Effect of disinfectant residuals on infection risks from *Legionella pneumophila* released by biofilms grown under simulated premise plumbing conditions

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Conghui Huang[a], Yun Shen[b], Rebecca L. Smith[c], Shengkun Dong[d,*], Thanh H. Nguyen[a,*]

[a]*Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States*

[b]*Department of Environmental and Occupational Health, George Washington University, Washington, DC 20052, United States*

[c]*Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, United States*

[d]*Guangdong Engineering Technology Research Center of Water Security Regulation and Control for Southern China, Key Laboratory of Water Cycle and Water Security in Southern China of Guangdong Higher Education Institute, School of Civil Engineering, Sun Yat-sen University, Guangzhou, Guangdong 510275, China*

[*]Authors to whom correspondence should be addressed:
Thanh H. Nguyen (thn@illinois.edu) and Shengkun Dong (dongshk5@mail.sysu.edu.cn)

Abstract

The ubiquitous presence of biofilms in premise plumbing and stagnation, which commonly occurs in premise plumbing, can exacerbate the decay of chlorine residual in drinking water. Using biofilms grown in a simulated premise plumbing setup fed directly with freshly treated water at two full-scale water treatment plants, we previously determined the mass transfer coefficients for chlorine decay in premise plumbing. These coefficients coupled with inactivation kinetics of *L. pneumophila* released from biofilms reported previously were integrated into a Monte Carlo framework to estimate the infection risk of biofilm-derived *L. pneumophila* from 1 to 48 hours of stagnation. The annual infection risk was significantly higher when water stayed stagnant for up to 48 hours in pipes covered internally with biofilms, compared to clean pipes without biofilms. The decay of residual chlorine due to biofilms during 48-hour stagnation led to up to 6 times increase in the annual infection risk compared to the case where biofilms was absent. Global sensitivity analysis revealed that the rate of *L. pneumophila* detachment from biofilms and the decay of chlorine residual during stagnation are the two most important factors influencing the infection risks. Stagnation caused by water use patterns and water-saving devices in the premise plumbing can lead to increased infection risk by biofilm-derived *L. pneumophila*. Overall, this study's findings suggested that biofilms could induce chlorine decay and consequently increase *L. pneumophila* infection risk. Thus, reducing stagnation, maintaining residual chlorine, and suppressing biofilm growth could contribute to better management of *L. pneumophila* infection risk.

Keywords: residual chlorine, stagnation, biofilm, *Legionella pneumophila*

1. Introduction

Legionella pneumophila is a causative agent for Legionnaires' disease, which occurs at increasing frequency in the United States (Beer et al., 2015; Shah et al., 2018). *L. pneumophila* contributed to 67% of the drinking water-associated disease outbreaks from 2013 to 2015 in the US, according to surveillance reports from the Centers for Disease Control and Prevention (Beer et al., 2015; Shah et al., 2018). More than 340 million USD was charged annually to US Medicare by hospitals treating patients with Legionnaires' disease between 1991 and 2006 (Naumova et al., 2016). In addition, the presence of *L. pneumophila* in building plumbing systems was one of the most identified causes in drinking water-associated outbreaks in the US (Garrison et al., 2016). High occupancy buildings, such as hospitals and hotels, were identified as common locations of Legionnaires' disease outbreaks because of residual disinfectant depletion that is due to stagnation, pipe materials, and temperatures (Garrison et al., 2016; Shah et al., 2018). *L. pneumophila* has been detected in biofilms and drinking water collected from showerheads, faucets, and humidifiers in these high occupancy buildings and green buildings (Garrison et al., 2016; Ji et al., 2017; Proctor et al., 2018; Rhoads et al., 2016; Shah et al., 2018). Among Legionnaires' disease outbreaks in the US, 85% were attributed to deficiencies in water system maintenance, among which 70% of these outbreaks were associated with inadequate disinfectant residual (Garrison et al., 2016). In some European countries, including the Netherlands, Switzerland, and Norway, residual disinfectant is not maintained in DWDS (Rosario-Ortiz et al., 2016). In contrast, the US requires a residual disinfectant is maintained in 95% of the water samples from the DWDS to control growth and release of bacteria (EPA, 2016). In the US systems, however, the residual disinfectant may not be well maintained throughout the entire distribution pipeline due to the self-decay of the disinfectant and consumption of the disinfectant by pipe

materials, water organic matter, and biofilms growing inside the pipes (Buse et al., 2012; Lu et al., 2017; Rhoads et al., 2016; Xu et al., 2018). Long stagnation in premise plumbing led to the absence of disinfectant and promotes the growth of biofilm on the pipe internal surface (Lautenschlager et al., 2010; Ling et al., 2018; Rhoads et al., 2016). The presence of biofilms in the premise plumbing has been found to facilitate the survival of *L. pneumophila* (Buse et al., 2012; Cooper and Hanlon, 2010; Declerck, 2010; Declerck et al., 2009; Wang et al., 2017). However, the complex role of stagnation, disinfectant residual, biofilms, and opportunistic pathogens released from biofilms on public health has not been well characterized.

Intervention and surveillance for *L. pneumophila* is often difficult and depends on guidelines, sampling skills, and managers' judgment (Hamilton et al., 2019; OSHA). The decision-making process and interpretation of measurements obtained from the monitoring program often rely on a quantitative microbial risk assessment (QMRA) approach to evaluate factors influencing *L. pneumophila* infection risk under various scenarios. For example, QMRA was used to determine the critical *L. pneumophila* concentrations corresponding to a given risk level (Hamilton et al., 2019). Although biofilms were identified as one of the most important factors contributing to *L. pneumophila* growth in the premise plumbing (Buse et al., 2012; Rhoads et al., 2016), only one QMRA study assessed the critical concentration of *L. pneumophila* associated with sloughed-off biofilms (Schoen and Ashbolt, 2011). Previous studies also estimated the infection risk of *L. pneumophila* on a time-averaged approach without considering the inactivation by residual disinfectant (Hamilton et al., 2019; Schoen and Ashbolt, 2011). Although both residual disinfectant levels and stagnation in premise plumbing can facilitate the survival of *L. pneumophila* (Buse et al., 2012), their effects on the infection risk of *L. pneumophila* released from biofilms in premise plumbing have not been fully evaluated. QMRA models that consider the effect of time-

dependent residual chlorine concentration on the inactivation and release kinetics of *L. pneumophila* from biofilms have not been developed.

