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Poopedi, Evida

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Evaluation of occupational health risks from bacterial contaminants at wastewater treatment plants in Gauteng Province, South Africa

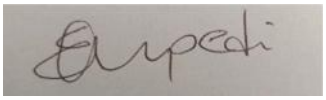
Evida Poopedi (350208)

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

June 2025

Declaration

I, Evida Poopedi, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. This work has not been previously submitted for any degree or examination at this or any other University.

Signature:  Date: ----------18 June 2025-----

Evida Poopedi

(student no: 350208)

Dedication

To my mom, your love and strength continue to guide me.

Presentations arising from this study

1. Poopedi, E., Singh, T., and Gomba, A. Genome sequencing unravels antibiotic resistance profiles and mutations that might affect drug susceptibility in *Legionella pneumophila* and *Aeromonas* species from municipal wastewater. **School of Dentistry Research Day, Sefako Makgatho Health Sciences University, 02 August 2024.** Oral presentation.
2. Poopedi, E., Singh, T., and Gomba, A. Preliminary findings on the presence of respiratory and enteric pathogens in wastewater: A potential risk of infection for municipal wastewater treatment plant workers. **National Institute for Occupational Health Biennial Research Day, 30 November 2022**. **Best Poster presentation award**.
3. Poopedi, E., Singh, T., and Gomba, A. Potential occupational exposure to respiratory and gastrointestinal bacterial pathogens at wastewater treatment plants, South Africa. **33rd International Congress on Occupational Health, 6 -10 February 2022**. **E-poster presentation –runner up award.**
4. Poopedi, E., Singh, T., and Gomba, A. Title: Potential exposure to enteric and respiratory bacterial pathogens at wastewater treatment plants: A preliminary study. **Pathology Research and Development Congress, 19-22 August 2021**, National Health Laboratory Service, Johannesburg, South Africa. Oral presentation.
5. Poopedi, E., Singh, T., and Gomba, A. Title: Potential worker's exposure to respiratory and gastrointestinal bacterial pathogens at wastewater treatment plants**. 12th Wits Cross Faculty Postgraduate Symposium, 26th July 2021,** University of the Witwatersrand, Johannesburg, South Africa. Poster presentation.
6. Poopedi, E., Singh, T., and Gomba, A. Title: Evaluation of occupational health risks from bacterial contaminants at wastewater treatment plants in Gauteng, SA. **Immunology and Microbiology Research Day, 4th September 2020**, National Institute of Occupational Health, Johannesburg, South Africa. **Best oral presenter - emerging researcher award.**

Publications arising from this study

1. Poopedi, E., Pierneef, R., Singh, T. and Gomba, A., 2025. Opportunistic bacterial pathogens in bioaerosols emitted at municipal wastewater treatment plants, South Africa. *Scientific Reports, 15*, p.10318. https://doi.org/10.1038/s41598-025-95484-y

2. Poopedi, E., Singh, T. and Gomba, A., 2025. Respiratory and enteric bacterial pathogens in municipal wastewater: A potential risk of infection to workers. Water, 17(2), p.268. https://doi.org/10.3390/w17020268

3. Poopedi, E., Singh, T. and Gomba, A., 2023. Potential exposure to respiratory and enteric bacterial pathogens among wastewater treatment plant workers, South Africa. *International journal of environmental research and public health*, *20*(5), p.4338. https://doi.org/10.3390/ijerph20054338

4. Poopedi, E., Pierneef, R., Singh, T. and Gomba, A., 2025. Antibiotic resistance profiles and mutations that might affect drug susceptibility in metagenome-assembled genomes of Legionella pneumophila and Aeromonas species from municipal wastewater. *BMC microbiology*, *25*(1), pp.1-9. https://doi.org/10.1186/s12866-025-03957-x

Abstract

Background: Workers at wastewater treatment plants (WWTPs) are routinely exposed to several health hazards, including bacteria due to frequent exposure to untreated wastewater. These occupational exposure increases the risk of infections, particularly respiratory and gastrointestinal illnesses.

Aim: This study aimed to provide data on the potential health risks associated with occupational exposure to selected respiratory and enteric bacterial pathogens among South African WWTP workers.

Methodology: 16S rRNA sequencing was used to screen and identify bacterial pathogens in wastewater and bioaerosol samples. Quantification of selected bacterial pathogens was determined using both culture method and qPCR. Subsequently, metagenome-assembled genomes (MAGs) *Legionella pneumophila* and *Aeromonas* spp. were reconstructed to identify antibiotic resistance genes (ARGs) and spontaneous mutations in key ARGs conferring resistance to commonly used drugs in *L. pneumophila.* Self-administered questionnaires were used to investigate the prevalence of chronic respiratory and gastrointestinal health issues among WWTP workers. Finally, quantitative microbial risk assessment (QMRA) was used to estimate the risk of infection associated with occupational exposure to *L. pneumophila* and *A. hydrophila*.

Key results: A total of 36 pathogenic genera were identified during the screening of influent samples. Culturable *L. pneumophila* was detected in 92% of the samples (2 to 5.4 $\log_{10}$ MPN/100 mL). *Mycobacterium* spp. had 100% detection rate, with concentrations ranging from 2.8 -8.9 $\log_{10}$ GC/mL. Enteric pathogens *A. butzleri* and *A. hydrophila* were detected in 96% and 82% of samples, respectively, with comparable GC levels. Bioaerosols from mechanical aeration tanks (72%, 26/36) had a relatively higher richness and diversity of airborne bacterial pathogens than diffused aeration tanks (17%, 6/36). In *L. pneumophila,* 138 nonsynonymous SNVs were identified in genes associated with antimicrobial resistance, with *gyrA* and *gyrB* showing the highest prevalence of variants (85 and 17, respectively). In *Aeromonas* spp., two (*aph(6)-Id* and *aph(3'')-Ib*) and five (*bla*MOX−4, *bla*OXA−1143, *bla*OXA−724, *cepH*, and *imiH*) ARGs conferring resistance to aminoglycosides and β-lactams were identified, respectively. Commonly reported chronic respiratory symptoms were wheezing (23.5%) and shortness of breath (7.4%) among WWTP workers compared to the control group (16.9 and 0%, respectively). *Legionella pneumophila* infection risks were

minimal for 0.5-hr but exceeded the USEPA threshold for exposure durations of 2-hrs or more across all scenarios and WWTPs. Similarly, infection risks posed *A. hydrophila* for the 0.5-hr exposure time were acceptable across all scenarios.

Conclusion: Findings of this study suggest that WWTP workers are at increased risk of respiratory and gastrointestinal health issues, potentially linked to occupational exposure to bacterial pathogens. The study emphasises the urgent need for improved PPE practices, targeted safety training, and increased awareness to mitigate these risks. Therefore, the combined data (hazard identification, health surveys, and QMRA) generated in this study can contribute to the understanding of microbial exposure risks at WWTPs, offering a foundation for risk mitigation strategies to safeguard worker health.

Keywords: sanitation workers, occupational health, biological hazards, antibiotic resistance, airborne pathogens, microbial risk assessment

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List of abbreviations and symbols

Abbreviation	
ACE	Abundance-based coverage estimator
AR	Antibiotic resistance
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
ASV	Amplicon sequence variant
BC	Background control
bp	base pair
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
Covid-19	Coronavirus disease 2019
DAER	Diffused aerator
DALY	Disability-adjusted life years
DNA	Deoxyribonucleic acid
FQR	Fluoroquinolone resistance
GC	Gene copy
GU	Genomic units
HBA	Hazardous biological agent
HIV	Human immunodeficiency virus
IE	Infective endocarditis
LD	Legionnaires ‘disease
LMICs	Low- and middle-income countries
LOQ	Limit of quantification

MAER	Mechanical aerator
MAG	Metagenome-assembled genome
MBL	Metallo-β-lactamases
Mbp	Mega base pair
MPN	Most probable number
NCBI	National center for biotechnology information
NCD	Non-communicable disease
NTM	non-tuberculous mycobacteria
OEL	Occupational exposure limits
OP	Opportunistic pathogen
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential hydrogen
PPE	Personal protective equipment
pppy	Per person per year
QMRA	Quantitative Microbial Risk Assessment
qPCR	Quantitative polymerase chain reaction
RA	Risk assessment
rDNA	ribosomal DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SNV	Single nucleotide variant

SST	Secondary settling tank
USEPA	United states environmental protection agency
UTI	Urinary tract infection
WHO	World health organization
WWTP	Wastewater treatment plant

Symbols and units

β	Beta
ºC	Degree Celsius
=	Equal
fmol	Femtomole
x *g*	Gravity at the earth's surface
>	Greater than
≥	Greater than or equal
hr/hrs	Hour/hours
km	Kilometre
<	Less than
≤	Less than or equal
L	Litre
ML/D	Mega litres per day
µL	Microlitre
µm	Micrometre
mL	Millilitre
µM	Micromolar
min	Minute

-	Minus
mol	Mole
ng	Nanogram
nM	Nanomolar
%	Percentage
sec	Second

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CHAPTER 1: INTRODUCTION

1.1 General introduction

Workers at wastewater treatment plants (WWTPs) provide an invaluable public service; however, they are exposed to several occupational health risks, particularly hazardous biological agents (HBAs) (WHO, 2019). Epidemiological studies on work-related health issues have revealed commonly reported symptoms in WWTP workers, including fatigue, headaches, gastrointestinal and respiratory problems. Collectively, these symptoms are referred to as "sewage workers' syndrome" (Rylander *et al*., 1976; Heldal *et al*., 2010; Chandra and Arora, 2018. Muzaini *et al*., 2021; Oza *et al*., 2022). Despite the known health risks, there are currently no standardised safety guidelines or occupational health measures in place to protect WWTP workers, particularly in low- and middle-income countries (LMICs) (LeChevallier *et al*., 2020). Therefore, workers in these regions may face increased risk of exposure to HBAs, underscoring the need for comprehensive safety protocols and protective interventions to mitigate these risks and ensure the well-being of WWTP workers, especially in resource-constrained settings (WHO, 2019).

Wastewater is a complex mixture containing several types of contaminants, including microbial pathogens (Chahal *et al*., 2016). However, the risk of illnesses or contracting infectious diseases from wastewater among WWTP workers remains an understudied area that is still being explored. Scientific interest on occupational infection risks among WWTP workers began to gain momentum only after the global COVID-19 pandemic (Chen *et al*., 2021; Dada and Gyawali, 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Gui *et al*., 2022; Morales *et al*., 2023). These studies mainly investigated the risk of inhaling aerosols containing enteric bacterial pathogens among WWTP workers. Therefore, there is an urgent need to expand research efforts to assess the infection risks from other prevalent and emerging waterborne pathogens in this occupational group.

Waterborne diseases represent a significant global health burden, with high mortality rates, particularly in developing countries, posing a serious threat to public health in these regions (Ritchie and Roser, 2019). According to the Global Burden of Disease report in 2019, unsafe drinking water, sanitation, and hygiene (WASH) were major contributors to global mortality, causing 744,000 deaths from acute respiratory infections and 1.5 million deaths from diarrheal diseases. The mortality rates are disproportionately higher in LMICs (WHO, 2023). Among HBAs, bacterial pathogens are of primary concern due to their diversity and significant role in waterborne illnesses worldwide (Ramírez-Castillo *et al*., 2015; Chahal *et al*., 2016). Bacteria are the most prevalent and diverse group of microbial pathogens found in wastewater (Chahal

et al., 2016), and various pathogenic bacterial species have been detected in wastewater (Cai and Zhang, 2014; Numberger *et al*., 2019; Oluseyi Osunmakinde *et al*., 2019; Huang *et al*., 2021; Bonetta *et al*., 2022). Despite this, pathogenic bacterial species are often not routinely monitored in wastewater, with water quality surveillance typically limited to microbial indicators. This highlights the need to assess health implications associated with bacterial pathogens in wastewater, particularly for individuals at increased risk of exposure, such as WWTP workers.

Existing research suggests that WWTP workers face higher risk of infectious illnesses compared to the general population due to occupational exposure (Heldal *et al*., 2010; Han *et al*., 2020; Oza *et al*., 2022; Lu *et al*., 2020). Exposure to such pathogenic microorganisms among WWTP workers mainly occurs through inhalation of contaminated aerosols, hand-to-mouth ingestion, and dermal absorption (Heldal *et al*., 2010; Lu *et al*., 2020). However, specific microbial pathogens responsible for these illnesses among WWTP workers remain poorly understood. Therefore, the current study assessed potential occupational health risks associated with bacterial pathogens in WWTP workers using an environmental assessment, self-administered questionnaires, and quantitative microbial risk assessment (QMRA). The study specifically focused on identifying respiratory and gastrointestinal bacterial pathogens present in municipal wastewater and estimating the risk of infection for WWTP workers. The findings of this study are intended to inform the development of targeted interventions aimed at reducing occupational exposure to HBAs, particularly in regions with suboptimal wastewater treatment infrastructure, thereby promoting safer working environments and improving the overall health and wellbeing of WWTP workers.

1.2 Aim of the study

The study's aim was to provide empirical evidence on the potential health risks associated with occupational exposure to selected respiratory and enteric bacterial pathogens among WWTP workers.

1.3 Specific objectives and thesis structure

1.3.1 To conduct an initial screening of bacterial contaminants in grab samples and select key bacterial pathogens for identification (Chapter 3).

Municipal wastewater is typically contaminated with a diverse range of microorganisms (Chahal *et al*., 2016). However, the composition of microbial communities in municipal wastewater varies depending on several factors including population served, technology used, and seasonal variations, among others (Kim *et al*., 2019). Therefore, non-targeted screening of microbial contaminants was performed using 16S rRNA amplicon sequencing to characterise bacterial diversity and identify potential pathogens that could pose an occupational health risk from exposure to municipal wastewater. For this purpose, grab influent samples from five municipal WWTPs in the city of Tshwane, South Africa, were screened to identify priority bacterial pathogens for further in-depth studies.

1.3.2 Environmental assessment to determine the occurrence and levels of selected bacterial pathogens in grab samples (Chapter 4).

Most studies on bacterial pathogens in municipal wastewater have focused on final effluent to assess treatment efficacy and safeguard public and environmental health (Cui and Liang, 2019; Bonetta *et al*., 2022; Skwor *et al*., 2023). However, this focus often overlooks the health risks faced by workers exposed to potentially pathogenic bacteria present at earlier treatment stages.This chapter quantified selected bacterial pathogens associated with respiratory (*Legionella pneumophila* and *Mycobacterium* spp.) and enteric (*Arcobacter butzleri* and *Aeromonas hydrophila*) health risks, based on findings from Objective 1. The quantities of these target pathogens were determined using both culture methods and real-time PCR. Grab samples were collected bi-weekly over six months from treatment points where workers are highly exposed (influent, activated sludge, and secondary settling tank effluent) at five municipal WWTPs in South Africa.

1.3.3 To determine the diversity of airborne bacteria in WWTP (Chapter 5).

Treatment processes in WWTPs, such as aeration tanks, bar screens, grit chambers, and sludge dewatering, are major sources of bioaerosols (Yang *et al*., 2019; Wan *et al*., 2023). These bioaerosols contain harmful components, including bacteria (Lu *et al*., 2020; Wan *et al*., 2023). However, there is a scarcity of studies investigating characteristics of bioaerosols emitted from WWTPs in most countries, except China (Yang *et al*., 2019; Han *et al*., 2020). This chapter examined the diversity of bacteria in bioaerosols emitted from municipal WWTPs in South Africa. Bioaerosol samples collected at aeration tanks utilising diffused and mechanical

aerators were assessed using 16S rRNA gene amplicon sequencing. The study aimed to characterise bacterial diversity and identify airborne pathogenic bacteria that could pose occupational health risks to WWTP workers.

1.3.4 To identify and characterise antimicrobial resistance determinants of the selected bacterial pathogens (Chapter 6).

Wastewater treatment plants are recognised as key reservoir for the emergence and spread of antimicrobial resistance (Zhang *et al*., 2019; Sambaza and Naicker, 2023). Metagenome-assembled genomes of four *L. pneumophila* and three *Aeromonas* spp. were reconstructed with a focus on identifying antibiotic resistance genes (ARGs). Furthermore, spontaneous mutations in key ARGs conferring resistance to commonly used antimicrobial drugs in *L. pneumophila*.

1.3.5 To investigate the prevalence of illnesses and disease associated with exposure of bacterial pathogens among WWTP workers (Chapter 7).

The literature indicates that sanitation workers, including WWTP workers, are at increased risk of respiratory and gastrointestinal illnesses compared to the general population, potentially associated with occupational exposure to pathogenic microorganisms (Lu *et al*., 2020; Oza *et al*., 2022). This chapter investigated the prevalence of chronic respiratory symptoms and illnesses such as cough, wheeze, shortness of breath, bronchitis, pneumonia and asthma among WWTP workers in South Africa using self-administered questionnaires. The study also assessed the prevalence of gastrointestinal symptoms, risk factors, and compliance with PPE use.

1.3.6 To estimate the infection risk attributed to *Legionella pneumophila* and *Aeromonas hydrophila* in wastewater using quantitative microbial risk assessment (Chapter 8).

Although numerous pathogenic microorganisms have been identified WWTPs (Chahal *et al*., 2016; Bonetta *et al*., 2022), scientific information regarding the associated infection risks, particularly for WWTPs workers, remains limited. In this chapter, QMRA was used to estimate infection risks associated with exposure to *L. pneumophila* and *A. hydrophila* at critical treatment stages with increased risk of exposure. These risks arise from potential inhalation or accidental ingestion of wastewater by municipal WWTP workers.

1.4 Thesis outline

The thesis is structured into nine chapters. Chapter 1 provides a brief introduction to the occupational health risks faced by WWTP workers, focusing on bacterial pathogens as key

etiological agents potentially associated with the commonly reported symptoms and illnesses in this occupational group. This chapter also outlines the aims and specific objectives of the study, establishing the framework for subsequent chapters.

Chapter 2 presents a literature review, providing a brief overview of typical municipal wastewater treatment processes, the sources of bacteria in municipal wastewater, and the pathogenic bacterial species identified at critical treatment stages where there is increased risk of worker exposure. The chapter further explores the health risks faced by WWTP workers, with a particular emphasis on respiratory and gastrointestinal illnesses, providing evidence of bacterial associations where available.

Chapter 3, 4, and 5 present the results of screening for bacterial pathogens in municipal wastewater, the quantities of selected respiratory and enteric bacterial pathogens, and the genetic factors associated with the development of antimicrobial resistance in these pathogens, respectively. Chapter 6 reports on the identified airborne bacteria at two WWTPs using different aeration modes. Chapter 7 outlines the findings from the health survey, detailing the prevalence of chronic respiratory illnesses and gastrointestinal symptoms among WWTP workers in South Africa. Chapter 8 provides estimated infection risks posed by *L. pneumophila* and *A. hydrophila* following occupational exposure to untreated municipal wastewater. Finally, Chapter 9 concludes the thesis with a summary of the key findings from the previous chapters and recommendations for future research.

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CHAPTER 2: LITERATURE REVIEW

2.1 An overview of wastewater treatment processes

Historically, waste management received little attention, with wastewater (sewage) often disposed of near residential areas and open defecation widely practiced. These practices led to serious environmental and public health problems, facilitating the spread of infectious diseases such as cholera, typhoid fever, and other gastrointestinal infections through the contamination of drinking water and direct human exposure to pathogens (Lofrano and Brown, 2010; World Bank, 2019). However, it was not until the 19th century that the importance of proper wastewater management and disposal for the protection of public health was well understood (Brown, 2005; Lofrano and Brown, 2010). The first modern-day wastewater treatment plants (WWTPs) were built in the 1800s after scientists discovered that bacteria in wastewater and other waste streams were associated with infectious diseases and epidemics of the time (Brown, 2005; Lofrano and Brown, 2010).

A wastewater treatment system is a multistage process designed to remove contaminants such as organic matter, microbial agents, heavy metals, and other contaminants before the wastewater is discharged or recycled (Al-Gheethi *et al*., 2018). Traditional WWTPs typically consist of primary, secondary, and tertiary treatment processes (Samer, 2015).

Primary treatment is intended to remove large solid objects from raw wastewater that could damage the facility's pumps and other equipment. This stage also includes screening of trapped coarse and fine materials by gravity sedimentation. This phase of the treatment is known as the mechanical process, and it can reduce up to 30% of the biochemical oxygen demand (BOD) and 50 -60% of total suspended solids (Lofrano and Brown, 2010; Samer, 2015; Musa and Idrus, 2021).

The secondary treatment stage, also known as the biological process, treats primary effluent under aerobic or anaerobic conditions using microorganisms to break down biodegradable organic matter into carbon dioxide, water, and energy. Subsequently, secondary settling is used to separate microbial biomass and other residual pollutants, removing approximately 90% of the suspended solids and BOD (Samer, 2015; Musa and Idrus, 2021). Some WWTPs incorporate an additional step to remove nitrogen and phosphorus before tertiary treatment, depending on effluent requirements. The most commonly used biological treatment technologies include facultative lagoons, trickling filters, activated sludge process, and up-flow anaerobic sludge blanket (Lofrano and Brown, 2010; Samer, 2015; Musa and Idrus, 2021).

The purpose of tertiary treatment is to remove residual contaminants or impurities that remain after secondary treatment. At this stage, wastewater may still contain an array of harmful contaminants including microbial pathogens and heavy metals, which can pose risks to human and public health and lead to non-compliance with effluent discharge standards. To address these risks, disinfection methods such as chlorination, ultraviolet radiation, ozone, and bromine treatment are commonly employed before effluent discharge (Lofrano and Brown, 2010; Samer, 2015; Musa and Idrus, 2021). Through these processes, WWTPs play a critical role in mitigating public health risks associated with wastewater while also protecting the environment.

Despite the critical role of WWTPs in mitigating public health risks, population growth has led to a substantial increase in wastewater generation, particularly in low- and middle-income countries (LMICs) (World Bank, 2019). United Nations Water reports significant disparities in wastewater treatment rates between developed and developing regions, with approximately 70% of wastewater treated in high-income countries, compared to only 28% in lower-middle-income countries and 8% in low-income countries (Ryder, 2017). However, LMICs face serious challenges with inadequate wastewater treatment infrastructure as many facilities remain incomplete, uncommissioned, or underperforming (World Bank, 2019). Factors contributing to this suboptimal performance include malfunctioning technologies, design capacity overload, financial constraints, and a shortage of skilled personnel for operation and management (World Bank, 2019).

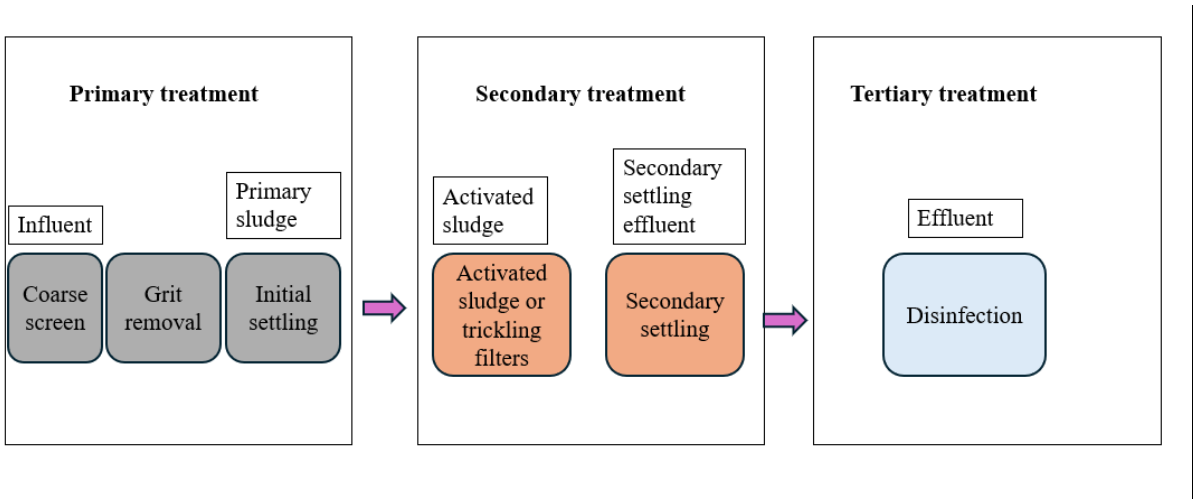

Figure 2.1: A schematic representation of a conventional wastewater treatment process.

2.2 Worker exposure to pathogenic microorganisms present in municipal wastewater

Municipal wastewater provides a conducive environment for microbial growth and proliferation (Abdel-Raouf *et al*., 2012). Different microorganisms, including bacteria, viruses, protozoan, and helminths have been detected in wastewater (Chahal *et al*., 2016). While most are harmless, pathogenic microbes excreted by symptomatic and asymptomatic individuals, pose serious health risks (García-Aljaro *et al*., 2019). Human pathogens commonly detected in wastewater include bacteria such as *Vibrio cholerae*, *Legionella pneumophila*, *Mycobacterium tuberculosis*; viruses such as adenovirus, rotavirus, hepatitis and other enteric viruses; helminths such as *Ascaris lumbricoides* and *Trichuris trichiura*; and protozoa such as *Entamoeba histolytica, Cryptosporidium* spp. (Fuhrimann *et al*., 2016; Osuolale and Okoh, 2017; Huang *et al*., 2018; Numberger *et al.,* 2019). Workers at WWTPs face a heightened risk of exposure to such pathogenic microorganisms (Heldal *et al*., 2010; Lu *et al*., 2020) through inhalation of contaminated aerosols, hand-to-mouth ingestion, and dermal absorption (Heldal *et al*., 2010; Zachary, 2017).

According to Water UK (2010), handling wastewater and sludge increases the likelihood of dermal exposure via splashes, while dry sludge handling increases the risk of inhaling airborne particles. Manual waste sorting further increases worker exposure to elevated microbial loads (World Bank, 2019; LeChevallier *et al*., 2020). Additionally, mechanical aerators at WWTPs generate significant amounts of aerosols (Niazi *et al*., 2015; Han *et al*., 2019), increasing the potential inhalation of infectious material while socialising or talking near aeration tanks or performing routine checks in these areas. Dermal contact frequently occurs as a result of direct contact with wastewater, sludge, or touching contaminated tools, handrails, and other surfaces, particularly when appropriate personal protective equipment (PPE) is not worn (LeChevallier *et al*., 2020). Microorganisms can penetrate the body via broken skin or mucous membranes, while ingestion may result from direct splashes, swallowing airborne particles, or hand-to-mouth contact following dermal exposure (Water UK, 2010; World Bank, 2019).

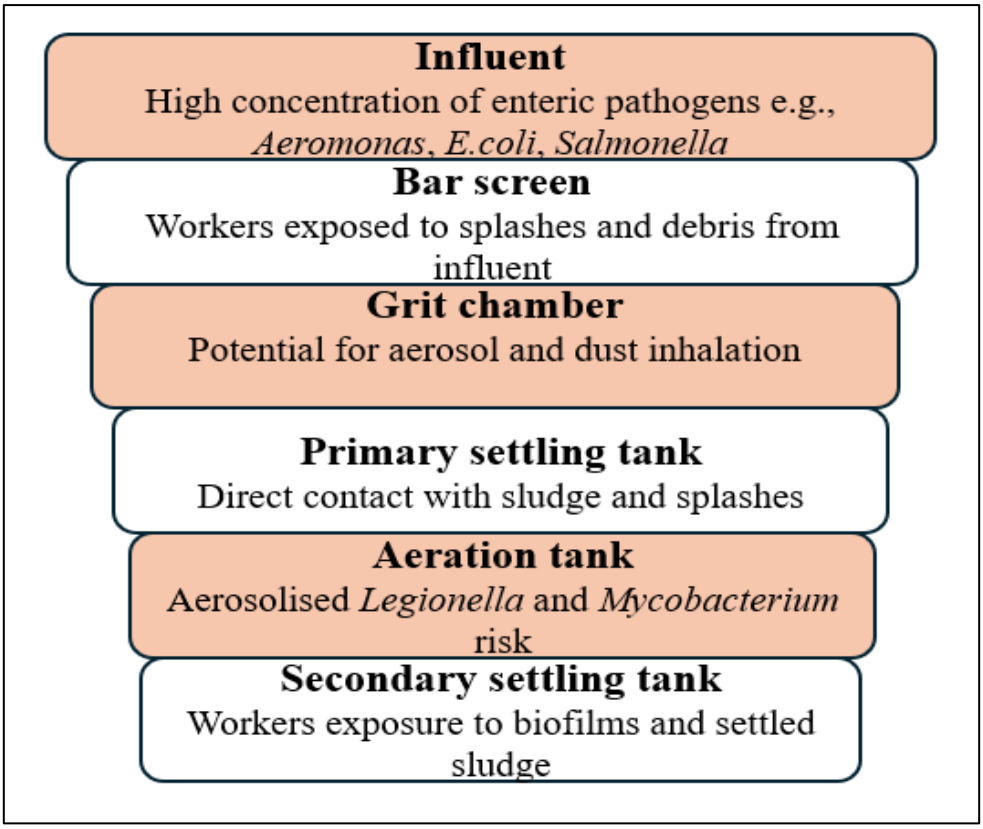

Figure 2.2: Key worker exposure points to pathogenic microorganisms in municipal WWTPs.

2.3 Origin and diversity of bacteria in municipal wastewater

Bacteria represent the most diverse group of pathogenic microorganisms detected in wastewater (García-Aljaro *et al*., 2019), with an estimated 538 human pathogenic bacterial species, 208 pathogenic virus types, 57 protozoan species, and fungi (Ramírez-Castillo *et al.,* 2015). The composition of bacterial communities and the prevalence of pathogenic species in wastewater are influenced by several factors including the health status of the population served, community size, proximity to hospitals, veterinary clinics, abattoirs, and industrial facilities, as well as treatment technology used (Santo Domingo and Edge, 2010; Newton *et al*., 2015).

Municipal wastewater, particularly from households, contains human excreta (e.g., faeces and urine), greywater, and many other contaminants. While faecal material is the main source of bacterial pathogens, wastewater also habours bacteria from human skin, the oral cavity, animal waste, food waste, industrial waste, household waste, and, to a lesser extent, stormwater intrusion (Chahal *et al*., 2016). These diverse inputs contribute to the complex microbial ecosystem within wastewater, posing challenges for effective treatment and pathogen control.

2.3.1 Human gut microbiota

Human faeces are the main source of microorganisms in municipal wastewater, containing approximately 10^{13} to 10^{14} microbial cells per 200 g wet weight in a healthy individual (Holcomb *et al*., 2020). However, microbial loads can vary due to pathological conditions such as diarrhea (Sender *et al*., 2016). The human colon hosts diverse microbial communities, including bacteria, protozoa, viruses, archaea and eukaryotes, with bacteria comprising the majority of the human gut microbiome (Sender *et al*., 2016). There is a consensus that the bacterial phyla *Firmicutes* and *Bacteroidetes* predominate in the human gut (Newton *et al*., 2015).

Wastewater influent reflects the human gut faecal microbiome and therefore provides a population-level snapshot of human microbial communities of the areas served by the treatment facilities (Newton *et al*., 2015). Although individual gut microbiomes differ depending on age, diet, environment, genetics, and socioeconomic status, microbial profiles of wastewater derived from populations with shared cultural and geographical settings exhibit temporal stability (De Filippo *et al*., 2010; Shanks *et al*., 2013). This stability allows comparative analyses to investigate demographic and lifestyle impacts on faecal microbiome.

In contrast, significant differences in the composition of gut microbiota have been observed among populations with distinct cultural, socioeconomic and geographical settings (De Filippo *et al*., 2012; Yatsunenko *et al*., 2012; Wu *et al*., 2019), making it challenging to define a typical human gut bacterial profile. Furthermore, human faeces may introduce pathogenic bacteria into wastewater, with concentration and diversity influenced by factors such as seasonal patterns, outbreak frequencies, and the prevalence of infectious diseases in specific locations. Many of the pathogenic bacteria shed in human faeces are transmitted through the faecal-oral route (García-Aljaro *et al*., 2019), highlighting the potential risks associated with untreated wastewater.

2.3.2 Animal microbiota

Another important aspect for water resource management is the composition of microbiota found in the colon of healthy animals, excreted through faeces and runoff from abattoirs. Bacterial phyla such as *Proteobacteria*, *Firmicutes* and *Bacteroidetes* are dominant in animal microbiota and show similar trends across different species (De Menezes *et al*., 2011; Isaacson and Kim, 2012; Oakley *et al*., 2014). For example, a recent study by Boukerb et al. (2021)

recently demonstrated that bacterial phyla *Firmicutes* and *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were consistently present in faecal bacterial communities from wild waterbirds, poultry, pigs, cattle, and influent wastewater. However, phylogenetic analysis revealed differences at the genus and species level, despite similar dominance at the phylum level.

Municipal wastewater often contains bacterial genera originating from animals, some of which harbor potential pathogens. For example, Diallo et al. (2013) detected atypical enteropathogenic *Escherichia coli*, extraintestinal pathogenic *E. coli*, and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in municipal WWTPs receiving slaughterhouse effluents. Similarly, Savin et al. (2020) detected clinically significant bacteria, such as *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *enterococci*, *P. aeruginosa,* and *Citrobacter* spp. These pathogens were also present in slaughterhouse operational areas (e.g., delivery and production areas), highlighting the potential occupational risks and possible zoonotic disease transmissions.

Slaughterhouse effluents are significant contributors of livestock-associated bacteria into municipal wastewater, with potential implications for zoonotic disease transmission. According to WHO, there are more than 200 different types of zoonotic diseases, including anthrax, bubonic plague, tuberculosis, brucellosis, campylobacteriosis, leptospirosis, *Arcobacter* infections, listeriosis, bordetellosis, lyme disease, Actinomycosis, Q fever and enterohemorrhagic *E. coli* infections. (Blancou *et al*., 2005). Given that several human pathogenic species have been identified from slaughterhouse effluents, effective treatment is necessary to reduce biological contaminants and prevent the spread of zoonotic diseases through contaminated wastewater.

2.3.4 Stormwater

Stormwater, in general, collects excess nutrients and pollutants accumulated during dry periods from various sources, including rooftops, roads, and natural environments such as soil and plants (García-Aljaro *et al*., 2019). This runoff is typically transported to WWTPs through sewer systems. Urban wildlife, domestic animals, and domestic waste are potential sources of biological contaminants in stormwater in urban settings. For instance, Monteiro et al. (2021) monitored faecal indicator bacteria in stormwater runoff collected from Lisbon streets, identifying human sources as the primary contributors of contamination, followed by domestic

animals such as dogs and cats. Additionally, faecal contamination was detected even in the absence of identifiable sources, suggesting that there are other important sources of contamination excluding humans and domestic animals.

Interestingly, the presence of pathogenic bacteria in stormwater has been increasingly documented (Jongman *et al*., 2016; Ahmed *et al*., 2019; Bae *et al*., 2019). A study by Sidhu et al. (2012) investigated human pathogens in stormwater runoff in Australia, detecting *Campylobacter* spp., *Salmonella enterica*, *Adenovirus* and *Polyomavirus*. Based on these findings, the authors recommended disinfection treatment for stormwater prior to reuse for non-potable purposes. Similarly, Steele et al. (2018) used digital PCR to determine pathogen concentrations in stormwater samples from southern California. The study reported *Campylobacter* spp. at concentrations ranging from 16 to 504 gene copies (GC) per 100 mL and *Salmonella* at 6 to 86 GC per 100 mL. Notably. *Campylobacter* spp. were the most detected potential pathogen in these samples.

It should be noted that microbial composition of stormwater runoff is highly influenced by the characteristics of specific geographic areas (Ahmed *et al*., 2019). However, the potential presence of pathogenic microorganisms in urban stormwater highlights the urgent need for effective control and treatment strategies to mitigate health risks for humans and protect the environment.

2.3.5 Sewer infrastructure

Studies revealed that municipal influent contains bacterial assemblages that differ from typical composition of human gut microbiota (Newton *et al*., 2015; Fisher *et al*., 2015; LaMartina *et al*., 2021). In fact, human faecal microbiota accounts only for up to 20% of the bacterial community in WWTP influent (Newton *et al*., 2015; Guo *et al*., 2019; LaMartina *et al*., 2021), while approximately 80% is dominated by genera such as *Arcobacter*, *Aeromonas*, *Trichococcus*, and *Acinetobacter* (Vandewalle *et al*., 2012; Fisher *et al*., 2015; LaMartina *et al*., 2021). The enrichment of non-faecal bacteria suggests additional bacterial sources in municipal wastewater other than humans.

The municipal sewer system represents a complex microbial habitat, continually infiltrated by microbes from diverse sources, including domestic, industrial, and agricultural waste (McLellan *et al*., 2015). This dynamic environment fosters the growth and diversification of microbial communities, influencing bacterial communities in receiving WWTPs. Studies have

shown differences in bacterial communities of influent wastewater, sewer pipe biofilms, and human faeces, suggesting significant microbial transformations during transport to WWTPs (McLellan *et al*., 2015; Newton *et al*., 2015; Jensen *et al*., 2016). Specifically, non-faecal microbes, such as those originating from sewer pipes and biofilms, tend to dominate the influent microbial communities (Frigon and Wells, 2019, McLellan and Roguet, 2019).

High wastewater flow rates have been shown to enhance the transport of resident sewer microbiota through mechanisms such as biofilm sloughing and sediment resuspension, further altering influent microbial composition (Guo *et al*., 2019). Furthermore, it has been demonstrated that the presence of loose sediments in the sewer system also plays a critical role in shaping bacterial communities. For example, Liu and co-workers demonstrated that the unique microenvironments within loose sediments create favorable conditions for bacterial growth, resulting in higher bacterial diversity in flowing water with sediments compared to biofilms on pipe surfaces (Liu *et al*., 2014). These findings underscore the complexity of microbial dynamics in wastewater systems and highlight the importance of understanding these processes to better manage wastewater treatment and environmental impacts.

2.3.6 Human bacterial pathogens in untreated municipal wastewater

There has been continuous global efforts to profile bacterial communities including pathogens in municipal wastewater using advanced molecular technologies (Ye and Zhang, 2011; Cai and Zhang, 2014; Kumaraswamy *et al*., 2014; Lu *et al*., 2015; Ahmed *et al.,* 2017; Huang *et al*., 2018; Kulkarni *et al.,* 2018; Numberger *et al.,* 2019; Oluseyi Osunmakinde *et al.,* 2019; Yasir, 2021; Jalowiecki *et al.,* 2021). Table 2.1 show key studies that detected bacterial pathogens in municipal wastewater prior to the final treatment stage, specifically influent and activated sludge. Pathogens identified include members of the genus *Mycobacterium, L. pneumophila, Aeromonas, Arcobacter, Bacillus, Campylobacter, Clostridium, Escherichia, Clostridium, Listeria, Salmonella, Shigella, Vibrio, Staphylococcus, Yersinia Bordetella, Chlamydia, Corynebacterium, Bacteroides, Enterobacter, Enterococcus, Haemophilus, Helicobacter, Klebsiella, Leptospira, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Streptococcus, Treponema, Brucella, Coxiella,* and *Acinetobacter*. Enteric pathogens were generally prevalent in wastewater; however, non-enteric pathogens such as *Legionella pneumophila*, *Mycobacterium* spp., and *Coxiella* have also been detected, which can cause illnesses unrelated to the gastrointestinal system.

Some pathogenic genera were frequently highlighted due to their high relative abundances in municipal influent. Ahmed et al. (2017) reported that *Arcobacter* and *Pseudomonas* were the most abundant genera in influent from four WWTPs in Australia, with relative abundances of 8.37–28.6% and 10.3–38.5%, respectively. Similar trends have been observed globally, including in China, Singapore, Denmark, Canada, and the United States, where *Arcobacter* was identified as the dominant pathogenic genus in municipal influent (Ye and Zhang, 2011; Lu *et al*., 2015; Kristensen *et al*., 2020). Further analysis at the species level by Lu et al. (2015) identified *Arcobacter butzleri*, with concentrations reaching 2.57×10^4 sequences per mL of wastewater. The genus *Arcobacter* is an emerging pathogen associated with diarrhoeal illnesses and gastroenteritis in humans (Ramees *et al*., 2017). The source of *Arcobacter* spp. in municipal wastewater is not well understood; however, it is hypothesized that this organism forms biofilms to attach to the surfaces of sewer pipes (McLellan and Roguet, 2019). Other studies suggests that the presence of *Arcobacter* in environmental water is often linked with faecal contamination (Lee *et al*., 2012). These findings underscore the importance of monitoring microbial communities in wastewater to mitigate health risks associated with emerging bacteria.

Additionally, research has identified dominant potentially pathogenic bacteria in activated sludge, highlighting significant trends across geographic locations (Ye and Zhang, 2011; Cai and Zhang, 2014; Lu *et al*., 2015; Huang *et al*., 2018). Ye and Zhang (2011) conducted one of the most comprehensive studies to date, spanning four countries (e.g., China, United States, Canada and Singapore), and reported that *Aeromonas* and *Clostridium* were the most prevalent genera in activated sludge samples. Interestingly, despite geographic differences, no significant differences were observed in the predominant pathogenic genera. Similarly, a study in China consistently identified *A. hydophila* as one of the major organisms in activated sludge using three different detection technologies (Lu *et al*., 2015).

In contrast, other studies have reported a prevalence of different pathogens. For instance, Cai and Zhang (2014) reported that *Mycobacterium tuberculosis* and *Shigella flexneri* were the most common pathogenic bacteria in activated sludge, accounting for 78% and 35% of the total relative abundance, respectively. Studies in the United States (Bibby *et al*., 2010; Kulkarni *et al.,* 2018) have corroborated these findings, reporting that most pathogenic bacteria in digested sludge belonged to the genera *Clostridium* and *Mycobacterium* (Bibby *et al*., 2010). Furthermore, *Legionella pneumophila* and *Mycobacterium* spp. were also identified as the predominant pathogens in activated sludge (Kulkarni *et al.,* 2018).

Although bacterial diversity in municipal wastewater is mostly influenced by factors such as community health and demographic characteristics (Al-Gheethi, *al*., 2018), these studies suggest that predominant clinically relevant bacterial genera in municipal wastewater are relatively stable across geographic locations. However, this remains a growing area of research, and further studies are necessary as these trends in bacterial communities and pathogens may evolve over time. Nevertheless, it has been highlighted that municipal wastewater prior to final treatment remains a significant reservoir of several pathogens, posing health risks, particularly to WWTP workers. This highlights the need for ongoing surveillance and further research to better understand microbial dynamics and inform effective risk mitigation strategies.

