Water Research X 21 (2023) 100201

Contents lists available at ScienceDirect

Water Research X

journal homepage: www.sciencedirect.com/journal/water-research-x

Extended water stagnation in buildings during the COVID-19 pandemic increases the risks posed by opportunistic pathogens

Casey K Huang [a,b], Anjani Weerasekara [a,b], Ji Lu [a], Robyn Carter [c], Karen D. Weynberg [d], Rachel Thomson [c,e], Scott Bell [f,g,h], Jianhua Guo [a,*]

[a] Australian Centre for Water and Environmental Biotechnology (ACWEB, formerly AWMC), The University of Queensland, QLD 4072, Australia
[b] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, QLD 4102, Australia
[c] Respiratory Research Unit, Gallipoli Medical Research Institute, QLD 4120, Australia
[d] Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Brisbane, QLD 4072, Australia
[e] Greenslopes Clinical Unit, The University of Queensland, QLD Australia
[f] Adult Cystic Fibrosis Centre, The Prince Charles Hospital, Australia
[g] Child Health Research Centre, Faculty of Medicine, The University of Queensland, QLD Australia
[h] Translational Research Institute, Australia

ARTICLE INFO

Keywords:
COVID-19 pandemic
Opportunistic pathogens
Premise plumbing
Building microbiome
Nontuberculous mycobacteria (NTM)
Legionella

ABSTRACT

The regrowth and subsequent exposure of opportunistic pathogens (OPs) whilst reopening buildings that have been locked down due to the stay-at-home restrictions to limit the spread of COVID-19, is a public health concern. To better understand such microbiological risks due to lowered occupancy and water demand in buildings, first and post-flush water samples (n = 48) were sampled from 24 drinking water outlets from eight university buildings in two campuses (urban and rural), with various end-user occupancies. Both campuses were served with chlorinated water originating from a single drinking water distribution system in South-East Queensland, situated 14 km apart, where the rural campus had lower chlorine residuals. Culture-dependent and culture-independent methods (such as flow cytometry, qPCR and 16S rRNA gene amplicon sequencing) were used concurrently to comprehensively characterise the OPs of interest (*Legionella* spp., *Pseudomonas aeruginosa*, and nontuberculous mycobacteria (NTM)) and the premise plumbing microbiome. Results showed that buildings with extended levels of stagnation had higher and diverse levels of microbial growth, as observed in taxonomic structure and composition of the microbial communities. NTM were ubiquitous in all the outlets sampled, regardless of campus or end-user occupancy of the buildings. qPCR and culture demonstrated prevalent and higher concentrations of NTM in buildings (averaging 3.25 $\log_{10}$[estimated genomic copies/mL]) with extended stagnation in the urban campus. Furthermore, flushing the outlets for 30 minutes restored residual and total chlorine, and subsequently decreased the levels of *Legionella* by a reduction of 1 log. However, this approach was insufficient to restore total and residual chlorine levels for the outlets in the rural campus, where both *Legionella* and NTM levels detected by qPCR remained unchanged, regardless of building occupancy. Our findings highlight that regular monitoring of operational parameters such as residual chlorine levels, and the implementation of water risk management plans are important for non-healthcare public buildings, as the levels of OPs in these environments are typically not assessed.

1. Introduction

Water stagnation is a characteristic of premise plumbing systems, where water flow may be interrupted and outlets in buildings can remain unused from days to weeks, due to variations in end-point outlet usage (Ling et al., 2018). As a result, this may contribute to the deterioration of water quality, through changes to water chemistry (such as lead and copper levels (LeChevallier et al., 2003)) and increased microbial growth, including opportunistic pathogens (OPs) (Wang et al., 2017; Wang et al., 2015). OPs such as *Pseudomonas aeruginosa* (Bedard et al., 2016), *Legionella* species (including *Legionella pneumophila* (Bartley et al., 2016) and *L. anisa* (van der Mee-Marquet et al., 2006)), and

* Corresponding author.
E-mail address: jianhua.guo@uq.edu.au (J. Guo).

https://doi.org/10.1016/j.wroa.2023.100201
Received 21 December 2022; Received in revised form 5 September 2023; Accepted 9 September 2023
Available online 9 September 2023

nontuberculous mycobacteria (NTM; including *M. avium* complex (MAC) (Falkinham, 2011; Falkinham et al., 2001; Whiley et al., 2012), and *M. abscessus* complex (MABC) (Thomson et al., 2013a)) have several common adaptive traits and mechanisms (Falkinham, 2015; Falkinham et al., 2015), allowing them to survive and persist in nutrient-limiting environments. Furthermore, OPs such as NTM and *L. pneumophila* can transmit as aerosols released by contaminated drinking water outlets (Feazel et al., 2009; Prussin et al., 2017), leading to exposure of downstream users and infection in vulnerable individuals (Mercante and Winchell, 2015).

Studies of the chemical and microbiological perspective impact of short-term stagnation (ranging from days to weeks) (Ling et al., 2018; Proctor et al., 2018) have supported the development of guidance and management strategies for public and regulatory organisations during periods of extended stagnation (Proctor et al., 2020). However, there remain knowledge gaps of the effect of long-term closure occurring within building plumbing systems, particularly those posed by OPs, and the effectiveness of these maintenance strategies when returning to these facilities (Hozalski et al., 2020). Water risk management plans in public buildings have largely focused on healthcare environments rather than in non-healthcare contexts (Hozalski et al., 2020), such as for the detection of *L. pneumophila* specifically as a model OP, (Grimard-Conea et al., 2022; Liang et al., 2021) or expanding to include subsets of waterborne indicators and opportunistic bacterial pathogens, such as coliforms, *Escherichia coli, Enterococcus,* along with *P. aeruginosa* and *L. pneumophila* (De Giglio et al., 2020). Limited studies have assessed the impact of occupancy, water usage and potential correlations with OPs and the collective premise plumbing microbiome in non-healthcare public facilities. Thus, our aim was to determine if extended stagnation during the lockdowns increased the risk of harbouring higher levels of OPs and microbial concentrations within these systems. To this end, 24 water samples were collected and analysed for microbiological and physicochemical parameters. The sampling took place in two university campuses, located in urban (campus 1) and rural-residential (campus 2) areas in Brisbane, respectively. The buildings sampled from each of the campuses had various end-user frequencies during the COVID-19 lockdown period of 4 months (June – September 2020), where the outlets individually and the building collectively were classified. We also sought to assess whether this risk could be mitigated by implementing building maintenance strategies such as remedial flushing, as advised by the Centers for Disease Control and Prevention (CDC), plumbing regulators and scientific associations (National Academies of Sciences and Medicine, 2020; Proctor et al., 2020). Thus, we sampled first and post-flush tap water and characterised using various culture-dependent and culture-independent approaches (such as qPCR and microbial community sequencing) to gain a comprehensive understanding from a microbiological point of view of these systems under various levels of stagnation.

2. Materials and methods

2.1. Study sites, sampling, and building characteristics

Twenty-four tap water outlets (sampled from cold water faucets, including washbasin taps, laundry sinks, showerheads) were sampled from 8 buildings (3 outlets per building, 4 buildings per campus) across two university campuses based in Brisbane, from June to October 2020. Campus 1, which undertakes predominantly teaching and research activities, was located in an urban area (buildings A-D), in comparison to campus 2, which focusses on agricultural and research activities and was located in a semi-rural outer suburban area (buildings E-H). For simplicity, campus 1 will be referred to as the urban campus, and campus 2 as the rural campus. Both campuses were served with chlorinated water originating from the same drinking water distribution system (DWDS) in South-East Queensland, situated 14 km from each other. Although each campus received chlorinated drinking water originating from the same DWDS, due to differences such as distances, plumbing configurations, and end-user demand/usage, the characteristics of drinking water reaching the end user differed between each campus. Thus, the comparisons were intended to be made between the buildings located in each campus, rather than a comparison between both campuses. To draw comparisons between the buildings, a range of buildings varying in size, age, and purposes were selected. Both the buildings and the outlets sampled were chosen based on end-user occupancy during the COVID-19 lockdown period, after consulting facility management personnel, Queensland Health, and university directives. At the time of collection, each outlet and building were designated in terms of end-user water usage – i.e. accessed at least weekly (such as core research or administration facilities), intermittent stagnation (including buildings affected by the COVID-19 pandemic-related lockdowns such as teaching facilities, unused for $\leq$ 4 months) and buildings under extended stagnation (such as buildings undergoing long-term constructions and renovations, unused for $\geq$ 6 months). Buildings and their corresponding end-user occupancy/water usage details during the pandemic are outlined in Figure S1, and further details about the buildings and their characteristics can be found in the supplementary materials, Table S1.