To better address these knowledge gaps in quantifying the risk of *L. pneumophila* released from biofilms grown in premise plumbing, we developed a time-dependent QMRA. We estimated the time-dependent infection risk of biofilm-derived *L. pneumophila*, factoring in the consumption of residual chlorine by biofilms during stagnation. Specifically, we used experimental measurements of the chlorine decay by biofilms grown in simulated premise plumbing (Xu et al., 2018) and the kinetics of biofilm-derived *L. pneumophila* release and inactivation (Shen et al., 2017). We chose to focus on the role of stagnation on residual chlorine decay by biofilms because stagnation was a key factor in promoting the growth of microbes in distribution systems (Prest et al., 2016) and biofilm-derived *L. pneumophila* are known to be more resistant to disinfectants than planktonic cells (Cooper and Hanlon, 2010; Declerck, 2010; Shen et al., 2017). The findings on infection risk under different stagnation durations and chlorine residual conditions may facilitate decision making to reduce premise plumbing infection risk from *L. pneumophila.*

2. Methods

2.1 Overview

In this study, we quantitatively compared *L. pneumophila* infection risks accounting for stagnation-dependent chlorine consumption by premise plumbing biofilms. These biofilms were grown from two different drinking waters directly collected at two drinking water treatment plants. We calculated the risk from *L. pneumophila* cells that were incorporated into the biofilms during stagnation and subsequently released when the biofilms were exposed to chlorine-containing water flow. We chose this scenario because we previously showed substantial *L. pneumophila*

attachment to drinking water biofilms and lower inactivation effectiveness of the attached *L. pneumophila* by disinfection compared to *L. pneumophila* cells that float in water (Shen et al., 2017). Additionally, we previously found that the shear stress exerted by water flow can release *L. pneumophila* from biofilms (Shen et al., 2015). Under stagnation without the water shear stress, the release of *L. pneumophila* from biofilms are likely to be much smaller. Therefore, to be conservative in risk estimation, we opted for the scenario in which *L. pneumophila* cells were released from biofilms under shear stress exerted from the water flow.

First, we selected the simulation scenarios relevant to premise plumbing systems. Second, mass transfer modeling of chlorine in contact with biofilms (grown in PVC pipe-loops at two full-scale water treatment plants (WTPs)) from 1 to 48 hours was performed and incorporated as one of the model inputs to simulate biofilm-derived chlorine decay in a model household premise plumbing. The stagnation time of 1-48 hours was chosen based on the surveys on water use patterns in buildings (DeOreo and Mayer, 2014). The demand peaks are mostly in the morning (8-9 am) with a lower peak in the evening (7 pm) (DeOreo and Mayer, 2014), which showed potential stagnation periods of 13 to 14 hours during daily water use. Shorter stagnation time could happen when the occupants use water throughout the day (DeOreo and Mayer, 2014). Low flow or stagnant conditions in the premise plumbing became much more common because of the sustainable and water-saving design in indoor water fixtures and green buildings (Ahmed et al., 2016; DeOreo and Mayer, 2014). Third, based on the experimentally determined inactivation kinetics of biofilm-derived *L. pneumophila* and the calculated residual chlorine profiles, the concentrations of live *L. pneumophila* that were released from the biofilms were determined. Coupling these concentrations with the dose-response relationship of an inhalation (followed by deposition in the lungs) scenario, the annual or per event infection risk of *L. pneumophila* that

were released from biofilms was determined. We only considered the aerosols of 1-10 μm in diameter because larger aerosols are not able to reach the alveolar region via mouth and nose inhalation (Allegra et al., 2016; Schoen and Ashbolt, 2011). Stagnation (1-48 hours) occurred when building occupants or home residents were not using water during workdays or weekend trips. The shower duration used in simulations was from 4-14 minutes (5-95% confidence interval (CI)) with a median of 7 minutes. During shorter shower events, residual chlorine concentration was assumed to be constant. During each shower event, the inactivation of *L. pneumophila* was based on the pseudo first-order relationship between residual chlorine concentration and shower time.

In summary, we made the following assumptions in the simulations: 1. the premise plumbing system in the building was large enough that no freshwater with higher residual chlorine concentration reached the shower hose during a shower event; 2. biofilm-derived *L. pneumophila* cells were released predominantly by the shear stress exerted by the water flow after stagnation; 3. only culturable *L. pneumophila* was considered to be live and infectious; 4. *L. pneumophila* cells were aerosolized in the ranges of inhalable aerosols and subsequently deposited in the human lung; 5. no regrowth of biofilms during shower events in the premise plumbing was considered in the risk assessment.

2.2 Simulation scenarios for premise plumbing

We conducted simulation to determine the effects of the residual chlorine decay after stagnation on the infection risk of *L. pneumophila* released from biofilms. The simulation was conducted under two scenarios: with (scenario 1a and 1b) and without (scenario 2) residual chlorine (**Figure 1)**. Scenario 1a and 1b represent premise plumbing in an occupied building where residual chlorine is present in fresh drinking water coming from the distribution system and is

decayed to different extents after stagnation. The effects of biofilms grown inside the premise plumbing pipes on chlorine decay and infection risk of biofilm-derived *L. pneumophila* were accounted for with the concentration profiles of the residual chlorine in the pipes covered by biofilms grown from two different drinking water sources. The chlorine concentration profile of water in contact with plant A biofilm and plant B biofilm corresponds to scenario 1a and 1b, respectively. Scenario 2 represents a temporarily vacant building where residual chlorine is consumed completely after being stagnated from days to months. Thus, in this scenario, neither biofilms nor biofilm-derived *L. pneumophila* were influenced by residual chlorine. A scenario representing a premise plumbing without biofilms (scenario 3) was used to investigate the effects of the presence of biofilms on *L. pneumophila* infection risk. In this scenario, the planktonic *L. pneumophila* in premise plumbing was inactivated in water with the residual chlorine concentration after stagnation without biofilms.

