	66	63
Total	169	117	130
	%	69	77

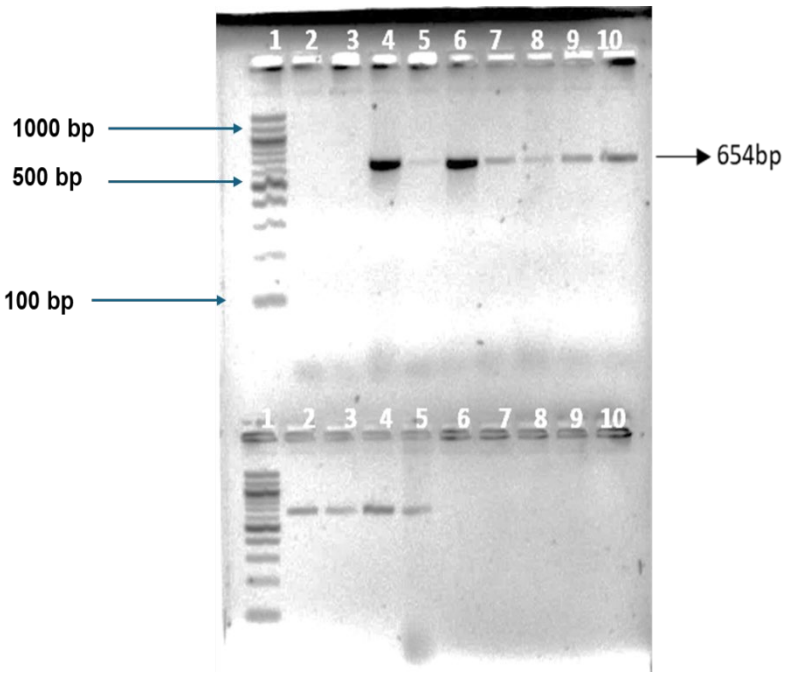


Figure 5-10. Agarose gel picture showing examples of positive *Legionella* spp. Amplicons of size 654 bp, Top wells Lane 1: DNA ladder, Lane 2: Negative control, Lane 3 Extraction negative, Lane 4: positive control, Lane 5-10 and bottom wells Lane 2-5: Legiolert™ samples.

5.6.2 Legionella pneumophila PCR

All the samples (n=169) were then tested for the macrophage infectivity potentiator *(mip)* gene, which indicates the presence of *Legionella pneumophila*. A total of 130 samples tested positive for *Legionella pneumophila*. The high positive rate observed in this study aligns with the findings of Ratcliff et al. (1998), who emphasized the reliability of the *mip* gene in identifying *Legionella pneumophila* in environmental samples. This high detection rate suggests that the water systems tested may provide conditions conducive to the growth of *Legionella*, such as warm temperatures and biofilm formation. An example gel picture is shown in Figure 5.11.

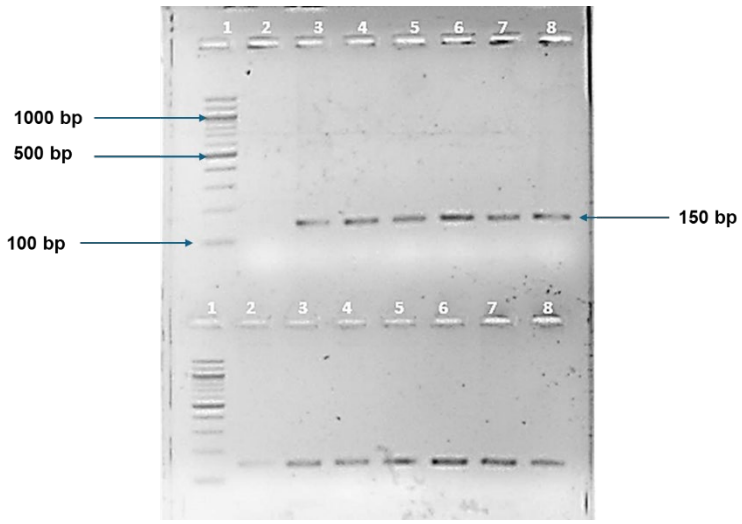


Figure 5-11. Agarose gel picture showing positive *Legionella pneumophila* at 150 bp. Top wells Lane 1: DNA ladder, Lane 2: Negative control, Lane 3 : PCR positive control, Lanes 4-8 and bottom wells Lanes 2-8: Legiolert™ ® samples positive for *Legionella pneumophila*.

Overall, 69% of samples tested positive for *Legionella* spp. DNA, while a higher 77% of the samples tested positive for *L. pneumophila* DNA from the Legiolert™ ® assay (Tabel 5.2). The presumptive culturable *Legionella* DNA from BCYE did not yield positive results. This discrepancy also highlights the sensitivity of DNA-based detection methods such as Legiolert™ ®, which can identify genetic material from both viable and non-viable bacteria. However, it is crucial to understand that these results are qualitative by allowing the detection of the presence or absence of *L. pneumophila* DNA, and so while they reflect the presence of *Legionella pneuphila*, they may not directly reflect the number of live, culturable bacteria present in the environment, as would be detected using BCYE agar. Nonetheless, the study results highlight a concerning pattern where *L. pneumophila* is as or more prevalent than the broader *Legionella* genus, posing a possible increased risk for Legionnaires' disease affecting these communities. The high detection rates of *L. pneumophila* DNA across the sites may indicate that the bacteria are in a viable but non-culturable (VBNC) state or are present as remnants of dead cells, which can still pose a potential health risk if aerosolized and inhaled.

These results emphasise the importance of using multiple detection methods for a comprehensive assessment of *Legionella* contamination, combining DNA-based methods with microbiological culturing to better understand the presence and viability of the bacteria in water systems.

5.6.3 Real-time qPCR results for *L. pneumophila*

A standard curve was obtained for *L. pneumophila* qPCR to ensure efficiency while performing qPCR reactions, as shown in Figure 5.12. To validate the qPCR assays before application of environmental samples, the detection limit and amplification efficiency of each reaction were determined. The nucleic acids were standardized by preparing standard curves from 10-fold serial dilutions of DNA controls assayed in triplicate. The PCR amplification efficiency (E) for each assay was calculated from the slope of the standard curves as 97. Results obtained by real-time PCR for *L. pneumophila* (from the Legiolert™ ® assay) showed 13/31 (42%) positive and the remaining 18/31 (58%) of samples were negative for *L. pneumophila*. These results are expressed per building in Table 5.3. The CT values for samples ranged between 16 and 30, and each value of qPCR was only considered positive when the threshold cycle (CT) of the sample was less than 35.

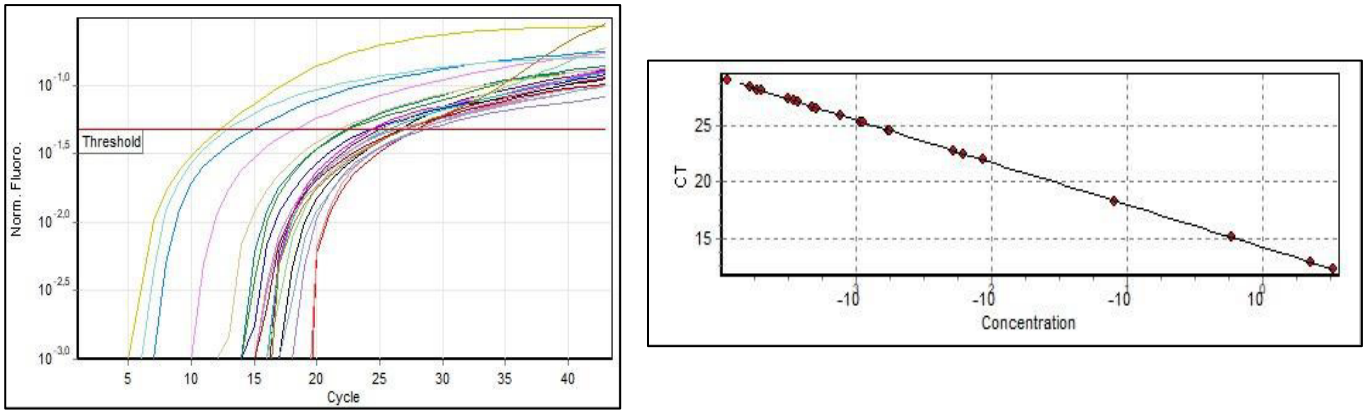


Figure 5-12. qPCR Standard curve for ATCC strain of *Legionella pneumophila*

Table 5.3. Genome copies of *L. pneumophila* detected by qPCR in Hillbrow, Zandspruit (Z), Vhembe (V) and Atteridgeville (A) building water samples building water samples.

Building Sample	Sample with the threshold cycle (CT) < 35	Copy Number
Positive Control	22.35	3,24E-08
Negative Control	14.47	1,13E-04
HB27	18,33	8,16E-02
HB29	25,83	7,81E-04
HB30	28,3	1,69E-04
HB35	21,93	8,76E-03
HB41	25,22	1,14E-03
HB44	27,12	3,51E-04
HB47	17,48	1,38E-01
HB52	26,95	7,95E-07
HB60	26,22	6,13E-04
Z1	20.75	1,69E-07
Z2	8.15	7,78E-02
Z3	23.05	1,57E-08
Z4	27.18	2,18E-10
Z5	23.15	1,42E-08
Z6	30.17	9,99E-12
Z7	30.00	1,19E-11
Z8	17.24	6,43E-06
Z9	19.95	3,86E-07
Z10	24.76	2,67E-09
Z11	29.39	2,22E-11
Z12	29.46	2,07E-11
Z13	14.51	1,08E-04
Z14	26.93	2,83E-10
Z15	24.69	2,89E-09
Z16	23.71	7,95E-09
Z17	24.16	4,98E-09
Z18	20.17	3,09E-07
Z19	21.51	7,76E-08
Z20	29.53	1,93E-11
Z21	26.05	7,05E-10
Z22	26.10	6,67E-10
Z23	15.79	2,88E-05
Z24	23.86	6,77E-09
Z25	30.63	6,20E-12
Z26	23.48	1,00E-08

Z27	30.65	6,03E-12
Z28	28.09	8,58E-11
Z29	18.24	2,28E-06
Z30	23.30	1,22E-08
V1	19.16	8,79E-07
V2	15.62	3,41E-05
V3	23.53	9,55E-09
V4	27.66	1,33E-10
V5	29.00	3,34E-11
V6	19.20	8,46E-07
V7	20.96	1,37E-07
V8	24.66	2,98E-09
V9	31.60	2,25E-12
V10	25.38	1,40E-09
V11	28.05	8,92E-11
V12	28.51	5,56E-11
V13	20.44	2,33E-07
V14	10.03	1,11E-02
V15	34.13	1,66E-13
V16	15.08	6,00E-05
V17	16.95	8,68E-06
V18	24.74	2,74E-09
V19	23.08	1,52E-08
V20	20.11	3,29E-07
V21	21.25	1,01E-07
V22	29.64	1,71E-11
V23	22.67	2,34E-08
V24	29.21	2,67E-11
V25	20.60	1,98E-07
V26	29.31	2,42E-11
V27	22.18	3,87E-08
V28	27.31	1,91E-10
V29	24.94	2,21E-09
V30	25.58	1,15E-09
A1	31.44	2,66E-12
A2	26.81	3,20E-10
A3	25.04	2,01E-09
A4	24.84	2,47E-09
A5	17.29	6,11E-06
A6	19.30	7,57E-07
A7	18.98	1,06E-06

Table 5.3 represents the quantification of *Legionella pneumophila* detected via qPCR across various building samples, with each sample identified by its threshold cycle (Ct) value and corresponding copy number. The copy numbers, expressed in scientific notation, give a precise measure of the genomic concentration of the pathogen. The positive control yielded a Ct value of 22.35 and a high copy number of 3,24E+08, confirming the accuracy and sensitivity of the qPCR assay used. This serves as a benchmark for the rest of the samples. Despite being a control, the negative sample showed a Ct value of 14.47 and a non-zero copy number of 1,13E+04. This may indicate possible cross-contamination or the presence of background amplification.

Geographic Location Breakdown:

- Hillbrow (Samples HB): Most Hillbrow samples showed low or negligible contamination. For example, HB27 displayed a Ct value of 18.33 and a copy number of 8,00E-02, indicating a low level of *L. pneumophila*. However, HB47 (Ct = 17.48, Copy Number = 1,40E-01) suggests a slightly higher concentration of the bacteria in this area.

- Zandspruit (Samples Z): Zandspruit samples showed a wide range of bacterial presence. Z2 exhibited an extremely low Ct value of 8.15, with a high copy number of 7,78E+01, indicating significant contamination. Conversely, Z5, Z10, and Z28 displayed moderate bacterial levels, with Ct values around 24–30, and lower copy numbers, indicating varying degrees of contamination across this location.
- Vhembe (Samples V): The Vhembe samples, such as V2 and V16, showed elevated bacterial presence, with copy numbers of 3,41E-05 and 5,99E-05, respectively. Samples such as V14 and V9, with copy numbers of 1,11E-02 and 2,98E-09, respectively, suggest a need for careful monitoring of the water systems in Vhembe, as the variation in bacterial load is evident.
- Atteridgeville (Samples A): Atteridgeville had a notable sample, A5, with a Ct value of 17.29 and a copy number of 6,11E-06, which signals a relatively higher contamination level compared to other locations. Other samples, like A1 and A10, displayed high Ct values above 30, with correspondingly low bacterial loads, suggesting better water quality control in certain areas of Atteridgeville.

The qPCR results from Table 5.3 indicate varying levels of target DNA across the building samples, suggesting differences in contamination levels. Most important to note was that samples with Ct values below 35 generally confirmed the presence of *L. pneumophila*. High variability in the data, particularly across different geographic locations, suggests that the water safety and microbial quality differ significantly between these urban and peri-urban environments. The presence of *L. pneumophila* in higher quantities in areas like Zandspruit and Vhembe requires further investigation, potentially indicating public health risks associated with contaminated water sources. Further analysis for the Melusi sites is ongoing.

5.7 AMOEBA-ASSOCIATED *L. PNEUMOPHILA* PCR RESULTS

5.7.1 Molecular detection of amoebae

Water and swab samples were concentrated and cultured onto non-nutrient agar, seeded with heat-killed *E. coli* and incubated for up to 21 days for the growth of amoeba. Amoebal trophozoites and cysts were recovered from some of the seeded sample cultures. Those samples that did not show any amoeba growth were incubated further and these plates were recorded as negative after three weeks of observation and absence of growth. All positive amoebae samples were stored for further downstream analysis. Figure 5.13 highlights positive results for amoebae growth formation and PCR analysis. The results of PCR analysis of amoebae are indicated in the electrophoresis gel as shown in Figure 5.14.

In Hillbrow, 28 water samples were positive for amoeba by culture, and after sub-culturing until pure and DNA extraction, five (n=5) amoeba colonies tested positive by PCR amplification. In Zandspruit, 20 water samples were positive for amoeba by growth in culture and four (n=4) amoebae were positive from PCR amplification. In Melusi, no water sample but some swab samples tested positive following amoeba culture. Of the samples collected in Atteridgeville, eight (n=8) water samples tested positive for amoeba by culture and two (n=2) tested positive for amoebae following PCR amplification of amoebae DNA. In Vhembe, presumptive amoeba positive for trophozoites and cysts from water samples were confirmed with PCR. Out of 18 presumptive, 3 (16,66%) were confirmed positive for free-living amoeba (data not shown). None were positive when subjected to PCR from swab samples.

