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Occurrence and diversity of nontuberculous mycobacteria affected by water stagnation in building plumbing

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ABSTRACT

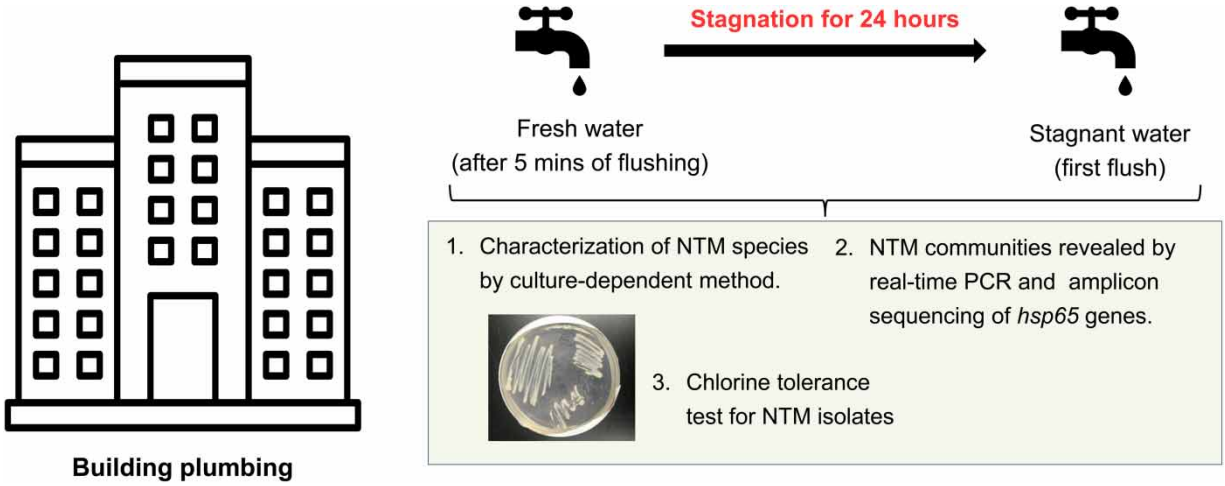
Nontuberculous mycobacteria (NTM) refer to mycobacteria other than *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Some NTM are known to cause pulmonary and skin diseases. As NTM are prevalent in water supply systems, the health burden of waterborne NTM is concerned. However, little is known about NTM in building plumbing. This study revealed the abundance and diversity of NTM in building plumbing by using culture-dependent and culture-independent approaches. Moreover, the chlorine susceptibility of NTM isolates in tap water was evaluated. The increase in the abundance of *Mycobacterium* spp. was observed in some taps after 24 h of stagnation, indicating that NTM could have the potential to regrow in building plumbing. While *Mycobacterium* mucogenicum and *Mycobacterium* avium were detected by cultivation, amplicon sequencing of NTM-specific hsp65 genes revealed that *Mycobacterium* gordonae was dominant in most of the samples, which was not detected by cultivation. The chlorine susceptibility experiment demonstrated that NTM strains related to *M. avium* and *M. mucogenicum* were 119 and 426 times more tolerant to chlorine than *Escherichia coli*, respectively. As *M. mucogenicum*, *M. avium*, and *M. gordonae* are regarded as opportunistic pathogens, intensive monitoring of NTM in tap water in building plumbing is necessary.

Key words: building plumbing, chlorine tolerance, nontuberculous mycobacteria, water stagnation

HIGHLIGHTS

- NTM in building plumbing had the potential to increase after water stagnation.
- Potentially pathogenic *M. avium* and *M. mucogenicum* were isolated from building plumbing, which were more tolerant to chlorine than *E. coli*.
- Integration of both culture-dependent and culture-independent approaches is necessary for a comprehensive understanding of NTM diversity in building plumbing.

GRAPHICAL ABSTRACT



INTRODUCTION

Nontuberculous mycobacteria (NTM) are environmental mycobacteria in soil and water other than *Mycobacterium tuberculosis* and *Mycobacterium leprae*, and some species are known as opportunistic pathogens (Falkinham *et al.* 2015). Exposure to pathogenic NTM through inhalation, ingestion, and skin contact can result in pulmonary and skin/soft tissue infection, particularly for individuals with compromised immune systems (Hamilton *et al.* 2017). The incidence of NTM infection has increased in many countries, including Japan, leading to a significant healthcare burden, particularly among the elderly population (Harada *et al.* 2020). Waterborne transmission of NTM is a major concern, and the Centers for Disease Control and Prevention (CDC) has reported a high number of hospitalizations and deaths related to waterborne NTM infections in the U.S. between 2010 and 2015 (Collier *et al.* 2021). Nosocomial NTM infection was related to the occurrence of NTM in the hospital water supply system (Zlojtro *et al.* 2014). A significant correlation was also found between the abundance of pathogenic NTM species (e.g., *Mycobacterium avium* complex and *Mycobacterium abscessus* complex) in household showerhead biofilm and the prevalence of NTM disease from medicare beneficiaries and cystic fibrosis patients (Gebert *et al.* 2018). These studies highlighted the necessity to control NTM in water supply systems.

While culture-dependent methods have been established for several NTM species in drinking water (Le Dantec *et al.* 2002; Pfaller *et al.* 2022), NTM cultivation can be time-consuming, especially for slow-growing species. In contrast, culture-independent characterization using next-generation sequencing has emerged as a valuable tool to comprehensively assess the diversity of NTM communities in drinking water (van der Wielen *et al.* 2013; Gebert *et al.* 2018; Waak *et al.* 2019). For NTM characterization, the use of *hsp65* genes instead of 16S rRNA genes has demonstrated enhanced resolution for species identification of NTM (van der Wielen *et al.* 2013). Amplicon sequencing of mycobacterial *hsp65* genes has provided valuable insights, such as the predominance of *Mycobacterium gordonae* in a chlorinated drinking water distribution system (Waak *et al.* 2019). Considering that NTM is resistant to chlorine (Taylor *et al.* 2000; Wang *et al.* 2019) and has the potential for regrowth during water stagnation in building plumbing (Rahmatika *et al.* 2022), it is possible that disinfection conditions can influence the diversity of NTM in drinking water.

Despite *M. avium* and *M. abscessus* being listed on the contaminant candidate list (CCL-5) for drinking water (USEPA 2021), our understanding of the abundance, diversity, and characteristics of NTM in building plumbing is still limited due to the lack of routine monitoring for NTM. While the culture-independent method can provide valuable information on NTM, the throughput is low and it generally takes a long time for cultivation of NTM. On the other hand, culture-independent methods have the advantage of rapid and comprehensive analysis. Therefore, the integration of both culture-dependent and culture-independent approaches is useful for monitoring NTM. This study aims to characterize NTM in tap water in building plumbing by using culture-dependent and culture-independent methods. Additionally, the chlorine tolerance of indigenous NTM strains isolated from tap water was also evaluated.