Infection risk of biofilm-derived *L. pneumophila* after five stagnation periods (1, 6, 12, 24, 48 hours of stagnation) was calculated, respectively, based on the experimentally measured release and inactivation kinetics by free chlorine from a previous study (Shen et al., 2017) and the time-dependent residual chlorine concentration profiles. These profiles after 1 to 48 hours of stagnation were simulated using a previously developed model for chlorine decay in contact with biofilms fed by waters at two WTPs (Xu et al., 2018). The release kinetics of biofilm-derived *L. pneumophila* were obtained under flow conditions at a flow rate of around 7 L/min, which is similar to the median flow rate measured during shower events (DeOreo and Mayer, 2014). The biofilms used for chlorine decay simulation during stagnation were grown from drinking water treated at two plants A and B, as described in the previous study (Xu et al., 2018). These two water plants used surface water from two rivers and reservoirs. Plant A had more than two times plant

185 production and serviced about three times more population than plant B. The surface water treated
186 by physical treatment (coagulation, sedimentation, and filtration) fed the PVC pipe-loops. The
187 pipe-loops were made up of the same pipe materials used in premise plumbing. The biofilms were
188 grown from water free of disinfectants before exposing to chlorine. Water before disinfection was
189 used to grow biofilms to simulate the end of the premise plumbing, where the residual
190 concentration of chlorine is usually low during stagnation (Buse et al., 2012; Ling et al., 2018).
191 We assumed that the water tank supplying the premise plumbing was large enough that no fresh
192 water with free chlorine from the water main reached users during a shower event. This assumption
193 was made based on the calculated retention time of water in the premise plumbing with water tanks
194 (**SI section 2**).
195
196 **Figure 1**. Modeling framework of scenario-specific infection risk quantification from biofilm-
197 derived *L. pneumophila* exposure by shower event on a daily or annual basis.
198
199 **2.3 Modeling framework**
200 We calculated the infection risk related to showering because exposure to *L. pneumophila*
201 during shower events resulted in the highest estimated infection risk among common household
202 activities (Hamilton et al., 2019). When water passes through the pressurized shower head during
203 the shower, some *L. pneumophila* cells can partition into the air phase, a portion of which was in
204 the respirable size range of 1 to 10 μm (Allegra et al., 2016). Aerosolized *L. pneumophila* cells are
205 introduced into the human lungs by inhalation. A fraction of the inhaled *L. pneumophila* cells are
206 then deposited in the alveolar, with a certain probability of causing infection, as described

previously using a dose-response model (Armstrong and Haas, 2007; Muller et al., 1983). Modeling parameters are listed in **Table 1**.

To estimate the infection risk of biofilm-derived *L. pneumophila*, we started with estimating the total number of *L. pneumophila* cells released from biofilms over time (**equation 1**). We fit the total released biofilm-derived *L. pneumophila* concentration measured previously (Shen et al., 2017) using a two-phase first-order decay model segmented at the fifth minute (**Figure 1S** and **Table 1S)**. Two sets of first-order release rate constants (k_f for the first 5 minutes and k_{f_5} for after 5 minutes) were obtained and used for biofilm-derived *L. pneumophila*, with or without disinfectant exposure (scenario 1 or 2). As shown previously, higher flow rate and long-term exposure to free chlorine increased the release of *L. pneumophila* from biofilms (Shen et al., 2017; Shen et al., 2015). Biofilms can be exposed to residual chlorine with a median of 1.3 mg/L (0.36-3.7 mg/L, 5-95% CI) in the fresh drinking water. To account for the effect of long-term chlorine exposure on *L. pneumophila* release from biofilms, we used a *L. pneumophila* release rate from biofilms that were exposed to 2 mg/L free chlorine. Another release rate of *L. pneumophila* from biofilms without previous exposure to free chlorine was also used to simulate a chlorine free scenario (scenario 2). No release kinetics were used in scenario 3 because only inactivation occurred on planktonic *L. pneumophila* cells in the premise plumbing without biofilms.

The release rate constants (k_f and k_{f_5}) are shown in **Table 1S** and **Figure 1S**. The total concentration of *L. pneumophila* released from biofilms at time t, $N_{total}(t)$ in cells/L, is calculated using **equation 1**. The exposure time t ranged from 0 to T (shower duration, in minutes).

$$N_{total}(t) = \begin{cases} N_0 \times e^{k_f \times t} & 5 \geq t \geq 0 \\ N_{total}(t=5) \times e^{k_{f\_5} \times (t-5)} & t > 5 \end{cases} \qquad \textbf{equation 1}$$

The value of the *L. pneumophila* concentration at time zero (N_0 in **equation 1**) was randomly chosen from the distributions of the *L. pneumophila* concentrations ranging from 1 to 30054 CFU/L, as reported in previous studies (Borella et al., 2004; Borella et al., 2005; LeChevallier, 2019; Totaro et al., 2017). This method of selecting the *L. pneumophila* concentration at time zero considered the *L. pneumophila* concentrations from hot chlorinated and non-chlorinated drinking water systems in Italy (Borella et al., 2004; Borella et al., 2005; Totaro et al., 2017) and both hot and cold chlorinated drinking water systems in the US (LeChevallier, 2019). These *L. pneumophila* concentrations were measured in water flushed out of the drinking water systems. These concentrations were selected to include representative *L. pneumophila* concentration ranges in drinking water. The variation in the *L. pneumophila* concentration distributions can be due to the heterogeneity of the premise plumbing systems, the sampling methods, and the detection methods. A case study on the effect of these four distributions of initial *L. pneumophila* concentration distributions on the annual infection risks in scenario 1a is shown in SI.