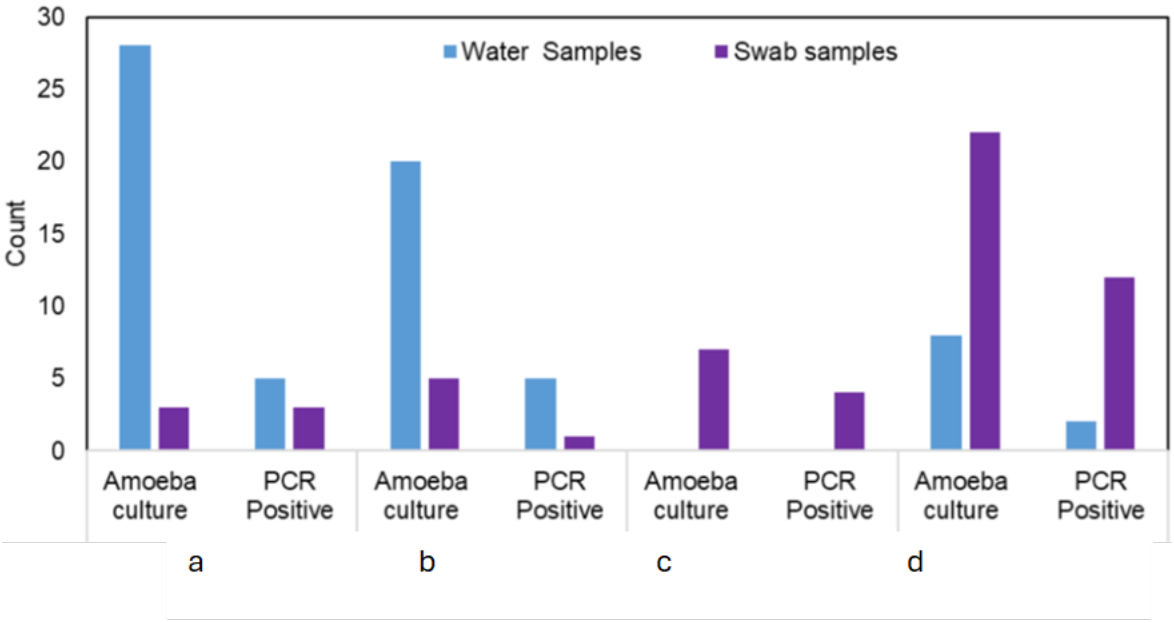


Figure 5-13. Schematic representation of amoeba culture and PCR results from a) Hillbrow, b) Zandspruit, c) Melusi and d) Atteridgeville

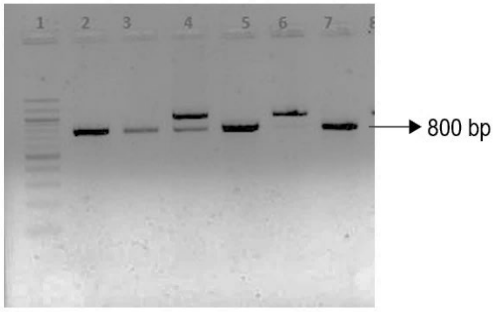


Figure 5-14. Agarose gel confirming the detection of free-living amoeba (800-1300 bp). Lane 1:100 bp ladder, Lane 2: Positive control, Lanes 3-7: Amplified amoeba DNA from collected samples.

5.7.2 Amoeba-associated *Legionella* culture results

To induce the release of *Listeria* spp. cells from inside amoebae cells, the amoeba cells from the positive amoeba growth plates, were lysed and the lysate was cultured on BCYE agar. Fewer than 10% (2/19) of the lysates then tested positive for amoeba-associated *Legionella*. These lysates were further tested by extracting their DNA and performing PCR for the mip gene. Figure 5.15 shows the amoeba growth as observed microscopically, and Figure 5.16 shows the amoeba after Giemsa staining with bacterial cells.

After culturing and DNA isolation from presumptive *Legionella cells that were* extracted from amoebae, the *mip* gene of DNA isolates was amplified using PCR. Only three (n=3) of the four (n=4) isolates were confirmed as positive for *L. pneumophila.*

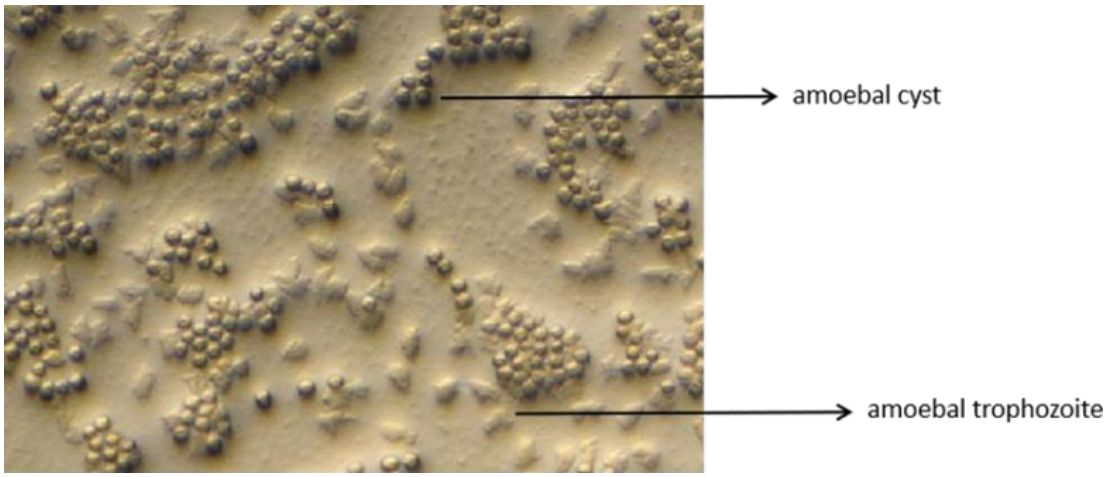


Figure 5-15. Typical morphology of presumptive *Amoeba* cysts and trophozoites (arrows) (40 x magnification, Olympus Microscope).

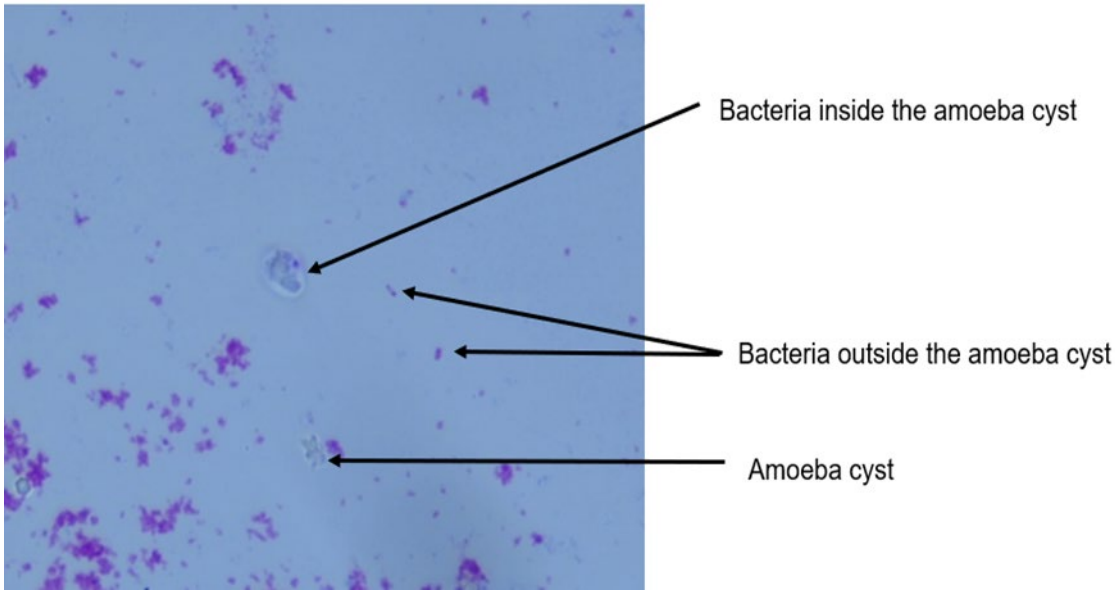


Figure 5-16. Giemsa-stained image showing amoeba cyst with bacteria inside and outside the amoeba cyst (purple and blue colour, respectively).

5.7.3 Amoeba-associated bacterial isolation

The amoebal lysates were cultured for non-*L. pneumophila* bacteria, to further investigate the presence of amoeba-associated bacteria like the ESKAPEs and *E. coli.* bacteria. From these presumptive isolates, the following bacteria were identified *Pseudomonas* spp. (n=15), *E. coli/Enterobacter* (n=47), *Klebsiella* spp. (n=16), *Enterococcus* spp. (n=15), *Acinetobacter* spp. (n=14), *Staphylococcus* spp. (n=3). These results are shown in Figure 5.17. The identity of the presumptive isolates was confirmed using the VITEK® 2 compact system as well and classify their antimicrobial resistance profiles. The following were identified; *E. coli, Klebsiella pneumoniae and Klebsiella oxytoca species, Enterobacter cloacae complex and Enterobacter asburiae, Enterococcus species, E. faecalis, E. durans E. galinarum, Aeromonas hydrophilia, Staphylococcus saprophyticus,* and *Raoultella planticola.*

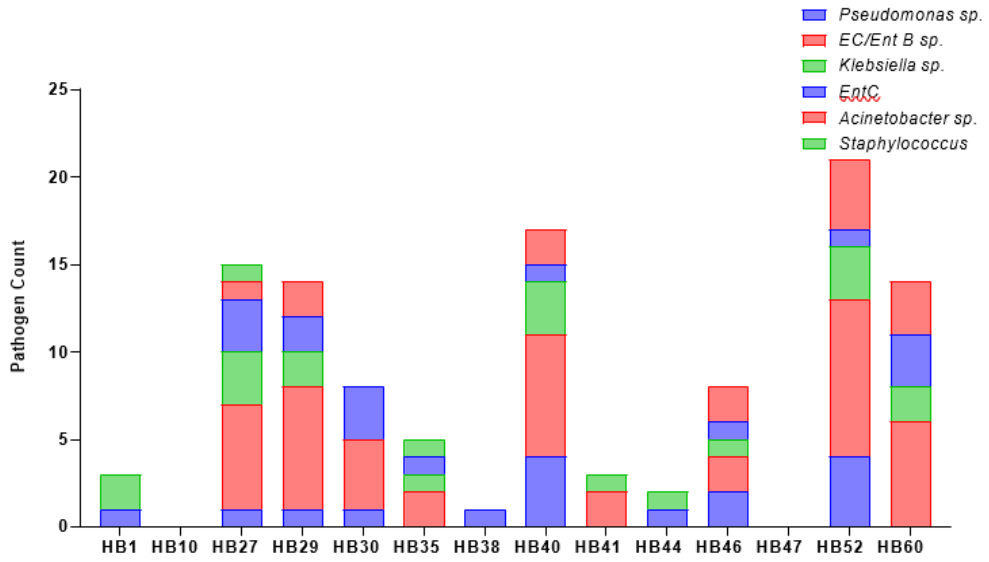


Figure 5-17. Amoeba-associated bacteria, excluding *L. pneumophila*, detected in Hillbrow water samples.

5.8 SEQUENCING

A subset of samples from each site that tested positive using PCR amplification of the *mip* gene, including those associated with amoeba-related *Legionella*, was selected for sequencing. The resulting sequences were subjected to BLAST analysis against the NCBI database to identify sequences with high homology or similarity to known *Legionella pneumophila* sequences. The BLAST search results showed relatively low E-values and high percentages, from 97%-100%, indicating identity with the partial sequence of the *Legionella pneumophila mip* gene isolated from 92-03764/. The alignment results confirmed the identity and relatedness of the samples to *L. pneumophila* as shown in Table 5.4.

Table 5.4. NCBI BLAST Sequence similarities for *L. pneumophila*.

Sample	Description	Query cover	E- Value	Accession number
H3	*Legionella pneumophila* strain LEG1117 chromosome	98%	3,00E-51	LS483410.1
H11	*L. pneumophila* isolate L1860 macrophage infectivity potentiator surface protein (mip) gene, partial cds	98%	3,00E-51	KC410215.1
Z1	*Legionella pneumophila* strain Edelstein isolate SI7 macrophage infectivity potentiator (*mip*) gene, partial cds	98%	3,00E-51	MW524769.1
Z2	*Legionella pneumophila* strain MIP1 macrophage infectivity potentiator (*mip*) gene, partial cds	98%	3,00E-51	KJ160890.1
Z4	*Legionella pneumophila* strain H3 chromosome, complete genome	98%	3,00E-51	CP114576.1
Z5	*Legionella pneumophila* partial mip gene for macrophage infectivity potentiator,	98%	3,00E-51	AJ810195.1

CHAPTER 6: EVALUATION OF RISKS ASSOCIATED WITH LEGIONELLA AEROSILIZATION

6.1 INTRODUCTION

Quantitative Microbial Risk Assessment (QMRA) is a powerful tool used to evaluate the risk of infection from exposure to waterborne pathogens such as *Legionella pneumophila.* This method leverages both empirical data on pathogen concentrations in environmental samples and mathematical models to estimate the probability of infection under various exposure scenarios. In this analysis, the implementation of the QMRA method based on the average *Legionella* concentrations in water samples from Legiolert™ ® was as described by Denissen et al. (2023).

6.2 OVERVIEW OF THE QMRA APPROACH

The QMRA process typically involves four key steps:

6.2.1 Hazard identification

Water samples from Hillbrow and Vhembe were tested for *Legionella pneumophila* to provide a baseline level.

6.2.2 Exposure assessment

This step models different exposure scenarios, including activities such as showering and toilet flushing. These scenarios utilize inhalation and ingestion rates from existing literature to estimate the dose of *Legionella* inhaled by individuals during each event. For example, showering involved inhalation rates of 20-30 L/min over 5-10 minutes (Nicas and Hubbard, 2002). Toilet flushing typically results in much lower aerosol exposure rates, with some studies identifying this as a minor source of aerosolized bacteria (Zhang et al., 2022).

For this study, the following exposure scenarios were evaluated:

- Hillbrow scenario: Involves showering using cold tap water.
- Vhembe scenario: Involves showering using water from a geyser/boiler.

6.2.3 Dose-response assessment

The beta-Poisson model was applied to determine the probability of infection based on the dose of pathogens to which an individual is exposed. The dose is calculated using the following formula:

$$\mathbf{Dose} = \mathbf{Concentration} \times \mathbf{Inhalation\ Rate} \times \mathbf{Exposure\ Duration} \qquad \textbf{Equation 1}$$

Where:

- Concentration is in MPN/100 mL
- Inhalation rate is in L/min
- Exposure duration is in minutes.

Parameters such as the pathogen dose, N50 and α values are extracted from previous studies (Haas et al., 1999; Hamilton et al., 2018b). These parameters allow for accurate capture of variability in individual susceptibility and dose-response relationships. Once the dose data were collected, the beta-Poisson model was used to calculate the probability of infection for a single exposure event. This is done by inputting the dose data into the model along with values for N50 and α, which are derived from existing literature on Legionella

infections (Haas et al., 1999). The beta-Poisson model is widely used in microbial risk assessments to describe the relationship between the dose of a pathogen and the probability of infection. The key parameters of this model are as follows:

- Concentration data (MPN/100 mL): Specific concentration levels of *Legionella* contamination in the water samples collected from Hillbrow and Vhembe. For the exposure scenarios selected, the concentrations were as follows:
    - Hillbrow:
        - Cold tap: 14 samples were in the 11-100 MPN/100 mL range, and one sample was in the 101-1000 MPN/100 mL range. No extreme contamination (>2272.6 MPN/100 mL) was detected.
        - Geyser/boiler: 10 samples showed <1 MPN/100 mL, and 13 samples were in the 11-100 MPN/100 mL range, with no significant contamination in stored water.
    - Vhembe:
        - Cold tap: Low contamination, with most samples <1 MPN/100 mL. Two (n=2) samples were in the 11-100 MPN/100 mL range, with one (n=1) sample exceeding 2272.6 MPN/100 mL.
        - Geyser/boiler: Relatively higher contamination, with 40 samples <1 MPN/100 mL, 11 samples between 11-100 MPN/100 mL, and one (n=1) sample exceeding 101-1000 MPN/100 mL.
        - Stored water: Variable results, with some samples reaching up to 101-1000 MPN/100 mL .

- Inhalation rate (L/min): Specific inhalation rates depending on the exposure scenario, particularly for showering.

- Exposure duration (minutes): Estimated time spent showering or interacting with contaminated water.

The model is represented by the following equation:

$$\mathbf{P}inf = \mathbf{1} - (\mathbf{1} + \frac{\mathbf{d}}{\mathbf{N50}}\left(\mathbf{2}^{\frac{\mathbf{1}}{\alpha}} - \mathbf{1}\right))^{-\alpha} \qquad \textbf{Equation 2}$$

Where:

- P_{inf} is the probability of infection from a single exposure
- d is the dose of microorganisms (ingested or inhaled)
- N_{50} is the median infective dose (the dose at which 50% of exposed individuals are expected to become infected)
- and α is a shape factor that determines the steepness of the dose response curve.

The beta-Poisson model is particularly useful for *Legionella pneumophila* because it can account for the variability in individual susceptibility to infection, as well as the variability in exposure dose. Three common scenarios contributing towards susceptibility to infection are toilet flushing, showering, and pouring water over the body. Table 6.1 shows a summary of inhalation/ingestion rates for QMRA from the literature. The typical inhalation and ingestion rates associated with these activities include:

1. Toilet flushing

- Inhalation of aerosols:
    - Rate: The inhalation of aerosols generated from toilet flushing is usually low. Studies have estimated that about 0.1 to 1 L of air is inhaled during the time spent near a toilet that is flushed (Hamilton et al., 2018a,b). While specific inhalation rates during toilet flushing are not well-

documented, the generation of aerosols during flushing can pose a risk. The potential for exposure exists, as flushing can aerosolize *L. pneumophila*, leading to inhalation risks (Couturier et al., 2020).

