PNAS Nexus, 2022, **1**, 1–14
https://doi.org/10.1093/pnasnexus/pgac145
Advance access publication date: 10 November 2022
Research Report

Shower water contributes viable nontuberculous mycobacteria to indoor air

Yun Shen[a,1], Sarah-Jane Haig[a,2], Aaron J. Prussin, II.[b], John J. LiPuma[c], Linsey C. Marr[b] and Lutgarde Raskin[a,*]

[a]Department of Civil and Environmental Engineering, University of Michigan, 1351 Beal Avenue, Ann Arbor, MI 48109, USA
[b]Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall, Blacksburg, VA 24061, USA
[c]Department of Pediatrics, University of Michigan Medical School, 1500 E. Medical Center Dr., Ann Arbor, MI 48109, USA
*To whom correspondence should be addressed: Email: raskin@umich.edu
[1]Present address: Department of Chemical and Environmental Engineering, University of California, Riverside, 900 University Avenue, Riverside, CA 92521, USA.
[2]Present address: Department of Civil and Environmental Engineering, University of Pittsburgh, 3700 O'Hara Street, Pittsburgh, PA 15261, USA.
Edited By: Karen E. Nelson

Abstract

Nontuberculous mycobacteria (NTM) are frequently present in municipal drinking water and building plumbing, and some are believed to cause respiratory tract infections through inhalation of NTM-containing aerosols generated during showering. However, the present understanding of NTM transfer from water to air is insufficient to develop NTM risk mitigation strategies. This study aimed to characterize the contribution of shower water to the abundance of viable NTM in indoor air. Shower water and indoor air samples were collected, and 16S rRNA and *rpoB* genes were sequenced. The sequencing results showed that running the shower impacted the bacterial community structure and NTM species composition in indoor air by transferring certain bacteria from water to air. A mass balance model combined with NTM quantification results revealed that on average 1/132 and 1/254 of NTM cells in water were transferred to air during 1 hour of showering using a rain and massage showerhead, respectively. A large fraction of the bacteria transferred from water to air were membrane-damaged, i.e. they had compromised membranes based on analysis by live/dead staining and flow cytometry. However, the damaged NTM in air were recoverable as shown by growth in a culture medium mimicking the respiratory secretions of people with cystic fibrosis, implying a potential infection risk by NTM introduced to indoor air during shower running. Among the recovered NTM, *Mycobacterium mucogenicum* was the dominant species as determined by *rpoB* gene sequencing. Overall, this study lays the groundwork for future pathogen risk management and public health protection in the built environment.

Keywords: shower water, indoor air, pathogen, nontuberculous mycobacteria, aerosol

Significance Statement:

Nontuberculous mycobacteria (NTM) are a group of environmental bacteria commonly present in municipal drinking water. Some NTM can cause respiratory tract infections in susceptible individuals. NTM can be transferred from water to indoor air during showering, thus posing a potential infection risk through inhalation of NTM-containing aerosols generated during showering. We quantified the proportion of NTM that were transferred from water to indoor air during shower running and determined that many had damaged membranes. However, these damaged NTM could be recovered in a bacterial culture medium that simulated sputum of susceptible individuals, implying NTM introduced to indoor air by showering pose a potential infection risk. The results lay the groundwork for controlling NTM infection risk through showering.

Introduction

Nontuberculous mycobacteria (NTM) are environmental bacteria within the genus *Mycobacterium*, but do not include *Mycobacterium leprae* and *Mycobacterium tuberculosis* (1). A subset of NTM are opportunistic pathogens that can cause human infections, most often of the respiratory tract in susceptible persons, including individuals with obstructive lung diseases such as cystic fibrosis (CF), non-CF bronchiectasis, and chronic obstructive pulmonary disease (COPD) (2). Although the exact route of NTM respiratory tract infection is not established with certainty, aspiration and inhalation of aerosols containing NTM (e.g. generated from pools, spas, and showers) are believed to be the primary modes of acquisition (3, 4). In 2010, the number of NTM lung disease cases in the United States was estimated to be 86,244, resulting in health care costs of $815 million (5). From 1999 to 2014, almost 10,000 US deaths were attributed to NTM infection (6), and NTM-related disease has been increasing in recent years (7). While the sources of NTM infections are not always clear, municipally treated drinking water and building plumbing are recognized as important reservoirs for NTM (8), and surveys of tap water and showerhead biofilms across the United States have reported NTM to be present in the majority of samples collected (9, 10). Even when a disinfectant residual is maintained throughout the drinking water distribution system, it is possible for the water quality in buildings to deteriorate due

Competing Interest: The authors declare no competing interest.
Received: December 20, 2021. **Accepted:** November 7, 2022
© The Author(s) 2022. Published by Oxford University Press on behalf of National Academy of Sciences. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (https://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

to long stagnation times, elevated temperatures, and low disinfectant levels (11). Moreover, NTM have a lipid-rich cell wall, can multiply in amoebae, and tend to grow in biofilms. These characteristics make NTM quite resistant to disinfection (12, 13). As a result, the levels of NTM in building plumbing have been found to be higher than those in distribution systems (14–16). In addition, NTM have been detected in air near hot tubs, pools, and showers (17–20), implying that NTM may transfer from water to air by aerosol formation (aerosolization). These previous studies suggest that the aerosolization of NTM present in building plumbing might be an important path to transfer NTM from water to air and enable infection through the inhalation exposure route. Understanding the NTM transmission route from building plumbing to air to a human host and the corresponding health risk is of great importance for developing NTM control strategies.

Since humans spend most of their time indoors, taking into account the indoor environment is essential when considering how respiratory tract infections are acquired (21). Building plumbing has been suggested to be important in influencing the bacterial community in indoor air (22, 23). Yet, little is known about how water use influences indoor air quality and how opportunistic pathogens transfer from water to air. Specifically, showering produces aerosols and significantly increases the aerosol concentration in indoor air (24). A previous study reported the presence of *Mycobacterium* spp. in air during showering by sequencing 16S rRNA genes (25). However, the experimental shower unit used in the previous study did not represent realistic showering conditions since it had a much smaller volume (2.5 m^3) and less ventilation than actual shower rooms. Therefore, it is unclear if showering under realistic conditions can alter the microbial community in indoor air. Furthermore, no studies have determined the amount of NTM transferred from shower water to air during shower use. The limited information on NTM aerosolization makes it difficult to estimate the health risk caused by NTM in building plumbing. Previous studies have shown that challenges with quantifying water–air transfer for bacteria contributed substantially to uncertainty in pathogen risk assessment (26, 27). In addition, it has not been determined whether pathogenic NTM species or strains can transfer from water to air. While methods have been developed to identify NTM species and strains in drinking water (15), such methods have not been applied to examine air samples. Understanding the health risk associated with NTM in building plumbing is critical for the development of water quality regulations and water system and building design. Therefore, investigating how water use events (e.g. showering) affect microbial indoor air quality is a necessary first step toward appropriately assessing and managing such risk.

In addition to quantifying and identifying the NTM transferred from water to air, determining NTM viability is important to assess risk. Due to the presence of disinfectants in drinking water and evaporation of water from aerosols carrying microorganisms (28), bacterial cells may be damaged when they transfer across the water–air interface. Flow cytometry combined with live/dead staining has been used extensively to quantify membrane-intact bacterial cells in aqueous environments, including in drinking water systems (29–32). Conventional culture-based methods cannot detect viable but nonculturable bacteria, which are prevalent in environmental samples (33). Therefore, culture-based methods typically underestimate the number of live bacteria and often fail to isolate environmental bacteria. Flow cytometry provides a promising way to estimate the viability of bacteria in environmental samples (30, 31, 34). However, to identify NTM (or other bacterial taxa) in environmental samples, live/dead staining needs to be combined with fluorescence-activated cell sorting (FACS) followed by appropriate DNA sequencing methods (35). While these techniques have been used in aqueous environments and outdoor air (35, 36), to the best of our knowledge, the viability of bacteria in indoor air has not yet been studied using this approach. To better understand the health risk associated with NTM, a systematic investigation of NTM abundance and viability in building plumbing and indoor air is needed.

This study aimed to characterize the transfer of NTM in building plumbing from water to indoor air. Specifically, the objectives of this study were: (1) to understand how shower use influences the bacterial community structure in indoor air, (2) to identify potentially pathogenic NTM species that can be transferred from water to air, (3) to quantify NTM cells transferred from water to air during shower use, and (4) to determine the viability of bacteria and NTM in water and indoor air.

Results

Mycobacterium is one of the dominant members of the shower water microbial community

We determined the bacterial community structure in shower water by 16S rRNA gene sequencing. Water samples were collected from a shower room by using either "rain" or "massage" showerhead spray patterns (Figure S1) during 60 min of running the shower water (although there was no person taking a shower during sample collection, the term "showering" in the text refers to this shower running process). The most abundant classified genera in all 70 water samples from 10 sampling events included *Hydrogenophaga*, *Herminiimonas*, and *Mycobacterium*, and their average relative abundances were 7.0%, 6.0%, and 5.8%, respectively (Figure 1a and Figure S2). In addition, *Pseudomonas* (relative abundance = 4.4%) and *Legionella* (relative abundance = 0.5%), genera that contain opportunistic pathogen species, were also found in the water samples, but the resolution of the method was insufficient for species-level identification. The *Mycobacterium* relative abundance in the water decreased from 9.6% at the start of showering (0 min) to 2.5% after 50 min of showering (Figure 1a). In addition to 16S rRNA gene sequencing, qPCR targeting the *atpE* gene, which is present as a single copy in *Mycobacterium* spp. genomes, was selected to quantify NTM in water samples due to its high specificity and sensitivity (37). The NTM concentrations ranged from 1.08×10^4 to 4.93×10^6 gene copies/L. Consistent with the relative abundance data, the qPCR results also showed a decreasing trend of the NTM concentrations as a function of shower time, with an average concentration of 2.16 (± 1.67) $\times 10^6$ gene copies/L at 0 min decreasing to 5.32 (± 5.28) $\times 10^4$ gene copies/L at 50 min (Figure 2).