Inactivation rate constants (k_i for inactivation in the first 5 minutes and k_{i_5} for inactivation after 5 minutes) were obtained by fitting a two-phase pseudo first-order rate equation (Chick-Watson model) using the ratios of live *L. pneumophila* among total biomass released from biofilms over 30 minutes of chlorine exposure. These ratios were measured and reported previously (Shen et al., 2017). The fittings of the inactivation rate constants are shown in **Figure 2S** and **Table 2S**. We found that the inactivation rate constants were 0.46 and 0.1 $(\text{mg } Cl_2/\text{L} \times \text{min})^{-1}$ for the first 5 min and between 5-30 min, respectively. The inactivation rate constant of the planktonic *L. pneumophila* was simulated by a first-order rate equation as shown in SI. The concentrations of culturable biofilm-derived *L. pneumophila* (in cells/L) after exposure to a certain free chlorine

concentration post stagnation was calculated based on the simulated concentrations of residual free chlorine. For this simulation, we used the free chlorine concentration distribution in fresh drinking water (AWWA, 2018) as the initial chlorine concentration, Cl_0, and our previously developed mass transfer model (Xu et al., 2018). The coefficients obtained from this mass transfer model (as shown in **SI section 4**) was then used in this study to estimate free chlorine decay in contact with biofilms after the stagnation of 1, 6, 12, 24, and 48 hours. To simulate the free chlorine decay after stagnation without biofilms, a pseudo first-order decay, as shown in the SI, was used. The same distribution of initial chlorine concentration was used in this simulation.

The concentrations of culturable biofilm-derived *L. pneumophila* (N(t) in cells/L) after inactivation with free chlorine based on the released *L. pneumophila* (estimated in **equation 1**) as a function of time is shown in **equation 2a**. The culturable *L. pneumophila* concentrations were estimated from the inactivation ratios of biofilm-derived *L. pneumophila* with free chlorine by **equation 2a**.

$$N(t) = \begin{cases} N_{total}(t) \times e^{k_i \times C \times t} & 5 \geq t \geq 0 \\ N_{total}(t=5) \times e^{k_{i\_5} \times C \times (t-5)} & t > 5 \end{cases} \qquad \textbf{equation 2a}$$

Inactivation kinetics were modeled by a two-phase pseudo first-order rate equation (Chick-Watson model) segmented at the fifth minute with inactivation rate constants developed from experimentally measured inactivation ratio values. Note that under the shear stress from the disinfectant-containing water, *L. pneumophila* cells were released from the biofilm and were exposed to the disinfectant. The cells that survived inactivation under disinfectant exposure were cultured. The inactivation ratios were defined as the ratios of culturable *L. pneumophila* cells released from biofilms by water containing disinfectant divided by the total concentration of released biofilm-derived *L. pneumophila* cells. The inactivation ratios were determined by both residual chlorine concentrations after stagnation (C) and total shower time (T). To calculate the

accumulated dose of *L. pneumophila* cells through inhalation during showers, we divided the total shower time (T) into multiple time points (t) with increments of 0.001 min (Δt) from 0 to T. The concentrations of culturable *L. pneumophila* cells at each time point (t) were calculated by **equation 2a** to estimate the accumulation of *L. pneumophila* exposure during time spent in a shower event (T) for each iteration.

$$N(t) = N_0(t) \times e^{k_{i\_b} \times C_b \times t} \quad \textbf{equation 2b}$$

In scenario 3, the concentration of culturable *L. pneumophila* was calculated using **equation 2b** because only inactivation occurred. The fitting of inactivation rate constant in **equation 2b** is shown in **Figure 3S**.

The air-water partition function (PFi(t) in m^{-3} for every liter of water) was used to model the accumulation of water droplets in the air that was generated from the showerhead at each time point t. This partition function was calculated based on the aerosol accumulation in the air in a simulated shower event (Davis et al., 2016) and was determined by the aerosol generation rate (G in mg × min^{-1}), aerosol removal rate (d_i in min^{-1}, including both deposition onto the wall and removal by fan), and shower room volume (V_s in m^3), as shown in **equation 3**,

$$PF_i(t) = \frac{G}{\rho_w \times d_i \times V_s}(1 - e^{-d_i \times t}) \quad \textbf{equation 3}$$

where d_i was the aerosol removal rate for aerosol size ≤ 2 μm (d_1) and > 2 μm (d_2). These parameters are listed in **Table 1**. Water density (ρ_w) was 10^6 mg/L. These shower-related parameters (G, d_i, and V_s) were measured from aerosol deposition at 1.5 m above ground over more than 20 minutes during a shower event in a shower stall (Xu and Weisel, 2003). These parameters were chosen because of the relevant flow rate (7 – 10 L × min^{-3}) using hot drinking water. Since the removal rate for smaller aerosols was less than larger aerosols, two removal rates

were used to simulate the size of the aerosol less than or greater than 2 μm (Davis et al., 2016; Xu and Weisel, 2003).

The density of bacteria in the air at each time point t ($N_{air}(t)$, in cells/m^3) was estimated by multiplying the density of bacteria in the water (N(t)) by the partitioning of *L. pneumophila* between air and water, as shown in **equation 4**. The partitioning coefficient ($\sum_{i=1}^{2} PF_i \times F1_i$) was calculated by multiplying the proportion of *L. pneumophila* cells entrapped in aerosols of size range i ($F1_i$) (Allegra et al., 2016) and aerosol concentration in the air at each time point t ($PC_i(t)$). Values of $F1_1$ and $F1_2$ were randomly drawn from the truncated normal distributions bounded by a minimum of 0, with means and standard deviations as listed in **Table 1** and divided into two groups: 1-2 μm and 2-10 μm (Allegra et al., 2016). Details on the categorization for the ranges of aerosols' size can be found in the SI. Aerosols within the size range of 1-10 μm diameter were considered to be able to reach the alveolar region by mouth and nose inhalation (Schoen and Ashbolt, 2011).

$$N_{air}(t) = N(t) \times \sum_{i=1}^{2} PF_i \times F1_i \qquad \textbf{equation 4}$$

The cumulative dose (D, in cells) of *L. pneumophila* deposited in the alveolar region was estimated by the product of inhalation rate and time increment (IR × Δt) and density of bacteria in the air ($N_{air}(t)$) at each time point (Schoen and Ashbolt, 2011), as shown in **equation 5**,

$$D = \sum_{t=0}^{t=T-\Delta t} N_{air}(t) \times IR \times \Delta t \times CF \qquad \textbf{equation 5}$$

where Δt is the time increment (0.001 min) for each $N_{air}(t)$ calculated in **equation 4**; CF is the conversion factor between minutes and hours. The inhalation rate was simulated according to parameters listed in **Table 1**.

