A valuable experimental setup to model exposure to Legionella's aerosols generated by shower-like systems

Séverine Allegra, Serge Riffard, Lara Leclerc, Françoise Girardot, Magalie Stauffert, Valérie Forest, Jérémie Pourchez

A valuable experimental setup to model exposure to *Legionella's* aerosols generated by shower-like systems

Séverine Allegra[1]*, Serge Riffard[1], Lara Leclerc[2], Françoise Girardot[1], Magalie Stauffert[1], Valérie Forest[2], Jérémie Pourchez[2].

[1-] University of Lyon, University Jean Monnet of Saint-Etienne, CNRS, EVS-ISTHME UMR 5600, F-42023 Saint-Etienne, France

[2-] Mines Saint-Etienne, University of Lyon, University Jean Monnet of Saint-Etienne, INSERM, U 1059 Sainbiose, Centre CIS, F - 42023 Saint-Etienne France

*Corresponding author:
UMR 5600 EVS-ISTHME
6 rue Basse des Rives
42000 St Etienne, France
Tel: +33477463356
Fax: +33477421924
E-mail: severine.allegra@univ-st-etienne.fr

Abstract

The mechanism underlying *Legionella* aerosolization and entry into the respiratory tract remains poorly documented. In previous studies, we characterized the aerodynamic behaviour of *Legionella* aerosols and assessed their regional deposition within the respiratory tract using a human-like anatomical model. The aim of this study was to assess whether this experimental setup could mimic the exposure to bioaerosols generated by showers. To achieve this objective we performed experiments to measure the mass median aerodynamic diameter (MMAD) as well as the emitted dose and the physiological state of the airborne bacteria generated by a shower and two nebulizers (vibrating-mesh and jet nebulizers). The MMADs of the dispersed bioaerosols were characterized using a 12-stage cascade low-pressure impactor. The amount of dispersed airborne bacteria from a shower was quantified using a Coriolis® Delta air sampler and compared to the airborne bacteria reaching the thoracic region in the experimental setup. The physiological state and concentration of airborne *Legionella* were assessed by qPCR for total cells, culture for viable and cultivable *Legionella* (VC), and flow cytometry for viable but non-cultivable *Legionella* (VBNC). In summary, the experimental setup developed appears to mimic the bioaerosol emission of a shower in terms of aerodynamic size distribution. Compared to the specific case of a shower used as a reference in this study, the experimental setup developed underestimates by 2 times (when the jet nebulizer is used) or overestimates by 43 times (when the vibrating-mesh nebulizer is used) the total emitted dose of airborne bacteria. To our knowledge, this report is the first showing that an experimental model mimics so closely an exposure to *Legionella* aerosols produced by showers to assess human lung deposition and infection in well-controlled and safe conditions.

Keywords: *Legionella*; bioaerosols; shower systems; exposure.

Introduction

Members of the genus *Legionella* (Gram negative bacilli) are ubiquitous in natural and anthropogenic aquatic ecosystems. These bacteria are responsible for severe pneumonia, which may be fatal in 30% of cases when considering nosocomial infections. *L. pneumophila* is, by far, the most frequent species associated with Legionnaires' disease (LD). *Legionella* is now the number one cause of reported waterborne disease in the United States. The latest epidemiological data showed an increase in LD worldwide (Centers for Disease Control and Prevention, 2018; InVS, 2018) with the identification of new sources of contamination by *Legionella* aerosol dispersion (*i.e.,* car washing stations (Baldovin et al., 2018), street cleaning trucks (Valero et al., 2017), aerosols from biologic wastewater treatment plants (Loenenbach et al., 2018), reclaimed water used for spray irrigation (Pepper and Gerba, 2018), etc.). To assess the risk of infection of LD, researchers have used the quantitative microbial risk assessment (QMRA) method to provide models for aerosol dispersion (Thomas W. Armstrong and Haas, 2007; T. W. Armstrong and Haas, 2007; Buse et al., 2012; Hamilton and Haas, 2016; Schoen and Ashbolt, 2011). However, these risk models are only based (i) on the fluidic characteristics of water systems that could generate bioaerosols and (ii) on the infectious doses extrapolated to humans from animal experiments using inhalation, intraperitoneal injections or tracheal instillation for infection of the animal model.

In previous works, we used a medical nebulization device to generate *Legionella* aerosols. Thus, we extensively characterized the bioaerosols produced by a vibrating-mesh nebulizer in terms of aerodynamic features and airborne *Legionella* emitted dose (Allegra et al. 2016). We also experimentally assessed the deposition of these bacteria in the pulmonary region using a 3D replica of the upper airways (Pourchez et al., 2017) and using an *ex vivo* ventilated porcine lung as an innovative human-like respiratory model (Perinel et al., 2018). We showed that the

developed experimental setup can be used to mimic bacterial inhalation by an anatomical model of the respiratory tract to assess the *Legionella* dose reaching the thoracic region for a given bioaerosol source. Therefore, an interesting further step would consist of demonstrating that nebulizers can be used to satisfactorily mimic bioaerosol exposure during showering events. Indeed, if cooling towers are the most significant sources of *Legionella* outbreaks at a community level, spa pools and showers from public facilities are the most significant sources of *Legionella* nosocomial outbreaks (K. A. Hamilton et al., 2018).

For this purpose, we compared (in terms of aerodynamic size distribution and bacteria concentration) two different technologies of bioaerosol generation: a vibrating-mesh nebulization system and a jet nebulizer. The jet nebulizer has been in continuous development since medicinal aerosol delivery started in the 19th century. Today, the majority of jet nebulizers are inexpensive devices operated via compressed gas. The gas passes through a small aperture in the nebulizer to collect and atomize the liquid. The aerosol generated by atomization contains large and small droplets and is driven to a baffle. Large droplets are impacted by the baffle, while small droplets are transported out of the nebulizer. In contrast, the vibrating-mesh nebulization system is a recent technology for aerosol generation using an annular piezo element to produce mesh vibration to push the liquid through the mesh. Holes in the mesh have a conical structure, with the largest cross-section of the cone in contact with the liquid. The mesh deforms into the liquid side, thus pumping and loading the holes with liquid. This deformation on the other side of the liquid-drug reservoir ejects droplets through the holes. We hypothesize that with these two technologies, our experimental setup will be able to reproduce many different shower-like systems on the market place.

For adaptation of our previous model to showering facilities, the original replica of the human upper airways (El Merhie et al., 2015; Leclerc et al., 2014) was placed in an experimental sealed enclosure mimicking a shower cubicle and connected to a filter mimicking the thoracic region

(Perinel et al., 2018; Pourchez et al., 2017). A respiratory pump was used to fit the breathing parameters corresponding to adult male physiology at rest: breathing rate of 15 breaths per minute and tidal volume of 500 mL (Gradon and Marijnissen, 2003; NF EN 13544-1, 2002). The results obtained using this experimental setup were compared with the MMAD and the emitted dose of bioaerosols collected by the Coriolis® Delta air sampler in the lab's shower that is routinely used by the staff. The lab shower will be our reference in this work and hereafter referred to the "real" shower.

