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

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22 **Abstract**

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

44

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

46

48 **Introduction**

49 Members of the genus *Legionella* (Gram negative bacilli) are ubiquitous in natural and
50 anthropogenic aquatic ecosystems. These bacteria are responsible for severe pneumonia, which
51 may be fatal in 30% of cases when considering nosocomial infections. *L. pneumophila* is, by far,
52 the most frequent species associated with Legionnaires' disease (LD). *Legionella* is now the
53 number one cause of reported waterborne disease in the United States. The latest
54 epidemiological data showed an increase in LD worldwide (Centers for Disease Control and
55 Prevention, 2018; InVS, 2018) with the identification of new sources of contamination by
56 *Legionella* aerosol dispersion (*i.e.*, car washing stations (Baldovin et al., 2018), street cleaning
57 trucks (Valero et al., 2017), aerosols from biologic wastewater treatment plants (Loenenbach et
58 al., 2018), reclaimed water used for spray irrigation (Pepper and Gerba, 2018), etc.). To assess
59 the risk of infection of LD, researchers have used the quantitative microbial risk assessment
60 (QMRA) method to provide models for aerosol dispersion (Thomas W. Armstrong and Haas,
61 2007; T. W. Armstrong and Haas, 2007; Buse et al., 2012; Hamilton and Haas, 2016; Schoen and
62 Ashbolt, 2011). However, these risk models are only based (i) on the fluidic characteristics of
63 water systems that could generate bioaerosols and (ii) on the infectious doses extrapolated to
64 humans from animal experiments using inhalation, intraperitoneal injections or tracheal
65 instillation for infection of the animal model.

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

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

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

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

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

104

105 **Materials and methods**

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

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

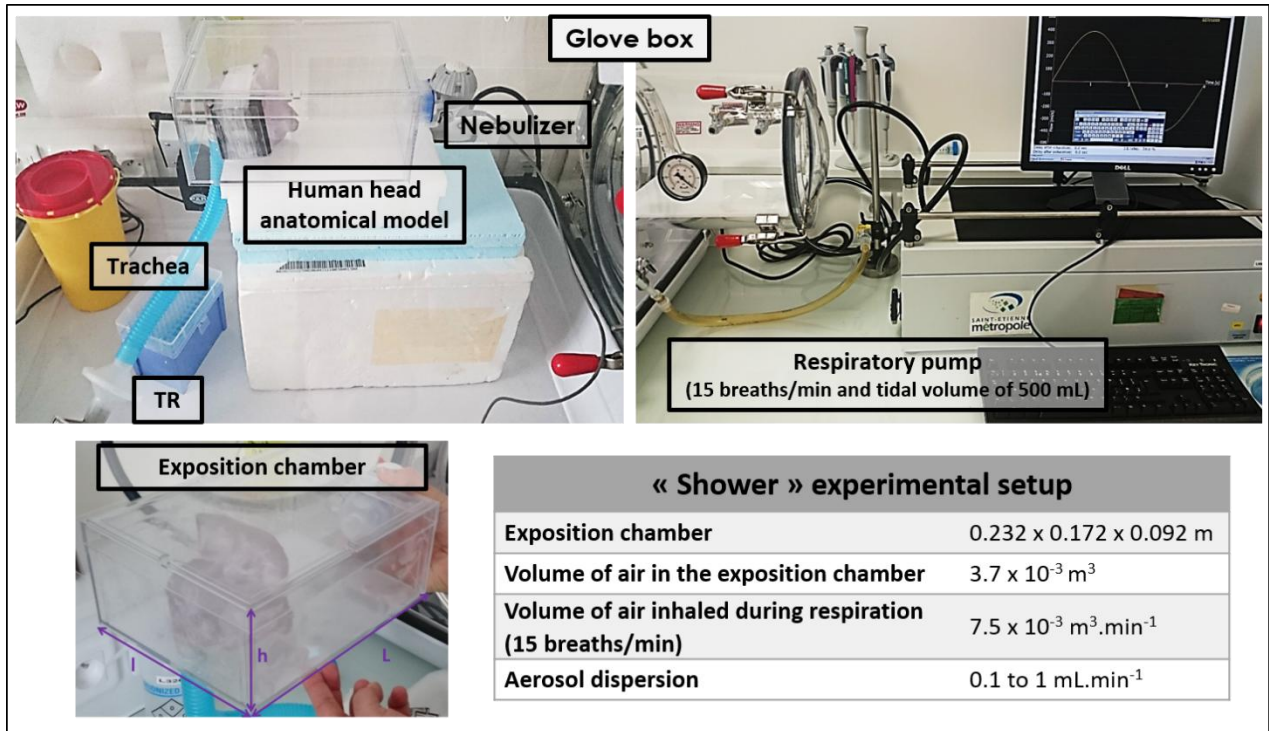
110 Three independent experiments were carried out. The concentration of bacteria in the water
111 network was determined by sampling 1 litre of water from the first flow and 1 litre during a
112 thirty-minute showering event respectively at 10, 20 and 30 minutes. The quantity of bioaerosols
113 dispersed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