312 The infection risk of *L. pneumophila* (P_{inf}) per person per shower event (pppse) was
313 estimated by a dose-response model described in **equation 6**.

$$P_{inf} = 1 - e^{k \times D} \quad \textbf{equation 6}$$

314 where the exponential parameter (k, in cells^{-1}) was reported from the dose-response model based
315 on the infection number over the dose reported in the infection experiment using guinea pigs
316 (Armstrong and Haas, 2007; Muller et al., 1983).

317 The infection risk per person per year (P_{annual} in pppy) can be estimated by **equation 7**
318 (Karavarsamis and Hamilton, 2010),

$$P_{annual} = 1 - \prod_{i=1}^{n} (1 - P_{inf,i}) \quad \textbf{equation 7}$$

319 where n, the number of shower events per year, is 365 assuming that the shower was a daily event.
320 The annual risk for 48 hours of stagnation was calculated with n = 182 events per year. The
321 infection risks per person per shower event ($P_{inf,i}$) were randomly drawn from the estimated
322 infection risk from **equation 6** for n (365 for the stagnation within 24 hours and 182 for the
323 stagnation of 48 hours) times (i denoted individual infection risk pppse). This risk model was
324 simulated using the Monte Carlo method with 10^4 iterations in R v3.2.3 using the model parameters
325 described in **Table 1**.

326 We estimated the annual infection risk based on the detection rate of *L. pneumophila* in the
327 relevant premise plumbing by **equation 8**. The annual probability of both *L. pneumophila*
328 detection and infection, P(Detc and Inf), was estimated based on the detection rate of *L.*
329 *pneumophila* in the premise plumbing (P(Detc)) and the annual infection risk of *L. pneumophila*
330 when it is detected (P_{annual}). P(Detc) was reported from 4% to 28%, which was the percent of

positive *L. pneumophila* found in the collected samples (Borella et al., 2004; Borella et al., 2005; LeChevallier, 2019).

$$P(Detc\ and\ Inf) = P(Detc) \times P_{annual} \quad \textbf{equation 8}$$

Table 1: Parameters used in risk assessment

2.4. Sensitivity analysis

To determine the key variables in the model on the biofilm-derived *L. pneumophila* infection risk, we conducted a global sensitivity analysis using the Latin Hypercube Sampling (LHS) method. The sampling of model parameters (**Table 1**) was implemented within the package 'lhs' in R (Carnell, 2016). The full range of each model parameter was sampled independently for 1000 intervals by LHS, and the infection risk of *L. pneumophila* per event was estimated by the risk model described before (**equations 1-5**). Then, partial rank correlation coefficients (PRCC) were calculated between each model parameter and the corresponding infection risk of *L. pneumophila* (Marino et al., 2008). The calculation of PRCC was carried out with R package "sensitivity" (Pujol, 2016). The calculation and distribution functions used were included in the following R packages: "mcm", "mc2d", "readxl", and "rriskDistributions". Nonparametric methods, the Kruskal-Wallis and Kolmogorov–Smirnov (KS) tests, were used to compare infection risks between scenarios or variables to identify possible significant differences. The difference was considered significant if the p-value was less than 0.05.

3. Results

3.1 Decay of residual chlorine in the presence of biofilms

We showed the simulated time- and biofilm-dependent chlorine concentrations in **Figure 2** and **Table 4S**. These simulation results revealed that the residual chlorine concentration decreased when drinking water stayed stagnant in PVC pipes coated with biofilms grown at drinking water treatment plant A or B. The rate of chlorine decay without biofilms was much lower than with biofilms (**Figure 2**). The median chlorine concentration (indicated as purple line in **Figure 2**) decreased from 1.3 to 1.2 mg/L after 48 hours of stagnation without biofilms. The decay of the residual chlorine during the first 12-hour stagnation was faster with plant A biofilms than with plant B biofilms. Plant A biofilms caused the residual chlorine to rapidly decrease from 1.3 to 0.05 mg/L during the first 12 hours of stagnation, then steadily to 0.02 mg/L between 12 to 48 hours. The residual chlorine in contact with plant B biofilms decreased rapidly from 1.3 to 0.08 mg/L during the first 24 hours of stagnation and then steadily to 0.05 mg/L. The simulation results agreed with field measurements showing complete decay of chlorine to below the detection limit (Lautenschlager et al., 2010; Ling et al., 2018; Rhoads et al., 2016). These results suggested that the decay of chlorine residual during stagnation depended on the presence and reactivity of the biofilms grown from different water sources or treated by different treatment trains. We then determined the effect of residual chlorine decay during stagnation on the infection risk of *L. pneumophila* released from biofilms.