Table 2.1: Metagenomic studies on bacterial pathogens in wastewater (influent and at different stages of WWTPs)

Study country	Bacterial pathogens	Wastewater type	Sequencing platform used	Reference
China, United States, Canada and Singapore	[1]*Legionella, Mycobacterium.* [2]*Aeromonas, Arcobacter, Bacillus, Campylobacter, Clostridium, Escherichia, Listeria, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia.* [3]*Bordetella, Chlamydia, Corynebacterium, Enterobacter, Enterococcus, Haemophilus, Helicobacter, Klebsiella, Leptospira, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Streptococcus, Treponema.*	Influent, activated sludge	454 pyrosequencing	Ye and Zhang, 2011
China	[1]*M. tuberculosis, L. pneumophila.* [2]*Brucella melitensis, Shigella flexneri, Vibrio cholerae, Salmonella enterica, Yersinia pestis, E. coli, Clostridium perfringens.* [3]*Enterococcus faecalis, Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria meningitides.*	Influent, activated sludge	Shotgun sequencing	Cai and Zhang, 2014
United Arab Emirates	[1]*M. tuberculosis.* [2]*S. enterica, E. coli, C. perfringens, Y. pestis, V. cholera.* [3]*Streptococcus agalactiae, S. pneumonia, Pseudomonas aeruginosa, E. faecalis.*	Influent, SST effluent	16S rRNA sequencing	Kumaraswamy *et al*., 2014

Zhengzhou City, central China	[2]*Aeromonas, Arcobacter, Clostridium, Bacillus.* [3]*Enterobacter, Enterococcus, Klebsiella, Pseudomonas, Streptococcus, Treponema, Mycoplasma, Neisseria.*	Influent, activated sludge, SST effluent	16S rRNA sequencing and 454 pyrosequencing	Lu *et al*., 2015
Australia	[1]*Coxiella, Legionella, Mycobacterium.* [2] *Escherichia/Shigella, Clostridium, Aeromonas, Arcobacter Salmonella, Staphylococcus, Yersinia.* [3] *Acinetobacter, Bordetella, Neisseria, Pseudomonas*, *Rickettsia, Streptococcus, Treponema.*	Influent, secondary treated wastewater	16S rRNA sequencing	Ahmed *et al*., 2017
China	[1] *L. pneumophila, M. tuberculosis.* [2]*E. coli, Arcobacter butzleri, Bacillus cereus, C. perfringens, Shigella boydii, Helicobacter pylori, Yersinia enterocolitica, V. cholerae, Clostridium jejuni*, *Listeria monocytogenes, S. enterica, Staphylococcus aureus, Shigella dysenteriae.* [3]*Bacteroides vulgatus, P. aeruginosa, Enterobacter aerogenes, Bacteroides fragilis, Enterobacter cloacae, P. aeruginosa, N. meningitides, Haemophilus influenzae.*	Influent, activated sludge	Shotgun sequencing	Huang *et al*., 2018
United Stated	[1] *Legionella spp, Mycobacterium spp.* [2]*Clostridium* [3]*Acinetobacter, Pseudomonas, Streptococcus*	Influent, activated sludge	16S rRNA sequencing	Kulkarni *et al.,* 2018

Germany	[2]*Escherichia/Shigella, Leptospira alstonii, C. perfringens, Clostridium botulinum, Clostridium butyricum, Yersinia massiliensis, Y. frederiksenii, Y. enterocolitica and Yersinia media.* [3]*Acinetobacter beijerinckii, Acinetobacter haemolyticus, Acinetobacter baumannii*, *Aerom*onas *sharmana, Pseudomonas alcaligenes and P. aeruginosa.*	Influent	Single molecule real time (SMRT) sequencing	Numberger *et al.,* 2019
South Africa	[1]*Mycobacterium.* [2]*Aeromonas, Clostridium, Staphylococcus, Vibrio, Yersinia, E. coli.* [3]*Acinetobacter, Prevotella, Pseudomonas, Roseomonas, Rickettsia, Streptococcus, Treponema, Klebsiella.*	Influent	16S rRNA sequencing	Oluseyi Osunmakinde *et al.,* 2019
Saudi Arabia	[2]*Campylobacter jejuni, Campylobacter fetus, C. botulinum, E. coli, Vibrio parahaemolyticus, V. cholera.* [3]*A. baumannii, Bacteroides fragilis, Burkholderia pseudomallei, Enterococcus faecium, Fusobacterium nucleatum, H. pylori, Klebsiella pneumonia, Treponema denticola, P. aeruginosa, Porphyromonas gingivalis, Leptospira interrogans.*	Influent	Shotgun sequencing	Yasir, 2021
Poland	[2]*Campylobacter, Salmonella, Shigella, Helicobacter pylori, S. aureus*	Influent, primary settling tank effluent,	16S rRNA sequencing	Jalowiecki *et al.,* 2021

	[3]*A. baumannii, P. aeruginosa, E. faecium, H. influenzae, S. pneumoniae, Neisseria gonorrhoeae*	SST effluent		
Italy	[1] *Mycobacterium* [2] *Escherichia-Shigella* [3]*Acinetobacte, Enterococcus, Pseudomonas, Streptococcus, Klebsiella*	Influent, effluent	16S rRNA sequencing	Ferraro *et al.,* 2024

Note: 1- respiratory pathogens, 2 - enteric pathogens, 3 - opportunistic pathogens

2.4 Selection of priority bacterial pathogens in this study

Prioritisation and risk ranking of bacterial pathogens requires detailed information to define the burden of the biological hazard in humans. Specific guidelines for this process are available for some settings, such as food safety, chemical hazards and communicable diseases. There is, however, no consensus on the most effective criteria and methodological guidelines for prioritising pathogenic biological agents or infectious diseases (WHO, 2017).

In the present study, the following criteria were used to establish a priority list of bacterial pathogens, providing motivation for in-depth study of the selected pathogens:

- Pathogenic bacteria should be present in wastewater, ideally detected in South Africa.
- Persistent in wastewater environments.
- Clinical impact of infection after potential occupational exposure. Prioritised bacterial pathogen should cause mild disease and/or require medical treatment or life-threatening infections.
- Potential to cause an outbreak.

Table 2.2: Priority pathogens in this study

Legionella pneumophila	**Causes serious lung infection** • Legionnaires' disease and Pontiac fever. • Outbreaks are frequently linked to large or complex bodies of water. • 1 out of 10 people will die from Legionnaires' disease.
Mycobacterium species	**Non-specific clinical manifestation** • Non-tuberculous mycobacteria can cause infections and immunocompromised and healthy people • Rising cases in most countries related to population ageing, chronic diseases, and comorbidities
Arcobacter butzleri	**An emerging human pathogen** • Severe diarrhoea, enteritis, bacteremia and wound infections. • Food and waterborne pathogen.

Aeromonas hydrophila	**Enteric pathogen of serious public concern** • Watery diarrhoea to dysenteric or bloody diarrhoea. • Inhabits aquatic environments.

2.5 Typical characteristics of the selected priority bacterial pathogens

2.5.1 *Legionella pneumophila*

Legionella species are aerobic, gram negative, non-spore forming bacteria that can be found in both natural and artificial water systems. The genus has more than 50 species, approximately half of which are considered potential human pathogens. Infection occurs exclusively through inhalation of contaminated aerosols, which can result in an influenza-like illness called Pontiac fever or a serious type of pneumonia known as Legionnaire's disease (LD). *L. pneumophila* is the most common *Legionella* species isolated from clinical specimens, accounting for more than 90% of confirmed LD cases with a 15% mortality rate (Kozak-Muiznieks *et al*., 2014). *Legionella* species are fastidious organisms with stringent growth requirements. Amino acids are the primary source of carbon and energy. This genus has developed strategies to survive in nutrient limited environments by multiplying intracellularly within numerous protozoan genera such as *Acanthamoeba*, *Hartmannella*, *Naegleria*, and *Tetrahymena* and *Echinamoeba*, as well as in complex matrices such as biofilms. These associations protect *Legionella* species from hostile environmental conditions such as high temperatures, dryness, antibiotics and disinfection agents, and may also induce virulent phenotypes (Mercante and Winchell, 2015; Jia and Zhang, 2020).

2.5.2 *Mycobacterium* species

The genus *Mycobacterium* is characterised by its acid-fastness, the presence of long-chain mycolic acids, a high GC content, and the ability to grow optimally at temperatures from 25 to 45°C, depending on the species (Magee and Ward, 2015). Nontuberculous mycobacteria (NTM) are naturally occurring environmental organisms that have been isolated from various natural water sources such as rivers, lakes, and soil (Falkinham, 2015). The term NTM refers to all *Mycobacterium* species other than the *Mycobacterium tuberculosis* complex and *M. leprae*. These organisms were identified in the late 19th century but not widely recognised as human pathogens until the late 1950s (Jeon, 2019). In recent years, NTMs have emerged as

important pathogens worldwide, with an increasing incidence and prevalence of associated diseases in both immunocomprised and healthy individuals (Koh, 2017).

2.5.3 *Arcobacter butzleri*

Arcobacter has 29 known species, and members of this genus are gram negative, non-spore forming helical rods that are typically 0.2–0.9 μm wide and 1–3 μm long. They are also motile, with a polar flagellum that moves in corkscrew pattern. This genus can grow aerobically at temperatures ranging from 15 to 37 °C, but it requires microaerobic conditions for optimal growth or primary isolation. Four species of *Arcobacter* namely *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. thereius* are commonly associated with clinical outcomes in humans. In particular, *A. butzleri* in humans has been associated to severe diarrhoea, bacteremia and enteritis (Ferreira *et al.,* 2019). *A. butzleri* can be found in vegetables, animal products, water sources such as wastewater, rivers, lakes, surface water, and recreational water. This pathogen causes symptoms similar to *Campylobacter jejuni*, but with more persistent watery diarrhoea (Chieffi *et al*., 2020).

2.5.4 *Aeromonas* species

Aeromonas are gram negative, oxidative positive bacilli, non-spore forming facultative anaerobic bacteria that occur naturally in aquatic environments including drinking water, wastewater, swimming pools, and reclaimed water (Fernández-Bravo and Figueras, 2020). There are 36 species in the *Aeromonas* genus. *Aeromonas* species have the potential to cause a wide range of diseases in both humans and animals. However, in humans, it is commonly associated with diarrhoea, wound infections, and bacteremia. Interestingly, this genus has been ranked third as the leading cause of diarrhoea globally (Igbinosa *et al*., 2012; Fernández-Bravo and Figueras, 2020). Clinically, the most common species isolated from human specimens are *A. hydrophila*, *A. caviae*, *A. dhakensis*, and *A. veronii* (Figueras and Beaz-Hidalgo, 2015).

2.6 Potential occupational health hazards for WWTP workers

According to WHO, occupational health is defined as a multidisciplinary aimed at protecting and promoting worker's health by preventing and controlling diseases and accidents and eliminating hazardous occupational factors in the workplace (WHO, 2002). Workers at WWTPs provide a crucial public service by ensuring optimal maintenance and functioning of these facilities (World Bank, 2019). However, WWTP workers may be exposed to multiple occupational and environmental health hazards associated with handling wastewater and sludge, operating heavy machinery, and working in confined spaces (World Bank, 2019). The National Institute of Occupational Safety has established a hierarchy of controls for managing hazardous occupational exposures, prioritising elimination, substitution, engineering controls, administrative controls, and PPE in descending order of effectiveness (Morris and Cannady, 2019). Although hazard elimination is the most effective strategy, it is not always feasible in most workplaces' settings, including WWTPs (Spellman, 2015; Morris and Cannady, 2019). It is important to note that the implementation of engineering and administrative control, such as improved ventilation, regular training for workers, and proper use of PPE, has been shown to significantly reduce the risk of occupational illnesses and injuries (Morris and Cannady, 2019).

Workers at WWTPs are exposed to various occupational health hazards including microorganisms, chemicals, gases, and physical hazards (World Bank, 2019). Repeated exposure to these hazards has been linked to several work-related illnesses and adverse effects. Since the 1970s, symptoms collectively termed "sewage worker's syndrome" have been reported among WWTP workers including headaches, fatigue, fever, gastrointestinal and respiratory symptoms, skin irritation, and musculoskeletal disorders (Rylander *et al*., 1976; Heldal *et al*., 2010). These findings underscore the need for effective occupational health and safety protocols to mitigate health risks in WWTP environments.

2.6.1 Gastrointestinal illnesses among WWTP workers

Exposure to partially treated or untreated wastewater has been linked to increased risk of gastrointestinal illnesses among WWTP workers (Fuhrimann *et al*., 2017; Adegoke *et al*., 2018). Commonly reported symptoms include diarrhoea, stomachache, indigestion, burping, abdominal pain, and nausea (Van Hooste *et al*., 2010; Fuhrimann *et al*., 2016; Hassanein *et al*., 2019). The main route of exposure is faecal-oral transmission, often resulting from poor hygiene practices such as eating, drinking or touching the face with contaminated hands or

gloves (Fuhrimann *et al*., 2016). This highlights the urgent need for improved hygiene protocols and protective measures to mitigate infection risks among WWTP workers.

Research on bacterial aetiologies of gastrointestinal illnesses among WWTP workers remains limited. Existing studies have primarily focused on leptospirosis (Samsudin *et al*., 2015; Fernandes *et al*., 2019; Jeffree *et al*., 2020), *Tropheryma whipplei* (Schöniger-Hekele *et al*., 2007), and *Helicobacter pylori* infections (Van Hooste *et al*., 2010; Hassanein *et al*., 2019). These studies reported increased seropositivity rates and presence of specific enteric bacteria in the stool samples of WWTP or sanitation workers highlights increased risk of gastrointestinal illnesses in this neglected occupational group (Schöniger-Hekele *et al*., 2007; Fernandes *et al*., 2019; Hassanein *et al*., 2019). Therefore, it is important to provide health education to these workers emphasising infection transmission and the critical role of protective measures in reducing exposure to enteric pathogens.

2.6.2 Respiratory health risks for WWTP workers

Occupational respiratory diseases remain a significant global health concern. According to the International Labour Organisation, occupational respiratory diseases and lung cancers account for one-third of the 2.5 billion worker deaths reported annually (Muzaini *et al*., 2021). Respiratory illnesses are commonly reported among sanitation workers including WWTP workers and can be associated with exposure to various occupational respiratory hazards (Chandra and Arora, 2018; Muzaini *et al*., 2021). Despite scientific evidence indicating that sanitation workers face higher risk of developing respiratory health issues, there has been limited research specifically focusing on WWTP workers (Heldal *et al*., 2010; Fahim and El-Prince, 2012; Cyprowski *et al*., 2015; Chandra and Arora, 2019).

Some of the prevalent respiratory symptoms and illnesses commonly reported among WWTP workers include asthma, wheezing, chronic obstructive pulmonary disease bronchitis, shortness of breath, and pulmonary tuberculosis (Heldal *et al*., 2010; Tabrizi *et al*., 2010; Al-batanony and El-Shafie, 2011; Tschopp *et al*., 2011; Fahim and El-Prince, 2012; Singh and Ladusingh, 2017; Chandra and Arora, 2019; Muzaini *et al*., 2021). A study among municipal WWTP workers in Norway revealed significantly higher rates of cough, and nose irritation, with odd ratios ranging from 2.3 to 5.0 among workers handling dry sludge (Heldal *et al*., 2010). Similarly, Singh and Ladusingh (2017) found that sewage workers had an increased risk of chronic bronchitis compared to waste collectors and drivers, noting that the use of safety

equipment positively influenced workers' respiratory health. More recently, Chandra and Arora (2019) assessed chronic morbidity profiles of WWTP workers in India. Respiratory illnesses included pulmonary tuberculosis, asthma, and chronic obstructive pulmonary disease. However, available literature on respiratory health of WWTP workers have been conducted primarily in developed countries, specifically Poland, Norway, and Sweden (Heldal *et al*., 2010; Cyprowski *et al*., 2015; Muzaini *et al*., 2021). This gap highlights the need for comprehensive studies across different geographical areas to better understand occupational respiratory health burden faced by WWTP workers globally.

Workers at WWTP are potentially exposed to respiratory hazards that can result in adverse respiratory health effects if proper personal protective equipment and control strategies are not consistently implemented (LeChevallier *et al*., 2020). Key respiratory hazards in this environment include inhalable dust (Heldal *et al*., 2010), hydrogen sulfide (Al-batanony and El-Shafie, 2011; Austigard *et al*., 2018), endotoxin (Tabrizi *et al*., 2010; Tschopp *et al*., 2011), and airborne microorganisms (Gotkowska-Płachta *et al*., 2013; Cyprowski *et al*., 2018; Lu *et al*., 2020). Bioaerosols, airborne particles containing both living and dead microorganisms (e.g., bacteria, viruses, fungi), endotoxins, and other toxic substances, have received increased attention due to their detrimental health effects on human health (Niazi *et al*., 2015; Kim *et al*., 2018; Schlosser, 2019). Several studies have investigated the microbial composition of bioaerosols emitted at WWTPs, with an emphasis on identifying disease causing components (Niazi *et al*., 2015; Kowalski *et al*., 2017; Lu *et al*., 2020). Bacteria are mostly abundant in WWTPs bioaerosols, with concentrations reaching up to 10^4 CFU/m^3 in countries such as China, Denmark, Middle East, and Poland (Niazi *et al*., 2015; Kowalski *et al*., 2017; Han *et al*., 2019; LeChevallier *et al*., 2020, Lu *et al*., 2020). Predominant bacteria identified in bioaerosols emitted during treatment processes at municipal WWTPs are listed in Table 2.3. The differences in bacterial concentrations across studies can be attributed to factors such as treatment size, specific sampling stage and time, sampling methodology or instruments used, as well as environmental conditions and plant location. However, treatment processes involving mechanical agitation, aeration tanks, and sludge treatment stages are primary sources of bioaerosols in WWTPs (Niazi *et al*., 2015; Kowalski *et al*., 2017; Michałkiewicz, 2019).

Despite the known health risks associated with bioaerosol exposure, there is currently no universally accepted exposure limits for hazardous biological agents. However, a threshold value of no more than 5000 CFU/m^3 during an 8-hr exposure shift has been recommended for workplace environments (Li *et al*., 2013; Wang *et al*., 2018). Nevertheless, the health effects

of bioaerosols on exposed workers are influenced by several factors, including the exposure pathway (e.g., inhalation or ingestion), use of PPE, ventilation equipment, work activities, and individual susceptibility (Wang *et al*., 2018).

Additionally, studies have shown that WWTP workers face higher risk of inhaling bioaerosols due to the multiple emission sources within treatment facilities (Kowalski *et al*., 2017; Yang *et al*., 2019). Risk assessment studies using the hazard index (HI) indicate a HI below 1 is considered safe, while values above 1 suggest a potentially carcinogenic risk, warranting concern (USEPA, 2011). However, HI findings on inhalable airborne bacteria among WWTP workers are inconsistent (Han *et al*., 2019; Yang *et al*., 2019; Lu *et al*., 2020). For instance, Han *et al*. (2019) reported consistently high HI values (>1) during warmer seasons, indicating significant risks, with males showing greater susceptibility due to higher inhalation rates. Conversely, Yang *et al*. (2019) and Lu *et al*. (2020) found HI values below 1 in studies conducted in China and Denmark, respectively, suggesting minimal risk from airborne bacteria for WWTP workers. However, it should be noted that these HI values were based on bioaerosol quantities detected using culture -dependent approach, which may underestimate concentrations of airborne bacteria. Variations in sampling methodologies and exposure times further contributed to the discrepancies in reported results.

Overall, it is imperative to develop standardised protocols for bioaerosol characterisation, and to establish a comprehensive database of airborne infectious agents in WWTPs representing different regions. This will be facilitating the development of robust risk assessment methodologies and targeted control strategies, ultimately enhancing safety and reducing infection risks among WWTP workers.

Table 2.3: Predominant pathogenic bacteria identified in bioaerosols from municipal WWTPs

Location	Treatment site	Sampling instrument, and time	Identified bacteria	Bacterial aerosol (CFU/m^3)	Analytical method	Reference
China	Grit chamber, SST, sludge dewatering room, oxidation ditch,	Six-stage Andersen impactor	Total bacteria	10^2 -10^3	Culture	Li *et al.,* 2013
Middle East	Aeration tank, SST, trickling filters, grit chamber and PST, sludge storage	Bio Stage single-stage cascade impactor, 2 min	*Bacillus, Micrococcus*, and *Staphylococcus.*	10^2 -10^3	Culture	Niazi *et al*., 2015
Poland	Mechanical treatment, aeration tanks, clarifiers, and sludge post-processing	Six-stage Andersen impactor, 8 min	*Staphylococcus, Bacillus, Micrococcus, Kocuria varians, Microbacterium, Rothia mucilaginosa, Corynebacterium,* Mesophilic *Actinomycetes: Streptomyces, Nocardia,* and *Pseudomonas.*	10^1 -10^3	Culture	Kowalski *et al*., 2017
Poland	All treatment stages	MAS 100 Eco type air sampler, not mentioned	Mesophilic bacteria, *Staphylococcus*, *Pseudomonas fluorescens*, and *Actinobacteria.*	0 -10^4	Culture	Michałkiewicz, 2019

China	PST, aeration tank, and SST	Six-stage Andersen impactor, 3 min	*Bacillus, Arthrobacter, Micrococcus, Streptomyces*, *Psychrobacillus,* and *Psychrobacter* were dominant. 65 species and 18 potentially pathogenic such as *Chryseobacterium*, *Stenotrophomonas*, *Alcaligenes, Micrococcus, Pantoea, Enterobacter* and *Escherichia-Shigella.*	10^2 -10^4	Culture, and 16S rRNA sequencing	Han *et al*., 2019
China	Fine grit, sludge dewatering room, oxidation ditch, aeration tank	Six-stage Andersen impactor, 2 min	50 genera and 21 potential pathogens. Dominant pathogens were *Rhodococcus, Staphylococcus, Arcobacter, Pseudomonas,* and *Mycobacterium.*	Relative abundance	16S rRNA sequencing	Yang *et al*., 2019
China	Influent, oxidation ditch, aeration tank, sludge dewatering room	Particulate matter sampler, 4 hours	Dominant genus was *Serratia, Yersinia, Klebsiella,* and *Bacillus.* In total, 95 potential pathogens were detected.	Relative abundance	16S rRNA sequencing	Han *et al*., 2020
Denmark	Aeration tank and grit chamber	Personal and stationary GSP samplers, workday shift (~5 hours)	131, 91, and 94 species were identified from personal, grid chamber and aeration tank, respectively. Pathogenic species were *Staphylococcus aureus, Yersinia enterocolitica, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae*	Personal exposure, 10^1 -10^3 Stationary, 10^2 - 10^3	Culture	Lu *et al*., 2020

		Six-stage Andersen impactor, 5, 10, and 30 min	*Morganella morganii, Pseudomonas aeruginosa, Neisseria meningitidis and Providencia rettgeri.*			
China	Aeration tanks	Six-stages Andersen impactor, 3 min	Predominant bacteria were *Bacillus Paenibacillus, Lysinibacillus, Pseudomonas, Acinetobacter,* and *Paenarthrobacter.*	10^2 -10^3	Culture and 16S rRNA sequencing	Li *et al*., 2022

Note: SST- secondary settling tank, PST – primary settling tank, CFU – colony forming unit

2.7 Predicted infection risks associated with exposure to bacterial pathogens in WWTP workers using QMRA

Quantitative microbial risk assessment (QMRA) has received significant attention in recent years as a scientific approach for assessing human risks associated with microbial agents in water. The WHO has established a structured QMRA framework to prevent and control waterborne infectious diseases (WHO, 2016). This framework involves a systematic process of hazard identification, exposure assessment, dose-response assessment, and risk characterisation, to quantify infection risks from microbial exposure (Haas *et al*., 2014). Previous studies on infection risks among WWTP workers (summarised in Table 2.4) indicate that annual infection risks vary depending on the exposure point within the facility and type of infection (Mbanga *et al*., 2020; Chen *et al*., 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Heida *et al*., 2024). Although, infection risks are generally higher near raw wastewater, aeration tanks, sludge handling areas, and during wastewater sampling, with values often exceeding the USEPA acceptable limit (≤1E-04 per person per year (pppy)) for both aerosol inhalation and accidental ingestion (Mbanga *et al*., 2020; Chen *et al*., 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Heida *et al*., 2024). These findings highlight the need for enhanced protective measures to reduce infection risks among workers at WWTP.

Despite these concerns, QMRA studies specifically assessing infection risks among WWTP workers as an occupational group remain limited and have only gained traction since the COVID-19 pandemic (Haas *et al*., 2017; Fuhrimann *et al*., 2017; Carducci *et al*., 2018; Deepnarain *et al*., 2020; Chen *et al*., 2021; Dada and Gyawali, 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Gui *et al*., 2022; Morales *et al*., 2023). Additionally, most research focuses on inhalation risks associated with gram-negative enteric bacteria, particularly pathogenic *E. coli* from bioaerosols (Mbanga *et al*., 2020; Chen *et al*., 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Heida *et al*., 2024).

Wastewater treatment plant workers in LMICs face heightened health risks due to inadequate occupational guidelines, frequent exposure to pathogens during manual sorting tasks, immune system vulnerabilites associated with poor socioeconomic conditions, and limited access to quality healthcare (World Bank, 2019; Philippe *et al*., 2022). Taken together, these factors increase their susceptibility to infections from waterborne pathogens. Despite these risks, QMRA studies evaluating occupational risks from waterborne pathogens in wastewater in LMICs remain scarce. Addressing this gap is crucial for enhancing risk assessments to inform the development of evidence-based interventions to protect worker health and safety.

Table 2.4: Quantitative microbial risk assessment studies estimating infection risks associated with bacterial pathogens among WWTP workers

Reference organisms	Exposure points	Exposure route	Exposure time and frequency	Annual median infection risk (value in bold >1E-04 pppy limit)	Location	Reference
Escherichia coli	• Influent • Effluent	• Accidental ingestion	52 times/year	• Influent 0 -1.2% • Effluent 23.8 -100%	South Africa	Mbanga *et al*., 2020
Staphylococcus aureus and *E. coli*	• Aeration tank	• Aerosol ingestion	20 min, 183 times/year	• *S. aureus* **>1E04**; *E. coli* **>1E04**	China	Chen *et al*., 2021
Total coliform, faecal coliform and Enterococci	• Aeration tank inspection	• Aerosol ingestion	1 hr, 180 times/year	• Total coliform **1.39E03** • Faecal coliform 1**.42E03** • Enterococci **1.28E03**	China	Wang *et al*., 2021
S. aureus and gram-negative bacteria	• Field engineer • Aeration tank inspection	• Aerosol inhalation	• Field engineer 30 min, 177 times/year • Lab technician	• *S. aureus* • Field engineer **1.08E03 -4.49E02** • Lab technician **6.15E04** • **-2.57E02**	China	Yan *et al*., 2021

	• Lab technician • Sampling at the aeration tank		10 min, 302 times/year	• Gram-negative bacteria • Field engineer **2.08E02 -3.80E02** • Lab technician **1.19E02 -2.18E02**		
E. coli	• Bar screen • Aeration tank • clarifiers • sludge handling	• Accidental ingestion • Aerosol ingestion • Hand to mouth • Aerosol inhalation	30 min	• Bar screen • Hand to mouth **9.9E01**; aerosol ingestion 2E05 • Aeration tank • Aerosol ingestion 6.8E08; aerosol inhalation 7.7E09 • Clarifiers • Hand to mouth **>9.8E02**; aerosol inhalation 7.7E09 • Sludge handling	South Africa	Amoah *et al*., 2022

L. pneumophila and E. coli	• Walking the plant • Contact • Direct splash • Lagoon sludge sampling	• Aerosol inhalation • Accidental ingestion	12 hrs	• Accidental ingestion 1.9E07 • *L. pneumophila* • Walking the plant 1.3E-05; lagoon sampling **7.8E-04** • *E. coli* • Accidental ingestion **5.3E-03**	Canada	Heida *et al*., 2024

Note: pppy -per person per year

2.8 References

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CHAPTER 3: POTENTIAL EXPOSURE TO RESPIRATORY AND ENTERIC BACTERIAL PATHOGENS AMONG WASTEWATER TREATMENT PLANT WORKERS, SOUTH AFRICA

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Potential Exposure to Respiratory and Enteric Bacterial Pathogens among Wastewater Treatment Plant Workers, South Africa

Evida Poopedi [1,2], Tanusha Singh [1,2,3] and Annancietar Gomba [1,*]

[1] National Institute for Occupational Health, National Health Laboratory Service, Johannesburg 2000, South Africa
[2] Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, Johannesburg 2050, South Africa
[3] Department of Environmental Health, University of Johannesburg, Doornfontein 2028, South Africa
* Correspondence: noncyg@nioh.ac.za; Tel.: +27-117-126-404

Abstract: Wastewater handling has been associated with an increased risk of developing adverse health effects, including respiratory and gastrointestinal illnesses. However, there is a paucity of information in the literature, and occupational health risks are not well quantified. Grab influent samples were analysed using Illumina Miseq 16S amplicon sequencing to assess potential worker exposure to bacterial pathogens occurring in five municipal wastewater treatment plants (WWTPs). The most predominant phyla were *Bacteroidota*, *Campilobacterota*, *Proteobacteria*, *Firmicutes*, and *Desulfobacterota*, accounting for 85.4% of the total bacterial community. Taxonomic analysis showed a relatively low diversity of bacterial composition of the predominant genera across all WWTPs, indicating a high degree of bacterial community stability in the influent source. Pathogenic bacterial genera of human health concern included *Mycobacterium*, *Coxiella*, *Escherichia/Shigella*, *Arcobacter*, *Acinetobacter*, *Streptococcus*, *Treponema*, and *Aeromonas*. Furthermore, WHO-listed inherently resistant opportunistic bacterial genera were identified. These results suggest that WWTP workers may be occupationally exposed to several bacterial genera classified as hazardous biological agents for humans. Therefore, there is a need for comprehensive risk assessments to ascertain the actual risks and health outcomes among WWTP workers and inform effective intervention strategies to reduce worker exposure.

Keywords: domestic wastewater; sanitation workers; biological hazards; occupational exposure; waterborne diseases

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1. Introduction

Municipal wastewater naturally contains hazardous biological agents, including bacteria, fungi, and viruses [1]. A significant amount of municipal wastewater is generated by households and contains human excreta such as faeces and urine, including that from disease-carrying individuals. Waterborne infections and diseases caused by ingestion, inhalation, or dermal contact with hazardous biological agents remain a global public health concern [1]. Daily work activities performed by WWTP workers such as manual screening and desludging, plant maintenance, and emergency repairs increase the risk of exposure to preventable waterborne diseases [2]. Although the World Health Organization (WHO) recognises the occupational health risks of WWTP workers [2], specific guidelines to protect wastewater workers from workplace hazards remain elusive, particularly in low to middle-income countries (LMICs). Consequentially, workers at wastewater treatment plants (WWTPs) are at perpetual risk of exposure to waterborne pathogens through different exposure routes [3]. Higher prevalence of gastrointestinal [4–6] and respiratory [6–9] symptoms have been reported in WWTP workers compared to controls. For instance, in a study to investigate the impact of inhalable particles and gas exposure on the respira- tory system of WWTP workers from urban and rural sewage plants and the sewer net

system in Norway, Heldal and co-workers (2019) [9] observed a lower lung function and a higher prevalence of airway symptoms (33 and 11%, respectively) among WWTP workers compared to the control group [8]. Furthermore, Van Hooste et al. (2010) [4] reported a higher prevalence (37%) of gastrointestinal symptoms such as stomach ache, abdominal pain, indigestion, heartburn, and burping compared to the control group (14.7%) among WWTP workers in Belgium [4]. However, the precise cause of the reported symptoms and illnesses is not well studied.

Among the most commonly detected microbial contaminants in municipal wastewater with potential to cause health issues in exposed populations, bacteria and their components are a major concern, particularly for immunocompromised individuals [10]. While a lot of studies on wastewater bacteriology employ traditional culture tests and/or targeted molecular assays to provide absence/presence and quantified measurements of a few selected bacteria, the advent of advanced high throughput DNA sequencing technologies dramatically increases the scope to comprehensively identify even the fastidious microorganisms. Therefore, advanced sequencing technologies provide a more accurate representation of the microbial population in a given sample with high taxonomic resolution [11].

In recent years, researchers have employed high throughput molecular approaches such as targeted amplicon sequencing and shotgun metagenomics to profile bacterial communities in wastewater and sludge. These studies have revealed the presence of several pathogenic bacteria at community level, including *Streptococcus pneumoniae*, *Vibrio cholerae*, *Salmonella* spp., *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Arcobacter* spp., and *Acinetobacter* spp., among others [10,12–15]. Considering that WWTP workers are potentially exposed to a plethora of harmful bacterial genera at any given time, knowledge of occupational exposure risks at microbial community level is critical. Moreover, microorganisms have evolved together in communities, and certain health effects may be due to synergistic or antagonistic behaviors of specific pathogens [16]. Additionally, understanding microbial composition at community level enables microbial source tracking and comprehensive risk assessment to minimise exposure to harmful biological agents.

Microbial communities in municipal wastewater and the extent of worker exposure may differ depending on geographic location, season, time of the day, population served, facility treatment capacity, the technology used, and performed activities [17–19]. Yet there is a general lack of research in LMICs to characterise bacterial communities in untreated wastewater using high throughput sequencing technologies [15,20,21], with little to no data documented in most regions [22,23]. In South Africa, for example, previous studies in wastewater environments have mainly focused on pathogen removal and effluent quality, essential to protect public health and the environment. Consequently, the likelihood of occupational exposure to untreated or partially treated wastewater at WWTPs and the associated health risks in LMICs remain unclear. Hence, in this study we used targeted 16 rRNA gene amplicon sequencing to describe bacterial diversity and identify bacterial pathogens that could pose an occupational health risk from repeated exposure to untreated municipal wastewater.

2. Materials and Methods

2.1. Study Design

The study was an experimental design conducted at the National Institute for Occupational Health, Immunology and Microbiology Laboratory, South Africa. Grab influent samples were obtained from five participating WWTPs on 20 October (WWTP2, 3 and 4) and 21 October (WWTP1 and 5), 2020.

2.2. Site Description

Sampling was conducted at five municipal WWTPs in the City of Tshwane Metropolitan Municipality, South Africa. The characteristics of the sampling sites are summarised in Table 1. The selected WWTPs all receive wastewater from healthcare facilities; however,

the quantities are not measured. Non-sewered informal settlements were observed nearby WWTP1, 2, and 4 but no intensive animal (cattle or battery chicken) production or roaming domestic animals were evident near any of the sampling sites.

Table 1. Characteristics of WWTP sampling sites.

Site	WWTP1	WWTP2	WWTP3	WWTP4	WWTP5
Source of wastewater (%)	Mixed (domestic (90) and industrial (10)	Domestic (100)	Mixed (domestic (80) and industrial (20)	Domestic (100)	Domestic (100)
Population size served	366,709	600,000	236,580	1,041,200	472,000
Treatment capacity * (ML/day)	35	60	93	180	85
Treatment train	Raw influent, bar screens, grit removal chamber, primary clarifiers, surface aeration tank, secondary sedimentation	Raw influent, bar screens, grit removal chamber, primary clarifiers, surface aeration tank and trickling bio-filters, secondary sedimentation	Raw influent, bar screens, grit removal chamber, primary clarifiers, surface aeration tank, secondary sedimentation	Raw influent, bar screens, grit removal chamber, primary clarifiers, diffused aeration tank, secondary sedimentation	Raw influent, bar screens, grit removal chamber, primary clarifiers, diffused aeration tank, secondary sedimentation
Biological treatment	Activated sludge	Activated sludge and	Activated sludge	Activated sludge	Activated sludge
Tertiary treatment	Chlorine disinfection	bio-filters Chlorine disinfection	disinfection Chlorine	Chlorine disinfection	Chlorine disinfection
Intended reuse of treated effluent	Irrigation and housekeeping purposes	Irrigation and cooling water to a nearby power station	Housekeeping purposes	Discharged into a river	Agricultural purposes
Workforce	22	38	37	66	27

ML/day—mega litres per day.

2.3. Sample Collection

Grab influent samples were collected in 1 litre sterile polypropylene bottles (Sigma-Aldrich, Saint Louis, MO, USA). Samples were transported on ice and stored at 2–8 °C for not more than 24 h until analysis. Sample temperature and pH were measured during sampling using a digital water-resistant thermometer 125 mm (−50 to +200 °C) (Lasec, Cape Town, South Africa). Metadata such as environmental temperature, relative humidity, and CO_2 were measured using IAQ-CALC Indoor Air Quality Meter, Model 7545 (TSI Instruments Ltd., High Wycombe, UK)

2.4. Microbial Cell Concentration

Microbial cells were concentrated following the protocol by Kumar et al. (2020) [24]. Briefly, each 1 L grab sample was manually shaken by hand to allow adequate mixing and an aliquot of 200 mL in four batches of 50 mL was centrifuged at 5000 rpm for 45 min at 4 °C. The supernatant was carefully removed and each pellet was washed in 1 mL of sterile distilled water to remove deposited salts and other impurities. The four pellets were pooled and stored at −20 °C until further analysis.

2.5. Total Genomic DNA Extraction and 16 S Sequencing

Total genomic DNA (gDNA) was extracted from the pelleted biomass of influent samples using DNeasy Powersoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified using a Nanodrop 2000c spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA). The gDNA was amplified with primers targeting bacterial V3 and V4 regions, namely, 16S Amplicon PCR forward primer (5[1]-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and 16S Amplicon PCR reverse primer (5[1]-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) [25]. The PCR reaction contained 12.5 µL of 2x KAPA HiFi HotStart ready mix, 5 ng/µL gDNA template, and 5 µL of 1 µM each primer. Cycling conditions included initial denaturation at 95 °C for 3 min, 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 5 min. The 16S amplicons were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA), and paired-end libraries were attached using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) and Illumina sequencing adapters (Illumina, USA). Indexed amplicons were cleansed with AMPure beads and normalised with PhiX Control v3 (Illumina, USA). Purified PCR amplicons were then sequenced using the Miseq platform (Illumina, USA).

2.6. Data Analysis

Raw reads were quality controlled and filtered (Q > 20 and length > 50 bp) using fastqc (v0.11.8) and trimGalore (v0.6.4_dev; https://github.com/FelixKrueger/TrimGalore, accessed on 13 July 2022), respectively. TrimGalore was also used to remove adapters. Krona charts for interactive data visualisation were generated using Kraken2 [26] and Krona [27]. All downstream analyses, including classification, abundance estimations, statistical analysis, and visualisation, were carried out in R (v3.6.1 Dada2 package (v1.12.1) [28] which was used to pre-process clean reads, including quality inspection, trimming, de-replication, merging paired-end reads, and removal of chimeric sequences. The obtained amplicon sequence variants (ASVs) were taxonomically classified, and ASV abundance estimates were determined using training sequence sets based on the SILVA reference database (v138; https://zenodo.org/record/1172783#.XvCmtkUzY2w, accessed on 14 July 2022). Ordinations for beta diversity, abundance bar plots, alpha diversity, and richness estimates were generated using the phyloseq (v1.28.0) [29], ggplot2 (v3.2.1), and AmpVis2 (v2.6.4) [30] packages. To compare alpha diversity between groups, Wilcoxon and/or Kruskal–Wallis rank-sum tests were used. The UpSet plots were generated using UpsetR (v1.4.0) [31]. DESeq2 (v1.24.0) was used to perform differential abundance analysis between sample groups [32].

3. Results

3.1. Alpha Diversity

A total of 397,766 quality raw reads were generated from all the samples collected (Table 2). The alpha diversity indices revealed comparably similar community diversity across the sites, except for WWTP4, which showed the highest diversity and species richness. The values for species richness (Chao1 and Abundance-based coverage estimators) ranged between 534–957 and for evenness (Shannon) ranged between 5.85–6.32. These results were confirmed by the rarefaction curve estimates, with WWTP4 presenting considerably higher values than the other sites, followed by relatively similar low values between WWTP1 and 2, and WWTP 3 and 5.

Table 2. Community diversity of influent wastewater samples from five WWTPs.

Sample	Total Reads	ASV	Chao1	ACE	Shannon	Simpson
WWTP1	85,200	19,449	695	697	6.00	0.997
WWTP2	75,629	16,334	643	646	5.91	0.996
WWTP3	59,881	13,594	534	535	5.85	0.996
WWTP4	84,585	20,300	949	957	6.32	0.998
WWTP5	92,471	16,923	508	510	5.85	0.996

ASV: Amplicon sequence variant, ACE: Abundance-based coverage estimators.

The rarefaction curves for the observed operational taxonomic units (OTUs) for all samples plateaued at around 5000 reads (Figure 1), indicating adequate sequencing depth; hence, a good representation of the bacterial community was achieved as most of the abundant species and some rare species are represented.