Materials and methods

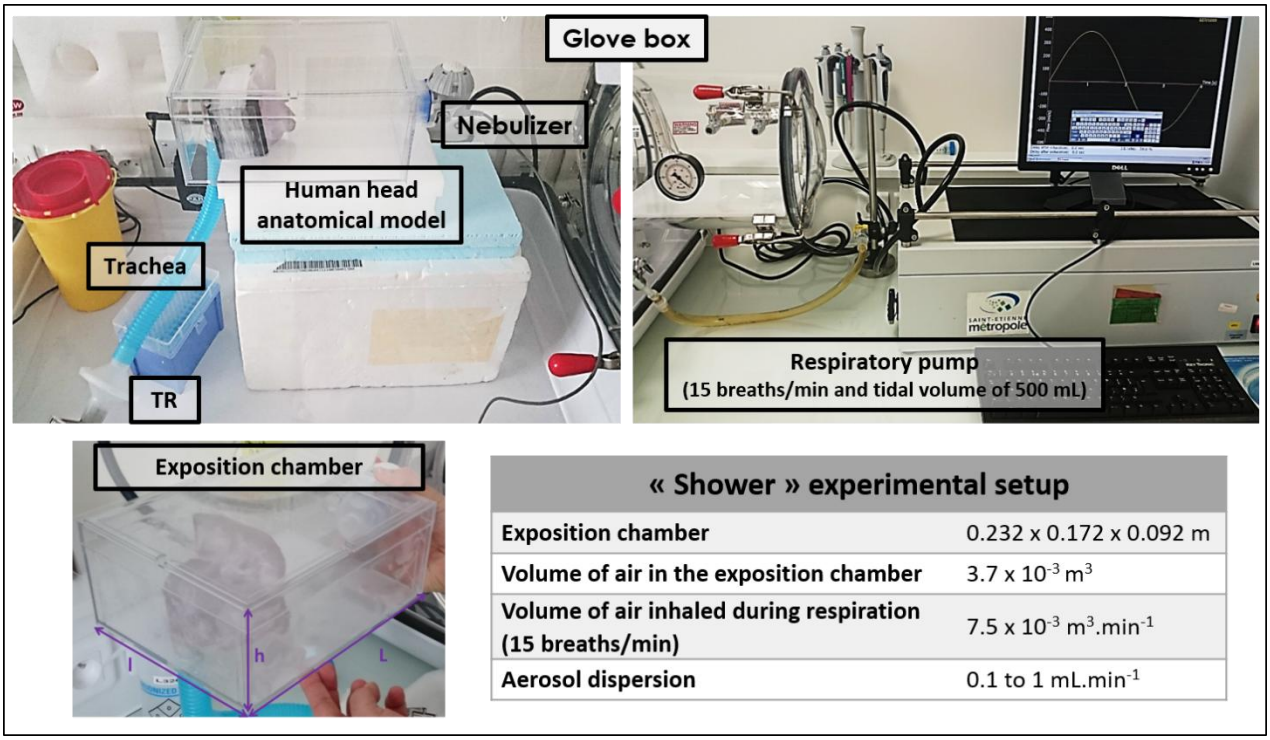
Description of the "real" shower experiments used as a reference

The shower analysed in this study is located on the ground floor of the building of the Center for Health Engineering of Mines Saint-Etienne (CIS, Saint-Etienne, France). This shower is regularly used by the academic staff and postgraduate students at lunchtime after sporting activities.

Three independent experiments were carried out. The concentration of bacteria in the water network was determined by sampling 1 litre of water from the first flow and 1 litre during a thirty-minute showering event respectively at 10, 20 and 30 minutes. The quantity of bioaerosols dispersed during 10, 20 and 30 minutes was measured by the Coriolis® Delta air sampler (Bertin Instruments, France) at a flow rate of 300 $L.min^{-1}$ in a Coriolis collection tube containing 5 mL of physiological sterile water. The water network samples (1 L) and the Coriolis® Delta collections (5 mL) were concentrated on a polycarbonate membrane by filtration. DNA from each polycarbonate membrane was extracted with the Power Water DNA extraction kit (MoBio). The total number of bacteria (in the water network and in bioaerosols) was quantified by qPCR targeting the 16S RNA gene (see qPCR section).

Description of the experimental setup to generate bioaerosol and to assess lung deposition

A previously validated and original respiratory model was used in this study (Pourchez et al., 2017). The setup was composed of a human 3D replica corresponding to the entire nasal cavities (nasal fossae and frontal, ethmoid and maxillary sinuses) (El Merhie et al., 2015; Le Guellec et al., 2014). This replica was manufactured in transparent water-resistant and non-porous resin. Qualitative analyses with endoscopy and CT-scans were performed on the replica to assess the anatomical reproducibility (Durand et al., 2011). As shown in Figure 1, this human upper airway replica was placed in an exposition chamber allowing aerosol dispersion between 0.6 and 0.9 $mL.min^{-1}$ of the calibrated suspensions. This replica was linked to a pump (model 420-2902 – 230 VAC, 50 Hz, 0.6 Amp, 1 PH, 0.02 HP, Thermo Scientific) to inhale the aerosol suspended in the exposition chamber. The thoracic region (TR) was simulated by a filter holder (filter kit and filter pad, PARI, PulmoMed) with a polycarbonate membrane (Nuclepore™ track-etched membrane filter, Whatman, GE Healthcare) that allowed optimal bacterial recovery. To avoid any bacterial contamination of the pump, a HEPA filter (Pall filter BB50TE, Pall Medical) was placed after the thoracic filters. Five millilitres and 10 mL of the *Legionella* calibrated suspensions were nebulized in the respiratory model by a vibrating-mesh nebulizer or jet nebulizer, respectively. For safety reasons, the experimental setup was placed in a glove box (815-PGB “La petite glove box”, Fisher Scientific). For quantification of the percentage of droplets reaching the filter holder, the polycarbonate membrane was weighed (1 g = 1 mL of water) before and after nebulization. After nebulization, the polycarbonate membrane was placed in 5 mL of physiological sterile water. After a 30 sec sonication (Bransonic 32 sonicator bath, Branson Instruments, Danbury, CT USA), the membrane was scraped using an automatic pipette tip. One millilitre was plated twice on adapted culture media to count the viable and culturable (VC) *Legionella*. One millilitre was placed in a tube for DNA extraction and qPCR analysis. One millilitre was placed in a flow cytometric tube for flow cytometry assays (FCAs).



« Shower » experimental setup	
Exposition chamber	0.232 x 0.172 x 0.092 m
Volume of air in the exposition chamber	3.7 x 10^{-3} m^3
Volume of air inhaled during respiration (15 breaths/min)	7.5 x 10^{-3} $m^3.min^{-1}$
Aerosol dispersion	0.1 to 1 $mL.min^{-1}$

Figure 1 Human-like shower experimental setup inside a glove box. This experimental setup mimics the dispersion and collection of *Legionella* aerosols under the breathing conditions of an adult male. TR: Thoracic region mimicked by a polycarbonate membrane inside a filter holder.

Legionella strain, culture and flow cytometry for the nebulization process

L. pneumophila serogroup 1 strain expressing green fluorescent protein (*Lp1 008-GFP*) was used. The strain was stored at -80°C in Cryobank tubes (Mast Diagnostic, Amiens, France). After thawing, *Lp1 008-GFP* was plated onto BCYE agar (Buffered Charcoal Yeast Extract, SR0110 C, Oxoid, France) supplemented with chloramphenicol (Sigma Aldrich, France) at 8 mg/mL (for GFP plasmid selection) for 72 h at 37°C. The samples were then re-plated onto the same media and incubated at 37°C for another 3 days to obtain *Legionella* EPF cells (Robertson et al., 2014). A suspension with an optical density of 0.2 at 600 nm (Biomate TM3, Avantec) was then used to achieve a 20 mL calibrated suspension (CS) in sterile normal saline (0.9% NaCl) solution at a concentration between 2.0 x 10^6 and 2.0 x 10^7 $CFU.mL^{-1}$.