METHODS

Sample collection

Fresh water (FW) and stagnant water (SW) samples were collected from four taps (A1–A4) in the buildings of the National Institute of Public Health in Saitama Prefecture, Japan (site A) in December 2020 (Supplementary material, Table S1). NTM in the samples were analyzed with culture-dependent and culture-independent approaches. Hot water was supplied at A1, while cold water was supplied at the other taps. Site A receives mixed drinking water derived from two plants: one plant treats surface water, while the other plant treats groundwater. From April 2020 to March 2021, standard plate counts (37 °C for 24 h) and heterotrophic plate counts (HPC, 20 °C for 7 days) were 0 and *Escherichia coli* were not detected in the mixed finished water from these plants, according to an annual water quality report of the waterworks. The faucets were flushed for 5 min at a flow rate of approximately 5 L/min before collecting the FW samples (4 L). The faucets were subsequently closed for 24 h. After 24 h of stagnation, the first 4 L of SW samples were immediately collected from the same faucets with the same flow rate. For reference, the groundwater used for non-potable use at site A was also collected to reveal NTM diversity. Additional SW samples were collected in the same manner from three faucets (B1–B3) at The University of Tokyo in Tokyo, Japan (site B) in four different seasons: Summer (May 2018), Autumn (November 2018), Winter (January 2019), and Spring (April 2019) (Rahmatika *et al.* 2022). Tap water in site B was supplied from a water treatment plant that treats surface water by advanced treatment using ozonation and biologically activated carbon filtration. The first 1 L of SW samples was collected after 24 h of stagnation from each faucet. These samples were used only for analyzing the diversity of NTM communities using the culture-independent method.

Water temperatures were measured on-site by a thermometer. Free chlorine was measured by *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (Hach, CO, USA) with a limit of quantification (LOQ) of 0.02 mg/L.

Cultivation and isolation of NTM

FW and SW samples as well as groundwater collected from site A were subjected to NTM cultivation. The samples were incubated with 0.04% cetylpyridinium chloride (CPC) (Sigma-Aldrich, MO, USA) for 30 min at room temperature to eliminate other microorganisms (Schulze-Röbbecke *et al.* 1991). Tap water samples (650 mL) and groundwater samples (1.3 L) were filtered through 0.45-μm EZ-Fit™ filtration unit (Merk-Millipore, MA, USA) in triplicate. The membrane filters were then rinsed with autoclaved Milli-Q water to remove the residual CPC. The first and second membrane filters were transferred to Middlebrook 7H10 agar (Becton, Dickinson and Company, NJ, USA) and R2A agar (Becton, Dickinson and Company), respectively. Middlebrook 7H10 agar contained 10% OADC enrichment (Becton, Dickinson, and Company) and 10% MGIT PANTA (Becton, Dickinson, and Company). These plates were incubated at 30–32 °C until the colonies were formed. The third membrane filter was preincubated in Middlebrook 7H9 broth (Becton, Dickinson, and Company) containing 10% OADC enrichment and 10% MGIT PANTA. The cultivation is conducted in aerobic conditions. After one week of enrichment, the broth was spread on 2% Ogawa medium (Kyokuto Pharmaceutical, Japan) and was incubated at 30–32 °C until the colonies were formed. Following 17–23 days of incubation, representative colonies were picked and subcultured using Middlebrook 7H10 agar for colony direct PCR and sequencing analysis.

Identification of NTM isolates

Colony-direct PCR was used to determine 16S rRNA genes and *hsp65* genes of the isolates. Amplification of 16S rRNA genes was performed with the primer set of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Heuer *et al.* 1997). Moreover, the primer set of Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTTGTCGAACCGCATACCCT-3′) were used to amplify *hsp65* genes of NTM (Waak *et al.* 2019). Each PCR reaction mixture (50 μL) was prepared using TaKaRa EX Taq HS version (TaKaRa Bio, Japan), consisting of 40.6 μL of nuclease-free water (Ambion Nuclease-Free Water, Invitrogen, MA, USA), 5.0 μL of 10× PCR buffer, 4.0 μL of 10 mM dNTPs, 0.25 μL of TaKaRa EX Taq polymerase, and 0.1 μL of each primer (100 pmol/μL). A small amount of a single colony was directly added to the reaction mixture. The thermal conditions for 16S rRNA genes were set as follows: 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min (Heuer *et al.* 1997). For *hsp65* genes, the thermal condition consisted of initial denaturation at 98 °C for 2 min, followed by 30 cycles at 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min (Waak *et al.* 2019). The final extension step at 72 °C for 10 min was added for both reactions. PCR products were purified by a MinElute PCR Purification kit (QIAGEN, Germany) and submitted to FASMAC (Japan) for sanger sequencing. A sequence homology search was conducted using BLAST. Sequences of NTM isolates were aligned using MUSCLE (Edgar 2004),

and the phylogenetic tree was generated using the Neighbor-Joining method by MEGAX software (Kumar *et al.* 2018). The reference sequences of characterized NTM obtained from the GenBank database were added to the phylogenetic tree. The sequences of 16S rRNA genes and *hsp65* genes of the isolates have been deposited on DDBJ/EMBL/GenBank databases under accession numbers LC637767–LC637776 and LC637933–LC637942, respectively.

DNA extraction

To harvest bacteria in the samples, 2 L (tap and groundwater samples from site A) and 1 L (tap water samples from site B) of samples were filtered through 0.2-μm polycarbonate filters (Isopore™, Merk-Millipore). DNA was extracted directly from the filter by FastDNA SPIN Kit for Soil following the manufacturer's instructions (MP Biomedicals, OH, USA). Before extraction, the filters were dissolved in phenol-chloroform-isoamyl alcohol (25:24:1) (Nippon Gene, Japan). The DNA concentration and purity (absorbance ratio at 260 nm/280 nm) were measured by Qubit Fluorometer (ThermoFischer Scientific, MA, USA) and NanoDrop spectrophotometer (ThermoFischer Scientific), respectively.

Real-time PCR

For FW and SW samples collected from site A, Myco637f (5′- GGGCGATACGGGCAGAC-3′), Myco841r (5′- GAAACCCA-CACCTAGTACC-3′), and Myco662p (5′-FAM-CAGGGGAGACTGGAATTCCTGGTGTA-MGBNFQ-3′) were used for quantification of 16S rRNA genes of *Mycobacterium* spp. with TaqMan-based real-time PCR (Feazel *et al.* 2009). The thermal condition consisted of initial denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 1 min (Feazel *et al.* 2009). All reactions were prepared in triplicate by LightCycler® Probes Master solution (Roche, Switzerland) and analyzed using a LightCycler® LC480 system (Roche). Standards were prepared using an artificially synthesized plasmid containing 16S rRNA genes of *Mycobacterium* spp. with the concentration of 5.0×10^{0}–5.0×10^{6} gene copies/μL. The LOQ was 2.5×10^{-1} gene copies/mL. To evaluate the significant difference ($p < 0.05$) in the abundance of *Mycobacterium* spp. between FW and SW, paired student's *t*-test was performed for log-transformed data by R statistical software (ver. 3.4.1).