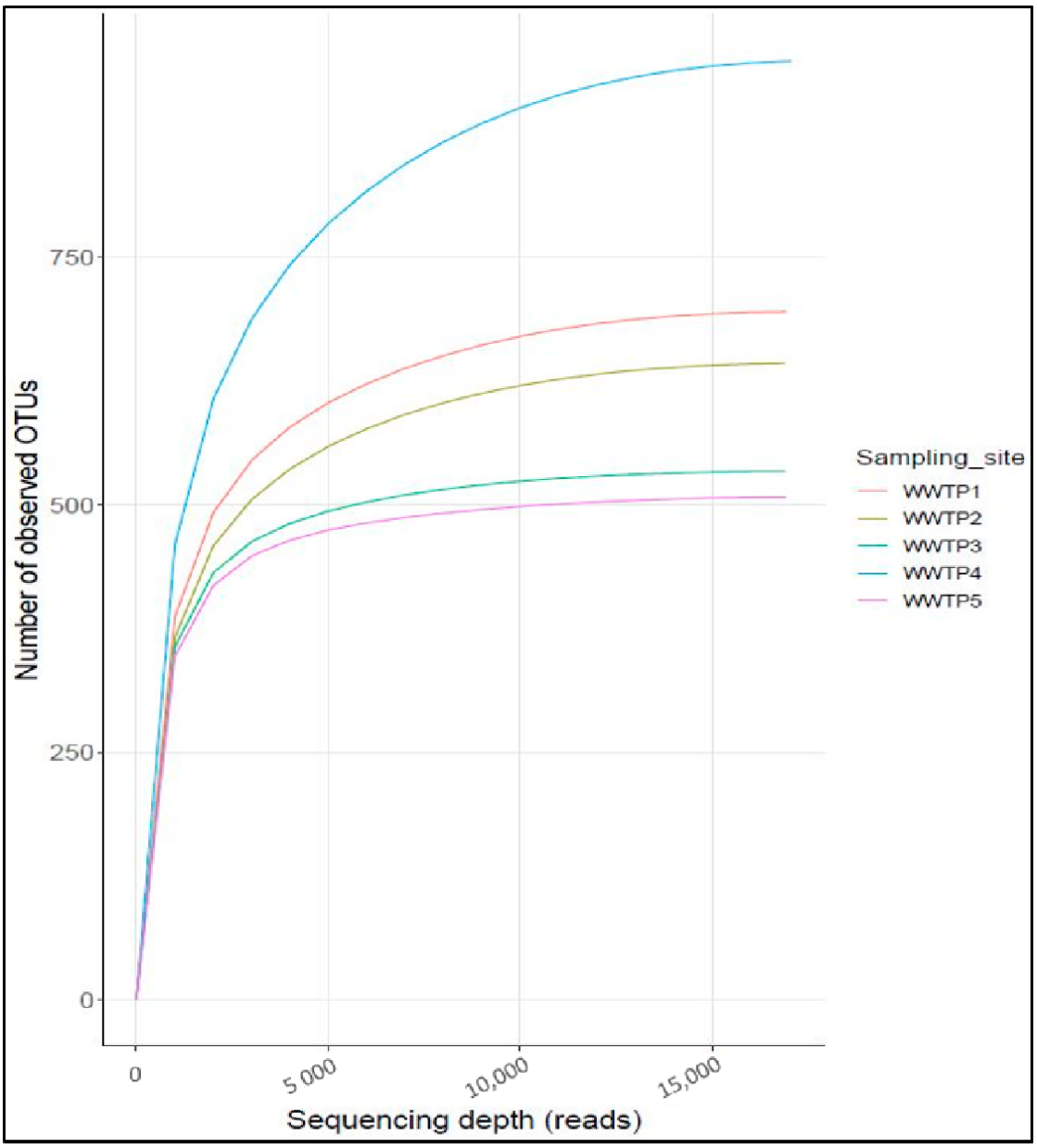

Figure 1. Rarefaction curves showing bacterial richness of influent wastewater from five WWTPs.

3.2. Taxonomic Comosition at Phylum, Family, and Genera Levels

A total of 24 phyla were identified, with fourteen (*Acidobacteriota, Actinobacteriota, Bacteroidota, Campilobacterota, Cyanobacteria, Desulfobacterota, Elusimicrobiota, Firmicutes, Fusobacteriota, Patescibacteria, Proteobacteria, Spirochaetota, Synergistota, Verrucomicrobiota*) appearing across the five WWTPs (Figure 2). Of these, the five most predominant phyla were *Bacteroidota* (31.7%), *Campilobacterota* (18.4%), *Proteobacteria* (20.2%), *Firmicutes* (8.6%), and *Desulfobacterota* (6.5%).

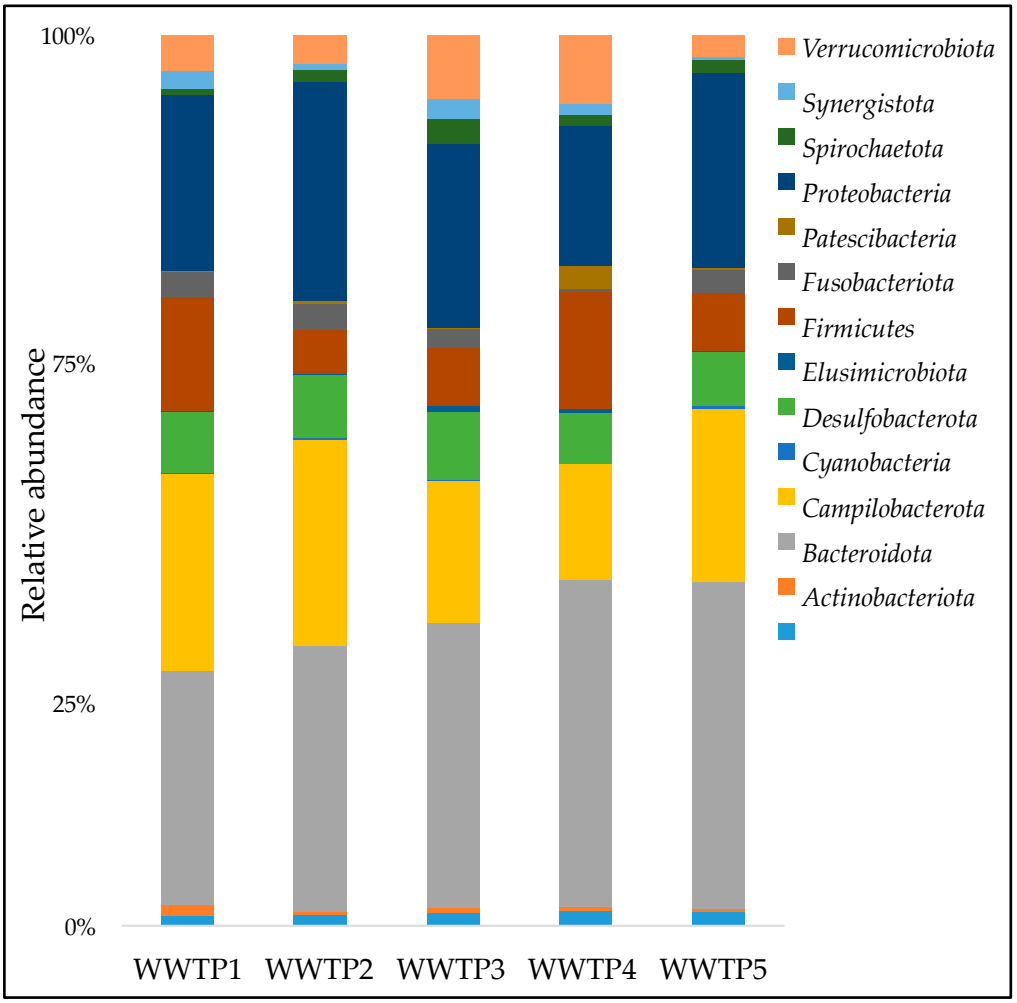

Figure 2. Relative abundance of the predominant phyla in influent wastewater.

A total of 122 families were identified across all samples. Families of the top three dominant phyla contributing at least 1% to total abundance in the five WWTPs influent samples are shown in Figure 3. For the phylum *Bacteroidota*, the predominant families were *Bacteroidaceae*, *Paludibacteraceae*, *Prolixibacteraceae*, *Tannerellaceae*, and *Williamwhitmaniaceae*. The phylum *Proteobacteria* was dominated by *Pseudomonadaceae*, and *Rhodocyclaceae* and *Campilobacterota* were dominated by *Arcobacteraceae* and *Sulfurospirillaceae*.

At the genus level, 253 genera were identified from all samples accounting for the majority of the classified sequences, with approximately 25% of the total communities remaining unidentified. Genera that were detected at all sites with a relative abundance of at least 1% are presented in Figure 4. *Sulfurospirillum*, *Macellibacteroides*, and *Bacteroides* were the three most abundant genera identified from the influent samples, accounting for 12.8, 5.2, and 5.1% of total genera, respectively.

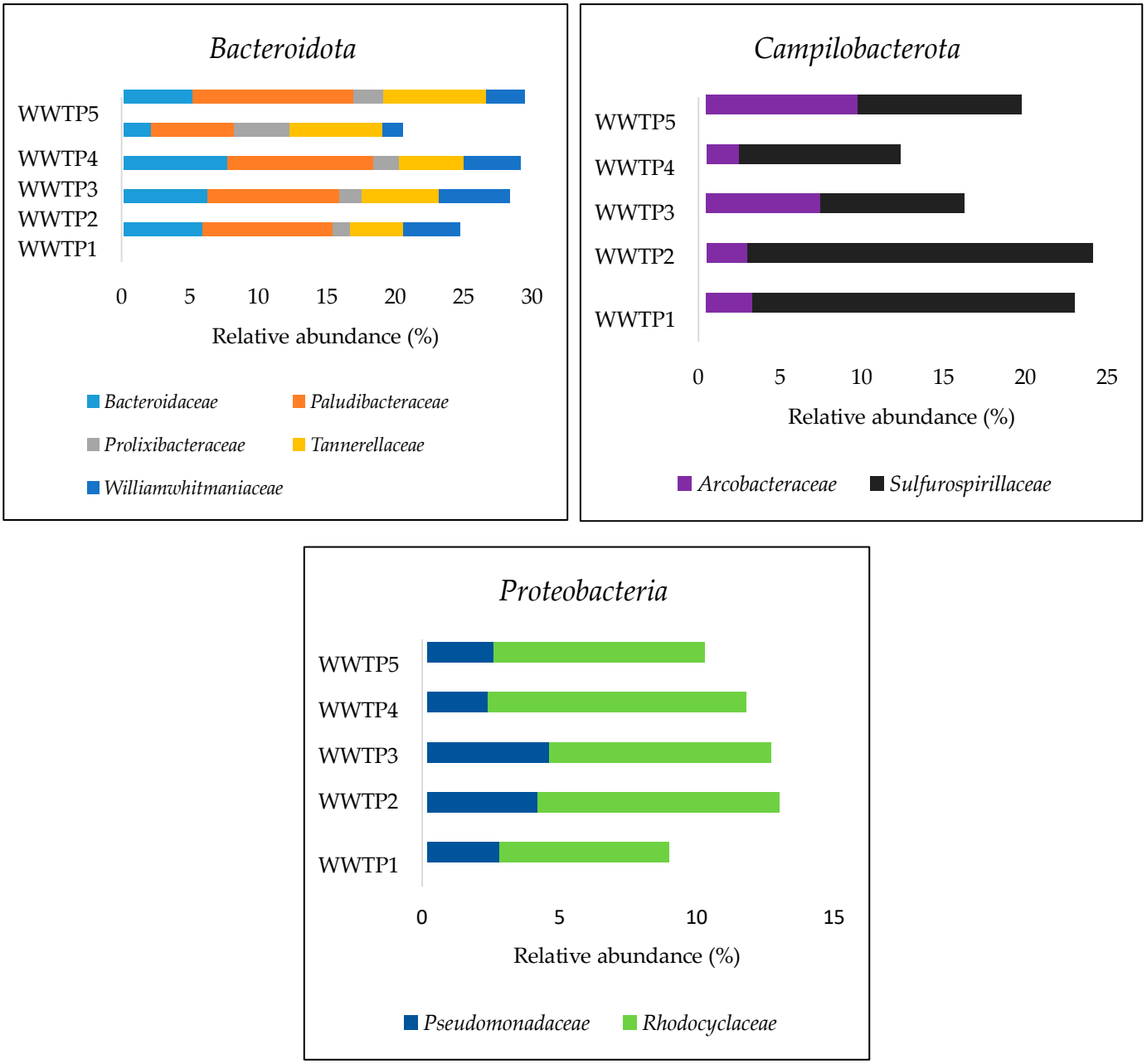

Figure 3. Relative abundance of the families of the top three dominant phyla *Bacteroidota*, *Campilobacterota*, and *Proteobacteria* from the WWTP influent samples.

3.3. Shared and Distinct Bacterial Genera

Shared bacterial genera (those that appeared in two or more WWTPs) and distinct genera (those that occurred at one WWTP) are presented in Figure 5. Of the 253 genera identified, 40 were shared by all five sites, 24 shared by four sites, 32 by three sites, and 29 by two sites. WWTP4 had the highest bacterial richness while WWTP3 showed the least richness in the number of genera detected. We also observed genera that were unique to specific sites with 62 distinct genera observed at WWTP4, 29 at WWTP1, 15 at WWTP2, and 14 at WWTP5. The least diverse bacterial genera, with only eight distinct genera, were observed in WWTP3.

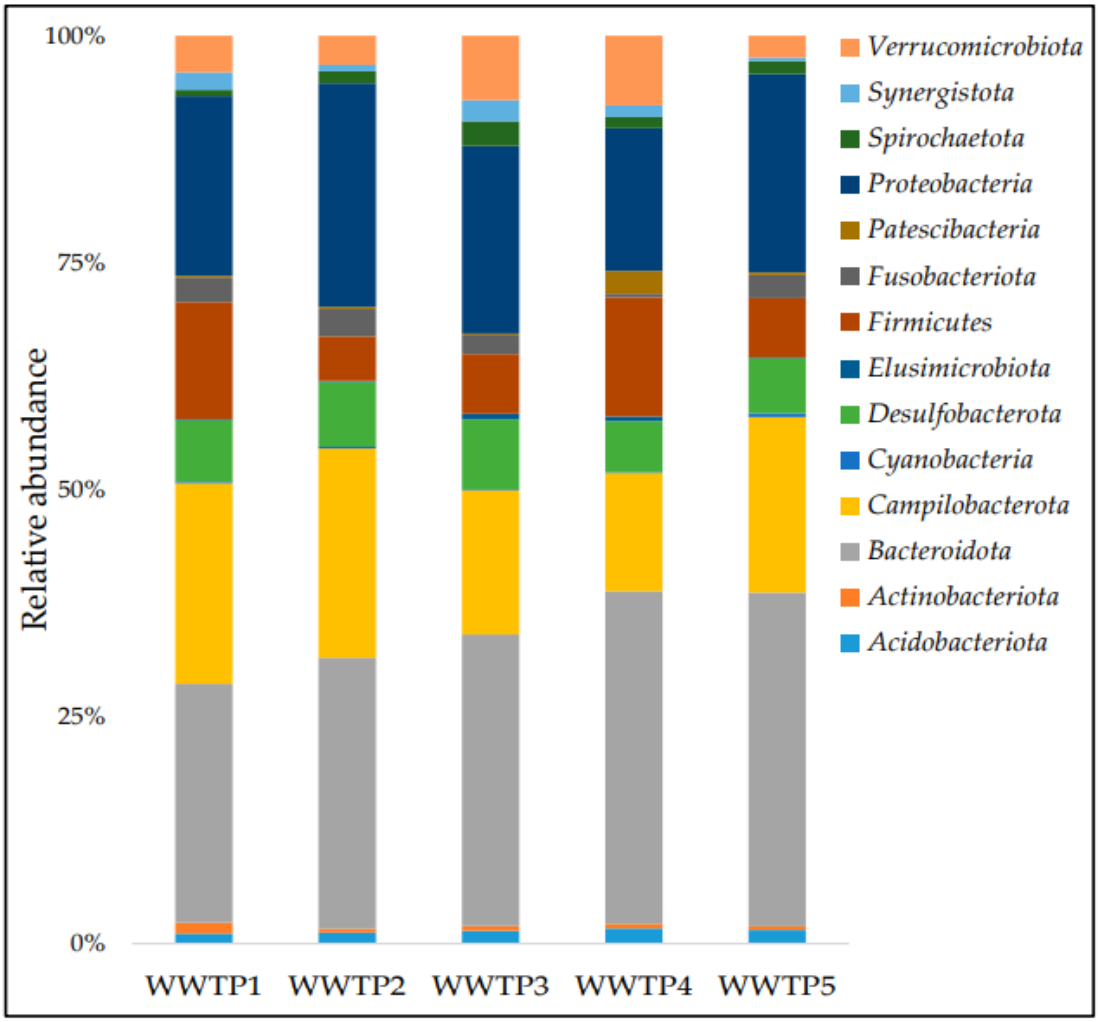

Figure 4. Relative abundance of predominant genera across the five WWTPs.

3.4. Genera That Contain Potential Pathogenic Species in Influent Samples

A total of 36 genera (approximately 20% of total abundance) of medical importance to human health were identified (Table 3) and belonged to the major phyla *Bacteroidota*, *Campilobacterota, Proteobacteria, Firmicutes, Synergistota, Fusobacteriota*, and *Actinobacteriota*. The dominant pathogenic genera were *Bacteroides* (5.1%), *Pseudomonas* (2.9%), *Aeromonas* (2.8%), *Arcobacter* (2.6%), *Leptotrichia* (1.4%), *Treponema* (0.9%), *Streptococcus* (0.6%), *Enterobacter* (0.5%), *Shewanella* (0.5%), and *Acinetobacter* (0.4%).

Pathogenic Genera and Their Potential Health Outcomes

Table 3 shows the relative abundance of pathogenic genera from the bacterial community as represented by influent samples of the five WWTPs. Potentially pathogenic bacterial genera were classified into three main groups according to the type of infection they cause in humans: (1) respiratory pathogens, (2) enteric pathogens, and (3) opportunistic pathogens commonly associated with nosocomial infections and multidrug resistance.

Respiratory: *Mycobacterium* and *Coxiella* were the most abundant respiratory tract-associated pathogens contributing up to 0.1% and 0.05% of the total bacterial community of influents, respectively. *Mycobacterium* was detected only at WWTP1 and WWTP2, with a relative abundance of 0.2% at each plant, while *Coxiella* was only detected at WWTP4, accounting for approximately 0.2% of the total bacterial community at this site.

Enteric: Enteric pathogens detected were *Escherichia/Shigella*, *Laribacter*, *Arcobacter*, and *Aeromonas* with total relative abundances of 0.1%, 0.2%, 2.6%, and 2.8%, respectively.

Aeromonas was the most prevalent enteric pathogen, with a relative abundance ranging between 0.7% and 5.3%, followed by *Arcobacter* with the highest abundance (9.7%) at WWTP5 and the lowest at WWTP3 (0.9%). It is also important to mention that *Aeromonas* and *Arcobacter* were detected at all sites. *Escherichia/Shigella* were rare genera detected only at WWTP1 and WWTP2, with abundances of 0.3% and 0.1% at the two sites, respectively.

Opportunistic: Thirty opportunistic bacterial genera were identified in the present study, with an overall abundance of 14.4% in the total bacterial community (Table 3). The top three opportunistic pathogens were *Bacteroides, Pseudomonas*, and *Leptotrichia* contributing (5.1%, 2.9%, and 1.4%, respectively, of the total bacterial community. The other opportunistic genera were <1% in relative abundance. A total of 13 opportunistic genera (*Acinetobacter, Aeromonas, Arcobacter, Bacteroides, Comamonas, Dysgonomonas, Enterobacter, Leptotrichia, Pseudomonas, Pseudoxanthomonas, Shewanella, Streptococcus*, and *Treponema*) were detected in at least four of the five WWTPs.

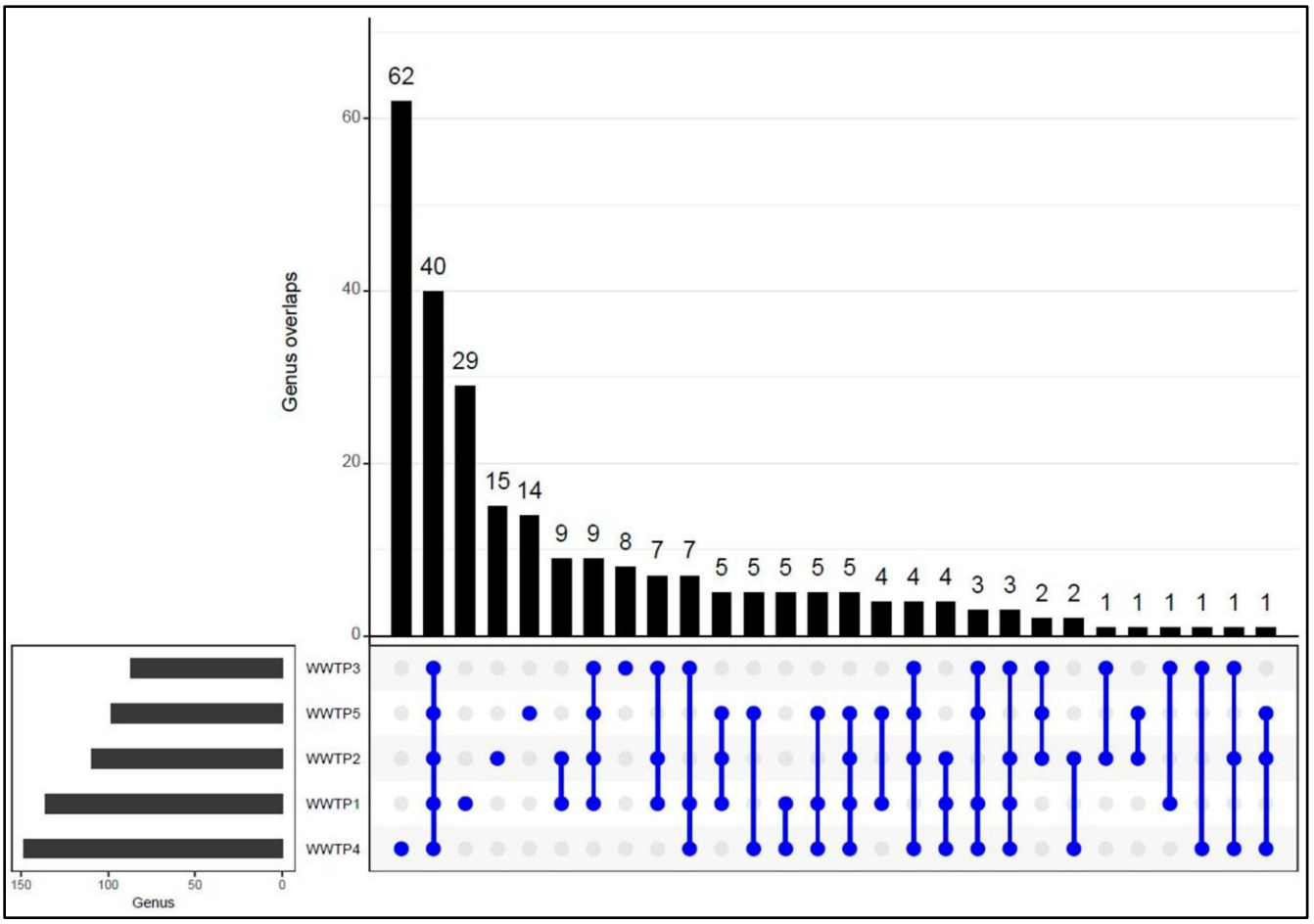

Figure 5. UpSet plot showing shared and distinct bacterial genera in influent wastewater from five WWTPs. Numbers above the bars indicate the number of overlapping genera between different WWTPs appearing in the left panel display.

Table 3. Overall relative abundance of pathogenic genera and their risk evaluation by type of infection.

Genera by Type of Infection	Relative Abundance (%)	HBA Risk Group
Respiratory:		
Coxiella	0.05	3
Mycobacterium	0.1	2/3
Enteric:		
Aeromonas	2.8	2
Arcobacter	2.6	unclassified
Escherichia/Shigella	0.1	2/3
Laribacter	0.2	2
Opportunistic:		
Acinetobacter	0.4	2
Actinomyces	0.1	2
Atopobium	0.05	unclassified
Bacteroides	5.1	2
Blastomonas	0.05	unclassified
Brachybacterium	0.05	unclassified
Chryseobacterium	0.05	unclassified
Citrobacter	0.05	unclassified
Comamonas	0.3	unclassified
Dysgonomonas	0.3	unclassified
Empedobacter	0.05	unclassified
Enterobacter	0.5	2
Enterococcus	0.05	2
Erysipelothrix	0.1	2
Finegoldia	0.05	unclassified
Gordonia	0.05	unclassified
Klebsiella	0.05	2
Leptotrichia	1.4	unclassified
Leuconostoc	0.05	unclassified
Ochrobactrum	0.05	unclassified
Prevotella	0.2	2
Pseudomonas	2.9	2/3
Pseudoxanthomonas	0.1	unclassified
Roseomonas	0.05	unclassified
Shewanella	0.5	unclassified
Sphingobacterium	0.05	unclassified
Streptobacillus	0.05	2
Streptococcus	0.6	2
Synergistes	0.1	unclassified
Treponema	0.9	2

3.5. Risk Characterisation of Potentially Pathogenic Bacteria

The observed pathogenic genera were classified into different risk groups according to the revised South African Regulation for Hazardous Biological Agents, 2022 [33] (Table 3). Thirteen of the 36 genera (36%) belong to HBA Risk Group 2 (may cause disease, are unlikely to spread to the community, and effective treatment is available), and three genera belong to HBA Group 2 or 3 (HBA Risk Group 3, may cause severe disease, present a risk of spreading to the community but effective treatment is available) depending on the species type (Table 3). *Coxiella* belongs to HBA Risk Group 3 and has only one member, *C. burnetii*. In summary, close to 50% (17/36) of pathogenic genera identified were classified as hazardous biological agents, indicating that these organisms can cause human diseases and may pose a health risk to WWTP workers.

4. Discussion

4.1. Bacterial Community Composition in Influent Samples

Municipal WWTPs receive wastewater from different sources but mostly households, surface runoff, and industrial activities, contributing to the complexity of bacterial communities in wastewater [34]. The phyla *Bacteroidota*, *Campilobacterota*, *Proteobacteria*, *Firmicutes*, and *Desulfobacterota* were predominant in the present study. Except for *Campilobacterota*, the top dominant phyla in the current study have previously been reported in high abundance in influent wastewater [35,36]. Wu and colleagues (2019) [37] recently provided a comprehensive wastewater analysis on a global scale, representing 23 countries from six continents including Africa, Asia, Australia, Europe, North America, and South America. Their findings revealed that despite the considerable diversity in bacterial communities between samples, a core global community (28 OTUs) exists. A majority of these members belonged to the phyla *Proteobacteria* and *Bacteroidota*, implying some degree of bacterial community conservation in municipal wastewater at higher taxa rank [37]. In addition, the phyla *Bacteroidota*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* have consistently been reported as the predominant bacterial community members in the human microbiome, suggesting that a large proportion of bacterial members in the samples analysed originated from human faecal material [38]. When comparing bacterial communities in other wastewater types, in particular, activated sludge, previous studies highlighted a distinct microbial ecosystem with high bacterial diversity and a high concentration of biomass in activated sludge samples [39,40]. Begmatov and co-workers [40] recently reported a dominance of *Proteobacteria*, *Chlorofexi*, *Myxococcota*, *Firmicutes*, *Patescibacteria*, and *Nitrospirota* in activated sludge [40]. A comparison between the activated sludge community identified in Moscow with that identified globally by the Global Water Microbiome Consortium show clustering, emphasising that influent characteristics, which are largely influenced by cultural, social, and environmental factors in each region, are more important than WWTP operating conditions [40].

It is noteworthy that at the genus level in the current study, bacterial composition exhibited some degree of diversity across the five WWTPs. The highest bacterial richness was recorded at WWTP4, whereas WWTP3 showed the least richness in the number of genera detected. The lowest bacterial richness was found at WWTP3, which could be explained by the fact that this plant also treated industrial wastewater, whereas the other four WWTPs received municipal wastewater primarily from households. Chemical substances in industrial wastewater have been shown to negatively impact microbial community structures, resulting in reduced bacterial richness and diversity compared to municipal wastewater [41,42]. Interestingly, WWTP4 contained many distinct genera compared to the other plants, which may be attributed to it being the largest treatment plant in the area, serving approximately seven different communities. Overall, these findings suggest that while some WWTPs harboured exclusive genera, the predominant genera did not vary considerably regardless of plant location, indicating a high degree of bacterial community stability in the influent source.

4.2. Potentially Pathogenic Genera

This study grouped bacterial genera with known disease-causing species into three major infection categories: respiratory, enteric, and opportunistic pathogens.

Coxiella and *Mycobacterium* were the only medically important genera identified in the present study with the potential of causing respiratory tract infections. Although the total relative abundance of these genera was much lower than that of the majority of the identified genera, *C. burnetii* and *M. tuberculosis* have extremely low infectious doses, requiring less than 10 living organisms to cause an infection [43,44]. Respiratory pathogens cause infections of the upper and lower respiratory tract. By far, the most serious respiratory infections involve the lower respiratory tract such as bronchitis and pneumonia, and are the leading cause of high mortality rates worldwide [45]. The abundance of *Mycobacterium* in influent samples was comparable to previous studies [10,12,46]. For instance, studies

conducted in Germany and Australia found that the overall abundance *Mycobacterium* in influent and effluent was less than 0.02% [12,46]. In comparison to other types of wastewater, studies on *Mycobacterium* in influent are not common. However, *Mycobacterium* has primarily been studied in effluents to assess the efficacy of wastewater treatment processes in removing biological agents and the safety of treated effluents [21,36,47]. Humans become infected with *Coxiella* by inhaling aerosols from contaminated animal waste, soil, or food products, and veterinarians, slaughterhouse, and farm workers are generally considered to be at increased risk of occupational exposure to *Coxiella* [43]. Given that WWTPs receive wastewater from various institutions and farms, it raises the question of whether WWTP workers are at risk of occupational exposure to this pathogen.

Five enteric genera, namely, *Escherichia/Shigella*, *Laribacter*, *Arcobacter*, and *Aeromonas* were identified in the study, accounting for 5.7% relative abundance of the total bacterial community. Enteric pathogens normally reside in the intestines of humans and can utilise their pathogenic mechanisms to cause gastrointestinal tract infections [48]. Enteric organisms are typically transmitted via the faecal–oral route, and illness symptoms are caused by consuming contaminated food or water [48]. Using metagenomics analysis, previous studies have reported the prevalence of *Aeromonas* to be noticeably high in wastewater with counts similar to those of faecal coliforms [47,49,50]. Ye and co-workers [47] analysed potentially pathogenic bacteria in sixteen samples comprising of influent, activated sludge, and effluent from 14 municipal WWTPs across, China, Canada, United States, and Singapore, and reported that *Aeromonas* were among the most dominant genera occurring at least in ten samples across four countries [47]. Other metagenomics studies [17,51–53] have also reported a high abundance of *Aeromonas* in various wastewater types. *Aeromonas* genus has been ranked third as the leading cause of diarrhoea after *Camplylobacter* and *Salmonella* [54], and incidences of gastroenteritis linked to *Aeromonas* have been reported across the globe, with cases more common in developing countries [54]. Two *Arcobacter* species, *A. butzleri* and *A. cryaerophilus*, are considered emerging pathogens threatening human health [55]. Human-associated *Arcobacter* species have been consistently isolated from human sewage systems [35,55]. In the present study, the genus *Arcobacter* was observed in high abundance exclusively at WWTP5 (9.7%), compared to the other WWTPs that had an abundance ranging from 0.9% to 2.5%. This inconsistency could not be explained further. In the present study, the genus *Laribacter* was 0.2% relative abundance, which is comparable with that reported in municipal influent (≤0.1% relative abundance) from a study conducted at three WWTPs in Western Australia [56]. The genus *Laribacter* has one species, *L. hongkongensis*, which is associated with traveller gastroenteritis and diarrhoea [57]. It should be noted that, even though *Laribacter* has not been previously reported among the most prevalent genus in wastewater, if ingested, this bacterium can cause gastroenteritis in humans [56].

The present study identified 32 genera that could cause opportunistic infections in humans. Although opportunistic pathogens pose little risk to healthy WWTP workers, they can cause serious illnesses in individuals with weakened immune systems and the elderly. The main concern with opportunistic organisms is that they are typically resistant to commonly used antimicrobial treatments, posing a serious problem for public health [58]. Five pathogenic genera (*Enterococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacter*) that could contain species belonging to the group of ESKAPE pathogens were identified in the present study with *Pseudomonas* topping the list. Members of the ESKAPE pathogens are well known for their ability to develop multidrug resistance and account for most nosocomial infections [59]. Moreover, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Streptococcus pneumonia* are on the WHO priority list of antibiotic-resistant pathogens as they can cause fatal infections [60]. Resistance to commonly used antibiotics is a serious global problem. Current efforts to monitor the development of antimicrobial resistance have mainly focused on clinical settings; however, interest has grown in recognising the importance of antibiotic resistance in the environment and water supply [57]. In fact, WWTPs have been implicated as key reservoirs, incubators, and source for disseminating antimicrobial resistance and virulence genes [61].

4.3. Risk Characterisation of Identified Potential Pathogens

In this study, 17 genera were classified as potentially hazardous biological agents to human health. Overall, the risk characterisation exercise revealed that workers at WWTP may be exposed to genera that cause airway obstruction, gastrointestinal problems, and opportunistic infections in the workplace. Except for *Mycobacterium* and a few Gram-positives such as *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Erysipelothrix*, *Atopobium*, *Brachybacterium*, *Finegoldia*, *Gordonia*, and *Actinomyces*, a majority (28/36 genera) of the classified pathogenic genera identified at WWTPs were Gram-negative. Gram-negative bacteria express endotoxins as their main component of the outer membrane [62], and the presence of numerous and diverse Gram-negative bacteria at WWTPs may be a major contributor to workers' exposure to elevated levels of endotoxins. Long-term exposure to inhalable endotoxins has been linked to inflammatory responses in the lungs, leading to symptoms such as chronic bronchitis, organic toxic dust syndrome, or asthma [63]. Therefore, the findings of this study suggest that workers at the selected five WWTPs may be exposed to pathogens, including endotoxins from Gram-negative bacteria, which could compromise their respiratory health.

Waterborne diseases are expected to rise with climate change and a growing global population [64]. Consequently, WWTP workers remain at risk from waterborne diseases and outbreaks irrespective of the country's economic status (i.e., developed or developing) or whether in the tropics or temperate, highlighting the importance of regular monitoring of wastewater microbiomes using advanced but cost-effective detection techniques, precise disinfectant procedures, and proper management of operations to minimise worker exposure. The morbidity and mortality of waterborne diseases are enormous [64], and they can only be controlled by providing workers with a microbiologically safe environment. WHO has established a list of priority waterborne pathogens with moderate to high health significance [65]. In the present study, *Mycobacterium* and *Escherichia/Shigella* genera were detected in wastewater and these organisms are listed on the WHO priority pathogens. Additionally, emerging waterborne pathogens of importance *Aeromonas* and *Leptotrichia* were detected in the study. Therefore, the findings of this study warrant further investigation into incidences of infections in WWTP workers associated with exposure to these pathogens. Workers at WWTP play an important role in urban communities, making sure that the treatment plants function optimally. However, the working conditions for sanitation workers, including WWTP workers, expose them to significant health risks such as waterborne diseases, injuries, and even death [22].

4.4. Limitations of the Study

Data presented in this study are from a single type of wastewater (influent), and samples were collected once over one month from five municipal treatment plants in South Africa. Hence, the small sample size is a limitation. Furthermore, sample collection during and outside the respiratory infection season was impractical as some COVID-19 symptoms were similar to respiratory influenza and occurred throughout the year; hence, defining the respiratory infection season was challenging and thus omitted. Therefore, more research is needed to monitor trends and changes in the diversity of bacterial pathogens in various types of wastewater on a large scale over a long period to assess temporal and spatial variation. Although the current study provides local baseline data on potential pathogens circulating in WWTP environments, further research to provide impact of exposure on workers' health is needed. Therefore, a follow-up study assessing the associations between work activities and the incidence of gastrointestinal and respiratory infections among WWTPs in South Africa is underway. This information is currently non-existent in South Africa, making it difficult to reform occupational health and safety policies and practices.

5. Conclusions

The study found evidence of several potentially pathogenic bacteria in untreated municipal wastewater, which may pose a health risk to WWTP workers. The major phyla identified in the study were *Bacteroidota*, *Proteobacteria*, *Campilobacterota*, *Firmicutes*, and

Desulfobacterota and the dominant pathogenic genera were *Bacteroides*, *Pseudomonas*, and *Aeromonas*. Risk characterisation of the identified pathogenic genera revealed that the assigned genera are capable of causing gastrointestinal illnesses, airway obstruction, and some are intrinsically resistant to commonly used antibiotics. However, further studies are imperative to establish an association between the identified pathogenic bacterial pathogens and the commonly reported symptoms among WWTP workers. Despite the preliminary nature of the results, intervention strategies should focus on raising awareness of bacterial contaminants present at WWTP and improving personal protective equipment (PPE) compliance in workers to mitigate health risks.

WWTP workers are vulnerable to increased occupational and environmental health hazards, barriers to healthcare, access to personal protective equipment, legal protection, and other safeguards. Molecular profiling of bacterial communities generates scientific-based evidence on the abundance of potential pathogenic bacteria in untreated wastewater that WWTP workers may be exposed to. With a lack of such information for workers in LMICs, comparison with high income countries is impracticable. Therefore, the findings of this study contribute to the body of knowledge, the relevance of region-specific data for a low-income countries given that bacterial communities in wastewater differs between geographical locations and are influenced by factors such as cultural, social, and environmental conditions. This may lead to the identification of region-specific diseases thus tailoring interventions that are specific and targeted to local settings.

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Informed Consent Statement: Not applicable.

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CHAPTER 4: RESPIRATORY AND ENTERIC BACTERIAL PATHOGENS IN MUNICIPAL WASTEWATER: A POTENTIAL RISK OF INFECTION TO WORKERS

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Respiratory and Enteric Bacterial Pathogens in Municipal Wastewater: A Potential Risk of Infection to Workers

Evida Poopedi [1,2,3,*], Tanusha Singh [2,4] and Annancietar Gomba [2]

[1] Department of Oral and Maxillofacial Pathology, Oral Microbiology and Oral Biology, Sefako Makghato Health Sciences University, Pretoria 0204, South Africa
[2] National Health Laboratory Service, National Institute for Occupational Health, Johannesburg 2000, South Africa; tanushas@uj.ac.za (T.S.); noncyg@nioh.ac.za (A.G.)
[3] Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, Johannesburg 2050, South Africa
[4] Department of Environmental Health, University of Johannesburg, Doornfontein 2028, South Africa
* Correspondence: evida.poopedi@smu.ac.za

Abstract: Investigating human pathogens in wastewater is crucial for identifying and predicting potential occupational health risks faced by wastewater treatment plant (WWTP) workers. This study aimed to determine the occurrence and levels of *Legionella pneumophila*, *Mycobacterium* spp., *Arcobacter butzleri*, and *Aeromonas hydrophila* in untreated municipal wastewater. Grab influent, activated sludge, and secondary settling tank (SST) effluent samples were collected bi-weekly over 6 months from 5 WWTPs in Tshwane, South Africa. *Mycobacterium* spp., *A. butzleri*, and *A. hydrophila* were detected using quantitative PCR (qPCR), while *Legionella* was detected using both a culture method and qPCR. The four pathogens were identified in most samples at varying levels. *Legionella pneumophila* had a positivity rate of 92%, ranging from 2 to 5.4 $\log_{10}$ MPN/100 mL. Detection rates of *Legionella* spp., *L. pneumophila*, and *L. pneumophila* serogroup 1 were 97%, 75%, and 69%, respectively, with up to 5.3 $\log_{10}$ gene copies (GC)/mL. Importantly, this study demonstrates molecular typing of *L. pneumophila* serogroup 1 in wastewater, a topic that has been rarely documented. *Mycobacterium* spp. were detected in all samples at varying levels ($\log_{10}$ GC/mL) in influent (2.8–7.6), activated sludge (4.8–8.9), and SST effluent (3.8–8.9) samples. *Arcobacter butzleri* and *A. hydrophila* were detected in 96% and 82% of the samples, respectively, with GC levels in influent, activated sludge, and SST effluent ranging from 0.8 to 6.6, 1.5 to 6.5, and 0.7 to 6.6 $\log_{10}$ GC/mL for *A. butzleri*, and similar levels for *A. hydrophila*. These findings underscore the presence of respiratory and enteric pathogens at various treatment points, suggesting potential occupational exposure for WWTP workers. This emphasises the need for microbiological risk assessments (RAs) or reviewing existing RAs and implementing necessary control measures to protect WWTP workers.

Keywords: municipal wastewater; workplace exposure; occupational health; opportunistic pathogens; microbial risk assessment

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1. Introduction

The composition of municipal wastewater has become increasingly complex due to population growth and economic development associated with higher living standards [1]. In many cities, wastewater comprises a diverse range of contaminants from various sources, including domestic, office, healthcare, and industrial facilities [2]. Microorganisms found in municipal wastewater are known or suspected to pose health risks, contributing to occupational disease burden among WWTP workers [3,4]. Occupational exposure to pathogenic

microorganisms is a notable concern for WWTP workers who face an elevated risk of direct contact with wastewater. The existing literature suggests that WWTP workers may be exposed to increased levels of pathogenic microorganisms in their work environment [5–8]. A higher prevalence of respiratory and gastrointestinal illnesses, assumed to be associated with microbial exposure, along with elevated levels of antibodies against bacteria, viruses, and parasites among WWTP workers have been reported [4,6,9].

The sources of pathogenic microorganisms in municipal wastewater are diverse. Most pathogens originate from human and animal excreta, with soil intrusions contributing to a lesser extent [1,10,11]. Enteric and zoonotic pathogens can also enter wastewater systems, posing significant risks to WWTP workers. Recent studies have shown the persistence of microbial pathogens in various environments, emphasising the importance of their consideration in wastewater treatment [11,12]. Environmental factors such as temperature, nutrient availability, salinity, and light play an important role in the survival and spread of pathogens [13]. Climate change presents serious threats to the environment, ecosystems, and human health, and its effects are predicted to intensify as global warming continues [14]. Microbial biodiversity is sensitive to environmental changes, which can alter the distribution and abundance of disease-causing microorganisms [13]. Therefore, climate change-induced disruptions of microbial communities have increased the incidence of infectious diseases, particularly those caused by environmental pathogens, such as waterborne illnesses [15,16].

Advances in molecular methods, such as quantitative polymerase chain reaction (qPCR) and next-generation sequencing, provide a unique opportunity to detect and quantify opportunistic pathogens (OPs) that are difficult to grow using traditional culture methods [17]. Consequently, metagenomics studies have identified numerous opportunistic bacterial pathogens in municipal wastewater that could potentially contribute to the commonly reported health issues among WWTP workers [18–21]. Some of the frequently identified opportunistic bacterial pathogens include *L. pneumophila*, *Mycobacterium* spp., *Arcobacter* spp., and *A. hydrophila*, among others [18,20,22]. Despite these findings, limited information exists on the incidence of specific pathogens in wastewater environments, which is critical for understanding the risk of infectious diseases or illnesses among WWTP workers. *Legionella* infections, primarily transmitted through inhalation [23], pose a potential occupational health risk to WWTP workers due to bioaerosol emissions at these facilities [24–26]. However, only a few studies have investigated *Legionella* occurrence at municipal WWTPs [27–31]. Furthermore, outbreaks of Legionnaires' disease (LD) associated with WWTPs (both industrial and municipal) in countries such as Norway, Germany, the Netherlands, and Finland [32,33], and transmission of *Legionella*-laden bioaerosols over great distances (up to 10 km) [34], highlight the importance of *Legionella* monitoring in wastewater.