The culturability (determination of the number of viable and culturable *Legionella* - VC) was assessed in duplicate by plating on BCYE agar, supplemented with chloramphenicol, 100 µL and 500 µL of the suspension retrieved from the polycarbonate membrane. After incubation for 72 h at 37°C, all the BCYE plates were observed under UV at 366 nm to quantify the *Legionella* colonies (CFU) expressing GFP.

The physiological states of the airborne *Legionella* collected in the thoracic region (TR) were quantified by flow cytometry assays (FCAs) as previously described (Allegra et al., 2016, 2008). Briefly, the FCAs profiles of the samples were obtained by using a combination of GFP green fluorescence (viable cells expressing GFP) and propidium iodide (PI) red fluorescence for cells with damaged membranes. Flow cytometric measurements were performed using a CyFlow Cube6 instrument (Sysmex Partec) equipped with an air-cooled argon laser (488-nm emission; 20 mW). The green fluorescence emission from GFP was collected in the FL1 channel (500 to 560 nm), and the red fluorescence from PI was collected in the FL3 channel (670 nm). A threshold was applied onto the FL1 channel to eliminate background signals. Analyses were performed at a low-flow-rate setting. The results were analysed with FCS Express software (De Novo Software).

Quantification by qPCR

The total number of bacteria in the shower water network and in bioaerosols was quantified using the 16S RNA gene as a target. Each reaction tube contained 5 µL of DNA extract, 2.5 µL of each primer at 10 pmol/µL (5'-AGG GTT GAT AGG TTA AGA GC-3' and 5'-CCA ACA GCT AGT TGA CAT CG-3') and 10 µL of the 2X Power SYBR® Green PCR Master Mix (Life Technologies). The standard curve was established using a mix of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and the *L. pneumophila* sg1 strain isolated from water (strain collections of the teaching laboratory of Technical University Institute of Saint-Etienne). A suspension of an optical density of 0.2 at 600 nm (Biomate TM3, Avantec) was made for each bacterial strain. One

187 millilitre of each suspension was mixed and DNA was extracted from 1 mL of this latter
188 suspension (Power Water DNA extraction kit - MoBio). 1:10 serial dilutions of this DNA were
189 prepared and 5 µL of each dilution were used as template to generate the standard curve.
190 Meanwhile, the total number of bacteria in CFUs was determined by culture.
191 The number of *Legionella* during the nebulization process was determined using the GFP mut2
192 sequence of the *Lp1 008-GFP* strain (template DNA 5 µL). The forward and reverse primers were
193 5'-AGAGTGCCATGCCCGAAGG -3' and 5'-AAGGACAGGGCCATCGCCAA-3', respectively (2.5 µL of
194 each primer at 10 pmol/µL). Ten microliters of the 2X Power SYBR® Green PCR Master Mix (Life
195 Technologies) were used for each reaction. A standard curve was generated with *Lp1 008-GFP*
196 suspensions ranging from $2.0x10^7$ to $2.0x10^1$ CFU.mL^{-1}. Plasmid DNA extraction from all samples
197 was performed with a NuCleoSpin Plasmid kit (Macherey-Nagel) following the manufacturer's
198 instructions.
199 qPCR analyses were carried out on an ABI Prism 7500 automated system (Applied Biosystems),
200 as follows: initial denaturation for 15 min and a two-step cycle consisting of 15 s denaturation, 1
201 min annealing and elongation at 60 °C. At the end of each elongation step, the fluorescence of
202 the incorporated SYBR green dye was measured. At the end of 45 cycles of amplification, a
203 melting curve programme was incorporated to identify primer dimers or other non-specific
204 amplifications. Each sample was run in duplicate. The results were analysed using Sequence
205 Detection Software version 1.4 (ABI 7500 System Software, Applied Biosystems).
206 The results were expressed in genomic units (GU). When a comparison with cell counts was
207 necessary, and to a better understanding, data was expressed in colonies forming units (CFUs)
208 according to the standard curves.
209
210 <u>Biological characterization and aerodynamic features of aerosols generated by a jet nebulizer</u>

We compared two different technologies used for bioaerosol generation: a vibrating-mesh nebulization system and a jet nebulizer. A vibrating-mesh nebulizer, a medical device (eRapid® Nebulizer System by PARI, Cystic Fibrosis Services) used to aerosolize the bacterial suspensions, was linked to a small compressor and delivers aerosols with a vibrating membrane technology. The jet nebulizer used was a Bio-Aerosol Nebulizing Generator (BANG, VitroCell®). This system is specifically designed for the generation of aqueous aerosols at a low air flow rate. Based on the aerosolization principle of the Collison nebulizer, the design of the BANG has been modified to minimize foaming of protein solutions and thus increase the viability of microorganisms in bioaerosols while maximizing aerosol output.

The aerodynamic features of the jet nebulizer were determined as previously described (Allegra et al., 2016). Briefly, the airborne particle size distribution was assessed using a 12-stage cascade low pressure impactor (DLPI, Dekati, Finland). The size classification in DLPI was made from 30 nm up to 10 µm with evenly distributed impactor stages. Each stage of the cascade impactor was covered with a polycarbonate membrane (Nuclepore™ track-etched membrane filters, Whatman, GE Healthcare) to collect *Legionella* aerosols. After nebulization, each membrane was placed in 5 mL of physiological sterile water. After a 30 sec sonication (Bransonic 32 sonicator bath, Branson Instruments, Danbury, CT, USA), the bacterial suspensions of each stage were analysed by GFP mut2 qPCR. The mass median aerodynamic diameter (MMAD) was calculated. The MMAD was defined as the median of the distribution of airborne particle mass with respect to the aerodynamic diameter.

Results

Quantification of the exposure to airborne bacteria during one minute in the shower as a reference

The technical features of the "real" shower are presented in Figure 2. As determined by qPCR, the total bacteria concentration in the water network was $3.3 \pm 1.0 \times 10^4$ bacteria.L^{-1}. Assuming that the aerosols are evenly distributed in the shower space, when extrapolating the numbers, we found that the mean number of bacteria collected per minute by the Coriolis® Delta air sampler was $8.6 \pm 3.2 \times 10^2$. As the shower cubicle has a volume of 1.125 m^3, the Coriolis® system (300 L.min^{-1}) can collect 26.7% of the air per minute. The actual number of bacteria per minute in the shower cubicle was therefore 3.2×10^3, and the number of aerosolized bacteria in the shower cubicle was 2.9×10^3 bacteria.m^3. The volume of air inhaled by an adult male at rest is 7.5×10^{-3} m^3.min^{-1}. Therefore, in the specific case studied, we can estimate that a human male at rest inhales approximately 22 bacteria per minute during a shower.

Characteristics of the shower use as a reference	
The shower cubicle size	0.75 x 0.75 x 2 m
The aerosols are spreading out in	1.125 m^3
Volume of water dispersed in shower	11.5 ± 0.3 L.min^{-1}
C_w: bacteria source water concentration in sanitary hot water (qPCR)	$3.3 \pm 1.0 \times 10^4$ bacteria.L^{-1}
Coriolis® Delta flow rate	300 L.min^{-1}
Air fraction of shower cubicle collected by Coriolis® Delta	26.7%
Number of bacteria collected by Coriolis® Delta (qPCR)	$8.6 \pm 3.2 \times 10^2$ bacteria.min^{-1}
C_A: bacterial air concentration in shower cubicle	2.9×10^3 bacteria.m^{-3}
Flowrate of air inhaled during respiration (15 breaths/min)	7.5×10^{-3} m^3.min^{-1}
Number of inhaled bacteria	22 bacteria.min^{-1}

Figure 2 Summary of the experimental and calculated data from shower analysis. The number of inhaled bacteria was calculated based on results obtained by qPCR under the breathing conditions of an adult male and extrapolated from the characteristics of the shower and Coriolis air sampler. The results are from 3 independent experiments.

