Preprint of article accepted for publication in Energy for Sustainable Development

A potential source of undiagnosed Legionellosis: *Legionella* growth in domestic water heating systems in South Africa

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Abstract

Legionella is a genus of pathogenic bacterial mesophiles that cause a range of diseases collectively referred to as Legionellosis, with immunocompromised individuals being particularly susceptible. Water heaters, a potential domestic niche for these pathogens, are heavy energy consumers, causing cost-sensitive users to employ energy-saving initiatives, such as scheduling and lower temperature set points. However, lower heated water temperatures allow *Legionella* to flourish. This paper uses computational fluid dynamics modelling to show that the pipes downstream of a horizontal electric water heater provide an environment that is conducive to *Legionella* growth, not the heater itself. The presence of *Legionella* in water heaters is established through water sampled from five in-field water heaters, of which the temperatures and heating schedules are known. Microbiological techniques (PCR and weight-based qRT-PCR) are used to assess *Legionella* and *L. pneumophila* presence at point-of-use taps. A model is used to determine the potential infection rate from these concentrations, demonstrating that undiagnosed Legionellosis infection is likely. In low- and middle-income countries, like South Africa, misdiagnosis of Legionellosis may be common due to the shadow cast by HIV and TB prevalence.

Keywords: Legionellosis, *Legionella pneumophila*, Electric water heaters.

1. Introduction

The occurance of waterborne *Legionella* and the Legionellosis-causing pathogenic bacterium *Legionella pneumophila* in domestic water heaters in South Africa (SA) is not known. In SA, Legionellosis is a notifiable disease, yet rarely reported.

Several studies have related waterborne disease outbreaks to the growth of *Legionella* in large plumbing systems (Schoen and Ashbolt, 2011; Borella et al., 2004; Zacheus and Martikainen, 1994). A small number of

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recent studies indicate that a strong possibility exists for *Legionella* growth and infection in single-households (Schoen and Ashbolt, 2011; Armstrong et al., 2014). Legionnaires' disease is usually only diagnosed when an outbreak occurs at public institutions, leading to the possibility of *Legionella* growth in single-households being largely overlooked in SA.

Tuberculosis (TB) remains a global health concern. Of the 10.4 million new cases in 2015, one third were never diagnosed and of those diagnosed, only a minority were bacteriologically confirmed (WHO, 2016). Furthermore, of the 600,000 cases with rifampicin resistance, only 120,000 were diagnosed.

South Africa is faced with the dual epidemics of HIV (a prevalence of 12.7 % in 2016) and TB (781 cases per 100,000 population in 2016), as well as resource and medical care limitations, making it a potential incubator for drug-resistant *M. tuberculosis*. This is evidenced by the country having one of the highest burdens of multi drug resistant TB in the world (WHO, 2016). In South Africa, approximately 5 % of all TB cases are believed to be multi drug resistant TB of which one-tenth are extremely drug resistant TB (NIfCD, 2016). Highest rates of multi drug resistant TB and extremely drug resistant TB were notified for the Western Cape, Eastern Cape and KwaZulu-Natal provinces (NIfCD, 2016). This heavy burden creates both a diagnosis bias hiding many other diseases, as well as an immuno-compromised population susceptible to many other diseases. This might explain the imbalance in reported Legionellosis cases in comparison to developed countries.

The *Legionella* genus comprises of more than 50 gram-negative bacterial species that are ubiquitous in soil and water, at least 20 of which are pathogenic (EPA, 2001; WHO, 2007; Fields et al., 2002; Diederen, 2008; Burstein et al., 2016). *L. pneumophila* is the most notorious species, responsible for respiratory diseases such as the milder Pontiac's fever and the more severe Legionnaires' disease. These organisms are thermophyllic, with optimal growth temperatures ranging from 37 to 42°C (Piao et al., 2006), while temperatures around 45 °C stimulate biofilm growth (Rogers et al., 1994).

Infections are often reported in immunocompromised patients due to ubiquitous environmental exposure. *L. pneumophila* infects patients via droplet inhalation, rather than the typical ingestion or patient-to-patient routes.

A prior study of the prevalence of *Legionella* spp. infections in SA demonstrated that 21 of 1805 (1.2 %) patients tested were polymerase chain reaction (PCR) positive for *Legionella* spp. (Wolter et al., 2016). Within this group, 9 of the 21 (43 %) tested positive for TB, while 75 % were HIV positive. HIV or TB or both were detected in 18 of 20 (90 %) of these patients. Symptomatic Legionellosis presents as community-acquired pneumonia in common with several other potential opportunistic bacterial infections and is often associated with immunosuppression. Thus, evidence such as the above would suggest that under normal circumstances, i.e. those in which it is not actively tested for, diagnoses of Legionellosis might be missed due to the shadow

cast by HIV and TB. This seems particularly likely in resource-constrained settings in SA, where the burdens of HIV and/or TB infections are high, and clinicians lack access to appropriate diagnostic testing[1]. This picture is complicated by the fact that antimicrobial treatment for community-acquired pneumonia and TB, for example rifampicin, has demonstrated efficacy against *Legionella* (Klein and Cunha, 1998; Vesely et al., 1998). However, effective treatment normally requires the addition of macrolides or fluoroquinolone (Phin et al., 2014). As a result, morbidity due to Legionellosis may remain underestimated in SA.

Despite recent advances, South Africa still has high incidence of poverty (Burger et al., 2017), resulting in financially-constrained consumers resorting to various means to limit the cost of water heating. Water heating is responsible for 32 % of household energy consumption in South Africa, where water is predominantly heated with horizontally-oriented cylindrical electric water heaters. Water heaters nominally heat water to 65 °C, although temperatures of as low as 40 °C are considered sufficiently warm for user satisfaction (Belov et al., 2015; Nel et al., 2018a). The energy consumed by a domestic water heater can be reduced by 29 % through schedule control and lowering the thermostat's target temperature (Booysen and Cloete, 2016; Nel et al., 2018b). Despite the financial benefit to the user of operating at these lower temperatures, the heater and its hot water distribution system could be creating ideal temperature niches for the growth of the *L. pneumophila* pathogen. *Legionella* is often proposed to be a threat only to immuno-compromised individuals, and yet is repeatedly reported in association with widespread outbreaks related to water cooling systems, water distribution systems, spas and whirlpools, largely in developed countries (EPA, 2001; WHO, 2007; European Agency for Safety and Health at Work, 2011). Confirming the presence of *Legionella* in general and *L. pneumophila* in particular is key to validating the temperature results and understanding the risk to immunucompromised individuals, at this interface between immunity, load of exposure and financial heating considerations.

This paper evaluates the presence and survival of *Legionella*, and the pathogenic bacterium *L. pneumophila* in horizontal domestic water heaters, which are ubiquitous in South Africa.

A computational fluid dynamics (CFD) approach is used to evaluate whether the horizontal electric water heater provides an environment that is conducive to the growth of *Legionella* in biofilms inside the heater even under thermostat control. The analysis is also used to determine the streamlines for particles that exit the heater during a shower. Linking the potential risk of proliferation to the potential risk of infection, an existing infection model is also improved to determine the probability of an immunocompromised individual contracting Legionellosis. This aims to add to the international epidemiological work feeding into these

[1] A counterfactual to this hypothesis is the determination of the cause of the unexpected passing of the prominent Minister of Environmental Affairs, Edna Molewa. The cause of death was determined to be a *Legionella* infection (The South African, 2018)

questions, in terms of the balance between energy consumption and sanitation of water heaters (Armstrong et al., 2014), heating and cooling systems (Zhao et al., 2015), the biofilm proliferation of *Legionella* (Murga et al., 2001), the impact of materials on the control of these biofilms (Rogers et al., 1994; Buse et al., 2014), as well as water quality (Bargellini et al., 2011) and temperature and hydraulics (Boppe et al., 2016).