Amplicon sequencing of *hsp65* genes

The diversity of NTM communities in FW and SW samples from site A and SW samples from site B was characterized using amplicon sequencing targeting *hsp65* genes. The fragments of *hsp65* genes (441 bp) were amplified with Tb11 and Tb12 with an adapter sequence (Waak *et al.* 2019). Amplicon sequencing was conducted on an Illumina MiSeq platform at the Bioengineering Lab (Japan). Sequences generated from the Illumina MiSeq analysis were processed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) (Bolyen *et al.* 2019). After eliminating noise and chimera sequences, the feature-classifier (QIIME 2 plugin) was used to affiliate OTUs to an in-house database of 149 different *hsp65* genes of *Mycobacterium* spp. Operational taxonomic units (OTUs) were clustered with 100% identity. To evaluate the significant difference of NTM communities between samples in sites A and B, analysis of similarity (ANOSIM) was performed based on the Bray–Curtis dissimilarity index at the OTU level using vegan package (ver. 2.5-7) in R statistical software (ver. 3.4.1). The sequences of representative OTUs were analyzed using a phylogenetic tree together with the sequences of the isolates. All data obtained from amplicon sequencing were submitted to the DDBJ Sequence Read Archive under accession number DRA012200.

Chlorine tolerance test

Two NTM strains isolated from site A (T1 and T8) were subjected to a chlorine tolerance test. Strain T1 (Accession number: LC637941) and T8 (Accession number: LC637937) were incubated in Middlebrook 7H9 broth (Becton, Dickinson Company) containing 10% OADC enrichment 30 °C for 7 days. In addition, *Escherichia coli* strain K-12 as a reference was incubated in LB broth (Becton, Dickinson Company) overnight. The cultures were then collected by centrifugation (6,000× *g* for 10 min) and washed with phosphate-buffered saline (PBS) three times, then finally suspended in PBS. Triplicate sets of cell suspension in PBS (10^{5} CFU/mL) were prepared in 300 mL chlorine demand-free Erlenmeyer flasks for chlorine disinfection test. Free chlorine with a concentration of 0.60–0.85 mg/L was added to the cell suspension. The experiments were performed at room temperature with gentle stirring (60 rpm). 10 mL of samples were collected during the experiment to measure free chlorine residual by DPD colorimetric method (Hach, Colorado, USA). In parallel, samples were also collected and quenched with sodium thiosulfate to enumerate NTM on Middlebrook 7H10 agar containing 10% OADC enrichment or *E. coli* on LB agar. Log reduction values of the NTM strains and *E. coli* were summarized with Ct value which is defined as the product of free chlorine concentration (mg/L) and contact time (min).

RESULTS AND DISCUSSION

Changes in NTM abundance during water stagnation

The increase in temperature and decrease of free chlorine was observed after 24 h of water stagnation in the samples in site A (A1–A4) (Supplementary material, Table S1). 16S rRNA genes of *Mycobacterium* spp. were quantified for samples collected from A1 to A4 (Figure 1). FW contained 1.3×10–4.8×10^2 gene copies/mL. After 24 h of stagnation, the abundances were 3.9×10–2.5×10^2 gene copies/mL. The apparent increase of *Mycobacterium* spp. was observed at A1–A3 and the increases at A2 and A3 were significant (Student's *t*-test; $p < 0.05$). The abundance slightly decreased after stagnation at A4 where NTM abundance before stagnation was higher compared to the others. The initial abundance before stagnation and the regrowth potential of NTM after stagnation may depend on water usage frequency and various factors such as temperature and pipe condition. The abundance of 16S rRNA genes of NTM in the groundwater sample was 2.0×10 gene copies/mL, indicating that NTM abundance in groundwater was less than that in stagnant tap water. It is possible that NTM could adapt more in building plumbing environments. The concentration of NTM at site B before and after stagnation in different seasons was 2.5×10^0 and 8.8×10 gene copies/mL, respectively, indicating that regrowth after stagnation was reproducibly observed (Rahmatika *et al.* 2022). The increase in NTM abundance after stagnation can be attributed to their characteristics, such as resistance to chlorine, biofilm formation ability, and association with free-living amoeba (Falkinham *et al.* 2015). The level of *Mycobacterium* spp. after stagnation in site A and site B was almost equivalent to the abundance of 16S rRNA genes of *Mycobacterium* spp. in the chlorinated drinking water in Paris (average: 1.3×10^2 gene copies/mL) (Perrin *et al.* 2019).

HPC as well as other opportunistic pathogens should be monitored to obtain a more comprehensive view of microbial regrowth in building plumbing. Investigating the relationship between NTM and HPC or other opportunistic pathogens can provide valuable information into the occurrence and dynamics of NTM.

Identification of NTM isolates

Eight colonies (T1–T8) were isolated from tap water samples in site A. T1–T3 were isolated from FW at A1. T4 and T5 were isolated from FW and SW at A2, respectively. T6 and T7 were isolated from FW and SW at A3, respectively. T8 was isolated from FW at A4. Two colonies (G1 and G2) were isolated from the groundwater sample. They were identified as *Mycobacterium* spp. by sequencing their 16S rRNA genes and *hsp65* genes (Supplementary material, Table S2). Figures 2 and 3 show the phylogenetic trees based on 16S rRNA genes and *hsp65* genes, respectively. The isolates obtained from tap water were closely related to *Mycobacterium mucogenium* for T2 and T4–T8 and *M. avium* for T1 and T3, which were supported by both 16S rRNA genes and *hsp65* genes.