Showering altered the bacterial community structure and NTM species composition in indoor air

We collected air samples before (pre-shower air), during (during-shower air), and after (post-shower air) each shower event. *Bacillus* and *Tepidiphilus* were the most abundant genera in all air samples (Figure 1b, Figure 1c, and Figure S3), constituting more than half of the indoor air bacterial community. *Mycobacterium*, *Pseudomonas*, and *Staphylococcus* were also present in the air samples, and the relative abundances of *Mycobacterium* in the air samples collected during showering were significantly higher than in the pre-shower air samples (Figure 1b and c, paired t test, $P = 0.01$ and

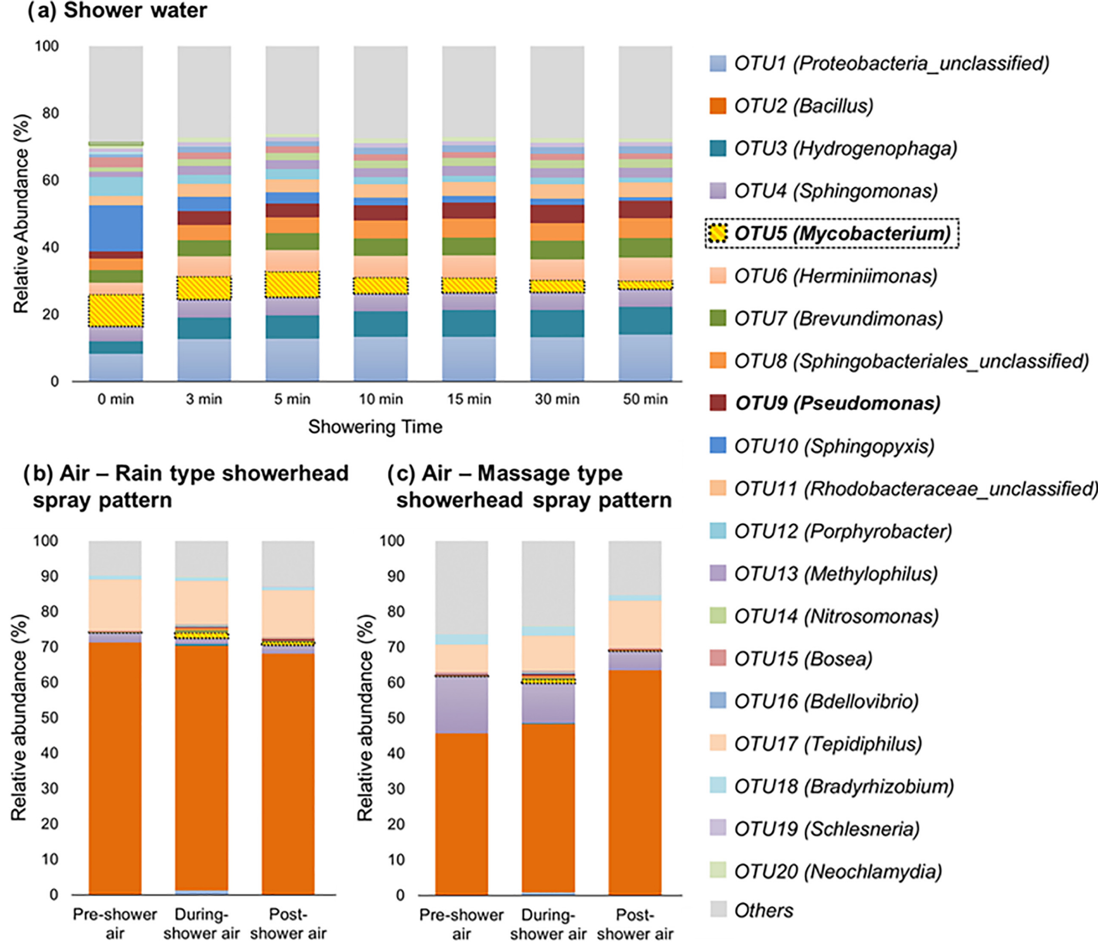

Figure 1. Relative abundance of bacterial taxa at genus or lowest identifiable taxonomic level in shower water samples collected at different shower times (a) and indoor air samples collected for 60 min each before, during, and after showering with a rain showerhead (b) and a massage showerhead (c). The 16S rRNA gene sequences from each sample were clustered to operational taxonomic units (OTUs) based on 97% identity and the top 20 most abundant OTUs in shower water and air samples combined are shown. Each colored segment represents the average relative abundance of each OTU in the same type of samples ($n = 10$ for shower water samples and $n = 5$ for air samples).

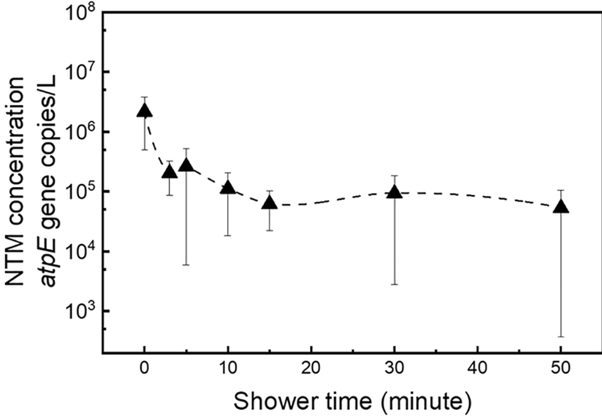

Figure 2. Average NTM concentrations in water as a function of shower time. The error bars represent the standard deviation of measurements from 10 shower events.

0.04 for rain and massage showerheads, respectively), suggesting that showering transferred NTM from water to air. To evaluate the effect of showering on the indoor air bacterial community, the Abundance-based Coverage Estimator (S_{ACE}), Shannon diversity index (H), and Pielou's evenness index (J) were calculated to estimate the bacterial community richness, diversity, and evenness, respectively (Table S1). Compared with the pre-shower air samples, the during-shower air samples showed higher richness, diversity, and evenness (paired t test, $P = 0.002$, 0.00002, and 0.005, respectively). For example, with the rain showerhead, S_{ACE}, H, and J increased from 284 ± 181, 1.26 ± 0.31, and 0.23 ± 0.04, respectively, before showering to 424 ± 154, 1.60 ± 0.40, and 0.27 ± 0.06, respectively, during showering. The increased richness indicates that showering introduced new bacterial taxa to indoor air. The increased diversity and evenness implys that the newly added taxa reduced the relative abundance of the taxa present in indoor air before showering. Therefore, showering clearly altered the indoor air bacterial community.

A source tracking method based on the 16S rRNA gene sequencing results was performed to compare the contribution of shower water to indoor air bacterial communities before and during showering (38). In this analysis, the shower water samples collected during each shower event served as the "source," while the air sample collected during the same experiment served as the "sink." The proportion of the "sink" taxa that originated from the "source" was then calculated by SourceTracker (38). This analysis suggested that shower water contributed 7% ± 3% of the bacterial communities in pre-shower air

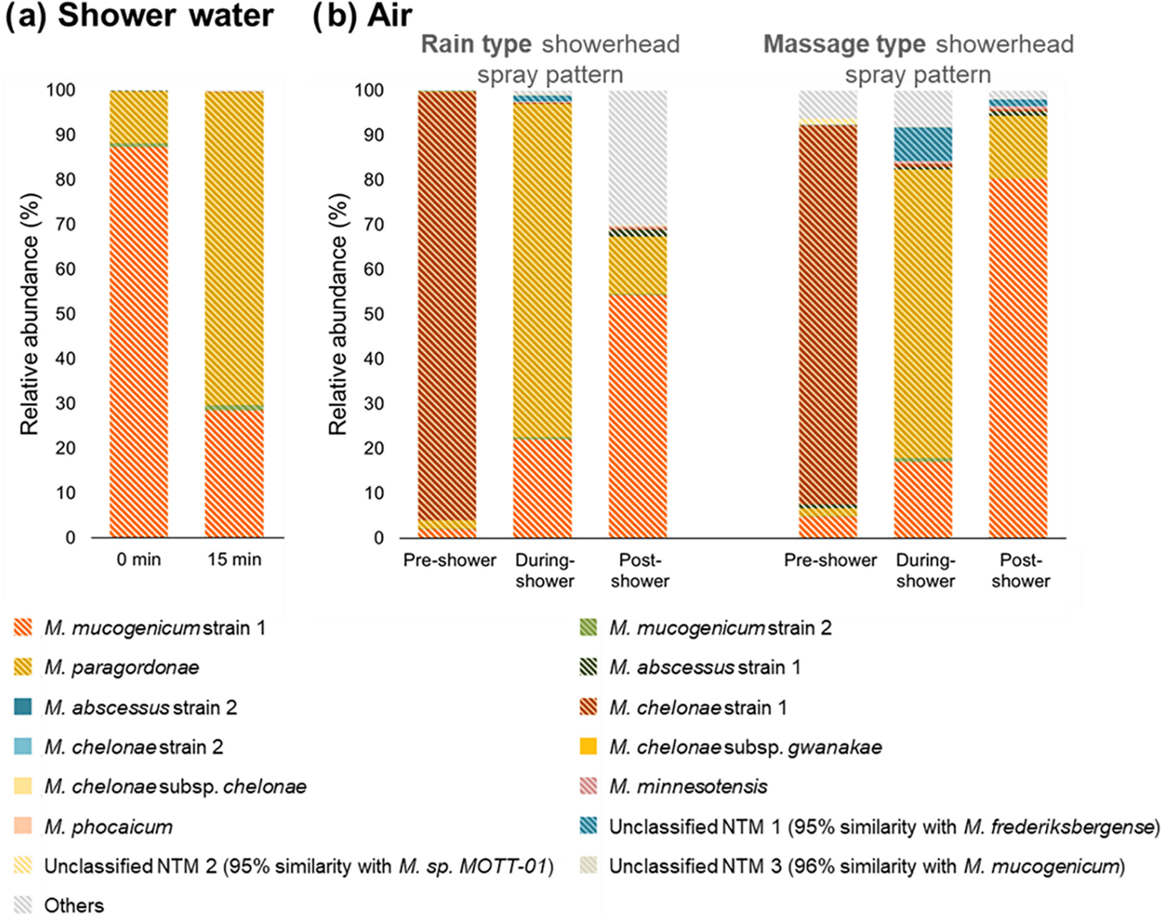

Figure 3. Relative abundance of NTM species in shower water samples collected at 0 and 15 min during showering (a) and in indoor air samples collected for 60 min each before, during, and after showering with rain and massage showerheads (b). The NTM were classified by sequencing the *rpoB* gene with PacBio and clustered based on 97% identity. Each bar represents overall relative abundance of each NTM species by pooling five replicate samples.

samples. During showering, shower water contributed 12% ± 1% and 9% ± 2% of the bacterial communities with use of rain and massage showerheads, respectively. Therefore, the proportion of air taxa that originated from shower water increased significantly during showering (paired t test, $P = 0.03$ and 0.04), further indicating that showering transferred bacteria from water to air.