Grappling with real-world challenges to inform the models demanded environment-driven culturing and molecular techniques. Samples and scrapings are taken from decommissioned water heaters to determine the presence of *Legionella*, and the microbial loads at point-of-use in tap water from five active heaters are evaluated in comparison to cold water from the same source. Culturing and a PCR-based technique are used to demonstrate the presence of *Legionella*, while Quantitative Real Time PCR (qRT-PCR), quantified against a weight-based standard curve, is used to quantify the *L. pneumophila* present. These results are used to calculate an infection probability, and relevance related to other disease in South Africa, and low- to medium-income countries in general.

2. Materials and Methods

2.1. Computational fluid dynamics model

A domestic cylindrical water heater in SA has a heating element (typically 2 to 4kW), controlled by a thermostat that is mounted near the element. Importantly, electric water heaters in South Africa are mounted horizontally, with the inlet on the lower end near the element, and the outlet at the upper end on the opposite horizontal side.

A CFD model of a horizontal heater is developed in this paper to simulate temperature stratification and determine the velocity fields which influence the motion and growth of resident microbes. The CFD model represented a horizontal heater operating at 600 kPa using a 2 kW element. The simulated heater had a length and diameter of 1 m and 0.4 m, respectively. Particular attention was paid to the detailed geometry of the heating coil as it has a direct influence on natural convection. The resulting mesh consisted of more than 280 000 elements and passed all typical mesh quality metrics.

Natural convection was simulated using the Boussinesq approximation for buoyancy driven flow (Tritton, 2012). This involves solving the incompressible Navier-Stokes equations in conjunction with a linear approximation for thermal expansion to model the buoyancy force. Heat flux boundary conditions were applied throughout, with temperature dependent heat loss at the tank walls and a fixed heat flux at the heating element to ensure an overall heat supply of 2 kW. Two flow conditions were simulated: (1) no flow occurred into the heater, and (2) 5 L/ min flow into (and out of) the heater and a pressure specified outlet.

The CFD implementation described above was extremely computationally intensive and it was not possible to simulate flow patterns over the duration of an entire day. To this end, a second CFD model was developed using a coarse mesh. The heat flux as well as the inlet flow boundary conditions were set based on field measurements from heater controllers. The coarse CFD model was able to simulate entire days of usage. While it is unlikely that the flow patterns simulated using the coarse model is accurate, the dynamic temperature profiles presented a fair approximation.

All simulations were implemented using ANSYS® CFD software.

2.2. Sterilization model

The CFD model (from the previous section) produced a vector-valued velocity field and a scalar temperature field which was used to predict the thermal exposure experienced by planktonic- and biofilm associated microbes. Multiple seeding points were selected and the velocity field was used to track the movement of the microbe through the heater. Interpolating the temperature with respect to the microbes' position in the heater tank yielded a temperature profile which was subsequently used to predict the viability of microbes leaving the heater outlet. Microbial viability was estimated using eqns. 1 and 2:

$$\frac{df_{\mathrm{X}}}{dt} = \mu - k_d \tag{1}$$

$$k_d = k_0 \exp(-E_A/RT) \tag{2}$$

Where f_{X} represents the fraction of microbes remaining viable at time t, μ and k_d represent microbial growth and decay, respectively. The rate of decay is estimated using an Arrhenius-type equation (eq. 2) with pre-exponential coefficient $k_0 = \exp(95.7)\ \mathrm{s}^{-1}$ and activation energy $E_A = 276\ \mathrm{kJ/(mol.K)}$. A specific growth rate of $\mu = 1.04\ \mathrm{hr}^{-1}$ was used . These parameters were chosen to ensure a decimal reduction rate of 80 min at 50 °C and 2 min at 60 °C (Bartram, 2007), while maintaining a specific hourly growth rate of $\mu = 0.86$ at 45 °C, corresponding to an estimated maximum growth rate (Sharaby et al., 2017).

2.3. Remote controller and failed water heaters

The five in-field water heaters used in the study are part of a larger field trial of water heaters, in which users were shown water and energy consumption information and given heating schedule control through an online platform. The temperature is measured at the outlet, using a temperature sensor that is strapped onto the pipe with self-fusing silicon tape, and reported as an average temperature every 1 min (Fig. A.1). The electricity supply to the heating element is controlled by a cloud-based Set Point Controller (SPC). The

SPC controls the heating element based on a control schedule and a target temperature, both set by the user on the online interface. More information on the control system can be found in (Roux and Booysen, 2017).

All the water heaters are pressurised, horizontally mounted, had a volume of 150 L, and are manufactured from mild steel with a thermo-fused porcelain enamel. This is the most common set-up found in South Africa.

2.4. Legionella quantification: relative and absolute

Two approaches were taken to empirically evaluate *Legionella* in water heaters. The first approach was to cut open random water heaters that failed mechanically ("burst"), and to take water samples and biofilm scrapings from these, shortly after the failure. In these samples, culturing was employed for relative quantification of *Legionella* in comparison to general heterotrophic plate counts. The method is described in Section 2.4.1.

The second approach, described from Section 2.4.2, was to at take three water samples at the point-of-use in the water distribution systems of five active household water heaters, of which the user-chosen heating control schedule was known through the controllers. In these point-of-use samples, real time PCR was employed for absolute quantification of *Legionella* exposed to water users. A cold water sample was taken as a control; a first hot water sample was taken to establish the presence and quantity of *Legionella* in the piping downstream from the heater; and a second hot sample was taken to establish the presence and quantity of *Legionella* in the heater tank itself.

2.4.1. Direct sampling and relative quantification (culture-based)

Four domestic water heaterss that recently failed mechanically, were cut open on site approximately 12 to 24h post-decommissioning, and (a) grab samples and (b) biofilm scrapings were taken from inside the heaters.

Grab samples were collected in sterile 50 mL bottles, and scrapings were collected in sterile Petridishes. Biofilm scrapings were taken near the outlet and the inlet, focusing on regions likely to see the least flow disturbance, as well as directly from the base of the elements which had notable precipitate deposition (Fig. A.2).

Coupons were cut from the various heater tanks (copper, steel and plastic; inlet and outlet regions), for direct incubation on agar. Samples were transported on ice and processed within 6 hours.

All liquid (four, one per heater) and biofilm (eight, two per heater) samples were diluted in physiological saline solution (0.9 % w/v NaCl; Sigma Aldrich, Modderfontein, South Africa) and dilution ranges (undiluted - 10^7) plated on (a) *Legionella* CYE agar base, with *Legionella* BCYE growth supplement (Chatfield and Cianciotto, 2013), and (b) Tryptic Soy agar. Cultures were grown and isolated at 35 °C. Single colonies grown

on *Legionella*-specific medium were isolated on the same medium (every 3 days over a month-long interval), and subsequently cultured on *Legionella* CYE agar base with BCYE growth supplement without L-cysteine. *Legionella* have a unique absolute metabolic requirement for L-cysteine, thus all isolates that did not survive the transfer to *Legionella* media sans L-cysteine were tentatively positively identified as *Legionella* species. All media was purchased from Thermo-Scientific, Johannesburg, South Africa.

All species tentatively identified as *Legionella* via the culture-based technique were confirmed by DNA sequencing, employing standard primers to amplify a 386-bp fragment of the V3–V5 region of the 16S rRNA gene, specific to *Legionella* spp. (Parthuisot et al., 2010). Primers include JRP (5'-AGG GTT GAT AGG TTA AGA GC-3') and JFP (5'-CCA ACA GCT AGT TGA CAT CG-3'). Microbial DNA was extracted from individual isolates, scraped directly from the agar plates, with the Zymo Quick DNA Fungal/Bacterial Kit according to manufacturer's instructions (Inqaba Biotechnical Industries, Pretoria, South Africa). Each 25 µL Polymerase Chain Reaction (PCR) contained 3 to 5 ng DNA, 1 µM primers, 0.8 mM deoxynucleoside triphosphates (dNTPs), and 1 to 1.5 U of Taq DNA polymerase. Reagents were purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). The PCR protocol included an initial denaturation of 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 57 °C, and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were amplified in a BioRad T100 Thermal Cycler, confirmed with gel electrophoresis and sequenced on an Applied Biosystems 3500XL Genetic Analyzer (Thermo Fischer). Sequences were positively or negatively identified as *Legionella* by cleaning up the sequences on 4Peaks Software (Nucleobytes, 2004), and subsequent comparison against the international BLAST database (BLAST, nd).