Mycobacterium spp., known for their adaptability and survival in different eco- logical niches and hostile environments, are a significant public health concern. The *Mycobacterium tuberculosis* complex accounts for approximately two million deaths worldwide each year [35]. Additionally, non-tuberculous mycobacteria (NTM) cause the majority of bacterial infections in immunocompromised individuals [36]. Recent findings indicate that NTM infection cases almost surpass tuberculosis in most countries due to population ageing, chronic diseases and comorbidities, and other immunodeficiency disorders [37]. Several metagenomic studies have detected the genus *Mycobacterium* in wastewater [18,20,22]; however, there is a lack of geographical characterisation and limited research on *Mycobacterium* spp. specific quantities in wastewater [30,38], presenting a gap in understanding their potential health impacts.

Aeromonas is a common genus in wastewater bacterial communities, of which four *species* (*A. hydrophila*, *A. veronii*, *A. caviae*, and *A. dhakensis*) are currently recognised as clinically relevant [21,39,40]. Quantifying *Aeromonas* spp. in wastewater environments using molecular methods remains understudied [41,42]; therefore, more accurate identification is critical in understanding and predicting the potential risk of *Aeromonas* infections in persons exposed to wastewater.

Arcobacter butzleri has been classified as a serious emerging threat to human health by the International Commission on Microbiological Specifications for Foods [43], as it is frequently associated with bacteraemia and acute gastrointestinal illnesses such as watery diarrhoea and gastroenteritis in humans [43,44]. *Arcobacter* spp. are highly abundant in wastewater and typically predominate in influent [45,46] and have been correlated with high levels of faecal pollution [43]. Moreover, Webb et al. (2016) and Kristensen et al. (2020) have highlighted the detection of a substantial fraction of *A. butzleri* in the final treated effluent despite a noticeable reduction during wastewater treatment, emphasising the need for continued monitoring to understand its persistence in wastewater [46,47].

Given the increasing importance of bacterial pathogens and the global health threat posed by waterborne diseases, a critical need exists to determine their presence and distribution in wastewater. Insights into pathogen levels hold value for estimating disease risk and implementing appropriate mitigation measures for exposed WWTP workers. Despite this imperative, few studies have explored the presence and levels of opportunistic bacterial pathogens at critical points in wastewater before the final treatment stage, where exposure risks for WWTP workers are most significant [4,6]. The existing research has predominantly focused on pathogen removal efficiency given the public and environmental health impacts, resulting in significant knowledge gaps regarding the risks faced by workers during earlier treatment processes [31,40,42,48–50]. Consequently, this study sought to investigate the occurrence of *L. pneumophila*, *Mycobacterium* spp., *A. butzleri*, and *A. hydrophila* in wastewater across various treatment processes to improve the understanding of occupational exposure risks for WWTP workers potentially exposed to these pathogens if appropriate control measures are not implemented. The findings of this study provide valuable insights into an underexplored yet relevant intersection of occupational safety and environmental microbiology.

2. Materials and Methods

2.1. Sampling Sites and Sample Collection

This cross-sectional study comprised 176 wastewater samples collected from five municipal WWTPs that primarily treat municipal wastewater in the Tshwane Metropolitan Municipality, South Africa. Table 1 summarises the characteristics and treatment processes used at each sampling site. Poopedi et al. (2023), previously outlined a detailed description of the treatment processes used by the participating WWTPs [21]. An ethics waiver was obtained from the Research Ethics Committee of the University of the Witwatersrand for collecting wastewater samples (Ref: W-CBP-201120-01). Grab samples were collected in sterile 1 L bottles every second week over a six-month period (November 2021 to April 2022) at different treatment stages, including influent, activated sludge, and secondary settling tank (SST) effluent. All samples were transported on ice to the National Institute of Occupational Health Waterborne Pathogen Unit laboratory, maintaining a cold chain (2–8 °C) until processing within 24 h.

Table 1. Characteristics of sampling sites.

Site	Treatment Capacity (ML/D)	Treatment Train	Aeration Technology	Source of Wastewater	Effluent Treament	Employee Number
WWTP1	35	Bar screens, grit removal chamber, primary clarifiers, biological treatment, secondary sedimentation	Surface aeration	Domestic and industrial	Chlorine	22
WWTP2	60	Bar screens, grit removal chamber, primary clarifiers, biological treatment and trickling bio-filters, secondary sedimentation	Surface aeration	Domestic	Chlorine	38
WWTP3	93	Bar screens, grit removal chamber, primary clarifiers, biological treatment, secondary sedimentation	Surface aeration	Domestic and industrial	Chlorine	37
WWTP4	180	Bar screens, grit removal chamber, primary clarifiers, biological treatment, secondary sedimentation	Diffused aeration	Domestic	Chlorine	66
WWTP5	85	Bar screens, grit removal chamber, primary clarifiers, biological treatment, secondary sedimentation	Diffused aeration	Domestic	Chlorine	27

Note: ML/D: mega litres per day.

2.2. Laboratory Analysis

2.2.1. Detection of Culturable *L. pneumophila*

Culturable *L. pneumophila* quantities in wastewater were determined using the Legiolert testing method for non-potable water according to the manufacturer's instructions (IDEXX Laboratories, Inc., Westbrook, ME, USA). A number of wells (brown colour or turbidity) were considered positive for *L. pneumophila,* and enumeration was carried out using the Legiolert most probable number (MPN) table provided by the manufacturer.

2.2.2. Total Genomic DNA (gDNA) Extraction

Microbial cells were concentrated following the protocol by Kumar et al. (2019) [51]. Briefly, a 1 L grab sample was thoroughly shaken by hand to allow for adequate mixing and an aliquot of 200 mL was centrifuged (5000 rpm, 5 min; 4 °C) (Eppendorf 5804R, Germany). The supernatant was carefully removed without disturbing the pellet, after which the pellet was washed in 1 mL sterile distilled water to remove deposited salts and other impurities. The pellets were pooled and gDNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Eluted gDNA was stored at −20 °C until further analysis.

2.2.3. qPCR Standard Curves

Standard curves were prepared to optimise and determine the efficiency of the assays and were constructed using a synthetic oligonucleotide, gBlocks® Gene Fragment (Integrated DNA Technologies, Coralville, IA, USA), inserted with known amplicon sequences

(Supplementary Table S1). Reconstitution of the lyophilised gBlocks fragment provided 9×10^9 gene copies (GC). The target GC was estimated using the equation below [52]:

$$(C) \times (M) \times (1 \times 10^{-15} \text{ mol/fmol}) \times (\text{Avogadro's number}) = \text{copy number}/\mu\text{L}$$

where

C = initial concentration of the gBlocks Gene Fragment in ng/µL,
M = molecular weight in fmol/ng, as provided by the manufacturer.

The gBlock fragment was serially diluted to create a standard ranging from 9×10^6 to 9×10^1 GC per µL of the PCR products. For each standard curve, the concentration of GC was plotted against the threshold cycle (Cq value). The amplification efficiency was estimated by the formula $E = (10^{-1/\text{slope}}) - 1$. Slope and correlation coefficient values were used to determine the performance of the reaction. The acceptable metrics included were $R^2 = 0.9$ to 1, indicating that the data almost perfectly fit the linear model, and efficiency of 90 to 110% ($-3.8 \leq$ slope ≤ -3.3), indicating a perfect doubling of the template at every cycle. Amplification performance was analysed using the QuantStudio™ Design and Analysis Software version v 2.5.1 (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.4. Real-Time Quantitative PCR (qPCR) Multiplex Assays for *Legionella*, *A. hydrophila*, and *A. butzleri*

In this study, qPCR was used to determine gene copy levels of *L. pneumophila*, *Mycobacterium* spp., *A. butzleri*, and *A. hydrophila* in wastewater samples. Molecular methods, particularly qPCR, were chosen due to their high sensitivity and specificity, enabling the detection and quantification of target organisms even at low concentrations. This is particularly importantly in matrices like wastewater, which contains various inhibitors that can compromise and render traditional culture methods ineffective in isolating the organisms of interest. Additionally, qPCR allows for the rapid and simultaneous detection of multiple target organisms, making it especially suitable for the timely identification of pathogens in wastewater environments, where contamination levels and pathogen diversity can fluctuate [17,53]. The reaction mixture was prepared in a total volume of 25 µL and contained the following: 12 µL Quanta PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), 500 nM primers (see Table 2 for sequences) (Integrated DNA Technologies, Coralville, IA, USA), 100 nM probes (Integrated DNA Technologies, Coralville, IA, USA) according to the protocol by Benitez and Winchell (2013), 1 µL of Low ROX Reference Dye (Quanta Biosciences, Gaithersburg, MD, USA), 1 µL of nuclease-free water, and 7 µL of DNA template. The assay was performed on a QuantStudio™ 5 Real-Time PCR System (Thermo Fischer Scientific, Waltham, MA, USA) with the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C at 15 sec, and annealing at 60 °C for 1 min. A positive control (9×10^3 GC/µL) and a non-template control (NTC) PCR-grade water (Integrated DNA Technologies, Coralville, IA, USA) were included in all runs. All samples and controls were run in duplicate. Both multiplex qPCR assays 1 and 2 (Supplementary Table S2) had amplification efficiencies ranging from 91 to 101% and a correlation coefficient of 0.99. The slopes of the multiplex standard curves ranged from −3.3 to −3.6. Real-time quantitative PCR performance characteristics for individual assays are shown in Supplementary Table S2. The limit of quantification (LOQ) for qPCR was determined using the Cq values obtained for the standard range (9×10^2 to 1 GC). The qPCR LOQ was defined as the lowest amount of GC from the standard series detected in all samples from three independent runs [54]. The lower limit of quantification for *Legionella* spp., *L. pneumophila*,

L. pneumophila serogroup (sg) 1, and *A. butzleri* was determined to be 2 GC/reaction. The LOQ for *A. hydrophila* was 9 GC/reaction. The GC numbers in wastewater samples were

normalised to sample volume and expressed as $\log_{10}$ GC/mL. Values below the limit of detection after normalisation to GC/mL were reported as result/$\sqrt{2}$ [55].

2.2.5. *Mycobacterium* spp. qPCR Assay

Real-time qPCR for *Mycobacterium* spp. was performed using a commercially available LightMix Kit (TIB Molbiol GmbH, Berlin, Germany) according to the manufacturer's instructions. The assay amplifies a conserved 1000 bp fragment of the 16S ribosomal RNA gene specific to the *Mycobacteria* genus [56]. The reaction mix comprised 2 µL ofpremixed primers and probes, 4 µL of hot start reaction mixture (LightCycler FastStart DNA Master HybProbe, Roche Products (Pty) Ltd., Randburg, South Africa), 9 µL of PCR grade water, and 5 µL of DNA template. Samples were amplified as follows: 10 min at 95 °C (denaturation), followed by 50 cycles of denaturation (95 °C for 5 s), annealing (64 °C for 5 s), and extension (72 °C for 40 s). A melting curve analysis was carried out at 40 to 85 °C with a transition rate of 0.2 °C/s. Melting temperature values were assigned automatically based on a plot generated by the instrument (LightCycler 1.5, Roche Diagnostics International, Rotkreuz, Switzerland). The melting peak of 64 °C indicated *Mycobacterium* spp,

2.2.6. PCR Inhibitors

A ten-fold serial dilution (1:10) of the DNA extracts from wastewater samples was performed to reduce the concentration of PCR inhibitors in samples that initially showed no amplification or detectable DNA when tested with neat DNA [57].

Table 2. Oligos used in the detection of *Legionella*, *A. hydrophila*, and *A. butzleri*.

Target Organism	Gene Description	Sequence Direction (5′-3′)	Reference
		Multiplex assay 1	
Legionella species	*ssrA*, transfer messenger RNA	Lspp Forward-GGC GAC CTG GCT TC	[58]
		Lspp Reverse-GGT CAT CGT TTG CAT TTA TAT TTA	
		FAM-ACG TGG GTT GCA A-3MGB-NFQ	
L. pneumophila	*mip*, outer membrane protein	Lpn Forward-TTG TCT TAT AGC ATT GGT GCC G	[58]
		Lpn Reverse-CCA ATT GAG CGC CAC TCA TAG	
		Cy5-CG GAA GCA A-TAO-T-GGC TAA AGG CAT GCA -3IAbRQSp	
L. pneumophila sg1	*wzm*, transport permease of the O-antigenic polysaccharide of LPS	Lsg1 Forward-GC CTC TGG CTT TGC AGT TA	[58]
		Lsg1 Reverse-CAC ACA GGC ACA GCA GAA ACA	
		SUN-TT TAT TAC TCC ACT CCA GCG AT-3MGB-NFQ	
		Multiplex assay 2	
A. hydrophila	*aer*, aerolysin	Aer Forward-CAAGAACAAGTTCAAGTGGCCA	[59]
		Aer Reverse-ACGAAGGTGTGGTTCCAGT	
		Cy5-ATGAGTTCAAGGCCGATGTCAGCT-BHQ2	This study
A. butzleri	16S rDNA	16S Forward-CCTGGACTTGACATAGTAAGAATGA	[60]
		16S Reverse-CGTATTCACCGTAGCATAGC	
		FAM-ACGGTGACGTGGAGCAAATCTCAA-lowaBlackFQ	This study

2.3. Statistical Analysis

The data were captured and organised using Microsoft Excel Version 2403 (Microsoft Corporation, Redmond, WA, USA) and then exported into STATA software (version 17). Most probable number values and GC were log-transformed to provide a normal distribution of the data. The two-way ANOVA and Bonferroni post hoc test were used to determine the mean quantity differences between sampling sites and sample type. The p value ≤ 0.05 was considered statistically significant.

3. Results

The present study detected varying concentrations of the target opportunistic bacterial pathogens. Figures 1 and 2 show the results for cultured and GC of *L. pneumophila*,

respectively. Gene copy levels of *Mycobacterium* spp., *A. butzleri*, and *A. hydrophila* are displayed in Figures 3, 4A, and 4B, respectively.

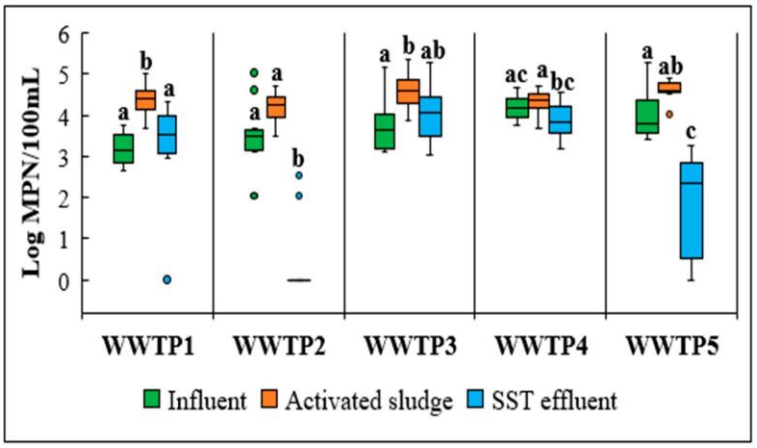

Figure 1. Concentration of culturable *L. pneumophila* in wastewater at different treatment stages across five WWTPs over a six-month sampling period. The whiskers illustrate the minimum and maximum; the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different letters on the graph indicate significant differences ($p \leq 0.05$) in means within a site, as determined by Bonferroni post hoc test following a two-way ANOVA.

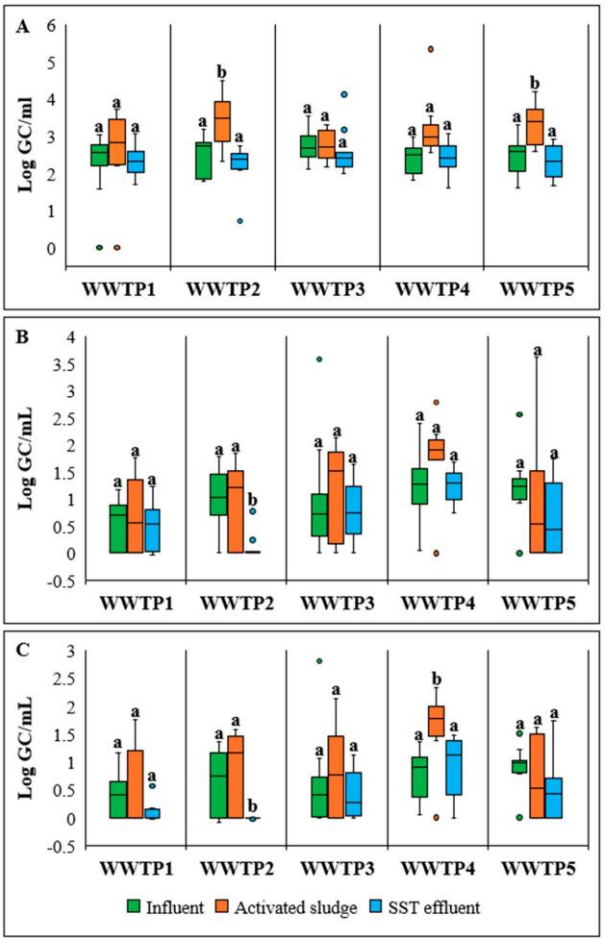

Figure 2. Gene copy levels of *Legionella* spp. (**A**) *L. pneumophila,* (**B**) and *L. pneumophila* sg1 (**C**) in wastewater at different treatment stages over six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different letters on the graph indicate significant differences ($p \leq 0.05$) in means within a site, as determined by Bonferroni post hoc test following a two-way ANOVA.

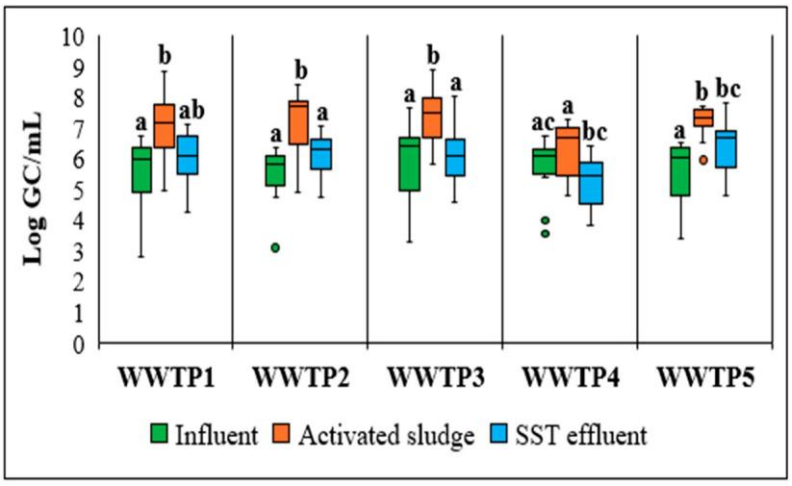

Figure 3. Gene copy levels of *Mycobacterium* spp. in wastewater at different treatment stages over a six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different letters on the graph indicate significant differences ($p \leq 0.05$) in means within a site, as determined by Bonferroni post hoc test following a two-way ANOVA.

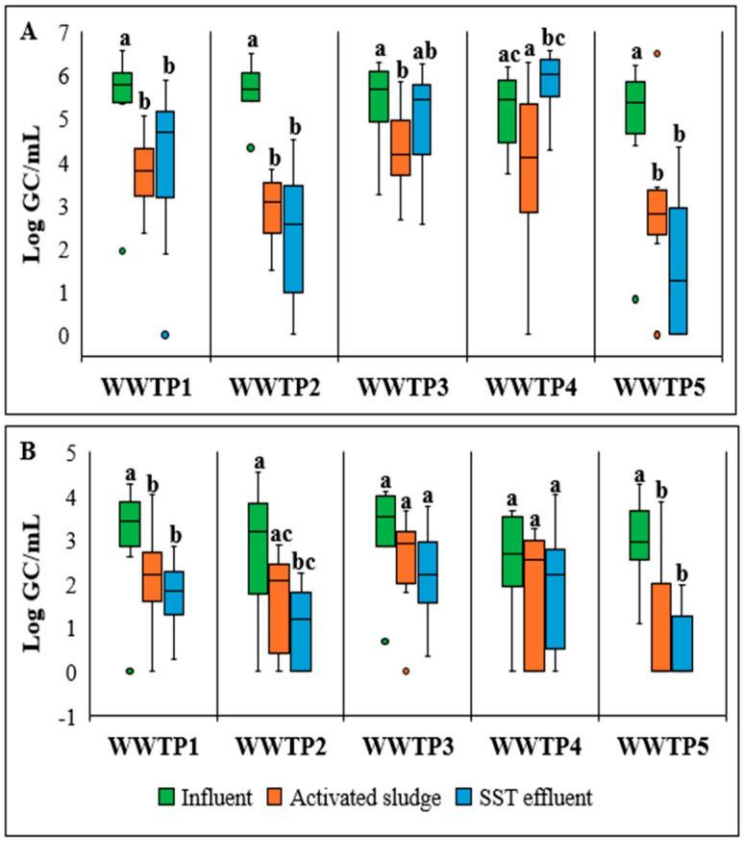

Figure 4. Gene copy levels of *A. butzleri* (**A**) and *A. hydrophila* (**B**) in wastewater at different treatment stages over a six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different letters on the graph indicate significant differences ($p \leq 0.05$) in means within a site, as determined by Bonferroni post hoc test following a two-way ANOVA.

3.1. Detection of culturable L. pneumophila

Culturable *L. pneumophila* was detected in 92% (n = 162) of the tested samples, and only 14 samples (8%) exclusively from the SST effluent were negative. Figure 1 depicts the

levels of *L. pneumophila* at different sampling points with overall concentrations ranging from 110 (SST effluent at WWTP2) to 227,260 (activated sludge at WWTP3) MPN/100 mL (2.0 to 5.4 $\log_{10}$ MPN/100 mL).

Data from the three sampling points (influent, activated sludge, and SST effluent) showed a similar trend across all five WWTPs, with activated sludge having the highest levels of *L. pneumophila* compared to the other two sampling points. However, only WWTP1 and WWTP3 showed a statistically significant increase in *L. pneumophila* counts from influent to activated sludge. When comparing *L. pneumophila* levels in incoming municipal wastewater (influent) and partially treated SST effluent, only WWTP2 and WWTP5 showed a significant reduction. A similar trend was observed when comparing

L. pneumophila levels in activated sludge and SST effluent, showing significant difference at WWTP2 and 5. *Legionella pneumophila* concentration levels were relatively consistent across WWTP1, WWTP3, and WWTP4 at all sample points, even after the biological treatment step. Furthermore, *L. pneumophila* levels in SST effluent from WWTP1, 3, and 4 were comparable to, if not higher than, those found in influent, although not statistically significant ($p > 0.05$).

When comparing *L. pneumophila* levels across the five WWTPs (Supplementary Table S3), influent samples from WWTP4 and WWTP5 were similar and exhibited significantly higher concentrations of *L. pneumophila* compared to WWTP1. Additionally, influent levels in WWTP4 were significantly greater than those observed in WWTP2. No significant difference was observed when WWTP3 influent was compared with other WWTPs. In activated sludge samples, the only significant difference was observed between WWTP2

and WWTP3, and no significant difference across all WWTPs in SST effluent samples.

3.2. Gene copies of Legionella spp., L. pneumophila and L. pneumophila sg1

Legionella spp. was detected in 97% of the samples (171/176). The GC of *Legionella* spp. in wastewater ranged from 0.7 (SST effluent at WWTP2) to 5.3 (activated sludge at WWTP4) $\log_{10}$ GC/mL (Figure 2A). In both WWTP 2 and 5, activated sludge had significantly higher *Legionella* spp. GC, then, influent, and SST effluent. Furthermore, comparisons of *Legionella* spp. at corresponding sampling points across all WWTPs revealed no statistically significant difference (Supplementary Table S4).

The detection rate of *L. pneumophila* was 75% (132/176), and overall GC ranged from

0.3 (SST effluent at WWTP1) to 3.6 (activated sludge at WWTP5) $\log_{10}$ GC/mL (Figure 2B). Significantly lower levels of *L. pneumophila* were found in SST effluent compared to other sample types at WWTP2 only. A comparison across all WWTPs (Supplementary Table S5) indicated that WWTP4 had significantly higher *L. pneumophila* GC in activated sludge than WWTP1. In SST effluent samples, *L. pneumophila* levels at WWTP4 remained significantly elevated compared to WWTP1 and WWTP5. Moreover, significantly lower *L. pneumophila* GC were recorded in SST effluent at WWTP2 than at WWTP3 and 4 (Supplementary Table S5).

The detection rate of *L. pneumophila* sg1 was found to be 69% (121/176). The overall GC of *L. pneumophila* sg1 were from 0.3 (influent at WWTP2) to 2.8 (influent at WWTP3) $\log_{10}$ GC/mL (Figure 2C). *Legionella pneumophila* sg1 gene copy levels in SST effluent were significantly lower than in influent and activated sludge at WWTP2. However, at WWTP4, activated sludge GC were significantly higher than other wastewater types. Across sites, levels *L. pneumophila* sg1 in activated sludge were significantly higher at WWTP1 than at WWTP4. For SST effluent, *L. pneumophila* sg1 levels were significantly higher at WWTP4 than at WWTP1, 2 and 3. Furthermore, in SST effluent, *L. pneumophila* sg1 GC at WWTP2 were significantly lower than those observed at WWTP5 (Supplementary Table S6).

3.3. Gene copies of Mycobacterium spp.

Gene copies of the *Mycobacterium* genus representing non-tuberculous mycobacteria (NTM) are presented in Figure 3. *Mycobacterium tuberculosis* was not detected in this study. *Mycobacterium* spp. were consistently detected across all sampling points (100% detection rate) ranging from 2.8 (influent at WWTP1) to 8.9 (activated sludge at WWTP3) $\log_{10}$ GC/mL (Figure 3). Notably, *Mycobacterium* spp. levels were significantly higher in activated sludge than in influent at WWTP1, 3, and 5. Although a slight decrease in *Mycobacterium* spp. gene copy numbers was observed in partially treated SST effluent, the levels were not significantly different from incoming raw wastewater ($p > 0.05$) at all WWTPs except WWTP5 where GC were significantly higher in SST effluent than in influent. Significant differences between activated sludge and SST effluent were observed at WWTP2, 3 and 4. Across all sites, activated sludge exhibited the highest GC levels of *Mycobacterium* spp., followed by SST effluent, with influent samples showing the lowest levels. However, significant difference across sites were observed only in SST effluent from WWTP4 and WWTP5 (Supplementary Table S7).

3.4. Gene Copies of the Enteric Pathogens, Arcobacter butzleri and Aeromonas hydrophila

Arcobacter butzleri was detected in 96% (169/176) of the wastewater samples, with overall GC ranging from 0.11 (SST effluent at WWTP2) to 6.57 (SST effluent at WWTP4) $\log_{10}$ GC/mL (Figure 4A). Except for WWTP4, *A. butzleri* gene copy levels reduced significantly in all WWTPs following biological treatment compared to initial levels in GC in WWTP1, 3, and 5. Similarly, *A. butzleri* GC in SST effluent at WWTP 3 and 4 were similar to those in the influent ($p > 0.05$). The levels of *A. butzleri* GC at the three sampling points across the five WWTPs (Supplementary Table S8) showed a significant difference only in SST effluent. Significantly lower *A. butzleri* levels were observed at WWTP2 and WWTP5 compared to WWTP1, 3, and WWTP4. Conversely, significantly higher *A. butzleri* GC levels in SST effluent were detected at WWTP4 compared to all other WWTPs, except for WWTP 3.

Aeromonas hydrophila was detected in 82% (144/176) of the samples, with overall mean GC ranging from 0.2 (SST effluent at WWTP4) to 4.5 $\log_{10}$ GC/mL (influent at WWPT2). A general decline in *A. hydrophila* GC from influent to SST effluent was observed across all WWTPs studied, with a significant decrease between influent and activated sludge samples at WWTP1 and 5. However, both plants had similar overall *A. hydrophila* GC in activated sludge and SST effluent ($p > 0.05$). At WWTP2, there was no significant difference ($p > 0.05$) in *A. hydrophila* GC between other sample types except for influent and SST effluent. Similarly, no significant difference was observed across the sampling points for WWTP3 and 4. A comparison of *A. hydrophila* GC across different sampling sites (Supplementary Table S9) showed evidence of significantly higher GC in activated sludge samples from WWTP3 than WWTP5. Furthermore, SST effluent at WWTP5 had significantly lower *A. hydrophila* GC than at WWTP1, WWTP3, and WWTP4. Similarly, *A. hydrophila* GC in SST effluent were significantly lower at WWTP2 compared to WWTP3.

4. Discussion

Municipal wastewater systems harbour diverse pollutants, including opportunistic bacterial pathogens thriving in environments conducive to their growth and persistence [8], thereby raising concerns regarding potential health risks for WWTP workers. This study explored the detection rates and levels of respiratory and enteric opportunistic bacte- rial pathogens in municipal wastewater across different treatment stages where there is increased likelihood of worker exposure over a six-month sampling period.

This study revealed varying detection rates of culturable *L. pneumophila* in WWTPs, which concurs with findings (between 83% and 93%) from previously reported studies [23,61].

Additionally, the current study present GC numbers of *Legionella* spp., *L. pneumophila,* and *L. pneumophila* sg1 in municipal wastewater. The high occurrence of *Legionella* spp. coupled with relatively low levels of *L. pneumophila* in wastewater corresponds with international studies conducted in Norway [27], Israel [28], Germany [29], and Italy [31]. While there is information on the occurrence of *Legionella* spp., and *L. pneumophila* in wastewater, it is important to highlight the scarcity of data particularly on *Legionella* quantities in municipal wastewater [28,31,62]. The highest levels of both culturable and *Legionella* GC were identified in activated sludge samples compared to other sampling points. Similar results were previously reported, with *Legionella* levels reaching up to 10 log genomic units (GU)/mL in activated sludge samples [27,29]. Therefore, bioaerosols containing *Legionella* generated by aerobic tanks in WWTPs may play a significant role in the transmission of *Legionella* infections. Of the 60 different species in the *Legionella* genus [63], *Legionella pneumophila,* being the most clinically relevant, accounts for approximately 90% of the reported LD cases worldwide, with a majority of the cases attributed to *L. pneumophila* serogroup1 [64]. Using molecular typing, the current study highlights potential health risk to *L. pneumophila* sg1 among WWTP workers, a topic that has been rarely documented. While Bolufer et al. (2024) is the only similar study we found [65], to the best of our knowledge, studies addressing this important topic specifically in wastewater remain limited. Therefore, our study adds valuable insights to the growing landscape of research on *L. pneumophila* in wastewater environments, especially as it may affect workers' health.

The high occurrence of *Mycobacterium* spp. found in this study (100%) correlates with what has been reported in other studies [30,38]. In addition, overall GC *Mycobacterium* spp. detected in the present study are comparable with previous studies that observed similar levels (4 to 8 log GU/mL) in wastewater [38,42]. *Mycobacterium* spp. in the present study demonstrated consistent GC levels across the five sampling sites, with activated sludge presenting higher GC at all sampling events. *Mycobacterium* spp. are dominant in activated sludge as they may have a role in the degradation of organic compounds in wastewater [66], accounting for up to 4% of the total bacterial community [67]; however, their occurrence in activated sludge samples has not been systematically studied. The GC levels of *Mycobacterium* spp. in activated sludge could therefore be higher consider- ing the low copy number of the ribosomal RNA in the *Mycobacterium* genome [56,68]. Furthermore, current findings demonstrated that the secondary sedimentation process significantly reduced *Mycobacterium* spp. GC at three WWTPs compared to the GC observed in the activated sludge. This is probably due to the hydrophobic nature of *Mycobacterium*, which promotes adhesion to large particles in the sedimentation tanks [69]. However, GC levels in SST effluent were similar to those initially observed in the incoming influent. *Mycobacterium* spp. are common in natural and anthropogenic-related environments, and some members are pathogenic, causing a wide range of diseases such as tuberculosis, lung infection, skin and soft tissue infection, and others [70]. The high occurrence and detection rate of *Mycobacterium* spp. in this study, particularly in the aeration tanks where wastewater may become aerosolised, could pose a risk of human mycobacteriosis through respiratory exposure and skin contact in WWTP workers.

This study has demonstrated a high occurrence of *A. butzleri* in wastewater especially in the influent, which is in agreement with previous studies [46,47,71]. However, the biological treatment processes in this present study, significantly reduced *A. butzleri* GC in three of the four WWTPs studied with no further reduction following secondary sedimentation. Similarly, *A. butzleri* GC were significantly reduced following biological treatment at a WWTP in Canada, although the bacteria could still be detected in the final effluent [47]. Additionally, Tang et al. (2016) indicated that *A. butzleri* was barely detectable in activated sludge samples, indicating significant removal following biological treatment.

While *A. butzleri* GC were greatly reduced during biological treatment at most of the plants in this study, a significant proportion of *A. butzleri* could still be detected after sedimentation in the secondary settling tanks [70]. The significant increase in *A. butzleri* after secondary sedimentation at one of the studied plants was unexpected but could be attributed to factors such as poor retention time and high organic matter, resulting in reduced efficiency in bacterial removal. Therefore, current primary and secondary treatment processes may not be effective in reducing the densities of *A. butzleri*, suggesting that its presence in wastewater could pose gastrointestinal risks to WWTP workers if ingested.

A high occurrence and detection rate of *A. hydrophila* was observed in this study compared to that previously reported by Drk et al. (2023), and Skwor et al. (2023), with up to 18% prevalence rate using culture method [40,72]. These disparities could be related to the inherent characteristics and limitations of the culture method used in previous studies. This includes the presence of competing microorganisms, viable but nonculturable bacteria, and the intrinsic poor sensitivity of culture techniques, resulting in an underestimation of the presence of *A. hydrophila* in wastewater by the previous studies. Members of the *Aeromonas* genus are commonly associated with gastroenteritis, wound infections, and bacteraemia, with immunocompromised individuals being the most affected [40]. According to numerous metagenomics studies, *Aeromonas* spp. are prevalent in wastewater [21,39,40]. *Aeromonas hydrophila* GC have been reported to be 4 to 9 log copies per litre, comparable to the GC found in this study [42]. In the present study, a significant decrease in *A. hydrophila* was noted in activated sludge samples after biological treatment at two of the five plants studied although there was no further significant reduction after biological treatment. These results suggest that, while there was a general decline in *A. hydrophila* GC throughout the treatment process, only the biological treatment process at the aforementioned WWTPs was able to significantly reduce *A. hydrophila* in wastewater. Similarly, previous studies that investigated removal efficiency of *A. hydrophila* in wastewater recovered equivalent, if not higher, quantities of this organism in treated effluents than in influent [41,42,73]. For example, Popovic et al. (2015) found equal cell numbers of *A. hydrophila* in raw wastewater and effluent [73]. Cui and Liang, in 2019, conducted a five-month microbiological investigation of municipal wastewater in China and found that *A. hydrophila* GC did not differ significantly between influent and final treated effluents [42]. These findings are consistent with the general consensus that the conventional biological treatment does not significantly reduce *Aeromonas* spp. levels. Therefore, additional tertiary treatment is critical, and workers should don personal protective equipment (PPE) to minimise exposure [39].

While the present study primarily focused on a selected group of bacterial pathogens, municipal wastewater harbours a wide range of other important human pathogens, including viruses, protozoa and helminths [18,74,75]. Globally, studies have reported *Cryptosporidium* and *Giardia* as the most prevalent parasites detected in wastewater, with concentrations often correlating with infection and excretion rates in the served population [76,77]. Similarly, waterborne viruses like Adenovirus, Norovirus, and Rotavirus are frequently detected in wastewater and are associated with cases of acute gastroenteritis [78], further complicating the pathogen landscape in wastewater and the health risks to exposed workers. For example, a recent five-year study in Egypt reported a 53% detection rate of *Adenovirus* in wastewater samples, with concentrations ranging from 10^3 to 10^5 GC/L and 10^1 to 10^3 in influent and effluent samples, respectively [79]. Additionally, numerous waterborne pathogens have shown resistance to commonly used disinfectants, presenting serious challenges for treatment facilities in effectively eliminating these organisms [10,79]. The presence of diverse and high loads of pathogens in municipal wastewater underscores the need for continuous monitoring to mitigate transmission and protect WWTP workers from occupational exposure risks, globally.

In this study, opportunistic bacterial pathogens (*Legionella* spp., *Mycobacterium* spp., *A. butzleri*, and *Aeromonas* spp.) were detected at different wastewater treatment stages. These pathogens are associated with respiratory and gastrointestinal illnesses, suggesting the potential occupational health risks for workers. Workers at WWTPs can be exposed to the identified potential bacterial pathogens mainly through inhaling contaminated bioaerosols and accidentally ingesting them during routine work activities or while walking around the treatment plant. All four opportunistic bacterial pathogens identified in this study are classified as hazardous biological agents by the revised South African microbial risk group classification system [80]. The genus *Mycobacterium* contains organisms classified as Risk Group 2 and 3 depending on the species type, whereas *Legionella* is designated as a Risk Group 2 agent which can cause diseases but are unlikely to spread to the community as effective treatment is available. Risk Group 3 species can cause severe disease and present a risk of spreading to the community; however, effective treatment is available. While *Legionella* species are primarily transmitted through inhalation, *Mycobacteria* are transmitted via inhalation or ingestion [70]. Consequently, the presence of these pathogens in WWTP environments may pose an occupational health risk, particularly due to the aerosolization of activated sludge, which was found to have the highest GC levels of respiratory pathogens (*Legionella* and *Mycobacterium* species) [25,26]. These aerosols can disperse into the air, increasing the likelihood of worker exposure through inhalation. *A. butzleri* and *Aeromonas* spp. are both classified as Risk Group 2 [80], and mostly cause gastroenteritis and, to a smaller extent, other illnesses such as bacteraemia and wound infections. Enteric pathogens may not present life-threatening risks to healthy individuals; however, elderly and immunocompromised WWTP workers could face serious complications. The occupational health impact on workers includes an increased likelihood of respiratory infections (e.g., LD, tuberculosis-like conditions) and gastrointestinal illnesses that can lead to prolonged sick leave, reduced work capacity, and, in severe cases, long-term health complications [81]. Therefore, these findings underscore the need for stringent health protection measures for WWTP workers, such as the use of appropriate PPE, regular monitoring of bioaerosols for pathogenic contamination, and targeted health surveillance to mitigate potential risks.

The consistent detection of the four targeted opportunistic bacterial pathogens in municipal wastewater has significant implications for wastewater treatment methodologies and occupational safety protocols [10]. The elevated GCs of *L. pneumophila* and *Mycobacterium* spp. in activated sludge samples highlight the need for improved treatment processes. For example, strategies such as replacing mechanical surface aerators with submerged aerators could substantially reduce bioaerosol emissions [25,26]. Additionally, enclosing or covering aeration tanks, where feasible, could further mitigate the release of airborne pathogens, enhancing worker safety and environmental containment. Although this study did not directly evaluate effluent quality, the detection of significantly high GC levels of the targeted respiratory and enteric pathogens in SST effluent highlights the necessity for advanced disinfection processes to reduce bacterial pathogen levels in treated wastewater effectively [10]. Additionally, wastewater surveillance plays a critical role in monitoring pathogens and their genetic material, providing a valuable tool for infectious disease prevention and containment strategies [2]. Recent advances in pathogen detection technologies enable timely and more accurate identification, offering opportunities to effectively mitigate health risks in wastewater environments [53]. Importantly, robust preventive measures, such as raising awareness of occupational risks and improving PPE compliance, are critical in minimising exposure risks protecting WWTP workers' health [80].

Although this study focused on assessing the presence and quantities of bacterial pathogens in wastewater before the final treatment stage, where risks of worker exposure

are highest, existing research has demonstrated that certain pathogens can persist throughout various stages of wastewater treatment [65]. This persistence is a key factor influencing public health and environmental safety. While our study did not directly assess the effectiveness of treatment processes, the detection of pathogens in influent, activated sludge, and SST effluent highlights the potential need to implement and adhere to regular equipment maintenance plans, increase security at WWTPs to prevent theft and vandalism of electric infrastructure, as well as strengthen disinfection practises. Such interventions could improve the overall performance of treatment processes, ultimately reducing pathogen persistence, thereby mitigating both worker exposure risks and environmental contamination. Future research could build upon these findings by investigating the correlation between treatment method efficacy, pathogen persistence, and occupational and public health risks.

Limitations of the Study

The use of qPCR in this study may have overestimated the occurrence and levels of targeted opportunistic pathogens by detecting DNA from nonviable bacterial cells. Therefore, qPCR with photoactivatable DNA intercalators such as ethidium monoazide and propidium monoazide could help distinguish viable from dead bacterial cells, thereby improving occupational exposure risk evaluation of the targeted pathogens. Pathogen detection does not necessarily equate to human infections because the actual risk will depend on factors such as host immune status, pathogen characteristics (e.g., infection dose, virulence), exposure duration, and control measures in place. Therefore, it is critical to evaluate possible health risks posed by these opportunistic pathogens of interest to better understand their health burden in WWTP workers. Furthermore, while this study assessed bacterial pathogens in wastewater over six months (twelve-time points), the limited timeframe constrained our ability to assess seasonal variations in pathogen prevalence. Future research should increase the frequency of sampling and investigate the incidence and possible occupational exposures to other pathogenic microorganisms such as viruses, protozoa, and helminths, which are also major contributors to waterborne illnesses in wastewater environments worldwide. Despite limitations, this study confirmed the presence of four clinically important opportunistic bacterial pathogens in wastewater at different critical points along the wastewater treatment channel before reaching the final effluent. These findings provide valuable insights into potential occupational respiratory and gastrointestinal health risks for WWTP workers. Due to the small sample size, the findings cannot be generalised; however, they offer valuable insights into the presence of opportunistic bacterial pathogens that could pose risks to WWTP workers.