Legionella exposure quantification in the experimental setup

253 The amount of *Legionella* reaching the thoracic region was assessed by culture, FCA and qPCR.
254 The number of viable and culturable *Legionella* (VC) was determined by culture. FCA allowed the
255 quantification of viable and potentially pathogen bacteria (VC and VBNC), and qPCR was used to
256 quantify the total DNA from VC, VBNC and dead cells (DCs).
257 Inside the experimental setup used with the vibrating-mesh nebulizer or with the jet nebulizer to
258 generate bioaerosols, the volume per minute of calibrated suspension dispersed around the
259 human 3D replica of the upper airways was 1.00 ± 0.25 $mL.min^{-1}$ and 0.16 ± 0.06 $mL.min^{-1}$,
260 respectively. As determined by qPCR, only 2.0 ± 0.3% of the total *Legionella* DNA dispersed from
261 the jet nebulizer could reach the thoracic region compared to 11 ± 9.6% from the vibrating-mesh
262 nebulizer. In the experiments with the jet nebulizer, no significant values were obtained in the
263 culture and FCA experiments. The amount of culturable and viable bacteria that could be
264 detected was below the detection limits of the culture and FCA methods. Table 1 shows all the
265 results obtained with the vibrating-mesh nebulizer.
266 qPCR was used to quantify all *Legionella* physiological states, including dead cells. We found that
267 11 ± 9.6% of the total aerosolized *Legionella* DNA could reach the thoracic region. From the
268 culture results, only 0.5 ± 0.7% of the culturable aerosolized *Legionella* was detected in the
269 thoracic region. As expected, the culture method underestimates the real *Legionella* risk. Finally,
270 with the FCA results, we observed that through the human anatomical model, the thoracic
271 region was exposed to 4.3 ± 4.0% of the viable (VC + VBNC) aerosolized *Legionella*.
272
273 **Table 1** Quantifications of aerosols generated by a vibrating-mesh nebulizer and reaching the TR
274 of the shower-like experimental setup. Weighing experiments show the quantity of droplets
275 reaching the TR. CS: calibrated suspension; TR: thoracic region; VC: viable and culturable
276 bacteria; VBNC: viable but not culturable bacteria; DC: dead cells; CFU: colony forming unit; GU:
277 genomic unit; SD: standard deviation. The results are from 20 independent experiments.

			Mean	SD	Min	Max
		Time of nebulization (min)	3.9	0.8	2.4	5.4
		Volume of aerosolized CS (mL)	3.7	0.3	3.1	4.2
		% of water reaching TR	21	5.3	14	30
	Culture	**Amount of aerosolized VC (CFU)**	9.3×10^{7}	2.5×10^{7}	5.3×10^{7}	1.4×10^{8}
		Amount of VC on membrane (CFU)	5.1×10^{5}	7.7×10^{5}	0.0	2.6×10^{6}
		% of VC reaching TR	0.5	0.7	0.0	1.9
FCA	**Viable bacteria (VC+VBNC)**	**Amount of aerosolized Viable bacteria (CFU)**	3.7×10^{7}	3.1×10^{7}	7.5×10^{5}	1.0×10^{8}
		Amount of Viable bacteria on membrane (CFU)	2.1×10^{6}	3.4×10^{6}	1.0×10^{4}	1.3×10^{7}
		% of Viable reaching TR	4.3	4.0	0.9	17
qPCR	**Total bacteria (VC+VBNC+DC)**	**Amount of aerosolized Total bacteria (GU)**	2.0×10^{7}	2.0×10^{7}	1.1×10^{6}	7.1×10^{7}
		Amount of Total bacteria on membrane (GU)	1.1×10^{6}	9.0×10^{5}	3.4×10^{5}	2.6×10^{6}
		% of Total reaching TR	11	9.6	2	34

Comparison of the exposure to bacteria

To determine if the shower-like experimental setup satisfactorily mimics a bioaerosol shower exposure, we compared the number of inhaled bacteria for a one-minute shower event (Table 2). As we used the "real" shower as a reference, the number of bacteria in the nebulizer's reservoir (5 mL or 10 mL for the vibrating-mesh and jet nebulizer, respectively) was fixed at 3.3×10^{4} bacteria. The results in Table 2 indicate that the experimental setup developed underestimates by 2 times (if the jet nebulizer is used) or overestimates by 43 times (if the vibrating-mesh nebulizer is used) the emitted dose of total (VC, VBNC and DC) airborne bacteria.

Table 2 Comparison of bacterial exposure between a "real" shower event and a one-minute shower-like experimental setup. VC: viable and culturable bacteria; viable (VC + VBNC); TR: thoracic region. See Supplementary data for equations and calculations used.

	Shower as a reference (SS)	**Experimental setup (SE)**

		Vibrating-mesh nebulizer			Jet nebulizer
	Total	**VC**	**Viable**	**Total**	**Total**
Exposition chamber (dimensions, volumes)	0.75 m x 0.75 m x 2 m =1.125 m^3	0.232 m x 0.172 m x 0.092 m = 3.7 x 10^{-3} m^3			
Water flowrate of the source (L.min^{-1})	11.5	1 x 10^{-3}			1.6 x 10^{-4}
C_W (bacteria.L^{-1}): source water concentration	3.3 x 10^4				
C_A (bacteria.m^{-3}): bacterial air concentration	2.9 x 10^3	8.9 x 10^3			1.4 x 10^3
Flowrate of bacteria coming from the source (bacteria.min^{-1})	3.8 x 10^5	33			5
Inhaled bacteria (in % of bacteria coming from the source)	0.006	0.5	4.3	11	2
Number of inhaled bacteria (bacteria.min^{-1})	22	41	3.8 x 10^2	9.4 x 10^2	11
Ratio: SE / SS for inhaled bacteria or bacteria reaching TR	**1**	2	17	**43**	**0.5**

Features of the aerosols generated by nebulization

The aerodynamic features of the aerosols generated by the jet nebulizer were compared to those of the vibrating-mesh nebulizer that were previously determined (Allegra et al., 2016). Figure 3 shows the repartition of *Legionella* depending on the aerosol size. Table 3 presents the proportions of viable and potentially pathogenic airborne bacteria. Both nebulizer profiles were examined for the aerosol fraction that could reach the thoracic region (0.26 µm to 2.4 µm).

From the literature and our previously published data with the vibrating-mesh nebulizer (Allegra et al., 2016; Perinel et al., 2018; Pourchez et al., 2017), we confirmed that *Legionella* EPF cells (Robertson et al., 2014) are approximately 1–2 µm long and 0.3–0.9 µm wide. Several studies have shown that bioaerosol droplets of more than 2.5 µm in diameter cannot reach the pulmonary alveoli (Leclerc et al., 2015; Pourchez et al., 2013; Schlesinger, 1989). In this study, depending on the cut-off diameters of the cascade impactor (DLPI), we defined the fraction of viable *Legionella* bioaerosol diameters ranging from 0.26 µm to 2.4 µm as the fraction that can reach the pulmonary alveoli and cause infection.

First, we obtained similar results (by qPCR) between the percentage of bacteria reaching the thoracic region (Table 1) and the percentage of bacteria collected in the DLPI (Table 3): 2.0 ± 0.3% compared to 2.4 ± 0.1% for the jet nebulizer and 11 ± 9.6% compared to 12.8 ± 1.1% for the vibrating-mesh nebulizer (p>0,5 for the two tests).