- Exposure pathway: Aerosols generated during toilet flushing can contain *Legionella* if the water system is contaminated. The risk increases in public restrooms or locations with high turnover and potentially contaminated water supplies. Studies have linked toilet flushing to *Legionella* transmission, particularly in public restrooms and healthcare settings where contaminated water systems may contribute to exposure (Couturier et al., 2020; National Academies of Sciences, Engineering, and Medicine, 2020).

2. Showering

- Inhalation of aerosols:
  - Rate: During showering, individuals typically inhale between 20 to 30 L of air per minute, depending on the intensity of the activity and individual respiration rate (Nicas and Hubbard, 2002). The fine aerosols produced during showering can efficiently transport *L. pneumophila* to the lower respiratory tract (Niculita-Hirzel et al., 2022).
  - Exposure pathway: Showers are a well-documented source of *Legionella* exposure due to the generation of fine aerosols and the potential for biofilm development in showerheads and plumbing systems. The risk is heightened by the prolonged duration of exposure and close proximity to the water source.

3. Pouring water over the body

- Inhalation of aerosols:
  - Rate: Similar to showering, but typically less intense and shorter in duration, the inhalation rate is estimated to be around 10 to 20 L per minute; however, specific inhalation rates for this activity are not well-documented.
  - Exposure pathway: Pouring water over the body, such as during bucket baths or rinsing in non-shower settings, can still generate aerosols, especially if the water is splashed or poured from a height. The risk is generally lower compared to showering but still present, particularly in areas with poor water quality (Niculita-Hirzel et al., 2022).

Table 6.1. Summary of inhalation/ingestion rates for QMRA from the literature.

Activity	Inhalation Rate (L/min)	Duration	Risk	References
Toilet flushing	Low (varies, ~1-2 L/min)	Single flush event	Minimal aerosol exposure from splash/aerosol plumes	Zhang et al. (2022); (Couturier et al., 2020); Hamilton et al. (2018b)
Faucet usage	N/A	Short, ~1-3 minutes	Minimal aerosol exposure	Hamilton et al. (2018b)
Showering	20 - 30 L/min	5 - 10 minutes	High: aerosols generated close to the face, prolonged exposure	Nicas and Hubbard (2002)
Pouring water over the body	10 - 20 L/min	2 - 5 minutes	Moderate: lower aerosol generation	(Niculita-Hirzel et al., 2022); Allegra et al. (2020)

These inhalation rates are critical for accurately assessing the dose of *Legionella pneumophila* that individuals are likely to inhale during these common activities. By integrating these rates into the QMRA model, along with concentration data and dose-response parameters, the risk of infection can be estimated for various exposure scenarios.

6.2.4 Risk characterisation

The probability of infection for each exposure scenario is calculated using Monte Carlo simulations. A total of 500,000 iterations per scenario account for variability and uncertainty in the data, providing robust risk estimates. Monte Carlo methods are widely used in microbial risk assessment for their ability to handle uncertainty and variability in complex systems (Sylvestre et al., 2024).

6.3 QMRA RESULTS

The beta-Poisson dose-response model and Monte Carlo simulations were applied to estimate the infection risk in Hillbrow and Vhembe. In applying this model *to Legionella pneumophila*, a substantial amount of data on *Legionella* concentrations in water samples was gathered. These data serve as the basis for estimating the dose (d) that individuals are exposed to during different scenarios, such as using showers or toilet flushing.

The Monte Carlo simulation setup was as follows:

- Run 500,000 iterations to account for variability.
- Concentration values are randomly sampled within the provided ranges.
- Inhalation rates and exposure durations are constant for simplicity.

The simulation produced the following results:

- ***Hillbrow – Cold Tap Water***
    - Mean probability of infection: 0.0204 (2.04%)
    - Median probability of infection: 0.0206 (2.06%)
    - 95th percentile of probability of infection: 0.0346 (3.46%)

- ***Vhembe – Geyser/boiler water***
    - Mean probability of infection: 0.0205 (2.05%)
    - Median probability of infection: 0.0206 (2.06%)
    - 95th percentile of probability of infection: 0.0346 (3.46%)

The results indicate that the probability of infection from *Legionella pneumophila* is similar in both Hillbrow and Vhembe under the given scenarios. Both areas show a mean and median probability of infection around 2.05%, with the upper end (95th percentile) reaching around 3.46%.

Despite the similarity in risk levels, the specific conditions in each area, such as infrastructure quality and water management practices, may influence the actual risk experienced by residents. In Hillbrow, the higher density and older infrastructure may exacerbate exposure, whereas in Vhembe, the risk may be influenced more by the maintenance and operation of water heating systems. This QMRA represents a preliminary analysis based on available data and assumptions. The risk assessment should be further refined for larger data sets.

Figure 6.1 represents a conceptual model for *Legionella* exposure in a bathroom setting within a household. According to this model, first, *Legionella* multiply within the premise's plumbing biofilm, potentially by colonising the biofilm or within a protozoan host (A). The biofilm-associated *Legionella* detach from the biofilm (B) during a showering event, it is transported to the shower head and is aerosolized or during a toilet flushing event the biofilm is transferred from the plumbing to the cistern, to the toilet bowl and then aerosolized (this can occur even with the toilet lid closed) (C). Finally, the aerosolized *Legionella* is inhaled (D) and a fraction of that inhaled dose is deposited in the alveolar region of the lungs (E) (Schoen and Ashbolt, 2011).

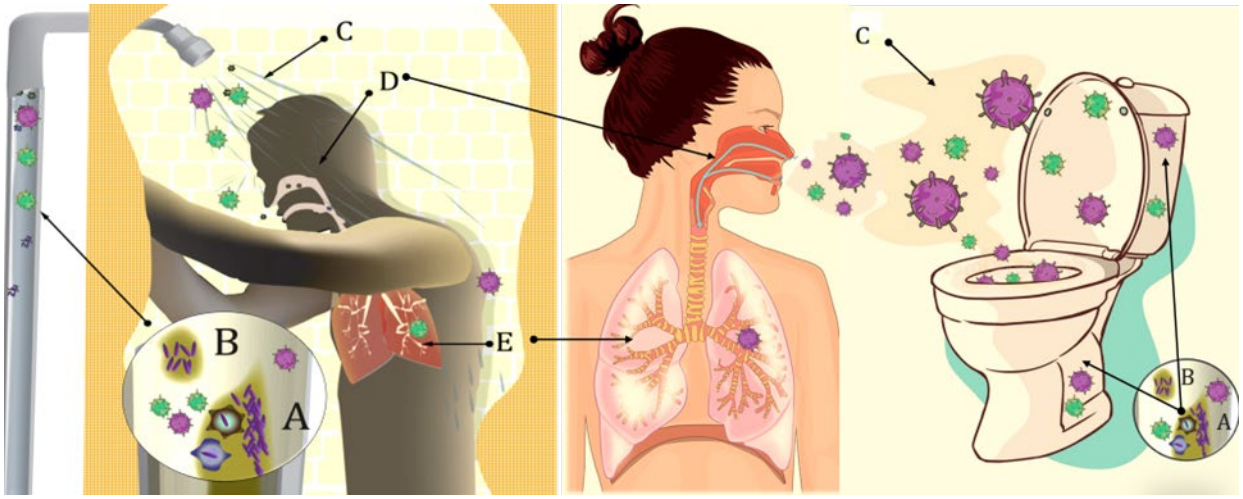


Figure 6-1. Conceptual model for *Legionella* exposure from inhalation of shower and toilet aerosols containing *Legionella* derived from the in-premise plumbing of buildings. (A) Biofilm; (B) Biofilm detachment; (C) Aerosilization and (D) Inhalation. Figure adapted with permission from Schoen and Ashbolt (2011).

CHAPTER 7: DISCUSSION AND SUMMARY OF FINDINGS

7.1 INTRODUCTION

The analysis of *Legionella pneumophila* contamination across different sites, Hillbrow (low-income urban areas), Melusi and Zandspruit (informal settlements), and Atteridgeville and Vhembe (rural areas), reveals significant insights into how socio-economic and environmental factors impact waterborne pathogen risks. Most studies on *Legionella* spp. focused on healthcare facilities, cooling towers, and large-scale water systems (Sciuto et al., 2021; Stone et al., 2019), with less attention given to residential environments such as community households and residential high-rise buildings. This discussion integrates socio-economic contexts, water quality data and the occurrence of *L. pneumophila* to provide a comprehensive understanding of the conditions that favour bacterial contamination in these diverse settings.

7.2 STUDY SITE: URBAN AND RURAL BUILDING CLASSIFICATION

The use of the SANS 10400A:2022 Occupancy or Building Classification to categorise the buildings provided a comprehensive understanding of how different residential setups may influence public health risks.

7.2.1 Hillbrow and Vhembe High-Rise Buildings (Class H5: Hospitality)

The classification of high-rise buildings in this study site suggests a transient population renting furnished rooms. This transient nature can lead to inconsistent use of water patterns, increasing the risk of stagnation within the plumbing systems in the buildings, providing a conducive environment for *L. pneumophila* to thrive (Nisar et al., 2020; 2023).

7.2.2 Atteridgeville (Urban) and Vhembe (rural) (Class H4: Dwelling house)

The classification of dwelling houses in this study site suggests the likelihood of consistent water usage compared to high-rise buildings. However, inadequate residents' responsibility for water management, such as plumbing maintenance and renovations, may increase the risk of *Legionella* spp. contamination within the water system (Szwetkowski and Falkinham, 2020).

7.2.3 Zandspruit and Melusi (Informal settlements)

The inability to classify buildings in these study sites reflects the unregulated nature of rural settlements. These sites often lack adequate water and sanitation facilities, which are crucial in mitigating the occurrence and spread of waterborne pathogens like *L. pneumophila* (Weinbren, 2020). These settlements are vulnerable to public health crises due to poor hygiene, reliance on untreated or shared water sources and water storage practices (Slavik et al., 2020).

7.3 WALK-THROUGH ASSESSMENTS

Walk-through site assessments conducted across study sites where applicable showed notable trends in plumbing maintenance, storage and sanitation practices, which together influence the risk of *L. pneumophila* and amoeba.

7.3.1 Plumbing maintenance and infrastructure

Inadequate plumbing maintenance and renovation across study sites indicate significant *Legionella* spp. contamination risks. Hillbrow, a densely populated area with old buildings, showed limited records of plumbing renovations and irregular maintenance. Poorly maintained plumbing systems, especially in aging buildings, contribute to biofilm formation and water stagnation. This is in agreement with (Szwetkowski and Falkinham, 2020), who indicated that aging infrastructure and inadequate renovations promote microbial contamination.

The plumbing materials recorded across the study sites varied, with Hillbrow using iron pipes, Vhembe and Atteridgeville using various materials, including PVC, PEX, Iron, and copper. These plumbing materials can have effects on the growth of opportunistic pathogens, including *Legionella* spp. in premise plumbing. Depending on the material, pipes can influence these pathogens by providing nutrient that promotes growth and consuming secondary disinfectants, allowing for microbial growth and proliferation (Cullom et al., 2020). Moreover, reports on copper pipes on the growth of *L. pneumophila* are controversial; some studies indicating that copper inhibited *L. pneumophila* growth, while others found the bacteria to grow better on copper than on other plumbing materials (van der Kooij et al., 2020). Copper possesses inherent antimicrobial properties, effectively reducing *Legionella* populations when used in plumbing systems (Song et al., 2024). However, its efficacy can be compromised by factors such as orthophosphate presence, which reduces copper bioavailability, and the development of copper-resistant microbial strains (Song et al., 2024; Cullom et al., 2020).

The current study aligns with studies indicating that copper promotes *L. pneumophila* growth. PVC pipes are associated with lower disinfectant demand and less interaction with water chemistry, which can lead to increased organic carbon leaching, potentially supporting pathogen growth (Cullom et al., 2020). Stagnation in PVC systems has been linked to a rebound in *Legionella* populations, indicating a risk for pathogen proliferation under certain conditions (Cullom et al., 2020). Iron pipes can provide nutrients for opportunistic pathogens and exhibit high disinfectant demand, which may facilitate biofilm formation and pathogen survival. The complex interactions between iron, water chemistry, and microbial communities can create environments conducive to *Legionella* growth, particularly when combined with stagnation (Cullom et al., 2020).

In Atteridgeville and Hillbrow, despite being urban areas, the aging infrastructure and high population density contribute to the persistence of *L. pneumophila* in water systems. These areas are often characterized by older buildings with complex plumbing networks, where biofilms can form and protect *L. pneumophila* from eradication efforts. Additionally, inconsistent water heating and the potential for water stagnation in these densely populated areas further exacerbate the risk of contamination.

7.3.2 Water Storage

Storage tanks are known to cause a loss of disinfection and the subsequent disinfection by-product formation, can increase the pH of the water, this coupled with the increase in Iron and Manganese promote corrosion of the material, promotes biofilm formation thus posing a potential a risk to human health (Slavik et al., 2020). Hillbrow high-rise buildings had the most storage tanks in place that served a reservoir for water supply to the apartments within the building. In Hillbrow, the location of the storage tank was on the roof for 60% of the buildings, where water was pumped from the source up to the roof, then down to the residences. The position of the storage tank on the roof has an impact on water quality in that when the storage tank is not under any shade and in direct sunlight, this leads to unregulated water temperature increase that promotes microbial growth (Slavik et al., 2020). The material of the storage tank used by these buildings was plastic (93%), whilst only one building had a metal storage tank (7%). This creates an issue when the plastic material is leached into the water and provides organic carbon that induces microbial growth, thus raising concerns for human health (Proctor et al., 2017).

7.3.3 Sanitation facilities and potential aerosolization

In Hillbrow, all buildings were equipped with flushing toilets with seat covers, highlighting a reduced risk of aerosolization. In contrast, the lack of seat covers in Zandspruit and Atteridgeville households increases the aerosolization risks. Literature has shown that while evidence for the transmission of *L. pneumophila* from flushing toilets is lacking, there is evidence that aerosolized toilet water may contain *L. pneumophila*, and this may have been the source of infection in two cases of Legionnaires' disease (Couturier et al., 2020). Residents in Melusi had no access to sanitation facilities, having only pit toilets, which are less likely to cause aerosolization. However, pit toilets are well-documented to contribute to environmental contamination by leaching nutrients, pathogens and emerging contaminants into surface and groundwater systems (Gwenzi et al., 2023).

7.3.4 Water heating practices

Unspecified water heating periods were observed predominantly across all study sites. This raises concerns about the potential periods of water stagnation within the temperatures that may exacerbate *L. pneumophila* growth and proliferation. Continuous heating is an effective practice to reduce *Legionella* spp. risks by maintaining water temperature consistently high. However, the reliance on electric kettles rather than centralized systems like geysers suggests that heating is not incorporated into the broader water distribution system, allowing the occurrence of microbial contamination within the plumbing infrastructure. On-demand heating poses an increased risk due to inconsistent water heating and water stagnation, leaving the plumbing system vulnerable to *Legionella* spp. contamination. Electric kettles are widely used in Zandspruit and Melusi, indicating the lack of centralized water heating infrastructure. Although kettles boil water for immediate use, they do not address the risks within the plumbing systems, resulting in a conducive environment for bacterial growth. However, the use of kettles indicates that a substantial number of households rely on less effective water heating methods. Geysers and boilers are used in Hillbrow and Vhembe. These water heating tools are part of the centralised heating infrastructure, which, if well maintained, can reduce *Legionella* spp. risk within the plumbing systems. Boilers are exclusively used in Atteridgeville. These tools, which are similar to geysers, can reduce *Legionella* spp. across the plumbing system. However, it should be noted that at the time of sampling during this study, the areas sampled experienced intermittent water and electricity supply, which significantly altered the water heating practices.

7.3.5 Effect of temperature on *Legionella pneumophila* proliferation

Temperature plays a pivotal role in the proliferation of *L. pneumophila* (Bédard et al., 2016). However, the results from the analysed sites indicate that *L. pneumophila* can persist even at lower temperatures, particularly in areas with complex water systems or aging infrastructure. Water temperature regulation plays a role in lowering the risk of *Legionella* growth within those water systems (Sciuto et al., 2021). To control *Legionella* colonization in water distribution systems, it is important to maintain low temperatures for cold water (<20°C) and elevated temperatures for hot water (>60°C) with the circulation not falling below 49°C (CDC, 2024; Finnish Institute for Health and Welfare, 2023)

The temperature range associated with positive *L. pneumophila* occurrences in Vhembe was between 16.9°C and 49.1°C. This broad range reflects the varied environmental conditions typical of rural areas, where water storage and heating infrastructure may not be as rigorously controlled or maintained. The presence of *L. pneumophila* at these temperatures suggests that rural water systems, which often lack consistent monitoring, can harbor bacterial contamination, especially in stagnant or inadequately treated water (Moffa et al., 2023).