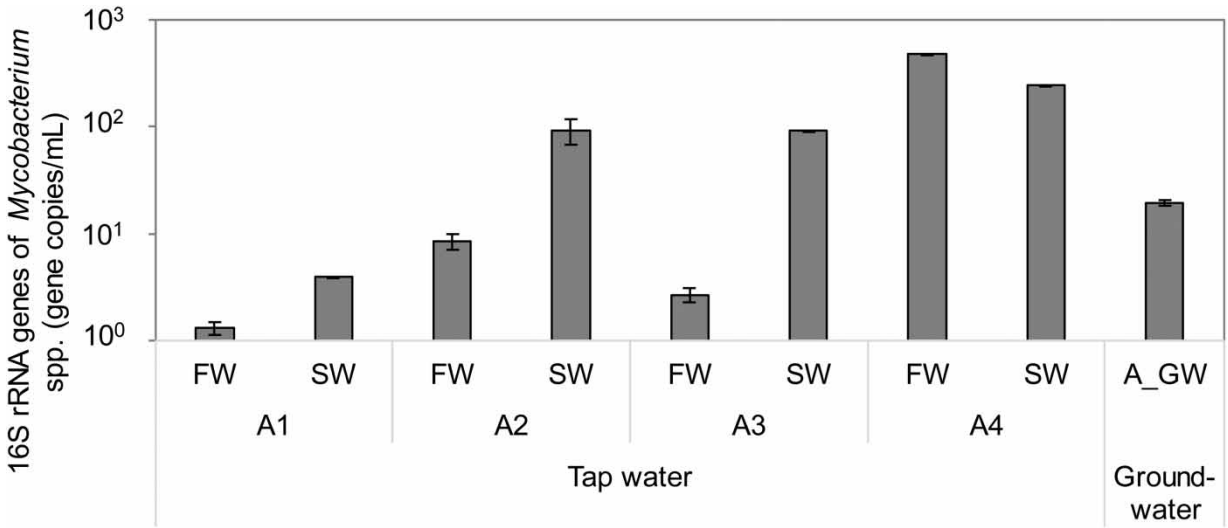


Figure 1 | The copy number of 16S rRNA genes of *Mycobacterium* spp. in the samples collected from site A. Error bars indicate the standard deviation of triplicate measurement of real-time PCR. FW, fresh water before stagnation; SW, stagnant water after 24 h stagnation.

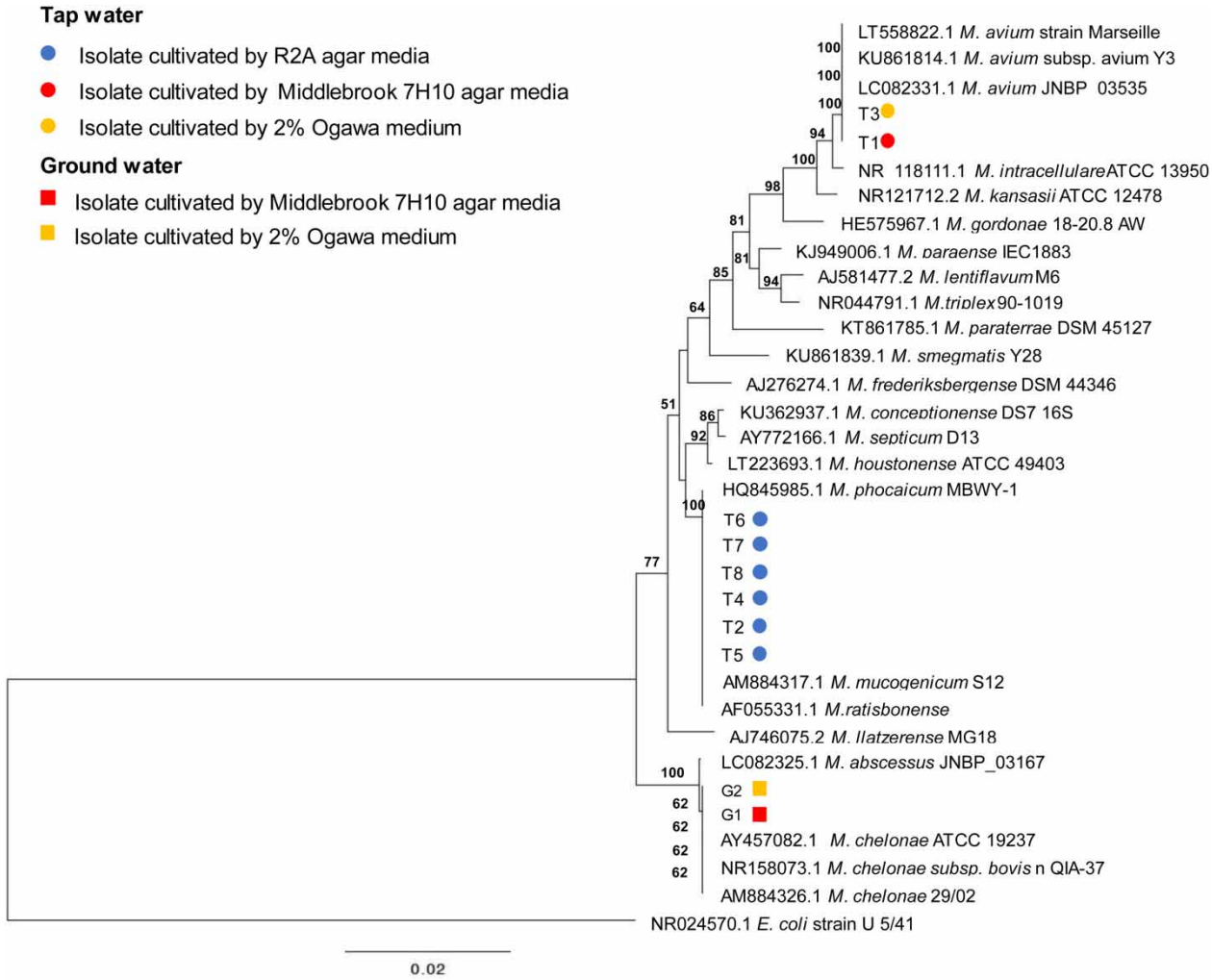


Figure 2 | Phylogenetic tree of 16S rRNA gene sequences of NTM isolates using the Neighbor-Joining method. The tree is rooted with the outgroup (*Escherichia coli*). The bootstrap values higher than 50% are shown.

The closest relative of T1 and T3 was identified as *M. avium* JNBP_03535 (16S rRNA gene) and *M. avium* ATCC25291 (*hsp65* gene). *M. avium* JNBP_03535 was isolated from the sputum of a patient in Japan (Mizuno *et al.* 2016), while *M. avium* ATCC25291 was a clinical isolate collected from Korea (Kim *et al.* 2005). *M. avium* is well-known as a major pathogenic NTM that causes pulmonary disease. In Japan, *M. avium* has been reported to be one of the most pathogenic agents that caused NTM disease (Morimoto *et al.* 2017). Interestingly, T1 and T3 were obtained from fresh tap water at A1, which supplied hot water with a temperature of 34.4–55.0 °C. *M. avium* is relatively resistant to elevated temperatures up to 50 °C for 60 min (Schulze-Röbbecke & Buchholtz 1992). Previous studies reported the occurrence of *M. avium* from shower water and bathtub water from the bathroom of patient with NTM disease in Japan (Nishiuchi *et al.* 2009), suggesting that a hot water environment could be preferable for *M. avium*.

T2 and T4–T8 were closely related to *M. mucogenicum* S12 (16S rRNA gene) and *M. mucogenicum* ATCC 49651 (*hsp65* gene). *M. mucogenicum*-like strains were isolated from both FW and SW samples at A2 and A3. On the other hand, G1 and G2 isolated from groundwater were affiliated with *Mycobacterium chelonae*, which has been recently subclassified as new emended genera, *Mycobacteroides chelonae* (Gupta *et al.* 2018). *M. mucogenicum* is associated with catheter-related infections, respiratory infections, and skin and soft tissue infections (Adékambi 2009). These findings suggested that building a plumbing environment could affect culturable NTM diversity.