Second, the results obtained with the jet nebulizer confirmed that (i) VC cells were detected with the 262 nm cut-off diameter and (ii) the *Legionella* physiological states were not modified by the nebulization process or by its transportation on the 12-stage DLPI (same % of VC, VBNC and DC were obtained by FCA – data not shown). The jet nebulizer generated smaller airborne droplets than the vibrating-mesh nebulizer (MMAD of 1.2 ± 0.4 µm for the jet nebulizer *versus* 3.4 ± 0.4 µm for the vibrating-mesh nebulizer), but the same proportions of viable and potentially pathogen airborne bacteria (42 ± 3.2% and 48 ± 5%, respectively) can reach the deep lung.

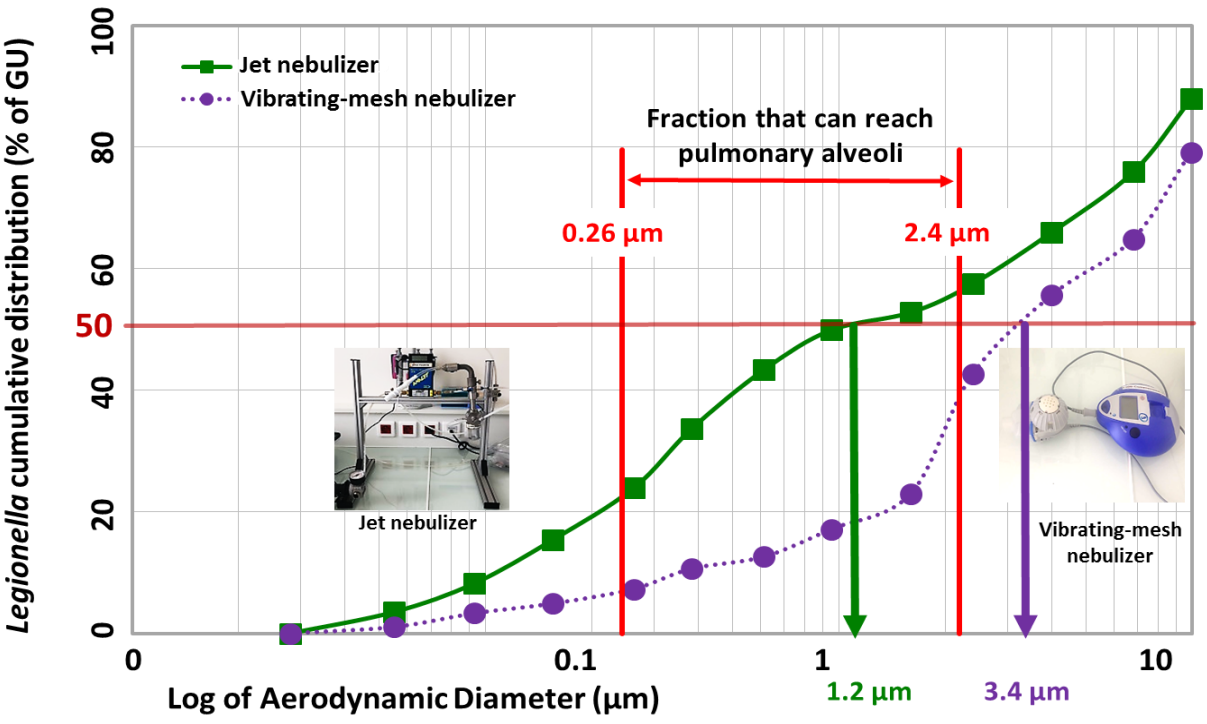


Figure 3 Cumulative distribution of *Legionella* aerosols generated by the 2 types of nebulizers. GU: genomic units. Results from 10 and 30 independent experiments for the jet nebulizer and vibrating-mesh nebulizer, respectively.

Table 3 Characteristics of *Legionella* aerosols generated by the 2 types of nebulizers. GU: genomic units. MMAD: mass median aerodynamic diameter. DLPI: low pressure cascade impactor. Results from 10 and 30 independent experiments for the jet nebulizer and vibrating-mesh nebulizer, respectively.

Aerosols containing Legionella	**Vibrating-mesh nebulizer**	**Jet nebulizer**
MMAD for 50% of GU	3.4 ± 0.4 µm	1.2 ± 0.4 µm
Percentage of aerosols dispersed in DLPI from nebulizer reservoir	12.8 ± 1.1%	2.4 ± 0.1%
Total 12-stage DLPI	100%	100%
Trachea (2.4-10µm)	44.4 ± 4.6%	34 ± 4.3%
The pulmonary region		
Viable bacteria (0.26-2.4µm)	48.3 ± 5%	42.1 ± 3.2%
Cellular fragments (0.03-0.26µm)	7.3 ± 4.5%	23.9 ± 1.1%

Discussion

QMRA provides a very useful methodology to manage the risk of infections by foodborne or some waterborne pathogens with simple cases of contamination. *Legionella* is currently a well-documented human waterborne pathogen, but as shown by Bentham and Whiley (Bentham and Whiley, 2018; Whiley et al., 2014), persistent and indeterminable uncertainties remain regarding *Legionella* epidemiology, frequency of exposure, aerosol characterization (quantity, size, content

of *Legionella*), strain infectivity, infectious dose and detection methods. No risk acceptance threshold is currently validated. However, in the context of global change and diminishing water resources, the need to use various water sources will lead to a need for the development and use of LCA (life cycle assessment) and QMRA methodologies (Harder et al., 2017; National Academies of Sciences and Medicine, 2019). To optimize these risk assessment and prevention methods, our studies using different detection methods of *Legionella* (Allegra et al., 2008; Allegra et al., 2011; Allegra et al., 2011), aimed to characterize the aerosol generation (Allegra et al., 2016; Pourchez et al., 2017) and the inhaled aerosols that can reach pulmonary cells (Perinel et al., 2018). The experimental setup developed in this study proposes a model as close as possible to the human anatomy and physiology, respecting the 3R rule (Replacement, Reduction and Refinement) and consequently does not raise ethical issues. The model is easy to handle and has physiological similarities to human ventilation. Some factors, such as the nasal and bronchial mucosa, the ciliated cells, the secretion of mucus/surfactant and the body temperature, could not be taken into account in the model. Nevertheless, depending on the use of the vibrating-mesh or jet nebulizer, we can adapt the experimental configuration to mimic the exposure to *Legionella* aerosols generated by different shower-like systems.

- Bioaerosol generation from shower-like systems

Regarding aerosol dispersion (quantity, size), the discrepancy between the results from the different studies is due to the different methods and apparatuses used to measure the air bacteria concentration and to numerous other factors, such as ambient humidity, type of shower stall, design of the showerhead, water temperature, piping materials, human occupants or bacterial communities and biofilm development. Several publications (Chattopadhyay et al., 2017; Cowen and Ollison, 2006; Estrada-Perez et al., 2018; O'Toole et al., 2009; Perkins et al., 2009; Zhou et al., 2007) have shown that (i) a shower with the low-flow showerhead generates a significantly higher number of aerosols than a high-flow shower. (ii) A hot shower (approximately