Attempts were made to harness molecular techniques for direct identification and quantification of *Legionella* in the heaters' planktonic and biofilm biomass, using the above-mentioned kit for DNA extraction, as well as manual protocols, including adding bovine serum albumin to PCR reactions to minimize inhibition. However, the biofilm samples and liquid samples were red with precipitate, likely containing heavy metals such as iron (Fig. A.3), and thus the lack of molecular success due to PCR inhibitors was not surprising.

2.4.2. Distribution system sampling and molecular quantification

Since direct molecular quantification is a more robust and reliable technique for measuring microbial loads in water, and point-of-use bacterial concentrations are of greater infectious relevance than concentrations in the heater tank, samples from household taps were analysed for *Legionella* presence and *L. pneumophila* concentrations using PCR and quantitative Real-Time PCR (qRT-PCR), respectively.

Samples (2 L) were taken aseptically from each of the five heaters in sterile screw-top glass bottles from cold water taps 3 min after opening (CT), hot water taps directly after opening whilst water is still cold (HT1)

and hot water taps after running at maximum heat for 1 min (HT2). Water was transported immediately to the laboratory and processed within 1 h. Microbial cells in the samples were concentrated by filtration (2 L) and released from the filters into suspension by incubation in an acidic buffer according to Dobrowsky et al. (2015). The samples were flocculated by the addition of 2 mL/L $CaCl_2$ (1M) and 2 mL/L Na_2HPO_4 (1M) and subsequent stirring (5 min). Flocculated tap water samples were filtered ($\pm$50 mL/ min /cm) through non-charged, mixed-ester membrane filters (47 mm diameter, 0.45 m pore size; Whatman GmbH, Germany). Filters were incubated for 3 min in 4 mL citrate buffer (0.3 M, pH 3.5; in 9 cm Petridishes), with occasional shaking. The membrane was rubbed gently with a pipette tip, the citrate buffer solution containing the bacterial cells and DNA transferred to 2 mL centrifuge tubes, centrifuged, combined and re-suspended in 200 µL phosphate buffered saline (1X PBS).

Microbial DNA was extracted from the concentrated tap water samples with the Zymo Quick DNA Fungal/Bacterial Kit, according to manufacturer's instructions (Inqaba Biotechnical Industries, Pretoria, South Africa). Quality of DNA was assessed by comparison to a standard DNA ladder (1 kb Plus O'Gene Ruler, ThermoFischer Scientific, Johannesburg, South Africa) via agarose gel electrophoresis, as well as quantification and quality assessment with A260/A280 ratios on an ND1000 NanoDrop spectrophotometer (Inqaba Biotec).

A standard PCR, using the JFP and JRP primers as described above, was employed to determine presence or absence of *Legionella* spp. in cold (CT), cold hot (HT1) and hot hot (HT2) tap water. A 98 % homology was used to classify organisms as *Legionella*. Whereas these primers amplified a genomic region common to most *Legionella* species Parthuisot et al. (2010), quantification was narrowed down to include only *L. pneumophila*, the species most often responsible for pneumonia outbreaks (Welti et al., 2003; EPA, 2001; Yu et al., 2002).

For qRT-PCR quantification of *L. pneumophila*, primers were selected that amplified a 73 bp region of the gene encoding the macrophage infectivity potentiator (MIP, GenBank accession number AF022336), according to Welti et al. (2003), directly correlated to colony forming units in *L. pneumophila* serotypes (Welti et al., 2003; Behets et al., 2007). Primers LPTM1 (5'-AAA GGC ATG CAA GAC GCT ATG-3'), LPTM2 (5'-TGT TAA GAA CGT CTT TCA TTT GCT G-3') and an LP probe (5'-FAM-TGG CGC TCA ATT GGC TTT AAC CGATAMRA-3') were purchased from Inqaba Biotechnical Industries (Pretoria, South Africa). Reactions of 25 µL were set up, with 3 to 5ng DNA, 0.1 µM primers, and Taqman Universal qPCR Mastermix according to the manufacturer's protocol (Roche Industries, Sandton, South Africa). Thermal cycling ran for 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles of 15 sec at 95 °C and 1 min at 60 °C, and was detected in real time on a Roche LightCycler 96 System.

2.4.3. Quantification of L. pneumophila against a standard curve

Isolating and growing individual *L. pneumophila* colonies to set up a standard curve is expensive, tedious and undesirable due to the infection potential of these Biosafety Level 2 organisms. Thus, a standard curve was set up using conventional PCR from environmental samples, based on the fact that the primers are highly specific for a single-copy gene in *L. pneumophila*. Welti and colleagues (2003) rigorously demonstrated single-copy genes for quantification, using multiple controls in monoplex and multiplex identification experiments for quality control. These included (1) negative (no template), (2) inhibition (IPC block control) and positive (plasmid) for each pathogen in each experiment, as well as verifying quantification in pure culture experiments. The melting temperature (Tm) of the probes was also chosen at 10 degrees C higher than the primer Tm, for optimal extension hybridization, as described by authors. Thus, this quantification is based on the assumption of a single MIP gene per cell, but this assumption was demosntrated during method development (Welti et al., 2003).

A conventional PCR was set up with the above-mentioned qRT-PCR primers, LPTM1 and LPTM2, using DNA extracted from river water according to the sampling and filtering protocol described above. Also as described above, each reaction contained 3 to 5 ng DNA, 1 µM primers, 0.8 µM deoxynucleoside triphosphates (dNTPs), and 1 to 1.5 U of Taq DNA polymerase. Thermocycling was as described for qRT-PCR, but in a conventional BioRad T100 Thermal Cycler.

The amplified fragments were separated from PCR reagents via agarose gel electrophoresis (Fig. A.4), extracted from the gel using the QIAquick Gel Extraction kit according to manufacturer's instructions (Whitehead Scientific, Cape Town, South Africa), and quantified using an ND1000 NanoDrop spectrophotometer (Inqaba Biotec). The fragment concentration in the amplified and isolated solution was calculated using the known molecular weight of each fragment.

As described by Dr. John Hildyard (Royal Veterinary College, London, UK, ResearchGate communication), the solution was used to make a dilution range ($0 - 10^8$ fragments/mL), and qRT-PCR was performed on each sample to set up a standard curve (in duplicate) for quantification. The cutoff (Ct) value for each dilution was determined with the LightCycler 96 System Application and Instrument Software, and plotted against the known fragment concentration, calculated based on weight. As there is one MIP gene per cell, as demonstrated by the authors described above, the number of cells is directly equal to the number of molecules in solution. Typically, this concentration is halved in quantifying cDNA for gene expression, but since these PCR products are double-stranded DNA, the Ct values were used directly to plot the standard curve (Fig. A.5). The Ct values in a biological duplication of the standard curve did not vary more than 5 % per dilution. Ct values of the unknown samples were quantified, in terms of concentration, against this standard curve.

2.5. Infection model

Legionella infection requires the deposition of pathogenic microbes in the alveolar region of the lungs (Schoen and Ashbolt, 2011). The risk of infection is greatest during shower events where water is aerosolized. Schoen and Ashbolt (2011) outlined a method to determine critical *Legionella* concentrations in the water supply which would lead to microbial infection during a shower event, as shown in eqns. 3 and 4:

$$DD = \left[\sum_i F_i^{(1)} \times F_i^{(2)}\right] \times n_l \tag{3}$$

$$n_l = V_{air} \times PC \times c_w \tag{4}$$

The total deposited dose (DD as colony-forming-units, CFUs) depends on the number of *Legionella* microbes inhaled (n_l in CFUs) as well as the size distribution of the inhaled aerosol: specifically, the product of the fraction $F_i^{(1)}$ of *Legionella* cells that partition to aerosols in size range i and the fraction $F_i^{(2)}$ of aerosols in size range i that are deposited in the alveolar region of the lungs. The number of *Legionella* microbes inhaled is a product of the volume of air inhaled during a typical shower event (V_{air}, $1/\mathrm{m}^3$), the partition coefficient (PC as $\mathrm{CFU/m}^3$ in air / CFU/L in water) describing the likelihood of *Legionella* partitioning into the aerosol phase, and the concentration of *Legionella* in the water (c_w, CFU/L).