Based on these details, the water usage of the building was designated with an overall ranking based on the allocations of the individual outlets. Prior to flushing, an initial water sample was collected ('first flush'). All tap water outlets in the building were then flushed concurrently for 30 min based on recommendations by local plumbing guidelines as a preventative measure. Subsequent water samples collected were designated as 'post-flush'. Two bulk water samples (2 $\times$ 10 L) for each of the 24 outlets, corresponding to first flush and post-flush, were collected in sterile polypropylene water storage containers. The physicochemical parameters were measured onsite as described in section 2.2. Apart from 1 L of water for the culturing of the NTM outlined in section 2.5, the remaining water samples were immediately quenched with sodium thiosulfate (to a 1% final concentration) to dechlorinate the water samples and transported at 4°C to the laboratory for further microbiological analyses within 4 hours.

2.2. Water chemistry – physicochemical parameters

All physicochemical parameters were measured and recorded onsite, including dissolved oxygen (DO), pH, and temperature, using a digital oxygen meter (Lutron Electronic, Taiwan) and a pH meter (Horiba Scientific, Japan). Free chlorine (the concentration of residual chlorine in water) and total chlorine (comprising the sum of combined (including chloramines) and free chlorine) levels were measured using a chlorine meter (Hach, USA), using the DPD Free Chlorine and Total Reagent Powder Pillows (Methods 8021 (free chlorine) and 8167 (total chlorine), with detection limits ranging from 0.02 – 2.00 mg/L), adapted from the (Standard Methods for the Examination of Water and Wastewater, 2017), Method 4500-Cl, which has been accepted by the USEPA for drinking water and wastewater analysis. To assess the levels of ammonium, nitrate, nitrite and phosphate, 100 mL of the water samples were filtered using 0.22 μm filter units (Merck Millipore, United States) and stored at -20°C for subsequent analyses. The nitrogen species levels were determined by the Lachat QuickChem 8000 Flow Injection analyser (FIA). All measurements were performed in technical replicates, as triplicates.

2.3. Live/dead staining and flow cytometry measurements

Live/dead staining was performed on 500 μL of the water samples using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (L7007; Invitrogen, Australia), with SYTO 9 and propidium iodide (PI) stains, as per manufacturer's instructions. Further details about the gating strategies can be found in the Supplementary Information, Text S1. Flow cytometry measurements were conducted using the CytoFLEX S flow

cytometer (Beckman Colter, USA) with excitation/emission maxima at 480/500nm and 561/610nm, respectively, and at a flow rate of 60 μL/min. Based on the controls, for each detected event, all signals were collected in log area mode with biological replicates ($n \geq 3$).

2.4. Heterotrophic Plate Counts (HPC)

HPCs for the samples were determined in triplicate by the spread plate method on Reasoner's 2A (R2A) agar (Oxoid, Australia) (ISO, 1999; Reasoner, 2004). Specifically, 100 - 500 μL of the water samples directly and their 10-fold serial dilutions (up to 10^{-3} dilution) were spread-plated on the solid media and incubated at both 30 and 37°C, which corresponded to the incubation temperatures for drinking water microorganisms and human pathogens, respectively. All plates were incubated for 10 days.

2.5. Culture-based enumeration of Legionella spp., Pseudomonas spp., and nontuberculous mycobacteria

Enumeration of *Legionella* spp. was performed as per the ISO 11731:2017 Water quality — Enumeration of *Legionella* standards (ISO, 2017). Briefly, 500 – 1000 mL water samples were concentrated through vacuum filtration using autoclave-sterilised membrane filters (0.22 μm pore size, 47mm diameter, mixed cellulose esters; Merck Millipore). The membrane filters were subjected to pre-reatment with acid buffer for 5 min and plated on buffered charcoal yeast extract (BCYE) and Glycine Vancomycin Polymyxin Cycloheximide (GVPC) agar plates as duplicates. All agar plates were incubated at 37°C for 7-10 days. *Legionella* positive colonies were identified based on morphology and sub-cultured on both BCYE and Horse Blood Agar (HBA) for verification.

Enumeration of *Pseudomonas* spp., particularly *P. aeruginosa*, was performed as per the ISO 16266:2006 Water quality — Detection and enumeration of *P. aeruginosa* method by membrane filtration standards (ISO, 2006). Briefly, 100 – 1000 mL of each water sample was concentrated through vacuum filtration using autoclave-sterilised membrane filters (0.45 μm pore size, 47mm diameter, mixed cellulose esters; Merck Millipore), and plated directly onto agar plates containing *Pseudomonas* agar base supplemented with *Pseudomonas* CN selective supplement (Oxoid, CM0559B and SR0102) as duplicates. All agar plates were incubated at 37°C for 48 h.

Enumeration of NTM and isolation was performed as previously described (Thomson et al., 2013b). Briefly, 500 mL of each water sample was filtered on sterile membrane filters (0.45 μm pore size, 47mm diameter, Millipore S-pak® membrane filters, HAWG047S6) using in-house agar plates supplemented with oleic albumin dextrose catalase growth supplement (OADC), and Mycobacterial Growth Indicator Tubes (MGIT). Plates were incubated at 37°C and checked weekly for the presence of colonies up to eight weeks. MGIT tubes were incubated at 35°C. Representative colonies were subcultured, based on morphology and Ziehl-Neelsen (ZN) stain to presumptively identify acid-fast bacilli. Positive acid-fast bacilli colonies were then further sub-cultured on M7H11 agar and incubated at 37°C in preparation for identification.

2.6. Identification of NTM isolates through Sanger sequencing

To identify each NTM isolate (Fedrizzi et al., 2017), DNA extracted from a subcultured NTM colony using 10 μL sterile loops was performed using the Qiagen DNeasy UltraClean Microbial kit (Qiagen, Germany). The following modifications were made to the manufacturer's instructions to optimise the DNA extracted from the NTM isolates: by including an initial heat kill/inactivation step for 80°C for 10 min to lyse the mycobacterial cell wall, and incubating the supernatant with solution IRS for 10 min. Extracted DNA was amplified by PCR using the GoTaq Flexi DNA Polymerase kit (Promega, USA), in 50 μL final volumes containing a final concentration of 1X Green Buffer, 4 mM MgCl2, 0.2 mM dNTPs, 0.5 M of forward and reverse primers BF (5' – GAGTTGGATCCTGGCTCAG – 3') and R2 (5' – CCTACGAGCTCTTTACG – 3'), based on the forward and reverse region associated with the hypervariable regions A and B of the 16S rRNA gene (Carter, 2018), 1 unit of GoTaq DNA Polymerase with 1 μL of template DNA. Thermocycling conditions were based on manufacturer guidelines, with an initial denaturation for 2 min at 95°C, followed by 35 cycles of 30 s denaturation at 95°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C. The final extension step was carried out at 72°C for 5 min. The PCR products were detected by gel electrophoresis on a 1% agarose gel stained by SYBR Safe (Invitrogen, USA) and imaged using the Gel Doc XR+ gel documentation system (Bio-Rad, USA). The unpurified PCR products were then sent to the Australian Genome Research Facility (AGRF) for dual-direction Sanger sequencing using the Applied Biosystems 3730 and 3730xl capillary sequencers in conjunction with Big Dye Terminator (BDT) chemistry version 3.1 (Applied Biosystems), under standardised cycling PCR conditions. The sequencing data was analysed using Geneious Prime (version 2022.0.1) by trimming and assembling the forward and reverse reads as a consensus sequence. Subsequently, the consensus sequences were aligned using the basic local alignment tool (BLAST) against the GenBank database for identification (Sayers et al., 2022). A phylogenetic tree was constructed using default parameters and based on the unweighted Pair Group Method with arithmetic Mean (UPGMA) tree building method (Michener and Sokal, 1957).

2.7. Total DNA extraction

Water samples (500 – 1000 mL) were concentrated on 0.22 μm cellulose ester membrane filter using vacuum filtration. Total genomic DNA was extracted from the filters using the Qiagen DNeasy PowerWater kit (Qiagen, Germany), with modifications made to the manufacturers' protocols in the final DNA elution step, where a reduced volume of 50 μL of elution buffer (Solution EB) was used to concentrate the DNA. Negative controls were also processed in parallel as part of the DNA extraction workflow, using molecular grade nuclease-free water as a DNA extraction reagent control. DNA concentration and purity were measured using the Nanodrop ND-1000 (ThermoFisher Scientific, USA) and Qubit 4 Fluorometer (Invitrogen, USA).

2.8. Quantitative PCR (qPCR)

In addition to enumerating OPs of interest through culturing, qPCR was performed by using NTM, *Legionella* and *Pseudomonas* genus specific primers (further details on primer sequences and amplification efficiency can be found in Table S2). QuantiNova SYBR® Green PCR Kit in an Applied Biosystems ViiA7 Real-Time PCR System. These were performed in 10 μL reaction volumes and contained a final concentration of 1X QuantiNova PCR master mix, 1X QN ROX Reference dye, 0.7 μM of forward and reverse primers, and 3 μL of template DNA. Thermocycling conditions were per manufacturer guidelines, with an initial activation for 2 min at 95 °C, followed by a combined 2-step cycling step for 35 cycles consisting of denaturation for 5 s at 95 °C and a combined annealing/extension step for 10 s at 60 °C on the pre-set maximal/fast mode ramp rate. All assays were performed in triplicate, and mean C_T (cycle threshold) values were used for estimating gene copies per mL (GU/mL).