5. Conclusions

In conclusion, this study provided a comprehensive exploration of four opportunistic bacterial pathogens in wastewater using a combination of both culture-dependent and culture-independent methods. The consistent detection of *L. pneumophila*, *Mycobacterium* spp., *A. hydrophila*, and *A. butzleri* in wastewater across different treatment stages underscores the potential health risks faced by WWTP workers who are routinely exposed to wastewater. Although the detection of potential human bacterial pathogens in munici- pal wastewater does not directly equate to infection or provide data on health burdens, these findings suggest the potential presence and footprint of potentially harmful bacterial pathogens in the studied WWTPs. Therefore, the presence of these opportunistic bacterial pathogens in wastewater should be evaluated thoroughly with complementary approaches such as Quantitative Microbial Risk Assessment, allowing for a nuanced understanding of the long-term effects of occupational exposure in WWTP workers. Furthermore, the present study accentuates a critical concern of the notably higher detection and levels of

respiratory pathogens, specifically *Legionella* and *Mycobacterium* species, in activated sludge samples from aeration tanks than in other sample types. This emphasises the urgent need for heightened attention to prevent the concentrations and spread of airborne microorganisms by implementing or upgrading existing ventilation systems at WWTPs based on a comprehensive risk assessment. Findings from the present study provide valuable information to inform decision-making and management strategies for reducing and addressing occupational microbial risks for WWTP workers. Key recommendations include equipping workers with appropriate PPE, particularly when working around aeration tanks or during sludge handling, to minimise the risk of inhaling infectious organisms. Protective clothing should also be provided to the workers to reduce skin contact with contaminated wastewater and prevent accidental ingestion. Additionally, regular training should be provided to educate and reinforce awareness of health risks associated with wastewater, proper use of PPE, and hygiene practises [80]. Finally, WWTPs should expand monitoring efforts to include specific microbial pathogens to better understand pathogen trends and potential health risks for workers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w17020268/s1, Table S1: Gene fragments used to construct a synthetic oligonucleotide gBlocks for PCR standards; Table S2: Amplification efficiency, correlation coefficient, slope and coefficient of variance (CV) of the standard curves for qPCR; Table S3: Comparison of viable *L. pneumophila* across all plants; Table S4: Comparison of *Legionella* spp. gene copies across all plants; Table S5: Comparison of *L. pneumophila* gene copies across all plants; Table S6: Comparison of *L. pneumophila* serogroup 1 gene copies across all plants; Table S7: Comparison of *Mycobacterium* spp. gene copies across all plants; Table S8: Comparison of *A. butzleri* gene copies across all plants; Table S9: Comparison of *A. hydrophila* gene copies across all plants.

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CHAPTER 5: OPPORTUNISTIC BACTERIAL PATHOGENS IN BIOAEROSOLS EMITTED AT MUNICIPAL WASTEWATER TREATMENT PLANTS, SOUTH AFRICA

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Opportunistic bacterial pathogens in bioaerosols emitted at municipal wastewater treatment plants, South Africa

Evida Poopedi[1,3]✉, Rian Pierneef[5,6], Tanusha Singh[2,4] & Annancietar Gomba[2]

Aeration tanks at wastewater treatment plants (WWTPs) emit significant amounts of bioaerosols containing potentially hazardous infectious material. Occupational exposure to airborne pathogens can pose health risks to WWTP workers. Bioaerosol samples collected at aeration tanks of two typical municipal WWTPs that use different aeration modes were analysed to investigate the composition and diversity of airborne bacteria in wastewater environments, using the Illumina MiSeq platform. Thirty-six potential airborne bacterial pathogens were identified in the air samples, and these were dominated by *Bacillus*, *Enterococcus*, *Clostridium*, *Streptococcus*, *Acinetobacter*, *Enterobacter*, *Pseudomonas*, *Bacteroides fragilis*, *Acinetobacter baumannii*, and *Escherichia/Shigella*. Bioaerosols from mechanical aeration tanks (72%, 26/36) had a relatively higher richness and diversity of airborne bacterial pathogens than diffused aeration tanks (17%, 6/36). Furthermore, most of the identified airborne bacterial pathogens (78%, 28/36) were classified as Risk Group 2 according to the revised South African Regulation for Hazardous Biological Agents, 2022, and up to 70% of these were gram-negative bacteria. The presence of potentially pathogenic bacteria in the ambient air at WWTPs suggests an elevated risk of bioaerosol exposure for workers. Therefore, further research and site-specific risk assessments are recommended to guide the implementation of effective bioaerosol strategies to protect workers' health, with special attention paid to WWTPs that use mechanical aerators.

Keywords Airborne bacteria, Opportunistic pathogens, Occupational exposure, Municipal wastewater

Wastewater treatment plants (WWTPs) are a typical source of hazardous airborne biological particles, also known as bioaerosols[1]. Bioaerosols are primarily emitted from aeration tanks due to external forces such as aeration, mechanical agitation, and sludge dewatering. Other important sources of bioaerosols include bar screens, grit chambers, primary settling tanks, pump stations as well as sludge storage sites[1–3]. However, the conventional layout of WWTPs is not designed to prevent aerial dispersion of wastewater contaminants, thus posing an unavoidable risk to human health[2,4].

Bioaerosols emitted from wastewater contain different types of biologically harmful components, including bacteria, fungi, viruses, spores, allergenic pollen, and toxins among others[5]. Human exposure to bioaerosols has been associated with respiratory symptoms and impaired lung function[5]. Additionally, other studies have reported adverse health effects such as infections[6,7], allergies[8], acute toxic effects[9], and cancer[5]. Due to their microscale size (aerodynamic diameter smaller than 5 µm), bioaerosol particles can be disseminated by wind over distances of up to 10 km, and easily deposited into the respiratory tract[2]. Consequently, bioaerosol emissions at WWTPs could pose serious health risks to WWTP workers as well as residents of nearby communities[2].

A plethora of opportunistic bacterial pathogens have been identified in bioaerosols originating from WWTPs, including those belonging to the genera *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, *Klebsiella*, *Mycobacterium*, and *Enterococcus* among others[4,10–13]. Various factors such as wastewater source, aeration

[1]Department of Oral and Maxillofacial Pathology, Oral Microbiology and Oral Biology, Sefako Makgatho Health Sciences University, Pretoria 0204, South Africa. [2]National Health Laboratory Service, National Institute for Occupational Health, Johannesburg 2000, South Africa. [3]Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, Johannesburg 2050, South Africa. [4]Department of Environmental Health, University of Johannesburg, Doornfontein 2028, South Africa. [5]Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria 0028, South Africa. [6]Centre for Bioinformatics and Computational Biology, University of Pretoria, Pretoria 0028, South Africa. ✉email: evida.poopedi@smu.ac.za

technology used, inactivation rate of the bacteria, the amount of aerosolised material, meteorological conditions (e.g., temperature, sunlight, wind), and humidity may influence the composition, load, dispersion, and survival of aerosolised bacteria[14,15]. However, comprehensive analysis of bacterial communities in WWTP generated bioaerosols using high throughput sequencing technologies are scarce in many developing countries[10,11,16,17]. Since the 1970s, WWTP workers have commonly reported symptoms, also termed 'sewage worker's syndrome', fatigue, unexplained tiredness, headache, respiratory diseases, and fever among others ([18,19]. Most importantly, research has found a correlation between sewage worker's syndrome and bioaerosol emission from WWTPs[20]. Therefore, a comprehensive identification of pathogenic bacterial species in bioaerosols from WWTPs is crucial to provide scientific evidence on the causal agents associated with health outcomes among workers, and also provide a basis for the control and prevention of potential health risks posed by bioaerosols for protection of WWTP workers and nearby communities. Hence, the aim of the present study was to investigate bacterial communities in bioaerosols emitted from two municipal WWTPs in South Africa using high throughput sequencing to better understand potential occupational exposure to airborne bacteria. This approach provides a broad profile of bacterial diversity, including pathogenic bacteria.

Materials and methods

Study site description

Bioaerosol samples were collected at two municipal WWTPs in the City of Tshwane Metropolitan Municipality, South Africa. The two WWTPs primarily receive domestic wastewater and, to a lesser extent, wastewater from industrial and healthcare facilities. Poopedi et al.[21], previously outlined a detailed overview of the studied WWTPs. Briefly, the WWTPs comprise the following treatment stages: coarse screening, primary settling, biological treatment, secondary settling, and chlorine disinfection. The two WWTPs use different aeration systems for the biological degradation of organic contaminants. At WWTP1, mechanical aerators (MAER) are used for aeration in activated sludge tanks, where rotating blades introduce oxygen into the water by creating negative pressure on the rear side, causing the activated sludge to splash out into the air. This process releases droplets containing microorganisms, that may dehydrate and atomise to form bioaerosols. In contrast, at WWTP5, diffused aerators (DAER) are used, which provide oxygen through air bubbles released from the bottom of the aeration tank. As these bubbles rise through the water, they carry activated sludge flocs and rupture at the water–air interface, thus forming bioaerosols[11,22].

Bioaerosol sampling

Sampling was carried out on 18th October and 1st November 2022 at WWTP1, while WWTP5 was sampled on the 9th and 15th of November 2022. Airborne biological particles were collected into a sterile 20 mL phosphate buffered saline (PBS) (Sigma-Aldrich, MO, USA) using a stationary SKC Bio-Sampler (SKC Ltd, Blandford Forum Dorset, UK) at a flow rate of 12.5 L/min for 150 min per sample. The Bio-Sampler, mounted securely on a tripod stand 1.5 m above the ground to simulate the breathing zone of WWTP workers, was placed within 1 m to the aeration tank. The SKC Bio-Sampler collects bioaerosol particles with aerodynamic diameters ranging from 0.3 to 8 µm[23]. Air samples upwind of each plant were collected as background controls (BC). For quality assurance purposes, the pump flow rate was calibrated using an SKC rotameter (SKC Ltd, Blandford Forum Dorset, UK) before and after each sampling visit. A field control consisting of 20 mL PBS was processed alongside the samples to ensure the integrity of the sampling procedure. In addition, all sample collecting tools were decontaminated with 75% ethanol and rinsed with sterile water before every sampling event. Samples were transported to the National Institute of Occupational Health Waterborne Pathogen Unit laboratory on ice and stored at − 20 °C until further analysis. Meteorological conditions (e.g., temperature, relative humidity, and wind speed) at the sampling sites were obtained from the South African weather Service (Supplementary Table S1). These data were averaged over the region including the study site.

DNA extraction

Aliquots (2 mL) of the collected PBS samples were centrifuged (Mikro 22R, Hettich, Germany) at 14,000 × *g* for 10 min at 4 °C to concentrate bacterial cells. The supernatant was carefully removed, and genomic DNA was isolated from the pellet using DNeasy Powersoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Eluted DNA was stored at −20 °C until further analysis.

16S rRNA sequencing and data analysis

The V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the universal primers (16S Forward Primer =TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and 16S Reverse Primer=5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC)[24]. The libraries were sequenced on the Illumina MiSeq instrument (Illumina, USA) using MiSeq reagent kit v3 (Illumina, USA) and 2 × 300 bp paired-end sequencing reads were generated. Raw reads were processed using DADA2 workflow[25]. The non-chimeric sequences were analysed for taxonomic classification with the silva_nr99_v138.1_wSpecies_train_set.fa.gz database (https://zenodo.org/record/4587955/files/silva_nr99_v138.1_wSpecies_train_set.fa.gz?download=1). The number of amplicon sequence variants (ASV) and alpha diversity indices (Shannon, Simpson and InvSimpson) were calculated using PhyloSeq R package.

Results

Alpha diversity of the air samples

A total of 657,203 quality reads were obtained for further downstream analysis. The number of Amplicon Sequence Variants (ASVs) generated per sample ranged from three (WWTP1-BC1) to 29 (WWTP1-MAER2).

Sample ID	Quality reads	ASV	Shannon	Simpson	InvSimpson
WWTP1-MAER1[a]	76,734	19	2.10	0.83	5.98
WWTP1-BC1[a]	113,638	3	0.10	0.04	1.04
WWTP1-MAER2[b]	81,995	29	2.29	0.87	7.42
WWTP1-BC2[b]	86,826	8	1.60	0.76	4.21
WWTP5-DAER1[c]	28,872	6	1.22	0.61	2.53
WWTP5-BC1[c]	90,791	7	0.93	0.56	2.26
WWTP5-DAER2[d]	102,117	4	0.12	0.04	1.04
WWTP5-BC2[d]	76,230	5	0.69	0.45	1.81

Table 1. Bacterial community diversity of air samples. Note: MAER: mechanical aerators, DAER: diffused aerators, BC: background control, ASV: amplicon sequence variants. [a]Sample collection date, 18th October 2022. [b]Sample collection date, 1st November 2022. [c]Sample collection date, 9th November 2022. [d]Sample collection date, 15th November 2022.

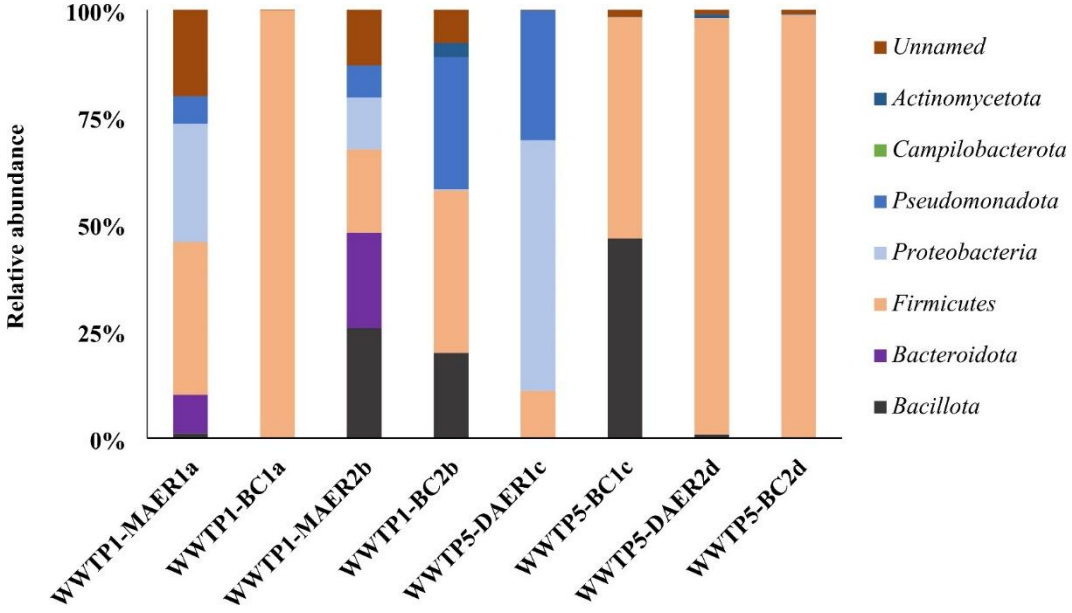

Fig. 1. Relative abundance (%) of phyla identified in bioaerosols from two municipal WWTPs. MAER: mechanical surface aerators; DAER: submerged aerators; BC: background control. [a]Sample collection date, 18th October 2022. [b]Sample collection date, 1st November 2022. [c]Sample collection date, 9th November 2022. [d]Sample collection date, 15th November 2022.

Analysis based on Shannon diversity revealed that the aeration tank (MAER) at WWTP1 had higher species diversity than aeration tank (DAER) at WWTP5 (Table 1). The Simpson and InvSimpson values for species richness and evenness ranged from 0.039–0.865 to 1.041–7.420, respectively. Both the Simpson and InvSimpson indexes revealed higher bacterial richness and evenness in the WWTP1 aeration tank (MAER) compared to DAER at WWTP5, and higher values were found for background control at WWTP1 than at WWTP5.

Airborne bacterial community composition

Seven phyla were identified across the eight bioaerosol samples as shown in Fig. 1. The three predominant phyla were *Firmicutes* identified in all the samples, followed by *Proteobacteria* and *Bacillota*, identified in six and five samples, respectively.

Figure 2 depicts nineteen families identified in the bioaerosol samples studied. *Bacillaceae* (8/8), *Moraxellaceae* (5/8), and *Clostridiaceae* (4/8) were the most frequently identified families detected in at least four samples. The remaining sixteen bacterial families were only identified in three or fewer air samples.

The results for bacterial communities at the genus level are illustrated in Fig. 3, highlighting the most abundant genera (more than 1% relative abundance in at least one sample). *Bacillus* was the predominant genus identified in all samples, followed by *Acinetobacter* (5/8), *Clostridium,* and *Enterococcus*, which were identified in four of the samples. Genera with relative abundance of at least 5% within samples were *Streptococcus* (29.2%), *Enterobacter* (14.8%), *Bacteroides* (9.0%), *Klebsiella* (6.4%), *Escherichia/Shigella* (6.2%), and *Acinetobacter* (6.0%) in WWTP1-MAER1; *Bacillus* (99.9%) was the only identified genera in WWTP1-BC1; *Bacteroides* (22.1%),

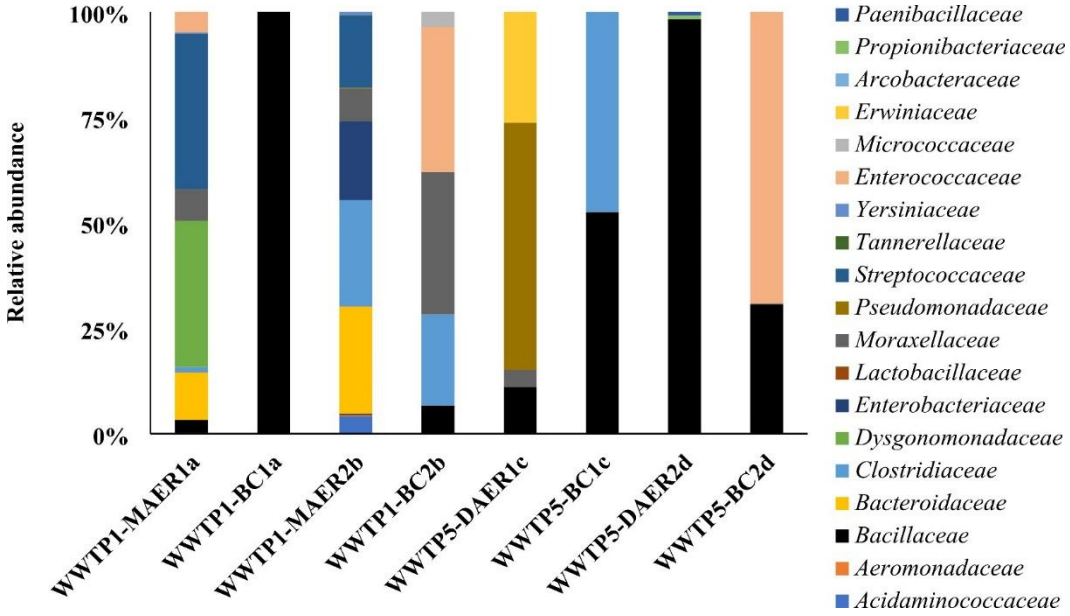

Fig. 2. Relative abundance (%) of the families identified in bioaerosols from two municipal WWTPs. MAER: mechanical surface aerators; DAER: submerged aerators; BC: background control. [a]Sample collection date, 18th October 2022. [b]Sample collection date, 1st November 2022. [c]Sample collection date, 9th November 2022. [d]Sample collection date, 15th November 2022.

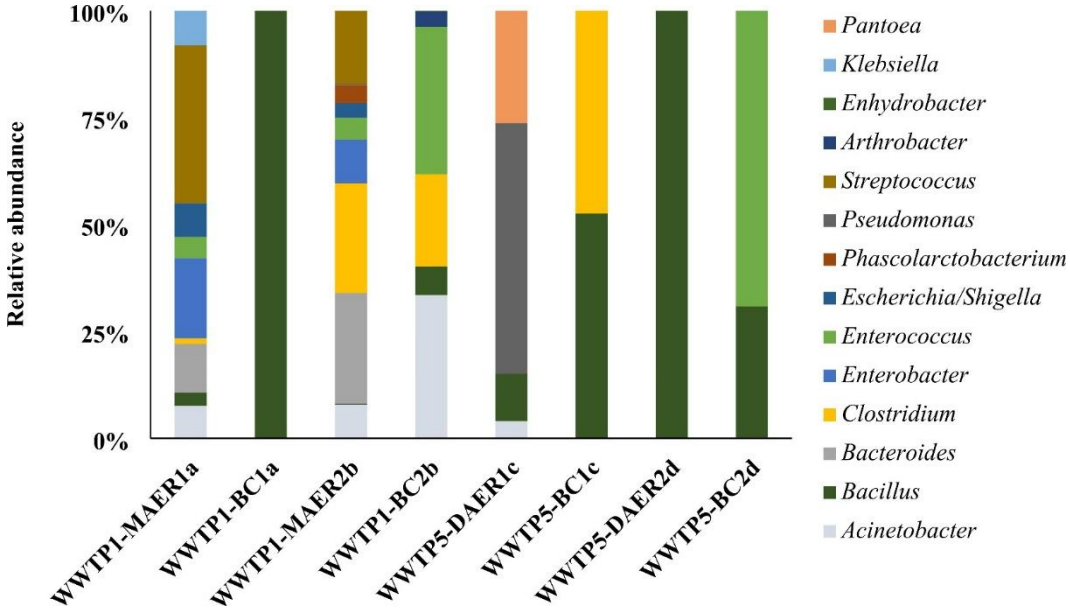

Fig. 3. Most abundant genera (>1% relative abundance) in bioaerosols from two municipal WWTPs. MAER: mechanical surface aerators; DAER: submerged aerators; BC: background control. [a]Sample collection date, 18th October 2022. [b]Sample collection date, 1st November 2022. [c]Sample collection date, 9th November 2022. [d]Sample collection date, 15th November 2022.

Clostridium (21.9%), *Streptococcus* (14.7%), *Enterobacter* (8.7%), and *Acinetobacter* (6.8%) were dominant in WTP1-MAER2; *Enterococcus* (31.8%), *Acinetobacter* (30.9%), *Clostridium* (19.9%), and *Bacillus* (6.2%) were dominant in WWTP1-BC2. The genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Bacteroides*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Escherichia/Shigella*, *Lactococcus*, *Serratia*, and *Streptococcus* were the most dominant bacteria in the bioaerosol samples at WWTP1 identified during both sampling events. In addition, *Acinetobacter*, *Bacillus*, *Clostridium* and *Enterococcus* were also identified in the background control at WWTP1, while *Arthrobacter* and *Enhydrobacter* were only identified in the background control. Regarding WWTP5 (DAER),

the dominant genera in WWTP5-DAER1 were *Pseudomonas* (58.5%), *Pantoea* (26.3%), and *Bacillus* (11.0%). *Bacillus* and *Clostridium* were the most abundant genera in WWTP5-BC1, accounting for 51.6 and 46.6% of the total relative abundance, respectively. *Bacillus* (97.1%) dominated in WWTP5-DAER2, *Enterococcus* (68.3%), and *Bacillus* (30.3%) were dominant in WWTP5-BC2. In general, bacterial communities in the bioaerosol samples at WWTP1 which uses mechanical aerators exhibited the most diverse genera, with 15 distinct genera compared to diffused aerators.

Shared and distinct genera in bioaerosols

A Venn diagram was used to explore shared and distinct bacterial genera in bioaerosols, and the results are presented in Fig. 4 and Supplementary Table S2 (genera list). A total of 26 genera across all samples were identified in the present study. Of these genera, *Acinetobacter* and *Bacillus* appeared across all sampling sites, *Clostridium* and *Enterococcus* were shared by WWTP1 employing mechanical aerators and the background control samples from both WWTPs. The genus *Pseudomonas* was shared among three samples namely, bioaerosol samples from both bioreactors utilising mechanical aerators (WWTP1) and diffused aerators (WWTP5), as well as background control samples from WWWT5. On the other hand, *Pantoea* was only common in samples from WWTP5. All sampling points had unique genera, albeit the MAER bioaerosol samples had the most, with 15 unique genera detected at this sampling point. Both background control samples had one unique genus with *Enhydrobacter* and *Arcobacter* identified at WWTP1 and WWTP5, respectively.

Potential opportunistic airborne bacterial pathogens present in the air at municipal WWTPs

Several potential opportunistic pathogenic genera were identified in the bioaerosol samples at the two WWTPs and in the background control. This study identified 36 potential airborne bacterial pathogens, which included 22 different genera and 18 species (Table 2). Twenty-eight potential opportunistic pathogens were detected in mechanical aerators bioaerosol samples at WWTP1, while six potential pathogens were identified at WWTP5 (diffused aerators). At WWTP1 and WWTP5, seven and ten potential pathogens, respectively, were identified in the background air samples. The most abundant (>1% relative abundance) potential airborne pathogens in this study were *Bacillus* spp. (44.6), *Enterococcus* spp. (13.2%), *Clostridium* spp. (8.8%), *Streptococcus* spp. (5.2%), *Acinetobacter* spp. (4.3%), *Enterobacter* spp. (2.8%), *Pseudomonas* spp. (2.4%), *Bacteroides fragilis* (2.1%), *Acinetobacter baumannii* (1.2%), and *Escherichia/Shigella* (1.1%) (Table 2).

As shown in Table 2, different potential opportunistic airborne pathogens dominated in individual bioaerosol samples. Opportunistic airborne pathogens *Streptococcus*, *Enterobacter*, *Bacteroides fragilis*, *Acinetobacter baumannii* and *Escherichia/Shigella* were abundant in WWTP1 aeration tank (MAER) samples. However, *Bacillus* and *Pseudomonas* were the main pathogens in WWTP5 aeration tank (DAER) samples. Background controls from the two WWTPs were dominated by pathogens *Bacillus* and *Clostridium*. Three pathogenic genera, *Acinetobacter*, *Bacillus* and *Pseudomonas*, were identified in the bioaerosol samples from the aeration tank area at both WWTPs. *Acinetobacter radioresistens*, *Arcobacter butzleri*, *Bacillus cereus*, *Clostridium perfringens* and *Arthrobacter* spp. were detected in the background control only and not in any of the samples from the aeration tanks. Furthermore, similar genera (*Acinetobacter*, *Bacillus*, *Clostridium*, and *Enterococcus*) were detected in the background control from both WWTPs.

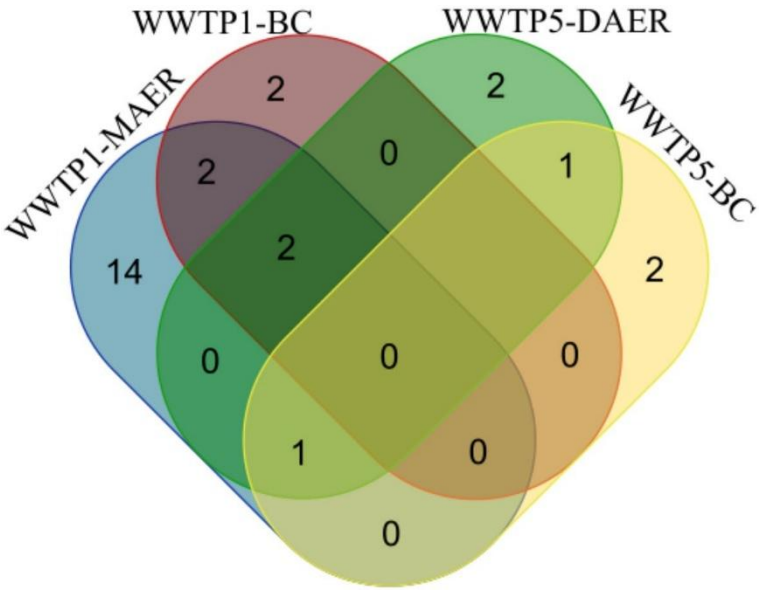

Fig. 4. Venn diagram showing airborne bacterial genera from aeration tanks with mechanical aerators and diffused aerators at WWTP1 and WWTP5, respectively. MAER: mechanical aerators; DAER: diffused aerators; BC: background control. Overlap indicates genera identified in the different sample locations.

Genera	Species	Total relative abundance (%)	Gram stain	HBA Risk group	WWTP5-DAER	WWTP5-BC	WWTP1-MAER	WWTP1-BC
1. *Acinetobacter*	*radioresistens*	0.2	-	2				
2. *Acinetobacter*	*baumannii*	1.2	-	2				
3. *Acinetobacter*	*soli*	0.04	-	2				
4. *Acinetobacter*	*junii*	0.03	-	2				
5. *Acinetobacter*	spp.	4.3	-	2				
6. *Aeromonas*	spp.	0.1	-	2				
7. *Arcobacter*	*butzleri*	0.02	-	2				
8. *Arthrobacter*	spp.	0.4	+	2				
9. *Bacillus*	spp.	44.6	+	2				
10. *Bacillus*	*cereus*	0.3	+	2				
11. *Bacteroides*	*uniformis*	0.02	-	2				
12. *Bacteroides*	*fragilis*	2.1	-	2				
13. *Bacteroides*	*ovatus*	0.03	-	2				
14. *Bacteroides*	*thetaiotaomicron*	0.2	-	2				
15. *Bacteroides*	spp.	0.3	-	2				
16. *Buttiauxella*	spp.	0.3	-	unclassified				
17. *Clostridium*	spp.	8.8	+	2				
18. *Clostridium*	*perfringens*	0.7	+	2				
19. *Enterobacter*	spp.	2.8	-	2				
20. *Enterococcus*	*faecium*	0.02	+	2				
21. *Enterococcus*	spp.	13.2	+	2				
22. *Escherichia/Shigella*	spp.	1.1	-	2 or 3				
23. *Klebsiella*	*pneumoniae*	0.3	-	2				
Klebsiella	spp.	0.5	-	2				
Lactobacillus	spp.	0.003	+	unclassified				
Lactococcus	spp.	0.03	+	unclassified				
Pantoea	*ananatis*	0.6	-	unclassified				
Pantoea	spp.	0.5	-	unclassified				
Propionibacterium	*acnes*	0.1	+	2				
Pseudomonas	*aeruginosa*	0.01	-	2				
Pseudomonas	spp.	2.4	-	2				
Raoultella	*ornithinolytica*	0.01	-	unclassified				
Raoultella	spp.	0.02	-	unclassified				
Serratia	spp.	0.1	-	2				
Streptococcus	spp.	5.2	+	2				
Vagococcus	*fluvialis*	0.01	+	unclassified				

Table 2. Relative abundance of potential opportunistic airborne bacterial pathogens and their risk group evaluation.
Note: Red shade = detected with > 1% relative abundance; orange shade = detected with < 1% relative abundance; no shade or color = not detected.

Risk group classification of the identified opportunistic bacterial pathogens

Twenty-eight bacterial pathogens (89% of the total relative abundance) identified were classified as hazardous biological agents (HBAs) according to the revised South African Regulation for Hazardous Biological Agents, 2022 (Table 2)[26]. Out of the identified HBAs, 27 organisms belong to Risk group 2 agents (organisms that may cause disease but are unlikely to spread to the community and have effective treatment in place). *Escherichia/Shigella* belong to Risk group 2 or 3 depending on the species types. Risk Group 3 HBAs may cause severe disease, presenting a risk of spreading to the community; nevertheless, effective therapy is available. The identified airborne pathogens were further differentiated based on their cell wall properties. Up to 67% (24/36) of the identified bacteria in the bioaerosol samples from aeration tanks and background control of the studied WWTPs were gram-negative bacteria.

Discussion

Utilising high-throughput sequencing, this study assessed the bacterial communities of bioaerosols emitted from two municipal WWTPs that use different aeration modes. The findings shed light on a broad profile of

bacteria, including potential opportunistic pathogenic bacteria, present in the air at WWTPs. The results of this study demonstrated that bacterial composition of bioaerosols generated by MAER was diverse when compared to bioaerosols generated by DAER. These findings are further supported in a recent study where significantly higher bacterial diversity was observed in bioaerosols produced by MAER than the background air[27]. The study further indicated that rotor speed influences species composition, size distribution, and concentration of bioaerosols, and that the levels of bacteria in the air increased with accelerated rotational speed[27]. The secondary treatment step is a key component of the wastewater treatment process, aimed at biologically removing organic components in wastewater[28]. Mechanical (e.g. rotors, fountains, agitators and others) and diffused (e.g. fine and coarse bubble diffusers) aerator systems are commonly utilised to aerate wastewater in aeration tanks, resulting in bioaerosol emission due to intense mixing and turbulence. The type of aeration system used determines the size of bubbles formed, which in turn influences the size of the bioaerosol and its microbial load[16,22]. Additionally, distinct genera were identified in the background controls compared to aeration tanks samples. Differences in bacterial composition across different treatment stages in WWTPs are primarily influenced by factors including mechanical processes (e.g., aeration and agitation), meteorological conditions (e.g., temperature, humidity, solar radiation, and wind), and sources of bacteria (e.g., wastewater, sludge, and background atmospheric sources)[13,15]. Therefore, it is plausible that bacteria from other sources unrelated to wastewater may have been dominant in the background control samples, contributing to the observed differences in bacterial species.

Although the bioaerosol data in this study are presented as relative abundances rather than quantitative measurements, the inclusion of environmental metadata provides a valuable context for interpreting the results. Environmental factors such as relative humidity, temperature, wind and UV index are known to influence bioaerosol dynamics and could partially explain the observed shifts in relative abundance[13,15]. For example, a study investigating bioaerosols from six municipal WWTPs in China demonstrated that meteorological factors, particularly temperature, solar radiation, and relative humidity influence the survival and dispersion of airborne microorganisms[29]. Temperature and relative humidity directly affect bioaerosol dispersal and viability of airborne microorganisms, thus affecting the concentration of bioaerosols. Wind dilute and reduce bioaerosol concentrations, whereas poor ventilation may lead to significant accumulation of microbial aerosols in indoor treatment units[13,15]. Since the environmental metadata is averaged over a larger area, it may not perfectly represent microclimatic conditions at the study site. However, the data provides a reasonable approximation of the prevailing environmental conditions at the studied areas during the sampling period.

In this study, majority of potentially opportunistic pathogenic bacterial genera and species were identified in MAER bioaerosols. These findings are comparable with previous studies that explored bacterial composition in bioaerosols from WWTPs using different aeration modes[16,22,27]. Intestinal bacteria in bioaerosols from six municipal WWTPs in China were substantially higher in mechanical aerators than biochemical reaction tanks utilising diffused aerators[11]. Similarly, Han and co-workers (2020) indicated that mechanical aerators (horizontal rotors) contributed the most total microorganisms and specific pathogens in bioaerosols when compared to fine bubble diffusers in the same WWTP[22]. In a recent study, Lu et al.[4] collected air samples from the grit chamber house and near the aeration tank at a municipal WWTP in Denmark, identifying 91 and 94 bacterial species, respectively, with five Risk Group 2 species (*Enterobacter cloacae*, *Klebsiella oxytoca*, *K. pneumoniae*, *Escherichia coli*, and *Morganella morganii*) present at both sites.

Potentially pathogenic genera in this study were dominated by *Bacillus* spp., *Enterococcus* spp., *Clostridium* spp., *Streptococcus* spp., *Acinetobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Bacteroides fragilis*, *Acinetobacter baumannii*, and *Escherichia/Shigella*. These bacterial species can cause skin, community acquired pneumonia, gastrointestinal, and urinary tract infections (UTI) in humans. Previous studies have found similar pathogenic bacteria in bioaerosols from WWTPs[4,11–13,16,17]. *Bacillus* species are common in soil and wastewater, and the species of medical importance are *Bacillus anthracis* and *Bacillus cereus*, which are responsible for anthrax and food poisoning, respectively[30]. *Enterococcus* species can cause UTI, meningitis, infective endocarditis (IE), and wound infections[31]. Clostridial infections, on the other hand, are typically associated with food poisoning, gas gangrene, botulism, and soft tissue infections[32]. *Acinetobacter* species can cause infections of the respiratory tract, blood and urinary tract, particularly in immuno-compromised individuals. Of note, *A. baumannii* is one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens primarily associated with hospital-acquired infections as well as antimicrobial resistance[33]. *Streptococcus* species can cause invasive diseases such as sepsis, meningitis, IE, and pneumonia[34], whereas the *Enterobacter* genus is a well-known nosocomial pathogen that causes UTI, osteomyelitis, IE, respiratory, and soft tissue infections[35]. *Pseudomonas aeruginosa* causes the majority of *Pseudomonas* infections, and can infect virtually any tissue, including the blood, central nervous system, and lungs, among others[36]. *Bacteroides fragilis* can cause peritonitis, lung and brain abscesses, bacteremia, soft tissue infections, and toxin-associated diarrhoea[37]. Common infections of *Escherichia* species include UTI, pneumonia, bacteremia, and acute enteritis[38], whereas *Shigella* is a well-known cause of acute gastrointestinal infections[39].

Twenty-seven human bacterial pathogens identified in this study are classified as Risk Group 2, whereas *Escherichia/Shigella* belong to Risk group 2 or 3[26]. Overall, this study suggests that WWTP workers are potentially exposed to airborne bacterial pathogens that can cause a range of diseases such as respiratory and gastrointestinal infections, UTI, and skin and soft tissue infections. Five species (*E. faecium*, *A. baumannii*, *K. pneumoniae*, *P. aeruginosa, and Enterobacter* spp.) that are highly virulent and associated with multidrug resistance were also identified. Furthermore, a majority of the classified bacterial pathogens were gram-negative bacteria, suggesting that workers at WWTPs may be exposed to higher levels of inhalable endotoxins present in their ambient air, potentially leading to chronic diseases such as bronchitis, asthma, wheeze, and organic toxic dust syndrome after long-term exposure[40]. This highlights the importance of effective bioaerosol control strategies to safeguard the health of WWTP workers. This could include engineering controls such as covering aeration tanks, particularly at WWTPs using mechanical aerators, to minimise bioaerosol generation and reduce

the concentration of bioaerosols in the air. In addition, implementing worker exposure controls such as strict adherence to PPE and administrative measures, such as work schedule adjustments and regular training, can further mitigate the risk of bioaerosol exposure among WWTP workers.

Conclusion

The diversity of bacterial composition in bioaerosols varied depending on the mode of aeration at the WWTPs. Furthermore, mechanical aeration tanks exhibited relatively higher richness and diversity of airborne bacterial communities and specific pathogens compared to diffused aeration tanks. Twenty-seven human bacterial pathogens near aeration tanks and background control, classified as Risk Group 2, with *Escherichia/Shigella* potentially belonging to Risk Group 3 were identified. Among these, highly virulent species associated with multidrug resistance were identified, underscoring the urgent need for effective bioaerosol control strategies and worker exposure protocols to safeguard the health of WWTP workers. The predominance of gram-negative bacteria suggests a higher risk of inhalable endotoxin exposure, potentially leading to chronic respiratory conditions. Overall, our findings underscore the critical importance of addressing bioaerosol exposure in WWTP environments to mitigate health risks and ensure worker safety. Future studies should conduct comprehensive investigations to identify additional potentially harmful components in bioaerosols such as viruses, fungi, allergens, and antibiotic-resistant genes, as these may pose serious health risks to humans. Additionally, long-term studies are important for understanding the spatial and temporal variation of bioaerosols. Finally, conducting site-specific risk assessments with a few selected dominant bacterial pathogens determined by culture methods will enable a complementary and more accurate evaluation of the health risks posed by bioaerosols to WWTP workers.

Data availability

The datasets generated and analysed during the current study are available in the NCBI BioProject repository (BioProject ID: PRJNA1182964), https://www.ncbi.nlm.nih.gov/PRJNA1182964.

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Author contributions
E.P: Conception and design of study, Methodology, Acquisition of data, Analysis of data, Writing—original draft, and Writing—review and editing. R.P: Analysis of data, and Writing—review and editing. T.S: Conception and design of study, Resources, Supervision, and Writing—review and editing. A.G: Conception and design of study, Funding acquisition, Methodology, Resources, Supervision, and Writing -review and editing. All authors reviewed and approved the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to E.P.

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CHAPTER 6: ANTIBIOTIC RESISTANCE PROFILES AND MUTATIONS THAT MIGHT AFFECT DRUG SUSCEPTIBILITY IN METAGENOME-ASSEMBLED GENOMES OF *LEGIONELLA PNEUMOPHILA* AND *AEROMONAS* SPECIES FROM MUNICIPAL WASTEWATER

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Antibiotic resistance profiles and mutations that might affect drug susceptibility in metagenome-assembled genomes of *Legionella pneumophila* and *Aeromonas* species from municipal wastewater

Evida Poopedi[1,3*], Rian Pierneef[5,6], Tanusha Singh[2,4] and Annancietar Gomba[2]

Abstract

Antibiotic resistance (AR) has emerged as a significant global health issue. Wastewater treatment plants (WWTPs) contain diverse bacterial communities, including pathogens, and have been identified as crucial reservoirs for the emergence and dissemination of AR. The present study aimed to identify antibiotic resistance genes (ARGs)and screen for the presence of mutations associated with AR in *Legionella pneumophila* and *Aeromonas* spp. from municipal wastewater. Metagenome-assembled genomes (MAGs) of *L. pneumophila* and *Aeromonas* spp. were reconstructed to investigate the molecular mechanisms of AR in these organisms. A total of 138 nonsynonymous single nucleotide variants (SNVs) in seven genes associated with AR and one deletion mutation in the *lpeB* gene were identified in *L. pneumophila*. In *Aeromonas* spp., two (*aph(6)-Id* and *aph(3")-Ib*) and five (bla_{MOX-4}, $bla_{OXA-1143}$,$bla_{OXA-724}$, *cepH*, and *imiH*) ARGs conferring resistance to aminoglycosides and β-lactams were identified, respectively. Moreover, this study presents β-lactam resistance genes, $bla_{OXA-1143}$ and $bla_{OXA-724}$, for the first time in *Aeromonas* spp. from a municipal WWTP. In conclusion, these findings shed light on the molecular mechanisms through which clinically relevant pathogenic bacteria such as *L. pneumophila* and *Aeromonas* spp. found in natural environments like municipal wastewater acquire AR.

Clinical trial number

Not applicable.

Keywords Antibiotic resistance, Metagenome-assembled genome, Mutations, Molecular mechanisms, Municipal wastewater

*Correspondence:
Evida Poopedi
evida.poopedi@smu.ac.za
[1]Department of Oral and Maxillofacial Pathology, Oral Microbiology and Oral Biology, Sefako Makgatho Health Sciences University, Ga-Rankuwa, South Africa
[2]National Institute for Occupational Health, National Health Laboratory Service, Johannesburg, South Africa
[3]Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, Johannesburg, South Africa
[4]Department of Environmental Health, University of Johannesburg, Doornfontein 2028, South Africa
[5]Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa
[6]Centre for Bioinformatics and Computational Biology, University of Pretoria, Pretoria, South Afric

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Introduction

Antibiotic resistance (AR) has increased significantly, primarily due to the excessive and inappropriate use of antibiotics in clinical and veterinary settings, increased infection rates, and inadequate hygiene and disinfection practices [1]. Historically, clinical settings were believed to be the primary reservoir of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [2]. However, ARB and ARGs have recently become ubiquitous contaminants in other environments including wastewater treatment plants (WWTPs) [2, 3]. Numerous studies have implicated WWTPs as key reservoirs and sources of AR in the environment [2, 4]. As such, different AR types and subtypes of almost all ARGs are consistently detected in different types of wastewaters [5, 6].