120

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

122 A previously validated and original respiratory model was used in this study (Pourchez et al.,
123 2017). The setup was composed of a human 3D replica corresponding to the entire nasal cavities
124 (nasal fossae and frontal, ethmoid and maxillary sinuses) (El Merhie et al., 2015; Le Guellec et al.,
125 2014). This replica was manufactured in transparent water-resistant and non-porous resin.
126 Qualitative analyses with endoscopy and CT-scans were performed on the replica to assess the
127 anatomical reproducibility (Durand et al., 2011). As shown in Figure 1, this human upper airway
128 replica was placed in an exposition chamber allowing aerosol dispersion between 0.6 and 0.9
129 mL.min⁻¹ of the calibrated suspensions. This replica was linked to a pump (model 420-2902 – 230
130 VAC, 50 Hz, 0.6 Amp, 1 PH, 0.02 HP, Thermo Scientific) to inhale the aerosol suspended in the
131 exposition chamber. The thoracic region (TR) was simulated by a filter holder (filter kit and filter
132 pad, PARI, PulmoMed) with a polycarbonate membrane (Nuclepore™ track-etched membrane
133 filter, Whatman, GE Healthcare) that allowed optimal bacterial recovery. To avoid any bacterial
134 contamination of the pump, a HEPA filter (Pall filter BB50TE, Pall Medical) was placed after the
135 thoracic filters. Five millilitres and 10 mL of the *Legionella* calibrated suspensions were nebulized
136 in the respiratory model by a vibrating-mesh nebulizer or jet nebulizer, respectively. For safety
137 reasons, the experimental setup was placed in a glove box (815-PGB “La petite glove box”, Fisher
138 Scientific). For quantification of the percentage of droplets reaching the filter holder, the
139 polycarbonate membrane was weighed (1 g = 1 mL of water) before and after nebulization. After
140 nebulization, the polycarbonate membrane was placed in 5 mL of physiological sterile water.
141 After a 30 sec sonication (Bransonic 32 sonicator bath, Branson Instruments, Danbury, CT USA),
142 the membrane was scraped using an automatic pipette tip. One millilitre was plated twice on
143 adapted culture media to count the viable and culturable (VC) *Legionella*. One millilitre was
144 placed in a tube for DNA extraction and qPCR analysis. One millilitre was placed in a flow
145 cytometric tube for flow cytometry assays (FCAs).

146



147
 148 **Figure 1** Human-like shower experimental setup inside a glove box. This experimental setup
 149 mimics the dispersion and collection of *Legionella* aerosols under the breathing conditions of an
 150 adult male. TR: Thoracic region mimicked by a polycarbonate membrane inside a filter holder.

151
 152 *Legionella* strain, culture and flow cytometry for the nebulization process

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

162 The culturability (determination of the number of viable and culturable *Legionella* - VC) was
163 assessed in duplicate by plating on BCYE agar, supplemented with chloramphenicol, 100 µL and
164 500 µL of the suspension retrieved from the polycarbonate membrane. After incubation for 72 h
165 at 37°C, all the BCYE plates were observed under UV at 366 nm to quantify the *Legionella*
166 colonies (CFU) expressing GFP.

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

177

178 Quantification by qPCR

179 The total number of bacteria in the shower water network and in bioaerosols was quantified
180 using the 16S RNA gene as a target. Each reaction tube contained 5 µL of DNA extract, 2.5 µL of
181 each primer at 10 pmol/µL (5'-AGG GTT GAT AGG TTA AGA GC-3' and 5'-CCA ACA GCT AGT TGA
182 CAT CG-3') and 10 µL of the 2X Power SYBR® Green PCR Master Mix (Life Technologies). The
183 standard curve was established using a mix of *Escherichia coli*, *Pseudomonas aeruginosa*,
184 *Staphylococcus aureus* and the *L. pneumophila* sg1 strain isolated from water (strain collections
185 of the teaching laboratory of Technical University Institute of Saint-Etienne). A suspension of an
186 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 μ L 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 μ L). The forward and reverse primers were
193 5'-AGAGTGCCATGCCCCGAAGG -3' and 5'-AAGGACAGGGCCATCGCCAA-3', respectively (2.5 μ L of
194 each primer at 10 pmol/ μ L). 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 2.0×10^7 to 2.0×10^1 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 Biological characterization and aerodynamic features of aerosols generated by a jet nebulizer