In addition to altering the indoor air bacterial community structure, showering also led to a change in the NTM species composition in indoor air as determined by sequencing of the NTM *rpoB* gene. In shower water, *Mycobacterium mucogenicum* and *Mycobacterium paragordonae* dominated (Figure 3). *Mycobacterium chelonae* was the dominant NTM species in indoor air before showering. However, during showering, *M. mucogenicum* and *M. paragordonae* became the dominant NTM species in indoor air. *M. paragordonae* accounted for 74.5% and 64.5% of NTM species during showering using rain and massage showerheads, respectively. The change in dominant NTM species in air suggests that shower water was the major source of NTM in air during showering. After showering, *M. mucogenicum* became the most abundant NTM species in air, indicating that *M. mucogeniucm* (or at least its DNA) remained in indoor air for a longer time after showering than *M. paragordonae*.

Showering led to increased NTM concentration in air

We monitored the NTM concentration in indoor air before, during, and after showering by performing qPCR targeting the *atpE* gene. NTM concentrations in air during showering were significantly higher than before showering (Figure 4, paired t test, $P = 0.003$ and 0.0008 for rain and massage showerheads, respectively). After showering, the NTM concentrations were similar to those before showering (Figure 4, paired t test, $P = 0.17$ and 0.22 for rain and massage showerheads, respectively). The observation of higher NTM concentrations during showering confirmed that showering transferred NTM from water to air.

During showering, the indoor air NTM concentration of 3.80 (± 1.32) $\times 10^3$ gene copies/m^3 for the rain showerhead was significantly higher than the corresponding value of 2.03 (± 0.94) $\times 10^3$ gene copies/m^3 for the massage showerhead (paired t test, $P = 0.01$), suggesting the rain showerhead released more NTM than the massage showerhead. To further explore the difference in NTM released from the different showerheads, we measured the size-resolved concentration of total aerosols during showering (Figure S4). For all size ranges (0.3 to 0.5, 0.5 to 1, 1 to 2.5, 2.5 to 5, 5 to 10, and 10 to 25 μm), the concentrations of aerosols were higher when using the rain showerhead compared to the mas-

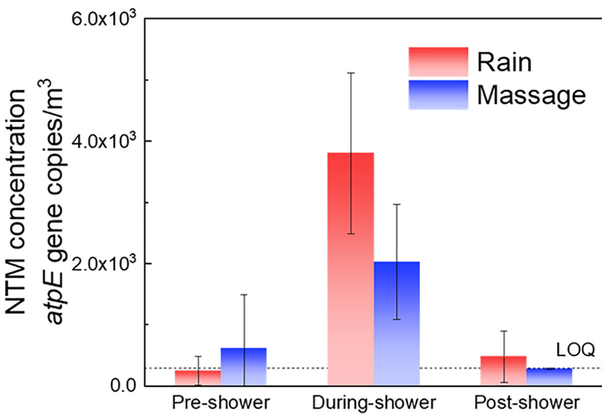

Figure 4. NTM concentrations in pre-, during-, and post-shower air samples with a rain or massage showerhead. The error bars represent the standard deviation of measurements from five replicate experiments. The limit of quantification (LOQ) of qPCR is represented by the black dashed line. Concentrations below the limit of detection (LOD) were represented by 1/2 LOD, while the concentration values between LOD and LOQ were represented by 1/2 (LOD + LOQ). The fan was kept on during air sampling, and the air exchange rate was 14.4 air changes/hour.

sage showerhead. It appears that the production of more aerosols by the rain showerhead resulted in greater transfer of NTM from water to air.

A mass balance model was developed to estimate the emission rate of NTM to air, which combined with the release rate of NTM present in shower water, allowed estimation of the fraction of NTM in water that were transferred to air. In this model, the shower room was assumed to be a well-mixed space with a running fan that provided a constant high air exchange rate of 14.4 air changes/hour (Figure S5). During 1 hour of showering, the total numbers of NTM released to air from rain and massage showerheads were 9.24 (± 3.70) $\times 10^5$ and 3.65 (± 1.08) $\times 10^5$ gene copies, respectively. The total numbers of NTM in the shower water flowing from the rain and massage showerheads were calculated to be 1.11 (± 0.57) $\times 10^8$ and 0.93 (± 0.50) $\times 10^8$ gene copies, respectively. The values in air significantly differed by showerhead type ($P = 0.01$), but the values in water did not ($P = 0.13$). Based on the estimates for total NTM released to air and total NTM present in the shower water, the water–air transfer ratio for the rain showerhead was calculated to be 1/(132 ± 73). In other words, on average, one out of 132 NTM cells in the shower water transferred to indoor air. For the massage showerhead, the water–air transfer ratio was calculated to be 1/(254 ± 126), which was significantly lower than for the rain showerhead (paired t test, $P = 0.01$).

Membrane-intact bacteria in water were damaged after transferring to air

The total and membrane-intact bacterial cells in water and indoor air samples were quantified by live/dead staining and flow cytometry. Distinct populations of membrane-intact and membrane-damaged cells were observed in all shower water samples (Figure 5a), and the ratio of membrane-intact to total cells ranged from 0.36 to 0.57. The bacterial community structure for the membrane-intact cell fraction was characterized using 16S rRNA gene sequencing after performing FACS for three additional experiments with the rain showerhead. Compared with the overall bacterial community, the membrane-intact bacterial community in water samples exhibited lower species richness and diversity (Figure 6). Specifically, at the start of showering ($t = 0$ min), the membrane-intact bacterial community had a S_{ACE} of 211 ± 129 and H of 3.1 ± 0.1 ($n = 3$), compared to a S_{ACE} of 718 ± 269 and H of 4.2 ± 0.2 ($n = 3$) for the overall bacterial community. This observation suggests that different taxa in the shower water exhibit different viabilities. For example, *Pseudomonas* was present at a relative abundance of 2.5% ± 0.8% ($n = 3$) in the overall bacterial community at the start of showering ($t = 0$ min), whereas its relative abundance in the membrane-intact fraction was only 0.3% ± 0.3% ($n = 3$), suggesting that a large fraction of *Pseudomonas* cells in shower water were damaged or dead. In contrast, the relative abundance of *Mycobacterium* in the membrane-intact fraction was comparable with, or higher than, that in the overall bacterial community (Figure 6). For instance, at the start of showering ($t = 0$ min), the relative abundance of *Mycobacterium* in the overall bacterial community was similar to that in the membrane-intact fraction 7.5% ± 1.2% vs. 5.0% ± 5.2%, paired t test, $P = 0.2$). NTM species composition profiles, determined with *rpoB* gene sequencing, were similar for the overall bacterial community and the membrane-intact fraction (Figure S6), suggesting that different NTM species exhibited similar viability.

The flow cytometry density plots for air samples (Figure 5b) showed very different patterns than the ones for water samples (Figure 5a). For the air samples, no distinct membrane-intact and membrane-damaged bacterial cell fractions were observed. The results of 16S rRNA and *rpoB* gene sequencing and qPCR targeting NTM presented above indicated that bacteria were transferred from shower water to air during showering. The flow cytometry results suggest that bacterial cells, including NTM, upon transferring from shower water to air were damaged.

Membrane-damaged NTM present in indoor air can be recovered and grow in a simulated human respiratory tract environment

As NTM appeared damaged after transferring from water to air (Figure 5b), it was unclear if such damaged cells could be recovered and potentially cause respiratory tract infection. To address this question, we incubated air samples collected during shower use with the rain showerhead in a simulated human sputum medium (i.e. a medium designed to mimic sputum from persons with CF, a patient population that is particularly susceptible to NTM respiratory tract infection (39)) for 14 days and quantified the abundance of NTM using qPCR at 0, 5, 10, and 14 days (Figure 7a). The NTM concentrations in the culture medium substantially increased from 1.30 (± 0.63) $\times 10^2$ gene copies/mL on Day 0 to 2.08 (± 1.85) $\times 10^5$ gene copies/mL on Day 5. The NTM concentrations did not increase further as observed on Day 10. Two of the replicates showed a decrease in NTM concentration by Day 14. The increase of NTM concentrations during the first 5 days of incubation indicates that the damaged NTM cells recovered and propagated in the simulated human sputum medium. NTM concentrations did not increase during incubation of air samples collected before showering (Figure S7).

NTM speciation during the incubation of air samples in the simulated sputum medium was examined by *rpoB* gene sequencing (Figure 7b). On Day 0, *M. paragordonae*, *M. mucogenicum*, unclassified NTM 3, and *M. chelonae* were the most abundant NTM species, with relative abundances of 65.5%, 16.4%, 10.3%, and 5.0%, respectively. With longer incubation times, the relative abundance of *M. paragordonae* decreased and ranged between 26.6% and 27.6% from Days 5 to 14, while the relative abundance of *M. mucogenicum* increased and reached 63.8% on Day 10.