Figure 2. The decay of residual chlorine simulated for biofilms grown from water treated by drinking water treatment plants (plant A in orange and B in green) after stagnation from 1 to 48 hours. The chlorine decay without biofilms are shown in purple. Colored areas represent the 10^{th}

374 to the 90th percentile of chlorine concentration. Lines represent the medians of chlorine
375 concentration.
376
377 **3.2 Influence of chlorine consumption by different biofilms during stagnation on annual**
378 **infection risk**
379 We first determined the role of biofilms on chlorine decay and subsequently the infection
380 risks. As shown in **Figure 3**, the annual infection risk of planktonic *L. pneumophila* was
381 significantly lower than the risk of biofilm-derived *L. pneumophila* ($p < 0.05$). The differences in
382 medians of the annual infection risk between planktonic and biofilm-derived *L. pneumophila*
383 increased over the stagnation from 1 to 24 hours for plant A and plant B biofilms, respectively.
384 The lower annual infection risks by planktonic *L. pneumophila* is due to the much slower chlorine
385 decay without biofilms and greater inactivation of planktonic *L. pneumophila* compared to biofilm-
386 derived *L. pneumophila*. Because the biofilm-derived *L. pneumophila* was exposed to lower
387 chlorine concentration. The median annual infection risk of biofilm-derived *L. pneumophila*
388 increased rapidly from 2.3×10^{-4} to 4.0×10^{-4} per person per year (pppy) within the first 12 hours
389 of stagnation for plant A biofilms. After 12 hours of stagnation, the increase in annual risk became
390 much slower with time. For plant B biofilms, the median annual risk increased continuously from
391 2.1×10^{-4} to 3.8×10^{-4} pppy within 24 hours of stagnation. The annual infection risks of biofilm-
392 derived *L. pneumophila* due to the decay of residual chlorine by plant A biofilms were significantly
393 higher than that by plant B biofilms ($p < 0.05$). After 48 hours of stagnation, the distribution of
394 annual risks associated with plant A biofilms had a second-order stochastic dominance over that
395 with plant B biofilms with median risks of 2.0×10^{-4} and 1.9×10^{-4} pppy, respectively ($p < 0.05$).
396 In summary, the presence of biofilms led to higher annual risks of *L. pneumophila* over stagnation

events, compared to the scenario without the presence of biofilms. This finding suggested that suppressing biofilm growth can reduce the associated annual infection risks.

Figure 3. Distributions of annual infection risk of biofilm-derived *L. pneumophila* in the presence of residual chlorine after the stagnation of 1, 6, 12, 24, and 48 hours. Dashed lines represent medians and straight lines represent the 25th and the 75th percentiles.

3.3 Comparison of annual infection risk of biofilm-derived *L. pneumophila* with vs. without residual chlorine exposure

From 2.0 to 4.6 times higher annual infection risk was estimated for *L. pneumophila* released from biofilms that had not been exposed to residual chlorine compared to infection risk of *L. pneumophila* released from biofilms under residual chlorine exposure (**Figure 4**). In addition to the lack of chlorine to inactivate biofilm-derived *L. pneumophila*, the number of *L. pneumophila* detached from biofilms without previous chlorine exposure (950-84000 cells within 95% prediction interval) was higher than from biofilms previously exposed to chlorine (620-55000 cells within 95% prediction interval) after the first 5 minutes of detachment. These findings suggest that maintaining appropriate residual chlorine concentrations can reduce the annual infection risk of *L. pneumophila*. Next, we evaluated the influence of chlorine concentration on the infection risks per shower event of biofilm-derived *L. pneumophila*.

Figure 4. Annual infection risk of *L. pneumophila* released from biofilms under residual chlorine contact after stagnation (scenario 1) vs. no residual chlorine scenario (scenario 2). Scenario 2 simulated the premise plumbing in a vacant building from days to months. Straight lines represent median annual infection risks. Boxes within each distribution figure are the 25th and the

421 75^{th} percentile of annual infection risks. Asterisks indicate significant comparisons according to
422 the Kruskal-Wallis test and KS test ($p \leq 0.001$).
423
424
425 **3.4 Influence of chlorine concentration on infection risk per shower event**
426 To determine the correlation between infection risk per shower event and residual
427 chlorine concentration, we estimated the infection risk per shower event as a function of residual
428 chlorine from 0 to 5 mg/L, as shown in **Figure 5**. Model parameters except residual chlorine
429 concentrations were kept identical as listed in **Table 1**. The median infection risk per shower
430 event decreased by more than five times from 6.6×10^{-7} to 1.0×10^{-7} as chlorine concentration
431 increased from 0 to 5 mg/L. In addition, the $\log_{10}$ values of the median infection risk per event
432 linearly decreased with increasing chlorine concentration ($R^2 = 0.97$), as shown in **Figure 5**. The
433 median free chlorine concentration ranged from 1 to 1. 5 mg/L at water treatment plant effluents
434 (AWWA, 2018). After traveling through the distribution systems, the residual chlorine in the
435 premise plumbing can be much lower and in the range of 0.2 to 0.5 mg/L (AWWA, 2018). The
436 most reported residual chlorine concentration range of 0.5-1.5 mg/L (AWWA, 2018)
437 corresponds to a median infection risk of 2.9×10^{-7} to 4.7×10^{-7} pppse. The corresponding risk
438 would be 2.9×10^{-7} to 3.6×10^{-7} pppse for water leaving the treatment plant and 5.5×10^{-7} to
439 5.8×10^{-6} pppse for water in premise plumbing. Note that an annual risk of 1 infection per
440 10,000 people (Hamilton et al., 2019; Regli et al., 1991) corresponds to an infection risk of
441 approximately 2.7×10^{-7} pppse according to **equation 7.** All median infection risks calculated
442 using residual chlorine from 0 to 1.7 mg/L exceeded this risk level (1×10^{-4} pppy). Thus, in our
443 simulated shower events, a residual chlorine concentration of 1.7 mg/L was recommended to

maintain a median annual risk lower than the targeted risk of 10^{-4} pppy. This finding suggests that maintaining an appropriate residual chlorine concentration can effectively reduce the infection risk per shower event from biofilm-derived *L. pneumophila*.

Figure 5. The infection risk of exposure to biofilm-derived *L. pneumophila* per shower event decreased with increasing residual chlorine concentration from 0 to 5 mg/L Cl_2. Triangles represent median infection risks. Colored areas represent the 10^{th} to the 90^{th} percentile of infection risk. The orange line is fitted linearly with an R^2 of 0.97. The black line represents the target infection risk per person per shower event that resulted from 1 infection per 10^4 people per year, according to **equation 7**.