While *M. mucogenium* was isolated using R2A agar, *M. avium* and *M. chelonae* were recovered from Middlebrook 7H10 agar and 2% Ogawa medium after preincubation in Middlebrook 7H9 broth. Because *M. mucogenicum* and *M. chelonae* are

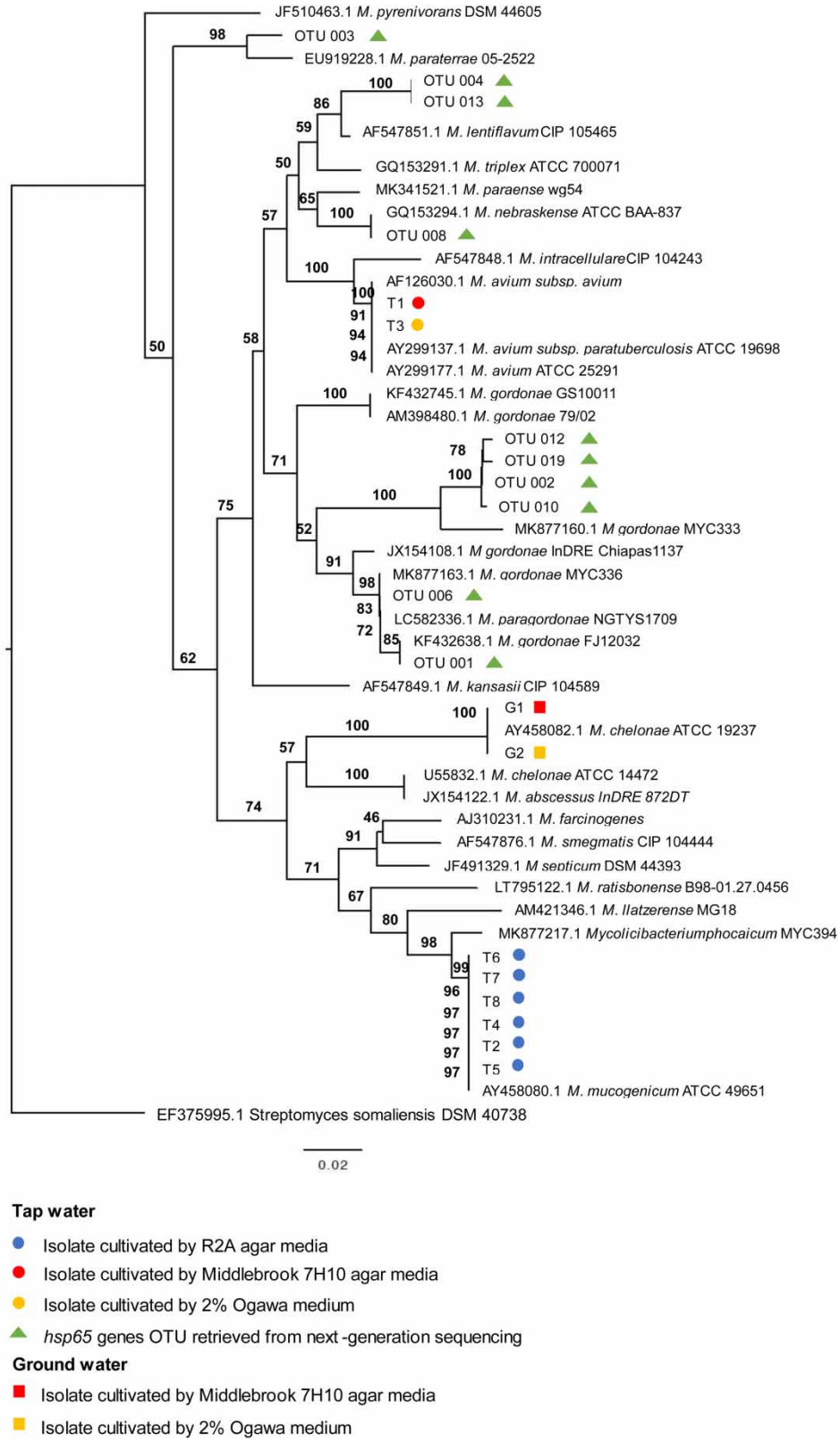


Figure 3 | Phylogenetic tree of *hsp65* gene sequences of NTM isolates and the representative OTUs retrieved by next-generation sequencing using the Neighbor-Joining method. The tree is rooted with the outgroup (*Streptomyces somaliensis*). The bootstrap values higher than 50% are shown.

rapid growers (van Ingen *et al.* 2009), it is possible that they could outcompete other NTM during cultivation. This result indicates that preferable cultivation conditions could be different for these NTM species.

Although the occurrence of NTM related to *M. avium* and *M. mucogenicum* in tap water was noteworthy, it is essential to support future studies with evidence of water-linked infections, by incorporating patient data on the incidence and prevalence of NTM infections to establish a connection between the isolated strains and their potential role in causing diseases. In addition, previous studies reported the occurrence of *M. mucogenicum*, *M. avium,* as well as other opportunistic pathogens (e.g., *Legionella spp.,* and *Pseudomonas aeruginosa*) from the outlet of drinking water treatment plants (Dávalos *et al.* 2021; Huang *et al.* 2021b). Though their abundance at the outlet of the treatment plant was low, we should pay more attention to the persistence of these opportunistic pathogens along the treatment process. To reveal the comprehensive dynamics of NTM and other opportunistic pathogens, their occurrence at the outlet of drinking water treatment plants and pipe networks should be studied.

The diversity of NTM communities revealed by next-generation sequencing

NTM communities in tap water samples from site A and site B were analyzed using next-generation sequencing targeting *hsp65* genes. In total, 41,073–60,974 reads were determined for each sample. After eliminating noise and chimera sequences, 26,168–45,748 qualified reads were used for further analysis. All of them were 86.8–100% identical to *hsp65* genes of *Mycobacterium* spp. and were classified into 94 OTUs in *Mycobacterium* spp. ANOSIM revealed that NTM communities between samples in sites A and B were statistically different (R-value $= 0.8253$, $p < 0.05$). *M. gordonae* was the most dominant NTM species, with an average abundance of 75.6 ± 27.7 and 78.7 ± 28.8% of the total reads in the samples from site A and site B, respectively (Figure 4). *M. avium* and *M. mucogenicum* were not detected by next-generation sequencing, demonstrating that cultivation could have a great selection bias on the diversity of NTM communities. For practical monitoring of NTM, both culture-dependent and culture-independent methods should be implemented to comprehensively reveal NTM communities in fresh and stagnant drinking water. *M. gordonae* can cause pulmonary infection in immunocompromised patients (Mazumder *et al.* 2010). The dominance of *M. gordonae* was reported in chloraminated and chlorinated drinking water (Waak *et al.* 2019). Moreover, *M. gordonae* was frequently detected in the distribution system supplied by surface water (Le Dantec *et al.* 2002). *Mycobacterium paraterrae* was also abundant in some samples of site A (0–66.3%). While *M. paraterrae* was isolated from patients with symptomatic pulmonary infection in Korea (Lee *et al.* 2010), information on the occurrence of *M. paraterrae* in drinking water is limited. *Mycobacterium lentiflavum* was the second most dominant NTM in site B (0–84.7%). *M. lentiflavum* was also reported to cause pulmonary infection (Yagi *et al.* 2018). *M. lentiflavum* was detected in chlorinated drinking water distribution systems and chlorinated treated water (King *et al.* 2016). Different water sources and different building plumbing conditions between sites A and B could probably affect NTM diversity.