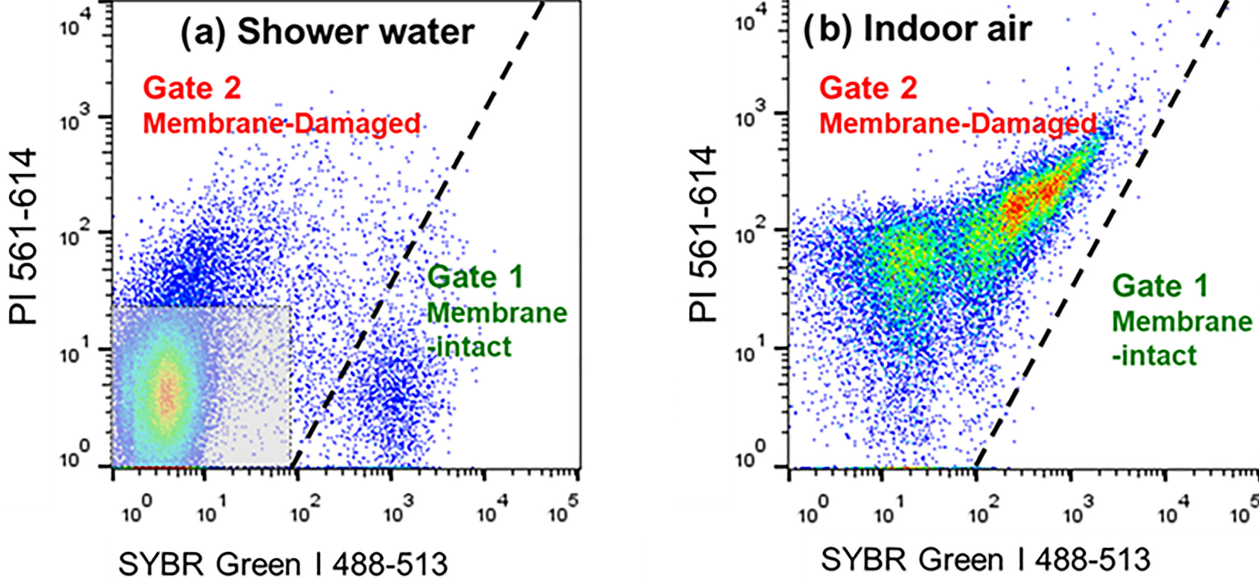

Figure 5. Flow cytometry analysis of typical shower water sample (a) and indoor air sample (b) collected during showering. The dots in Gate 1 represent membrane-intact bacterial cells. The dots in Gate 2 represent membrane-damaged bacterial cells. The gray color covered area in panel (a) represents the background signal in shower water.

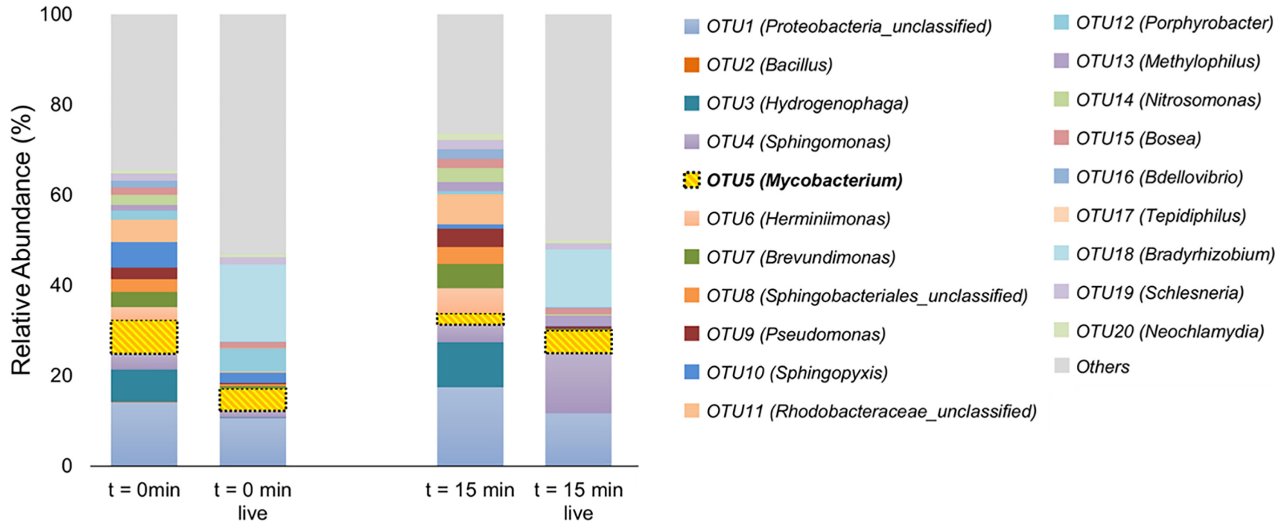

Figure 6. Relative abundance of bacterial taxa at the genus or most resolved taxonomic level in the overall bacterial communities (left bar) and membrane-intact bacterial fractions (right bar labeled "live") of shower water samples collected at the start of showering ($t = 0$ min) and after 15 min. The 16S rRNA gene sequences from each sample were clustered to operational taxonomic units (OTUs) based on 97% identity and the top 20 most abundant OTUs for all samples combined are shown. Each bar represents three replicate samples.

In addition, the relative abundance of *Mycobacterium abscessus* increased from 0.4% on Day 0 to 3.4% on Day 14. On Day 14, *M. mucogenicum* became the most abundant NTM species, followed by *M. paragordonae* and *M. abscessus*. Therefore, NTM species appear to exhibit differential growth rates differently in a culture medium simulating respiratory secretions of humans susceptible to NTM infection.

Discussion

Previous studies have suggested that plumbing fixtures (e.g. shower, toilet, and faucet) are potential sources of microorganisms in indoor air (22, 23). In the current study, we demonstrated, by simultaneously characterizing shower water and indoor air samples collected in an actual shower room, that showering altered the bacterial community structure and NTM species composition of indoor air. We report that showering transferred bacterial taxa from water to air and increased the bacterial community richness and diversity in indoor air. Therefore, microbial water quality in building plumbing systems is a critical component of maintaining a healthy indoor air environment. We also demonstrated that showering altered the relative proportions of NTM species in air. The dominant NTM species in air changed from *M. chelonae* before showering to *M. mucogenicum* and *M. paragor-*

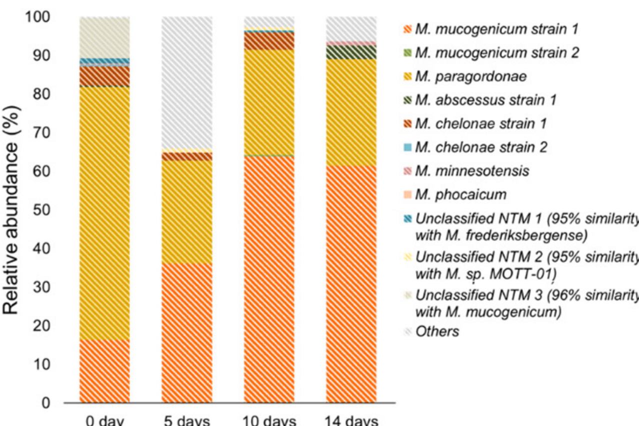

Figure 7. NTM concentrations as a function of incubation time in the mixtures of air samples collected during showering and the simulated sputum medium (a). Relative abundance of NTM species in the mixture of air samples collected during showering and simulated sputum medium as a function of incubation time (b). Results of the control culture experiments, including the pre-shower air sample in sputum medium and during-shower air sample in buffer, are shown in Figure S7. The NTM concentration values below LOD were represented by 1/2 LOD, while the concentration values between LOD and LOQ were represented by 1/2 (LOD + LOQ). Note that in the recovery tests, the LOD and LOQ at Day 0 were 16 and 36 gene copies/mL, respectively, while the LOD and LOQ at Days 5 to 14 were 326 and 474 gene copies/mL, respectively. The different LOD/LOQ levels at different incubation times were due to using different sample volumes. In Figure 7b, each bar represents overall relative abundance of each NTM species by pooling three replicate samples.

donae during showering. Both *M. mucogenicum* and *M. paragordonae* strains have been found in patients with pulmonary disease (40, 41). In fact, *M. mucogenicum* was among the top five NTM species isolated from human specimens in four states in the United States (40), and has been described as causing catheter-related infections as well as pulmonary infections (2). Although the role of *M. paragordonae* in contributing to disease is not known, this species has been found in patient and hospital environmental samples in Japan (41, 42). Taken together, these results indicate that showering has the potential to transfer respiratory tract pathogens from water to air.

Our results showed that showering can significantly change the NTM concentrations in air during showering. However, we note that NTM concentrations in building plumbing can vary greatly, depending on water treatment processes, dissipation of disinfectant residual, and nutrient availability (8). In our study, NTM concentrations in shower water were higher than in building plumbing water from systems that use free chlorine as the disinfectant (43, 44), but consistent with NTM levels in building plumbing water originating from distribution systems operated with chloramine as the disinfectant (15). Therefore, future work should evaluate the impact of showering on NTM concentration and species composition in indoor air for buildings fed with water from various types of water supplies. In addition, our results showed that the NTM concentrations decreased soon after showering, likely due to the high air exchange rate of 14.4 air changes/hour. Within three air changes (i.e. 13 min), the NTM concentration should fall by 95% due to dilution with background air. Moreover, the NTM concentration in the first flush of shower water was higher, suggesting that there would be more NTM released at the beginning of showering. While the high air exchange rate can help to dilute the higher NTM concentration, letting the water run for some time before entering the shower might reduce the exposure to NTM during the shower process, especially for susceptible populations. The results of this study could also provide information for future investigations on NTM exposure and corresponding risk assessment for other types of water use activities (e.g. running bath water or tap water).