3.5 Global sensitivity analysis

To determine the governing factors for the infection risk, we conducted a global sensitivity analysis on input variables (**Table 1**). We calculated the PRCC that represented the magnitude of linear association between the model input variables and the biofilm-derived *L. pneumophila* infection risk (output). Positive PRCC indicated that the infection risk increased with the corresponding input variables, while negative PRCC indicated lowered infection risk as input variables increased. The sensitivity analysis results (**Figure 6** and **Table 6S**) revealed that the infection risk increased with the rate of *L. pneumophila* cell released from biofilms and decreased with chlorine concentration after stagnation. The detachment rate (k_{f_5}) and inactivation rate of biofilm-derived *L. pneumophila* after 5 minutes of detachment (k_{i_5}) did not affect the infection risk significantly within the range of fitting based on the detachment and inactivation experiments in previous studies (Shen et al., 2017). These results were likely caused by the steady detachment and inactivation of *L. pneumophila* from biofilms after 5 minutes. The top three influential

variables on infection risk in this model (**Figure 6**) were the initial *L. pneumophila* concentration in the premise plumbing (N_0), the residual chlorine concentration (C), and the shower stall volume (V_s). The time required to drain stagnant water in the premise plumbing with water tank, i.e., the premise plumbing retention time (t_1), did not affect the infection risk of biofilm-derived *L. pneumophila* with residual disinfectant exposure. The value of PRCC for t_1 is below 0.2, while the values of PRCC for initial *L. pneumophila* concentration (N_0) and chlorine residual (C) are +0.96 and -0.81, respectively. The magnitude of the PRCC indicates the strength of the relationship between the parameters and the calculated infection risk. These results suggested that the retention time (t_1) had a less significant effect on infection risk compared to N_0 and C. If *L. pneumophila* is found in the premise plumbing, residual chlorine concentration and stagnation time should be preferentially controlled by engineering measures to reduce the infection risks.

Figure 6. Mean partial rank correlation coefficients (PRCC) of input variables on infection risk per person per shower event in the model. Colored bars are the PRCC associated with infection risk, while the ticks are standard deviations. The labels of the variables are listed below and can be found in **Table 1**: residual chlorine decay rate constant without biofilms (k_b), residual chlorine concentration after stagnation (C), shower stall volume (V_s), shower duration (T), inactivation rate of biofilm-derived *L. pneumophila* after 5 minutes (k_{i_5}), detachment rate of biofilm-derived *L. pneumophila* after 5 minutes (k_{f_5}), premise plumbing retention time (t_1), inactivation rate of biofilm-derived *L. pneumophila* in the first 5 minutes (k_i), partition of *L. pneumophila* in the aerosols ($F1_i$; i can be 1 or 2), aerosol generation rate (G), inhalation rate (IR), dose-response coefficient (k), detachment rate of biofilm-derived *L. pneumophila* in the first 5 minutes (k_f), and *L. pneumophila* concentration at time zero (N_0).

4. Discussion

In this work, a novel bottom-up approach accounting for the effects of stagnation and residual chlorine concentration in premise plumbing was used to estimate the infection risks of *L. pneumophila* released from biofilms. To demonstrate the relevance of our estimation, we compared our estimated risks with values reported in previous studies. The median annual risks (2.0×10^{-4} to 4.0×10^{-4} pppy (scenario 1a) and 1.9×10^{-4} to 3.8×10^{-4} pppy (scenario 1b)) from biofilm-derived *L. pneumophila* were in the range of the annual risks (10^{-6} to 10^{-2} pppy) that were estimated for shower events using the initial *L. pneumophila* concentration from 1 to 10^4 CFU/L (Hamilton et al., 2019). In another study, an initial *L. pneumophila* concentration in roof-harvested rainwater ranging from 7.1×10^2 to 2.0×10^3 CFU/L resulted in the estimated infection risk of 3.0 $\times 10^{-6}$ to 8.6×10^{-6} pppse (Ahmed et al. 2010). These infection risk estimates were in the range for the case of *L. pneumophila* released from biofilms without chlorine exposure (7.9×10^{-8} to 7.9×10^{-6} pppse in 5-95% PI). Our estimate of annual infection risk without residual chlorine was about a hundred times lower than the risk due to *L. pneumophila* exposure in shower events using roof-harvested rainwater without disinfection (Hamilton et al., 2017). Our lower estimated risks may be related to the lower *L. pneumophila* concentration in the roof-harvested rainwater by Hamilton et al. compared to those used in our simulations (2018). We used the *L. pneumophila* concentration detected in the premise plumbing (1 to 10^4 CFU/L) and the maximum and minimum of the detection rate (28% and 4%, respectively) reported previously (Borella et al., 2004; Borella et al., 2005; LeChevallier, 2019) to estimate that the annual infection risk from exposure to *L. pneumophila* released from biofilms was from 2.9×10^{-5} to 2.5×10^{-4} (without residual chlorine) and from 6.8×10^{-6} to 1.2×10^{-4} (with residual chlorine). This range includes the reported incidence rate of Legionnaires' disease cases from the Netherlands in 2016 (2.7 per 10^5 people or

514 risk of 2.7×10^{-5} per person) (ECDC, 2019). The reported incidence rate of Legionnaires' disease
515 cases in the US in 2015 (1.9 per 10^5 people or risk of 1.9×10^{-5} per person) (Shah et al., 2018) was
516 in the range of the annual infection risk (6.8×10^{-6} to 1.2×10^{-4}). This range was calculated using
517 **equation 8** based on the maximum and minimum detection rate from Borella et al. (2005) and
518 LeChevallier (2019) and the highest and lowest 95% prediction interval of the annual infection
519 risk estimated under scenario 1 and 2. In general, our estimation of biofilm-derived *L. pneumophila*
520 infection risks was consistent with other studies.