Using parameters obtained from an extensive literature review, the authors predicted a critical density of *Legionella* in the water supply based on a required DD of 1 CFU (low estimate) or 10 CFU (best estimate) for infection. However, the estimated concentrations are higher than reported concentrations associated with cases of Legionellosis. A more appropriate approach is to predict a probability of infection dependent on the microbial density c_w. Specifically, given the probability p of a single aerosol droplet leading to deposition of *Legionella* on the alveoli, and given that n aerosol droplets are inhaled during a shower event, the probability P_s that k *Legionella* microbes will be deposited on the alveoli during a single shower event is described by a Poisson distribution (eq. 5, (Beers, 2006)):

$$P_s(k; p, n) = \frac{(pn)^k}{k!} e^{-pn} \tag{5}$$

The probability of deposition p can be estimated as the product of the fraction of inhaled aerosols being deposited on the lungs $\left(\sum_i F_i^{(1)} \times F_i^{(2)}\right)$ and the fraction of aerosol droplets containing *Legionella* ($F_l = n_l/n$). However, calculating pn yields DD as calculated in eq. 3. Equation 5 can therefore be simplified as

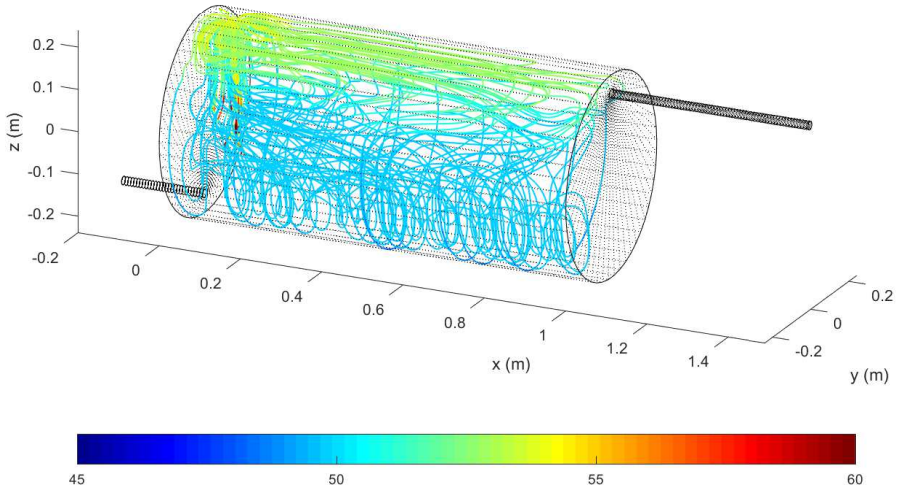

Figure 1: CFD results showing the temperature distribution superimposed on flow streamlines in the tank in the absence of flow. Notice the high temperatures $> 60\,^{\circ}\mathrm{C}$ directly adjacent to the heating element.

shown in eq. 6:

$$P_s(k; DD) = \frac{DD^k}{k!} e^{-DD} \tag{6}$$

Infection is dose-dependent. For a healthy individual, a minimum number of deposited *Legionella* microbes $k_{min} > 10$ may be required for infection. However, it is possible that immunocompromised individuals can be infected by $k_{min} > 1$. The probability of infection P_i can be defined in terms of k_{min} (eq. 7):

$$P_i(k_{min}; DD) = P_i(k > k_{min}; DD) = \sum_{k \geq k_{min}} P_s(k; DD) \tag{7}$$

Finally, P_i is the probability of infection during a single shower event. Assuming a person showers every day, the probability of being infected over a period of one year P_{yr} is given by eq. 8:

$$P_{yr}(k_{min}; DD) = 1 - (1 - P_i(k_{min}; DD))^{365} \tag{8}$$

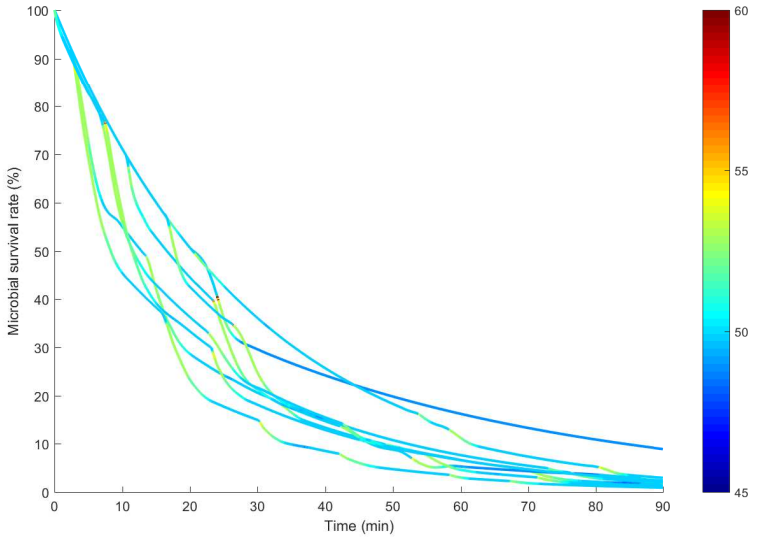

Figure 2: Sterilization model results showing a minimum microbial reduction of 80 % after 60 min.

3. Results and discussion

3.1. CFD model results

Planktonic cells may also grow in the tank, but would be subjected to varying temperatures as the microbes circulate through the tank. The velocity field generated by natural convection in the tank is shown in Fig. 1. The streamlines shown in Fig. 1 are coloured according to the local temperature. Assuming *Legionella* cells will follow these streamlines, a temporal temperature profile can be generated for individual cells and used to predict cellular growth or sterilization as per eq. 1. These growth profiles (2) show that planktonic cells are exposed to high temperatures at an adequate frequency to ensure a decimal reduction time of approximately 85 min. The likelihood of planktonic *Legionella* surviving in a heater with the element turned on is low.

A coarse mesh CFD simulation was used to determine the effect of controlled heater scheduling on planktonic *Legionella* survival. The temperature distribution in an tank was approximated over the course of 24 hours, based on usage data obtained from controller field units. The average temperature as a function of time, in conjunction with eq. 1, was used to estimate the growth of planktonic *Legionella* in a controlled heater. The results from the coarse mesh simulation confirm that the survival of planktonic *Legionella* is unlikely during the course of an average day (data not shown). However, both the detailed- and the coarse-mesh CFD results clearly show that the lower surfaces of the heater remain at temperatures below 45 °C, creating an ideal environment for *Legionella* growth (Video provided in the Supplementary material). Thus, it is likely that only biofilm-associated *Legionella* survive within a heater tank.