Melusi and Zandspruit (informal settlements): In these informal settlements, samples testing positive for *L. pneumophila* were found predominantly within lower temperature ranges, particularly in Zandspruit, where temperatures ranged from 14.8°C to 18.4°C. This narrow temperature range highlights the challenges of maintaining water quality in informal settings where water is often stored in makeshift containers that are

exposed to the elements. These conditions create a conducive environment for *L. pneumophila* growth, especially when water temperatures are not elevated sufficiently to inhibit bacterial proliferation (Gómez-Valcárcel et al., 2021).

Atteridgeville, Vhembe High-rise and Hillbrow High-rise (low-income urban areas): In these urban areas, *L. pneumophila* was detected across a broad temperature range, including temperatures above 50°C. The presence of the bacteria at such high temperatures could indicate the formation of biofilms within older or poorly maintained plumbing systems. Biofilms provide a protective environment for *L. pneumophila*, allowing these bacteria to survive even in less favourable conditions (Falkinham et al., 2020). The findings from Atteridgeville and Hillbrow suggest that the complex and often stressed water infrastructure in low-income urban areas can lead to increased risks of contamination of water with *L. pneumophila*, particularly in high-density housing where water systems may not be consistently maintained (Ramlal et al., 2022). The socio-economic conditions of the analysed sites play a crucial role in the observed patterns of *L. pneumophila* contamination. The quality and maintenance of water infrastructure are directly influenced by the economic status of the area, which in turn affects the risk of bacterial contamination.

Rural and informal settings: In areas such as Vhembe households, Melusi, and Zandspruit, the lack of robust infrastructure and consistent water quality management leads to higher risks of contamination of water with *L. pneumophila*. Rural areas often rely on outdated water systems, while informal settlements may lack formal water infrastructure entirely, leading to the use of improvised storage systems that are prone to contamination (Bartram et al., 2020; Hunter et al., 2022). These findings are consistent with those described in the literature that highlight the vulnerability of low-income and rural communities to waterborne diseases due to inadequate infrastructure and public health resources (Moffa et al., 2023).

L. pneumophila exhibits the ability to acclimate to elevated temperatures, with certain strains demonstrating resistance to brief exposures at 59°C attributed to genetic mutations in heat-shock response elements (Liang et al., 2023). Moreover, temperature significantly influences the composition of microbial communities within biofilms, with elevated thermal conditions facilitating the proliferation of *L. pneumophila* in association with protozoan hosts.

7.4 PREVALENCE OF *LEGIONELLA PNEUMOPHILA* ACROSS STUDY SITES

7.4.1 Diagnostic and analytical methods used for *Legionella* detection

The study evaluated various diagnostic and analytical methods for detecting *Legionella pneumophila* in water samples from five sites in South Africa. The methods included Buffered Charcoal Yeast Extract (BCYE) agar culture, Legiolert™ , serological agglutination tests, and PCR analysis.

In this study, water and swab samples collected were tested for *Legionella* growth using the Legiolert™ method and on Buffered Charcoal Yeast Extract (BCYE) agar and cysteine dependency. The BCYE medium provides the essential nutrients required for Legionella spp. growth, such as cysteine and ferric pyrophosphate, as recommended by the SANS 11731:2017 standard for *Legionella* isolation (Ditommaso et al., 2021; 2023; ISO, 2017). According to WHO, US EPA, and EU guidelines, *Legionella* concentrations should be <1 MPN/100 mL for drinking and safe water usage.

Results from the present study contrast with those from a study conducted by Boczek et al. (2021), who showed that the Legiolert™ and BCYE methods detected *L. pneumophila* in 83% and 85% of the samples, respectively. Bacteria, including *Legionella* in water distribution systems, have been reported to exist in a viable but non-culturable (VBNC) state, which might explain the low detection with the SANS: 11731 methods. Starvation, chlorine residuals and heat treatment in engineered water systems present stress conditions for Legionella, which decreases their culturability (Kirschner et al., 2016; Muchesa et al., 2018). Additionally,

Legionella detection on BCYE agar may not capture all species, and non-culturable *Legionella* species may be present but undetected. Also, plate culture agar has been optimised to select for the growth of *L. pneumophila*, and as such, other species that rarely cause disease may not be recovered (Walker and McDermott, 2021). Logan-Jackson and Rose (2021) highlighted that the culture method has limitations, including that they take time to yield results, the process requires highly trained personnel to accurately identify colonies, making it technically challenging, and the sensitivity of culture methods can be suboptimal, potentially missing cases with low bacterial counts.

Serology testing was conducted on all presumptive *L. pneumophila* isolates. However, none were positive for agglutination and hence cannot be serotyped. Serology tests for *Legionella* are primarily used to detect specific serogroups of the bacterium, with a significant focus on *L.pneumophila* serogroup 1 (SG1) due to its prevalence in causing Legionnaires' disease. However, serogroups 2- 14 are important, with studies showing the presence of these serogroups in environmental samples, and the detection of these serogroups is important for comprehensive epidemiological analysis. Legiolert™ methods are highly specific for *L. pneumophila*, but due to the presence of other bacteria capable of interfering with the enzymatic reaction, they can give false-positive results. These non-Legionella organisms may not be detectable by serology tests (Hirsh et al., 2021; Rech et al., 2018).

The *Legionella* spp. PCR results indicated a relatively low prevalence of general *Legionella* species. This discrepancy may arise from the higher sensitivity of PCR methods specifically targeting *L. pneumophila* or due to potential PCR inhibition affecting the detection of other Legionella species (Sylvestre et al., 2024; Yin et al., 2022; Walker and McDermott, 2021).

L. pneumophila was detected in amoeba samples. The detection of amoeba in water systems poses a health risk, as amoeba provides a nutrient-rich and protective environment for *L. pneumophila* against acidification and lysosomal digestion. This protozoon supports the replication of *L. pneumophila* and has been known to increase its virulency and the ability to resist environmental stresses (Pereira et al., 2021), such as desiccation and changes in pH, osmolarity, or temperature, and can even survive chlorination or other disinfection methods (Croze et al., 2021).

7.4.2 Sensitivity, Specificity, and Applicability of Methods Used to Detect *Legionella*

The choice of detection method depends on the balance between sensitivity, specificity, cost, and feasibility. In developed nations, a combination of culture, PCR, and molecular methods is used for accuracy, while in developing regions like South Africa, BCYE culture remains the primary tool due to cost constraints, despite its lower sensitivity compared to molecular techniques. This study was conducted as part of a capacity-building initiative aimed at enhancing research skills among three MSc students, focusing on the detection and analysis of *Legionella* in water systems.

The BCYE agar culture method, supplemented with cysteine, is widely used for *Legionella* isolation and recommended by SANS 11731:2017 and ISO 11731:2017 (Ditommaso et al., 2021). This method allows for direct colony identification but has low sensitivity due to the presence of *Legionella* in a viable but non-culturable (VBNC) state, environmental stressors, or chlorine residuals (Kirschner et al., 2016; Muchesa et al., 2018). Additionally, not all *Legionella* species grow well on BCYE, which means certain strains may go undetected (Walker and McDermott, 2021). BCYE cultures also take 5–15 days to yield results, making them less practical for rapid detection (Logan-Jackson and Rose, 2021).

The Legiolert™ method is a colorimetric, enzyme-based assay that detects *L. pneumophila* with higher sensitivity and faster turnaround (48-72 hours) compared to BCYE culture. However, Legiolert™ can produce false positives due to the presence of other bacteria that trigger the enzymatic reaction (Hirsh et al., 2021;

Rech et al., 2018). Legiolert™ is increasingly used in the USA and Europe as a faster alternative to culture methods.

Studies show Legiolert™ detects *L. pneumophila* in up to 85% of samples, comparable to PCR methods (Petrisek and Hall, 2018). However, confirmation using PCR is still recommended to reduce false positives (Scaturro et al., 2020).In South Africa, the cost of the test kits is high, which could limit their widespread adoption in resource-constrained laboratories.

Serology-based agglutination tests were performed in this study, but failed to detect *L. pneumophila*. This is expected since serology primarily targets *L. pneumophila* serogroup 1 (SG1), whereas other serogroups (SG2-14) also play a role in environmental infections (Monistero et al. 2024). Additionally, non-Legionella organisms cannot be detected by serology, further limiting their diagnostic value (Hirsh et al., 2021).

PCR was used to detect *Legionella* spp. in this study, with results showing lower prevalence than expected. This may be due to PCR inhibitors in water samples or higher specificity for *L. pneumophila* over other species (Sylvestre et al., 2024; Yin et al., 2022). PCR is widely recognized as the most sensitive method, but cost and infrastructure requirements limit its widespread use.

Developed countries invest in metagenomic sequencing to study Legionella-amoeba interactions, but cost limitations make this approach impractical in South Africa (Trigui et al., 2024). In a developing country context, BCYE culture remains the most feasible due to its low cost, but PCR and Legiolert™ could significantly improve detection accuracy in well-funded settings. While developed countries combine multiple methods for accuracy, financial and infrastructure constraints continue to limit the adoption of advanced techniques in South Africa (Monteiro et al., 2021).

7.4.3 *Legionella pneumophila* contamination ACROSS FIVE SITES

The data reveal significant differences in *L. pneumophila* contamination of water samples collected across the five sites, which can be attributed to variations in water infrastructure, maintenance practices, and socio-economic conditions:

Urban low-income areas (Hillbrow and Atteridgeville): These areas showed moderate to high levels of *Legionella* spp. contamination of water in geyser/boiler systems, likely due to biofilm development in older or poorly maintained heating systems. Cold tap water also shows significant contamination, particularly in Hillbrow.

Rural area (Vhembe): Vhembe exhibits high contamination with *Legionella* spp. of cold tap water, which may be due to less frequent monitoring and maintenance in rural water systems. Geyser/boiler systems in Vhembe also show high contamination levels involving *Legionella* spp., indicating a need for improved water heating and storage practices.

Informal settlements (Zandspruit and Melusi): These areas demonstrated variable contamination levels involving *Legionella* spp., with Melusi showing relatively lower contamination in cold tap water but higher levels in stored water. This variability highlights the challenges of managing water quality in informal settlements, where infrastructure is often inadequate.

7.5 INFLUENCE OF POPULATION DENSITY ON THE *LEGIONELLA PNEUMOPHILA* CONTAMINATION LEVELS

7.5.1 Increased water usage and stagnation

High population density often leads to increased water usage in residential buildings, resulting in fluctuating water pressure. This fluctuation can cause water stagnation in parts of the plumbing system, such as in pipes, tanks, and cooling towers. *L. pneumophila* thrives in stagnant water, especially at temperatures between 20°C and 50°C, increasing the likelihood of bacterial growth in densely populated areas (Schoen and Ashbolt, 2011). Conversely, low population density areas might not use water systems as intensively, leading to longer periods of stagnation in individual households, which can also foster *L. pneumophila* growth if the water temperature is favourable (Whiley et al., 2014). Detecting *Legionella* in diverse water sources, such as boreholes, taps (bathroom and kitchen), and storage tanks, suggests potential contamination at multiple points in the water distribution or storage process. Studies have shown that *Legionella* often proliferates in stagnant or inadequately disinfected water, particularly in biofilm-rich systems or environments (Margot et al., 2024; Sciuto et al., 2021; Yu et al., 2019).

7.5.2 Overburdened infrastructure

In areas with high population density, especially in informal settlements or older urban neighbourhoods, such as Hillbrow, the infrastructure may be overburdened. Thus, old, deteriorating pipes, tanks, and heating systems are more prone to biofilm formation where *L. pneumophila* can colonise (van der Kooij et al., 2017). The presence of biofilms in water systems can protect *L. pneumophila* from disinfectants, making it harder to eradicate such bacterial colonization once established. In contrast, areas with lower population density might have less strain on infrastructure, but they may also have less frequent maintenance or updates to water systems, which can similarly result in conditions that favour *L. pneumophila* growth (Rhoads et al., 2017).

7.5.3 Water system complexity

High-density areas often have complex water distribution systems with numerous junctions, diverse plumbing materials, storage tanks, and long piping networks. This complexity can create more opportunities for water to stagnate and for temperature fluctuations to occur, both of which are conducive for *L. pneumophila* proliferation (Lu et al., 2020). Lower-density areas generally have simpler water distribution systems, but if these systems are not properly maintained or if they are used less frequently, similar risks of contamination can arise (Proctor et al., 2017).

7.5.4 Temperature and water quality control

In densely populated urban areas, maintaining a consistent water temperature and ensuring adequate chlorination throughout the entire water system can be challenging. This is particularly true in high-rise buildings where water needs to be pumped to various levels, leading to potential temperature differences that can create niches for contamination and colonization by *L. pneumophila* (Buse et al., 2012a,b). In lower-density areas, while water systems are simpler, the challenge might lie in maintaining sufficient water temperature and disinfectant levels over longer distances, particularly in rural settings where the infrastructure might not be as robust (Hamilton et al., 2018b). *L. pneumophila* exhibits an ability to acclimate to elevated temperatures, with certain strains demonstrating resistance to brief exposures reaching 59°C attributed to genetic mutations in heat-shock response elements (Liang et al., 2023). Furthermore, the Lqs-LvbR regulatory framework is integral, overseeing the initiation of growth and cell density across diverse thermal conditions, with peak growth at 40°C and absence of proliferation beyond 50°C (Hochstrasser and Hilbi, 2021). This study showed that the temperature wherein *Legionella pneumophila* proliferated through Legiolert™ ranged from 16.9°C to 43.4°C, which overlaps with the optimal growth range for *L. pneumophila*, which is between 25 °C and 46 °C, suggesting that the environmental conditions are conducive to *Legionella* growth. The mean temperatures fall

within the optimal growth range for *Legionella*, further indicating a potential risk. However, the most concerning aspect is the growth observed below 20°C, as *Legionella* can grow and survive in biofilms at these temperatures.

7.5.5 Socio-economic factors and plumbing maintenance

High-density areas, particularly in informal settlements, might have limited resources for proper maintenance and sanitation of water systems. This can lead to increased risks of contamination, as seen in areas like Zandspruit, where informal housing and overcrowding stress the already limited infrastructure (Falkinham et al., 2015). In contrast, more affluent low-density areas might have better resources for maintenance, though complacency in regular checks can still lead to the risk of contamination with bacteria such as *Legionella* spp. (Rhoads et al., 2017).

7.5.6 Case examples from the data

Hillbrow, with its high population density and older infrastructure, showed substantial contamination of water with *L. pneumophila*, especially in cold tap and geyser/boiler water sources, with several samples exceeding the 101-1000 MPN/100 mL range. This can be attributed to the complex and stressed water systems in densely populated, high-rise buildings (Stone et al., 2019). Vhembe and Melusi, although less densely populated than Hillbrow, still showed considerable contamination, particularly in stored water, which is often more prone to stagnation and biofilm formation due to lower usage rates (Ramlal et al., 2022).

Thus, population density plays a crucial role in *L. pneumophila* contamination, influencing water system dynamics, infrastructure stress, and the potential for bacterial contamination and growth in water. High-density areas, especially those with aging infrastructure, face greater risks of contamination due to water stagnation, temperature fluctuations, and biofilm formation. However, even in less densely populated areas, poor maintenance and water system complexities can contribute to contamination risks. Addressing these factors through targeted infrastructure improvements, regular maintenance, and consistent water quality monitoring is essential for mitigating *L. pneumophila* risks.

7.6 RISK ASSOCIATED WITH *L. PNEUMOPHILA* IN HOUSEHOLDS

Results from the present study highlight the importance of targeted interventions to reduce *Legionella pneumophila* contamination in diverse environments. Urban low-income areas and informal settlements are particularly vulnerable due to aging infrastructure and inadequate water management practices. Improving water system maintenance, monitoring, and public health education are crucial for mitigating the risks associated with *Legionella pneumophila* in these communities.

The widespread presence of *L. pneumophila* across varying temperature ranges and socio-economic contexts underscores the need for targeted public health interventions. In rural and informal settlements, improving water infrastructure, ensuring regular water quality monitoring, and implementing public health education programs are essential for mitigating the risks associated with *L. pneumophila* (Gómez-Valcárcel et al., 2021). In low-income urban areas, upgrading aging infrastructure, maintaining consistent water temperatures, and controlling biofilm formation are critical for reducing bacterial contamination (Falkinham et al., 2020).

It is important to note that single residences are sometimes shared spaces, where each living space (living room, bedroom and kitchen) is rented out separately to different tenants. As such, these spaces are cramped and place pressure on resources, infrastructure and they impact the personal hygiene of individuals (Matshediso and Wafer, 2015). The multiple-floor infrastructure of high-rise buildings in Hillbrow and Vhembe provides a high surface-to-volume ratio that promotes microbial growth. Dead-legs (stagnation points) in the system are likely to occur, and the multi-directional flow of the water provides ample room for biofilm formation.