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

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

231
232 **Results**
233 Quantification of the exposure to airborne bacteria during one minute in the shower as a
234 reference

235 The technical features of the “real” shower are presented in Figure 2. As determined by qPCR,
 236 the total bacteria concentration in the water network was $3.3 \pm 1.0 \times 10^4$ bacteria.L⁻¹. Assuming
 237 that the aerosols are evenly distributed in the shower space, when extrapolating the numbers,
 238 we found that the mean number of bacteria collected per minute by the Coriolis® Delta air
 239 sampler was $8.6 \pm 3.2 \times 10^2$. As the shower cubicle has a volume of 1.125 m³, the Coriolis®
 240 system (300 L.min⁻¹) can collect 26.7% of the air per minute. The actual number of bacteria per
 241 minute in the shower cubicle was therefore 3.2×10^3 , and the number of aerosolized bacteria in
 242 the shower cubicle was 2.9×10^3 bacteria.m³. The volume of air inhaled by an adult male at rest
 243 is 7.5×10^{-3} m³.min⁻¹. Therefore, in the specific case studied, we can estimate that a human male
 244 at rest inhales approximately 22 bacteria per minute during a shower.
 245



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 ± 1.0 x 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 ± 3.2 x 10 ² bacteria.min ⁻¹
C _A : bacterial air concentration in shower cubicle	2.9 x 10 ³ bacteria.m ⁻³
Flowrate of air inhaled during respiration (15 breaths/min)	7.5 x 10 ⁻³ m ³ .min ⁻¹
Number of inhaled bacteria	22 bacteria.min ⁻¹

246
 247 **Figure 2** Summary of the experimental and calculated data from shower analysis. The number of
 248 inhaled bacteria was calculated based on results obtained by qPCR under the breathing
 249 conditions of an adult male and extrapolated from the characteristics of the shower and Coriolis
 250 air sampler. The results are from 3 independent experiments.

251

252 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 $1.00 \pm 0.25 \text{ mL}\cdot\text{min}^{-1}$ and $0.16 \pm 0.06 \text{ mL}\cdot\text{min}^{-1}$,
260 respectively. As determined by qPCR, only $2.0 \pm 0.3\%$ of the total *Legionella* DNA dispersed from
261 the jet nebulizer could reach the thoracic region compared to $11 \pm 9.6\%$ 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 $11 \pm 9.6\%$ of the total aerosolized *Legionella* DNA could reach the thoracic region. From the
268 culture results, only $0.5 \pm 0.7\%$ 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 $4.3 \pm 4.0\%$ 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

279

280 Comparison of the exposure to bacteria

281 To determine if the shower-like experimental setup satisfactorily mimics a bioaerosol shower
 282 exposure, we compared the number of inhaled bacteria for a one-minute shower event (Table
 283 2). As we used the “real” shower as a reference, the number of bacteria in the nebulizer’s
 284 reservoir (5 mL or 10 mL for the vibrating-mesh and jet nebulizer, respectively) was fixed at $3.3 \times$
 285 10^4 bacteria. The results in Table 2 indicate that the experimental setup developed
 286 underestimates by 2 times (if the jet nebulizer is used) or overestimates by 43 times (if the
 287 vibrating-mesh nebulizer is used) the emitted dose of total (VC, VBNC and DC) airborne bacteria.

288

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

292

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

	Total	Vibrating-mesh nebulizer			Jet nebulizer
		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

293

294 Features of the aerosols generated by nebulization

295 The aerodynamic features of the aerosols generated by the jet nebulizer were compared to
296 those of the vibrating-mesh nebulizer that were previously determined (Allegra et al., 2016).