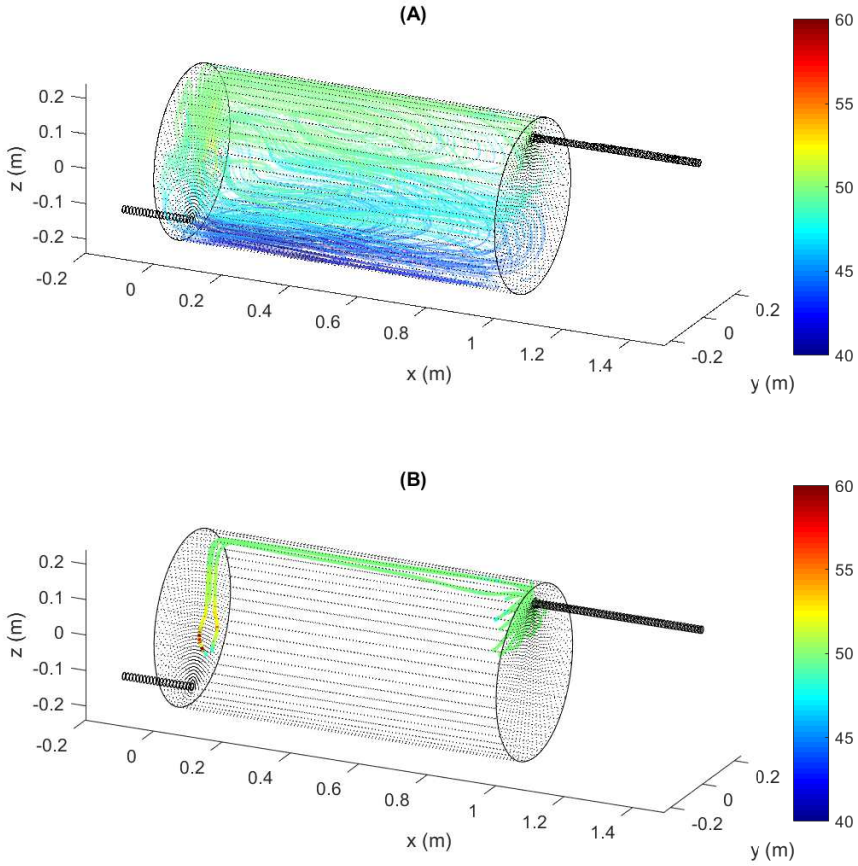

Figure 3: CFD results showing the temperature distribution superimposed on flow streamlines in the tank given a flow rate of 5 L/min. (A) All streamlines, (B) only streamlines associated with surface seed points that result in particles exiting the heater within 300 s.

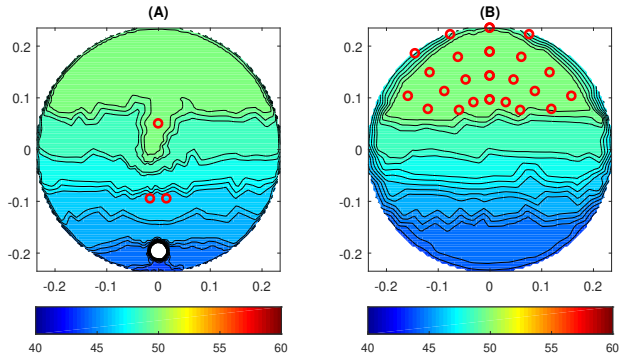

Figure 4: Relates to section 3.1. Starting positions of particles exiting the heater within a typical shower event with surface temperatures generated during no flow conditions: (A) cross-section at inlet and heating element, (B) cross-section at outlet. The starting positions near the heater outlet correspond to a no flow temperature of 49 °C

The flow rate through a heater during a typical shower event is approximately 5 L/ min. The cold water entering the system causes the temperature to drop significantly. The fluid dynamics are subject to both forced- and natural-convection.

Given the low probability of survival for planktonic cells, special attention was given to surface adherent cells which may detach and exit the tank within the timespan of a typical shower event. Figure 3 (A) shows the streamlines generated by particles seeded on the tank inner wall (corresponding to cells present in biofilms), while (B) is limited to particles which report to the outlet pipe within 5 min, taken as a representative time for a shower event. While a large surface area of the heater remains at a temperature conducive to *Legionella* growth, the surface area that allows cells to detach and exit the heater within the timespan of a shower event is quite limited: cells detaching from other regions of the heater are typically entrapped in eddies created by natural convection. Furthermore, heater surfaces corresponding to regions which could lead to cells exiting the tank within a shower event are subjected to temperatures exceeding the optimal temperature for *Legionella* growth under no flow conditions. These positions are shown in Fig. 4, superimposed on the temperature distribution of the pertinent surfaces..

It is improbable for surface adherent cells exposed to temperatures leading to optimal *Legionella* growth to exit the heater within the timespan of a typical shower event. In light of these results, it can be concluded that *Legionella* detected in plumbing systems are unlikely to originate in the heater, but rather in downstream piping. The decreasing temperature in the pipes leading away from the heater will ensure the existence of a thermally optimal region for *Legionella* growth. These biofilms will periodically be exposed to high temperatures during usage events, which may lead to sterilization, if the outlet temperatures are high enough. However, the average outlet temperature in schedule-controlled heaters are typically lower in comparison to those on thermostat control only. The short exposure times to lower temperatures during usage events may not be enough to sterilize biofilms in the piping system. These results are in line with a study of 452 hot water systems in two cities in Germany, which showed that the relationship between *Legionella* proliferation and piping systems as well as heater temperatures is statistically significant (Mathys et al., 2008), as well asa more recent study showing preferential biofilm growth on copper piping (Buse et al., 2014). This is further corroborated by field measurements, as described below.

3.2. Biological results

The heating schedules that were in effect at the time of taking the samples and the average temperature of the hot samples are shown in Table 1 on page 16.

A recent review compared culturing and molecular quantification (Whiley and Taylor, 2016), indicating that culturing techniques detect less than half of the *Legionella* quantified with genetic techniques but did

not mention the limitations of molecular techniques in environmental niches rich in PCR inhibitors such as metal ions, which were a significant challenge in the particular water heater environment (Fig. A.3). Because of these challenges in quantification standardization, there are few studies exploring the full arc of transfer and infection, from thermal simulation of the environment, to quantification of pathogen loads, to infection and public health.

Semi-quantitative assessment of *Legionella* prevalence was carried out by culturing direct heater samples, and molecular techniques were employed for detection and quantification at point-of-use in the water distribution system (household taps). The standard curve set up as described in the methods section produced a strong linear correlation (Fig. A.5, $R^2 = 0.97$), and proved an accessible, robust (less than 5 percent variation with a biological duplicate) technique for quantification, relying on the authors' thoroughly demonstrated claim that the gene is a single copy gene highly specific to *L. pneumophila* (Whiley and Taylor, 2016). The clean tap water as an environmental source thus permitted the use of PCR-based techniques to easily assess both the presence and concentrations of *Legionella* and *L. pneumophila*, respectively, in tap water sourced from controlled and well-characterised heaters.

Qualitative culturing of the scrapings demonstrated the presence of *Legionella* within the heater. However, even the selective media (BCYE) enriched for a plethora of organisms that were morphologically distinct from one another, and the relative percentage of *Legionella* within these samples was low (3.1 % of 62 isolates; Figures A.6 and A.7), based on the semi-quantitative selective media. *Legionella* species were identified by metabolic limitation on enriched BCYE media sans L-Cysteine, as *Legionella* are unique in their inability to synthesize this amino acid, needing it to survive. Isolates were subsequently sequenced, using the 16S rDNA to confirm genus identification. A heterogeneous community is critical for *Legionella* growth (EPA, 2001; Surman et al., 1994; Winn, 1998; Ensminger, 2016; Kwaik et al., 1998).

Whilst the models and qualitative data from within heaters provide information about this niche, as well as relative *Legionella* presence within the heaters, the true epidemiological impact lies in the infectious agents that reach point-of-use in the water distribution system, that is, household taps. The analysis of cold water (CT), hot water in the pipes prior to heating (HT1) and hot water running at maximum temperature from the heater (HT2), showed that *L. pneumophila* predominated in the hot taps prior to taking the water to maximum temperature (Table 1, columns 5-7). There were significant differences between the means of the HT1 group (water in hot taps, prior to heating) and both other groups ($p < 0.05$), as assessed with a 2-tailed Student's t-Test with independent variances (CT and HT1, $p = 0.022$; CT and HT2, $p = 0.226$; HT1 and HT2, $p = 0.049$).

The genus-specific *Legionella* primers showed relatively ubiquitous presence in most of the samples (Table 1, columns 2-4), however the primers unique to *L. pneumophila* were more source-specific (Table 1, columns

Table 1: PCR (Qualitative) results and qRT-PCR (Quantitative) cell count results from the water heaters. The presence of *Legionella* spp. was assessed qualitatively in cold taps (Cold: CT), hot taps prior to heating (Hot-Cold: HT1) and hot taps run at maximum temperature (Hot-Hot: HT2). The quantification of *L. pneumophila* was also carried out for each of these environments (columns 5-7).