This is supported by Donohue et al. (2022), who noted that buildings with many floor levels are at a higher risk of *L. pneumophila* growth in their water systems compared to single-resident homes. Building age, plumbing maintenance and the availability of a water quality monitoring system play a role in determining the risk of exposure, behaviour and remedial action to be taken in case of risk and monitoring of water systems. Research has established that *Legionella* and other OPPPs tend to recur after remedial action has been taken, and therefore, continuous monitoring is important (Lee-Masi et al., 2024). All buildings included in the present study were classified as old, and recorded recent renovation was more than two years prior to the study. This poses a threat of *Legionella* contamination because old buildings are prone to corrosion of plumbing materials, which promotes the formation of biofilms in the system(Nisar et al., 2020).

Decreased water flow and stagnation of water in the plumbing systems of buildings and homes (due to water shortages) provide an ideal environment for the unrestricted growth of *Legionella* bacteria. *Legionella* can survive phagocytosis and evade antimicrobial control agents, utilizing protozoa as a replication space and benefiting from the availability of nutrients, which promotes their growth and proliferation. The presence and prevalence of *Legionella pneumophila*, an opportunistic pathogen causing Legionnaires' disease, as well as its emergence as an antibiotic-resistant pathogen, pose significant challenges to public health and patient management. The screening and control of *Legionella* in water systems is complex, and it is impractical to assume that *Legionella* could be completely removed from treated drinking water systems prior to use; thus, regular detection is of great importance for risk assessment and disease prevention.

Legionella grow within free-living amoebae, which are hardy and tolerate a wide range of temperatures and other environmental conditions (Greub and Raoult, 2014). *Legionella* replicate between 20°C and 50°C, with fastest growth as temperatures approach 40°C when most bacteria are killed. However, *Legionella* continues to thrive within amoebae. The symbiotic relationship between *Legionella* and free-living amoebae presents a significant public health concern, particularly in water systems. This relationship enhances the resilience of *Legionella* to a wide range of environmental conditions, including temperature variations

Legionella-containing aerosols can be released from a variety of sources within a bathroom, including the shower, the bathtub, the faucet, and the flushing mechanism of the toilet (Azuma et al., 2013). The air–water partitioning coefficient has been recognised as a key source of uncertainty in the *Legionella* QMRA method (Armstrong and Haas, 2007; Schoen and Ashbolt, 2011). *Legionella* bacteria of various types have been found in engineered water systems; however, the QMRA risk that has been predicted may be inaccurate if a significant proportion of the *Legionella* species that are present do not have virulence characteristics that are comparable to those of the limited range of *L. pneumophila* strains that have been studied to this point (Buse et al., 2012).

7.7 POSSIBLE EXPLANATIONS FOR THE LOW INCIDENCE OF LEGIONNAIRES' DISEASE (LD) IN SOUTH AFRICA

Legionellosis is a significant health risk worldwide, particularly in areas with rapidly growing urban populations. Legionella bacteria thrive in man-made water systems, especially when maintenance and sanitation standards are inadequate. While it is recognized as a major public health threat in developed countries, data on its presence is limited in developing regions like South Africa. Gauteng is a densely populated province with expanding urban and rural areas. The lack of data on *L. pneumophila* hinders the development of effective strategies to mitigate potential Legionellosis outbreaks in the province. Given *Legionella's* extraordinary self-preservation mechanisms, including its ability to survive within amoebae and evade phagocytic immune responses, this absence of outbreaks raises interesting questions.

Several hypotheses could explain why LD is less frequently reported in South Africa:

7.7.1 Young Population and Lower Susceptibility

South Africa has a young demographic population, with nearly 75% of the population being under 50 years old. Since LD predominantly affects the elderly and immunocompromised individuals, young, immunocompetent individuals may have a more effective immune response, preventing progression to severe LD, unlike in developed nations where aging populations are more susceptible (Scaturro et al., 2020). However, sporadic cases may still be underreported due to diagnostic challenges and misdiagnosis as community-acquired pneumonia.

7.7.2 Role of HIV and Immune Modulation

South Africa has a high HIV prevalence, which could influence *Legionella* infection dynamics. A highly active immune system, despite immunosuppression, may stress intracellular *Legionella* less, potentially reducing virulence. Some studies suggest chronic immune activation in HIV may increase susceptibility to bacterial infections, but others debate that immune dysregulation alters bacterial persistence rather than triggering outbreaks (Donohue et al., 2023). It is also possible that LD is being underdiagnosed in immunocompromised patients, as *Legionella* testing is not routine in South African hospitals.

7.7.3 Cross-Reactive Immunity from Extensive Microbial Exposure

South Africans are continuously exposed to a broad spectrum of environmental pathogens, including *Mycobacterium tuberculosis*, *non-tuberculous mycobacteria* (NTM), and multiple enteric bacteria, which may prime the immune system, leading to cross-protective immunity against *Legionella*. Research suggests that past infections with bacteria can induce broad-spectrum immune responses, particularly through trained innate immunity and cross-reactive antibodies (Monteiro et al., 2021; Walker and McDermott, 2021). *Legionella* can replicate within amoebae and evade host immune responses, making it difficult to detect via standard methods (Stone et al., 2019; Trigui et al., 2024). While amoeba-associated *Legionella* may increase environmental persistence, their role in human infection remains poorly understood.

CHAPTER 8: CONCLUSION

8.1 INTRODUCTION

The findings from this analysis highlight the significant influence of temperature and socio-economic factors on *L. pneumophila* contamination in diverse environments. The ability of such bacteria to persist across a broad temperature range, particularly in areas with inadequate water infrastructure, poses a substantial public health risk. Addressing these risks requires a multi-faceted approach that includes infrastructure improvements, public health interventions, and ongoing research to better understand the environmental conditions that favor *L. pneumophila* proliferation.

8.2 STUDY OBJECTIVES AND RESULTS

Objective 1: To conduct a scoping study on determining the presence and diversity of Legionella spp. in selected buildings, in both urban and rural areas.

Relevant Observations and Results:

- The study confirmed the presence of *Legionella* across a variety of urban and rural settings. Urban areas, especially those with high population densities and older water systems, showed a higher prevalence of *Legionella*.
- There was a notable diversity in *Legionella* identified, with urban environments showing a greater number of isolates potentially linked to the variety and complexity of water system infrastructures in these areas.
- The bacteria's ability to persist across a broad temperature range, particularly in areas with inadequate water infrastructure, poses a substantial public health risk. Mitigating these risks requires a comprehensive approach, including infrastructure improvements, public health interventions, and further research on environmental factors promoting *L. pneumophila* growth and proliferation.
- Objective 1 was suitably addressed by highlighting critical areas for monitoring and intervention, which could aid in the development of tailored public health policies and practices.

Objective 2: To determine any genetic variations in Legionella sp. using next-generation molecular techniques and subsequently estimate which water samples posed the greatest health risk of exposure to Legionella strains.

Relevant Observations and Results:

- The analysis and results from this objective demonstrate that it was effectively met, providing foundational knowledge for enhancing disease surveillance and outbreak management systems.
- The use of advanced molecular techniques enabled precise identification of risk-related genetic traits of *Legionella*, enhancing predictive capabilities for public health strategies

Objective 3: To provide recommendations for water quality monitoring and safety management in buildings.

Relevant Observations and Results:

- The study underscored the need for regular monitoring and proactive management of building water systems to prevent Legionella growth and proliferation.

- This objective was comprehensively addressed through the development of actionable and evidence-based recommendations aimed at improving water safety and public health outcomes.

This study advances our understanding of *Legionella's* environmental presence and genetic diversity within South Africa's unique urban and rural landscapes. By aligning our findings with actionable recommendations, we aim to inform and enhance public health strategies, ultimately reducing the incidence and impact of *Legionella*-related health issues. The systematic approach adopted in this research addresses the complex dynamics of *Legionella* in varied settings and sets a precedent for future studies aiming to bridge the gap between scientific research and public health policy implementation.

8.3 STUDY LIMITATIONS

While the study provides substantial evidence on the presence and risks of *L. pneumophila* in water systems, it acknowledges certain limitations such as sample size constraints and inaccessible areas. Other limitations to this study include:

- The unsafe and inaccessible buildings in Hillbrow restricted the sample set and did not allow for a well-rounded result.
- Load-shedding (electricity cuts) limited the sample collection
- The unavailability of a building water distribution plan restricted the research because it would have allowed for the location of dead-legs and stagnation water points in the buildings.
- Storage tanks were inaccessible in most of the buildings, and this also restricted the diversity of the water samples collected and a larger sample set that would have allowed researchers to determine the impact of storage tanks on the water quality on a larger scale.
- Due to high electricity costs, many households with geysers are unable to afford heating water, leading to fewer hot water samples collected.
- A few swab samples were collected because household members did not allow access to collect swab samples within their premises.

8.4 CONCLUSION

This study has comprehensively explored the presence, diversity, and genetic variations of Legionella spp. across different urban and rural settings in South Africa. By systematically investigating these aspects, the research has illuminated the crucial interplay between environmental factors and public health risks associated with waterborne pathogens. The findings underscore the necessity for region-specific monitoring and tailored interventions to mitigate the risks posed by Legionella. Through a targeted approach informed by molecular and epidemiological data, this research supports the development of more effective public health strategies and water safety protocols, aiming to safeguard communities against the potential threats posed by Legionella infections. As we continue to refine these strategies, the insights gained here will undoubtedly contribute to the resilience and health security of urban and rural populations alike.

8.5 RECOMMENDATIONS

Based on the findings of this study, the following recommendations are proposed to mitigate the risks associated with *Legionella* contamination in South Africa's water systems:

Regular maintenance and monitoring:

- Implement a comprehensive maintenance schedule for water systems, particularly in high-risk areas such as hospitals, high-density urban neighbourhoods, and informal settlements.

- Ensure that water temperature and chlorination levels are consistently monitored and maintained to prevent the growth of *Legionella* .
- Regularly inspect and clean storage tanks, pipes, and other components of the water distribution system to reduce biofilm formation and water stagnation.

Enhanced public health surveillance:

- Strengthen the capacity for *Legionella* detection and diagnosis in public health laboratories across South Africa, with a focus on using next-generation molecular techniques for faster and more accurate identification of *Legionella* strains.
- Integrate *Legionella* testing into routine water quality monitoring programs, particularly in facilities serving vulnerable populations such as hospitals, care homes, and schools.
- Encourage reporting of *Legionella* cases to improve data collection and support better understanding of the epidemiology of Legionnaires' disease in the country.

Educational outreach and awareness:

- Develop and distribute educational materials aimed at building managers, healthcare providers, and the general public to raise awareness about the risks of *Legionella* and the importance of preventive measures.
- Facilitate training programs for maintenance staff and public health officials to improve the implementation of *Legionella* prevention and control strategies.
- Introduce educational programs, especially in the informal areas on how to practice hygienic storage practices, including closing their water storage containers unless water is in use and to use hot water with washing detergents including bleach and soap to wash the container, as well as to use clean cups or jars when collecting water from the storage containers.
- For researchers to combine traditional culture methods with molecular methods to detect both viable and non-cultural forms of bacteria.

REFERENCES

1. Abu Khweek, A., and Amer, O. (2018). Factors mediating environmental biofilm formation by *Legionella* pneumophila. Frontiers in Cellular and Infection Microbiology, 8. 38. https://doi.org/10.3389/fcimb.2018.00038
2. Agyeman, W. Y., Bisht, A., Gopinath, A., Cheema, A. H., Chaludiya, K., Khalid, M., Nwosu, M., Konka, S., and Khan, S. (2022). A systematic review of antibiotic resistance trends and treatment options for hospital-acquired multidrug-resistant infections. Cureus, 14(10).
3. Allegra, S., Riffard, S., Leclerc, L., Girardot, F., Stauffert, M., Forest, V., and Pourchez, J. (2020). A valuable experimental setup to model exposure to *Legionella*'s aerosols generated by shower-like systems. Water Research, 172:115496
4. Armstrong, P. M., Uapipatanakul, M., Thompson, I., Ager, D., and McCulloch, M. (2014). Thermal and sanitary performance of domestic hot water cylinders: Conflicting requirements. Applied Energy, 131, 171–179. https://doi.org/10.1016/J.APENERGY.2014.06.021
5. Armstrong, T. W., and Haas, C. N. (2007). Quantitative microbial risk assessment model for Legionnaires' disease: Assessment of human exposures for selected spa outbreaks. Journal of Occupational and Environmental Hygiene, 4(9), 634–646. https://doi.org/10.1080/15459620701487539
6. Assaidi, A., Ellouali, M., Latrache, H., Zahir, H., Karoumi, A. and Mliji, E. M., 2020. Chlorine disinfection against *Legionella pneumophila* biofilms. Journal of Water, Sanitation and Hygiene for Development, 10(4), 885-893.
7. Azuma, K., Uchiyama, I., and Okumura, J. (2013). Assessing the risk of Legionnaires' disease: The inhalation exposure model and the estimated risk in residential bathrooms. Regulatory Toxicology and Pharmacology, 65(1), 1–6. https://doi.org/10.1016/j.yrtph.2012.11.003
8. Barbosa, A., Azevedo, N. F., Goeres, D. M., and Cerqueira, L. (2024). Ecology of *Legionella pneumophila* biofilms: The link between transcriptional activity and the biphasic cycle. Biofilm, 100196.
9. Bartie, C., Muchesa, P., Barnard, T. G. and South Africa. Water Research Commission. (2016). An investigation into the presence of free living amoebae and amoeba resistant bacteria in drinking water distribution systems of health care institutions in Johannesburg, South Africa: Report to the Water Research Commission. WRC Report No. 2138/1/16. ISBN 978-1-4312-0764-0
10. Bartie, C., Venter, S. N., and Nel, L. H. (2003). Identification methods for *Legionella* from environmental samples. Water Research, 37(6), 1362–1370. https://doi.org/10.1016/S0043-1354(02)00220-8
11. Bartram, J., Cronk, R., Montgomery, M., Gordon, B., and Neira, M. (2020). Maintaining safe water and sanitation Infrastructure in urban settings: The role of effective governance. Journal of Water and Health, 18(4), 597-609.
12. Bédard, E., Paranjape, K., Lalancette, C., Villion, M., Quach, C., Laferrière, C., Faucher, S. P., and Prévost, M. (2019). *Legionella pneumophila* levels and sequence-type distribution in hospital hot water samples from faucets to connecting pipes. Water Research, 156, 277–286. https://doi.org/10.1016/j.watres.2019.03.019
13. Bentham, R., and Whiley, H. (2018). Quantitative microbial risk assessment and opportunist waterborne infections-are there too many gaps to fill? https://doi.org/10.3390/ijerph15061150
14. Bessong, P. O., Odiyo, J. O., Musekene, J. N., and Tessema, A. (2009). Spatial distribution of diarrhoea and microbial quality of domestic water during an outbreak of diarrhoea in the Vhembe district, South Africa. Journal of Health, Population, and Nutrition, 27(6), 652-659.
15. Boczek, L.A., Tang, M., Formal, C., Lytle, D. and Ryu, H. (2021). Comparison of two culture methods for the enumeration of *Legionella pneumophila* from potable water samples. Journal of Water and Health, 19(3), 468-477.
16. Bonetta, S., and Bonetta, S. (2020). Editorial comments to the special issue: "*Legionella* contamination in water environment." Pathogens, 9(12), 1–6. https://doi.org/10.3390/pathogens9121017
17. Bonetta, S., Pignata, C., Bonetta, S., Meucci, L., Giacosa, D., Marino, E., Gorrasi, I., Gilli, G., and Carraro, E. (2018). Effectiveness of a neutral electrolysed oxidising water (NEOW) device in reducing *Legionella pneumophila* in a water distribution system: A comparison between culture, qPCR and PMA-qPCR detection methods. Chemosphere, 210, 550–556. https://doi.org/10.1016/J.CHEMOSPHERE.2018.07.053