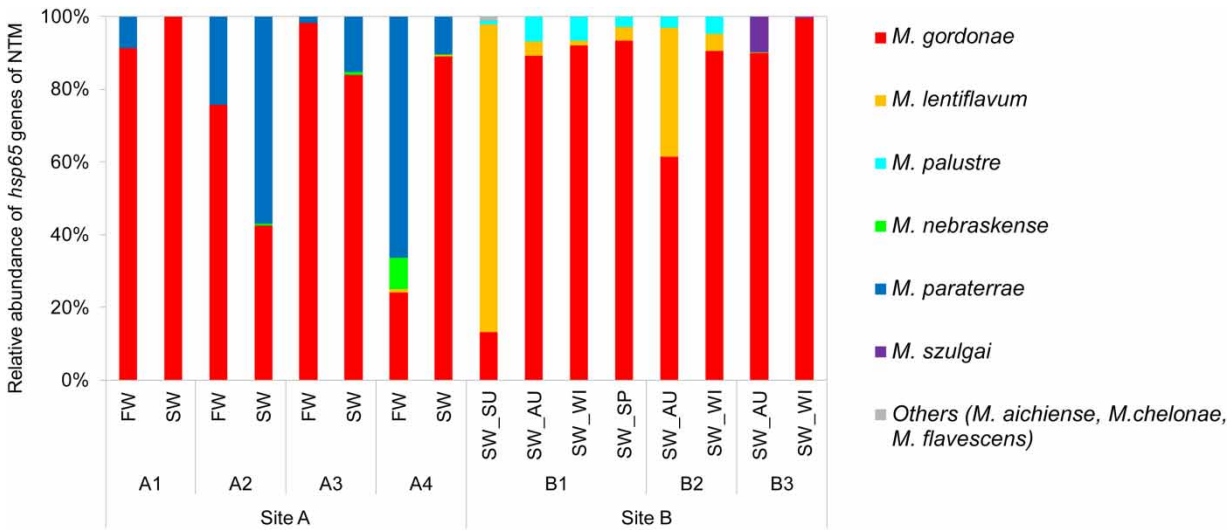


Figure 4 | The composition of NTM communities identified by *hsp65* genes from tap water samples in site A and site B. FW, fresh water before stagnation; SW, stagnant water after 24 h stagnation; SU, summer; AU, autumn; WI, winter; SP, spring.

Moreover, the relative abundance of *M. gordonae*, *M. paraterrae*, and *M. lentiflavum* differed among taps even in the same building, suggesting that NTM communities could be site-specific. The diversity of *Mycobacterium* spp. at site B were also analyzed using Nanopore sequencing targeting the full length of the 16S rRNA gene and only 18.7% of *Mycobacterium*-related sequences were assigned to species in *Mycobacterium* spp. (Rahmatika *et al.* 2022). This indicated that sequencing of *hsp65* gene provided better resolution for *Mycobacterium* spp. Identification in tap water.

Water stagnation not only increased NTM abundance (Figure 1), but also impacted the diversity of NTM communities at some faucets (Figure 4). For example, *M. gordonae* decreased from 75.7 to 42.5% and *M. paraterrae* increased from 24.2 to 57.0% after stagnation at site A2. However, the opposite trend was observed at A4. It is possible that changes in water quality could trigger the changes in NTM diversity and some selection may occur during water stagnation. On the other hand, *M. gordonae* was dominant in both fresh and SW samples at A1 and A3. Thus, changes in NTM communities during water stagnation could be dependent on the condition of each faucet. Seasonal variation of NTM communities was found in B1 in site B. In summer, *M. lentiflavum* was the most dominant, while *M. gordonae* was abundant in the other seasons.

M. gordonae, *M. paraterrae*, and *M. lentiflavum* were not detected by cultivation, probably because they are slow-growing NTM species (Lee *et al.* 2010; Mazumder *et al.* 2010; Yagi *et al.* 2018). It is possible that the growth of the slow-growing NTM species was outcompeted by rapidly growing NTM during cultivation. However, further evaluation is required to assess the selection pressure on slow-growing NTM during cultivation. Additionally, the decontamination process employed during cultivation should be evaluated to enhance the isolation of more NTM strains.

These findings highlighted the dynamics of NTM communities in building plumbing and the influence of various factors such as water stagnation on their abundance and diversity. However, it is important to note that the sample size in this study was limited, which could impact the overall understanding of the influence of water stagnation on the abundance and diversity of NTM. Future studies should consider collecting a larger number of tap water samples from various sites.

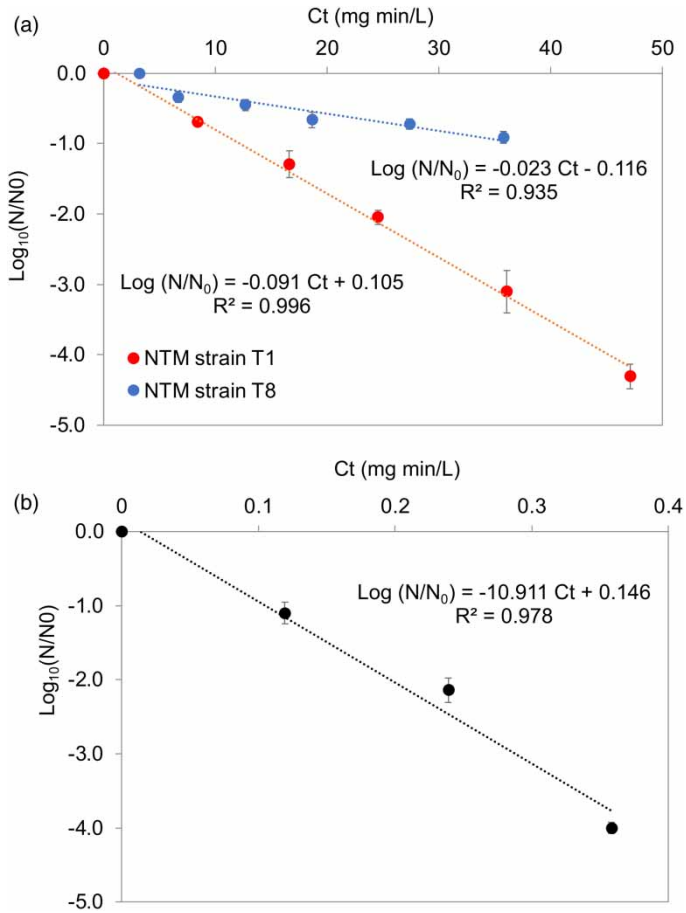


Figure 5 | Chlorine inactivation of (a) NTM strains (T1 and T8) in this study and (b) *E. coli* K12.