Standard curves based on genomic DNA extracted from representative strains from the American Type Culture Collection strains of *Legionella pneumophila* subsp. *pneumophila* Philadelphia-1 ATCC 33152, *Pseudomonas aeruginosa* PAO1, and *Mycobacterium abscesses* ATCC 19977 were used for the calculation of estimated genomic copies on a genus level for each respective target OP. Ten-fold serial dilutions of the standard curves, no template controls were included in each run, and the limit of detection (LOD) and limit of quantification (LOQ) were calculated for each of the target OPs, as per MIQE guidelines (Bustin et al., 2009). Details of the primers, amplification efficiency, and R^2 can be

found in Table S2.

2.9. 16S rRNA gene amplicon sequencing

To complement the OP-specific detection methods outlined in sections 2.5 to 2.8, broad microbial communities in the water samples were also detected using amplicon sequencing. For the urban campus, amplicon sequencing inclusive of bacterial, archaea, and eukaryotes was performed using universal 16S rRNA gene primers targeting the V4 and V8 regions of the hypervariable region, primer set 926F (5'- AAACTYAAAKGAATTGACGG-3') and 1392R (5'- ACGGGCGGTGWGTRC-3') (Engelbrektson et al., 2010). On the other hand, DNA extracts for the rural campus amplified poorly using the 926F-1392R universal primers, likely due to the presence of inhibitors in the extracted DNA, thus primers F-16S2 (5'-CCAGACTCCTACGGGAGGCA-3') and R-16S2 (5'-GTGGACTACCAGGGTATCTA-3') designed to specifically target waterborne bacterial pathogens in the V3-V4 hypervariable region were used instead (Chakravorty et al., 2007). As a result of the change of primers used between the two campuses, the microbial community sequencing outcomes cannot be directly compared between the buildings located on the two separate campuses, but interpretations of the outcomes can still be made for the buildings located in each campus.

DNA extracts from the water samples (n = 48) were submitted to the Australian Centre for Ecogenomics (ACE) for 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform (Illumina, USA), using paired-end sequencing (300 bp) with MiSeq Reagent Kit V4. All raw sequencing reads were processed in QIIME2 (QIIME2, version: 2021.8). The forward and assembled paired-end reads were both independently examined to confirm the taxonomic composition, and the reads were processed through the DADA2 pipeline and taxonomy was assigned based on the Silva 132 release database, as amplicon sequencing variants (ASVs). Statistical analyses including alpha and beta diversity metrics and significance were performed in QIIME2 (Bolyen et al., 2019), with the following pipelines and plugins (*q2-feature-classifier, q2-core-metrics, q2-diversity*). Statistical significance between groups (comparing first flush and post-flush, buildings and individual outlets) were determined using the beta-group-significance plugin (Anderson, 2001) ADONIS PERMANOVA test (permutation multivariate analysis of variance). Visualisation of taxonomic composition was performed in RStudio v4.0.3 (RStudio, 2020) and implementing ampvis2 (Andersen et al., 2018), which incorporates the packages ggplot2 (Wickham, 2011), phyloseq (McMurdie and Holmes, 2013), and vegan (Oksanen, 2011). Principal coordinate analysis (PCoA) using the weighted UniFrac approach (Lozupone et al., 2007) was used to visualise microbiome similarities for each campus.

3. Results

3.1. Water chemistry analysis

Water quality of the samples collected as indicated by key physico-chemical parameters are summarised in Table S2. In the first flush samples, the buildings in the urban campus in comparison to the rural campus had higher levels of both free chlorine (n = 12, ranging from 0.47-3.72 mg/L, average 1.81 ± 1.44 mg/L) and total chlorine (n = 12, ranging from 1.70-5.68 mg/L, average 4.25 ± 1.76 mg/L). Turbidity levels for first flush (n = 12, ranging from 0.11-1.36 NTU, average 0.51 ± 0.57 NTU) and post-flush (n = 12, ranging from 0.15-0.49 NTU, average 0.31 ± 0.14 NTU) were relatively similar, for the buildings that were frequently and intermittently occupied during the lockdown period, with the exception of Building D (located in the urban campus, under extended stagnation), which had the highest first flush turbidity at 1.36 NTU, and reduced to 0.49 NTU in post-flush. On the other hand, the post-flush free chlorine levels varied amongst the buildings (n = 12, ranging from 0.70-3.81 mg/L, average 1.86 mg/L ± 1.41), in comparison to the total chlorine, where it was higher than its first flush counterpart (n = 12, ranging from 2.77-5.88 mg/L, average 4.79 ± 1.38 mg/L). Other parameters remained stable in first and post-flush, such as pH (n = 24, ranging from 7.86-8.31), DO (n = 24, 7.97-9.03 mg/L), and temperature (n = 24, 19.7-22.6 °C).

The rural campus had low free and total chlorine, regardless of whether the outlets had been flushed (Table S2). The first flush samples collected from the buildings had free and total chlorine levels ranging from below the detection limit (0 mg/L) to 0.14 mg/L (n = 12) and 0.07-0.20 mg/L (n = 12), respectively. Although a flushing regime of 30 minutes reduced the initial turbidity levels observed in the first flush samples, free and total chlorine levels were not fully restored in the system in the rural campus (Table S3) the regulated free chlorine concentrations of 0.5 and 5 mg/L, as recommended by the Australian Drinking Water Guidelines (ADWG) (NHMRC, 2011). The post-flush counterparts had a slightly higher residual and similar total chlorine levels ranging from 0.02-0.14 mg/L (n = 12, average 0.08 ± 0.06 mg/L) and 0.14-0.18 mg/L (n = 12, average 0.15 ± 0.03 mg/L), respectively. However, the free and total chlorine levels measured at this campus were not sufficiently restored after flushing for 30 minutes, and were still lower than the aforementioned regulated levels (NHMRC, 2011). High levels of turbidity were observed in the first flush samples for buildings affected by long-term stagnation (Buildings F and G had turbidity levels (n = 3, for each building) averaging 1.43 and 1.49 NTU, respectively), in comparison to the frequently-accessed counterpart, Building E (averaging 0.45 NTU). However, once the flushing regime was introduced, the post-flush turbidity levels for Buildings F and G reduced to 0.30 and 0.38 NTU, respectively.

3.2. Microbial growth in buildings affected by water stagnation

The levels of microbial growth within the buildings were quantitatively determined by live/dead staining combined with flow cytometry and heterotrophic plate counts (HPC). Increased levels of microbial growth were observed in buildings affected by stagnation (Figure 1). In the first flush samples collected from the urban campus, total tap water cell count density in buildings affected by stagnation (Building B, C, D) were up to ~10^5 cells/mL, similar to the total tap water cell counts observed in the frequently-accessed Building A. However, the buildings affected by different levels of stagnation had higher proportions of live cells, averaging ~10^3 cells/mL, in comparison to frequently-accessed buildings that had less live cells, at ~10^2 cells/mL.

These differences in terms of microbial growth in buildings impacted by stagnation were also supported by HPC, obtained by incubation at two different temperatures – at 30°C and 37°C (Table S4). In the urban campus, buildings impacted by stagnation (Buildings B, C, D) had ~10^4 CFU/mL when incubated at 30°C, in comparison to Building A which was accessed frequently during the lockdowns (~10° CFU/mL). Furthermore, Building D, which was affected by extended stagnation (up to 6 months) had the highest level of microbial growth, at 3.83 × 10^4 CFU/mL at 30°C and 9.50 × 10^4 CFU/mL at 37°C.

Higher levels of microbial growth were observed in the rural campus in comparison to the urban campus, regardless of the building occupancy classification (Figure 1 and Tables S4, S5, S6). Significant differences in the total proportion of live cells were observed. In particular, higher levels of live cells of up to ~10^4 cells/mL were detected in all the first flush samples. This observation was also noted in the HPC counts (Tables S5 and S6), where the first flush samples had on average ~10^2 CFU/mL at both incubation temperatures, regardless of building occupancy.