18. Buchrieser, C., and Hilbi, H. (2019). *Legionella* Methods and Protocols Second Edition. Methods in Molecular Biology, 1921. http://www.springer.com/series/7651
19. Burillo, A., Pedro-Botet, M. L., and Bouza, E. (2017). Microbiology and epidemiology of Legionnaire's Disease. Infectious Disease Clinics of North America, 31(1), 7–27. https://doi.org/10.1016/J.IDC.2016.10.002
20. Buse, H. Y., Morris, B. J., Gomez-Alvarez, V., Szabo, J. G., and Hall, J. S. (2020). *Legionella* diversity and spatiotemporal variation in the occurrence of opportunistic pathogens within a large building water system. Pathogens, 9(7), 1–29. https://doi.org/10.3390/pathogens9070567
21. Buse, H. Y., Schoen, M. E., and Ashbolt, N. J. (2012a). *Legionella* in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Research, 46(4), 921–933). https://doi.org/10.1016/j.watres.2011.12.022
22. Buse, H. Y., Schoen, M. E., and Ashbolt, N. J. (2012b). *Legionella* ecology in engineering systems and an appreciation of disinfectant residual. Water Research, 46(4), 947-959.
23. Butterworth, F. (2024). Monochloramine – A new approach to disinfection of water systems. Online Journal for the Water Management Society. https://www.goodwater.co.uk/monochloramine-a-new-approach-to-disinfection-by-our-frank-butterworth/
24. Caicedo, C., Rosenwinkel, K.-H., Exner, M., Verstraete, W., Suchenwirth, R., Hartemann, P., and Nogueira, R. (2019). *Legionella* occurrence in municipal and industrial wastewater treatment plants and risks of reclaimed wastewater reuse: Review. Water Research, 149, 21–34. https://doi.org/10.1016/j.watres.2018.10.080
25. Centers for Disease Control and Prevention (CDC). (2022). Legionnaires' Disease Prevention Providing a Home for Guests, not *Legionella*. https://stacks.cdc.gov/view/cdc/135549
26. Centers for Disease Control and Prevention. 2024. Investigating Legionnaires' disease: Preventing Healthcare-associated Legionnaires' Disease. Accessed at: Preventing Healthcare-associated Legionnaires' Disease. Accessed at: https://www.cdc.gov/control-legionella/php/wmp/index.html
27. Centers for Disease Control and Prevention (CDC). (2020). About Water Disinfection with Chlorine and Chloramine. Accessed at: Water Disinfection with Chlorine and Chloramine.
28. Centers for Disease Control and Prevention (CDC) and National Center for Immunization and Respiratory Diseases (NCIRD). (2021). Developing a water management program to reduce *Legionella* growth and spread in buildings: A practical guide to implementing industry standards. www.cdc.gov/ *Legionella*
29. Cervero-Aragó, S., Rodríguez-Martínez, S., Puertas-Bennasar, A., and Araujo, R. M. (2015). Effect of common drinking water disinfectants, chlorine and heat, on free *Legionella* and amoebae- associated *Legionella* . PLoS ONE, 10(8). https://doi.org/10.1371/journal.pone.0134726
30. Chauhan, D., and Shames, S. R. (2021). Pathogenicity and virulence of *Legionella*: Intracellular replication and host response. Virulence, 12(1), 1122-1144.
31. Consonni, M., Grassi, A., Scuri, S., Gori, M., Tanzi, E., and Tesauro, M. (2021). Assessing the viability of *Legionella pneumophila* in environmental samples: Regarding the filter application of ethidium monoazide bromide. Annals of Microbiology, 71(1), 43. https://doi.org/10.1186/s13213-021- 01653-5
32. Cooper, I. R., and Hanlon, G. W. (2010). Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. Journal of Hospital Infection 74(2), 152-159.
33. Coovadia, H., Jewkes, R., Barron, P., Sanders, D., and McIntyre, D. (2009). The health and health system of South Africa: Historical roots of current public health challenges. The Lancet, 374(9692), 817-834.
34. Couturier, J., Ginevra, C., Nesa, D., Adam, M., Gouot, C., Descours, G., Campèse, C., Battipaglia, G., Brissot, E., Beraud, L., Ranc, A. G., Jarraud, S., and Barbut, F. (2020). Transmission of Legionnaires' Disease through Toilet Flushing. Emerging infectious diseases, 26(7), 1526–1528. https://doi.org/10.3201/eid2607.190941.
35. Cullom, A. C., Martin, R. L., Song, Y., Williams, K., Williams, A., Pruden, A., and Edwards, M. A. (2020). Critical review: Propensity of premise plumbing pipe materials to enhance or diminish growth of *Legionella* and other opportunistic pathogens. Pathogens, 9(11), 1–34. https://doi.org/10.3390/pathogens9110957
36. Danakumara, T., Kumar, N., Patil, B. S., Kumar, T., Bharadwaj, C., Jain, P. K., Nimmy, M. S., Joshi, N., Parida, S. K., Bindra, S., Kole, C., and Varshney, R. K. (2024). Unraveling the genetics of heat tolerance in chickpea landraces (Cicer arietinum L.) using genome-wide association studies. Frontiers In Plant Science, 15, 1376381. https://doi.org/10.3389/fpls.2024.1376381

37. De Giglio, O., Napoli, C., Lovero, G., Diella, G., Rutigliano, S., Caggiano, G., and Montagna, M. T. (2015). Antibiotic susceptibility of *Legionella pneumophila* strains isolated from hospital water systems in Southern Italy. Environmental Research, 142, 586-590.
38. Delgado-Viscogliosi, P., Solignac, L., and Delattre, J. M. (2009). Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Applied and Environmental Microbiology, 75(11), 3502–3512. https://doi.org/10.1128/AEM.02878-08
39. Ditommaso, S., Giacomuzzi, M., Memoli, G., Garlasco, J., and Zotti, C. M. (2023). Confirmation of presumptive *Legionella* colonies on culture media according to ISO 11731:2017: principles, problems, and practice. Journal of Applied Microbiology, 134(6), lxad100. https://doi.org/10.1093/jambio/lxad100
40. Ditommaso, S., Giacomuzzi, M., Memoli, G., Garlasco, J., and Zotti, C. M. (2021). Comparison of BCYEα+AB agar and MWY agar for detection and enumeration of *Legionella* spp. in hospital water samples. BMC Microbiology, 21(1), 48. https://doi.org/10.1186/s12866-021-02109-1
41. Ditommaso, S., Giacomuzzi, M., Ricciardi, E., and Zotti, C. M. (2016). Cultural and molecular evidence of *Legionella* spp. colonization in dental unit waterlines: Which is the best method for risk assessment? International Journal of Environmental Research and Public Health, 13(2). https://doi.org/10.3390/ijerph13020211
42. Ditommaso, S., Ricciardi, E., Giacomuzzi, M., Arauco Rivera, S. R., and Zotti, C. M. (2015). *Legionella* in water samples: how can you interpret the results obtained by quantitative PCR?. Molecular and Cellular Probes, 29(1), 7–12. https://doi.org/10.1016/j.mcp.2014.09.002
43. Ditommaso, S., Ricciardi, E., Giacomuzzi, M., Arauco Rivera, S. R., Ceccarelli, A., and Zotti, C. M. (2014). Overestimation of the *Legionella* spp. load in environmental samples by quantitative real-time PCR: Pretreatment with propidium monoazide as a tool for the assessment of an association between *Legionella* concentration and sanitary risk. Diagnostic Microbiology and Infectious Disease, 80(4), 260–266. https://doi.org/10.1016/j.diagmicrobio.2014.09.010
44. Dobrowsky, P. H., Khan, S., and Khan, W. (2017). Resistance of *Legionella* and *Acanthamoeba mauritaniensis* to heat treatment as determined by relative and quantitative polymerase chain reactions. Environmental Research, 158, 82–93. https://doi.org/10.1016/j.envres.2017.06.003
45. Domazetovska, A., Jensen, S. O., Gray, M., Radzieta, M., and Maley, M. (2022). Culture-Free Phylogenetic Analysis of *Legionella pneumophila* Using Targeted CRISPR/Cas9 Next-Generation Sequencing. Microbiology Spectrum, 10(4), e0035922. https://doi.org/10.1128/spectrum.00359-22
46. Donohue, M. J., Pham, M., Brown, S., Easwaran, K. M., Vesper, S., and Mistry, J. H. (2023). Water quality influences *Legionella pneumophila* determination. Water research, 238, 119989. https://doi.org/10.1016/j.watres.2023.119989
47. Eble, D., Gehrig, V., Schubert-Ullrich, P., Köppel, R., and Füchslin, H. P. (2021). Comparison of the culture method with multiplex PCR for the confirmation of *Legionella* spp. and *Legionella pneumophila*. Journal of applied microbiology, 131(5), 2600–2609. https://doi.org/10.1111/jam.15103
48. Edelstein, P. H. (2007). Urine antigen tests positive for Pontiac Fever: Implications for diagnosis and pathogenesis. Clinical Infectious Diseases, 229. https://academic.oup.com/cid/article/44/2/229/330131
49. Falkinham, J. O. (2015). Common features of opportunistic premise plumbing pathogens. International Journal of Environmental Research and Public Health, 12(5), 4533–4545. https://doi.org/10.3390/ijerph120504533
50. Falkinham, J. O., Hilborn, E. D., Arduino, M. J., Pruden, A., and Edwards, M. A. (2015a). Epidemiology and ecology of opportunistic premise plumbing pathogens: *Legionella pneumophila, Mycobacterium avium*, and *Pseudomonas aeruginosa*. Environmental Health Perspectives, 123(8), 749-758.
51. Falkinham, J. O., Pruden, A., and Edwards, M. (2015b). Opportunistic premise plumbing pathogens: Increasingly important pathogens in drinking water. Pathogens, 4(2), 373–386. https://doi.org/10.3390/pathogens4020373
52. Falkinham, J. O. (2020). Living with *Legionella* and other waterborne pathogens. Microorganisms, 8(12), 1–10. https://doi.org/10.3390/microorganisms8122026
53. Falkinham, J. O. , Pruden, A., and Edwards, M. A. (2020). Critical control points for *Legionella* in water systems: A public health perspective. International Journal of Environmental Research and Public Health, 17(4), 1495. https://doi.org/10.3390/ijerph17041495

54. Farhat, M., Shaheed, R. A., Al-Ali, H. H., Al-Ghamdi, A. S., Al-Hamaqi, G. M., Maan, H. S., Al-Mahfoodh, Z. A., and Al-Seba, H. Z. (2018). Legionella confirmation in cooling tower water. Comparison of culture, real-time PCR and next generation sequencing. Saudi Medical Journal, 39(2), 137–141. https://doi.org/10.15537/smj.2018.2.21587
55. Fields, B. S., Benson, R. F., and Besser, R. E. (2002). *Legionella* and Legionnaires' Disease: 25 Years of Investigation. Clinical Microbiology Reviews, 15(3), 506–526. https://doi.org/10.1128/CMR.15.3.506-526.2002
56. Filice, S., Sciuto, E. L., Scalese, S., Faro, G., Libertino, S., Corso, D., Timpanaro, R. M., Laganà, P., and Coniglio, M. A. (2022). Innovative antibiofilm smart surface against *Legionella* for water systems. Microorganisms, 10(5). https://doi.org/10.3390/microorganisms10050870
57. Finnish Institute for Health and Welfare., 2023. Environmental Health: *Legionella* bacteria in water systems. Accessed at: Legionella bacteria in water systems. https://thl.fi/en/topics/environmental-health/water/legionella-bacteria-in-water-systems
58. Gavaldà, L., Garcia-Nuñez, M., Quero, S., Gutierrez-Milla, C., and Sabrià, M. (2019). Role of hot water temperature and water system use on *Legionella* control in a tertiary hospital: An 8-year longitudinal study. WaterResearch,149,460–466. https://doi.org/10.1016/j.watres.2018.11.032
59. Gómez-Valcárcel, M., García-Álvarez, L., López-Sánchez, A., and Lomas-Martín, M. (2021). Water quality in low-income urban areas: Assessing the risks of *Legionella* and other pathogens. Water, 13(2), 134. https://doi.org/10.3390/w13020134
60. Graham, F. F., Hales, S., White, P. S., and Baker, M. G. (2020). Review Global seroprevalence of legionellosis - a systematic review and meta-analysis. Scientific Reports, 10(1). https://doi.org/10.1038/s41598-020-63740-y
61. Gwenzi, W., Marumure, J., Makuvara, Z., Simbanegavi, T. T., Njomou-Ngounou, E. L., Nya, E. L., Kaetzl, K., Noubactep, C., and Rzymski, P. (2023). The pit latrine paradox in low-income settings: A sanitation technology of choice or a pollution hotspot?. The Science of The Total Environment, 879, 163179. https://doi.org/10.1016/j.scitotenv.2023.163179
62. Hamilton, K. A., Pruden, A., Marr, L. C., and Edwards, M. A. (2018a). Operating conditions that influence *Legionella*, *Mycobacterium*, and *Pseudomonas aeruginosa* growth in household plumbing. Environmental Science: Water Research and Technology, 4(5), 750-761.
63. Hamilton, K. A., Hamilton, M. T., Johnson, W., Jjemba, P., Bukhari, Z., LeChevallier, M., and Haas, C. N. (2018b). Health risks from exposure to *Legionella* in reclaimed water aerosols: Toilet flushing, spray irrigation, and cooling towers. Water research, 134, 261–279. https://doi.org/10.1016/j.watres.2017.12.022Ji,
64. Haas, C. N., Rose, J. B., and Gerba, C. P. (1999). Quantitative Microbial Risk Assessment. New York: John Wiley and Sons.
65. Hirsh, M., Baron, J. L., Mietzner, S., Rihs, J. D., and Stout, J. E. (2021). Cross-reactivity of the IDEXX Legiolert method with other Gram-negative bacteria and waterborne pathogens leads to false-positive assay results. Letters in Applied Microbiology, 72(6), 750–756. https://doi.org/10.1111/lam.13469
66. Hochstrasser, R., and Hilbi, H. (2022). The Legionella Lqs-LvbR Regulatory Network Controls Temperature-Dependent Growth Onset and Bacterial Cell Density. Applied and Environmental Microbiology, 88(5), e0237021. https://doi.org/10.1128/aem.02370-21
67. Hsu, W. T., , B. M., Chang, T. Y., Hsu, T. K., Kao, P. M., Huang, K. H., Tsai, S. F., Huang, Y. L., and Fan, C. W. (2014). Surveillance and evaluation of the infection risk of free-living amoebae and *Legionella* in different aquatic environments. Science of the Total Environment, 499, 212–219. https://doi.org/10.1016/j.scitotenv.2014.07.116
68. Hunter, C. M., Salandy, S. W., Smith, J. C., Edens, C., and Hubbard, B. (2022). Racial Disparities in Incidence of Legionnaires' Disease and Social Determinants of Health: A Narrative Review. Public health reports (Washington, D.C. : 1974), 137(4), 660–671. https://doi.org/10.1177/00333549211026781
69. Kirschner, A. K. (2016). Determination of viable *Legionella* in engineered water systems: Do we find what we are looking for? Water Research, 93, 276-288.
70. LeChevallier, M. W. (2019). Monitoring distribution systems for *Legionella pneumophila* using Legiolert. AWWA Water Science, 1(1), e1122.

71. LeChevallier, M. (2023). Examining the efficacy of copper-silver ionization for management of *Legionella*: Recommendations for optimal use. AWWA Water Science, 5(2), e1327.
72. Lee-Masi, M., Coulter, C., Chow, S. J., Zaitchik, B., Jacangelo, J. G., Exum, N. G., and Schwab, K. J. (2024). Two-year evaluation of Legionella in an aging residential building: Assessment of multiple potable water remediation approaches. The Science of the total environment, 941, 173710. https://doi.org/10.1016/j.scitotenv.2024.173710
73. Leslie, E., Hinds, J., and Hai, F. I. (2021). Causes, Factors, and Control Measures of Opportunistic Premise Plumbing Pathogens—A Critical Review. Applied Sciences, 11(10), 4474. https://doi.org/10.3390/app11104474.
74. Lesnik, R., Brettar, I., and Höfle, M. G. (2016). *Legionella* species diversity and dynamics from surface reservoir to tap water: From cold adaptation to thermophily. The ISME Journal, 10(5), 1064–1080. https://doi.org/10.1038/ismej.2015.199
75. Li, L., Mendis, N., Trigui, H., Oliver, J. D., and Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. Frontiers in Microbiology, 5, 258. https://doi.org/10.3389/fmicb.2014.00258
76. Liang, J., Cameron, G., and Faucher, S. P. (2023). Development of heat-shock resistance in Legionella pneumophila modeled by experimental evolution. Applied and Environmental Microbiology, 89(9), e0066623. https://doi.org/10.1128/aem.00666-23
77. Logan-Jackson, A., and Rose, J. B. (2021). Water age effects on the occurrence and concentration of *Legionella* species in the distribution system, premise plumbing, and the cooling towers. Microorganisms, 10(1), 81. https://doi.org/10.3390/microorganisms10010081
78. Lombardi, A., Borriello, T., De Rosa, E., Di Duca, F., Sorrentino, M., Torre, I., Montuori, P., Trama, U., and Pennino, F. (2023). Environmental monitoring of *Legionella* in hospitals in the Campania Region: A 5-Year Study. International Journal of Environmental Research and Public Health, 20(8), 5526. https://doi.org/10.3390/ijerph20085526
79. Lu, J., Struewing, I., Yelton, S., and Ashbolt, N. J. (2020). *Legionella pneumophila* in engineered water systems: The role of biofilms and amoebae. Journal of Water and Health, 18(1), 1-13.
80. Magdzińska M, Cudzik-Dziurzyńska J, Błaszczyk A, Barwinek K. (2023). *Legionella pneumophila* as an important public health problem – epidemiology and clinical management of Legionnaires' disease. Medycyna Środowiskowa – Environmental Medicine. 26(3–4): 125–128. doi: 10.26444/ms/172892
81. Marchesi, I., Paduano, S., Frezza, G., Sircana, L., Vecchi, E., Zuccarello, P., Oliveri Conti, G., Ferrante, M., Borella, P. and Bargellini, A. (2020). Safety and effectiveness of monochloramine treatment for disinfecting hospital water networks. International Journal of Environmental Research and Public Health, 17(17), 6116.
82. Mansi, A., Amori, I., Marchesi, I., Marcelloni, A. M., Proietto, A. R., Ferranti, G., Magini, V., Valeriani, F., and Borella, P. (2014). *Legionella* spp. survival after different disinfection procedures: Comparison between conventional culture, qPCR and EMA-qPCR. Microchemical Journal, 112, 65–69. https://doi.org/10.1016/j.microc.2013.09.017
83. Moffa, M. A., Rock, C., Galiatsatos, P., Gamage, S. D., Schwab, K. J., and Exum, N. G. (2023). Legionellosis on the rise: A scoping review of sporadic, community-acquired incidence in the United States. Epidemiology and Infection, 151, e133. https://doi.org/10.1017/S0950268823001206
84. Monistero, V., Vicari, N., Prati, P., Bragoni, R., Gazzola, A., Sala, L., Maisano, A., Moroni, P., Bronzo, V., Luini, M. V., Castiglioni, B., and Cremonesi, P. (2024). A rapid and reliable method for early *Legionella pneumophila* identification and characterization in support of the epidemiology study. Frontiers in Microbiology, 15, 1452861. https://doi.org/10.3389/fmicb.2024.1452861
85. Monteiro, S. N., Robalo, A. M., and Santos, R. J. (2021). Evaluation of Legiolert™ for the Detection of *Legionella pneumophila* and Comparison with Spread-Plate Culture and qPCR Methods. Current Microbiology, 78(5), 1792–1797. https://doi.org/10.1007/s00284-021-02436-6
86. Mraz, A. L., and Weir, M. H. (2022). Knowledge to predict pathogens: *Legionella pneumophila* lifecycle systematic review part II growth within and egress from a host cell. Microorganisms, 10(1), 141
87. Muchesa, P., Leifels, M., Jurzik, L., Barnard, T., and Bartie, C. (2018). Detection of amoeba-associated *Legionella pneumophila* in hospital water networks of Johannesburg. Southern African Journal of Infectious Diseases, 33(3), 72–75. https://doi.org/10.1080/23120053.2018.1434060

88. Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P., and Wool, E. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. The lancet, 399(10325), 629-655.
89. Naicker, N; Mathee, A; Teare, J. (2015). Food insecurity in households in informal settlements in urban South Africa. South African Medical Journal, 1(105), 268-270
90. National Academies of Sciences. (2020). Technology Board and Committee on Management of *Legionella* in Water Systems, 2020. *Management of Legionella in water systems*. National Academies Press.
91. Nicas, M., and Hubbard, A. (2002). A risk analysis for airborne pathogens with low infectious doses: application to respirator selection against Coccidioides immitis spores. Risk analysis : an official publication of the Society for Risk Analysis, 22(6), 1153–1163. https://doi.org/10.1111/1539-6924.00279
92. Niculita-Hirzel, H., Vanhove, A. S., Leclerc, L., Girardot, F., Pourchez, J., and Allegra, S. (2022). Risk Exposure to Legionella pneumophila during Showering: The Difference between a Classical and a Water Saving Shower System. International Journal Of Environmental Research and Public Health, 19(6), 3285. https://doi.org/10.3390/ijerph19063285
93. Nisar, M. A., Ross, K. E., Brown, M. H., Bentham, R., and Whiley, H. (2020). *Legionella pneumophila* and protozoan hosts: Implications for the control of hospital and potable water systems. Pathogens, 9(4). https://doi.org/10.3390/pathogens9040286
94. Nisar, M. A., Ross, K. E., Brown, M. H., Bentham, R., Best, G., and Whiley, H. (2023). Detection and quantification of viable but non-culturable Legionella pneumophila from water samples using flow cytometry-cell sorting and quantitative PCR. Frontiers in Microbiology, 14, 1094877. https://doi.org/10.3389/fmicb.2023.1094877
95. Ohno, A., Kato, N., Sakamoto, R., Kimura, S., and Yamaguchi, K. (2008). Temperature-dependent parasitic relationship between *Legionella pneumophila* and a free-living amoeba (*Acanthamoeba castellanii*). Applied and Environmental Microbiology, 74(14), 4585–4588. https://doi.org/10.1128/AEM.00083-08
96. Ortí-Lucas, R. M., and Luciano, E. (2022). New immunomagnetic separation method to analyze risk factors for *Legionella* colonization in health care centres. Journal of Exposure Science and Environmental Epidemiology, 32(5), 744–750. https://doi.org/10.1038/s41370-022-00421-0
97. Palazzolo, C., Maffongelli, G., Lepore, L., Mariano, A., Vulcano, A., Ascoli Bartoli, T., Bevilacqua, N., Letizia Giancola, M., di Rosa, E., Nicastri, E., Claudia, P., Gaetano, M., Alessandra, A., Luciana, L., Andrea, M., Antonella, V., Tommaso Ascoli, B., Nazario, B., Maria Letizia, G., Emanuele, N. (2020). *Legionella* pneumonia: Increased risk after COVID-19 lockdown? Italy, May to June 2020. Eurosurveillance. https://doi.org/10.2807/1560-7917.ES.2020.25.30.2001372
98. Pappa, O., Chochlakis, D., Sandalakis, V., Dioli, C., Psaroulaki, A., and Mavridou, A. (2020). Antibiotic resistance of *Legionella pneumophila* in clinical and water isolates—A systematic review. International Journal of Environmental Research and Public Health, 17(16), 5809.
99. Pareek, A., Singhal, R., Pareek A., Chuturgoon, A., Sah, R., Apostolopoulos V. (2024) Global surge of Legionnaires' disease in 2024: Urgent call for heightened awareness and preparedness. Lancet Microbe . https://doi.org/10.1016/j.lanmic.2024.101031
100. Pécastaings, S., Bergé, M., Dubourg, K. M., and Roques, C. (2010). Sessile *Legionella pneumophila* is able to grow on surfaces and generate structured monospecies biofilms. Biofouling, 26(7), 809–819. https://doi.org/10.1080/08927014.2010.520159
101. Pepper, R. E., Riley, E. E., Baron, M., Hurot, T., Nielsen, L. T., Koehl, M. A. R., Kiørboe, T., and Andersen, A. (2021). The effect of external flow on the feeding currents of sessile microorganisms. Journal of the Royal Society Interface, 18(175), 20200953.
102. Pereira, A., Silva, A. R., and Melo, L. F. (2021). *Legionella* and biofilms—integrated surveillance to bridge science and real-field demands. Microorganisms, 9(6). https://doi.org/10.3390/microorganisms9061212
103. Petrisek, R., and Hall, J. (2018). Evaluation of a most probable number method for the enumeration of Legionella pneumophila from North American potable and nonpotable water samples. Journal of Water and Health, 16(1), 25–33. https://doi.org/10.2166/wh.2017.118
104. Proctor, C. R., Hammes, F., and Schwartz, T. (2017). Biofilms in drinking water distribution systems: A multifaceted future challenge. Current Opinion in Biotechnology, 47, 185-192.
105. Rafiee, M., Jahangiri-Rad, M., Hajjaran, H., Mesdaghinia, A., & Hajaghazadeh, M. (2014). Detection and identification of Legionella species in hospital water supplies through Polymerase Chain Reaction (16S

rRNA). Journal of Environmental Health Science and Engineering, 12, 83. https://doi.org/10.1186/2052-336X-12-83

106. Rahimi, B., and Vesal, A. (2017). Antimicrobial resistance properties of *Legionella pneumophila* isolated from the cases of lower respiratory tract infections. Biomedical and Pharmacology Journal, 10(1), 59-65.
107. Ramlal, P. S., Lin, J., Buckley, C. A., Stenström, T. A., and Amoah, I. D. (2022). Determinants of diarrhoeal infections among users of shared sanitation in informal settlements in Durban, South Africa. Journal of Water and Health, 20(10), 1517–1533. https://doi.org/10.2166/wh.2022.201
108. Ratcliff, R. M., Lanser, J. A., Manning, P. A., and Heuzenroeder, M. W. (1998). Sequence-based classification scheme for the genus *Legionella* targeting the *mip* gene. Journal of Clinical Microbiology, 36(6), 1560–1567.
109. Rech, M. M., Swalla, B. M., and Dobranic, J. K. (2018). Evaluation of Legiolert for Quantification of Legionella pneumophila from Non-potable Water. Current Microbiology, 75(10), 1282–1289. https://doi.org/10.1007/s00284-018-1522-0
110. Rhoads, W. J., Ji, P., Pruden, A., and Edwards, M. A. (2017). Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. Microbiome, 5(1), 130.
111. Rhoads, W. J., Keane, T., Spencer, M. S., Pruden, A., and Edwards, M. A. (2020). Did municipal water distribution system deficiencies contribute to a Legionnaires' Disease outbreak in Quincy, IL? Environmental Science and Technology Letters, 7(12), 896–902. https://doi.org/10.1021/acs.estlett.0c00637
112. Rhoads, W. J., Pruden, A., and Edwards, M. A. (2016). Survey of green building water systems reveals elevated water age and water quality concerns. Environmental Science: Water Research and Technology, 2(1), 164–173). Royal Society of Chemistry. https://doi.org/10.1039/c5ew00221d
113. Scaturro, M., Buffoni, M., Girolamo, A., Cristino, S., Girolamini, L., Mazzotta, M., Sabattini, M. A. B., Zaccaro, C. M., Chetti, L., Laboratory, M. A. N., Bella, A., Rota, M. C., and Ricci, M. L. (2020). Performance of Legiolert test vs. ISO 11731 to confirm *Legionella pneumophila* contamination in potable water samples. Pathogens, 9(9), 1–8. https://doi.org/10.3390/pathogens9090690
114. Scaturro, M., Fontana, S., Dell'eva, I., Helfer, F., Marchio, M., Stefanetti, M. V., Cavallaro, M., Miglietta, M., Montagna, M. T., de Giglio, O., Cuna, T., Chetti, L., Sabattini, M. A. B., Carlotti, M., Viggiani, M., Stenico, A., Romanin, E., Bonanni, E., Ottaviano, C., Ricci, M. L. (2016). A multicenter study of viable PCR using propidium monoazide to detect *Legionella* in water samples. Diagnostic Microbiology and Infectious Disease, 85(3), 283–288. https://doi.org/10.1016/j.diagmicrobio.2016.04.009
115. Schwake, D. O., Alum, A., & Abbaszadegan, M. (2021). Legionella Occurrence beyond Cooling Towers and Premise Plumbing. Microorganisms, 9(12), 2543. https://doi.org/10.3390/microorganisms9122543
116. Schoen, M. E., and Ashbolt, N. J. (2011). An imperative to apply regulatory frameworks to *Legionella* in drinking water systems. Environmental Science and Technology, 45(20), 8616-8617.
117. Schoen, M. E., and Ashbolt, N. J. (2011). An in-premise model for *Legionella* exposure during showering events. Water Research,45(18), 5826–5836. https://doi.org/10.1016/J.WATRES.2011.08.031
118. Sciuto, E. L., Laganà, P., Filice, S., Scalese, S., Libertino, S., Corso, D., Faro, G., and Coniglio, M. A. (2021). Environmental management of *Legionella* in domestic water systems: Consolidated and innovative approaches for disinfection methods and risk assessment. Microorganisms, 9(3), 577. https://doi.org/10.3390/microorganisms9030577
119. Shaheen, M. and Ashbolt, N.J. (2018). Free-living amoebae supporting intracellular growth may produce vesicle-bound respirable doses of *Legionella* within drinking water systems. Exposure and Health, 10(3), 201-209.
120. Slavik, I., Oliveira, K. R., Cheung, P. B., and Uhl, W. (2020). Water quality aspects related to domestic drinking water storage tanks and consideration in current standards and guidelines throughout the world - a review. Journal of Water and Health, 18(4), 439–463. https://doi.org/10.2166/wh.2020.052
121. Smith, D. B., Bennet, M., and Schlech, W. F. (2019). *Legionella* and drinking water: Improving the evidence base. Environmental Science: Water Research and Technology, 5(3), 492-499.
122. Söderberg, M. A., Rossier, O., and Cianciotto, N. P. (2004). The type II protein secretion system of *Legionella pneumophila* promotes growth at low temperatures. Journal of Bacteriology, 186(12), 3712-3720.

123. Song, Y., Mena-Aguilar, D., Brown, C. L., Rhoads, W. J., Helm, R. F., Pruden, A., and Edwards, M. A. (2024). Effects of Copper on Legionella pneumophila Revealed via Viability Assays and Proteomics. Pathogens (Basel, Switzerland), 13(7), 563. https://doi.org/10.3390/pathogens13070563
124. Spagnolo, A. M.; Cristina, M. L.; Casini, B.; Perdelli, F. (2013). *Legionella pneumophila* in healthcare facilities. Reviews in Medical Microbiology 24(3): 70-80. DOI: 10.1097/MRM.0b013e328362fe66
125. Springston, J. P., and Yocavitch, L. (2017). Existence and control of *Legionella* bacteria in building water systems: A review. Journal of Occupational and Environmental Hygiene, 14(2), 124–134. https://doi.org/10.1080/15459624.2016.1229481
126. Stadler, J., and Dugmore, C. (2017). "Honey, Milk and Bile": A social history of Hillbrow, 1894-2016. BMC Public Health, 17. https://doi.org/10.1186/s12889-017-4345-1
127. Steinert, M., Hentschel, U., and Hacker, J. (2002). *Legionella pneumophila*: An aquatic microbe goes astray. FEMS Microbiology Reviews, 26(2), 149–162. https://doi.org/10.1111/j.1574- 6976.2002.tb00607.x
128. Stewart, C. R., Muthye, V., and Cianciotto, N. P. (2012). *Legionella pneumophila* persists within biofilms formed by *Klebsiella pneumoniae*, *Flavobacterium* sp., and *Pseudomonas fluorescens* under dynamic flow conditions. PLoS ONE, 7(11), e50560. https://doi.org/10.1371/journal.pone.0050560
129. Stone, W., Louw, T. M., Gakingo, G. K., Nieuwoudt, M. J., and Booysen, M. J. (2019). A potential source of undiagnosed Legionellosis: *Legionella* growth in domestic water heating systems in South Africa. Energy for Sustainable Development, 48, 130–138. https://doi.org/10.1016/j.esd.2018.12.001
130. Sylvestre, É., Charron, D., Lefebvre, X., Bédard, E., and Prévost, M. (2024). Leveraging regulatory monitoring data for quantitative microbial risk assessment of *Legionella pneumophila* in cooling towers. medRxiv. doi: https://doi.org/10.1101/2024.05.19.24307585
131. Szwetkowski, K. J., and Falkinham, J. O., (2020). *Methylobacterium* spp. as Emerging Opportunistic Premise Plumbing Pathogens. Pathogens (Basel, Switzerland), 9(2), 149. https://doi.org/10.3390/pathogens9020149
132. Taguri, T., Oda, Y., Sugiyama, K., Nishikawa, T., Endo, T., Izumiyama, S., Yamazaki, M., and Kura, F. (2011). A rapid detection method using flow cytometry to monitor the risk of *Legionella* in bath water. Journal of Microbiological Methods, 86(1), 25–32. https://doi.org/10.1016/j.mimet.2011.03.012
133. Taviani, E., van den Berg, H., Nhassengo, F., Nguluve, E., Paulo, J., Pedro, O., and Ferrero, G. (2022). Occurrence of waterborne pathogens and antibiotic resistance in water supply systems in a small town in Mozambique. BMC Microbiology, 22(1), 243.
134. Taylor, M., Ross, K., and Bentham, R. (2009). *Legionella*, protozoa, and biofilms: Interactions within complex microbial systems. Microbial Ecology, 58, 538-547.
135. Thomas, V., Herrera-Rimann, K., Blanc, D. S., and Greub, G. (2006). Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. Applied and Environmental Microbiology, 72(4), 2428–2438. https://doi.org/10.1128/AEM.72.4.2428-2438.2006
136. Trigui, H., Matthews, S., Bedard, E., Charron, D., Chea, S., Fleury, C., Maldonado, J. F. G., Rivard, M., Faucher, S. P., and Prévost, M. (2024). Assessment of monitoring approaches to control *Legionella pneumophila* within a complex cooling tower system. The Science of the total environment, 950, 175136. https://doi.org/10.1016/j.scitotenv.2024.175136
137. Trnková, K., Kotrbancová, M., Špaleková, M., Fulová, M., Boledovičová, J., and Vesteg, M. (2018). MALDI-TOF MS analysis as a useful tool for an identification of *Legionella* pneumophila, a facultatively pathogenic bacterium interacting with free-living amoebae: A case study from water supply system of hospitals in Bratislava (Slovakia). Experimental Parasitology, 184, 97–102. https://doi.org/10.1016/j.exppara.2017.12.002
138. Turok, I., and Borel-Saladin, J. (2016). The theory and reality of urban slums: Pathways-out of poverty or cul-de-sacs? Urban Studies, 53(8), 1759-1775.
139. van der Kooij, D., Veenendaal, H. R., and Italiaander, R. (2020). Corroding copper and steel exposed to intermittently flowing tap water promote biofilm formation and growth of *Legionella pneumophila*. Water Research, 183, 115951. https://doi.org/10.1016/j.watres.2020.115951
140. van der Kooij, D., Veenendaal, H. R., and Schellart, J. A. (2017). Biofilm formation and *Legionella pneumophila* colonization in a model drinking water distribution system with copper and plastic pipes. Water Research, 112, 144-153.