362 40°C) increases the rate of evaporation; therefore, the number of small particles within the
363 breathable range is increased, and the culturability of cells is decreased with the turn off to the
364 VBNC state, as shown for *Pseudomonas* and *Legionella*. (iii) The number of bioaerosols
365 generated during a shower is not only dependent on the concentration of bacteria in the water
366 network. As an example, Zhou et al. (Zhou et al., 2007) demonstrated that the particle size
367 distribution of shower aerosols was only mildly affected by the water flow rate in the case of hot
368 water experiments showing droplet size in the 5 µm range. However, for cold water, the particle
369 distribution varied significantly with the flow rate, and the droplets generated were smaller,
370 approximately 2 µm. The two nebulization technologies used in this study generated similar
371 proportions of viable and potentially pathogenic airborne *Legionella* that could reach the lungs.
372 In addition, the droplet size generated by the vibrating-mesh nebulizer (MMAD of 3.4 µm)
373 appeared to be representative of a shower using lukewarm water. However, the jet nebulizer,
374 which generates smaller airborne droplets (MMAD of 1.2 µm) seems to be representative of a
375 shower using cold water. Moreover, these nebulizers could also mimic the bioaerosol
376 characteristics from new sources of LD, such as a spa (Moore et al., 2015) or bioaerosols
377 generated by wastewater treatment tanks (Wang et al., 2019).
378 Concerning aerosol content, we can identify 4 modes of transportation during the aerosolization
379 of *Legionella* (Allegra et al., 2016): cellular fragments of *Legionella* (entrapped or not) in droplets
380 (28–262 nm), airborne bacteria not entrapped in droplets and oriented by the air flow direction
381 during the respiration process (0.262–0.949 µm), airborne bacteria (entrapped or not) in
382 droplets or in amoeba vacuoles (0.949–1.6 µm), and airborne bacteria packaged in droplets or in
383 amoebae vacuoles (1.6–9.92 µm). VC *Legionella* was also detected as early as the 262 nm cut-off
384 diameter of DLPI, confirming that *Legionella* bacilli have a size of approximately 1–2 µm long and
385 0.3–0.9 µm wide and can reach the deep lung. In the context of this study, we demonstrated a
386 new data that the *Legionella* physiological states were not modified by the nebulization process

or by its transportation through the 12-stage DLPI or the anatomical model. Thermally or chlorine-induced VBNC *Legionella pneumophila* may still infect amoebae and pulmonary cells after resuscitation on amoebae (Cervero-Aragó et al., 2019; Epalle et al., 2015; Mustapha et al., 2015). VBNC cell concentrations in the water network must be included in the QMRA models.

- Exposure and dose-response modelling

There is currently no consensus on a threshold for the acceptability of microbiological risk. The acceptable risk of infection set by the WHO for waterborne pathogens is less than one infection per 10000 persons per year and stipulates that risk assessment be undertaken for waterborne pathogens to demonstrate microbiologically safe water (World Health Organization, 2016). The calculation and the expression of the result of the risk differ according to the authors (Azuma et al., 2013; Bouwknegt et al., 2013; Kerry A. Hamilton et al., 2018; Schoen and Ashbolt, 2011; Sharaby et al., 2019). Indeed, the annual risk estimation varies significantly between the scenario analyses: the dose response model chosen (clinical severity, incidence of LD or/and Pontiac fever, during or after an outbreak, etc.), the population at risk (residential or occupational), the detection method (culture-based, EMA-qPCR, or qPCR), the meteorological conditions, etc.

The exposure assessment (duration, frequency) consists of determining the inhaled quantity of potentially pathogenic *Legionella*. However, the amount of inhaled air will depend on the activity of the subject and the exposure scenario. It is therefore easier to evaluate and model the risk associated with the generation of aerosols by shower-like systems rather than cooling towers. Our study shows similarities and complementarities with 2 other recent studies from Chattopadhyay et al. (Chattopadhyay et al., 2017) and Estrada-Perez et al. (Estrada-Perez et al., 2018) in term of aerosol samplers, Gram-negative bacillus targets, and detection methods. Table 4 shows that the concentrations of *Legionella* aerosols generated by the vibrating-mesh nebulizer (8.9×10^3 CFU.m^3) or by the jet nebulizer (1.4×10^3 CFU.m^3.min^{-1}) in our experimental

setup effectively mimic an emitted dose of shower bacteria. The air bacteria concentrations measured in these three studies were of the same order of magnitude.

Table 4 Comparison of the experimental conditions and results between our study and 2 recent, close and complementary studies.

	Target organisms	C_A: Air bacteria concentration ($CFU.m^{-3}$)	MMAD of bioaerosols
Chattopadhyay et al. 2017	*Brevundimonas diminuta* (10^9 $CFU.L^{-1}$)	Cold: 5.2×10^3 to 1.8×10^4 Cool: 3.8×10^3 to 1.9×10^4 Hot: 7.1×10^2 to 5.7×10^3	[2.6-4.3 µm] Measured by MLI and ACI, respectively
	Pseudomonas aeruginosa (10^9 $CFU.L^{-1}$)	Cold: 68 to 9.5×10^3 Cool: 5.4×10^2 to 6.6×10^4 Hot: 2.2×10^2 to 4.4×10^4	[3.9-2.7 µm] Measured by MLI and ACI, respectively
Estrada-perez et al. 2018	All bacteria present in the residential city water (fluctuate between 4.9×10^4 and 5.7×10^5 during experiment)	6.2 and 3.2×10^3 for low-flow and high-flow showerheads, respectively	[2.7-3.7 µm] for both low-flow and high-flow showerheads
Present study	All bacteria present in the building city water (3.3×10^4 $CFU.L^{-1}$)	2.9×10^3	Not evaluated
	Legionella pneumophila (3.3×10^4 $CFU.L^{-1}$)	(jet nebulizer) 1.4×10^3	1.2 ± 0.4 µm
		(vibrating-mesh nebulizer) 8.9×10^3	3.4 ± 0.4 µm

Studies using animal models or manikins calculate the dose of inhaled *Legionella* from mathematical models. Here, bioaerosols were inhaled by a well-validated 3D replica of the upper airways (Le Guellec et al., 2014; Pourchez et al., 2017). A respiratory pump was used, to fit the breathing parameters corresponding to adult male physiology at rest: a breathing rate of 15 breaths per minute and a tidal volume of 500 mL. The number of viable bacteria reaching the thoracic region represents the dose of inhaled *Legionella* that can result in LD. As observed in Table 1, 4.3 ± 4.0% of the viable (VC + VBNC) *Legionella* aerosolized during the experimentation (experimental setup combined with the vibrating-mesh nebulizer) reached the thoracic region. In the context of the shower used as a reference (water network contaminated by 3.3×10^4 *Legionella*.L^{-1}), 22 viable and potentially pathogenic *Legionella* may reach the pulmonary region after a one-minute shower. Depending on the exposure scenario, we now have several experimental setups to mimic a large range of shower systems, by adjusting the concentration of

bacteria in the water network, the nebulizers settings and the time of experimentation, to provide robust data to scientific experts for QMRA development.

Conclusion

- Viable and cultivable aerosolized *Legionella* bacilli were detected as early as the 262 nm cut-off diameter of DLPI, confirming that *Legionella* bacilli are approximately 1–2 µm long and 0.3–0.9 µm wide and can reach the deep lung.
- The *Legionella* physiological states were not modified by the nebulization process or by its transportation through the 12-stage DLPI or the anatomical model.
- After a one-minute shower (used as a reference in this study), contaminated by 3 x 10^4 *Legionella* cells/L, 22 viable and potentially pathogenic *Legionella* cells may reach the pulmonary region of an adult male.
- The characteristics of the 2 nebulizers can allow the adaptation of our experimental setup to analyse the exposure to different shower-like systems.
- This original experimental setup will provide data to accurately quantify the *Legionella* risk exposure and the dose-response in the context of a *Legionella* infection from a water network to deposition in the lung.

Conflict of interest

The authors declare no conflict of interest.

Funding information

This study was supported by a grant from Jean Monnet University, France.