3.3. Microbiological indicators of OPs in first flush samples using qPCR

Culture-independent qPCR approach was used for detecting OPs of interest, such as *Legionella* spp., NTM, and *P. aeruginosa*. For both campuses, NTM was ubiquitously detected in all outlets and buildings sampled (Figure 2), while *P. aeruginosa* was not detected, or was below

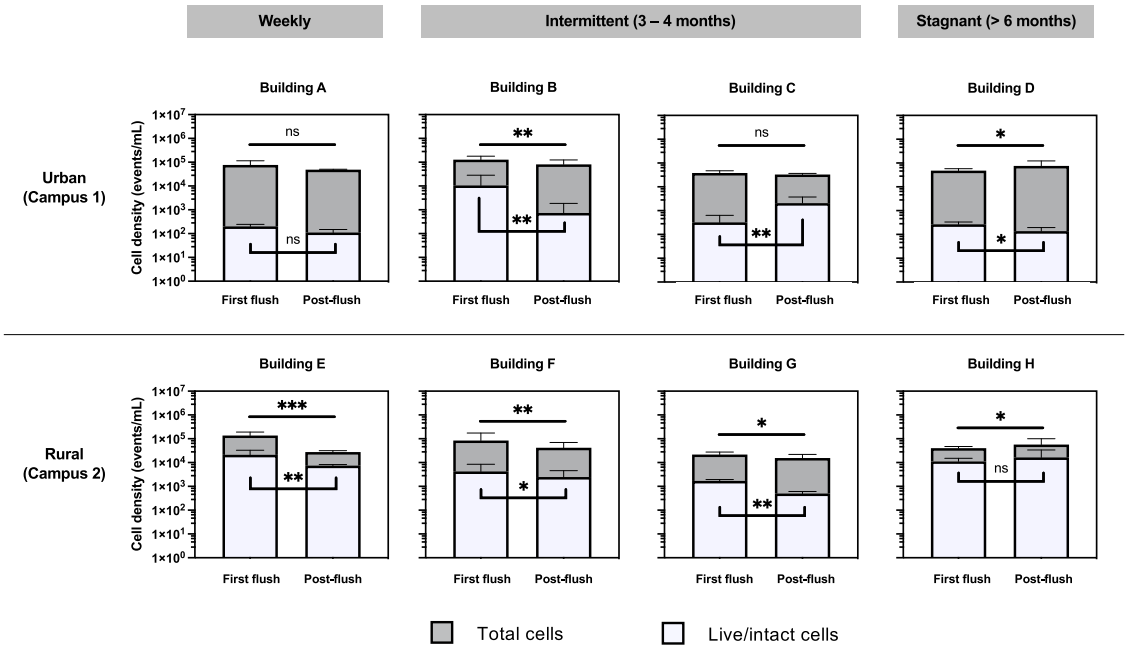

Figure 1. Microbial density measured by live and dead staining combined with flow cytometry, differentiated as subsets of live or intact cells, and total cells. Each figure represents the mean observations for the first and post-flush samples collected from the outlets (n = 3) for each building. Buildings A-D were located in the urban campus, whereas buildings E-H were located in the rural campus. Standard deviation was plotted as error bars. Statistical tests between first and post-flush samples were performed for each building using t tests, where statistical significance was denoted (*: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001), and non-statistical significance was denoted as ns.

the detection limit of the qPCR assays. In the urban campus, NTM was ubiquitously detected in all the outlets of the buildings. In particular, Building D (under extended stagnation of > 6 months) had the highest levels of NTM out of all the buildings, averaging 3.25 $\log_{10}$[estimated genomic copies/mL]. This was significantly different (p= 0.04) from the NTM levels found in the frequently-accessed Building A, averaging 1.7 $\log_{10}$[estimated genomic copies/mL]. In addition, *Legionella* spp. was selectively detected in outlets from Buildings B and D, but it was not detected in any of the other buildings. Contrastingly, NTM and *Legionella* spp. were both ubiquitously detected in all the outlets of the buildings in the rural campus. There were no specific trends with NTM levels in terms of building occupancy, with all buildings averaging 2.61 $\log_{10}$[estimated genomic copies/mL]. Similarly, there were no statistically significant differences in the *Legionella* levels amongst buildings, averaging 1.40 $\log_{10}$[estimated genomic copies/mL].

3.4. Sequencing and identification of NTM species

In addition to determining the total or the maximum concentration levels of OPs within the water using qPCR, culture-based detection was performed to identify viable OPs to species or strain level, particularly those that were human opportunistic pathogens. Based on morphology and the use of selective media, no *P. aeruginosa* or species corresponding to *Legionella* spp. were cultured from any of the water samples collected from either campus. Although *Legionella* was not detected through culture-based methods, it was detected using qPCR-based methods in several outlets on a genus level (Figure 2).

Several NTM species were cultured from buildings in both campuses, largely from first flush samples (Figure 3). It was observed that a number of these species corresponded to pathogenic species of concern, such as *M. kansasii* (Bailey et al., 1970, Luo et al., 2021), *M. szulgai* (Tsukamura, 1986), and *M. iranicum* (Shojaei et al., 2013). A small subset of isolates from the rural campus corresponded to *M. gordonae*, which is typically considered to be the least pathogenic and ubiquitous to both water and soil (Honda et al., 2018; Stout et al., 2016).

3.5. Microbial structure and taxonomic composition

Amplicon sequencing of the 16S rRNA gene was performed on the DNA extracted from the tap water samples collected. Taxonomic compositions of the microbial communities were determined up to the highest resolution possible – at genus level, where the top 20 genera are depicted in Figure 4.

Although both campuses were dominated by bacteria from the *Proteobacteria* phylum, it was observed that microbial compositions were highly distinct from one another. For the urban campus, human pathogens with biofilm-associated roles, including genera encompassing known and emerging OPs associated with healthcare settings corresponding to *Methylobacterium* (Kovaleva et al., 2014), *Sphingomonas* (Toh et al., 2011), and *Mycobacterium,* were highly abundant in the first flush sample subsets. Overall, *Methylobacterium* had the highest relative abundance (75%) amongst the buildings. Aside from *Mycobacterium, Legionella* and *Pseudomonas* were detected in low abundance and did not constitute the top 20 genera as part of the dataset.

Building A had the least amount of microbial diversity in comparison to all the other buildings that were subjected to various levels of stagnation (Figure 4A), and were largely dominated with *Methylobacterium* (75.3%), followed by *Mycobacterium* (7%) and *Sphingomonas* (3.2%). Furthermore, based on alpha diversity metrics (Table S7), the first flush samples from Building A had the lowest richness in terms of Shannon and Simpson indices (ranging from 1.11-1.83, and 0.37-0.67, respectively) and only had 34-53 observed OTUs.

Buildings affected by various levels of stagnation (B, C and D) had higher levels of microbial diversity, with *Methylobacterium, Porphyrobacter* and *Brevundimonas* as the dominant genera. This is supported by the alpha diversity metrics, where the observed OTUs from the samples of buildings affected by various levels of stagnation ranged 64-176, and Shannon and Simpson indices ranged from 1.95-4.07 and 0.66-0.92, respectively (Table S7).

Similarly, the rural campus (Figure 4B) was also dominated by bacteria belonging to the Phylum *Proteobacteria*. The first flush samples collected were largely dominated with *Sphingomonas, Brevudimonas* and