141. Walker, J. T., and McDermott, P. J. (2021). Confirming the Presence of *Legionella pneumophila* in Your Water System: A Review of Current Legionella Testing Methods. Journal of AOAC International, 104(4), 1135–1147. https://doi.org/10.1093/jaoacint/qsab003
142. Weinbren M. J. (2020). Dissemination of antibiotic resistance and other healthcare waterborne pathogens. The price of poor design, construction, usage and maintenance of modern water/sanitation services. The Journal of Hospital Infection, S0195-6701(20)30133-X. https://doi.org/10.1016/j.jhin.2020.03.034
143. Whiley, H., Bentham, R., and Brown, M. H. (2014). *Legionella* persistence in manufactured water systems: Pasteurization and long-term control. Journal of Applied Microbiology, 117(1), 144-155.
144. Whiley, H., and Taylor, M. (2016). *Legionella* detection by culture and qPCR: Comparing apples and oranges. Critical Reviews in Microbiology, 42(1), 65–74. https://doi.org/10.3109/1040841X.2014.885930
145. Wolter, N., Carrim, M., Cohen, C., Tempia, S., Walaza, S., Sahr, P., de Gouveia, L., Treurnicht, F., Hellferscee, O., Cohen, A. L., Benitez, A. J., Dawood, H., Variava, E., Winchell, J. M., and von Gottberg, A. (2016). Legionnaires' disease in South Africa, 2012–2014. Emerging Infectious Diseases, 22(1), 131–133. https://doi.org/10.3201/eid2201.150972
146. Wolter, N., Carrim, M., Walaza, S., Chandu, L., Morifi, M., Lawrence, C., Cohen, C., and von Gottberg, A. (2020). Issue 3 Notified Legionnaires' Disease In South Africa, 18.
147. World Health Organization. (2004). Guidelines for drinking-water quality (Vol. 1). WHO Regional Office Europe.
148. World Health Organization. (2006). Guidelines for safe recreational water environments. Volume 2, Swimming pools and similar environments. https://www.who.int/publications/i/item/9241546808
149. World Health Organization. (2007). Guide to Ship Sanitation. WHO Regional Office Europe. https://www.who.int/publications/i/item/9789241546690
150. World Health Organization. (2007). *Legionella* and the prevention of legionellosis https://www.who.int/publications/i/item/9241562978
151. World Health Organization. (2016). *Legionella* and the prevention of legionellosis. https://iris.who.int/bitstream/handle/10665/43233/9241562978_eng.pdf
152. Yadav, D., Patil-Takbhate, B., Khandagale, A., Bhawalkar, J., Tripathy, S., and Khopkar-Kale, P. (2023). Next-Generation sequencing transforming clinical practice and precision medicine. Clinica Chimica Acta; International Journal of Clinical Chemistry, 551, 117568. https://doi.org/10.1016/j.cca.2023.117568
153. Yin, X., Chen, Y. Z., Ye, Q. Q., Liao, L. J., Cai, Z. R., Lin, M., Li, J. N., Zhang, G. B., Peng, X. L., Shi, W. F., and Guo, X. G. (2022). Detection performance of PCR for *Legionella pneumophila* in environmental samples: a systematic review and meta-analysis. Annals of Clinical Microbiology and Antimicrobials, 21(1), 12. https://doi.org/10.1186/s12941-022-00503-9
154. Zhang, T., Yao, L., Gao, Z., and Wang, F. (2022). Particle exposure risk to a lavatory user after flushing a squat toilet. Scientific Reports, 12(1), 21088.

APPENDIX A: ETHICAL CLEARANCES TO CONDUCT THE STUDY

FACULTY OF HEALTH SCIENCES
RESEARCH ETHICS COMMITTEE

NHREC Registration: REC 241112-035

ETHICAL CLEARANCE LETTER
(RECX 2.0)

Student/Researcher Name	Keletso Emily Buthane	Student Number	222252800
Supervisor Name	Singh, Atheesha		
Department	Biomedical Technology		
Research Title	OCCURRENCE OF LEGIONELLA PNEUMOPHILA IN HIGH-RISE BUILDINGS IN HILLBROW, JOHANNESBURG SOUTH AFRICA		
Date	27 July 2022	Clearance Number	**REC-1656-2022**

Approval of the research proposal with details given above is granted, subject to any conditions under 1 below, and is valid until 2023/07/26.

FACULTY OF HEALTH SCIENCES
RESEARCH ETHICS COMMITTEE

NHREC Registration: REC 241112-035

ETHICAL CLEARANCE LETTER
(RECX 2.0)

Student/Researcher Name	Keletso Emily Buthane	Student Number	222252800
Supervisor Name	Dr Atheesha Singh	Co-Supervisor Name	Prof Tobias George Barnard
Department	Biomedical Technology- Water and Health Research Centre		
Qualification	Masters Degree in Biomedical Sciences		
Research Title	OCCURRENCE OF *LEGIONELLA PNEUMOPHILA* IN HIGH-RISE BUILDINGS IN HILLBROW, JOHANNESBURG SOUTH AFRICA		
Date	9 March 2023	Clearance Number	**REC-1656-2022**

Approval of the amended research proposal with details given above is granted, subject to any conditions under 1 below, and is valid until 9 March 2024.

ETHICS APPROVAL CERTIFICATE

FACULTY OF HEALTH SCIENCES
RESEARCH ETHICS COMMITTEE

NHREC Registration: REC 241112-035

ETHICAL CLEARANCE LETTER
(RECX 2.0)

Student/Researcher Name	Zandice Faith Mnisi	Student Number	217032070
Supervisor Name	Singh, Atheesha		
Department	Biomedical Technology		
Research Title	LEGIONELLA CIRCULATING IN URBAN AND RURAL WATER SYSTEMS IN GAUTENG, SOUTH AFRICA		
Date	28 November 2023	Clearance Number	**REC-2418-2023**

Approval of the research proposal with details given above is granted, subject to any conditions under 1 below, and is valid until 2024/11/26.

ETHICS APPROVAL CERTIFICATE

FACULTY OF SCIENCE, ENGINEERING AND AGRICULTURE
RESEARCH ETHICS COMMITTEE

NAME OF RESEARCHER/INVESTIGATOR: BC MULAUDZI

Student number: 18007737

PROJECT TITLE:

PREVALENCE OF LEGIONELLA SPP IN RESIDENCE AROUND THOHOYANDOU, SOUTH AFRICA

ETHICAL CLEARENCE No. FSEA/23/BMY/29

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
AN Traore	University of Venda, Biochemistry and Microbiology	Supervisor
N Potgieter	University of Venda; Dean's office, FSEA	Co-supervisor
A Singh	University of Johannesburg	Co-supervisor

Type: **Student Research**

Risk: **Minimal risk to humans, animals, or environment (Category 1)**

Approval Period: **November 2023 – October 2025**

APPENDIX B: Permission to Access Study Site

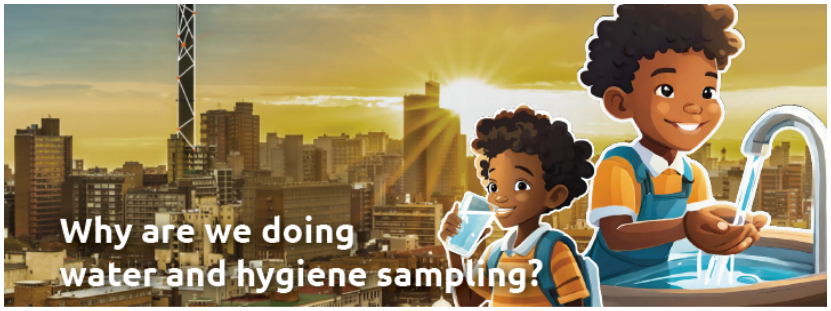
GAUTENG *Inspired* RESEARCH TRIANGLE

We are researchers from the University of Johannesburg working as part of the Gauteng Research Triangle inspired project. We are collecting water samples to check water quality in water systems of High-rise buildings in Hillbrow.

Participation will be anonymous and will be less than 15 minutes. You may withdraw from the study at anytime

Please consider participating in the study throughout the month of November as your inputs will add value towards the development of the Hillbrow community. Thank you for helping us

For More Info:
063 947 7559 tseome@uj.ac.za



Good morning,

We are a team from the University of Johannesburg Water and Health Research Centre, working with the Gauteng Research Triangle to study water and hygiene in households. We prepared this pamphlet to help you understand our work, but you can always ask more questions. Thank you for taking the time to think about this.

Greetings from Prof TG Barnard and Dr A Singh

Useful information:

Why are we doing this work?

We are looking at how people work with water, and what could impact water quality. This is important so that we can help develop training to protect your water quality and stop the spread of diarrhea.

Do I have to participate?

No, this study is voluntary, and you do not have to participate. You can withdraw from the study at any stage, and you only need to give samples you feel comfortable with.

What will we do when visiting your home?

When we visit the team will do the following:

1) We will take water samples to analyze the water quality.
2) We will ask you for a sample of any used water (greywater).
3) We will take samples from the kitchen and toilet surface using a wipe.
4) We would like to take a sample of the surface of your hand.
5) We would like to give you a new dishcloth in exchange of your used dishcloth.

What will you get from the visit?

If it is convenient, we will visit a second time to discuss your water quality data and help guide what the water can be used for, and how to treat it. We will also arrange activities in your community to show you how bacteria are transferred, challenge you to test your hand washing skills and have some fun competitions.

How will you identify us?

Students and staff will be visiting your house and will be accompanied by a member of the community. The UJ student and staff will wear UJ branded clothing and will have their UJ staff or student cards for identification. You can also contact us to confirm their identity if you feel uncomfortable, or ask the local GRT team members if they know us.

How to contacts us

Please feel free to WhatsApp us on 073 432 2686 if you have any queries.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF JOHANNESBURG

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

GRT inspired
GAUTENG RESEARCH TRIANGLE

APPENDIX C: Building Risk Assessment Checklist Urban

Name of Building: _____
Date Assessed: _____ Permission to come back and sample: _____
Flat Nos that provided permission: Ground Floor: _____ Middle Floor: _____ Top floor: _____

1. Building General Information checklist
2. What type of building is it?☐ Residential☐ Office☐ Warehouse☐ Other Specify:___
3. How many floors does the building have? _____
4. How many apartments or offices are on each floor? _____
5. How old is the building? _____
6. Has the building plumbing system undergone any renovations recently? ☐ Yes ☐ No
7. How often is maintenance done on the building plumbing system?☐ Weekly ☐ Monthly☐ Annually ☐ Other Specify: _
8. What is the occupancy of the building?☐ Fully Occupied ☐ Partially Occupied ☐ Other Specify: ___
9. Was the building or parts of it evacuated during the COVID-19 pandemic? ☐ Yes☐ No ☐ Partially
10. If yes for how long? __
11. Does the building have an air-cooling system such as:☐ Air Ventilation ☐ Air Conditioning ☐ Water Spray Coolers☐ None
12. Building Water General Information checklist
13. Does the building have any water feature or pool of water? ☐ Yes☐ No ☐ Other Specify:_____
14. Does the building have drinking water fountains for general use? ☐ Yes☐ No
15. If drinking water fountains are available for general use, how many are available and where are they situated?_
16. Is the plumbing distribution system plan available of ☐ Yes☐ No?
17. What type of plumbing material is used? ☐ Copper☐ Iron☐ PEX☐ PVC ☐ Other Specify:__
18. How is the water distributed (piped) in the building? ☐ Top to bottom or ☐ Bottom to Top ☐ Both ways
19. How is hot water distributed throughout the building?
20. ☐ Central Water Heating (geyser) shared →How many floors share? _____
21. ☐ Water heating/ Boiling on each floor ☐ Geyser in each apartment/office
22. ☐ Water Boiler in each apartment/office
23. When is the water heated? ☐ Continually throughout the day ☐ Only at peak h ☐ Depends on each tenant in the apartment/office
24. Is there a water storage tank in the building? ☐ Yes ☐ No If yes, Where is the tank situated? ___
25. What material is the storage tank made of?☐ Plastics☐ Metal Specify type: _____ ☐ Concrete
26. What is the water in the storage tank used for? _____
27. How often is the water changed in the storage tank? _____
28. What is the general quality of the water in the building (cold drinking water)? ☐ Good (clear colour and good taste)☐ Poor (discoloured and sour/ metallic taste)
29. What about smell of the water?☐ No smell ☐ Don't Know☐ Other Specify: _____
30. Does the building have other sources of drinking water (ground water, grey water, harvested rainwater, or borehole? ☐ Yes ☐ No If yes please specify:_____
31. Is there Water treatment on-site? ☐ Yes ☐ No
32. What is the Fixed water use frequency? _____
33. Is there aerosol generating devices/equipment in the building? ☐ Yes ☐ No Specify if yes._____
34. Is the water quality monitored? ☐ Yes ☐ No If yes specify frequency (weekly/ monthly)__
35. Is there a facility manager for the building, and specifically for water quality management? ☐ Yes ☐ No

36. Are there areas of stagnation in the building? (dead legs, vacant units/rooms, etc.) ☐ Yes ☐ No
37. Do all toilets have covers that can be closed when flushing? ☐ Yes ☐ No
38. Identify areas with hand-held showers, faucets with aerators/flow restrictors: _____
39. Identify areas where temperatures can support microbial growth: _ _____
40. Identify sinks and sink locations: _____
41. Identify electronic sinks/faucets and temperature setting for mixing valve: _____
42. Identify any other potential exposures to water: _____

OTHER COMMENTS: _____ _____

APPENDIX D :Building Risk Assessment Checklist Rural

COMMUNITY WATER RISK ASSESSMENT PLAN CHECKLIST
Community Name: _____
Date Assessed: _____ Permission to come back and sample: _____

Community and Water General Information checklist

1.1 What is the level of urbanization in the area?

☐Very urbanized ☐Somewhat urbanized ☐Most rural ☐Fully rural

1.2 What is the source of water for the area?

☐Pipe/Tap ☐River/streams ☐Rain harvesting ☐Boreholes ☐Municipal water trucks other, specify _____

1.3 How is water stored in the area?

☐Storage tank ☐Buckets ☐Clay pots ☐Other, specify_____

1.4 What material are the storage containers made of?

☐Plastic ☐Concrete ☐Galvanised steel ☐ Stainless steel ☐ Other, specify_____

1.5 What is the water in these storage containers used for?_____

1.6 How often the water is changed in these storage containers? _____

1.7 a. What is the general quality of the water within the household (Cold drinking water)?

☐Good (clear colour and good taste) ☐Poor (discoloured and sour/metallic taste)

1.8 b. What about the smell of the water?

☐No smell ☐Don't know ☐Other Specify_____

1.9 Has the household plumbing system undergone any renovations recently, if there is plumbing system?

☐ Yes ☐No

1.10 Does the community have any water feature or pool of water? ☐Yes ☐No ☐Other, specify____

1.11 Is the plumbing distribution system plan available? ☐Yes ☐No

1.12 What type of plumbing material is used?

☐Copper ☐Iron ☐PEX ☐PVC ☐Other, specify_____

1.13 How is hot water distributed within the house?

☐Geyser ☐Water heaters/boilers ☐Other, specify _____

1.14 When is the water heated?

☐Continually throughout the day ☐Only on peak hours ☐Depends on the water demand

1.17 Is there water treatment on-site? ☐Yes ☐No

1.18 What is the fixed water use frequency? _____

1.19 Is there aerosol generating devices/equipment in the household? ☐Yes ☐No specify if yes____

1.20 Is the water quality monitored? ☐Yes ☐No if yes, specify frequency (weekly/ monthly) _____

1.21 Does the toilet have a cover that can be closed when flushing? ☐Yes ☐ No

1.22 Identify areas with hand-held showers, faucets with aerators/flow restrictors:_____

1.23 Identify areas where temperatures can support microbial growth:_____

1.24 Identify sinks and sink locations:_____

1.25 Identify any other potential exposures to water:_____

OTHER COMMENTS: _____