Given our finding that the NTM species composition in indoor air changes during showering, a quantitative evaluation of NTM transfer from water to air became paramount. Therefore, we quantified the air NTM concentration increase during showering and found it to range from 0.8×10^3 to 5.3×10^3 gene copies/m^3. A previous study had provided an indirect estimation of the air NTM concentration change in an experimental shower unit to be slightly lower ((0.1 to 1.7) $\times 10^3$ gene copies/m^3) (25). To assess the health risk of bacterial pathogens aerosolization, water–air partitioning coefficients are used to estimate the quantity of pathogens aerosolized to indoor air (45–48). The water–air partitioning coefficient is defined as the ratio of pathogen concentration in water to that in air. Partitioning coefficients of *Legionella* spp., a more widely studied group containing waterborne opportunistic pathogens, have been reported to range from 2.0×10^{-7} to 5.7×10^{-4} L/m^3 (49–51), whereas the partitioning coefficients for NTM have been estimated to be higher (i.e. 2.7×10^{-3} L/m^3) (19). A previous study also suggested that NTM can preferentially partition into air compared with other bacteria, as the relative abundance of NTM in the bacterial communities of pool water was much lower than in air (5% vs. 84%) (52). This higher partitioning coefficient of NTM might be due to the high hydrophobicity of the lipid-rich cell wall of NTM (53). In our study, we calculated the average NTM partitioning coefficients to be even higher (3.8×10^{-2} L/m^3 and 2.9×10^{-2} L/m^3 for rain and massage showerheads, respectively). Partitioning coefficients defined in this way depend on the volume of air into which the aerosolized microorganisms are mixed and the ventilation rate of this space. For the small bathroom in this study, we might expect higher partitioning coefficients, although its high ventilation rate could offset the effect. We also attribute this difference to our NTM quantification method and experimental design. We used qPCR instead of a culture-based method to quantify NTM in water and air. Culture-based methods only recover a small portion of bacteria, especially

in low humidity and low nutrient environments (e.g. air). Therefore, the results obtained using culture-based methods may not best represent the true partitioning coefficient of NTM (54). In addition, we determined the partitioning coefficients using running shower water instead of using bulk water collected from hot tubs or pools (19). While the partitioning coefficient is widely adopted for risk assessment, it generally does not consider factors such as shower room dimension, ventilation conditions, and shower water flow rate, which may substantially influence pathogen aerosolization and have been identified as major sources of uncertainty for risk assessment (26). Therefore, we estimated the water–air transfer ratio in this study by integrating data on air NTM concentrations, shower water NTM concentrations, shower room dimension and ventilation conditions (air exchange rate), and shower water flow rate in a real shower room. In principle, this water–air transfer ratio is independent of the characteristics of the room and would apply for the same water, same showerhead, and same water flow rate in shower rooms with different dimensions or ventilation rates (i.e. different air exchange rates). We suggest that the NTM water–air transfer ratios can provide a more accurate tool for evaluating NTM infection risk associated with aerosolized pathogens during showering.

The showerhead spray pattern was found to affect the water–air transfer of NTM. This was not entirely surprising as a rain showerhead is designed to produce a wide water jet with relatively low velocity, while a massage showerhead produces a narrow jet with high velocity. These different showerhead spray patterns and corresponding flow conditions (velocity, dimension, water stream distribution, and so on), are thus expected to generate different amounts of aerosols (55). In this study, we report that the rain showerhead produced more aerosols than the massage showerhead, in terms of concentrations in all size ranges, and thus transferred more NTM cells from water to air. Therefore, the showerhead spray pattern might be a factor influencing NTM infection risk and should be considered in future risk assessment and control studies. In particular, caution should be applied when selecting showerheads for healthcare and long-term care facilities or for other buildings that house populations predisposed to NTM infection.

A substantial fraction of membrane-intact NTM were present in the bacterial community in water samples, but NTM in indoor air collected during showering were largely membrane compromised. We determined that 2.21×10^5 to 1.38×10^6 NTM cells (as determined by monitoring *atpE* gene copies) were transferred from water to air during our 1-hour shower studies. Using the live/dead ratio of bacteria and the relative abundance of NTM in water samples, we estimated that at least 8.7×10^3 membrane-intact NTM cells transferred from water to air during a typical 10-min shower. However, no membrane-intact bacterial cells were detected in air samples. Applying live/dead staining and flow cytometry to determine membrane integrity for bacteria in air samples has limitations due to the possibility that membrane damage may have occurred during transfer from water to indoor air, sample collection, and sample processing. Our experiments did not allow us to quantify the contributions to membrane damage by each of these steps. Nevertheless, several factors indicate that flow cytometry can detect viable bacteria in air samples: (1) the gelatin membranes we used for aerosol collection maintain better or comparable viability of opportunistic bacterial pathogens compared with other types of filters or liquid media (56, 57). (2) Although a fraction of viable bacteria might be damaged during sampling, flow cytometry analyzed a large number of particles and captured viable bacteria with intact membranes. (3) The live/dead staining and flow cytometry method determined the integrity of bacterial cell membranes, enabling the detection of more viable cells than culture-based methods (56). Therefore, the detection of a small fraction of membrane-intact bacterial cells in air samples suggested that many bacteria had been damaged after they were transferred to indoor air, collected, and processed for membrane integrity tests. Previous lab-scale studies also reported that pure cultures of bacteria lost their viability after aerosolization by using either live/dead staining or culture-based methods, although the lab-scale setups in these studies were different from the real shower room used in this study (58–61). The researchers proposed reasons for the loss of viability or membrane integrity, including the loss of water or shear stress formed during aerosolization. The loss of water can further induce substantial changes in osmotic pressure and membrane tension, thereby damaging bacterial cell membranes (58, 62, 63). Shear stress during aerosolization may also disturb membrane homeostasis, making the membrane the major site of bacterial cell damage (61, 64). However, it is noteworthy that membrane damage could be driven by different mechanisms under different experimental conditions. Therefore, the findings from previous lab-scale studies may not be completely transferable. In addition, our NTM recovery analysis indicates that aerosolized, membrane-damaged NTM can still propagate in a culture medium mimicking the respiratory secretions of a human population (persons with CF) that is particularly susceptible to NTM infection. We reasoned that use of this medium provided a more rigorous and meaningful assessment of the potential for aerosolized NTM to infect susceptible human hosts than the use of laboratory media designed to optimize recovery of NTM from other sources. If we assume a human inhalation rate of 12 L/min, then 134 to 652 NTM cells would be inhaled by a person during a 10-min shower. Although some of these NTM might be membrane-damaged, they are still viable and able to propagate, and thus could present a source of respiratory tract infection. We also note, however, that exposure to aerosolized nonviable NTM cells, while not capable of causing respiratory tract infection, has been associated with hypersensitivity pneumonitis (65). Thus, even nonviable NTM, acquired from aerosolized home building plumbing water, may present a human health risk.

Our findings are not inconsistent with previous studies that reported recovery of bacterial cells with membrane damage due to treatment with UV/chlorine, pressure, and plasma, but those recovery studies were performed with pure cultures in a nutrient-rich environment (66–68). Future studies are necessary to better understand the effect of environmental stressors on NTM viability and infectivity and the health risk associated with environmentally injured pathogens. Our recovery analysis further indicated that *M. mucogenicum*, a NTM species commonly associated with pulmonary disease (69, 70), became the dominant NTM species after 10 days of incubation. While NTM species exhibit varying degrees of pathogenicity, and thus pose different levels of health risk, our results imply that certain NTM species in air may recover more readily and propagate in the human respiratory tract. A greater understanding of NTM risk at the species level is needed for developing more effective pathogen control strategies in the built environment.

While the results of this study expand our understanding of the health risks associated with exposure to opportunistic pathogens during showering, this study has several limitations. First, although live/dead staining combined with flow cytometry and FACS has advantages over culture-based methods for determination of bacterial viability in environmental samples, different bacterial species exhibit differential responses to live/dead staining

(71). In addition, the sampling process was not able to completely recover all viable bacteria, likely resulting in the underestimation of bacterial cell viability. Improvements in bioaerosol sampling tools and viability determination methods are needed to characterize the viability of different bacterial species. In particular, several bioaerosol samplers showed good recovery of viable microorganisms in recent studies, which would be worthwhile to explore in the future (72–75). Second, the NTM recovery tests we used to employ in vitro conditions (simulated human sputum medium cultured at 37°C) that may not provide for optimal growth for all NTM species. Future studies also need to evaluate NTM recovery and growth at other temperatures and environmental conditions. Third, no human was taking a shower while we sampled, which may have influenced aerosol generation (water splashing off a human body may change the size distribution of aerosols). Future work should study how the human body influences aerosol formation and NTM release during showering. Fourth, we only focused on possible NTM exposure during the shower process and did not monitor the persistence of NTM long-term after showering. Future studies should also investigate the possible risk caused by the extended suspension and survival of NTM after showering. Finally, this study focused on the process of NTM transfer from shower water to air after water exited a showerhead. The transport of NTM within water distribution and plumbing systems is also of great importance. Future investigation of NTM identification, quantification, and release from biofilms is needed to develop improved strategies to mitigate the health risk associated with NTM in drinking water.

Materials and Methods

Water and indoor air sample collection in a shower room

Water and aerosol samples were collected from a shower room in an office building on the University of Michigan (Ann Arbor, MI) campus, which received chloraminated water from the City of Ann Arbor drinking water treatment plant (35). The building had its own hot water heater. The shower room had an area of 7.4 m^2 and a volume of 18.1 m^3. There were no windows in the shower room. This shower room was selected because it was not frequently used, so we could well control the showering frequency during the sampling period. Daily shower room cleaning on floor and table surfaces was conducted by the building janitors. The relative humidity before shower running ranged from 11% to 40%. The maximum relative humidity during shower running was 66%. For each sampling event, the shower ran for 60 min and 4 L water samples were collected at 0, 3, 5, 10, 15, 30, and 50 min. The water temperature was adjusted to 40 ± 2°C during sample collection. The chloramine concentrations in the shower water varied from 0.6 mg/L to 1.3 mg/L as Cl_2, and the pH ranged between 8.9 and 9.3. Water samples were filtered through a 0.2 μm polycarbonate membrane (EMD Millipore, Burlington, MA, USA) and a 0.45 μm backing membrane (EMD Millipore). Filtration took place within 4 hours of sample collection. The 0.2 μm membranes were stored at −20°C until DNA extraction.