Legionella spp. are gram-negative fastidious aerobic bacteria found ubiquitously in natural and artificial water systems, including WWTPs. *Legionella pneumophila* is the causative agent of severe atypical pneumonia also called Legionnaires' disease (LD), as well as an acute febrile illness known as Pontiac fever [7]. This organism is responsible for up to 90% of the reported LD cases worldwide, with most of these cases attributed to *L. pneumophila* serogroup 1 [7]. Macrolide and fluoroquinolone antibiotics (usually azithromycin and levofloxacin, respectively) are currently recommended for moderate and severe LD [8]. Despite AR historically not being a primary concern in *Legionella* infections, recent reports have identified resistance to first-line drugs in clinical strains and documented instances of treatment failure in patients [9, 10]. These findings highlight the possibility of reduced efficacy and the emergence of resistance to traditional therapies. Jia et al. (2019) reported that an efflux pump component (*lpeAB*) primarily confers azithromycin resistance in *L. pneumophila* [10]. Through sequencing approaches, several resistance mediating mechanisms have been revealed in *L. pneumophila*, including enzymatic modifications and mutations in genes encoding 23S rRNA, DNA gyrase and topoisomerase IV, as well as ribosomal accessory proteins [11, 12].

The genus *Aeromonas* comprises gram-negative rod-shaped bacteria commonly found in diverse aquatic habitats such as rivers, drinking water, wastewater, soil, and animals. Members of this genus have been implicated in a wide range of human diseases, including gastroenteritis, wound infections, and bacteremia [13]. Notably, *Aeromonas* spp. serve as reservoirs of ARGs in aquatic settings [13]. Moreover, members of the genus *Aeromonas* harbour a plethora of AR determinants located on mobile genetic elements, including plasmids, transposons, integrons, and genomic islands, facilitating the horizontal transfer of ARGs even to phylogenetically distant bacterial species [14]. Notwithstanding the significance of *Aeromonas* in the dissemination of AR, few studies have characterised the genotypic profiles of AR determinants in *Aeromonas* species within WWTP environments [15, 16]. Understanding these genotypic profiles is crucial, as it provides valuable insights into the mechanisms by which ARGs are disseminated in WWTPs and implications for WWTP workers' health.

In 2019, it was approximated that antimicrobial resistant infections led to nearly 1.27 million deaths globally [17]. Moreover, forecasts indicate that by 2050, AR could result in 10 million deaths annually and a 3.8% decline in the global gross domestic product (GDP) [18]. Therefore, efforts to identify the emergence and spread of AR require a broader and non-invasive approach, especially in countries with limited resources and poor surveillance strategies [19]. Antibiotic-resistant bacterial species that are commonly studied in WWTPs predominantly include culturable enteric bacteria such as *Klebsiella pneumoniae*, *Enterobacteriaceae*, *Escherichia coli*, *Citro- bacter* spp., and *Serratia* spp [3, 20]. However, culture- dependent methods have drawbacks, as only 1% of total environmental bacteria are culturable. Additionally, some of these bacteria are fastidious or slow growing and are thus difficult to characterise using culture methods [21]. While *L. pneumophila* is an established pathogen, *Aeromonas* is emerging as a potential public health concern because of its presence in water systems and resistance to multiple antibiotics [13, 14]. Therefore, the aim of this study was to characterise metagenome-assembled genomes (MAGs) of *L. pneumophila*, and *Aeromonas* spp. from municipal wastewater and identify their AR genotypes. Additionally, this study investigated the pres- ence of spontaneous mutations in select ARGs known to confer resistance to commonly used antimicrobial drugs in *L. pneumophila*. To the best of our knowledge, this is the first study to perform genome analysis of wild type *L. pneumophila* detected from municipal wastewater to better understand the potential health risks of environ- mental pathogenic organisms with no link to outbreaks or clinical cases.

Materials and methods

Wastewater sample collection and culture

Wastewater samples were collected from municipal WWTPs in Tshwane, South Africa. The characteristics of the sampling sites were previously described by Poopedi et al. (2023), see Supplementary Table S1 [22]. Sampling sites were selected based on treatment capacity, representing small (WWTP1: 35 mega litres per day (ML/day)), medium (WWTP1: 60 ML/day), and large (WWTP3: 93 ML/day) WWTPs. Additional selection criteria included the use of surface aeration technology, proximity to the laboratory, and willingness of site management to participate in the study. The Legiolert method (IDEXX Laboratories, Inc., Westbrook, U.S.) was used

Table 1 Sources and type of wastewater used in this study

Sample ID	Source	Sampling point	Collection date
WP1INF4	WWTP1	Bar screen	30 November 2021
WP1INF9	WWTP1	Bar screen	30 November 2021
WP2AS8	WWTP2	Aeration tank	11 January 2022
WP2SST1	WWTP2	Secondary settling tank effiuent	25 January 2022
WP3INF4	WWTP3	Bar screen	16 November 2021
WP3INF2	WWTP3	Bar screen	16 November 2021
WP3AS1	WWTP3	Aeration tank	08 February 2022

to analyse 1 mL of wastewater in accordance with the manufacturer's instructions specified for non-portable water. The details of the wastewater samples are outlined in Table 1.

DNA extraction

The positive Legiolert trays were decontaminated with 70% ethanol, and the contents of the wells were extracted with a sterile disposable syringe (Merck, Darmstadt, Germany). The contents were placed into a 50 mL centrifuge tube and concentrated for 45 min at 5000 rpm (Eppendorf, Hamburg, German). Genomic DNA was then extracted from the pellet biomass using a DNeasy Powersoil Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

Sequencing, metagenome-assembled genomes (MAGs) assembly and taxonomic annotation

Sequencing was conducted at the Biotechnology Platform of the Agricultural Research Council in Onderstepoort, South Africa, using the MGI DNBSEQ-G400 sequencing platform according to the manufacturer's instructions, with 150 bp paired-end reads specified. Raw data were subjected to quality control, including adapter removal using BBDuk v.38.91 (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/;sourceforge.net/projects/bbmap/). De novo assembly of individual samples was performed with metaSPAdes v.3.15.3 [23], and only assembled contigs exceeding 1,500 bp were retained for subsequent analysis. The resulting contigs were then grouped into MAGs using Metabat v.2.15 [24]. Evaluation of MAG completeness and contamination was performed using CheckM v.1.1.3 [25], and only MAGs with completeness ≥ 50% and contamination ≤ 10% (classified as medium to high quality MAGs) were retained for further analyses [26]. Taxonomic classification of the MAGs using the Genome Taxonomy Database-Tk v.1.7.0 [27] identified *Legionella pneumophila* and *Aeromonas* species among the assembled genomes.

Antibiotic resistance genes and point mutations

Genome assemblies were assessed using the National Center for Biotechnology Information (NCBI) AMRFinderPlus (https://github.com/tseemann/abricate) for the in silico prediction of ARGs. This analysis identified ARGs in both *Legionella pneumophila* and *Aeromonas* species MAGs. The presence of the AR-associated genes *lpeA*, *lpeB*, *rplD*, *rplV*, *rrl/23 rRNA*, *gyrA*, *gyrB*, *parC*, *parE* and *rpoB* in the assembled *L. pneumophila* MAGs was screened using BLASTn querying the *L. pneumophila* strain Paris (NC_006368). The obtained gene sequences were subsequently aligned via multiple sequence alignment with the reference strain using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). BioEdit [28] was used to perform and visualise the alignments. Amino acid changes were determined by translating the nucleotide sequences using the bacterial genetic code table (*E. coli* numbering system).

Results

General features of the recovered metagenome-assembled genomes (MAGs)

Table 2 summarises the general characteristics of the MAGs classified as *L. pneumophila* and *Aeromonas* spp. In this study, high quality MAGs were recovered, defined by a completeness exceeding 50% and contamination

Table 2 Features of metagenome-assembled genomes (MAGs) classified as *L. pneumophila* and *Aeromonas* spp

Features	*L. pneumophila*				*A. caviae*		*A. hydrophila*
Sampling point	Bar screen	Aeration tank	SST effluent	Bar screen	Bar screen	Bar screen	Aeration tank
Sampling site	WWTP1	WWTP2	WWTP2	WWTP3	WWTP1	WWTP3	WWTP3
Sample ID	WP1INF4	WP2AS8	WP2SST1	WP3INF4	WP1INF9	WP3INF2	WP3AS1
Average coverage	548X	333X	1704X	710X	12X	64X	360X
Completeness (%)	95	99	99	99	95	69	59
Contamination (%)	0,19	0,19	0,19	0,19	0,00	0,00	0,00
Size (bp)	3,177,878	3,435,984	3,335,074	3,295,813	4,245,992	3,782,452	3,046,587
No. of Contigs	60	35	18	19	87	125	315
N50	101,252	160,164	341,210	278,643	85,178	50,913	14,488
GC (%)	38,2	38,2	38,2	38,2	61,9	62,3	62,2
No. of predicted genes	2,838	3,069	3,005	2,961	3,895	2,962	3,512

Note: ID: identification, bp: base pair, GC: guanine cytosine, %: percentage, SST: secondary settling tank

Table 3 Antibiotic resistance genes within the metagenome-assembled genomes (MAGs) classified as *L. pneumophila* and *Aeromonas* spp. from municipal wastewater

Antibiotic class	*L. pneumophila*				*A. caviae*		*A. hydrophila*
	WP1INF4	WP2AS8	WP2SST1	WP3INF4	WP1INF9	WP3INF2	WP3AS1
	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)
Aminoglycoside	*aph(9)-Ia* (89.8)	*aph(9)-Ia* (90.4)	*aph(9)-Ia* (90.3)	*aph(9)-Ia* (90.5)	*aph(6)-Id* (100) *aph(3")-Ib* (99.9)	*aph(6)-Id* (100) *aph(3")-Ib* (99.9)	-
ß-lactams	-	-	-	-	bla_{MOX-4} (95.7) $bla_{OXA-1143}$ (96.5)	bla_{MOX-4} (95.7) $bla_{OXA-1143}$ (96.5)	$bla_{OXA-724}$ (99.8) *cepH* (99.9) *imiH* (97.2)

Note: %: percentage

below 10%. Specifically, MAGs classified as *L. pneumophila* had completeness ranging from 95 to 99%, with contamination levels of less than 1%. Similarly, MAGs classified as *Aeromonas* spp. had completeness ranging from 59 to 95%, with no contamination (0%). The high level of completeness observed in these MAGs was con- sistent with high sequencing coverage achieved, highlighting the reliability of the genomic reconstructions.

The genome sizes of the WP1INF4, WP2AS8, WP2SST1, WP3INF4 *L. pneumophila* MAGs were approximately 3.18, 3.44, 3.34, and 3.30 Mbp, respectively, with identical low genomic G + C contents of 38.2% in all sequences. The assembled sequences of *L. pneumophila* WP1INF4, WP2AS8, WP2SST1, and WP3INF4 yielded 60, 35, 18, and 19 contigs with N50 values of 101,252, 160,164, 341,210, and 278,643, respectively. In addition, *L. pneumophila* WP1INF4, WP2AS8, WP2SST1, and WP3INF4 each had 2,838, 3,069, 3,005, and 2,961 predicted genes, respectively.

The sizes of the *Aeromonas caviae* MAGs WP1INF and WP3INF2 genomes were approximately 4.24 and 3.78 Mbp, with G + C contents of 61.9 and 62.3%, respectively. The assembly produced 145 and 87 contigs with N50 values of 85,178 and 50,913 in the *A. caviae* WP1INF and WP3INF2 genomes, respectively. The predicted gene counts for the two *A. caviae* genomes were 3,895 and 2,962. The genome of *A. hydrophila* MAGs had the following features: a genome size of 3.05 Mbp, a G + C con- tent of 62.2%, 315 contigs with N50 values of 14,488, and 3,512 predicted genes.

Potential antibiotic resistance mutations and genotype in *L. pneumophila*

Antibiotic resistance genotypes were searched in the NCBI's AMRFinderPlus tool. The gene encoding the aminoglycoside O-phosphotransferase *aph(9)-Ia*, which confers resistance to spectinomycin, was detected in all four *L. pneumophila* MAGs (Table 3). Additionally, point mutations in genes known to be responsible for resistance to macrolides (*lpeA*, *lpeB*, *rplD*, *rplV*, and *rrl* 23 rRNA), fluoroquinolones (*gyrA*, *gyrB*, *parC*, *parE*), and rifampicin (*rpoB*)) drugs used for the treatment and management of LD were explored (Supplementary Table S2). Several nonsynonymous single nucleotide variants (SNVs) in resistance-associated genes (*rpoB*, *lpeB*, *rplD*, *gyrA*, *gyrB*, *parC*, and *parE*) were detected compared to the reference *L. pneumophila* strain Paris. Detailed information regarding the positions and amino acid changes of these nonsynonymous SNVs that were identified are described in Supplementary Table S3. In total, 138 non- synonymous SNVs corresponding to seven genes and one deletion mutation in the *lpeB* gene were identified. Non-synonymous SNVs were highly prevalent in the genes *gyrA* and *gyrB*, which had 85 and 17 variants, respec- tively. No mutations were detected in the *lpeA* efflux pump gene, 23 rRNA gene *rrl* or ribosomal accessory protein gene *rplV*.

Antibiotic resistance patterns of *Aeromonas* species

The ARGs identified from *Aeromonas caviae* and *Aeromonas hydrophila* MAGs are presented in Table 3. Within these MAGs, resistance to the antimicrobial classes aminoglycoside and β-lactam was conferred by two and five ARGs, respectively. Specifically, the MAGs classified as *A. caviae* WP1INF9 and WP3INF2 were found to carry *aph(6)-Id* and *aph(3'')-Ib*, known for their strong affinity towards streptomycin and kanamycin, respectively, contributing to aminoglycoside gene resistance. Additionally, both *A. caviae* MAGs presented the β-lactam resistance genes bla_{MOX-4} and $bla_{OXA-1143}$. In contrast, *A. hydrophila* MAG WP3AS1 harboured dis- tinct β-lactam resistance genes, namely, $bla_{OXA-724}$, *cepH*, and *imiH*. The resistance profiles of the two *A. caviae* MAGs (WP1INF9 and WP3INF2) were similar, whereas *A. hydrophila* had unique ARGs. Furthermore, the iden- tified ARGs had a relatively high percent identity (above 95%), suggesting the potential completeness or functionality of the resistance genes.

Discussion

The present study comprehensively investigated AR profiles and point mutations associated with resistance in the MAGs of *L. pneumophila* and *Aeromonas* spp. from municipal wastewater. The aminoglycoside O-phosphotransferase gene (*aph(9)-Ia*) was detected in all *L. pneumophila* MAGs. This gene confers resistance to spectinomycin in *L. pneumophila.* Svetlicic et al. (2023) characterised the genomes of 39 *Legionella* isolates recovered during the environmental surveillance of man-made water systems, including showers, sinks, cooling towers, fountains, and irrigation tanks, across 13 coun- tries and identified the *aph(9)-Ia* gene in 25 strains of *L. pneumophila* and two strains of *Legionella gormanii* [29]. Although the *aph(9)-Ia* gene is currently not considered a serious risk due to the infrequent use of spectinomycin in LD treatment, spectinomycin-resistant *L. pneumophila* has the potential to horizontally transfer the gene to other clinically relevant pathogens.

This study identified several nonsynonymous SNVs in *L. pneumophila* AR genes associated with resistance to macrolide, fluoroquinolone, and rifampicin drugs. An inherent limitation of the present study is the unavailability of culturable isolates to explore the correlation between phenotypic antimicrobial susceptibility patterns and genomic characteristics due to resource constraints. However, genomic findings presented in this study offer valuable information on the potential resistance mechanisms in *L. pneumophila* and *Aeromonas* spp., providing a foundation for future studies that can link these geno- types to phenotypic resistance patterns. The SNV find- ings of this study were compared with those of previously reported in vitro target gene induced mutations associ- ated with macrolide, fluoroquinolone, and rifampicin in *L. pneumophila* [11–12, 30], but no similarities were found. It should be noted that unlike other ARB, limited studies have described gene mutations conferring AR in *L. pneumophila* [10, 31], resulting in limited data available for comparison. Among the identified mutations in the *L. pneumophila* MAGs recovered in this study, two- point mutations occurring at nucleotide positions 978 and 979 in the *lpeB* gene had previously been detected by Natås et al. (2019), and the other mutations could not be associated [31]. Natås et al. (2019) reported G978S and L979V variants in the *lpeB* gene in clinical and environmental *L. pneumophila* strains with reduced susceptibility to azithromycin [31]. Interestingly, the clinical *L. pneumophila* strain harboured both mutations, whereas the environmental strain possessed only the G978S variant [31]. Unlike clinical *Legionella* strains, the environ- mental MAGs of *Legionella* in this study may not have developed AR mutations affecting *L. pneumophila* sensitivity to treatment because of a lack of selective pressure. Notably, this study identified different amino acid changes (G978I and L979P), possibly due to distinct nucleotide substitutions resulting in different amino acid changes [32]. Unlike mutations induced by therapeutic interventions, humans can be exposed to resistant *Legionella* strains present in the environment. Macrolides, which are commonly used to treat respiratory infections in humans, are also naturally produced by *Streptomycetes* species in the environment [33]. These macrolides have been detected in various water systems, including both influent and treated effluent wastewater [34, 35]. In a recent one-year study by Senta et al. (2019) in Croatian WWTPs, macrolide concentrations of up to 25 micro- grams per liter were observed [34]. The widespread presence of macrolides in WWTPs, coupled with their poor removal through conventional WWTP processes, suggests that *Legionella* species are exposed to macrolides at relatively high levels in these settings, potentially contributing to the development of AR.

Detecting DNA gyrase mutations in *L. pneumophila* can help predict the presence and level of fluoroquinolone resistance (FQR). In this study, DNA gyrase genes harboured a high number of nonsynonymous SNVs compared to other target genes. While previously considered improbable, recent studies have revealed FQR in *L. pneumophila* from LD infected patients [36]. To date, the most common FQR mutation reported in clinical *L. pneumophila* occurs in *gyrA* codon 83 [9, 37]. Although the clinical significance of our findings on FQR could not be determined at this time, the alarming probability of FQR emergence associated with spontaneous mutations in environmental *L. pneumophila* warrants attention. This trend may complicate LD treatment in the future, particularly considering the efficacy of fluoroquinolones against broad spectrum bacteria.

Genetic mutation is one of the major mechanisms driving the development and acquisition of AR in bacteria. Antibiotic resistance caused by mutations frequently reduces drug binding or loss of function, thus promoting drug efflux activity and diminishing the intracellular drug concentration [38]. Spontaneous mutations, such as base pair substitutions, insertions, and deletions in genes encoding drug targets, are common mechanisms in acquired mutations [38]. Understanding mutational AR, especially in clinically relevant bacterial pathogens from the environment including WWTPs, is crucial, as AR can arise spontaneously even without strong selective pressure or antibiotic exposure [39]. Therefore, a more in- depth analysis to explore the mechanisms underlying the acquisition of nonsynonymous SNVs in environmental *L. pneumophila* is needed. Future studies should include sequencing of susceptible and resistant *L. pneumophila* isolates, which would help in determining the magnitude of the effect of the identified mutations.

The common ARGs detected among *Aeromonas* spp. MAGs in this study were β-lactams. This finding is in agreement with other studies indicating that this genus often exhibits high resistance to β-lactams and may serve as a potential reservoir for the dissemination of β-lactam ARGs in water environments [40, 41]. Class C β-lactamase genes are chromosomal or plasmid-encoded and confer resistance to cephamycins, oxyimino-β- cephalosporins, and aztreonam [13]. The main concern with plasmid mediated ARGs is their potential for horizontal transfer, including across different bacterial species. In this study, two β-lactamase genes from class C were identified: bla_{MOX-4}, and *cepH*. Previously, bla_{MOX-4} was identified in only an *Aeromonas* strain from an urban WWTP in Poland [40]. On the other hand, the *cepH* gene is specific to *A. hydrophila* and has been commonly identified in various environments including wastewater [40, 42], clinical samples [43], and fish [44].

Oxacillin-hydrolysing (OXA)-type β-lactamases (members of class D) are widely distributed among clinically relevant gram-negative bacteria, including *Aeromonas* spp [45]. Their diversity continues to expand to include new bla_{OXA} variants [45]. This study identified *bla-* $_{OXA-1143}$ and $bla_{OXA-724}$ in *A. caviae* and *A. hydrophila*, respectively. To the best of our knowledge, this is the first study to report the $bla_{OXA-1143}$ subtype in municipal wastewater, although it was previously reported in an unpublished study of a rectal swab sample from a patient in France (GenBank accession no. **NG_081795.1**). Conversely, $bla_{OXA-724}$ has been identified in different *Aeromonas* species isolated from faeces of gastroenteritis patients [40], poultry [46], chickens [47], and fish [48]. Notably, this study represents the first identification of $_{blaOXA-724}$ in an *Aeromonas* from a municipal WWTP.

Class B metallo-β-lactamases (MBLs) pose a serious AR threat because of their ability to enzymatically hydro- lyse most β-lactams, including carbapenems, which are reserved as a last resort for bacterial infections [49]. This study identified the MBL encoding gene *imiH*, which is specific to *A. hydrophila*. Previously, *imiH* was identified in *A. hydrophila* isolated from diverse sources, including a dairy farm in Texas [50], a recreational estuary in Brazil [51], Indian carp [48], and an activated sludge sample from a Polish urban WWTP [40]. Differences in resistance genotypes between *A. caviae* and *A. hydrophila* are associated with species variation. For instance, chromosomal β-lactamase is species-specific in *Aeromonas* species, with *A. caviae* possessing Class C and D β-lactamases, whereas *A. hydrophila* presents a broader spectrum with Class B, C, and D enzymes [13].

This study identified aminoglycoside resistance encoding genes, *aph(6)-Id* and *aph(3'')-Ib*, conferring resistance to streptomycin and kanamycin, in both *A. caviae* MAGs. These findings are consistent with prior research investigating circulating ARGs in wastewater, suggesting the widespread presence of *aph(6)-Id* and *aph(3'')-Ib* in such environments [52, 53]. It should be noted that aminoglycoside encoding genes are typically located on plasmids or transposons and have been reported in *Aeromonas* spp [41, 54]. Therefore, it is plausible that *A. caviae* could be an important reservoir for the dissemination of aminoglycoside encoding genes, including *aph(6)- Id* and *aph(3'')-Ib*, to other bacterial species, particularly those of clinical importance, thereby posing a significant public health risk.

Previous studies have suggested that WWTP workers are potentially exposed to ARB at work via different exposure routes, such as inhalation and/or accidental ingestion of water droplets [20, 55]. Further- more, an increased prevalence of respiratory and gastrointestinal diseases has been reported among WWTP workers [56], as well as elevated antibodies against certain bacteria [57], suggesting a link to bacterial exposure. Rodríguez-Molina and co-workers (2021) recently investigated ARB carriage in WWTP workers and noted the presence of extended-spectrum β-lactamase producing *E. coli* in the stool of WWTP workers and nearby residents in Romania, Germany, and the Netherlands, with the Romanian population having the highest carriage rate (28%) [58]. Therefore, AR studies are crucial for obtaining a better understanding of the exposure status of workers in WWTPs as well as developing preventive interventions to reduce potential occupational exposure.

The findings of this study highlight the role of municipal wastewater as a significant reservoir for AR, where environmental, human, and animal health intersect. The identified ARGs and SNVs reveal emerging AR pat- terns in municipal wastewater, providing early insights into potential threats from waterborne pathogens, specifically *L. pneumophila* and *Aeromonas* spp. Although preliminary, these findings are essential for guiding occupational, public health, and environmental monitoring efforts, highlighting the increasing risks posed by AR. Additionally, this study integrates the One Health approach, underscoring the importance of managing AR in the context of climate change, which is accelerating the spread of these pathogens across sectors.

Conclusion

This study characterised MAGs of *L. pneumophila* and *Aeromonas* spp. from municipal wastewater in South Africa. *Legionella pneumophila* MAGs harboured several nonsynonymous genetic variations in the genes *rpoB*, *lpeB*, *rplD*, *gyrA*, *gyrB*, *parC*, and *parE*, which are implicated in LD antibiotic resistance. Future phenotypic testing studies are needed to determine the clinical implications of these mutations in AR. Additionally, this study demonstrated the role of municipal wastewater as

a reservoir of *Aeromonas* spp. carrying multiple functional ARGs, including plasmid-mediated β-lactamase genes that can be easily disseminated among different species. Therefore, implementing large-scale surveillance programs is crucial to uncover AR strains and markers circulating in municipal wastewater, understand factors driving the spread of AR, and enable early detection and mitigation of the potential transmission of *L. pneumophila* and *Aeromonas* spp. from environmental sources to clinical settings and communities. In summary, our findings pave the way in elucidating how clinically relevant pathogenic bacteria such as *L. pneumophila* and *Aeromonas* spp. occurring in engineered water systems such as municipal wastewater can develop AR.

Supplementary Information

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Supplementary Material 1

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Author contributions
EP: Conception and design of study, Acquisition of data, Analysis of data, Writing -original draft, and Writing -review and editing. RP: Analysis of data, and Writing -review and editing. TS: Conception and design of study, Resources, Supervision, and Writing -review and editing. AG: Conception and design of study, Funding acquisition, Methodology, Resources, Supervision, and Writing -review and editing. All authors read and approved the final manuscript.

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Data availability
The datasets generated and analysed during the current study are available in the NCBI BioProject repository (BioProject ID: PRJNA1182933), https://www.ncbi.nlm.nih.gov/ PRJNA1182933.

Declarations

Ethical approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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CHAPTER 7: CHRONIC RESPIRATORY AND GASTROINTESTINAL HEALTH COMPLAINTS POTENTIALLY ASSOCIATED WITH MICROBIAL EXPOSURE IN MUNICIPAL WASTEWATER TREATMENT PLANT WORKERS, SOUTH AFRICAN

Abstract

Workers at wastewater treatment plants (WWTPs) are continuously exposed to potentially harmful microbial hazards present in wastewater. Despite this risk, limited research exists on the health implications associated with repeated exposure to wastewater containing potentially infectious microorganisms, particularly in low-income countries including South Africa. This study aimed to assess the prevalence of chronic respiratory and gastrointestinal health issues among South African WWTP workers. A cross-sectional study was conducted from October 2022 to April 2023, with 226 consenting participants, comprising 149 WWTP workers and 77 control group. A self-administered questionnaire was used to determine sociodemographic characteristics, chronic respiratory symptoms and illnesses, self-reported systemic and gastrointestinal symptoms, risk factors for infectious diseases, and use of personal protective equipment (PPE). The results showed that WWTP workers were mostly males (67.8%), aged 41 -50 (29.5%), had tertiary education (30.2%), and 2 -20 years of working experience (73.8%). Chronic respiratory symptoms such as wheezing (23.5%) and shortness of breath (7.4%) were commonly reported among WWTP workers, contrasting with lower percentages in the control group (16.9 and 0%, respectively). Systemic symptoms such as fatigue (35.6%) and headaches (35.6%) were common among WWTP workers, mirroring similar rates in the control group with 31.2% for fatigue and 40% for headache. Gastrointestinal complaints, including stomach pain (14.8%), bloating (12.8%), and heartburn (12.8%), were also prevalent in WWTP workers, but varied between the two groups. The control group reported bloating (19.5%), constipation (15.6%), heartburn (14.3%), and stomach pain (14.3%). Participants commonly used PPE such as safety boots, overalls and heavy-duty gloves; however, the majority reported no use of protective gear for the mouth, nose or eyes. The findings of this study highlight the urgent need for targeted policies and safety training programs to protect WWTP workers from occupational health risks associated with microbial exposure. Advocacy with governmental and industry stakeholders is essential to foster safer work environments for this crucial workforce.

Keywords: respiratory health, microbial hazards, exposure risks, sanitation workers, occupational health implications.

7.1 Introduction

Most countries are experiencing significant population growth, urbanisation, and economic development resulting in increased wastewater generation (WaterAid, 2019). Wastewater treatment plants (WWTPs) play an essential role in public health, removing contaminants from water sources from industrial plants, households, agriculture, and healthcare facilities, before it is disposed or recycled. The contaminants include organic matter, microbiological agents, heavy metals, and other pollutants (Chahal *et al*., 2016). Therefore, adequate treatment of wastewater in accordance with standards or guidelines is critical for the health of WWTP workers, nearby communities, and the environment (WaterAid, 2019).

However, this vital work comes with considerable occupational health risks, as WWTP workers encounter potentially hazardous biological agents daily (Spellman, 2013; Oza *et al*., 2022). Indeed, research has shown higher levels of oxidative stress and inflammatory markers among WWTP workers compared to the control group, which plays a key role in the onset of adverse health effects, especially impaired lung function (Shadab *et al*., 2014; Cyprowski *et al*., 2015; Rieger *et al*., 2018). Furthermore, individual risk factors such as underlying health conditions, poor hygiene, a lack of personal protective equipment (PPE), and living conditions associated with social vulnerability might further worsen their risk vulnerabilities (Poole and Basu, 2017; Wright *et al*., 2019; Leso *et al*., 2021). These health risks are often exacerbated by a lack of access to and training on PPE, education on occupational exposures or hazards (Wright *et al*., 2019). However, workers in low- or middle-income countries (LMICs) face serious occupational health and safety issues due to lack of adequate PPE, limited access to healthcare, and working in poorly performing facilities (WaterAid, 2019; Philippe *et al*., 2022). Therefore, workers in these regions are particularly vulnerable to work-related illnesses owing to a lack of policy frameworks or guidelines aimed at protecting WWTP workers.

Occupational health problems commonly reported among WWTP workers include respiratory illnesses (e.g., asthma, chronic obstructive pulmonary function, and reduced lung function), gastroenteritis, infectious illnesses (tuberculosis, hepatitis A, leptospirosis or tetanus), as well as nonspecific symptoms such as fatigue, and headache among others. Although less frequent, cancers and skin disorders have also been observed in this occupational group (Heldal *et al*., 2010; Van Hooste *et al*., 2010; Al-batanony and El-Shafie, 2011; Fahim and El-Prince, 2012; Toseva *et*

al., 2018; Chandra and Arora, 2019; Oza *et al*., 2022). The precise cause of these illnesses is still unknown. However, respiratory and gastrointestinal symptoms may be associated with prolonged or high level of exposure to infectious microorganisms and their components in the workplace (Heldal *et al*., 2010; Cyprowski *et al*., 2015; Niazi *et al*., 2015; Lu *et al*., 2020).

Work-related respiratory diseases have emerged as a serious public health concern, contributing to high rates of morbidity and mortality (GBD, 2020). According to the Global Burden of Disease research, occupational respiratory exposures are a significant cause of chronic work-related respiratory diseases. The study indicated about 500 000 deaths and an estimated burden of 13 million disability-adjusted life years from chronic respiratory disease induced by occupational respiratory hazards (GBD, 2020). A recent study conducted in India found a 22% incidence of newly diagnosed tuberculosis among WWTP workers. Additionally, majority of these workers (92%) were affected by at least one chronic respiratory illness including, asthma, chronic obstructive pulmonary disease (COPD), and asthma-COPD overlap syndrome (Chandra and Arora, 2019). These findings underscore the need for targeted screening and intervention strategies to address specifically respiratory health issues among WWTP workers as an occupational group. Furthermore, it has been shown that sludge handling can increase the risk of respiratory and systemic health issues in WWTP workers (Heldal *et al*., 2010; Amoah et al., 2022). Despite these consistent findings, disparities in research methodologies and regional contexts highlight the need for more comparative studies across diverse settings to better understand the full spectrum of health risks among WWTP workers globally. Though studies on WWTP workers' health risks exist in developed countries, research is scarce in LMICs, including South Africa (Chandra and Arora, 2019; Dannoun and Nouban, 2021; Huang *et al*., 2021; Oza *et al*., 2022). Therefore, the current study sought to fill this gap, examining chronic respiratory and gastrointestinal health issues in South African WWTP workers, while assessing the prevalence of non-communicable diseases (NCDs) and PPE compliance as additional risk factors.

7.2 Methods

7.2.1 Study setting and design

The study took place across 16 WWTPs in the City of Tshwane Metropolitan Municipality, Gauteng Province, South Africa, from October 2022 to April 2023. The cross-sectional design allowed for a snapshot of health outcomes among WWTP workers in comparison to a control group.

7.2.2 Study population

A non-probability, convenience sampling approach was used and included 149 WWTP workers and 77 control group members (office staff, security, and others with limited wastewater exposure). Workers who were present on the day of data collection and met eligibility criteria were invited to participate. The purpose of the study was explained to them in either English or Sepedi, an official South African language they understood. Workers at WWTPs were responsible for cleaning screens, operating equipment, unblocking and repairing sewage pumps, manual sorting of solids at the inlet works, desludging or sludge handling, and general maintenance of the treatment facility. Workers aged 18 years and above participated in the study and a written informed consent was obtained.

7.2.3 Data collection tool

A self-administered questionnaire was used to gather information on socio-demographic characteristics, chronic respiratory symptoms and illnesses (Supplementary S7.1), self-reported systemic and gastrointestinal symptoms, risk factors to infectious diseases (e.g., arthritis, cancer, diabetes, heart disease, hypertension, kidney disease, and lung disease), and PPE compliance (prepared in this study and piloted). The questions on respiratory health symptoms and illnesses were adapted from the American Thoracic Society Division of Lung Disease (ATS-DLD-78) questionnaire, which is recommended for use in epidemiological studies to assess the prevalence of chronic respiratory symptoms and illnesses in adults and children aged 13 and older. Table 7.1 presents clarification of respiratory health symptoms and illnesses. The questionnaire was pretested on nine WWTP workers before the commencement of the main study, and no major

changes were made based on the participant responses. Each questionnaire took an average of 20 to 30 minutes to complete.

Table 7.1: Clarification of chronic respiratory symptoms and illnesses.

Symptom or illness	Clarification
1. Persistent cough	I usually cough most days for three consecutive months or more during the year
2. Cough with phlegm	I usually cough up mucus most days for three consecutive months or more during the year
3. Mild wheeze	My chest makes whistling sound occasionally apart from colds
4. Severe wheeze	My chest makes whistling sound most days or nights
5. Shortness of breath	Was shortness of breath confirmed by a doctor?
6. Bronchitis	Was bronchitis confirmed by a doctor?
7. Pneumonia	Was pneumonia confirmed by a doctor?
8. Asthma	Was asthma confirmed by a doctor?

7.2.4 Statistical analysis

All data was captured in Research Electronic Data Capture (REDCap) (Vanderbilt University, United States). The data was checked to avoid missing values and discrepancies. The cleaned data was exported into STATA version 17 (StataCorp LLC, College Station, United States) for statistical analysis. Frequencies and percentages were calculated to summarise the data. The categorical variables were compared between WWTP workers and the control group using the chi-square test or Fisher's exact test, if the expected frequency value was below 5. A *p*-value less than 0.05 was considered as statistically significant.

7.2.5 Ethical clearance

This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Reference no: M210522), and permission to conduct the study at the WWTPs sites was granted by the Utility Services Department under the City of Tshwane Municipality.

7.3 Results

7.3.1 Sociodemographic characteristics

A total of 226 subjects participated in this study, comprising of 149 WWTP workers, and 77 control group. Table 7.2 shows the sociodemographic characteristics of both groups. Significant differences were observed in gender distribution between WWTP workers and the control group ($p < 0.001$). WWTP workers were predominantly male (67.8%), whereas the control group had a higher proportion of females (59.7%). No statistically significant differences were found in age distribution ($p = 0.130$); however, most participants in both groups were aged between 31-40 and 41-50 years.

Educational level differed significantly between WWTP workers and the control group ($p = 0.011$). Majority of WWTP workers (30.2%) had a tertiary qualification compared to 41.6% of the control group having completed a matric certificate. A significant difference was also observed in the distribution of working years ($p = 0.003$). A higher proportion of WWTP workers (36.9%) had been working for 2 -10 or 11 -20 years, whereas the control group (49.4%) had 2 -10 years of experience.

Regarding smoking habits, no significant difference was found in the two groups in terms of current smoking ($p = 0.434$). Majority of the respondents were nonsmokers, with 73.2% among WWTP workers and 77.9% in the control group. However, a significant difference was observed in past smoking history ($p = 0.017$), with 7.4% of WWTP workers reporting a history of smoking compared to 1.3% in the control group.

Table 7.2: Sociodemographic characteristics of municipal WWTP workers and control group in Gauteng Province, South Africa during October 2022 to April 2023.

Variables	**WWTP workers (n = 149)**		**Control group (n = 77)**		**All subjects (n = 226)**		***p*-value ^**
	(n)	**(%)**	**(n)**	**(%)**	**(n)**	**(%)**	
Gender							
Female	48	32.2	46	59.7	94	41.6	<0.001*
Male	101	67.8	31	40.3	132	58.4	

Age							
21 -30	21	14.1	4	5.2	25	11.1	0.130
31-40	42	28.2	26	33.8	68	30.1	
41-50	44	29.5	19	37.7	73	32.3	
51-64	42	28.2	18	23.4	60	26.6	
Ethnicity							
African	135	90.6	74	96.1	209	92.5	0.258
Mixed race	3	2.0	0	0.0	3	1.3	
White	11	7.4	3	4.0	14	6.2	
Level of education							
Primary school	19	12.8	4	5.2	23	10.2	0.011*
High school	38	25.5	30	40.0	68	30.1	
Matric	44	29.5	32	41.6	76	33.6	
Tertiary	45	30.2	10	13.0	55	24.3	
Don't know/don't want to answer	1	0.7	0	0.0	1	0.4	
Never went to school	2	1.3	1	1.3	3	1.3	
Smoking habits							
Current smoker	40	26.9	17	22.1	57	25.2	0.434
Non-smoker	109	73.2	60	77.9	169	74.8	
Past smoker	11	7.4	1	1.3	12	5.3	0.017*
Work contract							
Permanent	125	83.9	59	76.6	184	81.4	0.183
Fixed term	24	16.1	18	23.4	42	18.6	
Years of employment							
≤ 1 (all months)	22	14.8	22	28.6	44	19.5	0.003*
2 -10	55	36.9	38	49.4	93	41.1	
11 -20	55	36.9	13	16.9	68	30.1	
21 -30	10	6.7	2	2.6	12	5.3	
31 -43	7	4.7	2	2.6	9	4.0	

^ Chi square, * ($p < 0.05$)

7.3.2 Prevalence of chronic respiratory symptoms and illnesses

The prevalence of chronic respiratory symptoms and illnesses is presented in Figure 7.1. Wheezing (23.5%) was the most common chronic respiratory symptom experienced by WWTP workers, followed by shortness of breath (7.4%). Wheezing (16.9%), Bronchitis (2.6%), cough with phlegm (1.3%), and pneumonia (1.3%) were the only symptoms reported by the control group. Shortness of breath was found to be statistically significant between the groups studied (p = 0.014). When compared to the control group, the prevalence of chronic respiratory symptoms was generally higher in WWTP workers. There were similarities (no significant difference) in the prevalence of most chronic respiratory symptoms and illnesses between WWTP workers and the control group. However, WWTP workers showed a statistically significant higher prevalence of shortness of breath.

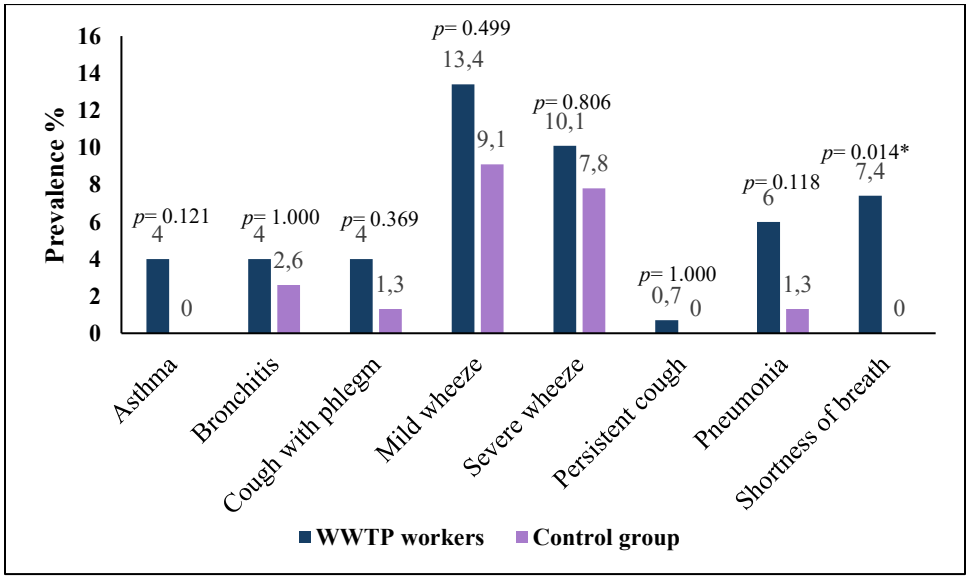

Figure 7.1: Prevalence of chronic respiratory symptoms and illnesses among WWTP workers and control group. * indicates $p < 0.05$.

7.3.3 Prevalence of self-reported systemic and gastrointestinal symptoms with recall period of two weeks

Self-reported systemic and gastrointestinal symptoms with a two-week recall period are shown in Figure 7.2. The most frequently reported gastrointestinal symptoms in both groups were stomach pain, bloating and heartburn. Among WWTP workers, stomach pain (14.8%), heartburn and bloating (12.8% each), and diarrhoea (11.4%) were commonly reported. In the control group, bloating, constipation, heartburn, and stomach pain were reported by 19.5, 15.6, 14.3 and 14.3% of the respondents, respectively.

In terms of systemic symptoms, majority of the WWTP workers reported headache, and fatigue, affecting about one-third (35.6% each) of the workers. Similarly, the control group also frequently reported headache (31.2%), and fatigue (40.0 %). Weight loss was the least commonly reported systemic symptom in both groups. Although most self-reported health complaints were statistically similar between WWTP workers and the control group, WWTP workers exhibited a significantly higher prevalence of shortness of breath ($p = 0.033$). Workers at WWTPs (12.8%) reported to have experienced shortness of breath in the past two weeks compared to the control group (4%).