Acknowledgments

We would like to thank: (i) Dr. C. Ginevra from the French Reference Center for *Legionella* for providing us with the GFP-modified strain. (ii) M. Landraud, P. Bourrassaud, A. Moravia, Z. Reynes, M. Pareux and M. Escot for the validation of the model during their internships and (iii) the teaching laboratory of Technical University Institute of Saint-Etienne for providing the environmental bacteria strains. Finally, we would like to dedicate this paper to the memory of Serge Riffard.

References

Allegra, S., Berger, F., Berthelot, P., Grattard, F., Pozzetto, B., Riffard, S., 2008. Use of flow cytometry to monitor Legionella viability. Appl. Environ. Microbiol. 74, 7813–7816. https://doi.org/10.1128/AEM.01364-08

Allegra, S., Girardot, F., Grattard, F., Berthelot, P., Helbig, J.H., Pozzetto, B., Riffard, S., 2011. Evaluation of an immunomagnetic separation assay in combination with cultivation to improve Legionella pneumophila serogroup 1 recovery from environmental samples. J. Appl. Microbiol. 110, 952–961. https://doi.org/10.1111/j.1365-2672.2011.04955.x

Allegra, Severine, Grattard, F., Girardot, F., Riffard, S., Pozzetto, B., Berthelot, P., 2011. Longitudinal evaluation of the efficacy of heat treatment procedures against Legionella spp. in hospital water systems by using a flow cytometric assay. Appl. Environ. Microbiol. 77, 1268–1275. https://doi.org/10.1128/AEM.02225-10

Allegra, S., Leclerc, L., Massard, P.A., Girardot, F., Riffard, S., Pourchez, J., 2016. Characterization of aerosols containing Legionella generated upon nebulization. Sci. Rep. 6, 33998. https://doi.org/10.1038/srep33998

Armstrong, T. W., Haas, C.N., 2007. A quantitative microbial risk assessment model for Legionnaires' disease: animal model selection and dose-response modeling. Risk Anal. Off. Publ. Soc. Risk Anal. 27, 1581–1596. https://doi.org/10.1111/j.1539-6924.2007.00990.x

Armstrong, Thomas W., Haas, C.N., 2007. Quantitative microbial risk assessment model for Legionnaires' disease: assessment of human exposures for selected spa outbreaks. J. Occup. Environ. Hyg. 4, 634–646. https://doi.org/10.1080/15459620701487539

Azuma, K., Uchiyama, I., Okumura, J., 2013. Assessing the risk of Legionnaires' disease: The inhalation exposure model and the estimated risk in residential bathrooms. Regul. Toxicol. Pharmacol. 65, 1–6. https://doi.org/10.1016/j.yrtph.2012.11.003

Baldovin, T., Pierobon, A., Bertoncello, C., Destefani, E., Gennari, M., Stano, A., Baldo, V., 2018. May car washing represent a risk for Legionella infection? Ann. Ig. Med. Prev. E Comunita 20, 57–65.

Bentham, R., Whiley, H., 2018. Quantitative Microbial Risk Assessment and Opportunist Waterborne Infections¯Are There Too Many Gaps to Fill? Int. J. Environ. Res. Public. Health 15. https://doi.org/10.3390/ijerph15061150

Bouwknegt, M., Schijven, J.F., Schalk, J.A.C., de Roda Husman, A.M., 2013. Quantitative risk estimation for a Legionella pneumophila infection due to whirlpool use. Risk Anal. Off. Publ. Soc. Risk Anal. 33, 1228–1236. https://doi.org/10.1111/j.1539-6924.2012.01909.x

Buse, H.Y., Schoen, M.E., Ashbolt, N.J., 2012. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 46, 921–933. https://doi.org/10.1016/j.watres.2011.12.022

Centers for Disease Control and Prevention, 2018. Legionnaires Disease and Pontiac Fever | Legionella | CDC [WWW Document]. URL https://www.cdc.gov/legionella/index.html (accessed 10.10.18).

Cervero-Aragó, S., Schrammel, B., Dietersdorfer, E., Sommer, R., Lück, C., Walochnik, J., Kirschner, A., 2019. Viability and infectivity of viable but nonculturable Legionella pneumophila strains induced at high temperatures. Water Res. 158, 268–279. https://doi.org/10.1016/j.watres.2019.04.009

Chattopadhyay, S., Perkins, S.D., Shaw, M., Nichols, T.L., 2017. Evaluation of Exposure to Brevundimonas diminuta and Pseudomonas aeruginosa during Showering. J. Aerosol Sci. 114, 77–93. https://doi.org/10.1016/j.jaerosci.2017.08.008

Cowen, K.A., Ollison, W.M., 2006. Continuous monitoring of particle emissions during showering. J. Air Waste Manag. Assoc. 1995 56, 1662–1668.

Durand, M., Pourchez, J., Aubert, G., Le Guellec, S., Navarro, L., Forest, V., Rusch, P., Cottier, M., 2011. Impact of acoustic airflow nebulization on intrasinus drug deposition of a human plastinated nasal cast: new insights into the mechanisms involved. Int. J. Pharm. 421, 63–71. https://doi.org/10.1016/j.ijpharm.2011.09.023

El Merhie, A., Navarro, L., Delavenne, X., Leclerc, L., Pourchez, J., 2015. A new Strategy to Improve Drug Delivery to the Maxillary Sinuses: The Frequency Sweep Acoustic Airflow. Pharm. Res. https://doi.org/10.1007/s11095-015-1851-7

Epalle, T., Girardot, F., Allegra, S., Maurice-Blanc, C., Garraud, O., Riffard, S., 2015. Erratum to: Viable but not Culturable Forms of Legionella pneumophila Generated After Heat Shock Treatment are Infectious for Macrophage-like and Alveolar Epithelial cells after Resuscitation on Acanthamoeba polyphaga. Microb. Ecol. 70, 576–578. https://doi.org/10.1007/s00248-015-0580-0

Estrada-Perez, C.E., Kinney, K.A., Maestre, J.P., Hassan, Y.A., King, M.D., 2018. Droplet distribution and airborne bacteria in an experimental shower unit. Water Res. 130, 47–57. https://doi.org/10.1016/j.watres.2017.11.039

Gradon, L., Marijnissen, J.C., 2003. Optimization of Aerosol Drug Delivery. Springer Science & Business Media.

Hamilton, K.A., Haas, C.N., 2016. Critical review of mathematical approaches for quantitative microbial risk assessment (QMRA) of Legionella in engineered water systems: research gaps and a new framework. Environ. Sci. Water Res. Technol. 2, 599–613. https://doi.org/10.1039/C6EW00023A

Hamilton, Kerry A., Hamilton, M.T., Johnson, W., Jjemba, P., Bukhari, Z., LeChevallier, M., Haas, C.N., 2018. Health risks from exposure to Legionella in reclaimed water aerosols: Toilet flushing, spray irrigation, and cooling towers. Water Res. 134, 261–279. https://doi.org/10.1016/j.watres.2017.12.022

Hamilton, K. A., Prussin, A.J., Ahmed, W., Haas, C.N., 2018. Outbreaks of Legionnaires' Disease and Pontiac Fever 2006-2017. Curr. Environ. Health Rep. 5, 263–271. https://doi.org/10.1007/s40572-018-0201-4

Harder, R., Peters, G.M., Ashbolt, N.J., Svanström, M., 2017. Using quantitative microbial risk assessment and life cycle assessment to assess management options in urban water and sanitation infrastructures: Opportunities and unresolved issues. Microb. Risk Anal., Quantitative Microbial Risk Assessment of Reclaimed Water 5, 71–77. https://doi.org/10.1016/j.mran.2016.11.004

