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

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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 ecological 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 of

premixed 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.

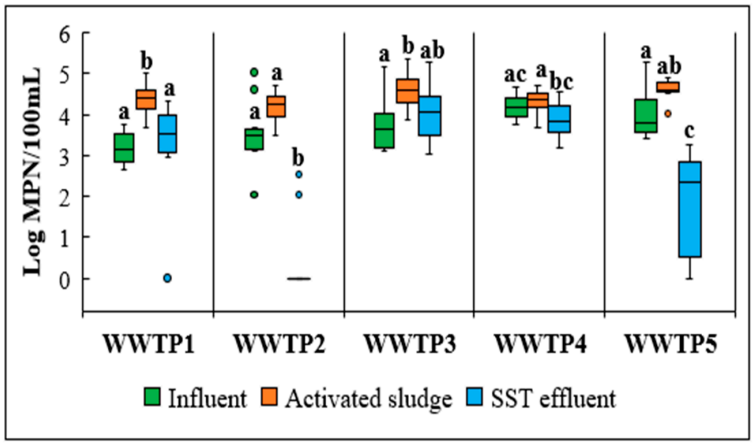


Figure 1. Concentration of culturable *L. pneumophila* in wastewater at different treatment stages across five WWTPs over a six-month sampling period. The whiskers illustrate the minimum and maximum; the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different 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.

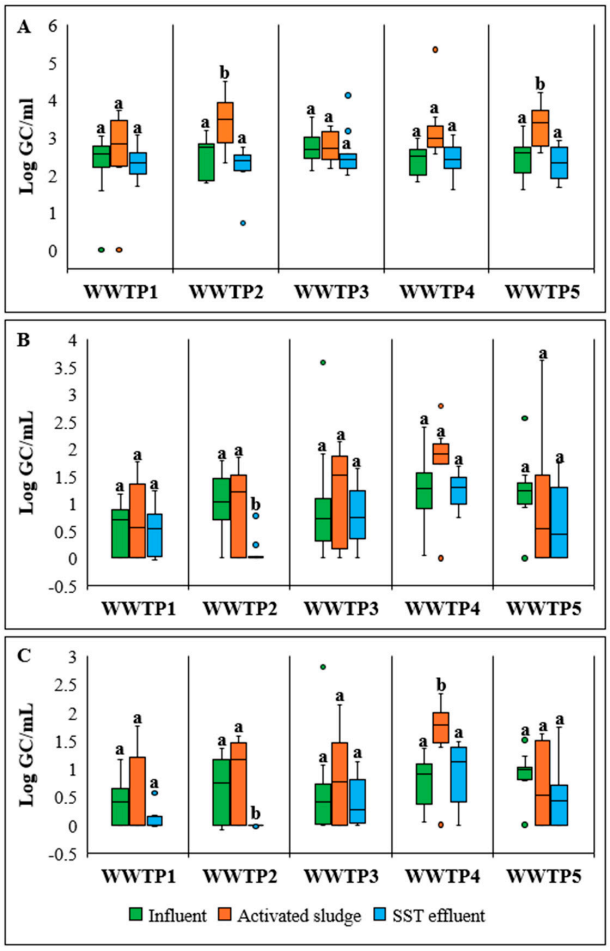


Figure 2. Gene copy levels of *Legionella* spp. (**A**) *L. pneumophila*, (**B**) and *L. pneumophila* sg1 (**C**) in wastewater at different treatment stages over six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner 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.

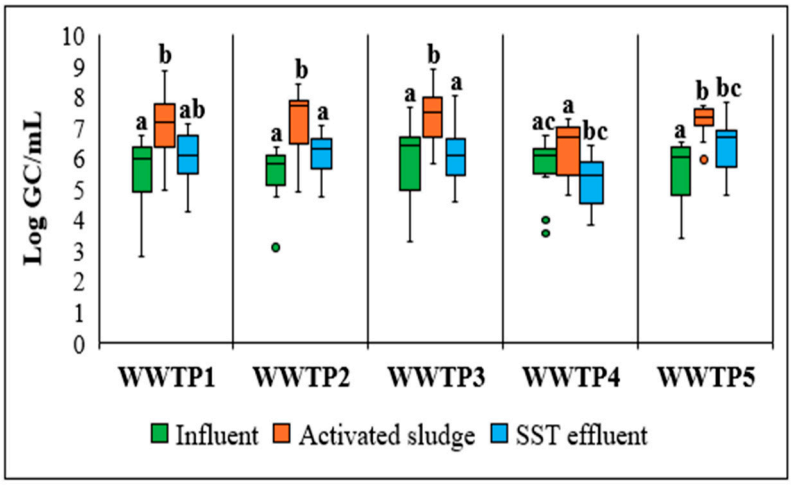


Figure 3. Gene copy levels of *Mycobacterium* spp. in wastewater at different treatment stages over a six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different letters on the graph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

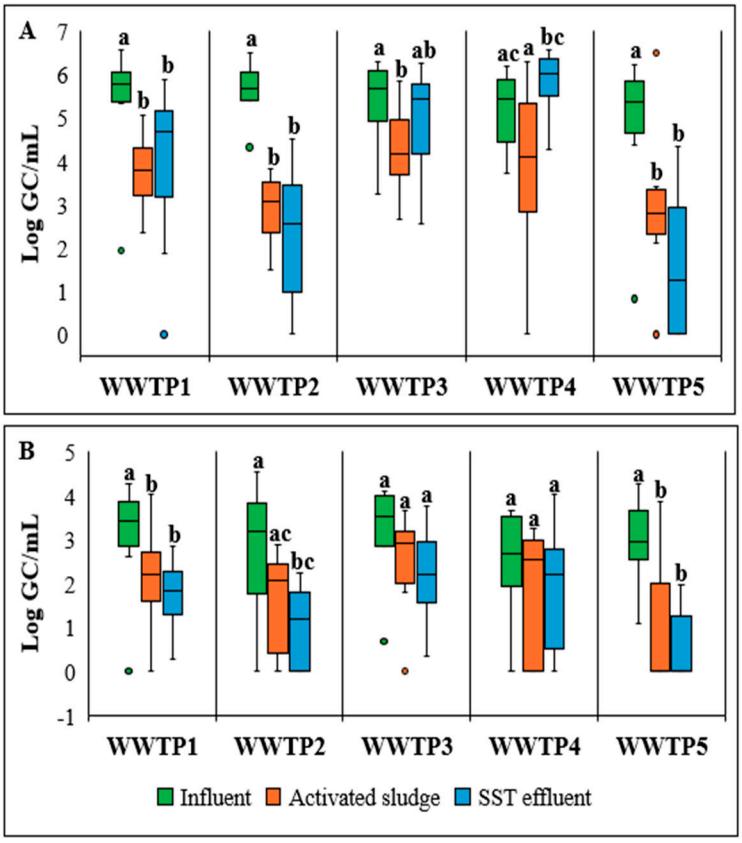


Figure 4. Gene copy levels of *A. butzleri* (**A**) and *A. hydrophila* (**B**) in wastewater at different treatment stages over a six-month sampling period. Whiskers illustrate the minimum and maximum, the outer box illustrates the 1st and 3rd quartiles, and the inner line illustrates the median. Different 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 bacterial 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 considering 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 municipal 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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