Chlorine tolerance of NTM

Chlorine tolerance of two NTM isolates (T1: M. *avium*-like, T8: M. *mucogenicum*-like) and *E. coli* were compared. As shown in Figure 5, NTM isolates were more resistant to chlorine compared to *E. coli*. The inactivation curves of T1, T8 and *E. coli* indicated that the inactivation rate constant was 0.091, 0.023, and 10.911 L mg^{-1} min^{-1} with the R^2 of 0.996, 0.935, and 0.979, respectively. T8 is more resistant than T1. CT values required for 3-log reduction ($CT_{99.9\%}$) of T1 and T8 were 34.2 and 122.7 mg min/L, respectively, which were 119 and 426 times greater than that of *E. coli*. A previous study reported the $CT_{99.9\%}$ of *M. avium* strain isolated from AIDS patients was 106 mg min/L (Taylor *et al.* 2000), which was higher than our finding. It was also reported that *M. avium* was resistant to other disinfectants, including monochloramine, chlorine dioxide and ozone (Taylor *et al.* 2000). $CT_{99.9\%}$ of *M. mucogenicum* in our study was higher than that of *M. mucogenicum* strains isolated from other distribution systems reported by Wang *et al.* (59.2 mg min/L) (Wang *et al.* 2019) and Chen *et al.* (29.6 mg min/L) (Chen *et al.* 2012).

The high hydrophobicity of the cell membrane of NTM could be one of the major factors influencing their tolerance to chlorine (Steed & Falkinham 2006; Wang *et al.* 2019). Furthermore, the biofilm formation could further increase the disinfectant resistance of NTM, including *M. avium* and *Mycobacterium phlei* (Steed & Falkinham 2006). It is also reported that the dominance of NTM in building plumbing biofilm (Huang *et al.* 2021a). Although disinfection is essential for maintaining microbial safety, it can consequently select chlorine-tolerant NTM that can survive in drinking water (Falkinham *et al.* 2015). Those factors likely contribute to the regrowth and persistence of NTM in tap water. Further study is thus required to control chlorine-resistance NTM completely in building plumbing.

CONCLUSIONS

NTM in tap water were characterized using culture-dependent and culture-independent approaches. Regrowth of NTM was observed after water stagnation. The cultivation method using R2A and Middlebrook 7H10 agar/Ogawa medium detected *M. mucogenicum* and *M. avium*, respectively. On the other hand, amplicon sequencing of *hsp65* genes of NTM could demonstrate that *M. gordonae* was the most prevalent in most samples, followed by *M. paraterrae* and *M. lentiflavum*. A significant difference between the results of culture-dependent and culture-independent methods indicated that NTM diversity should be carefully interpreted depending on the detection approaches. The chlorine tolerance test demonstrated that NTM strains related to *M. avium* and *M. mucogenicum* were 119 and 426 times more resistant to chlorine compared with *E. coli*, respectively. Since *M. mucogenicum*, *M. avium*, *M. gordonae*, *M. paraterrae*, and *M. lentiflavum* were reported as opportunistic pathogens causing pulmonary and respiratory infections as well as skin and soft tissue infections, we should pay attention to the occurrence of these NTM in building plumbing. Moreover, disinfection and residual disinfectant conditions should be revisited to control the occurrence of NTM in the water supply system.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Adékambi, T. 2009 Mycobacterium mucogenicum group infections: A review. *Clinical Microbiology and Infection* **15** (10), 911–918.

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, 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. *Nature Biotechnology* **37** (8), 852–857.

Chen, Y. Q., Chen, C., Zhang, X. J., Zheng, Q. & Liu, Y. Y. 2012 Inactivation of resistant Mycobacteria mucogenicum in water: Chlorine resistance and mechanism analysis. *Biomedical and Environmental Sciences* **25** (2), 230–237.

Collier, S. A., Deng, L., Adam, E. A., Benedict, K. M., Beshearse, E. M. & Blackstock, A. J., Bruce, B. B., Derado, G., Edens, C., Fullerton, K. E., Gargano, J. W., Geissler, A. L., Hall, A. J., Havelaar, A. H., Hill, V. R., Hoekstra, R. M., Reddy, S. C., Scallan, E., Stokes, E. K., Yoder, J. S. & Beach, M. J. 2021 Estimate of burden and direct healthcare cost of infectious waterborne disease in the United States. *Emerging Infectious Diseases* **27** (1), 140–149.

Dávalos, A. F., Garcia, P. K., Montoya-Pachongo, C., Rengifo, A., Guerrero, D., Díaz-Ordoñez, L., Díaz, G. & Ferro, B. E. 2021 Identification of nontuberculous mycobacteria in drinking water in Cali, Colombia. *International Journal of Environmental Research and Public Health* **18** (16), 8451.

Edgar, C. R. 2004 MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.

Falkinham, J. O., Pruden, A. & Edwards, M. 2015 Opportunistic premise plumbing pathogens: Increasingly important pathogens in drinking water. *Pathogens* **4** (2), 373–386.

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 of the United States of America* **106** (38), 16393–16399.

Gebert, M. J., Delgado-Baquerizo, M., Oliverio, A. M., Webster, T. M., Nichols, L. M., Honda, J. R., Chan, E. D., Adjemian, J., Dunn, R. R. & Fierer, N. 2018 Ecological analyses of mycobacteria in showerhead biofilms and their relevance to human health. *mBio* **9** (5), e01614–e01618.

Gupta, R. S., Lo, B. & Son, J. 2018 Phylogenomics and comparative genomic studies robustly support division of the genus mycobacterium into an emended genus mycobacterium and four novel genera. *Frontiers in Microbiology* **9**, 67.

Hamilton, K. A., Weir, M. H. & Haas, C. N. 2017 Dose response models and a quantitative microbial risk assessment framework for the Mycobacterium avium complex that account for recent developments in molecular biology, taxonomy, and epidemiology. *Water Research* **109**, 310–326.

Harada, K., Hagiya, H., Funahashi, T., Koyama, T., Kano, M. R. & Otsuka, F. 2020 Trends in the nontuberculous mycobacterial disease mortality rate in Japan: A nationwide observational study, 1997–2016. *Clinical Infectious Diseases* **73** (2), e321–e326.

Heuer, H., Krsek, M., Baker, P., Smalla, K. & Wellington, E. M. 1997 Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel–electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology* **63** (8), 3233–3241.