521 Wide ranges of bulk water *L. pneumophila* concentrations (10 to 10^5 CFU/L) have been
522 used as guidelines for building water system monitoring and safety management around the world
523 (Hamilton et al., 2019; OSHA). For example, an annual infection risk level of 10^{-4} (or 1 infection
524 per 10^4 people) was selected for indoor water fixture, spray irrigation, and cooling tower exposure
525 using a variety of water sources (drinking water, reclaimed water, and roof-harvested rainwater)
526 (Hamilton et al., 2018; Hamilton et al., 2019). This infection risk led to calculated critical *L.*
527 *pneumophila* concentrations of 1 to 10^3 CFU/L, depending on indoor exposure routes. This risk-
528 based approach allowed the development of guidelines for *L. pneumophila* infection risk control
529 but did not identify engineering measures to reduce the risk. Our bottom-up QMRA approach
530 allows for a quantitative comparison of infection risk related to *L. pneumophila* released from
531 biofilms at different levels of residual chlorine and biofilm types. This approach enables the
532 quantification of infection risks of *L. pneumophila* related to the level of residual chlorine, which
533 could be monitored and controlled.

534 In the US, residual chlorine is enforced in distribution systems to maintain drinking water
535 safety. Accidental low levels of chlorine in premise plumbing have already been linked to
536 incidences of Legionnaires' disease outbreaks (Zahran et al., 2018). In some European countries,

such as the Netherlands, residual disinfectant is not used. Instead, measures to control *L. pneumophila* and other bacterial growth, such as optimal pipe material choice and organic matter removal, are applied (Learbuch et al., 2019; Rosario-Ortiz et al., 2016). In such systems without residual chlorine, biofilms and *Legionella* growth can be supported by organic matter, which remains in the drinking water after conventional treatment or is released from certain pipe materials, such as PVC-P and PE (Learbuch et al., 2019). *L. pneumophila* can also propagate in amoebae, which feed on organic matter (natural organic matter in source water, biofilms aggregates, certain pipe materials, etc.). The release of *L. pneumophila* from amoeba cells can increase the *L. pneumophila* concentration (Buse et al., 2012). Warm temperatures between 20 and 50 ^{o}C have been found to encourage microbial growth, including *L. pneumophila* (Buse et al., 2012; Cervero-Aragó et al., 2019; LeChevallier, 2019), and accelerate the decay of residual chlorine compared to lower temperatures (Brazeau and Edwards, 2013). We estimated the infection risk based on the water retention time (t) in the premise plumbing systems that utilize 80 gallon (300 L) storage tanks, as reported by Rhoads et al. (2016). In the absence of the cold water storage, the cold water in the building pipe is mixed with the water from the hot water tank. We modeled the chlorine concentration in the stagnant water based on the measured chlorine decay in contact with biofilms at room temperature of about 25 ^{o}C. The chlorine concentration of the mixed water at the tap is likely to be lower than the concentration in the cold water. To address the issue of temperature-dependent chlorine concentration and the lack of cold water tanks in most US buildings, we conducted a sensitivity analysis for chlorine concentration ranging from 0.02 to 5 mg/L. This range was selected based on previous studies on chlorine residual concentrations (Rhoads et al., 2016; Salehi et al., 2018). The sensitivity analysis results showed an increased

559 infection risk at lower chlorine concentrations. Therefore, the infection risk from *L. pneumophila*
560 exposure might be higher at warmer temperatures, when the chlorine concentrations are lower.
561 This work has several limitations. The QMRA model was developed based on the
562 assumption that only the culturable *L. pneumophila* cells caused infection. The risk caused by *L.*
563 *pneumophila* in the viable but not culturable (VBNC) state was not considered due to the lack of
564 data on the aerosolized VBNC *L. pneumophila* concentration and the partition between water and
565 air. If both the culturable and the VBNC *L. pneumophila* cells can induce identical responses in
566 humans, the infection risk caused by both the culturable and the VBNC *L. pneumophila* cells will
567 be higher than the risk caused by the culturable cells alone. Another limitation of this study is the
568 assumption that all aerosolized *L. pneumophila* cells can infect the host. This assumption was made
569 due to the lack of information on the quantification of infectious *L. pneumophila* cells (both
570 culturable and VBNC cells) in aerosols over the size range of aerosols used in this model. The
571 third limitation is that we only considered the decay of free chlorine. About 71% and 21% of the
572 utilities in the US used free chlorine or monochloramine, respectively (AWWA, 2018). Although
573 monochloramine can be less effective on microbial inactivation, it can be more persistent in the
574 distribution system due to lower oxidation potential compared to free chlorine and produces fewer
575 regulated disinfection by-products (AWWA, 1993). Higher monochloramine concentration can be
576 found after stagnation than free chlorine concentration given the identical initial concentrations.
577 Since the infection risk of biofilm-derived *L. pneumophila* depends on the inactivation by
578 disinfectants, further study should consider scenarios with monochloramine. The fourth limitation
579 of this study is that we only considered the consumption of chlorine based on the measurement of
580 two drinking water biofilms. In practice, the chlorine consumption by different biofilms will
581 depend on many factors, including chemical composition of biofilms, building pipe materials, and

retention time of water in the premise plumbing. For this reason, the estimated risks found here should not be extrapolated. The fifth limitation of this work is that we did not differentiate the immunocompromised population from the general population. The sixth limitation is that the current QMRA model did not consider the mixing of cold and hot water and thermal inactivation of *L. pneumophila* released into the hot water due to the limited information on relevant parameters.

5. Conclusion

This study is the first to quantify the role of biofilm on the time-dependent infection risks of *L. pneumophila* released from premise plumbing after stagnation. These QMRA analysis results allowed for a quantitative comparison of infection risks associated with stagnation in premise plumbing. This knowledge is important because Legionnaires' disease incidence is among the most common drinking water-associated illnesses (Ashbolt, 2015; Shah et al., 2018). Based on the results of this study, we suggest reducing stagnation time, in agreement with the CDC recommendation (CDC, 2018). We also found that the infection risks of *L. pneumophila* released from biofilms may increase by various extents due to the consumption of residual chlorine by different biofilms. Under certain condition, even an hour of stagnation could lead to an annual risk above 10^{-4}, which was considered acceptable (Hamilton et al., 2018; Hamilton et al., 2019). The annual risks increase substantially with prolonged stagnation. Therefore, we also suggest monitoring and suppressing biofilm growth in premise plumbing where *L. pneumophila* is a concern, especially in dead-ends where stagnation commonly happens.

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