InVS, 2018. Bilan des cas de légionellose survenus en France en 2017. [WWW Document]. URL http://invs.santepubliquefrance.fr/Dossiers-thematiques/Maladies-

infectieuses/Infections-respiratoires/Legionellose/Donnees/Bilan-des-cas-de-legionellose-survenus-en-France-en-2017 (accessed 8.7.18).
Le Guellec, S., Le Pennec, D., Gatier, S., Leclerc, L., Cabrera, M., Pourchez, J., Diot, P., Reychler, G., Pitance, L., Durand, M., Jamar, F., Vecellio, L., 2014. Validation of anatomical models to study aerosol deposition in human nasal cavities. Pharm. Res. 31, 228–237. https://doi.org/10.1007/s11095-013-1157-6
Leclerc, L., Merhie, A.E., Navarro, L., Prévôt, N., Durand, M., Pourchez, J., 2015. Impact of acoustic airflow on intrasinus drug deposition: New insights into the vibrating mode and the optimal acoustic frequency to enhance the delivery of nebulized antibiotic. Int. J. Pharm. 494, 227–234. https://doi.org/10.1016/j.ijpharm.2015.08.025
Leclerc, L., Pourchez, J., Prevot, N., Vecellio, L., Le Guellec, S., Cottier, M., Durand, M., 2014. Assessing sinus aerosol deposition: benefits of SPECT-CT imaging. Int. J. Pharm. 462, 135–141. https://doi.org/10.1016/j.ijpharm.2013.12.032
Loenenbach, A.D., Beulens, C., Euser, S.M., van Leuken, J.P.G., Bom, B., van der Hoek, W., Husman, A.M. de R., Ruijs, W.L.M., Bartels, A.A., Rietveld, A., den Boer, J.W., Brandsema, P.S., 2018. Two Community Clusters of Legionnaires' Disease Directly Linked to a Biologic Wastewater Treatment Plant, the Netherlands. Emerg. Infect. Dis. 24, 1914–1918. https://doi.org/10.3201/eid2410.180906
Moore, G., Hewitt, M., Stevenson, D., Walker, J.T., Bennett, A.M., 2015. Aerosolization of respirable droplets from a domestic spa pool and the use of MS-2 coliphage and Pseudomonas aeruginosa as markers for Legionella pneumophila. Appl. Environ. Microbiol. 81, 555–561. https://doi.org/10.1128/AEM.02912-14
Mustapha, P., Epalle, T., Allegra, S., Girardot, F., Garraud, O., Riffard, S., 2015. Monitoring of Legionella pneumophila viability after chlorine dioxide treatment using flow cytometry. Res. Microbiol. 166, 215–219. https://doi.org/10.1016/j.resmic.2015.01.004
National Academies of Sciences, Engineering, Medicine, 2019. Management of Legionella in Water Systems. The National Academies Press, Washington, DC. https://doi.org/10.17226/25474
NF EN 13544-1, 2002. Respiratory therapy equipment - Part 1 : nebulizing systems and their components. [WWW Document]. URL https://www.boutique.afnor.org/norme/nf-en-13544-1/materiel-respiratoire-therapeutique-partie-1-systemes-de-nebulisation-et-leurs-composants/article/855050/fa029640 (accessed 11.24.19).
O'Toole, J., Keywood, M., Sinclair, M., Leder, K., 2009. Risk in the mist? Deriving data to quantify microbial health risks associated with aerosol generation by water-efficient devices during typical domestic water-using activities. Water Sci. Technol. J. Int. Assoc. Water Pollut. Res. 60, 2913–2920. https://doi.org/10.2166/wst.2009.722
Pepper, I.L., Gerba, C.P., 2018. Risk of infection from Legionella associated with spray irrigation of reclaimed water. Water Res. 139, 101–107. https://doi.org/10.1016/j.watres.2018.04.001
Perinel, S., Forest, V., Landraud, M., Pourchez, J., Girardot, F., Riffard, S., Stauffert, M., Vergnon, J.-M., Allegra, S., 2018. Deposition pattern of aerosolized Legionella using an ex vivo human-porcine respiratory model. Int. J. Hyg. Environ. Health 221, 252–259. https://doi.org/10.1016/j.ijheh.2017.11.002
Perkins, S.D., Mayfield, J., Fraser, V., Angenent, L.T., 2009. Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. Appl. Environ. Microbiol. 75, 5363–5372. https://doi.org/10.1128/AEM.00658-09
Pourchez, J., Albuquerque-Silva, I.M.D., Cottier, M., Clotagatide, A., Vecellio, L., Durand, M., Dubois, F., 2013. Generation and characterization of radiolabelled nanosized carbonaceous aerosols for human inhalation studies. J. Aerosol Sci. 55, 1–11. https://doi.org/10.1016/j.jaerosci.2012.07.011

595 Pourchez, J., Leclerc, L., Girardot, F., Riffard, S., Prevot, N., Allegra, S., 2017. Experimental
596 human-like model to assess the part of viable Legionella reaching the thoracic region
597 after nebulization. PloS One 12, e0186042.
598 https://doi.org/10.1371/journal.pone.0186042
599 Robertson, P., Abdelhady, H., Garduño, R.A., 2014. The many forms of a pleomorphic bacterial
600 pathogen-the developmental network of Legionella pneumophila. Front. Microbiol. 5,
601 670. https://doi.org/10.3389/fmicb.2014.00670
602 Schlesinger, R.B., 1989. Deposition and clearance of inhaled particles, in: R.O. McClellan, R.F.
603 Henderson (Eds.), Concepts in Inhalation Toxicology. Hemisphere Publishing Corp, New
604 York, pp. 163–192.
605 Schoen, M.E., Ashbolt, N.J., 2011. An in-premise model for Legionella exposure during showering
606 events. Water Res. 45, 5826–5836. https://doi.org/10.1016/j.watres.2011.08.031
607 Sharaby, Y., Rodríguez-Martínez, S., Höfle, M.G., Brettar, I., Halpern, M., 2019. Quantitative
608 microbial risk assessment of Legionella pneumophila in a drinking water supply system in
609 Israel. Sci. Total Environ. 671, 404–410. https://doi.org/10.1016/j.scitotenv.2019.03.287
610 Valero, N., de Simón, M., Gallés, P., Izquierdo, N., Arimon, J., González, R., Manzanares-Laya, S.,
611 Avellanes, I., Gómez, A., 2017. Street Cleaning Trucks as Potential Sources of Legionella
612 pneumophila. Emerg. Infect. Dis. 23, 1880–1882.
613 https://doi.org/10.3201/eid2311.161390
614 Wang, Y., Li, L., Xue, S., Han, Y., Yang, K., 2019. Characteristics and formation mechanism of
615 intestinal bacteria particles emitted from aerated wastewater treatment tanks. Water
616 Res. 163, 114862. https://doi.org/10.1016/j.watres.2019.114862
617 Whiley, H., Keegan, A., Fallowfield, H., Ross, K., 2014. Uncertainties associated with assessing the
618 public health risk from Legionella. Front. Microbiol. 5, 501.
619 https://doi.org/10.3389/fmicb.2014.00501
620 World Health Organization, 2016. Quantitative microbial risk assessment: application for water
621 safety management. Geneva Swiitzerland WHO.
622 Zhou, Y., Benson, J.M., Irvin, C., Irshad, H., Cheng, Y.-S., 2007. Particle size distribution and
623 inhalation dose of shower water under selected operating conditions. Inhal. Toxicol. 19,
624 333–342. https://doi.org/10.1080/08958370601144241
625