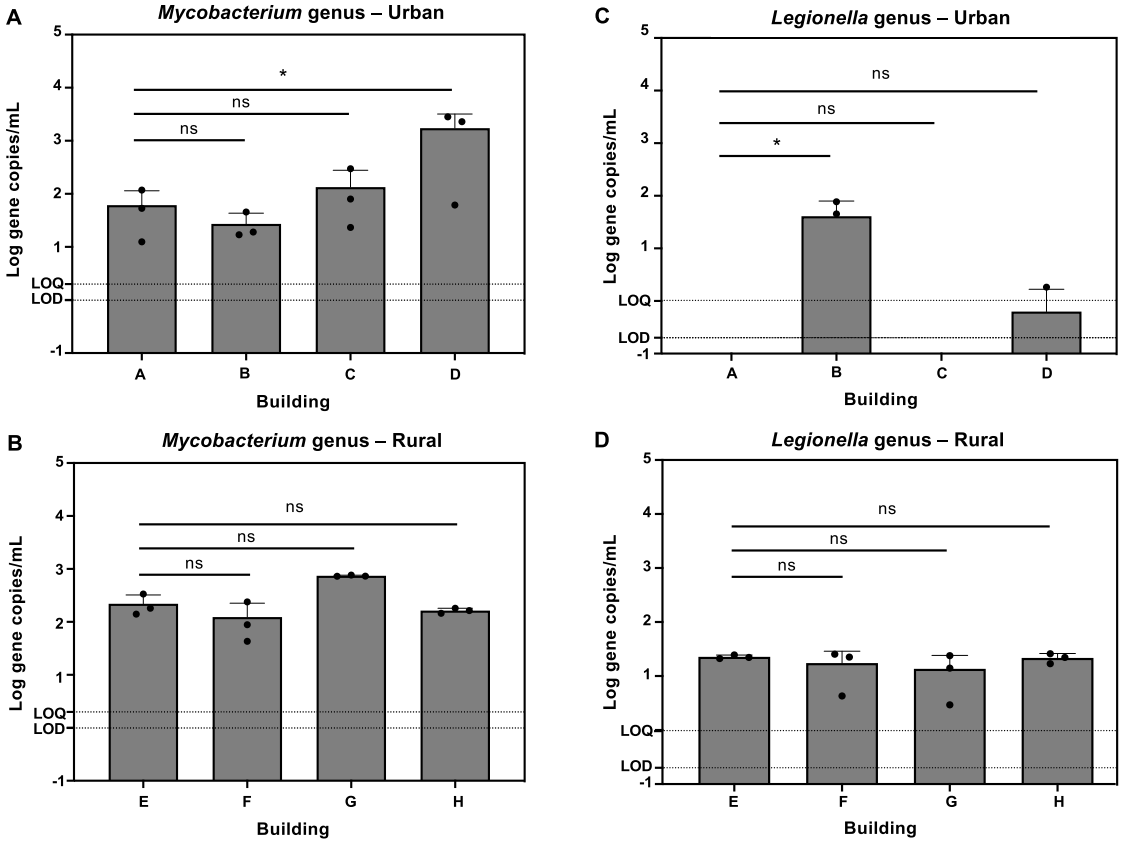

Figure 2. qPCR detection of *Mycobacterium* spp. and *Legionella* spp., for the buildings located in the urban campus (buildings A-D) and the rural campus (E-H) in the first flush samples. Each dot represents the mean (n = 3, analysed in triplicates) outcomes for an outlet sampled in each building (n = 3 outlets sampled in each building), shown as averages with standard deviation as the error bars. Statistical tests were performed with each building using the Mann-Whitney U / Wilcoxon non-parametric test, where statistical significance was denoted as * (p-value < 0.05), and non-statistical significance was denoted as ns. In the urban campus, *Legionella* spp. were only selectively detected in the first flush samples collected from buildings B and D, and were not detected in the outlets of buildings A and C.

Aquabacterium, which correspond to environmental genera typically associated with drinking water. Furthermore, based on alpha diversity metrics, all the first flush samples in this campus had higher microbial richness, supported by a higher number of OTUs observed (ranging from 195-395), Shannon and Simpson indices (3.76-4.00, and 0.81-0.93, respectively), regardless of the whether the buildings were influenced by stagnation (Table S8).

3.6. Microbial comparisons between first and post-flush samples

The post-flush samples collected from the buildings impacted by long-term stagnation had reduced HPC counts (Tables S4, S5, S6) and microbial cell density (Figure 1), for both the number of total and live cell populations. For example, both the total cell density and the live proportion in post-flush samples of Building B were statistically lower than that in the first flush samples (p-value < 0.05, Figure S3). Similarly, the post-flush samples of the buildings located in the rural campus also had similar trends, where both the total and live cell counts dropped at a statistically significant level for most of the buildings. However, the first flush and post-flush comparisons for Building H (under extended stagnation), particularly for the live cell populations were not statistically significant.

When comparing first and post-flush samples in the urban campus, it was observed that the qPCR levels of the pathogens of interest were variable (Figure S3). The levels of *Legionella* decreased post-flush for Building B and D in the urban campus (Figure S3C), and for most of the buildings in the rural campus (Figure S3D). Building F (in the rural campus), however, demonstrated a non-statistically significant increase in the *Legionella* levels in the post-flush samples. The reduction of NTM were not statistically significant (p-value = 0.04, t-tests) in post-flush samples in buildings affected by long-term stagnation in the urban campus (Buildings B, C, and D, Figure S3A), which were similar to the findings in the rural campus (Figure S3B).

3.7. Water samples cluster based on buildings, rather than by first or post-flush designations

Based on the principal coordinate analysis (PCoA) plots (Figures S4 and S5), it was observed that the samples clustered based on buildings. For example, all the samples (regardless of first or post-flush) originating from Buildings A (Figure S4) and E (Figure S5) clustered together based on their buildings for their respective plots, indicating high levels of similarity of taxonomic composition in the samples collected within each building. This finding was supported by the PERMANOVA pairwise comparisons of the various sample groups (Tables S9 and S10), where the microbial composition of the samples obtained from Building A were statistically significantly different (q-value < 0.05) from all the other buildings impacted by stagnation (Buildings B, C, D). This was similarly observed for the outcomes in the rural campus, where the microbial compositions of the samples from Building E were significantly different to the samples obtained from the other stagnation-impacted buildings (F and G); with the exception of Building H, where no statistical significance was observed (Table S9; q-value = 0.051).

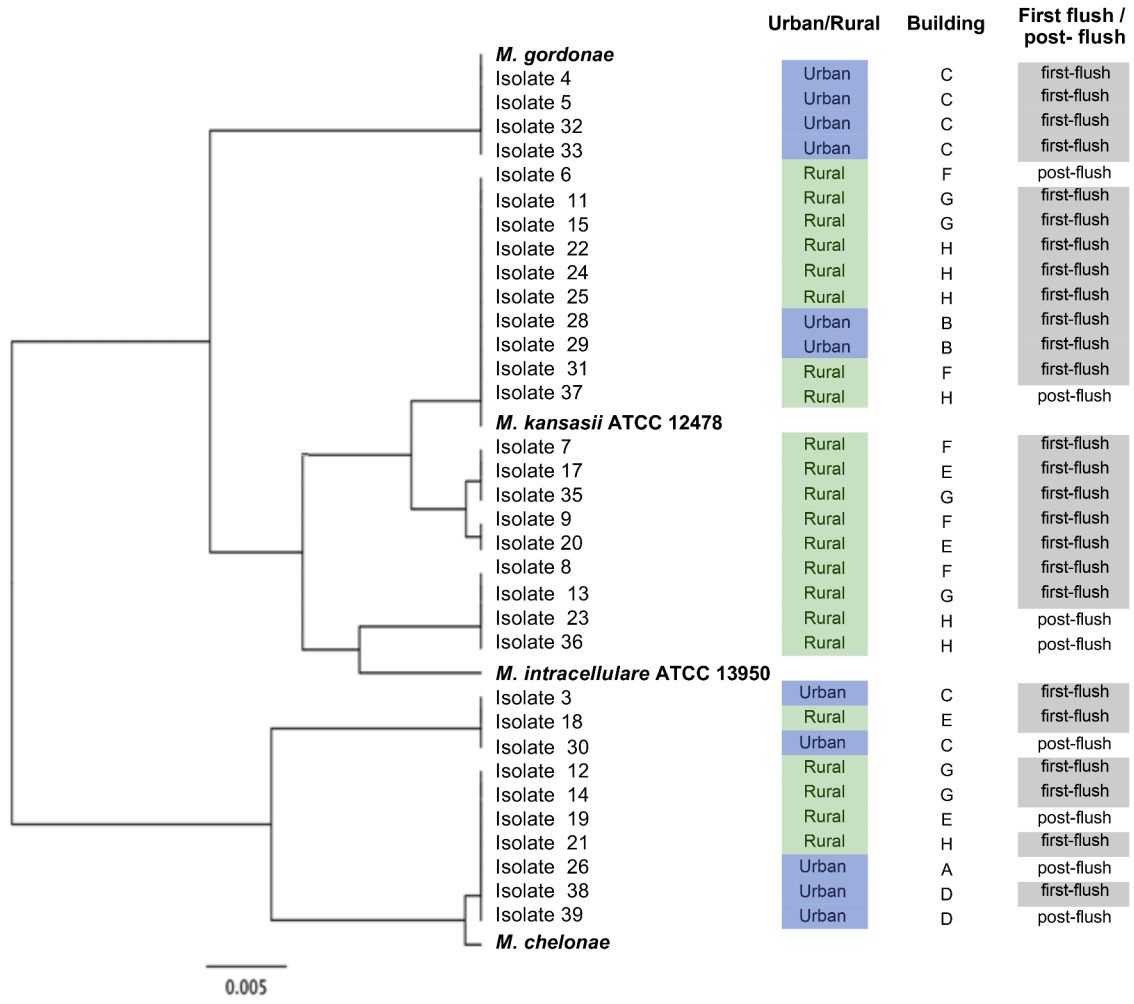

Figure 3. Phylogenetic tree of NTM isolates based on approximately 500 bp of the 16S rRNA gene sequences of isolates amplified using *Mycobacterium*-specific primers, BF-R2, using the UPGMA tree-building method, with a distance scale bar at the bottom indicating a branch length of 0.005. The data for the type and species/strains used as a reference for *Mycobacterium* were downloaded from NCBI GenBank and their descriptions and accession numbers are indicated in Table S11.