Heater no.	Heating schedule	Sample temp (°C)	PCR (Qualitative) *Legionella* spp.			qRT-PC (Quantitative) *Leg. pneum.* (cells/ml)		
			CT	HT1	HT2	CT	HT1	HT2
1	03:00 - 05:00;15:00 - 17:00	47	-	+	+	0	6	5
2	On (Thermostat)	42	+	+	+	0	7	0
3	04:00 - 07:00;16:00 - 19:00	45	+	+	+	0	7	0
4	02:00 - 06:00;15:00 - 20:00	46	-	-	+	0	0	2
5	18:00 - 21:00	44	+	+	+	0	10	0

5-7). This may be related to temperature (the less ubiquitous growth of *L. pneumophila*, or the growth of *L. pneumophila* in the pipes at the lower temperatures between heating events) or to flow dynamics (an initial sloughing event due to turbulence patterns as flow is initiated). The fact that the water stored in the pipes between uses has a higher *L. pneumophila* presence may indicate the pipes, rather than the heater, as a niche for *L. pneumophila* biofilm growth.

The cold tap showed no *L. pneumophila* during quantification, suggesting that the heater provides the temperatures necessary to stimulate growth, either within the heater or in the distribution system directly downstream of the heater. This supports reports of *Legionella*'s thermal preference (growth between 25 °C and 42 °C (EPA, 2001; Fields et al., 2002), and is confirmation of elegant research by Piao et al. (2006), which demonstrated that of 42 *Legionella* strains, *L. pneumophila* was most likely to form biofilms, and biofilm formation was temperature dependent, promoted at temperatures between 35 °C and 47 °C. This confirms the idea that the widely-reported ubiquity of *Legionella* might actually be species-dependent and temperature-dependent.

3.3. Infection model results

The infection model in section 2.5 was used to assess whether the detected concentrations of *Legionella* in the water supply may indeed lead to infections. The probability of infection occurring per year (calculated using eq. 8) is dependent on the minimum deposited dose k_{min} required for infection as well as the average deposited dose DD per shower event. The original infection model estimated a minimum of 10 CFUs for infection of otherwise healthy individuals (Schoen and Ashbolt, 2011). However, immunocompromised individuals may suffer infection if even a single CFU were to reach the lower alveolar region. The average deposited dose DD can be estimated based on previously determined parameters as well as the concentration of *Legionella* cells in the water supply (eqns. 3 and 4; Table A.2).

If only a single CFU would result in infection, there is a 19 % probability per year of Legionellosis occurring. This probability decreases dramatically as the required dosage increases, with the probability of infection

becoming negligible even if $k_{min} = 2$.

The results of the probabilistic form of the previously developed infection model combined with biological sampling results indicate that the probability of Legionellosis occurring in healthy individuals is negligible, which explains the fact that the disease commonly appears as a pandemic associated with public spaces which may be compromised. However, the probability of infection of immunocompromised individuals is much higher. If a single *Legionella* CFU could lead to Legionellosis in an immunocompromised individual, the probability of infection over a timespan of 10 years is approximately 88 %. These issues are of particular concern in low- to medium-income countries in light of the HIV/AIDS epidemic, and bear a striking resemblance to the transition of latent to active tuberculosis: the relative risk of latent tuberculosis infections progressing to the active stage is 10 to 110 times higher in patients with compromised immune systems (Ai et al., 2016).

4. Conclusions

The combination of the CFD model- and biological-results presents a strong case for the growth of *Legionella* in piping systems downstream of the water heater, although the infection model seems to indicate that Legionellosis from single household plumbing systems is unlikely except in the case of immunocompromised individuals. This work fits directly into the US EPA's identified research areas (EPA, 2001), trying to understand the reservoirs for this pathogen, as well as the transfer of the pathogen to the user.

Within economically-challenged communities, the regulation of water heating cycles is necessary for financial reasons. The balance between the regulation of the heaters and the energy cost has also been explored, however, the consideration of the post-heater distribution system has not been included in models. This work highlights the connection between heating regimes and *Legionella* proliferation. A further suggestion might be to explore distribution system materials that might prevent the spread of biofilms, if models can demonstrate that sloughing events play a role in *Legionella* distribution (Piao et al., 2006; Murga et al., 2001; Buse et al., 2014).

It must be added that, as with any pathogenic outbreak, the first and most effective point of resistance is the human immune system. Where economically and practically possible, the health of the individual is more effective in preventing outbreaks than design or habits. However, in the low- to medium-income countries context, there is already an extensive national nosocomial burden, in terms of economy, morbidity, mortality and resources (Klevens et al., 2007; Pooran et al., 2013). Water distribution system design, water

heater regulation habits and effective diagnosis all play a critical role in minimizing the burden of *Legionella* outbreaks, as part of managing the AIDS/TB crisis. Thus, building and testing models to understand

and regulate these pathogenic niches can assist with the management of these nosocomial burdens, through simple shifts in engineering and habits.

In summary, the Baas Becking phrase *"Alles is overals; maar het milieu selekteert"* (Everything is everywhere, but the environment selects) has been harnessed extensively in microbial ecology (De Wit and Bouvier, 2006), and this robust principle is particularly applicable at the intersection of microbiology and engineering. If we understand how the environment selects, we increase the possibility of manipulating it through engineering and management to protect the most vulnerable and prevent the selection of pathogens such as this genus. For instance, risk assessments based on temperature diagnostics for *Legionella* growth have been developed by Bédard et al. (2015), as well as elegant thermal regulation systems inspired by biomimicry according to Altorkmany et al. (2017). Models such as the one developed in this study can further inform such efforts.

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Appendix A. Supplementary material

The following supplementary material is provided:

- CFD model, parameters, and output datasets at `https://goo.gl/VKFzT6`.
- Visualisations (videos) of the EWH CFD models in action at `https://goo.gl/7A6zbV`.
- The following figures are provided as supplementary material.

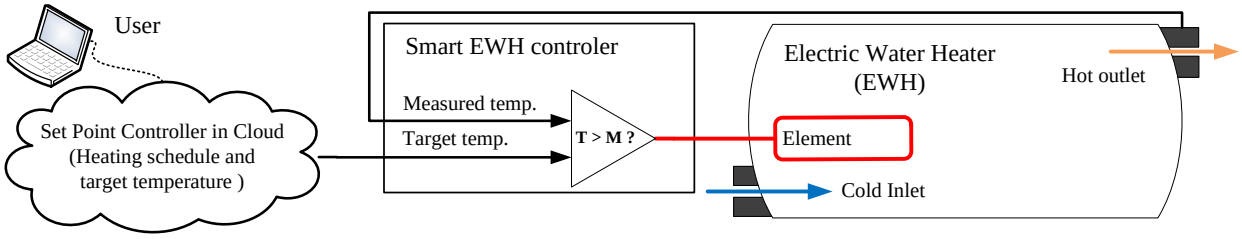

Figure A.1: Relates to section 2.3. Physical set-up used to apply control schedule and measure temperatures.

Figure A.2: Relates to section 2.4.2. Dark red-brown sludge formed against the base of the element (A), representative of the biofilm sludge sampled from the sides and elements in all EWHs within this study. Sampling was done by taking sludge scrapings in sterile petridishes or glass bottles and transporting to the lab on ice.

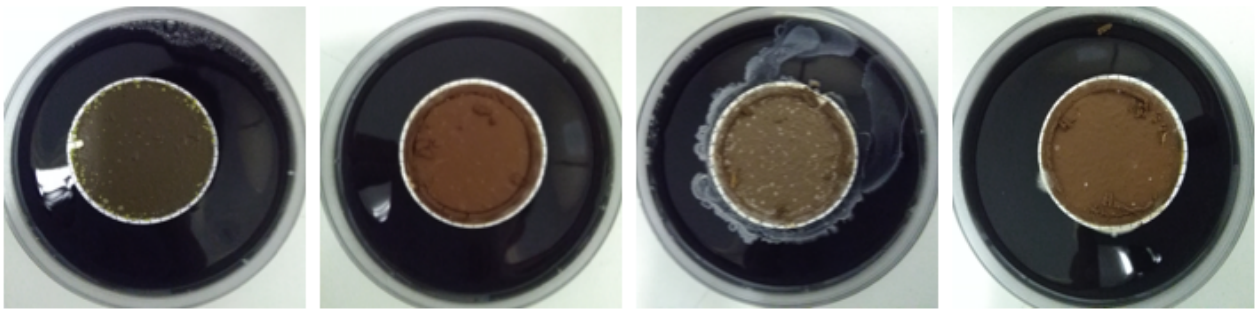

Figure A.3: Relates to section 2.4.2. Particulate matter filtered out of 100 mL water samples taken directly from the EWH and plated onto enriched BCYE media to monitor bacterial growth. The water samples were clearly contaminated with dense, likely metal-rich (red-brown) sediment.