Huang, C., Weerasekara, A., Bond, P. L., Weynberg, B. K. D. & Guo, J. 2021a Characterizing the premise plumbing microbiome in both water and biofilms of a 50-year-old building. *Science of The Total Environment* **798**, 149225.

Huang, J., Chen, S., Ma, X., Yu, P., Zuo, P., Shi, B., Wang, H., Alvarez, P. & J, J. 2021b Opportunistic pathogens and their health risk in four full-scale drinking water treatment and distribution systems. *Ecology Engineering* **160**, 106134.

Kim, H., Kim, S. H., Shim, T. S., Kim, M. N., Bai, G. H., Park, Y. G., Lee, S. H., Chae, G. T., Cha, C. Y., Kook, Y. H. & Kim, B. J. 2005 Differentiation of Mycobacterium species by analysis of the heat–shock protein 65 gene (hsp65). *International Journal of Systematic and Evolutionary Microbiology* **55** (Pt 4), 1649–1656.

King, D. N., Donohue, M. J., Vesper, S. J., Villegas, E. N., Ware, M. W., Vogel, M. E., Furlong, E. F., Kolpin, D. W., Glassmeyer, S. T. & Pfaller, S. 2016 Microbial pathogens in source and treated waters from drinking water treatment plants in the United States and implications for human health. *Science of the Total Environment* **562**, 987–995.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. 2018 Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **25**, 1547–1549.

Le Dantec, C., Duguet, J. P., Montiel, A., Dumoutier, N., Dubrou, S. & Vincent, V. 2002 Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Applied and Environmental Microbiology* **68** (11), 5318–5325.

Lee, H., Lee, S. A., Lee, I. K., Yu, H. K., Park, Y. G., Jeong, J., Lee, S. H., Kim, S. R., Hyun, J. W., Kim, K., Kook, Y. H. & Kim, B. J. 2010 Mycobacterium paraterrae sp. nov. recovered from a clinical specimen: Novel chromogenic slow growing mycobacteria related to Mycobacterium terrae complex. *Microbiology and Immunology* **54** (1), 46–53.

Mazumder, S. A., Hicks, A. & Norwood, J. 2010 Mycobacterium gordonae pulmonary infection in an immunocompetent adult. *North American Journal of Medicine and Science* **2** (4), 205–207.

Mizuno, T., Natori, T., Kanazawa, I., Eldesouky, I., Fukunaga, H. & Ezaki, T. 2016 Core housekeeping proteins useful for identification and classification of mycobacteria. *Microbial Resource System* **32** (1), 25–37.

Morimoto, K., Hasegawa, N., Izumi, K., Namkoong, H., Uchimura, K., Yoshiyama, T., Hoshino, Y., Kurashima, A., Sokunaga, J., Shibuya, S., Shimojima, M., Ato, M. & Mitarai, S. 2017 A laboratory-based analysis of nontuberculous mycobacterial lung disease in Japan from 2012 to 2013. *Annals of the American Thoracic Society* **14**, 49–56.

Nishiuchi, Y., Tamura, A., Kitada, S., Taguri, T., Matsumoto, S., Tateishi, Y., Yoshimura, M., Ozeki, Y., Matsumura, N., Ogura, H. & Maekura, R. 2009 Mycobacterium avium complex organisms predominantly colonize in the bathtub inlets of patients' bathrooms. *Japanese Journal of Infectious Diseases* **62** (3), 182–186.

Perrin, Y., Bouchon, D., Hechard, Y. & Moulin, L. 2019 Spatio–temporal survey of opportunistic premise plumbing pathogens in the Paris drinking water distribution system. *International Journal of Hygiene and Environmental Health* **222** (4), 687–694.

Pfaller, S., King, D., Mistry, J. H. & Donohue, M. 2022 Occurrence revisited: Mycobacterium avium and Mycobacterium intracellulare in potable water in the USA. *Applied Microbiology and Biotechnology* **106**, 2715–2727.

Rahmatika, I., Kurisu, F., Furumai, H. & Kasuga, I. 2022 Dynamics of the microbial community and opportunistic pathogens after water stagnation in the premise plumbing of a building. *Microbes and Environments* **37** (1), ME21065.

Schulze-Röbbecke, R. & Buchholtz, K. 1992 Heat susceptibility of aquatic mycobacteria. *Applied and Environmental Microbiology* **58** (6), 1869–1873.

Schulze-Röbbecke, R., Weber, A. & Fischeder, R. 1991 Comparison of decontamination methods for the isolation of mycobacteria from drinking water samples. *Journal of Microbiological Methods* **14** (3), 177–183.

Steed, K. A. & Falkinham, J. O. 2006 Effect of growth in biofilms on chlorine susceptibility of Mycobacterium avium and Mycobacterium intracellulare. *Applied and Environmental Microbiology* **72** (6), 4007–4011.

Taylor, R. H., Falkinham, J. O., Norton, C. D. & LeChevallier, M. W. 2000 Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. *Applied and Environmental Microbiology* **66** (4), 1702–1705.

USEPA. 2021 *Drinking Water Contaminant Candidate List 5 – Draft*. USEPA, Washington, DC, USA.

van der Wielen, P. W., Heijnen, L. & van der Kooij, D. 2013 Pyrosequence analysis of the hsp65 genes of nontuberculous mycobacterium communities in unchlorinated drinking water in The Netherlands. *Applied and Environmental Microbiology* **79** (19), 6160–6166.

van Ingen, J., Boeree, M. J., Dekhuijzen, P. N. R. & van Soolingen, D. 2009 Environmental sources of rapid growing nontuberculous mycobacteria causing disease in humans. *Clinical Microbiology and Infection* **15** (10), 888–893.

Waak, M. B., LaPara, T. M., Hallé, C. & Hozalski, R. M. 2019 Nontuberculous Mycobacteria in two drinking water distribution systems and the role of residual disinfection. *Environmental Science and Technology* **53** (15), 8563–8573.

Wang, J., Sui, M., Yuan, B., Li, H. & Lu, H. 2019 Inactivation of two Mycobacteria by free chlorine: Effectiveness, influencing factors, and mechanisms. *Science of the Total Environment* **648**, 271–284.

Yagi, K., Morimoto, K., Ishii, M., Namkoong, H., Okamori, S., Asakura, T., Suzuki, S., Asami, T., Uwamino, Y., Funatsu, Y., Fujiwara, H., Kamata, H., Nishimura, T., Betsuyaku, T., Kurashima, A. & Hasegawa, N. 2018 Clinical characteristics of pulmonary Mycobacterium lentiflavum disease in adult patients. *International Journal of Infectious Diseases* **67**, 65–69.

Zlojtro, M., Jankovic, M., Samarzija, M., Zmak, L., Jankovic, V. K., Obrovac, M., Zlojtro, I. & Jakopovic, M. 2014 Nosocomial pseudo–outbreak of Mycobacterium gordonae associated with a hospital's water supply contamination: A case series of 135 patients. *Journal of Water and Health* **13** (1), 125–130.

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