4. Discussion

Monitoring microbiological growth can be useful to assess water quality and evaluate the stability of the microbial ecosystems in premise plumbing systems, as they are sensitive to fluctuations of environmental conditions (Prest et al., 2016). Disruptions caused by the COVID-19 pandemic due to intermittent stagnation (ranging from 3-4 months and beyond) in these systems provided an opportunity to assess microbial growth and the risks posed by OPs. Understanding these microbial dynamics will support the development and implementation of water risk management plans prior to reopening and reoccupying these buildings (Proctor et al., 2020). Although several studies have examined the impacts of stagnation during the lockdowns, assessing representative OPs and waterborne bacterial pathogens in healthcare facilities (De Giglio et al., 2020; Grimard-Conea et al., 2022), limited studies have assessed the impact on non-healthcare public facilities such as university buildings and the approaches to deploy when returning to these facilities (Hozalski et al., 2020; Vosloo et al., 2023).

To expand upon the impacts on non-healthcare public buildings caused by stagnation during pandemic-related lockdowns, this study sought to gain a comprehensive understanding by evaluating buildings impacted by various levels of stagnation, in comparison to their frequently-accessed counterparts in two university campuses, situated in urban and rural locations. Using physicochemical parameters and both culture-dependent and -independent microbial analyses, it was observed that buildings impacted by intermittent stagnation (up to 3-4 months) had higher levels of microbial growth in terms of total cell concentrations. This could suggest that there was microbiome stability as the buildings were accessed frequently throughout the lockdown periods, in comparison to buildings impacted by various levels of stagnation, where fluctuations were caused by elevated levels of microbial growth and thus impacted taxonomic composition. This finding of microbial growth resulting in the instability of the drinking water microbiome in the plumbing of buildings has also been supported by previous studies that have assessed the impacts of long-term stagnation due to the COVID-19 lockdowns (Hozalski et al., 2020; Ye et al., 2021).

NTM were ubiquitous in all first and post-flush samples of all the outlets sampled in this study, using both culture-dependent and culture-independent microbiological methods. This includes isolating potentially pathogenic species of NTM, *M. kansasii*, which has been previously isolated from drinking water systems (Engel et al., 1980; Luo et al., 2021; Vaerewijck et al., 2005). In addition, the OPs of interest in this study and the ubiquity of NTM could be noted as the *Mycobacterium* genus was found as one of the top genera as part of the taxonomic composition. The prevalence of NTM in premise plumbing systems and their proliferation in these environments can be attributed to their biofilm-associating roles in distribution systems (Falkinham et al., 2015; Thomson et al., 2013b), and characteristics such as resistance to disinfectants and antibiotics (Wang et al., 2012).

On the other hand, *Legionella* was selectively detected in the first and

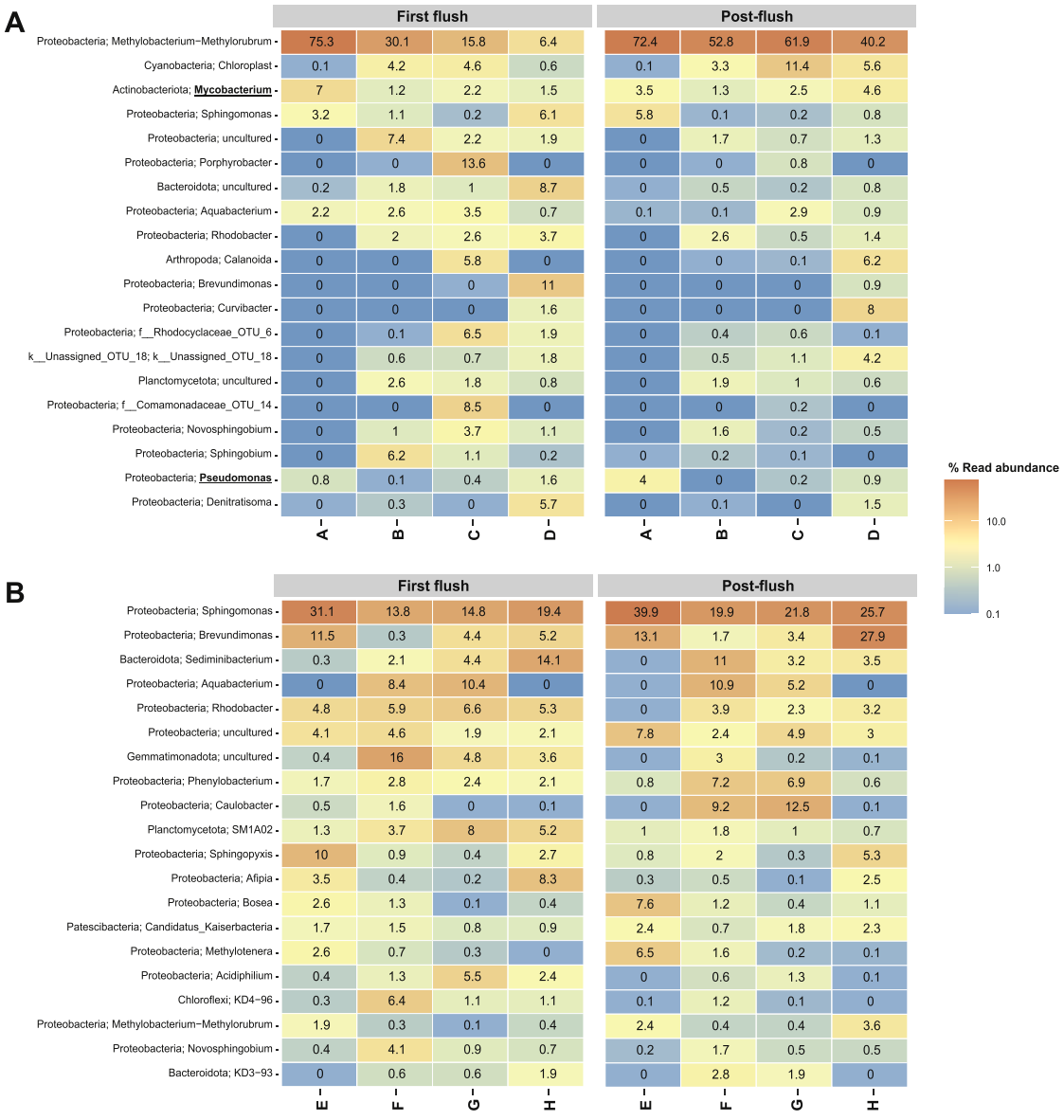

Figure 4. Taxonomic diversity composition of the first flush and post-flush tap water samples collected from the urban campus (A) and the rural campus (B), labelled by buildings. Heatmap of the relative abundance of the top 20 genera based on amplicon sequencing of the 16S rRNA gene targeting the V6-V8 and V3-V4 hypervariable region for the urban and the rural campuses, respectively. The taxonomy is labelled as phylum; genera, and relative abundances are indicated by the coloured scale (from blue, yellow to red). Genera that corresponded to the OPs of interest in the study, *Pseudomonas* and *Mycobacteria* (*Legionella spp.* were not part of the top 20 genera), are highlighted in bold and underlined.

post-flush samples of specific outlets collected in the urban campus, but was ubiquitously detected in all the samples from the rural campus using culture-independent qPCR approaches. The discrepancies between the culture-enumeration results and the qPCR approaches could be due to various factors, such as differences in the samples volumes analysed between both methods, as well as method-specific advantages and disadvantages. For example, populations of *Legionella* that may have entered a viable but non-culturable state (VBNC) (Whiley and Taylor, 2016), hence the number of culturable *Legionella* may be underestimated. On the other hand, whilst culture-independent qPCR methods were more sensitive than traditional culture-based methods, the main limitation was the technique's inability to discriminate between DNA which may also originate from non-viable cells and extracellular DNA that can persist in the environment (Nocker et al., 2009).

In addition to the OPs of interest, genera environmental bacterial genera, potential human pathogens with biofilm-associated roles including known and emerging OPs constituted the top genera as depicted in the taxonomic composition of the drinking water samples. In particular, *Methylobacterium* was the dominant genera in the urban campus, thus suggesting that this genus may have an integral role in seeding the drinking water infrastructure in the form of biofilms.

Furthermore, we examined the effectiveness of flushing as a remedial maintenance strategy in buildings. Levels of microbial concentration and OPs could be reduced after a 30-minute flush of the plumbing system of the buildings located in urban campuses, which was similar to the findings from previous studies that have sampled from urban campuses (Hozalski et al., 2020). These buildings typically had well-disinfected plumbing systems to resist the impacts caused by stagnation, as free and total chlorine levels were maintained or could be easily restored, which were consistent with the regulated free chlorine concentrations of

0.5 and 5 mg/L as recommended by the ADWG (NHMRC, 2011). However, there were some opposing trends where flushing caused an increase in microbial concentration and prevalence of OPs, as observed in Building C. This may be due to the dislodging of pre-existing biofilms within certain sections of the plumbing system (Declerck, 2010; Declerck et al., 2009; Lau and Ashbolt, 2009).