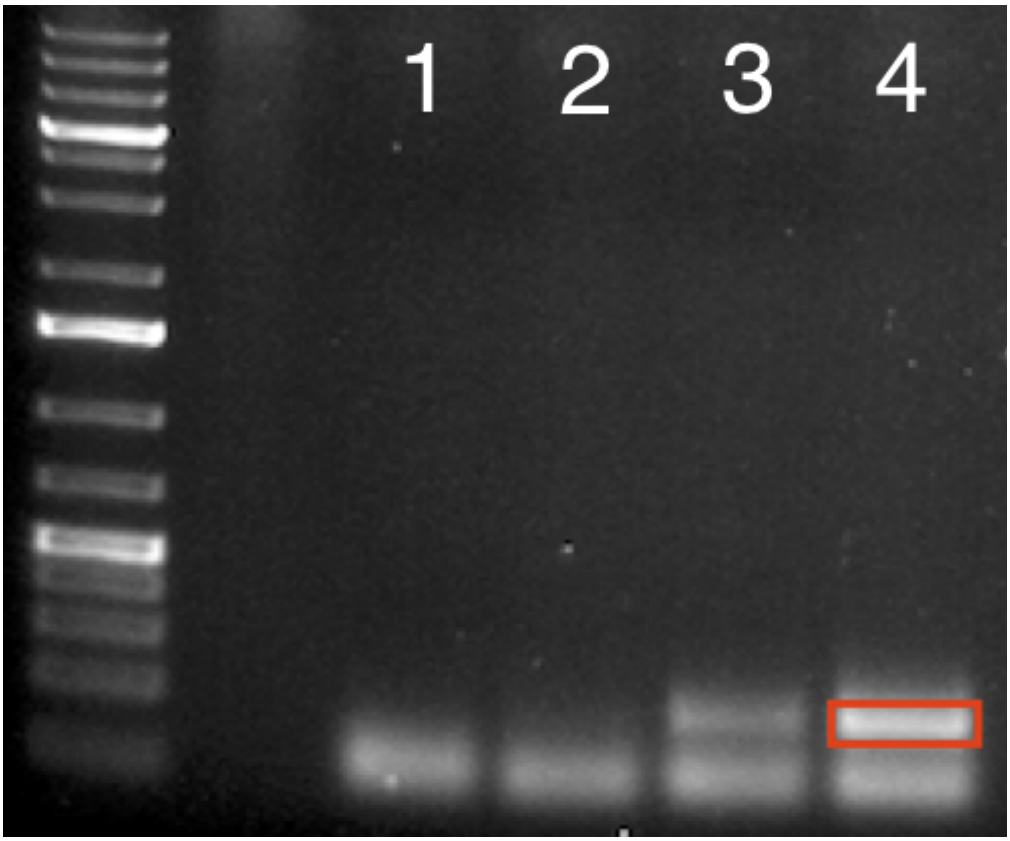

Figure A.4: Relates to section 2.4.3. The MIP region of *L. pneumophila* was amplified with the qRT-PCR primers LPM1 and LPM2, using standard PCR from 2 environmental samples (Lane 2, 3 and 4), with negative controls (Lane 1 and 2). The band from Lane 4 was extracted from the gel and used for the generation of a qRT-PCR standard curve (Figure A.5).

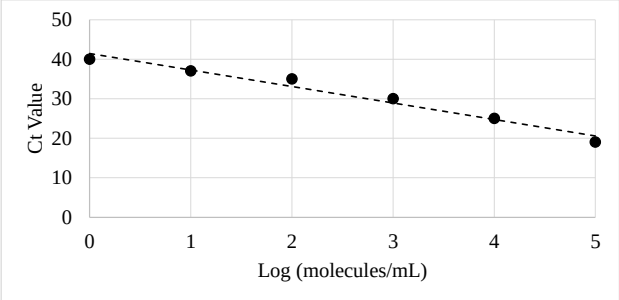

Figure A.5: A standard curve was set up for qPCR quantification by amplifying the DNA region of interest (MIP) using standard PCR, with qRT-PCR primers LPTM1 and LPTM2, extracting the PCR product from the gel, calculating the weight of the product, and creating a dilution series of the amplification product. The concentration of the product (molecules/L) was plotted against the fluorescent threshold (Ct) values generated by qRT-PCR. Quantification of unknown samples was by comparison to the linear log curve. *Note, this is not a calibration curve, but the result of the weight-based L. pneumophila quantification, the method of which is described here.*

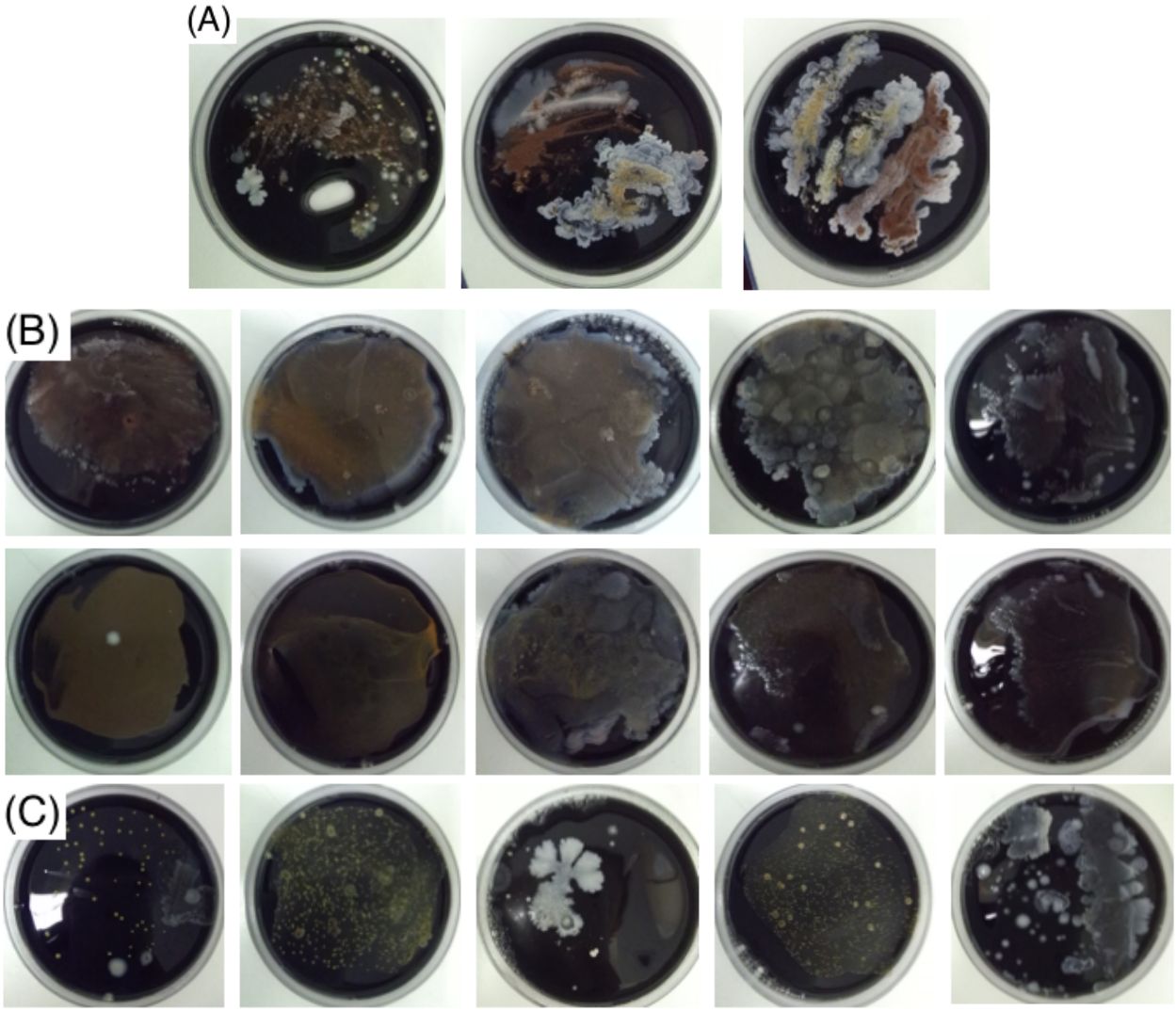

Figure A.6: Relates to section 3.2. Samples of water and biofilm scrapings taken from EWHs were grown on enriched BCYE agar at 35 °C for the selection of *Legionella* spp., using streak plates (A), Spread plates (B) and spread plates of dilutions (C). Morphologically distinct individual colonies were isolated from each of these for positive identification.