Air samples were collected onto gelatin membrane filters (Sartorius, Goettingen, Germany) using a handheld bioaerosol sampler (MD8 Airport, Sartorius) running at 50 L/min. According to a previous report using the same type of membrane manufactured by the same company, the gelatin membrane contained 46% to 49% water, which helped preserve the viability of captured microorganisms (56). For each sampling event, air samples were collected before showering, during showering, and after showering for 60 min each. After sample collection, the gelatin filter was dissolved in 10 mL phosphate-buffered saline (PBS) buffer, which was subjected to the same filtration process as described for the shower water.

A total of 10 sampling events were performed from March to April 2018. During the study period, the shower was sampled or flushed early in the morning every other day for 60 min to maintain a constant showerhead use frequency. The 10 sampling events (each event included the collection of three air samples and seven water samples) were performed to quantify the transfer of NTM from water to air and to investigate the effect of showerhead spray patterns on NTM transfer. Among the 10 sampling events, half of them were conducted with the rain showerhead setting, and the other half were conducted with the massage showerhead setting of a multifunction showerhead (Delta A112.18.1 M, Delta Faucet Company, Indianapolis, IN). The rain showerhead had 39 nozzles in the outer ring of the showerhead and generated an average water flow velocity of 0.11 m/s, while the massage shower head had eight nozzles in the center of the showerhead and delivered water with an average water flow velocity of 0.17 m/s (Figure S1). The flow velocity was calculated based on the flow rate and the nozzle sizes. The water stagnation time of the building plumbing system before each sampling event was 15 hours. Therefore, the temperature of the stagnant water in the building plumbing system was close to room temperature. All the water and air samples collected from the ten sampling events were analyzed for bacterial community structure, NTM concentration, and viability. Three additional sampling events were performed in December 2018 for live bacterial cell sorting and aerosolized NTM recovery tests. In each of these sampling events, two shower water samples were collected at $t = 0$ min and $t = 15$ min by using the rain showerhead and three air samples were collected for 60 min each before, during, and after showering.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from shower water and air samples using the Maxwell 16 LEV Blood DNA Kit (Promega, Madison, WI, USA) with modifications as previously described (76). Briefly, each membrane was dissolved in 500 mL of a 49:1 chloroform–isoamyl alcohol mixture. Three rounds of physical and chemical lysis were achieved by 2 minutes of bead beating with 0.5 g of zirconium beads (BioSpec Products, Bartlesville, OK, USA) in Maxwell lysis buffer. Extracted DNA was dissolved in 50 uL of molecular grade water and stored at −20°C until use. A blank control (sterile DI water) was also included for DNA extraction and subsequent gene sequencing.

The extracted DNA from the shower water and air samples were submitted to The University of Michigan Microbial Systems Molecular Biology Laboratory for paired end 250 bp 16S rRNA gene amplicon sequencing by an Illumina MiSeq platform using primers previously described (77). For each shower water and air sample, an average of 22,631 ± 7,966 sequence reads were obtained. The sequences were analyzed by mothur (Version 1.41.0). Specifically, the sequences were assembled into contigs and trimmed to the same size. Unique sequences were identified to save computation time for the subsequent alignment based on the SILVA bacterial reference database. The sequences were clustered into operational taxonomic units (OTUs) at 97% identity without rarefication, and the relative abundance of each OTU was calculated. In total, 786 OTUs were identified within all water and air samples. Each OTU was annotated according to the SILVA bac-

terial reference database. The bacterial community richness and diversity parameters, including S_{ace} and H, for the shower water and air samples were also estimated by mothur commands. The links of the standard operating procedure and OTU-based analysis calculations for mothur are listed in Table S2. The output of mothur, including the abundance of each OTU, was also used to estimate the contribution of shower water to bacterial communities in indoor air by a source tracking method (38). The R package, SourceTracker (Table S2), was used to estimate the proportion of air bacterial community that originated from shower water (38).

Determination of NTM community structure by *rpoB* gene sequencing

NTM species in the shower water and air samples were identified by *rpoB* gene sequencing performed with a PacBio Sequel System. The *rpoB* gene amplicon libraries were prepared as described previously (15). Briefly, a two-step PCR procedure was performed. The first step amplified *rpoB* gene fragments and added motif sequences to both ends of the fragments. The second step further added unique barcodes to each sample for future demultiplexing. The PCR reactions in both steps (30 μL for the first step and 10 μL for the second step) contained 1 × Phusion Flash High-Fidelity PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 μM forward and reverse primer (IDT, Coralville, IA, USA), 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), 0.625 mg/mL bovine serum albumin (Life Technologies, Inc., Waltham, MA, USA), 0.5 μM betaine (Sigma-Aldrich), and 10% of template. The primer sequences and thermal cycling conditions were optimized and reported previously (15). After the second PCR, the products (with the size of ~1,014 bp) were purified by a Qiagen Gel Cleanup kit (Qiagen, Germantown, MD, USA). The purified products were pooled together with even concentrations, and a final pool with DNA concentration of 12.5 ng/μL was sent to the Institute for Genome Science at the University of Maryland for SMRTbell library construction and sequencing with a 10-hour movie time. A blank control (sterile DI water) was also included for each batch of the two-step PCR run. In addition, the DNA extracted from sterile DI water was subjected to the PCR runs. No gel band representing the target *rpoB* gene fragment was observed for the blank control samples, and the DNA concentrations in the purified PCR products from the blank controls were all nondetectable. Therefore, there should be no substantial contamination during the library preparation. Due to the relatively low bacterial concentration in the environmental samples, the repeated shower water and air samples were pooled together to obtain higher number of reads. A control sample containing 97% of *M. abscessus* and 3% of *M. mucogenicum* DNA was also analyzed for quality control purpose. The sequencing results for the barcoded samples were pre-screened and demultiplexed by the Lima SMRT analysis tool (Table S2). The circular consensus sequences were generated using SMRT Link with default parameters including a minimal accuracy of 99% and at least three sequencing passes per molecule. The motif sequences were then removed by Cutadapt (Table S2). Next, USEARCH (Table S2) was used to clean up the sequences with error > 1 and to orient all the sequences to the same directions. The sequences were then further merged, screened (remove sequences with abnormal lengths), aligned, and clustered to OTUs (by 97% identity) in mothur. On average, 2,008 clean sequence reads were obtained for each sample. The OTUs were finally classified by NCBI BLAST (Table S2) (78). In particular, the NTM species were assigned to 11 OTUs at a sequence identity of ≥ 97%. Three other OTUs with sequence identity ≥ 95% were annotated with the most closely matched NTM species.

Quantification of NTM in shower water and air samples by qPCR

qPCR was performed using a CFX96 Real-time quantitative thermocycler (Biorad, Hercules, CA, USA). The abundances of the *atpE* gene for *Mycobacterium* were quantified in the shower water and air samples according to a previous study (37). Specifically, each 10 μL reaction contained 1 μL DNA template, 5 μL Fast Evagreen qPCR Master Mix (Biotium, Fremont, CA, USA), 0.625 mg/mL bovine serum albumin (Invitrogen, Waltham, MA, USA), and 500 nM forward and reverse primers (IDT, Coralville, IA, USA). Samples were quantified from a standard curve consisting of 10-fold serially diluted qPCR standards (10^1 to 10^6) gene copies/μL. The standards were prepared from the purified PCR products of DNA extracted from environmental samples. The limit of detection (LOD) and limit of quantification (LOQ) for this qPCR assay were 14 and 19 gene copies/reaction, respectively. Triplicate qPCR reactions were conducted for all samples and standards, while blank controls were carried out in duplicate for each qPCR run. More details on the qPCR assay can be found in the Supporting Information.

Assessment of water–air transfer ratios for NTM

A mass balance model was developed to assess the water to air transfer ratio of NTM. In particular, the shower room was assumed to be a well-mixed space where NTM were emitted into air from the shower water and removed by ventilation. Thus, the NTM concentration in air (C_{air}, gene copies/m^3) is described by Eq. 1.

$$V\,\frac{dC_{air}}{dt} = E - V \times C_{air} \times AER/60, \qquad ((1))$$

where V is the shower room volume (m^3), t is time, E is the NTM emission rate from the shower head to indoor air (gene copies/min), and AER is the air exchange rate (14.4 air changes/hour, estimated by CO_2 decay) (79). The air exchange rate of 14.4 air changes/hour in this bathroom corresponds to a volume flow rate of 70 L/s, which is considerably higher than ASHRAE's recommendation for residential bathrooms of 10 L/s. On the other hand, the Home Ventilating Institute, an industry trade association, recommends an air exchange rate of 8 air changes/hour for bathrooms, and a research study has recommended 10 air changes/hour (80). Because of the high air exchange rate, the characteristic time for concentrations of shower-generated aerosols to reach steady state in the room (4.3 min) is much smaller than the sampling period of 60 min; thus, the sample should be reasonably representative of steady-state conditions in the room. Here, we assumed steady-state conditions during the shower period for which a single-time integrated measurement of the NTM concentration was available and calculated the corresponding time-integrated NTM emission rate ($\bar{E}$) using Eq. 2.

$$\bar{E} = V \times \bar{C}_{air} \times AER/60, \qquad ((2))$$

where $\bar{C}_{air}$ is the average NTM concentration in air (gene copies/m^3). $\bar{C}_{air}$ was calculated by subtracting the background NTM concentration measured before showering from the NTM concentration measured during showering. Accordingly, the total number of NTM transferred from shower water to air (N_{air}, gene copies) within 1 hour could be calculated by multiplying $\bar{E}$ by 60 min.

The number of NTM (N_{water}, gene copies) in the shower water was calculated by Eq. 3.

$$N_{water} = \sum_{t=0\ min}^{t=60\ min} C_{water-t} \times \Delta t \times F\ , \quad (3)$$

where t is the time when water sample collection started (0, 3, 5, 10, 15, 30, and 50 min, C_t is the NTM concentration in the shower water collected at time t (gene copies/L), and F is the shower flow rate (L/min).