In addition, the effectiveness of flushing in the rural campus was not clear. Although microbial concentration was generally reduced after flushing, the reduction in the level of OPs was not statistically significant. Some first flush samples had high turbidity levels which exceeded the World Health Organisation (WHO) guidelines for turbidity levels of 1 NTU for drinking water. This could be caused by chemical and biological particles, such as by corrosion or the detachment of biofilms that had occurred during the long-term closure of the buildings. This suggests that the drinking water provided by the distribution system and the connected premise plumbing in the rural campus may not have been effectively disinfected and maintained at the time of collection. Despite turbidity levels reducing in the post-flush samples, free and total chlorine could not be restored back to the regulated chlorine concentrations of 0.5 mg/L and 5 mg/L (NHMRC, 2011). Collectively, this could indicate that there was low flow in the water distribution network in this rural campus and in large buildings with complex plumbing systems, where a 30-minute flush as recommended by local plumbing guidelines as a one-size fits all approach may not have fully removed the stagnant water within the system, requiring more time for fresh water containing residual disinfectant from the water mains to be delivered (Ling et al., 2018). This observation is similar to the findings reported from previous studies, where the recovery of the microbiological quality and the physicochemical parameters of water in buildings under long-term stagnation took 4-54 days to recover to routine levels (Ye et al., 2021), highlighting that, rather than short term remedial flushing, water demand had a key role in the recovery of the premise plumbing microbiome (Vosloo et al., 2023). As the water sampling took place during a snapshot in time, there were several limitations to the study. Some of these include: differences in the building sizes and networks, which would have contributed to the flushing regimes to be implemented, and the difficulties to draw comparisons between the buildings due to these underlying differences. There was no regular and ongoing monitoring of the outlets on a weekly or monthly basis to assess the fluctuations over time with the stability of the drinking water microbial communities, and there was no data collected prior to the lockdowns for the physicochemical parameters. In addition, there were limited number of outlets, buildings, and limited number of campuses that were surveyed and that all originated from a single drinking water network. Thus, further research would be invaluable to expand upon these limitations, to draw correlations between the heterogeneous microbial communities, chemical and physical parameters that occur within and between various premise plumbing systems for an improved understanding and effective management of these complex networks.

5. Conclusion

In summary, we found that buildings impacted by long-term stagnation, particularly buildings situated in rural areas, are at high risk for microbial growth and the proliferation of OPs. Further consideration, planning, and countermeasures should be put in place prior to reopening buildings to minimise potential health risks. Further research is required to draw correlations between the prevalence of OPs and building-specific biological, chemical, and physical factors. In addition, characterising clinically relevant species and strains, possible exposure routes, and determining the infectivity and dosage concentrations in these environments are required in order to develop rigorous microbial risk assessments models. In conjunction with designing building-specific water risk management plans, buildings impacted by stagnation situated in rural areas with low water demand should combine maintenance approaches with operational monitoring of physicochemical parameters such as free and total chlorine residuals, and turbidity, to evaluate whether flushing is an appropriate approach prior to reopening buildings.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

This study was supported by Queensland Health Leveraging Grant funds. JG would like to acknowledge the Australian Research Council for funding support through Australian Research Council Discovery Project (DP220101526). CKH would like to acknowledge scholarship support provided by the Australian Government Research Training Program (RTP), QAEHS and Water Research Australia (WaterRA – Project Number # 4533-17). JG, RT and SB would like to acknowledge the support provided by HeIDI and CSIRO IDR and AMR Mission-Affiliated Project Grant (HECSF-2021-007). We would like to thank Zhigang Yu and Lyman Ngiam, for their assistance and support with the water collections and flow cytometry, and Míriam Agulló Barceló for designing the F-16S2 and R-16S2 16S rRNA gene amplicon sequencing primers used in the study. We thank the members of the Respiratory Research Unit from the Gallipoli Medical Research Foundation (GMRF) and the Child Health Research Centre (CHRC) for all their guidance, expertise, and for the access of laboratory facilities for the NTM analyses. We would also like to thank Martin Bull, Chris Carney, Alison Moore, Michael Page, Barnaby Casson from the University of Queensland (UQ) for their insight and assistance with building access, surveying, and water sampling. Finally, we are grateful to the reviewers for their time and effort with providing detailed and valuable feedback, comments, and suggestions, which helped us to improve the quality of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2023.100201.

References

Andersen, K.S., Kirkegaard, R.H., Karst, S.M. and Albertsen, M. 2018. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. bioRxiv, 299537.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26 (1), 32–46.

Bailey, R.K., Wyles, S., Dingley, M., Hesse, F., Kent, G.W., 1970. The isolation of high catalase Mycobacterium kansasii from tap water. Am Rev Respir Dis 101 (3), 430–431.

Bartley, P.B., Ben Zakour, N.L., Stanton-Cook, M., Muguli, R., Prado, L., Garnys, V., Taylor, K., Barnett, T.C., Pinna, G., Robson, J., Paterson, D.L., Walker, M.J., Schembri, M.A., Beatson, S.A., 2016. Hospital-wide Eradication of a Nosocomial Legionella pneumophila Serogroup 1 Outbreak. Clin Infect Dis 62 (3), 273–279.

Bedard, E., Prevost, M., Deziel, E., 2016. Pseudomonas aeruginosa in premise plumbing of large buildings. Microbiologyopen 5 (6), 937–956.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodriguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T.,

Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson 2nd, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vazquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37 (8), 852–857.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55 (4), 611–622.

Carter, R., 2018. An investigation of the genotypes for Mycobacterium intracellulare in Queensland. Masters by Research thesis. Queensland University of Technology.

Chakravorty, S., Helb, D., Burday, M., Connell, N., Alland, D., 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69 (2), 330–339.

Declerck, P., 2010. Biofilms: the environmental playground of Legionella pneumophila. Environ Microbiol 12 (3), 557–566.

Declerck, P., Behets, J., Margineanu, A., van Hoef, V., De Keersmaecker, B., Ollevier, F., 2009. Replication of Legionella pneumophila in biofilms of water distribution pipes. Microbiol Res 164 (6), 593–603.

De Giglio, O., Diella, G., Lopuzzo, M., Triggiano, F., Calia, C., Pousis, C., Fasano, F., Caggiano, G., Calabrese, G., Rafaschieri, V., Carpagnano, F., Carlucci, M., Gesualdo, L., Ricci, M.L., Scaturro, M., Rota, M.C., Bonadonna, L., Lucentini, L., Montagna, M.T., 2020. Impact of lockdown on the microbiological status of the hospital water network during COVID-19 pandemic. Environmental Research 191, 110231.

Engel, H.W., Berwald, L.G., Havelaar, A.H., 1980. The occurrence of Mycobacterium kansasii in tapwater. Tubercle 61 (1), 21–26.

Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., Hugenholtz, P., 2010. Experimental factors affecting PCR-based estimates of microbial species richness and evenness. The ISME Journal 4 (5), 642–647.

Falkinham, J.O., 2015. Common Features of Opportunistic Premise Plumbing Pathogens. Int J Env Res Pub He 12 (5), 4533–4545.

Falkinham 3rd, J.O., 2011. Nontuberculous mycobacteria from household plumbing of patients with nontuberculous mycobacteria disease. Emerg Infect Dis 17 (3), 419–424.

Falkinham 3rd, J.O., Hilborn, E.D., Arduino, M.J., Pruden, A., Edwards, M.A., 2015. Epidemiology and Ecology of Opportunistic Premise Plumbing Pathogens: Legionella pneumophila, Mycobacterium avium, and Pseudomonas aeruginosa. Environ Health Perspect 123 (8), 749–758.

Falkinham 3rd, J.O., Norton, C.D., LeChevallier, M.W., 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67 (3), 1225–1231.

Feazel, L.M., Baumgartner, L.K., Peterson, K.L., Frank, D.N., Harris, J.K., Pace, N.R., 2009. Opportunistic pathogens enriched in showerhead biofilms. Proceedings of the National Academy of Sciences 106 (38), 16393–16399.

Fedrizzi, T., Meehan, C.J., Grottola, A., Giacobazzi, E., Fregni Serpini, G., Tagliazucchi, S., Fabio, A., Bettua, C., Bertorelli, R., De Sanctis, V., Rumpianesi, F., Pecorari, M., Jousson, O., Tortoli, E., Segata, N., 2017. Genomic characterization of Nontuberculous Mycobacteria. Scientific Reports 7 (1), 45258.

Grimard-Conea, M., Deshommes, E., Doré, E., Prévost, M., 2022. Impact of recommissioning flushing on Legionella pneumophila in a large building during the COVID-19 pandemic. Front Water 4.