Table A.2: Relates to section 3.3. Parameters used in the infection model (Schoen and Ashbolt (2011)).

Parameter	**Value**	**Description**
V_{air}	0.06 m^3	Average volume of air inhaled during a 5 min shower
PC	$10^{-5}\ \frac{CFU/m^3}{CFU/L}$	Partition coefficient of microbes from air to water
$F^1_{i=1,2,3}$	[0.75; 0.09; 0.14]	Fraction of aerosolized organism partitioning to aerosols in the size ranges of (1) 1 - 5 µm; (2) 5 - 6 µm; and (3) 6 - 10 µm
$F^2_{i=1,2,3}$	[0.2; 0.1; 0.01]	Fraction of aerosol deposited to alveoli in the size ranges of (1) 1 - 5 µm; (2) 5 - 6 µm; and (3) 6 - 10 µm

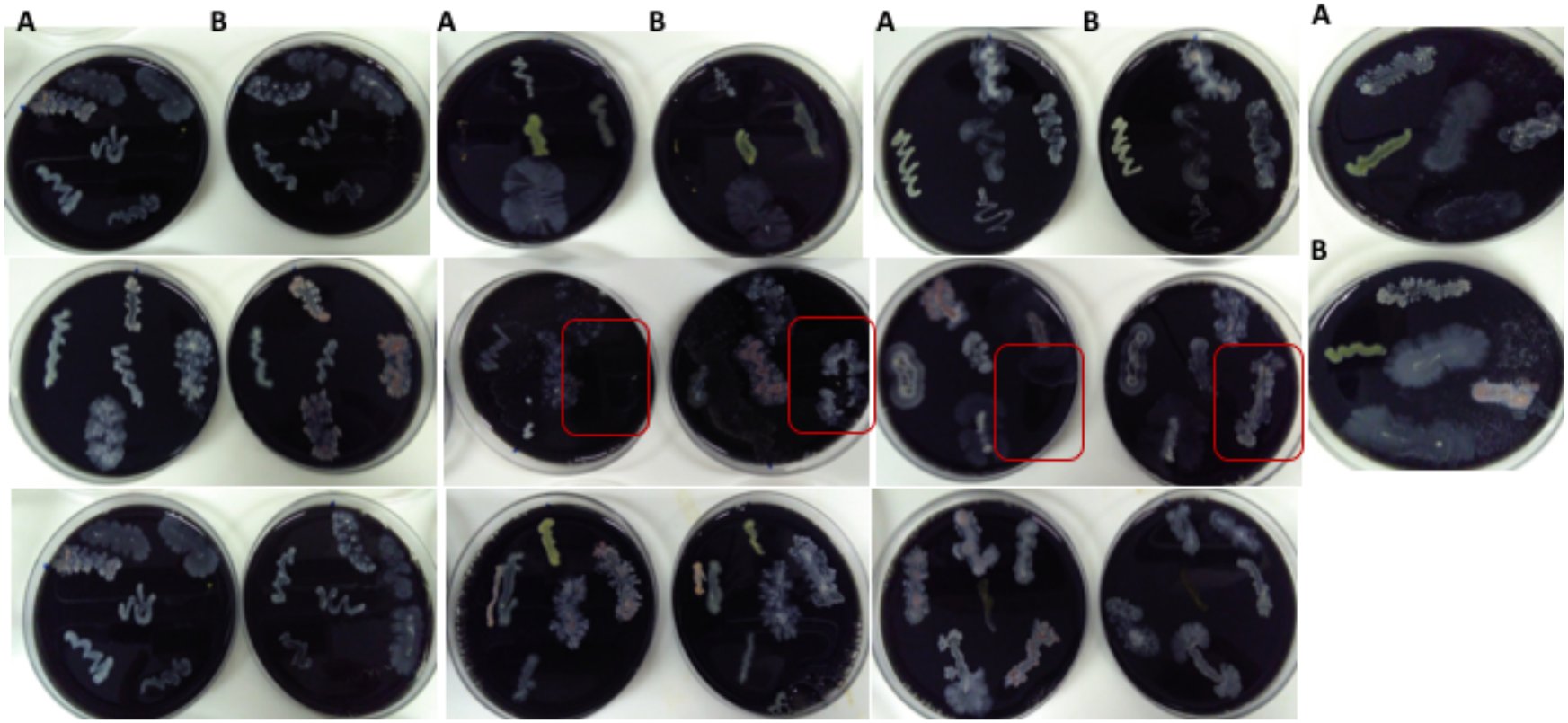

Figure A.7: Relates to section 3.2. Tentative positive identification of *Legionella* spp. from within EWHs was achieved by plating each isolate on enriched BCYE agar with (A) and without (B) L-Cysteine. *Legionella* species are unique in their inability to synthesize this amino acid, and thus need L-cysteine in the media to grow. The isolates that did not grow (red) were tentatively identified as *Legionella* spp. and further confirmed by sequencing the 16S rDNA region (JFP and JRP primers) and comparing it to the international BLAST database.

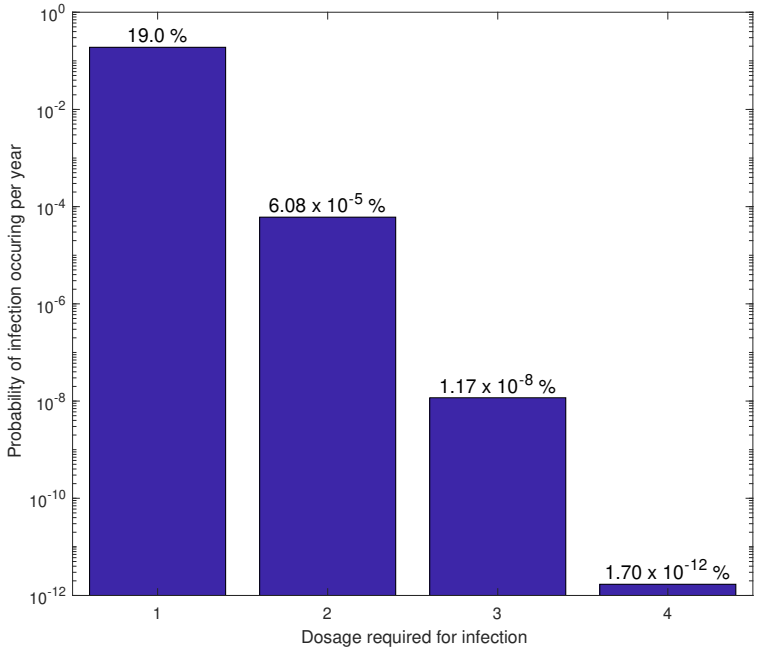

Figure A.8: Relates to section 3.3. The probability *Legionella* infection occurring in a year, given that infections are most likely to occur during a shower event, and *Legionella* concentrations of 6 CFU/ml in the water. Infection rate is dependent on the required dose to cause infections: this might be as low as 1 CFU for immunocompromised individuals, but at least 10 CFU for healthy individuals.