The water–air transfer ratio of NTM (R) was then calculated by Eq. 4.

$$R = \frac{N_{air}}{N_{water}}. \quad (4)$$

Bacterial viability estimation and live cell sorting by live/dead staining and flow cytometry

The determination of bacteria viability in environmental samples was described in previous studies (29, 30, 35, 81). Briefly, 1 mL of the collected shower water or PBS-dissolved air sample was mixed with SYBR® Green I (SGI; Molecular Probes, Eugene, OR, USA) and propidium iodide (PI) (Molecular Probes). SGI penetrates the bacterial cell membrane and stains both live and dead bacterial cells. PI can only penetrate permeabilized (with damaged cell membrane) cell membrane and thus has been used as a dead cell marker. The final SYBR® Green I and PI concentrations in the sample were 1X and 3 μM, respectively. The mixture was then incubated at 37°C in the dark for 15 min. Subsequently, a flow cytometer (MoFlo Astrios, Beckman Coulter Life Sciences, Indianapolis, IN, USA) equipped with six lasers (405, 488, 532, 561, 594, and 640 nm) was used for bacterial viability measurement. In this study, SGI was excited by the 488-nm laser and detected by the bandpass filter at 513/26 nm. PI was excited by the 561-nm laser and emitted light detected at 614/10 nm. In each measurement, three control samples were also tested, including a shower water or an air sample without any dye, the same sample stained with only SGI, and the same sample stained with only PI. These three controls helped to determine the background and gates for the live and dead cell fractions. In addition, the bacterial viability test was validated by adding 1.4 mg/L of free chlorine to a shower water sample. The shower water sample consisted of the initial 50 mL of water collected when turning on the shower after the water had been stagnant for 2 days. This sample had a high ratio of membrane-intact cells to total cells (64.3%). After 1 hour of exposure to free chlorine, the ratio of membrane-intact cells to total cells reduced to 9.5%, indicating that the live/dead staining and flow cytometry method captured the viability change in our samples.

For selected shower samples (six shower water samples collected in three sampling events in December 2018), the live bacterial fraction was sorted out by FACS for bacterial community analysis. Specifically, 2 L of the shower water sample was concentrated to 15 mL by a bench-scale cross/tangential flow cell (Sterlitech, Kent, WA, USA) and a microfiltration polyvinylidene fluoride (PVDF) membrane with 0.2 μm pore size. The sample was then mixed with SGI and PI and incubated at 37°C in the dark for 15 min. The same MoFlo Astrios flow cytometer mounted with a cell sorter was used to identify and sort out the live bacterial cell fraction. The cell sorting efficiency was maintained between 80% and 100%. The sorted live cells were filtered onto a 0.2-μm polycarbonate membrane and stored at −20°C until DNA extraction. All the bacterial viability and cell sorting was done within 10 hours of sampling.

NTM recovery in simulated human sputum medium

To test the recovery of damaged NTM in a medium that models human sputum from a susceptible host population, air samples collected in three sampling events in December 2018 were incubated in synthetic CF sputum medium (39). This medium contained minerals, free amino acids, glucose, lactate, DNA, lipids, and mucin to mimic sputum from persons with CF. For each air sample collected onto the gelatin membrane, 5 mL PBS was used to dissolve the membrane. For each PBS-dissolved air sample, 2 mL was used to determine the concentration of NTM by DNA extraction and qPCR. A volume of 1 mL of the PBS-dissolved air sample was used for the NTM recovery test by mixing with 9 mL of the simulated sputum medium and incubating at 37°C for 14 days. Another 1 mL of the PBS-dissolved air sample was mixed with 9 mL of PBS and incubated at 37°C as a negative control. At Days 5, 10, and 14, 2 mL sample was collected from each mixture for DNA extraction and qPCR to determine the NTM concentration in the sputum medium or PBS. The extracted DNA samples were also used for *rpoB* gene sequencing to analyze NTM community structure change during the recovery process.

Statistical analyses

To compare the sequencing and qPCR analysis results for air samples collected before and during showering, paired t tests were used to analyze the difference of pre-shower and during-shower air samples collected from the same shower event. In addition, paired t tests were also used to compare the qPCR and mass balance model analysis results from rain and massage showerheads. To minimize the effect of water quality variation on comparison of rain and massage showerheads, the shower water and air samples were alternately collected from rain and massage type. Each pair of sampling events from rain and massage showerheads occurred within one week. t tests were used in this study by assuming each measurement was independent and errors were random and followed normal distribution. The significance level used in t tests was 0.05.

Acknowledgments

We thank Jennifer Bomberger for providing the simulated human sputum medium. We also thank Liem Ellen Setiawan for her help on sampling collection, Nicole Rockey for her help with flow cytometry, and Jennifer Nord for providing access to the building with the shower room. In addition, we thank Maryland Genomics in the Institute for Genome Sciences at the University of Maryland School of Medicine for providing the sequencing services. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding sources.

Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

Funding

Y.S. was supported by an Alfred P. Sloan Foundation Microbiology of the Built Environment Fellowship (G-2016–7250). S.-J.H. was

supported by an Alfred P. Sloan Foundation Microbiology of the Built Environment fellowship (G-2014–13739) and a University of Michigan Dow Sustainability postdoctoral fellowship. A.J.P.II. was supported by the Virginia Tech's Institute for Critical Technology and Applied Science. This study was also supported by the MCubed program of the University of Michigan.

Authors' Contributions

Y.S. and L.R. designed the research. Y.S. performed the experiments and data analysis and drafted the manuscript. S.-J.H. developed and contributed the *rpoB* gene sequencing method. A.J.P.II. characterized the air exchange rate in the shower room and contributed to the development of air sampling method. J.J.L provided guidance on culture-based methods and interpretation of results. L.C.M. provided guidance on air sampling and aerosol characterization. L.R. provided guidance and supervision to the whole research. All authors provided suggestions for revisions.

Data Availability

All sequencing data are publicly available through figshare (DOI:10.6084/m9.figshare.19775551). All other data are provided in the manuscript or in the Supporting Information.

References

1. Koh W-J. 2017. Nontuberculous mycobacteria—overview. Microbiol Spectr. 5:653–661. Tuberculosis and nontuberculous mycobacterial infections.
2. Griffith DE, *et al.* 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 175(4):367–416.
3. Falkinham JO, III, Iseman MD, de Haas P, van Soolingen D. 2008. *Mycobacterium avium* in a shower linked to pulmonary disease. J Water Health. 6(2):209–213.
4. Halstrom S, Price P, Thomson R. 2015. Review: environmental mycobacteria as a cause of human infection. Int J Mycobacteriol. 4(2):81–91.
5. Strollo SE, Adjemian J, Adjemian MK, Prevots DR. 2015. The burden of pulmonary nontuberculous mycobacterial disease in the United States. Ann Am Thoracic Soc. 12(10):1458–1464.
6. Vinnard C, *et al.* 2016. Deaths related to nontuberculous mycobacterial infections in the United States, 1999–2014. Ann Am Thoracic Soc. 13(11):1951–1955.
7. Nishiuchi Y, Iwamoto T, Maruyama F. 2017. Infection sources of a common non-tuberculous mycobacterial pathogen, *Mycobacterium avium* complex. Front Med. 4:27.
8. Dowdell K, *et al.* 2019. Nontuberculous mycobacteria in drinking water systems–the challenges of characterization and risk mitigation. Curr Opin Biotechnol. 57:127–136.
9. Donohue MJ, *et al.* 2015. Increased frequency of nontuberculous mycobacteria detection at potable water taps within the United States. Environ Sci Technol. 49(10):6127–6133.
10. Gebert MJ, *et al.* 2018. Ecological analyses of mycobacteria in showerhead biofilms and their relevance to human health. MBio. 9(5):e01614–e01618.
11. Rhoads WJ, Pruden A, Edwards MA. 2014. Anticipating challenges with in-building disinfection for control of opportunistic pathogens. Water Environ Res. 86(6):540–549.
12. Falkinham JO. 2015. Environmental sources of nontuberculous mycobacteria. Clin Chest Med. 36(1):35–41.
13. Taylor RH, Falkinham JO, Norton CD, LeChevallier MW. 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. Appl Environ Microbiol. 66(4): 1702–1705.
14. Wang H, Edwards M, Falkinham JO, 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.
15. Haig S-J, Kotlarz N, LiPuma JJ, Raskin L. 2018. A high-throughput approach for identification of nontuberculous mycobacteria in drinking water reveals relationship between water age and *Mycobacterium avium*. mBio. 9(1):e02354–e02317.
16. Haig S-J, *et al.* 2020. Emerging investigator series: bacterial opportunistic pathogen gene markers in municipal drinking water are associated with distribution system and household plumbing characteristics. Environ Sci Water Res Technol. 6(11):3032–3043.
17. Morimoto K, *et al.* 2018. Prevention of aerosol isolation of nontuberculous mycobacterium from the patient's bathroom. ERJ Open Res. 4(3):00150–02017.
18. Thomson R, *et al.* 2013. Isolation of nontuberculous mycobacteria (NTM) from household water and shower aerosols in patients with pulmonary disease caused by NTM. J Clin Microbiol. 51(9):3006–3011.
19. Glazer CS, *et al.* 2007. Nontuberculous mycobacteria in aerosol droplets and bulk water samples from therapy pools and hot tubs. J Occup Environ Hyg. 4(11):831–840.
20. Perkins SD, Mayfield J, Fraser V, Angenent LT. 2009. Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. Appl Environ Microbiol. 75(16): 5363–5372.
21. Klepeis NE, *et al.* 2001. The National Human Activity Pattern Survey (NHAPS): a resource for assessing exposure to environmental pollutants. J Exposure Sci Environ Epidemiol. 11(3): 231–252.
22. Miletto M, Lindow SE. 2015. Relative and contextual contribution of different sources to the composition and abundance of indoor air bacteria in residences. Microbiome. 3(1):61.
23. Prussin AJ, Marr LC. 2015. Sources of airborne microorganisms in the built environment. Microbiome. 3(1):78.
24. Zhou Y, Benson JM, Irvin C, Irshad H, Cheng Y-S. 2007. Particle size distribution and inhalation dose of shower water under selected operating conditions. Inhalation Toxicol. 19(4): 333–342.
25. Estrada-Perez C, Kinney K, Maestre J, Hassan Y, King M. 2018. Droplet distribution and airborne bacteria in an experimental shower unit. Water Res. 130:47–57.
26. Buse HY, Schoen ME, Ashbolt NJ. 2012. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 46(4):921–933.
27. Armstrong T, Haas CN. 2007. A quantitative microbial risk assessment model for Legionnaires' disease: animal model selection and dose-response modeling. Risk Anal Int J. 27(6):1581–1596.
28. Vejerano EP, Marr LC. 2018. Physico-chemical characteristics of evaporating respiratory fluid droplets. J R Soc Interface. 15(139):20170939.
29. Ramseier MK, von Gunten U, Freihofer P, Hammes F. 2011. Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate (VI), and permanganate. Water Res. 45(3):1490–1500.