Honda, J.R., Virdi, R., Chan, E.D., 2018. Global Environmental Nontuberculous Mycobacteria and Their Contemporaneous Man-Made and Natural Niches. Frontiers in Microbiology 9.

Hozalski, R.M., LaPara, T.M., Zhao, X., Kim, T., Waak, M.B., Burch, T., McCarty, M., 2020. Flushing of Stagnant Premise Water Systems after the COVID-19 Shutdown Can Reduce Infection Risk by Legionella and Mycobacterium spp. Environ Sci Technol 54 (24), 15914–15924.

ISO 1999. ISO 6222:1999 Water quality — Enumeration of culturable micro-organisms — Colony count by inoculation in a nutrient agar culture medium.

ISO 2006. ISO 16266:2006 Water quality — Detection and enumeration of Pseudomonas aeruginosa — Method by membrane filtration.

ISO 2017. ISO 11731:2017 Water quality — Enumeration of Legionella.

Kovaleva, J., Degener, J.E., van der Mei, H.C., 2014. Methylobacterium and its role in health care-associated infection. J Clin Microbiol 52 (5), 1317–1321.

Lau, H.Y., Ashbolt, N.J., 2009. The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. Journal of Applied Microbiology 107 (2), 368–378.

LeChevallier, M.W., Gullick, R.W., Karim, M.R., Friedman, M., Funk, J.E., 2003. The potential for health risks from intrusion of contaminants into the distribution system from pressure transients. J Water Health 1 (1), 3–14.

Liang, J., Swanson, C.S., Wang, L., He, Q., 2021. Impact of building closures during the COVID-19 pandemic on Legionellainfection risks. American Journal of Infection Control 49 (12), 1564–1566.

Ling, F., Whitaker, R., LeChevallier, M.W., Liu, W.T., 2018. Drinking water microbiome assembly induced by water stagnation. ISME J 12 (6), 1520–1531.

Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R., 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. Appl Environ Microbiol 73 (5), 1576–1585.

Luo, T., Xu, P., Zhang, Y., Porter, J.L., Ghanem, M., Liu, Q., Jiang, Y., Li, J., Miao, Q., Hu, B., Howden, B.P., Fyfe, J.A.M., Globan, M., He, W., He, P., Wang, Y., Liu, H., Takiff, H.E., Zhao, Y., Chen, X., Pan, Q., Behr, M.A., Stinear, T.P., Gao, Q., 2021. Population genomics provides insights into the evolution and adaptation to humans of the waterborne pathogen Mycobacterium kansasii. Nat Commun 12 (1), 2491.

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE 8 (4), e61217.

Mercante, J.W., Winchell, J.M., 2015. Current and emerging Legionella diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev 28 (1), 95–133.

Michener, C.D., Sokal, R.R., 1957. A quantitative approach to a problem in classification1. Evolution 11 (2), 130–162.

National Academies of Sciences, E. and Medicine, 2020. Management of Legionella in Water Systems. The National Academies Press, Washington, DC.

NHMRC 2011. Australian Drinking Water Guidelines 6. National Water Quality Management Strategy.

Nocker, A., Mazza, A., Masson, L., Camper, A.K., Brousseau, R., 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J Microbiol Methods 76 (3), 253–261.

Oksanen, J. 2011. Vegan: community ecology package. R package version 1.17-9. https://cran.r-project.org/package=vegan.

Prest, E.I., Hammes, F., van Loosdrecht, M.C., Vrouwenvelder, J.S., 2016. Biological Stability of Drinking Water: Controlling Factors. Methods, and Challenges. Front Microbiol 7, 45.

Proctor, C.R., Reimann, M., Vriens, B., Hammes, F., 2018. Biofilms in shower hoses. Water Res 131, 274–286.

Proctor, C.R., Rhoads, W.J., Keane, T., Salehi, M., Hamilton, K., Pieper, K.J., Cwiertny, D. M., Prevost, M., Whelton, A.J., 2020. Considerations for Large Building Water Quality after Extended Stagnation. AWWA Water Sci e1186.

Prussin 2nd, A.J., Schwake, D.O., Marr, L.C., 2017. Ten Questions Concerning the Aerosolization and Transmission of Legionella in the. Built Environment. Build Environ 123, 684–695.

Reasoner, D.J., 2004. Heterotrophic plate count methodology in the United States. Int J Food Microbiol 92 (3), 307–315.

RStudio. 2020. RStudio: Integrated Development for R.

Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., Connor, R., Funk, K., Kelly, C., Kim, S., Madej, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z., Thibaud-Nissen, F., Murphy, T., Phan, L., Skripchenko, Y., Tse, T., Wang, J., Williams, R., Trawick, B.W., Pruitt, K.D., Sherry, S.T., 2022. Database resources of the national center for biotechnology information. Nucleic Acids Res 50 (D1), D20–d26.

Shojaei, H., Daley, C., Gitti, Z., Hashemi, A., Heidarieh, P., Moore, E.R.B., Naser, A.D., Russo, C., van Ingen, J., Tortoli, E., 2013. Mycobacterium iranicum sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents. Int J Syst Evol Microbiol 63 (Pt 4), 1383–1389.

Standard Methods for the Examination of Water and Wastewater, 2017. Standard Methods For the Examination of Water and Wastewater. American Public Health Association, Washington, D.C.

Stout, J.E., Koh, W.J., Yew, W.W., 2016. Update on pulmonary disease due to non-tuberculous mycobacteria. International Journal of Infectious Diseases 45, 123–134.

Thomson, R., Tolson, C., Sidjabat, H., Huygens, F., Hargreaves, M., 2013a. Mycobacterium abscessus isolated from municipal water - a potential source of human infection. BMC Infect Dis 13, 241.

Thomson, R.M., Carter, R., Tolson, C., Coulter, C., Huygens, F., Hargreaves, M., 2013b. Factors associated with the isolation of Nontuberculous mycobacteria (NTM) from a large municipal water system in Brisbane, Australia. BMC Microbiol 13, 89.

Toh, H.S., Tay, H.T., Kuar, W.K., Weng, T.C., Tang, H.J., Tan, C.K., 2011. Risk factors associated with Sphingomonas paucimobilis infection. Journal of Microbiology, Immunology and Infection 44 (4), 289–295.

Tsukamura, M., 1986. Differentiation of Mycobacterium gordonae from Mycobacterium scrofulaceum and Mycobacterium szulgai by susceptibility to enoxacin (antimycobacterial activity of enoxacin). Microbiol Immunol 30 (9), 931–933.

Vaerewijck, M.J., Huys, G., Palomino, J.C., Swings, J., Portaels, F., 2005. Mycobacteria in drinking water distribution systems: ecology and significance for human health. FEMS Microbiol Rev 29 (5), 911–934.

van der Mee-Marquet, N., Domelier, A.S., Arnault, L., Bloc, D., Laudat, P., Hartemann, P., Quentin, R., 2006. Legionella anisa, a possible indicator of water contamination by Legionella pneumophila. J Clin Microbiol 44 (1), 56–59.

Vosloo, S., Huo, L., Chauhan, U., Cotto, I., Gincley, B., Vilardi, K.J., Yoon, B., Bian, K., Gabrielli, M., Pieper, K.J., Stubbins, A., Pinto, A.J., 2023. Gradual Recovery of Building Plumbing-Associated Microbial Communities after Extended Periods of Altered Water Demand during the COVID-19 Pandemic. Environ Sci Technol 57 (8), 3248–3259.

Wang, H., Bedard, E., Prevost, M., Camper, A.K., Hill, V.R., Pruden, A., 2017. Methodological approaches for monitoring opportunistic pathogens in premise plumbing: A review. Water Res 117, 68–86.

Wang, H., Edwards, M., Falkinham 3rd, J.O., Pruden, A., 2012. Molecular survey of the occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa, and amoeba hosts in two chloraminated drinking water distribution systems. Appl Environ Microbiol 78 (17), 6285–6294.

Wang, H., Masters, S., Falkinham, J.O., Edwards, M.A., Pruden, A., 2015. Distribution System Water Quality Affects Responses of Opportunistic Pathogen Gene Markers in Household Water Heaters. Environmental Science & Technology 49 (14), 8416–8424.

Whiley, H., Keegan, A., Giglio, S., Bentham, R., 2012. Mycobacterium avium complex–the role of potable water in disease transmission. J Appl Microbiol 113 (2), 223–232.

Whiley, H., Taylor, M., 2016. Legionella detection by culture and qPCR: Comparing apples and oranges. Crit Rev Microbiol 42 (1), 65–74.
Wickham, H., 2011. ggplot2 WIREs Computational Statistics 3 (2), 180–185.
Ye, C., Xian, X., Bao, R., Zhang, Y., Feng, M., Lin, W., Yu, X., 2021. Recovery of microbiological quality of long-term stagnant tap water in university buildings during the COVID-19 pandemic. Sci Total Environ 806 (Pt 2), 150616.