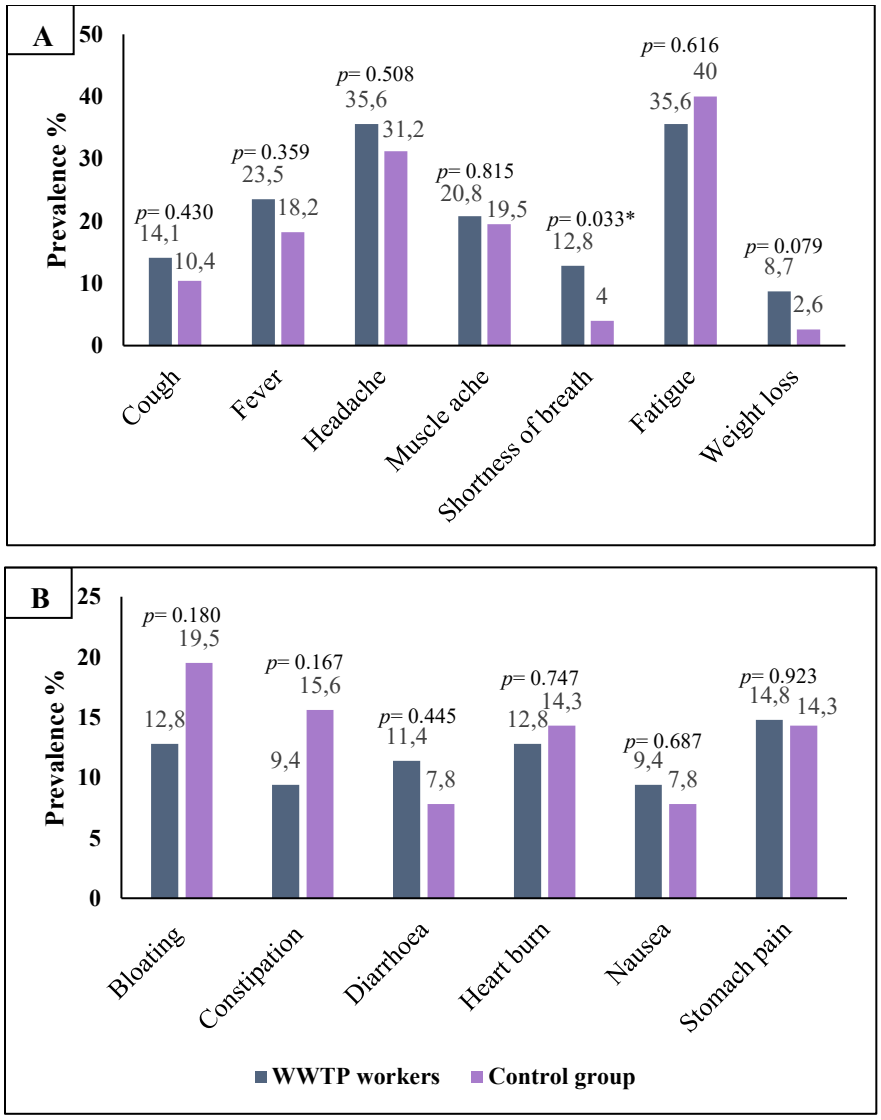

Figure 7.2: Prevalence of self-reported systemic (A) and gastrointestinal (B) symptoms in the past two weeks among WWTP workers and control group. * indicates $p < 0.05$.

7.3.4 Prevalence of non-communicable diseases

Figure 7.3 indicates the prevalence of chronic illnesses that could predispose workers to infectious diseases. Approximately half of the WWTP workers (54.4%) and one-third of the control group (31.2%) had chronic health conditions. Hypertension was the most prevalent chronic medical condition, accounting for 24.2 and 15.6% in WWTP workers and control group, respectively. Among WWTP workers, hypertension was followed by diabetes (8.1%), arthritis (7.4%) and others (6.7%). The control group reported four medical conditions: arthritis, diabetes,

hypertension, and others, with prevalence rates of 2.6, 5.2, 15.6 and 7.8%, respectively. However, there was no statistically significant difference in the occurrence of chronic medical conditions between the studied groups. Nonetheless, the slightly higher prevalence of certain morbidities among WWTP workers, particularly hypertension and diabetes, warrants further investigation to understand the specific occupational and environmental factors contributing to these health outcomes; as these NCDs, often exacerbated by occupational stressors, increase susceptibility to infectious diseases and may complicate health outcomes for WWTP workers.

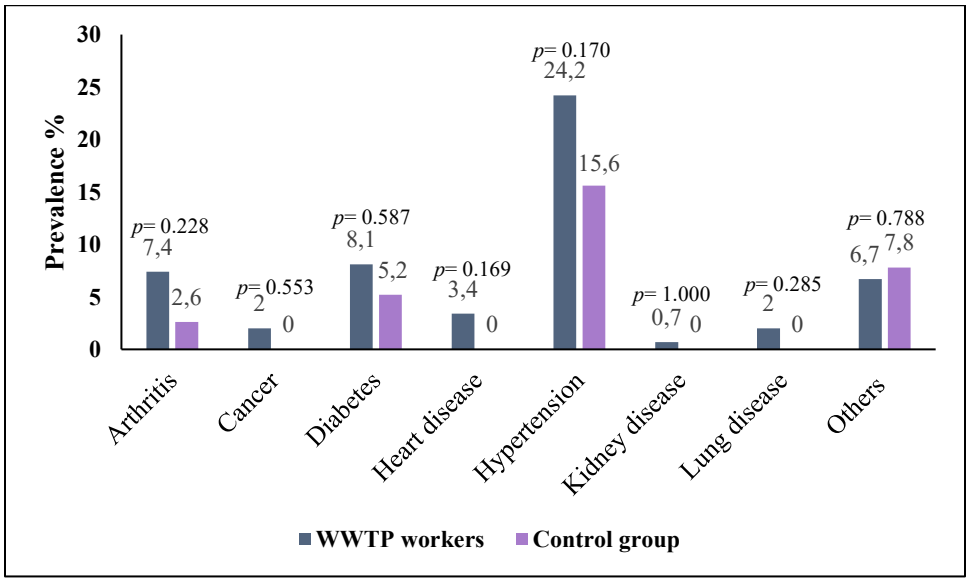

Figure 7.3: Prevalence of chronic illnesses among WWTP workers and control group.

7.3.5 Personal protective equipment (PPE) compliance and perceived knowledge on microbial occupational exposures

The participant's compliance to PPE use and knowledge of occupational exposures is presented in Table 7.3. Commonly used PPE among WWTP workers were safety boots (91.3%), overalls (88.6%), and heavy-duty gloves (50.3%). In comparison, the control group primarily reported the use of safety boots (75.3%), surgical gloves (59.7%), overalls (54.6%), and surgical masks (42.9%). Both groups reported no usage of PPE specifically for eye protection (WWTP workers: 72.5%, control group: 63.6%).

WWTP workers (50.4%) indicated having received no training on the safe use of PPE to protect against infectious agents, while the control group (66.2%) had received training. Despite this, a majority in both groups (WWTP workers: 76.3%, control group: 85.3%) expressed the belief that PPE training and education would be beneficial. Additionally, both groups acknowledged the importance of nose protection in their workplace (WWTP workers: 76.3%, control group: 67.7 %).

The majority in both groups reported being confident in using PPE to protect themselves (WWTP workers: 60.4%, control group: 72.1%). However. some individuals in both groups expressed lower confidence levels or uncertainty about using PPE effectively. While safety boots, overalls, and heavy-duty gloves were widely used, the low use of respiratory and eye protection highlights a critical gap in safety measures. Areas for improvement include increasing the use of specific PPE such as face masks, and eye shields, and enhancing confidence levels in the correct PPE usage through additional training and education. The data underscores the need for targeted PPE training to protect WWTP workers from microbial exposure, particularly in the context of limited resources in LMICs.

Table 7.3: Personal protective equipment compliance and knowledge of microbial exposures in WWTP workers and control group in Gauteng Province, South Africa during October 2022 to April 2023.

Variables	WWTP workers (n = 149)		Control group (n = 77)	
	Frequency (n)	Percent (%)	Frequency (n)	Percent (%)
Eye protection				
Face shield	12	8.1	9	11.7
Safety googles	31	20.8	18	23.4
None	108	72.5	49	63.6
Nose and mouth protection				
Cloth mask	19	12.8	15	19.5
Respirator e.g. N95, FFP2	25	16.8	11	14.3
Surgical mask	8	5.4	33	42.9

None	95	63.8	33	42.9
Hand protection				
Heavy duty gloves	75	50.3	19	24.7
Surgical gloves	36	24.2	46	59.7
None	25	16.8	23	29.91
Body protection				
Disposal apron	2	1.3	10	13.0
Long sleeves	14	9.4	7	9.1
Overalls	132	88.6	42	54.6
Personal clothing	25	16.8	16	20.8
Foot protection				
Closed shoes	19	12.8	20	26.0
Rubber boots	36	24.2	10	13.0
Safety boots	136	91.3	58	75.3
Training on the use of PPE to protect myself from infectious agents/germs				
Yes	69	49.6	45	66.2
No	70	50.4	23	33.8
I will benefit from PPE training and education				
Yes	106	76.3	58	85.3
No	12	8.6	5	7.4
Maybe	21	15.1	5	7.4
I believe I need nose protection daily when at work				
Yes	106	76.3	46	67.7
No	33	23.7	22	32.4
I know how to safely use PPE to protect myself				
A little confident	24	17.3	10	14.7
Confident	84	60.4	49	72.1
Not confident at all	6	4.3	7	10.3
Somewhat confident	25	18.0	2	2.9

7.4 Discussion

This study investigated the prevalence of chronic respiratory illnesses and gastrointestinal symptoms associated with exposure to wastewater containing potentially infectious microorganisms among WWTP workers in Gauteng Province, South Africa. The findings provide a snapshot of the health status, particularly respiratory health, among WWTP workers and highlight several significant observations. The males were predominant in this study among WWTP workers, which is consistent with the findings of previous studies (Toseva *et al*., 2018; Wright *et al*., 2019). Male dominance among WWTP workers is expected considering that typical work tasks in this sector are often physically demanding; therefore, the workforce continues to be dominated by male workers in most countries (Wright *et al*., 2019).

In this study, WWTP workers reported a higher prevalence of chronic respiratory symptoms (e.g., cough, wheeze, and shortness of breath) and illnesses (e.g., asthma, bronchitis, and pneumonia) than the control group. A statistically significant difference was observed in the prevalence of shortness of breath between the two groups. However, the lack of significant differences in other symptoms could be because of the small sample size in this study. Previous studies conducted in Italy, Iran, Greece, Germany, and Switzerland have reported similar respiratory symptoms and illnesses among this occupational group (Tschopp *et al*., 2011; Vimercati *et al*., 2016; Van Kampen *et al*., 2016; Aghaei *et al*., 2020). Occupational exposure to bioaerosols containing pathogenic microorganisms, bacterial endotoxins, allergens, inhalable dust, and other toxic substances has been suggested to play a role in the onset of respiratory symptoms and illnesses among WWTP workers (Austigard *et al*., 2018; Heldal *et al*., 2019; Muzaini *et al*., 2021; Lu *et al*., 2020). Additionally, endotoxin exposure has been linked to considerably reduced lung function among WWTP workers (Cyprowski *et al*., 2015; Heldal *et al*., 2019). Other potential contributing factors that should be considered include obesity and NCDs such as cardiovascular diseases, diabetes and COPD, all of which are commonly associated with shortness of breath (Gol *et al*., 2021; Guo *et al*., 2023). It has been suggested in the literature that exposure to hazardous gases, specifically hydrogen sulphide, may be the cause of respiratory among WWTP workers (Austigard *et al*., 2018; Heldal *et al*., 2019; Muzaini *et al*., 2021). Therefore, higher prevalence of respiratory distress such as wheezing and shortness of breath among WWTP workers may be attributed to exposure to incidental hydrogen sulfide in the workplace (Austigard *et al*., 2018). The higher prevalence of chronic respiratory symptoms in this study suggests increased vulnerability to

respiratory illnesses among WWTP workers compared to the control group. Therefore, it is crucial for WWTPs to implement preventive measures, such as wearing masks, to mitigate adverse effects on respiratory health among workers.

Predominant self-reported systemic health complaints in this study were headache and fatigue, which is agreement with other studies (Joy and Chitra, 2018; Aghaei *et al*., 2020; Muzaini *et al*., 2021). This could be attributed to the physical demands of WWTP work, which often involve carrying, lifting, sorting, and loading of waste and equipment. Additionally, longer working hours and exposure to hazardous materials such including bacteria may contribute to the fatigue and other systemic symptoms commonly reported among WWTP workers (Muzaini *et al*., 2021; Tshivhase *et al*., 2022). Commonly reported gastrointestinal symptoms in this study were stomach pain, bloating, and heartburn, which is in agreement with previous studies among WWTP workers (Oza *et al*., 2022; Tolera *et al*., 2023). It should be noted that WWTP workers reported a relatively lower prevalence, with no more than 15% in all reported gastrointestinal symptoms. The lower prevalence may be due to acquired immunity to specific pathogens over time. Therefore, it is plausible that the study population developed tolerance or immunity to enteric pathogens as a result of exposure to non-harmful levels of pathogens. However, new employees, immunocompromised workers, and those with minimal exposure to wastewater, may exhibit gastrointestinal symptoms more frequently (Amadou Amaniband Lang, 2020).

It was observed that WWTP workers were more predisposed to developing chronic illnesses as compared to the control group. Hypertension and diabetes topped the list of NCDs reported among WWTP workers in the current study. These results differ by three to four magnitudes (hypertension 67% and 43% diabetes) from what was previously reported in a similar study (Chandra and Arora, 2019). Non-communicable diseases among WWTP workers increase their risk and susceptibility to various infectious diseases. This concern was highlighted during the COVID-19 pandemic, where individuals with underlying health conditions faced greater health risks (Leso *et al*., 2021). Although NCDs may not directly arise from occupational exposure, they impact workplace health by increasing sick leaves, reducing productivity, and disrupting operational efficiency (Tolera *et al*., 2023). Furthermore, the high prevalence of NCDs, particularly hypertension and diabetes among WWTP workers, suggests that limited healthcare access may exacerbate health risks. Implementing targeted wellness program to manage these NCDs is crucial to mitigate absenteeism

and support overall worker's health. Studies have shown that untreated NCDs morbidities contribute to higher rates of absenteeism and increase health risks associated with occupational exposures (Gradidge *et al*., 2022; Tolera *et al*., 2023).

The study highlighted substantial gaps in PPE usage among WWTP workers. While safety boots, overalls, and heavy-duty gloves were frequently used, nose and mouth protection were notably underused, despite the high recognition of its necessity in workplaces where workers may be exposed to potentially inhalable infectious organisms (LeChevallier *et al*., 2020). The WHO recommends adequate health and safety measures for sanitation workers, including WWTP workers (WHO, 2018). However, lack of access and training in PPE use remains a challenge, particularly in developing countries such as South Africa (WaterAid, 2020; Philippe *et al*., 2022). While WWTP workers recognise the importance of PPE, inadequate training and limited resources may hinder effective use, placing workers at increased risk.

In summary, these findings indicate no significant differences in the prevalence rates of chronic respiratory symptoms, self-reported systemic and gastrointestinal symptoms, and NCDs between WWTP workers and the control group. It is worth noting that the low prevalence, together with zero reported symptoms in most cases in the control group (e.g., shortness of breath, cough, asthma, cancer, kidney disease, heart disease, and lung disease), hindered further statistical analysis to identify factors associated with specific symptoms studied. Therefore, it was not possible to statistically demonstrate whether WWTP workers are at an increased risk of respiratory symptoms or not in this study population. Further studies conducted over a longer period and with larger sample sizes would provide comprehensive understanding of the respiratory and gastrointestinal health statuses of WWTP workers, the long-term effects of wastewater exposure, and potential correlations between occupational exposures and specific health conditions.

This study provided a snapshot profile of respiratory and gastrointestinal health statuses among WWTP workers in South Africa. A validated questionnaire of the American Thoracic Society was used to assess the prevalence of respiratory symptoms and illnesses. However, there are some limitations inherent to cross-sectional studies. The findings of this study cannot be generalised to all 850 WWTPs in the country, as data were collected from sixteen WWTPs (DWS, 2023). Furthermore, it was not possible to provide correlation evidence because of low prevalence rates reported. The study relied on self-reported symptoms and doctor's diagnosis for chronic

respiratory symptoms and illnesses, which may have led to an underestimation of prevalence of these symptoms. Some workers could exhibit recall bias and be hesitant to seek medical attention (Walters *et al*., 2015; Fuhrimann *et al*., 2016). Additionally, workers may fear repercussions from management, leading them to provide socially desirable responses rather than providing truthful answers on the questionnaires. Endotoxin exposure have been linked to respiratory diseases in this occupational group (Farokhi *et al*., 2018; Muzaini *et al*., 2021). However, this study did not quantify airborne endotoxins or assess personal exposure levels. Data generally linked to NCDs such as obesity, lifestyle and diet was not collected. Lastly, the healthy worker effect may also influence results, as WWTP workers in this region typically perform manual labour and those who developed chronic respiratory issues may have quit their jobs.

7.5 Conclusion

This study found respiratory and gastrointestinal health issues as prevalent among WWTP workers, with key symptoms such as wheezing, shortness of breath, stomach pain, bloating and heartburn commonly reported. Furthermore, the majority of WWTP workers reported recent fatigue, headaches, and gastrointestinal symptoms, such as stomach pain, bloating and heartburn. Although most WWTP workers complied with PPE use, compliance was notably lower for mouth, nose and eye protection. This inadequate compliance with essential protective gear, such as masks, face shields, and disposable or reusable hand gloves, increases the risk of exposure to and transmission of infectious microorganisms. To mitigate health risks associated with microbial exposure, WWTP workers should strictly adhere to PPE protocols. Engineering controls such as proper ventilation systems and automated processes, should also be considered to minimise exposure risks. Administrative measures such as targeted safety training and risk assessments are important for ensuring compliance and enhancing workers' awareness of occupational biological hazards. Overall, the findings of this study can serve as a basis for advocacy to government and relevant stakeholders, urging the development of policies and training initiatives that addresses occupational risks associated with exposure to infectious microorganisms in WWTP workers. Such initiatives should include regular risk assessments, environmental monitoring of infectious microorganisms, and annual medical check-ups. Through increased awareness and training, the health and well-being of this essential workforce can be safeguarded, contributing to a more resilient public health infrastructure.

7.6 References

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CHAPTER 8: INFECTION RISK OF *LEGIONELLA PNEUMOPHILA* AND *AEROMONAS HYDROPHILA* FROM OCCUPATIONAL EXPOSURE TO UNTREATED MUNICIPAL WASTEWATER

Abstract

There is a growing concern about the health risks faced by wastewater treatment plant (WWTP) workers due to repeated occupational exposure to opportunistic pathogens in wastewater. This study applied Quantitative Microbial Risk Assessment (QMRA) to estimate infection risks for *L. pneumophila* and *A. hydrophila* via inhalation of bioaerosols and accidental ingestion of wastewater, respectively. Pathogen concentrations were determined at different stages of wastewater treatment where worker exposure is likely, including the bar screen, aeration tank, and secondary settling tank (SST). The duration of exposure ranged from 0.5 to 8-hrs per shift and the results were compared against the USEPA acceptable annual risk of infection threshold (≤1E-04 per person per year). The results indicated that the probability of *L. pneumophila* infection during a 0.5-hr exposure ranged from 1E-06 to 1E-05 at the bar screen and 1E-05 to 1E-04 at the aeration tank. Notably, the annual infection risk for *L. pneumophila* exceeded the benchmark threshold (1E-04 pppy), particularly during exposure times of 2-hrs or more at both the bar screens and aeration tanks across the five WWTPs. In contrast, *A. hydrophila* posed increased infection risks (>1E-04 pppy) when workers were exposed to influent near the bar screen. However, the infection risk remained minimal and consistently below the annual threshold at subsequent workstations despite extended exposure times. These findings suggest that prolonged occupational exposure to untreated municipal wastewater, particularly at the bar screen and aeration tank, may increase the risk of *L. pneumophila* and *A. hydrophila* infections among WWTP workers.

Keywords: Inhaled bioaerosols, occupational exposure, bacterial infections, health risks, QMRA

8.1 Introduction

Wastewater treatment plants (WWTPs) protect the general public from infectious diseases and epidemics by reducing microbiological contaminants and nutrients in final effluent before discharge into the environment (Gerba and Pepper, 2019). However, WWTP workers are at risk of exposure and contact with contaminants such as waterborne pathogens found in wastewater when manually performing regular tasks (Fuhrimann *et al*., 2017; Lu *et al*., 2020). Additionally, treatment processes, specifically biological treatment processes occurring in the aeration tanks generate copious amounts of aerosols harbouring a high bacterial load (Han *et al*., 2020; Li *et al*., 2022), posing the risk of pathogen inhalation for WWTP workers. Previous studies among WWTP workers have indicated high prevalence of respiratory and gastrointestinal symptoms including allergic alveolitis, dizziness, weakness, headache, and bronchitis, sometimes referred to as "sewage worker's syndrome" in this occupational group (Heldal *et al*., 2010; Han *et al*., 2020; Oza *et al*., 2022). These symptoms are likely attributed to occupational exposure to health hazards, such as pathogenic bacteria and endotoxins emitted during wastewater treatment processes (Heldal *et al*., 2010; Lu *et al*., 2020). Human pathogens, particularly *Legionella pneumophila* (Caicedo *et al*., 2016; Huang *et al*., 2021; Bonetta *et al*., 2022), and *Aeromonas hydrophila* (Al-Jassim *et al*., 2015; Popovic *et al*., 2015; Cui and Liang, 2019) have been identified in wastewater, implicating a potential risk of infection for the exposed workers at WWTPs.

Qualitative microbial risk assessment (QMRA) serves as a valuable tool for probabilistically estimating health risks associated with specific pathogenic microorganisms. It involves the assessment of pathogen concentrations and human exposure scenarios related to a specific activity or environment (Hamilton and Haas, 2016). The QMRA framework comprises four fundamental steps: hazard identification, exposure assessment, dose-response assessment, and risk assessment (Haas *et al*., 2014). Two widely recognised benchmarks for interpreting risk levels are established by the United States Environmental Protection Agency (USEPA) and World Health Organization (WHO). The USEPA set the acceptable annual infection risk (≤1E-04 per person per year (pppy)), while the WHO defines disease burden (≤1E-06 disability-adjusted life years (DALYs) pppy) for waterborne pathogens (Fuhrimann *et al*., 2017). Research interest in infection risks among WWTP has increased since the Covid-19 pandemic (Haas *et al*., 2017; Fuhrimann *et al*., 2017; Deepnarain *et al*., 2020; Chen *et al*., 2021; Dada and Gyawali, 2021; Yan *et al*., 2021; Amoah *et al*., 2022; Gui *et al*., 2022; Morales *et al*., 2023). Studies in China assessed health risks associated with

occupational exposure to aerosols containing *Staphylococcus aureus* and pathogenic gram-negative bacteria among WWTP workers. The results revealed that WWTP workers lacking personal protective equipment (PPE) such as face mask or N95 respirators, often exceeded the benchmark threshold set by the USEPA for *S. aureus* and gram-negative bacteria (Chen *et al*., 2021; Yan *et al*., 2021). A similar study investigating infection risks associated with accidentally ingestion of enteric bacteria (*Escherichia coli*, feacal coliforms and *Enterococcus* spp.) in aerosols found that WWTP workers responsible for maintaining aeration tanks faced a 1.4 times higher annual infection risk compared to workers sampling wastewater at the same site once a week, due to increased exposure frequency (Gui *et al*., 2022). Furthermore, studies among WWTP workers and farm workers irrigating with wastewater have reported a high incidence of gastrointestinal infections caused by parasites (*Giardia* spp., and *Cryptosporidium* spp.,), viruses (rotavirus, norovirus and enterovirus), and bacteria (*E. coli*, and *Campylobacter* spp.,), exceeding WHO threshold (Fuhrimann *et al*., 2017; Morales *et al*., 2023). It is important to highlight that existing QMRA studies among WWTP workers have predominantly focused on the risk aerosol particles entering the mouth resulting in gastrointestinal infections due to gram-negative bacteria and viruses. Therefore, there is a need to expand risk assessments for WWTP workers to include emerging waterborne pathogens.

The genus *Aeromonas*, a gram-negative opportunistic pathogen, is associated with gastrointestinal illnesses, bacteremia, as well as skin and wound infections (Fernández-Bravo and Figueras, 2020). Importantly, *Aeromonas* species are considered emerging pathogens and typically resistant to conventional wastewater treatment processes, posing a significant public health concern. *Aeromonas hydrophila* is frequently detected in diverse aquatic environments such as freshwater, drinking water, and municipal wastewater (Fernández-Bravo and Figueras, 2020). Research estimating the risk of *A. hydrophila* infections is limited, with only one study conducted in Saudi Arabia among farm workers to date (Al-Jassim *et al*., 2015). The study assessed infection risks associated with the reuse of wastewater including influent, effluent and chlorinated effluent for agricultural irrigation purposes. The findings indicated that only chlorinated effluent could be deemed safe for reuse, as it maintained an acceptable annual risk level (≤1E-04) for *A. hydrophila* infection (Al-Jassim *et al*., 2015). This raises concerns for WWTP workers, who are routinely exposed to raw and unchlorinated wastewater in the workplace. Therefore, further research is critical to better understand the prevalence, and infection risks of *A. hydrophila* particularly in

occupational settings such as WWTPs. Such efforts are crucial for informing the development of targeted interventions to mitigate exposure risks among WWTP workers and safeguard public health.

Legionella is a gram-negative waterborne opportunistic pathogen that causes a severe atypical pneumonia known as Legionnaires 'disease (LD) and a milder flu-like infection termed Pontiac Fever (Toplitsch *et al*., 2018). Currently, over 60 species of *Legionella* have been identified with *Legionella pneumophila* serogroup 1 accounting for more than 90% of the infections in LD patients (Gomez-Valero *al*., 2014; Toplitsch *et al*., 2018). The primary mode of transmission for *Legionella* infections in humans is through the inhalation of contaminated water aerosols. Legionnaires 'disease outbreaks linked to exposure to contaminated aerosols from man-made water sources such as cooling towers (George *et al*., 2016; Weiss *et al*., 2017), fountains (Haupt *et al*., 2012; Smith *et al*., 2015), and WWTPs (Olsen *et al.,* 2010; Maisa *et al.,* 2015; Loenenbach *et al.,* 2018) are well-documented. Specifically, several outbreaks of LD in Germany (Maisa *et al.,* 2015), Netherlands (Loenenbach *et al.,* 2018; Vermeulen *et al.,* 2021; Pijnacker *et al.,* 2024), and Norway (Olsen *et al.,* 2010) have been directly linked to WWTPs as the source of infection. Wastewater treatment plants provide optimal conditions, including appropriate temperatures and nutrients, for the proliferation of *Legionella* species (Caicedo *et al.,* 2019). Furthermore, aeration tanks in WWTPs generates significant bioaerosol, which may contain *Legionella*, contributing to the spread of this pathogen to the workers or the surrounding community (Caicedo *et al.,* 2019). Despite this, *Legionella* risk assessment remains a burgeoning field with limited studies investigating the risk of infection resulting from inhaling *Legionella*-contaminated aerosols. Most of these studies focused on drinking water distribution systems and reclaimed wastewater, driven by the global challenge of water scarcity (Blanky *et al*., 2017; Hamilton *et al*., 2018; Sharaby *et al*., 2019; Chen *et al*., 2022; Federigi *et al*., 2022; Kermani *et al*., 2022). Furthermore, these studies mainly addressed exposure scenarios such as toilet flushing, spray irrigation, and showers, with results generally indicating that the estimated annual risk of *Legionella* infection exceeds acceptable thresholds. Therefore, WWTP workers exposed to untreated wastewater, may face an even higher risk of infection. Globally, cases of LD have been increasing, a trend attributed to improved reporting practices, advanced diagnostic tools, and aging individuals, thereby increasing immunocompromised population (Moffa *et al*., 2023). Similarly, in South Africa, LD is a notifiable medical condition and reported cases have been increasing in the past decade (Wolter *et al*., 2016;

Wolter *et al*., 2020). There exists a scientific imperative to explore the potential infection risks associated with repeated exposure to untreated wastewater. Hence, the present study aimed to estimate the risk of infection associated with occupational exposure to *L. pneumophila* and *A. hydrophila* at different stages of the treatment process in municipal WWTP workers.

8.2 Methods

8.2.1 Hazard identification

Workers at WWTPs are exposed to health risks such as inhaling contaminated aerosols and/or accidental ingestion of wastewater via hand-to mouth or aerosol particles entering the mouth (Chen *et al*., 2021; Wang *et al*., 2021; Amoah *et al*., 2022). In this study, reference opportunistic waterborne pathogens of concern were *L. pneumophila* and *A. hydrophila* as they can cause atypical pneumonia and gastrointestinal illnesses, respectively. The characteristics of the sites and quantities (geometric mean) of the reference pathogens were previously detailed by Poopedi et al., (2025) in Chapter 4. *Legionella pneumophila* was determined using culture method, while *A. hydrophila* was quantified using quantitative real-time PCR (qPCR).

8.2.2 Exposure assessment

The treatment stages identified as having a high risk of worker exposure to the selected reference pathogens are outlined below. The exposure times were informed by literature findings (Yan *et al*., 2021; Amoah *et al*., 2022), which indicated that WWTP workers typically spend approximately 30 minutes to an hour collecting samples and inspecting aeration tanks during their shifts. It is important to note that in South Africa, WWTP workers are subjected to longer working hours (8-hr shift per day), and most of WWTPs often encounter operational challenges such as cable theft and load shedding. Therefore, in the present study, it was assumed that WWTP workers could be exposed to bioaerosols and wastewater for 0.5 hr (best-case scenario) and up to 8-hrs (worst-case scenario on days when the plant is malfunctioning) per working shift. This assumption translates to an annual exposure time ranging from 5 days (0.8 weeks per year) to a maximum of 87 days (12 weeks per year) for the best and worst-case scenarios, respectively. Below are key treatment stages and WWTP worker tasks:

a) Bar screen: This process is intended to remove large solid objects such as plastics, napkins, and paper from incoming influent to prevent damage to the facility's pumps and other equipment. Routine work tasks performed at this stage include daily inspection and monitoring of the inlet station, reading flood levels, high-pressure cleaning, as well as collecting and disposing of solid waste in designated bins. In addition, on workdays when the bar screen is malfunctioning due to several reasons such as cable theft, planned power outage, and flooding after heavy rain, workers are required to manually screen solids.
b) Aeration tank: This process employs microorganisms to degrade organic material present in the wastewater. Mechanical surface and submerged aerator systems are commonly used to aerate activated sludge in the aeration tanks, leading to substantial emission of aerosols due to vigorous mixing and turbulence. Work tasks conducted include daily inspection and monitoring of aeration tanks, lubrication of aerators, collection of wastewater samples, housekeeping, repairing pumps, and general maintenance.
c) Secondary settling tank: This process separates/clarifies microbial biomass and other pollutants from the treated wastewater. Work tasks include daily inspection and monitoring, channel cleaning using brooms, fixing broken equipment, and lubrication of the gearbox.

8.2.2.1 *Legionella pneumophila* exposure scenarios

a. Inhalation of bioaerosols generated by bar screens.

b. Inhalation of bioaerosols generated by aeration tanks.

8.2.2.2 *Legionella pneumophila* in the air using the partitioning coefficient approach

The concentration of *L. pneumophila* in the air (C_{air}) was determined using an emission factor (EF). The emission factor (EF) is the ratio between *L. pneumophila* air concentration and wastewater concentrations. As shown in Table 8.1, Medema et al. (2004) previously provided a partitioning coefficient value of aerosolised *Legionella* at WWTPs. Equation 1 (Kermani *et al*., 2022) was used to estimate *L. pneumophila* in the air based on the detected concentrations in wastewater samples (Armstrong and Haas, 2007).

$$C_{air} = C_{water} x\ EF \quad (1)$$

where:

C_{air} is *L. pneumophila* concentration in the air (most probable number (MPN)/m^3);
C_{water} is the *L. pneumophila* concentration in wastewater samples (MPN/L); and
EF is an emission factor (L/m^3).

Table 8.1: Input parameters used in qualitative microbial risk assessment model.

Parameter	Description	Value	Unit	Reference
EF	Emission factor	5.6E-06	Litre/m^3	Medema *et al*., 2004
ED	Daily exposure time	0.5, 2.0, 4.0, 6.0 and 8.0	Hr/day	This study
RR	Retention rate	0.5	-	Baskerville, 1981
IR	Inhalation rate	1	m^3/hr	USEPA, 2011

8.2.2.3 *Legionella pneumophila* inhalation exposure-dose assessment

Daily inhaled exposure dose was estimated based on *L. pneumophila* concentration in the air, exposure duration, retention rate of aerosols in the lungs, and inhalation rate according to Equation 2 (Kermani *et al*., 2022).

$$IED = C_{air} \, x \, ED \, x \, RR \, x \, IR \quad (2)$$

Where:

IED represents the daily inhaled exposure dose (MPN/day);
ED is a daily exposure dose (hrs);
RR is the retention rate of aerosols in the lungs; and
IR is the inhalation rate (m^3/hr)

8.2.2.4 *Aeromonas hydrophila* exposure scenarios

a) The risk of *A. hydrophila* infection due to accidental ingestion of wastewater, which can occur through splashing during work tasks, and talking or walking around the treatment plant, was estimated. An ingestion volume of 1 mL per 8-hr working shift was assumed

based on previously reported accidentally ingested volume of wastewater by workers at WWTPs (Sunger and Haas, 2015).

8.2.3 Dose-response assessment

Dose-response models are a set of mathematical functions that estimate the probability of adverse health outcomes following exposure to an infectious agent. The exponential dose-response model (Equation 3) was used to calculate the risk of infection associated with the presence of *L. pneumophila* (inhalation exposure) and *A. hydrophila* (ingestion exposure) in municipal wastewater.

$$P_{inf} = 1 - exp\ (-k \times dose) \quad (3)$$

Where P_{inf} refers to the probability of infection per daily exposure;

k is the probability of the pathogen surviving host defence and initiating the infection, 5.99E-02 and 3.90E-02 were used to calculate LD and Pontiac fever infection risk, respectively (QMRA wiki), and 1.39E-08 for *A. hydrophila* infection risk (Chakraborty *et al*., 1987). Dose is the number of inhaled or ingested target pathogen determined by the exposure assessment.

8.2.4 Risk assessment

Risk characterisation was determined using information provided from the previous steps of the risk assessment (hazard identification, exposure assessment, and dose-response assessment). Annual risk of infection was estimated using the formulae in Equation 4 (Haas *et al*., 2014; Sales-Ortells and Medema, 2014). This was expressed as the annual risk infection and were interpreted according to the United States Environmental Protection Agency (USEPA).

$$P_{ann} = 1-(1-P_{inf})^{n} \quad (4)$$

where P_{ann} is the annual probability of infection, and n is the frequency of exposure per year (number of working weeks according to exposure time).

8.3 Results

The study estimated the probability of infection per single work shift and annual infection risk among WWTP workers associated with two main exposure scenarios: inhalation of aerosols and accidental ingestion of wastewater. This assessment focused on occupational exposure to untreated wastewater containing opportunistic waterborne pathogens, *L. pneumophila* and *A. hydrophila* at different treatment stages. The annual risk of infection posed by the reference pathogens in WWTP workers while performing work tasks or in contact with untreated wastewater was compared to a hypothetical benchmark threshold of 1E-04 pppy recommended by the USEPA for waterborne pathogens (Fuhrimann *et al*., 2017).

8.3.1 Infection risk posed by inhalation of *L. pneumophila* in the best-case scenario.

The risk of LD infection from inhaling aerosols contaminated with *L. pneumophila* at the bar screens and aeration tanks at the five WWTPs is presented in Table 8.2. Secondary settling tanks were not included in the exposure assumptions due to the absence of aerosolisation or splashing at these tanks. For a single 0.5-hr exposure event at the bar screen, the median risk of LD infection ranged from 1E-06 to 1E-05. Workers at WWTP4 faced a higher likelihood of aerosol exposure and subsequent infection risk of 6.25E05 per work shift compared to workers at the other WWTPs, while workers at WWTP1 faced the lowest risk of 6.56E-06 per work shift. A similar trend was observed in the annual infection risk estimates, with the highest annual infection risk observed for WWTP4 (5E-05 pppy) and the lowest for WWTP1 workers (5.25E-06 pppy). These findings indicate that for a 0.5-hr exposure near the bar screens, the annual risk of LD infection remains below the USEPA's acceptable threshold of 1E-04 pppy across all WWTPs.

At the aeration tank, the median infection risk of LD per 0.5-hr work shift ranged between 7.39E-05 -1.70E-04. Across the five WWTPs, workers at WWTP3 had the highest estimated potential LD infection risk, while those at WWTP2 faced the lowest estimated risk. Annual infection risk estimates showed similar trends, with WWTP3 presenting the highest risk (1.36E-04) and WWTP2 the lowest (5.91E-05). Notably, the annual risk under the optimistic 0.5-hr exposure time could be deemed acceptable for workers at WWTP1, 2 and 4, as median values were below the 1E-04 benchmark. However, workers at WWTP3 and 5 faced annual infection risks that exceeded the benchmark, with median values of 1.36E-04 and 1.29E-04, respectively.

Comparatively, LD infection risks were generally higher for tasks associated with aeration tanks than those near bar screens. For WWTP1 workers, the median infection risk during a 0.5-hr work shift near aeration tanks was double that of bar screen tasks with annual infection risk estimated to be one-fold higher. Similarly, at WWTP3 and 5, single shift and annual risks were estimated to be one-fold higher for tasks performed near aeration tanks. On the other hand, median risk levels for workers at WWTP2 and 4 were of the same order of magnitude for both bar screen and aeration tank tasks.

The minimal risk of *L. pneumophila* resulting in Pontiac fever was also estimated, revealing a pattern consistent with the estimated infection risk for LD. Annual risk remained within the acceptable threshold (≤1E-04) across all WWTPs for work task at both bar screen and aeration tank during a 0.5-hr exposure time.

8.3.2 Infection risk posed by accidental ingestion of *A. hydrophila* in the best-case scenario.

Table 8.3 shows infection risk posed by *A. hydrophila* resulting from accidental ingestion of wastewater during a 0.5-hr exposure time in the five studied WWTPs. The median risk of *A. hydrophila* infection during a single shift ranged between 4.02E-07 -3.48E-06 near bar screens, 1.02E-07 -9.22E-07 near aeration tanks, and 2.82E-08 -1.78E-07 near SST. Workers at WWTP3 showed the highest risk of *A. hydrophila* infection near both bar screens and aeration tanks, compared to workers in other WWTPs. Additionally, occupational exposure near bar screens was generally observed to pose the highest risk of *A. hydrophila* infection. Conversely, risk assessment for subsequent treatment processes, particularly secondary treatment (aeration tank) and sedimentation (SST), revealed a gradual decrease in *A. hydrophila* infection risk when compared to the bar screens.

Annual estimates indicated that the overall risk of *A. hydrophila* infection in this study remained within the acceptable threshold (≤1E-04) for a 0.5-hr exposure time across all five WWTPs, regardless of the treatment stage.

Table 8.2 The median risk of infection from *L. pneumophila* for different exposure time and scenarios

***L. pneumophila* infection**	**Treatment stage**	**Exposure time (hr)**	**Infection risk (single event/work shift)**					**Annual infection risk (pppy)**				
			WWTP1	**WWTP2**	**WWTP3**	**WWTP4**	**WWTP5**	**WWTP1**	**WWTP2**	**WWTP3**	**WWTP4**	**WWTP5**
LD	Bar screen	**0.5**	6.56E-06	1.31E-05	1.87E-05	6.25E-05	2.63E-05	5.25E-06	1.05E-05	1.49E-05	5.00E-05	2.10E-05
		2	1.05E-04	2.10E-04	2.99E-04	1.00E-03	4.21E-04	3.15E-04	6.29E-04	8.95E-04	3.00E-03	1.26E-03
		4	4.20E-04	8.39E-04	1.19E-03	4.00E-03	1.68E-03	2.52E-03	5.02E-03	7.14E-03	2.38E-02	1.01E-02
		6	9.45E-04	1.89E-03	2.69E-03	9.00E-03	3.79E-03	8.47E-03	1.69E-02	2.39E-02	7.82E-02	3.36E-02
		8	1.68E-03	3.35E-03	4.78E-03	1.60E-02	6.74E-03	1.99E-02	3.95E-02	5.58E-02	1.76E-01	7.79E-02
	Aeration tank	**0.5**	1.06E-04	7.39E-05	1.70E-04	9.72E-05	1.62E-04	8.50E-05	5.91E-05	1.36E-04	7.78E-05	1.29E-04
		2	1.70E-03	1.18E-03	2.72E-03	1.56E-03	2.59E-03	5.09E-03	3.54E-03	8.13E-03	4.66E-03	7.74E-03
		4	6.80E-03	4.73E-03	1.09E-02	6.22E-03	1.03E-02	4.01E-02	2.81E-02	6.35E-02	3.67E-02	6.05E-02
		6	1.53E-02	1.06E-02	2.45E-02	1.40E-02	2.33E-02	1.30E-01	9.18E-02	2.00E-01	1.19E-01	1.91E-01
		8	2.72E-02	1.89E-02	4.35E-02	2.49E-02	4.14E-02	2.82E-01	2.05E-01	4.13E-01	2.60E-01	3.97E-01
Pontiac fever	Bar screen	**0.5**	4.27E-06	8.53E-06	1.21E-05	4.07E-05	1.71E-05	3.42E-06	6.83E-06	9.72E-06	3.26E-05	1,37E-05
		2	6.84E-05	1.37E-04	1.94E-04	6.51E-04	2.74E-04	2.05E-04	4.09E-04	5.83E-04	1.95E-03	8,22E-04
		4	2.73E-04	5.46E-04	7.78E-04	2.61E-03	1.10E-03	1.64E-03	3.27E-03	4.66E-03	1.55E-02	6,56E-03
		6	9.45E-04	1.89E-03	2.69E-03	9.00E-03	3.79E-03	8.47E-03	1.69E-02	2.39E-02	7.82E-02	3,36E-02
		8	1.09E-03	2.18E-03	3.11E-03	1.04E-02	4.39E-03	1.30E-02	2.59E-02	3.67E-02	1.18E-01	5,14E-02
			6.92E-05	4.81E-05	1.11E-04	6.33E-05	1.05E-04	5.53E-05	3.85E-05	8.85E-05	5.06E-05	8,42E-05

Aeration	**0.5**										
tank	**2**	1.11E-03	7.70E-04	1.77E-03	1.01E-03	1.68E-03	3.32E-03	2.31E-03	5.30E-03	3.04E-03	5,04E-03
	4	4.43E-03	3.08E-03	7.08E-03	4.05E-03	6.74E-03	2.63E-02	1.83E-02	4.17E-02	2.41E-02	3,97E-02
	6	9.96E-03	6.93E-03	1.59E-02	9.12E-03	1.52E-02	8.61E-02	6.07E-02	1.34E-01	7.91E-02	1,28E-01
	8	1.77E-02	1.23E-02	2.83E-02	1.62E-02	2.69E-02	1.93E-01	1.38E-01	2.91E-01	1.78E-01	2,79E-01

Note: aqua shade represents annual infection risk ≤1E-04, (minimal risk); tan shade represents annual infection risk above 1E-04 -1E-03, (exceeds 1E-04 threshold); lavender shade represents annual infection risk from 1E-02 -1E-01, (exceeds 1E-04 threshold).