30. Berney M, Hammes F, Bosshard F, Weilenmann H-U, Egli T. 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl Environ Microbiol. 73(10):3283–3290.
31. Grégori G, *et al.* 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. Appl Environ Microbiol. 67(10):4662–4670.
32. Van Nevel S, *et al.* 2017. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206.
33. Robertson J, McGoverin C, Vanholsbeeck F, Swift S. 2019. Optimisation of the protocol for the LIVE/DEAD® BacLightTM bacterial viability kit for rapid determination of bacterial load. Front Microbiol. 10:801.
34. Van Nevel S, Koetzsch S, Weilenmann H-U, Boon N, Hammes F. 2013. Routine bacterial analysis with automated flow cytometry. J Microbiol Methods. 94(2):73–76.
35. Kotlarz N, *et al.* 2018. Biofilms in full-scale drinking water ozone contactors contribute viable bacteria to ozonated water. Environ Sci Technol. 52(5):2618–2628.
36. Hernlem BJ, Ravva SV. 2007. Application of flow cytometry and cell sorting to the bacterial analysis of environmental aerosol samples. J Environ Monit. 9(12):1317–1322.
37. Radomski N, *et al.* 2013. *atpE* gene as a new useful specific molecular target to quantify *Mycobacterium* in environmental samples. BMC Microbiol. 13(1):277.
38. Knights D, *et al.* 2011. Bayesian community-wide culture-independent microbial source tracking. Nat Methods. 8(9):761–763.
39. Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. 2015. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. Proc Natl Acad Sci. 112(13):4110–4115.
40. Donohue MJ. 2018. Increasing nontuberculous mycobacteria reporting rates and species diversity identified in clinical laboratory reports. BMC Infect Dis. 18(1):163.
41. Kim B-J, Hong S-H, Kook Y-H, Kim B-J. 2014. *Mycobacterium paragordonae* sp. nov., a slowly growing, scotochromogenic species closely related to *Mycobacterium gordonae*. Int J Syst Evol Microbiol. 64(1):39–45.
42. Takajo I, *et al.* 2020. Pseudo-outbreak of *Mycobacterium paragordonae* in a hospital: possible role of the aerator/rectifier connected to the faucet of the water supply system. J Hosp Infect. 104(4):545–551.
43. Räsänen NH, Rintala H, Miettinen IT, Torvinen E. 2013. Comparison of culture and qPCR methods in detection of mycobacteria from drinking waters. Can J Microbiol. 59(4):280–286.
44. Zhang X, Xia S, Ye Y, Wang H. 2021. Opportunistic pathogens exhibit distinct growth dynamics in rainwater and tap water storage systems. Water Res. 204:117581.
45. Hamilton KA, *et al.* 2019. Risk-based critical concentrations of *Legionella pneumophila* for indoor residential water uses. Environ Sci Technol. 53(8):4528–4541.
46. Hamilton KA, Ahmed W, Toze S, Haas CN. 2017. Human health risks for Legionella and *Mycobacterium avium* complex (MAC) from potable and non-potable uses of roof-harvested rainwater. Water Res. 119:288–303.
47. Schoen ME, Ashbolt NJ. 2011. An in-premise model for *Legionella* exposure during showering events. Water Res. 45(18):5826–5836.
48. Huang C, Shen Y, Smith RL, Dong S, Nguyen TH. 2020. Effect of disinfectant residuals on infection risks from *Legionella pneumophila* released by biofilms grown under simulated premise plumbing conditions. Environ Int. 137:105561.
49. Bollin G, Plouffe J, Para MF, Hackman B. 1985. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets. Appl Environ Microbiol. 50(5):1128–1131.
50. Allegra S, *et al.* 2016. Characterization of aerosols containing Legionella generated upon nebulization. Sci Rep. 6(1):1–8.
51. Hines SA, *et al.* 2014. Assessment of relative potential for *Legionella* species or surrogates inhalation exposure from common water uses. Water Res. 56:203–213.
52. Angenent LT, Kelley ST, Amand AS, Pace NR, Hernandez MT. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci. 102(13):4860–4865.
53. Dufour A. 2004. Pathogenic mycobacteria in water: a guide to public health consequences, monitoring and management. Geneva: World Health Organization.
54. Nazaroff WW. 2016. Indoor bioaerosol dynamics. Indoor Air. 26(1):61–78.
55. Zhou Y, Mui KW, Wong LT, Tsui PH, Chan WK. 2019. Aerosol generation rates for showerheads. Build Serv Eng Res Technol. 40(5):595–610.
56. Chang CW, Chou FC. 2011. Methodologies for quantifying culturable, viable, and total *Legionella pneumophila* in indoor air. Indoor Air. 21(4):291–299.
57. Dabisch P, Bower K, Dorsey B, Wronka L. 2012. Recovery efficiencies for *Burkholderia thailandensis* from various aerosol sampling media. Front Cell Infect Microbiol. 2:78.
58. Lin K, Marr LC. 2019. Humidity-dependent decay of viruses, but not bacteria, in aerosols and droplets follows disinfection kinetics. Environ Sci Technol. 54:1024–1032.
59. Alsved M, *et al.* 2018. Effect of aerosolization and drying on the viability of pseudomonas syringae cells. Front Microbiol. 9:3086.
60. Heidelberg J, *et al.* 1997. Effect of aerosolization on culturability and viability of Gram-negative bacteria. Appl Environ Microbiol. 63(9):3585–3588.
61. Thomas RJ, *et al.* 2011. The cell membrane as a major site of damage during aerosolization of *Escherichia coli*. Appl Environ Microbiol. 77(3):920–925.
62. Nocker A, Fernández PS, Montijn R, Schuren F. 2012. Effect of air drying on bacterial viability: a multiparameter viability assessment. J Microbiol Methods. 90(2):86–95.
63. Wood JM. 2015. Perspectives on: the response to osmotic challenges: bacterial responses to osmotic challenges. J Gen Physiol. 145(5):381.
64. Zhen H, Han T, Fennell DE, Mainelis G. 2013. Release of free DNA by membrane-impaired bacterial aerosols due to aerosolization and air sampling. Appl Environ Microbiol. 79(24):7780–7789.
65. Cappelluti E, Fraire AE, Schaefer OP. 2003. A case of hot tub lung due to *Mycobacterium avium* complex in an immunocompetent host. Arch Intern Med. 163(7):845–848.
66. Bozoglu F, Alpas H, Kaletunç G. 2004. Injury recovery of foodborne pathogens in high hydrostatic pressure treated milk during storage. FEMS Immunol Med Microbiol. 40(3):243–247.
67. Dolezalova E, Lukes P. 2015. Membrane damage and active but nonculturable state in liquid cultures of *Escherichia coli* treated with an atmospheric pressure plasma jet. Bioelectrochemistry. 103:7–14.

68. Wang L, *et al.* 2021. Assessment of the UV/chlorine process in the disinfection of *Pseudomonas aeruginosa*: efficiency and mechanism. Environ Sci Technol.55. DOI: 10.1021/acs.est.1c00645.
69. Brown-Elliott BA, Philley JV. 2017. Rapidly growing mycobacteria. Microbiol Spectr. 5:703–723. Tuberculosis and Nontuberculous Mycobacterial Infection.
70. Adékambi T. 2009. *Mycobacterium mucogenicum* group infections: a review. Clin Microbiol Infect. 15(10):911–918.
71. Berney M, Hammes F, Bosshard F, Weilenmann H-U, Egli T. 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl Environ Microbiol. 73(10):3283–3290.
72. Degois J, Dubuis M-E, Turgeon N, Veillette M, Duchaine C. 2021. Condensation sampler efficiency for the recovery and infectivity preservation of viral bioaerosols. Aerosol Sci Technol. 55(6):653–664.
73. Pan M, *et al.* 2018. Collection of airborne bacteria and yeast through water-based condensational growth. Aerobiologia. 34(3):337–348.
74. Nieto-Caballero M, Savage N, Keady P, Hernandez M. 2019. High fidelity recovery of airborne microbial genetic materials by direct condensation capture into genomic preservatives. J Microbiol Methods. 157:1–3.
75. Lednicky J, *et al.* 2016. Highly efficient collection of infectious pandemic influenza H1N1 virus (2009) through laminar-flow water based condensation. Aerosol Sci Technol. 50(7):i–iv.
76. Webster TM, *et al.* 2016. Anaerobic microbial community response to methanogenic inhibitors 2-bromoethanesulfonate and propynoic acid. MicrobiologyOpen. 5(4): 537–550.
77. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 79:5112–5120. AEM. 01043-13.
78. Johnson M, *et al.* 2008. NCBI BLAST: a better web interface. Nucleic Acids Res. 36(suppl_2):W5–W9.
79. Claude-Alain R, Foradini F. 2002. Simple and cheap air change rate measurement using CO2 concentration decays. Int J Vent. 1(1):39–44.
80. Tung Y-C, Hu S-C, Tsai T-Y. 2009. Influence of bathroom ventilation rates and toilet location on odor removal. Build Environ. 44(9):1810–1817.
81. Barbesti S, *et al.* 2000. Two and three-color fluorescence flow cytometric analysis of immunoidentified viable bacteria. Cytomet J Int Soc Analy Cytol. 40(3):214–218.