Table 8.3: The median risk of infection from *A. hydrophila* for different exposure time and scenarios

Reference pathogen	Treatment stage	Exposure time (hr)	Infection risk (single event/work shift)					Annual infection risk (pppy)				
			WWTP1	**WWTP2**	**WWTP3**	**WWTP4**	**WWTP5**	**WWTP1**	**WWTP2**	**WWTP3**	**WWTP4**	**WWTP5**
A. hydrophila	Bar screen	**0.5**	2.64E-06	2.59E-06	3.48E-06	4.02E-07	7.94E-07	2.12E-06	2.07E-06	2.79E-06	4.09E-07	5.78E-07
		2	1.06E-05	1.03E-05	1.39E-05	1.61E-06	3.18E-06	3.17E-05	3.10E-05	4.18E-05	6.14E-06	8.68E-06
		4	2.12E-05	2.07E-05	2.79E-05	3.22E-06	6.35E-06	1.27E-04	1.24E-04	1.67E-04	2.46E-05	3.47E-05
		6	3.17E-05	3.10E-05	4.18E-05	4.83E-06	9.53E-06	2.85E-04	2.79E-04	3.76E-04	5.53E-05	7.81E-05
		8	4.23E-05	4.14E-05	5.57E-05	6.43E-06	1.27E-05	5.07E-04	4.97E-04	6.68E-04	9.83E-05	1.39E-04
	Aeration tank	**0.5**	1.37E-07	1.58E-07	9.22E-07	4.40E-07	1.02E-07	1.09E-07	1.27E-07	7.38E-07	3.52E-07	8.14E-08
		2	5.47E-07	6.33E-07	3.69E-06	1.76E-06	4.07E-07	1.64E-06	1.90E-06	1.11E-05	5.28E-06	1.22E-06
		4	1.09E-06	1.27E-06	7.38E-06	3.52E-06	8.14E-07	6.56E-06	7.60E-06	4.43E-05	2.11E-05	4.88E-06
		6	1.64E-06	1.90E-06	1.11E-05	5.28E-06	1.22E-06	1.48E-05	1.71E-05	9.96E-05	4.75E-05	1.10E-05
		8	2.19E-06	2.53E-06	1.48E-05	7.03E-06	1.63E-06	2.62E-05	3.04E-05	1.77E-04	8.44E-05	1.95E-05
	SST	**0.5**	5.98E-08	2.82E-08	1.48E-07	1.78E-07	1.45E-07	4.78E-08	2.26E-08	1.19E-07	1.43E-07	1.16E-07
		2	2.39E-07	1.13E-07	5.94E-07	7.14E-07	7.24E-08	7.17E-07	3.39E-07	1.78E-06	2.14E-06	2.17E-07
		4	4.78E-07	2.26E-07	1.19E-06	1.43E-06	1.45E-07	2.87E-06	1.36E-06	7.13E-06	8.56E-06	8.68E-07
		6	7.17E-07	3.39E-07	1.78E-06	2.14E-06	2.17E-07	6.46E-06	3.05E-06	1.60E-05	1.93E-05	1.95E-06
		8	9.56E-07	4.52E-07	2.38E-06	2.86E-06	2.89E-07	1.15E-05	5.42E-06	2.85E-05	3.43E-05	3.47E-06

Note: yellow shade represents annual infection risk >1E-04, (exceeds 1E-04 threshold); blue shade represents annual infection risk from 1E-05 -1E-06, (minimal risk); green shade represents annual infection risk from 1E-07 -1E-08, (minimal risk).

8.3.3 The impact of prolonged working hours on *L. pneumophila* and *A. hydrophila* infection risks for WWTP workers

This study extended exposure duration for WWTP workers to provide a more realistic assessment, especially relevant for low- and middle-income countries (LMICs) where treatment facilities are prone to malfunction or equipment breakdowns. Tables 8.2 and 8.3 present the median infection probabilities for *L. pneumophila* and *A. hydrophila* during a single exposure event, along with the estimated annual risks for different exposure durations (0.5, 2, 4, 6, 8 hrs).

Single-event and annual infection risk estimates for *L. pneumophila* (both LD and Pontiac fever) generally increased with longer exposure times, suggesting that extended shifts could elevate health risks for WWTP workers. In addition, the annual risk threshold of 1E-04 was exceeded for occupational exposure durations of 2-hrs or more across all scenarios and WWTPs. Furthermore, annual risk values for *L. pneumophila* were relatively consistent across WWTPs, suggesting that annual risks associated with occupational exposure near aerator tanks are comparable across the five WWTPs studied.

Extended exposure times also slightly increased the probability of *A. hydrophila* infections. Across all WWTPs, the median annual risk of *A. hydrophila* infection remained below the 1E-04 threshold for exposure times of 0.5 and 2-hrs near bar screens. However, for occupational exposure times of 4 to 8-hrs, median annual risk values were above the 1E-04 benchmark at WWTP1, 2 and 3. At WWTP5, only the 8-hr exposure time exceeded the benchmark threshold, while all exposure times remained within acceptable threshold at WWTP4.

In contrast, even with exposure time extending to 8-hrs, the median annual risk of *A. hydrophila* infection from accidental wastewater ingestion near both the aeration tank and SST consistently remained below the benchmark threshold across the five WWTPs. Therefore, these findings suggest minimal infection risk posed by *A. hydrophila* in WWTP workers when performing routine tasks near both aeration tanks and SST.

8.4 Discussion

This study quantified the probability of infection risks among WWTP workers, an occupational group that despite experiencing prevalent health issues such as respiratory and gastrointestinal illnesses, has received limited attention (Heldal *et al*., 2010; Oza *et al*., 2022). Infection risks for *L. pneumophila* were estimated for tasks performed near bar screens and aerator tanks, which are linked to aerosol emissions (Niazi *et al.,* 2015; Han *et al.,* 2019), as *L. pneumophila* primarily infects humans through inhalation (Diederen, 2008). Compared to the acceptable annual benchmark risk (≤1E-04 pppy), a 0.5-hr exposure at either the bar screen or aeration tank resulted in a relatively low infection risk of *L. pneumophila*. However, *L. pneumophila* infection risk exceeded the annual benchmark when occupational exposure time extended to 2-hrs or more, irrespective of the workstation. There is limited research on *Legionella* risk assessments involving untreated municipal wastewater exposure (Heida *et al*., 2024), with previous studies largely focusing on *L. pneumophila* infection risks in other water systems including drinking water (Sharaby *et al*., 2019; Kermani *et al*., 2022), recycled water (Blanky *et al*., 2017; Hamilton *et al*., 2018; Chen *et al*., 2022; Mori and Smith, 2023), and roof-harvested rainwater (Ahmed *et al*., 2010; Hamilton *et al*., 2017; Reyneke *et al*., 2020). These studies have particularly focused on exposure scenarios such as showering, cooling towers, toilet flushing, and irrigation, where aerosols generation presents a risk of inhaling contaminated water particles.

The probabilities estimated in this study suggest an increased risk of *L. pneumophila* infections for WWTP workers. In a similar study, Heida *et al*. (2024) assessed the risk of *L. pneumophila* infection among WWTP workers during different tanks (e.g., walking the plant, contact, direct splash, and lagoon sludge sampling), and found the highest risk for lagoon sampling (7.80E-04) and lowest for walking around the plant (1.30E-05). The study also indicated that face covering significantly reduced infection risk by 77%, demonstrating the effectiveness appropriate PPE in mitigating exposure to inhalable infectious organisms. Additionally, previous studies have implicated WWTPs aeration tanks in outbreaks of LD in Germany (Maisa *et al.,* 2015), Netherlands (Loenenbach *et al.,* 2018; Vermeulen *et al.,* 2021; Pijnacker *et al.,* 2024), and Norway (Olsen *et al.,* 2010), highlighting the potential role of WWTPs as sources of *Legionella* infections. Research exploring the probabilities of *L. pneumophila* infections particularly in occupational settings are currently limited with a few studies focused on occupational groups such as dental workers (Hamilton *et al*., 2021), miners (Madera-García *et al*., 2019), farm workers (Mori and Smith, 2023), and urban WWTP workers (Heida *et al*.

2024). These studies demonstrate significant variations in the occupational risk of *L. pneumophila* infection, probably due to differences in water sources and the devices used to aerosolise water containing *L. pneumophila* across different occupational settings. However, it is important to recognise WWTPs, particularly aeration tanks, as potential sources of *Legionella* infection considering that they emit copious amounts of aerosols (Niazi *et al.,* 2015), and have been implicated in LD outbreaks (Olsen *et al*., 2010; Maisa *et al.,* 2015; Vermeulen *et al.,* 2021). Measures should be implemented to control *Legionella* densities and prevent the release of wastewater contaminated with *Legionella* at WWTPs. Legionnaire's disease is a serious public health burden (Toplitsch *et al*., 2018). Workers at WWTPs may be exposed to *L. pneumophila* contaminated aerosols frequently and for extended periods, posing increased risks for vulnerable workers, particularly the elderly workers, smokers, and those with compromised immune systems (Toplitsch *et al*., 2018). Therefore, the findings of this study highlight the importance of strict adherence and use of appropriate PPE to minimise worker exposure. Additional control measures, such as covering of aeration tanks to limit *Legionella* aerosol emissions could further mitigate environmental and occupational risks.

The type of aeration system did not influence the findings of this study, which contrasts with previous studies suggesting that workers at WWTPs utilising mechanical aerators faced higher occupational health risks from airborne bacteria than those at diffused aerators (Chen *et al*., 2021; Wang *et al*., 2021). Mechanical aerators are expected to pose higher infection risks due to their vigorous wastewater mixing and increased aerosol production compared to diffused aerators (Wang *et al*., 2019; Han *et al*., 2020). However, in this study, extended occupational exposure time and frequency were associated with higher probabilities of *L. pneumophila* infection. Literature indicates that infection risk is primarily influenced by exposure duration and pathogen concentration (Chen *et al*., 2021; Morales *et al*., 2023), which may explain why aeration mode did not impact the estimated *L. pneumophila* infection risk. No significant differences were observed in culturable *L. pneumophila* across all plants (see Chapter 4), resulting in consistent infection risk estimates.

In this study, the risk of *A. hydrophila* infection was found to be high for tasks performed at the bar screens where influent is received. These results are comparable to two studies conducted in South Africa assessing occupational risks of ingesting enteric bacteria using pathogenic *E. coli* as a surrogate (Mbanga *et al.,* 2020; Amoah *et al*., 2022). Both studies found that the infection risk of pathogenic *E. coli* was highest for WWTP workers exposed to raw wastewater with decreasing risk in subsequent treatment stages, reflecting the contaminant reduction

expected during wastewater treatment (Mbanga *et al.,* 2020; Amoah *et al*., 2022). The variations observed in *A. hydrophila* risk levels among workers exposed at the bar screen at different WWTPs is likely due to the differences in *A. hydrophila* concentrations used in the simulation. This is consistent with previous studies indicating that pathogen concentration is an important factor contributing to variations in the estimated total infection risk (Chen *et al*., 2021; Morales *et al*., 2023). This underscores the importance of ongoing pathogen monitoring in wastewater to build larger datasets, facilitating more accurate health risk assessments. To the best of our knowledge, this study is the first to estimate the potential risk of *A. hydrophila* from accidental wastewater ingestion in WWTP workers. However, it is the second study that estimated the risk of *A. hydrophila* infection through QMRA simulations. Al-Jassim et al. (2015) previously explored occupational risk associated with wastewater reuse among agricultural workers during routine irrigation, focusing on dermal exposure.

This study also assessed the impact of prolonged occupational exposure time on infection risks among WWTP workers. Specifically, exposure to wastewater for a continuous period of 2-hrs or more near both bar screen and aeration tank showed that the annual risk for *Legionella* infections exceeded the established threshold. Similarly, prolonged exposure time of 4 or more continuous hours, particularly at the bar screen where wastewater containing *A. hydrophila* was present, were above the annual risk threshold. These findings suggest that prolonged working hours may present health risks in this occupational group, which is in agreement with previous studies (Dada and Gyawali, 2021; Amoah *et al*., 2022). Dada and Gyawali (2021) previously suggested that there may be an occupational risk of inhaling SARS-CoV-2 in WWTP workers involved in manual sludge handling for extended periods of up to 8 continuous hours. Similarly, Amoah et al. (2022) indicated that shorter occupational exposure periods of 0.5-hr or less resulted in reduced risk of microbial infections (e.g., *E. coli*, *Norovirus* and *Cryptosporidium* spp.) via inhalation or accidental ingestion in WWTP workers. These findings may be particularly relevant in LMICs where the majority of treatment facilities are not functioning properly, and workers are involved in manual screening and sludge dewatering. Thus, highlighting the importance of adhering to PPE practices and guidelines recommended for WWTP workers when handling untreated wastewater to reduce exposure to known and emerging pathogens (LeChevallier *et al*., 2020; CDC, 2021).

The findings of this study suggest that prolonged exposure times increases the risk *L. pneumophila* infections (both LD and Pontiac fever), with estimated annual risks exceeding the benchmark threshold (>1E-04). However, WWTP workers are typically expected to operate

at a specific treatment point for 0.5 to 2 hours, and rarely longer. At 4 hours of exposure, the estimated risk of *A. hydrophila* infection is 1 in 10 000 people. Given the relatively small number of WWTP workers in most countries and the low likelihood of prolonged exposure (4 hours or more), *A. hydrophila* is unlikely to pose serious occupational risks. Additionally, WWTP workers may develop stronger immune responses due to repeated exposure to non-harmful levels of pathogens, potentially deviating from the dose-response curve observed in the general population. The assumptions in this study are based on public health guidelines, which aim to protect vulnerable groups such as children, the elderly, and immunocompromised individuals. Occupational health limits are typically higher, as they are designed for a generally healthy adult workforce. The adult working-age population at WWTPs is generally healthy with more robust immune system, except for those with underlying health conditions such as tuberculosis, HIV, or diabetes, which could increase their susceptibility to infections.

Limitations

- In the assessment of *L. pneumophila* infection risk, this study focused on bar screens at inlet works and aeration tanks as the sources of aerosols contaminated with *L. pneumophila.* However, WWTP workers could also be exposed to *L. pneumophila* aerosols during activities such as sludge handling, maintenance or cleaning using high pressure hoses with treated effluent.
- Direct quantification of *Legionella* in the air is challenging with scarce data sets available (Medema *et al*., 2004). Consequently, this study used a partitioning coefficient approach to estimate *L. pneumophila* concentration in the air based on quantities detected in wastewater samples (Hines *et al*., 2014; Hamilton and Haas, 2016).
- QMRA relies on specific parameter values and incorporates several assumptions from the literature, introducing uncertainties which could result in either overestimation or underestimation of the calculated infection risks. In addition, the infection risk is influenced by the number of organisms required to induce an infection whether ingested or inhaled by the exposed population and pathogen's virulence factors (Bentham and Whiley, 2018). Other factors that should be considered for a more precise risk assessment include workers gender differences, health status, age and immunity.
- Due to the small sample size used to collect reference pathogen quantities in this study, the risk estimates should be considered preliminary and necessitate validation with

larger or longitudinal datasets for more accurate assessments of the risks posed by these reference pathogens in this occupational group. This study focused on specific reference pathogens, namely *L. pneumophila* and *A. hydrophila*, not accounting for other microbial agents present in wastewater that could adversely affect workers' health. Therefore, the control measures recommended for WWTP workers should consider the wide range of microbial hazards (e.g., bacteria, viruses, fungi and parasites) that workers could potentially be exposed to in the workplace. Notwithstanding, the results of this study provide a foundation for better understanding the level of infection risks in WWTP workers associated with exposure to *L. pneumophila* and *A. hydrophila* in municipal wastewater and will be helpful in the development of effective mitigation strategies.

8.5 Conclusion

This study used a QMRA approach to estimate potential infection risks in municipal WWTP workers associated with inhaling *L. pneumophila* contaminated aerosols or ingesting wastewater containing *A. hydrophila* while performing routine tasks at different workstations along the wastewater treatment channel. The results indicated an increased likelihood of infection risks due to exposure to *L. pneumophila* and *A. hydrophila*. The risk of *L. pneumophila* surpassed the acceptable annual threshold for waterborne pathogens defined by USEPA (≤1E-04 pppy), especially when exposed to or handling heavily contaminated wastewater, specifically influent and activated sludge; and increased with prolonged occupational exposure time. Similarly, *A. hydrophila* infection risk associated with exposure to influent exceeded the annual benchmark threshold, whereas exposure at subsequent workstations posed minimal risk even with prolonged exposure times. It is crucial that workers are provided with necessary protective measures, such as face masks and gloves, and are educated on the importance of compliance. This will reduce the risk of inhaling or ingesting pathogenic microorganisms and indirectly lowers the likelihood of developing work-related microbial infections. There are currently no established occupational exposure limits (OELs) for biological agents, thus challenging for decision-making and implementing effective risk mitigation measures in workplaces such as WWTPs where high levels of biological contaminants are expected. Therefore, infection risk estimates presented in the present study

can serve as a foundational reference when determining acceptable levels of waterborne pathogens in the work environment of WWTP workers.

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CHAPTER 9: KEY FINDINGS AND RECOMMENDATIONS

9.1 Key findings of the study

Workers at wastewater treatment plants (WWTPs) provide an essential service globally by ensuring proper operation of the treatment facilities, thereby protecting public health and the environment (WaterAid, 2019). However, WWTP workers, particularly in low- and middle-income countries (LMICs) including South Africa, Tanzania, Zimbabwe, Egypt, Bangladesh, Vietnam, Ghana and many others with suboptimal treatment infrastructure, are at risk of occupational exposure to various health hazards found in wastewater (WaterAid, 2019; Wright *et al*., 2019). This risk is further exacerbated by work tasks such as manual dislodging of screen bars due to equipment breakdown, sludge handling, general facility maintenance, and limited access to PPE (World Bank, 2019; LeChevallier *et al*., 2020).

Epidemiological studies have consistently shown that sanitation workers are at higher risk of infectious illnesses compared to the general population (Chandra and Arora, 2019; Oza *et al*., 2022). Respiratory and gastrointestinal illnesses, as well as systemic issues such as headaches and tiredness, are the most reported health issues in this occupational group (Chandra and Arora, 2019; Oza *et al*., 2022). Despite these concerns, there has been limited scientific focus on identifying and quantifying occupational health risks for WWTP workers globally. Additionally, there is a lack of data on specific health hazards at critical stages of wastewater treatment before final effluent discharge where worker exposure is likely due to frequent equipment breakdown and overflows during heavy rainfall (Cui and Liang, 2019; Chen *et al*., 2020; Kgopa *et al*., 2021; Bonetta *et al*., 2022). The present study addresses this gap by focusing on infection risks associated with exposure to bacterial pathogens in municipal wastewater. Bacteria are the most diverse group of microbial pathogens and are abundant in wastewater (Ramírez-Castillo *et al.,* 2015; Shayo *et al*., 2023). Occupational exposure to bacterial pathogens among WWTP workers can occur through multiple routes, including accidental ingestion, hand-to-mouth transfer, and inhalation of bioaerosols or splashes (Heldal *et al*., 2010; Zachary, 2017). Therefore, this study aimed to: **1)** screen for the predominant bacterial pathogens in South African municipal wastewater at selected WWTPs (Chapter 3); **2)** quantify the selected bacterial pathogens in wastewater (Chapter 4); **3)** identify antibiotic resistance genes (ARGs) and mutations in the selected bacterial pathogens linked to antimicrobial resistance (Chapter 5); **4)** assess the diversity of airborne bacteria emitted in typical municipal WWTPs **(**Chapter 6); **5)** investigate the prevalence of chronic respiratory and gastrointestinal health issues potentially associated with bacterial exposure among WWTP workers in the City of Tshwane Metropolitan Municipality, South Africa (Chapter 7); and **6)**

estimate the risk of infection associated with occupational exposure to untreated wastewater contaminated with *L. pneumophila* and *A. hydrophila* (Chapter 8). The following key findings were observed:

Chapter 3 presents the screening of predominant bacterial pathogens that pose potential occupational health risks to WWTP workers using 16S rRNA amplicon sequencing. Grab influent samples were collected from five municipal WWTPs in the City of Tshwane, South Africa revealing 253 bacterial genera, highlighting substantial microbial diversity within municipal wastewater. Despite the high microbial diversity, the predominant genera remained consistent across all WWTPs, indicating a stable bacterial community in the influent source.

The study identified 36 pathogenic genera, dominated by *Bacteroides*, *Pseudomonas*, *Aeromonas*, *Arcobacter*, *Leptotrichia*, *Treponema*, *Enterobacter*, *Streptococcus*, and *Acinetobacter*. These pathogenic genera were further classified based on their potential human health impacts, namely respiratory, enteric, and opportunistic infections. *Mycobacterium* and *Coxiella* were identified as respiratory pathogens, while *Escherichia/Shigella*, *Laribacter*, *Arcobacter*, and *Aeromonas* represented enteric pathogens. Additionally, 30 opportunistic genera were identified including *Enterococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, and *Enterobacter*. Some of these opportunistic genera include species belonging to the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), a cluster known for their multidrug resistance. Risk characterisation highlighted 17 pathogenic genera with the potential to cause airway obstruction, gastrointestinal problems, and opportunistic infections in exposed workers.

In conclusion, the findings indicate a stable microbial community across the studied WWTPs, suggesting that the workers are consistently exposed to a similar spectrum of bacterial pathogens. Importantly, several identified genera are known to cause gastrointestinal and respiratory illnesses, while some possess inherent resistance to commonly used antibiotics. These results highlight the urgent need for effective protective measures and regular health monitoring for workers in WWTPs.

Chapter 4 presents quantities of predominant respiratory and enteric opportunistic bacterial pathogens (*L. pneumophila*, *Mycobacterium* spp., *A. butzleri*, and *A. hydrophila*) at key treatment stages (influent, activated sludge, and SST effluent) in five municipal WWTPs. Over the study period (November 2021 to April 2022), 173 samples were collected and analysed using culture methods and quantitative PCR. Culturable *L. pneumophila* was detected in 92% of the samples, with concentrations ranging from 2.0 to 5.4 $\log_{10}$ MPN/100 mL. Notably, culturable *L. pneumophila* were often significantly higher in activated sludge compared to other sampling points. Molecular detection rates of *Legionella* spp., *L. pneumophila*, and *L. pneumophila* serogroup 1 were 97%, 75%, and 69%, respectively, with concentrations up to 5.3 $\log_{10}$ GC/mL. Importantly, this study demonstrates molecular typing of *L. pneumophila* serogroup 1, a strain responsible for up to 90% of Legionnaires' disease (LD) cases globally, highlighting potential health implications for WWTP workers.

Mycobacterium spp. were consistently detected in 100% of the samples, reaching concentrations up to 8.9 $\log_{10}$ GC/mL, with significantly higher GC levels in activated sludge samples. Enteric pathogens, *A. butzleri* and *A. hydrophila*, were detected in 96% and 82% of the samples, respectively. *Arcobacter butzleri* GC levels ranged from 0.11 to 6.5 $\log_{10}$ GC/mL, whereas *A. hydrophila* GC levels were from 0.2 to 4.5 $\log_{10}$ GC/mL. Both enteric pathogens showed significant reductions in GC levels following biological treatment (activated sludge) compared to influent, though GC levels remained the same thereafter, underscoring the potential importance of tertiary treatment for further pathogen removal in the final treated effluents.

In conclusion, this study provided evidence of four clinically relevant opportunistic bacterial pathogens in municipal wastewater at different treatment stages. This suggests potential exposure for WWTP workers, particularly those involved in manual waste screening, solids removal or handling, and cleaning tasks. Furthermore, the detection of significantly higher levels of respiratory pathogens *L. pneumophila* and *Mycobacterium* spp. in activated sludge samples, raises a potential concern regarding bioaerosol exposure. Therefore, there is an urgent need for enhanced safety measures to protect workers from inhaling bioaerosols potentially contaminated with these pathogens.

Chapter 5 presents the composition and diversity of airborne bacteria at two municipal WWTPs in South Africa with different aeration modes, using 16S rRNA amplicon sequencing.

Samples were collected from WWTP1, which uses mechanical aerators (MAER), and WWTP5, which uses diffused aerators (DAER), along with background controls from each site. Seven bacterial phyla were identified, with *Firmicutes, Proteobacteria,* and *Bacillota* being the most predominant. At the genus level, 26 genera were identified, with *Acinetobacter* and *Bacillus* detected across all sites. The study also identified distinct genera in the bioaerosol samples. Mechanical aerator samples showed the highest unique diversity with 15 genera, whereas DAER exhibited only two unique genera. The background control samples had one unique genus identified at each site.

A total of 36 potential bacterial pathogens were identified, comprising 22 genera and 18 species. Importantly, 28 and six potential opportunistic pathogens were detected at WWTP1 (MAER) and WWTP5 (DAER) samples, respectively. Background controls revealed seven potential pathogens at WWTP1 and ten at WWTP5. The most abundant bacterial pathogens (>1% relative abundance) included *Bacillus* spp. (44.6%), *Enterococcus* spp. (13.2%), *Clostridium* spp. (8.8%), *Streptococcus* spp. (5.2%), *Acinetobacter* spp. (4.3%), *Enterobacter* spp. (2.8%), *Pseudomonas* spp. (2.4%), *Bacteroides fragilis* (2.1%), *Acinetobacter baumannii* (1.2%), and *Escherichia/Shigella* (1.1%). Opportunistic pathogens such as *Streptococcus, Enterobacter, Bacteroides fragilis, Acinetobacter baumannii* and *Escherichia/Shigella*, dominated MAER air samples, while *Bacillus* and *Pseudomonas* were prevalent in DAER samples. Background controls from both WWTPs were dominated by *Bacillus,* and *Clostridium.*

In conclusion, the findings demonstrated that MAER bioaerosols had relatively higher bacterial composition and diversity when compared to DAER samples. Several hazardous biological agents known to cause infections including respiratory and gastrointestinal infections, UTI, and skin and soft tissue infections were identified, suggesting potential health risks for workers. In addition, five multidrug resistant bacterial species (*E. faecium*, *A. baumannii, K. pneumoniae, P. aeruginosa, and Enterobacter* spp.) were identified, emphasising the urgent need to address bioaerosol exposure in WWTPs. Therefore, implementing effective strategies to mitigate bioaerosol exposure is critical, particularly in WWTPs with mechanical aerators, to reduce bioaerosol release and safeguard worker health.

Chapter 6 presents metagenome-assembled genomes (MAGs) of *L. pneumophila* (n=4) and *Aeromonas* spp. (n=3) identified in Chapter 4, focusing on identifying ARGs and mutations

associated with drug resistance. This study represents the first genomic analysis of wild-type *L. pneumophila* identified in municipal wastewater, providing novel insights into the potential health risks posed by environmental pathogenic organisms with no link to outbreaks or clinical cases.

The *aph(9)-Ia* gene conferring resistance to spectinomycin was identified in all four *L. pneumophila* MAGs. Furthermore, 138 nonsynonymous SNVs were identified in genes associated with resistance to antibiotic drugs used for *L. pneumophila* infections. These included mutations in macrolide (*lpeB*, *rplD*), fluoroquinolone (*gyrA*, *gyrB*, *parC*, *parE*), and rifampicin resistance genes (*rpoB*), and one deletion mutation in the *lpeB* gene. Nonsynonymous SNVs were highly prevalent in *gyrA* (85 variants) and *gyrB* (17 variants), suggesting potential emerging fluoroquinolone resistance in environmental *L. pneumophila*.

Distinct resistance profiles were observed in *Aeromonas* spp. *Aeromonas caviae* MAGs harboured aminoglycoside resistance genes *aph(6)-Id* and *aph(3")-Ib*, along with β-lactam resistance genes *blaMOX-4* and *blaOXA-1143.* On the other hand, *A. hydrophila* MAGs carried additional β-lactam resistance genes, including *blaOXA-724, cepH*, and *imiH*. Beta-lactam ARGs were the most prevalent among *Aeromonas* spp. MAGs, an important finding as β-lactam resistance genes are potentially plasmid-mediated, facilitating horizontal gene transfer across different bacterial species. Notably, this study reported *blaOXA-1143* and *blaOXA-724* in *Aeromonas* spp. from municipal wastewater for the first time. Furthermore, all identified ARGs exhibited a high sequence identity (≥95%), suggesting their likely functionality and clinical relevance.

In conclusion, this study underscored the role of municipal wastewater as a critical reservoir for antimicrobial resistance, where environmental, human, and animal health intersect, emphasising the significance of a One Health approach to mitigate risks and protect public health. The detection of ARGs and nonsynonymous SNVs in *Aeromonas* spp. and *L. pneumophila* from municipal wastewater reveals the potential emergence of resistance patterns in waterborne pathogens. These findings contribute to understanding of how clinically relevant pathogenic bacteria, particularly *L. pneumophila* and *Aeromonas* spp. in aquatic environments such as wastewater, can develop drug resistance. Furthermore, they reinforce the need for monitoring and mitigating the health risks associated with waterborne pathogens.

Chapter 7 presents an assessment of the prevalence of chronic respiratory and gastrointestinal health issues, as well as risk factors specifically non-communicable diseases (NCDs) and PPE use in South African WWTP workers. Data were collected using self-administered questionnaires from 149 WWTP workers and 77 control group participants. Workers at WWTPs were predominantly males (67.8%), aged 41 - 50 (29.5%), with tertiary education (30.2%), and had 2 - 20 (73.8%) years of work experience.

Chronic respiratory symptoms were more prevalent among WWTP workers compared to controls, with wheezing (23.5%) and shortness of breath (7.4%) being the most frequently reported. Shortness of breath showed a statistically significant difference between the groups. Gastrointestinal symptoms, including stomach pain (14.8%), bloating (12.8%), and heartburn (11.4%) were also common among WWTP workers; their prevalence did not differ significantly from that observed in the control group.

Systemic symptoms, notably fatigue and headaches were reported by 35.6% of WWTP workers. The most prevalent NCDs were hypertension (24.2%), diabetes (8.1%), and arthritis (7.4%), and other conditions (6.7%), potentially increasing workers' susceptibility to infections. While most workers reported using PPE, primarily safety boots, overalls, and heavy-duty gloves, compliance with nose and mouth protection was poor.

In conclusion, this study revealed that chronic respiratory and gastrointestinal health issues were commonly reported among South African WWTP workers, possibly related to occupational exposures. Frequent systemic symptoms, such as fatigue and headaches, further underscore the health burden in this workforce. Poor adherence to respiratory PPE suggests increased risk of exposure to inhalable health hazards, highlighting the need for targeted interventions to mitigate health risks in WWTP workers.

Chapter 8 presents quantitative microbial risk assessment (QMRA) to estimate the risk of infection associated with exposure to *L. pneumophila* and *A. hydrophila* at different stages of the treatment process in municipal WWTP workers. Worker exposure durations ranged from 0.5 to 8-hrs per shift, and the estimated infection risks were compared to the benchmark threshold of 1E-04 per person per year (pppy) set by the USEPA for waterborne pathogens. In the best-case scenario (0.5-hr exposure), annual risk of infection posed by *L. pneumophila* (both LD and Pontiac fever) remained below the USEPA's acceptable threshold for most WWTPs. The annual infection risks ranged from 3.42E-06 to 5E-05 near bar screens and from

3.85E-05 to 1.36E-04 near aeration tanks. Similarly, annual risk of *A. hydrophila* infection was within the acceptable threshold across all WWTPs and exposure scenarios. Specifically, infection risks ranged from 4.09E-07 to 2.79E-06 near bar screens, 8.14E-08 to 7.38E-07 near aeration tanks, and 2.26E-08 to 1.43E-07 near secondary sedimentation tanks.

The analysis of occupational exposure time on infection risks revealed that the risk of both LD and Pontiac fever increased with prolonged working hours. The estimated annual risks exceeded the threshold for exposure durations of 2-hrs or more in all scenarios (bar screens and aeration tanks) across all WWTPs. On the other hand, annual risk of *A. hydrophila* infection was within the acceptable range for shorter exposure durations of 0.5 and 2-hrs near bar screens. However, extended exposure times (4-hrs or more) at the same working area posed higher risks, exceeding USEPA threshold. Notably, even with prolonged working hours of up to 8-hrs, *A. hydrophila* annual infection risks consistently remained below the benchmark threshold across the five WWTPs for tasks performed near aeration tanks and secondary sedimentation tanks.

In conclusion, these findings suggest an increased likelihood of infection risks among WWTP workers due to inhaling *L. pneumophila* contaminated aerosols or ingesting wastewater containing *A. hydrophila*. The risks were significantly higher, exceeding the USEPA threshold, for work tasks performed near treatment stages with high wastewater contamination, particularly near bar screens and aeration tanks. Additionally, increased risks were estimated for 2-hrs or more of exposure of *L. pneumophila* infections, while *A. hydrophila* posed increased risks for exposure durations of 4-hrs or more. These results suggest that prolonged occupational exposure at critical treatment stages may present serious health risks to WWTP workers.

9.2 Recommendations and future studies

Drawing from the findings of the current study, the following recommendations are proposed to better assess microbial health risks and protect the health of WWTP workers, particularly in LMICs, including South Africa:

- Conduct large-scale, long-term surveillance of microbial communities (e.g., bacteria, viruses, and fungi) at critical treatment points where worker exposure is expected. This surveillance will provide region-specific data and may reveal

temporal and spatial variations in pathogen diversity, thereby improving risk assessments in LMICs.

- Use viability PCR with photoactivatable DNA intercalators (e.g., ethidium monoazide and propidium monoazide) to distinguish viable from non-viable pathogens. This approach will enhance the accuracy of occupational risk evaluations for WWTP workers.
- Investigate a broader range of pathogenic microorganisms such as viruses, protozoa, helminths and potentially infectious viable but non-culturable (VBNC) *Legionella* at key occupational exposure points to assess potential health risks.
- Expand the current monitoring efforts (e.g. faecal indicator bacteria) to include specific bacterial and viral pathogens, thereby improving the understanding of pathogen trends and their implications for WWTP worker's health. Large-scale surveillance programs are needed to track antimicrobial resistant strains and determinants. This will provide critical insights into the spread of ARGs and support the development of mitigation strategies to address global antimicrobial resistance challenges.
- Conduct in-depth investigations into bioaerosol composition, focusing on other potentially harmful components in bioaerosols such as viruses, fungi, allergens, and ARGs, particularly in LMICs where research is limited. Additionally, long-term studies should assess spatial and temporal variations in bioaerosol exposure risks.
- Health surveys over a longer period and with larger sample sizes would provide comprehensive understanding of the health issues associated with prolonged occupational exposure among WWTP workers. This will help establish correlations between occupational exposures and specific health outcomes.
- Develop improved methods for direct quantification of inhalable biological hazards to improve the accuracy of exposure risk assessments. Future research should also consider other exposure scenarios and evaluate infection risks posed by other microbial agents in wastewater that could have significant health consequences for workers.

SUPPLEMENTARY MATERIAL

Supplementary S7.1: Questionnaire





Dear Participant,

I would like to ask you a few questions for my study and your answers will help to better understand the risks to bacterial exposure among wastewater treatment plant workers. The questionnaire is structured into different sections and will require you to provide information about yourself (for example, your age and education) and your work history. There are also questions about risk factors to infectious diseases, actual symptoms and respiratory health. Other questions are about the availability and use of personal protective equipment (PPE) such as gloves, overalls and masks.

Thank you again for taking your time to answer these questions. Please tick the appropriate box and where indicated, please type out your answer.

1. **Background information**

Gender

Male	○
Female	○

Age **……**

Ethnicity

African	○
India	○
White	○
Mixed race	○
Other	○

Level of education

Never went to school	○
Grade 1 to 7	○
Grade 8 to 11	○
Matric	○

Tertiary	○
Don't know /don't want to answer	○

2. Occupational background information (Current)

My work contract is

Permanent	○
Fixed term	○
Other	○

I have been working at this plant, please provide number of months or years …**……**

3. Risk factors to infectious diseases

The following questions will be regarding your current health status to understand your susceptibility to pathogenic infections.

I have been diagnosed with

Heart disease	○
Cancer	○
Lung disease	○
Diabetes	○
Kidney disease	○
High blood pressure	○
Arthritis	○
Others	○
None	○

I smoke cigarette or cigars or pipes

Yes	○
No	○

4. Actual disease signs and symptoms

Now I would like to ask you some questions about your health and symptoms that you may have experienced in the past 2 weeks.

I have had these symptoms in the past 2 weeks

Headache	○
Fever	○
Cough	○
Shortness of breath	○
Tiredness	○
Stomach pain	○
Loss of weight	○
Nausea	○

Muscle pain ○
None ○

I have had these (gastrointestinal) symptoms in the past 2 weeks

Stomach ache and cramps ○
Nausea ○
Vomiting ○
Constipation ○
Bloating (too much gas) ○
Heartburn ○
None ○

I have had diarrhoea episodes (3 or more loose or liquid stools per day) in the past 2 weeks.

Yes ○
No ○

5. Respiratory diseases

5.1 **Cough**

I usually have a cough

Yes ○
No ○

I usually cough 4 to 6 times a day

Yes ○
No ○

I usually cough 4 or more days a week

Yes ○
No ○

I usually cough when getting up, or first thing in the morning

Yes ○
No ○

I usually cough the rest of the day or at night

Yes ○
No ○

IF YES TO ANY OF ABOVE, ANSWER THE FOLLOWING. IF NO TO ALL, SKIP TO NEXT QUESTION.

I usually cough like this on most days for 3 consecutive months or more during the year

Yes ○
No ○
Does not apply ○

5.2 **Cough with mucus**

I usually cough up mucus from your chest

Yes	○
No	○

I usually cough up mucus like this as much as twice a day

Yes	○
No	○

I usually cough up mucus like this 4 or more days of the week

Yes	○
No	○

I usually cough up mucus when getting up, or first thing in the morning

Yes	○
No	○

I usually cough up mucus the rest of the day or at night

Yes	○
No	○

IF YES TO ANY OF ABOVE, ANSWER THE FOLLOWING. IF NO TO ALL, SKIP TO NEXT QUESTION.

I usually cough up mucus like this on most days for 3 consecutive months or more during the year

Yes	○
No	○
Does not apply	○

5.3 **Wheezing**

My chest makes whistling sound when I have cold

Yes	○
No	○

My chest makes whistling sound occasionally apart from colds

Yes	○
No	○

My chest makes whistling sound most days or nights

Yes	○
No	○

6. Past respiratory illnesses

I have had bronchitis attacks

Yes	○
No	○

If YES, please indicate if it was confirmed by a doctor

Yes	○
No	○

I have had pneumonia

Yes	○
No	○

If YES, please indicate if it was confirmed by a doctor

Yes	○
No	○

I have had shortness of breath

Yes	○
No	○

If YES, please indicate if it was confirmed by a doctor

Yes	○
No	○

I have had asthma

Yes	○
No	○

If YES, please indicate if it was confirmed by a doctor

Yes	○
No	○

7. Personal protective equipment (PPE)

I routinely wear this for eye protection at work

Safety googles	○
Face shield	○
None	○
Other	○

Please specify OTHER eye protection……

I put on this nose and mouth protection at work

Surgical mask	○
Cloth mask	○
Respirator e.g. N95, FFP2	○
None	○
Other	○

Please specify OTHER nose and protection ……

I routinely put on this hand protection at work

Surgical gloves to the wrist	○
Heavy duty gloves	○
None	○
Other	○

Please specify OTHER hand protection……

I routinely put on this body protection at work

Overalls	○
Personal clothing	○
Long sleeves	○
Disposal apron	○
Other	○

Please specify OTHER body protection……

I routinely put on this foot protection at work

Safety boots	○
Rubber boots	○
Closed shoes	○
Other	○

Please specify OTHER foot protection……

I have had formal training on the use of PPE to protect myself from infectious agents/germs

Yes	○
No	○

I will benefit from PPE training and education

Yes	○
Maybe	○
No	○

I believe I need respiratory/lung protection everyday when at work

Yes	○
No	○

I know how to safely use PPE to protect myself

Not confident at all	○
A little confident	○
Somewhat confident	○
Confident	○

……

……..

Thank you very much for your participation!

Appendix 1: Ethical waiver

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-201120-01 20/11/2020

TO WHOM IT MAY CONCERN:

Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Miss Evida Poopedi
Supervisor:	Dr Tanusha Singh and Dr Annancietar Gomba
Department:	Clinical Microbiology and Infectious Diseases
Project title:	Evaluation of occupational health risks from bacterial contaminants at wastewater treatment plants in Gauteng Province, South Africa
Reason:	*In vitro* laboratory study using environmental samples. No human participants, human data or human tissues will be used.

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

Appendix 2: Human ethics clearance

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms E Poopedi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210522

NAME: (Principal Investigator)	Ms E Poopedi
DEPARTMENT:	School of Pathology Dept of Clinical Microbiology & Infectious Diseases Medical School University
PROJECT TITLE:	*Evaluation of occupational health risks from bacterial contaminants at wastewater treatment plants in Gauteng Province, South Africa*
DATE CONSIDERED:	2021/05/28
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Drs T Singh and A Gomba
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/10/05

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in May and therefore reports and re-certification will be due in the month of **May** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

28.01.22
Date

Appendix 3: Turnitin report



Thesis for turn it in.docx

ORIGINALITY REPORT

15%	9%	12%	4%
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PRIMARY SOURCES

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3	Evida Poopedi, Rian Pierneef, Tanusha Singh, Annancietar Gomba. "Opportunistic bacterial pathogens in bioaerosols emitted at municipal wastewater treatment plants, South Africa", Scientific Reports, 2025 Publication	1%
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8	mcf.gsfc.nasa.gov Internet Source	<1%
9	Hemali Harish Oza, Madison Gabriella Lee, Sophie Boisson, Frank Pega, Kate Medlicott, Thomas Clasen. "Occupational health outcomes among sanitation workers: A systematic review and meta-analysis", International Journal of Hygiene and Environmental Health, 2022 Publication	<1%