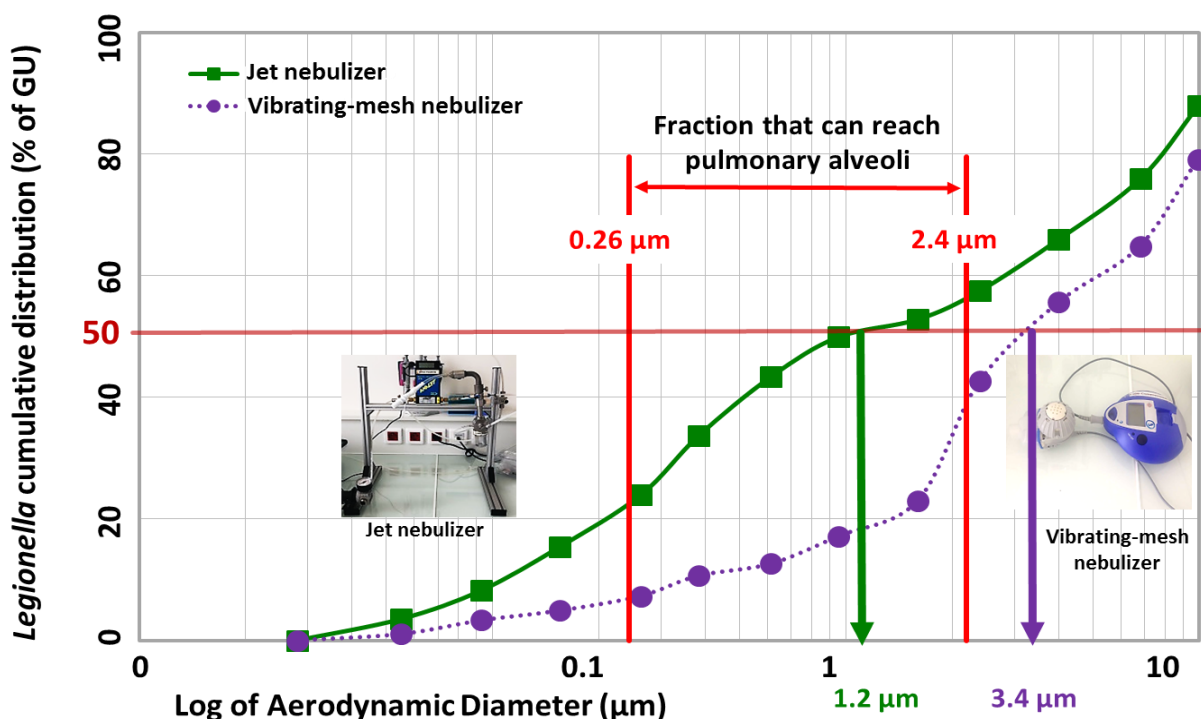
297 Figure 3 shows the repartition of *Legionella* depending on the aerosol size. Table 3 presents the
298 proportions of viable and potentially pathogenic airborne bacteria. Both nebulizer profiles were
299 examined for the aerosol fraction that could reach the thoracic region (0.26 µm to 2.4 µm).

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

308 First, we obtained similar results (by qPCR) between the percentage of bacteria reaching the
309 thoracic region (Table 1) and the percentage of bacteria collected in the DLPI (Table 3): $2.0 \pm$
310 0.3% compared to $2.4 \pm 0.1\%$ for the jet nebulizer and $11 \pm 9.6\%$ compared to $12.8 \pm 1.1\%$ for the
311 vibrating-mesh nebulizer ($p > 0,5$ for the two tests).

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

319



320

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

324
 325 **Table 3** Characteristics of *Legionella* aerosols generated by the 2 types of nebulizers. GU:
 326 genomic units. MMAD: mass median aerodynamic diameter. DLPI: low pressure cascade
 327 impactor. Results from 10 and 30 independent experiments for the jet nebulizer and vibrating-
 328 mesh nebulizer, respectively.

329

<i>Aerosols containing Legionella</i>	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%

330

331 Discussion

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

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

- 353 • Bioaerosol generation from shower-like systems

354 Regarding aerosol dispersion (quantity, size), the discrepancy between the results from the
355 different studies is due to the different methods and apparatuses used to measure the air
356 bacteria concentration and to numerous other factors, such as ambient humidity, type of shower
357 stall, design of the showerhead, water temperature, piping materials, human occupants or
358 bacterial communities and biofilm development. Several publications (Chattopadhyay et al.,
359 2017; Cowen and Ollison, 2006; Estrada-Perez et al., 2018; O'Toole et al., 2009; Perkins et al.,
360 2009; Zhou et al., 2007) have shown that (i) a shower with the low-flow showerhead generates a
361 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

387 or by its transportation through the 12-stage DLPI or the anatomical model. Thermally or
388 chlorine-induced VBNC *Legionella pneumophila* may still infect amoebae and pulmonary cells
389 after resuscitation on amoebae (Cervero-Aragó et al., 2019; Epalle et al., 2015; Mustapha et al.,
390 2015). VBNC cell concentrations in the water network must be included in the QMRA models.

391 • Exposure and dose-response modelling

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

402 The exposure assessment (duration, frequency) consists of determining the inhaled quantity of
403 potentially pathogenic *Legionella*. However, the amount of inhaled air will depend on the activity
404 of the subject and the exposure scenario. It is therefore easier to evaluate and model the risk
405 associated with the generation of aerosols by shower-like systems rather than cooling towers.
406 Our study shows similarities and complementarities with 2 other recent studies from
407 Chattopadhyay et al. (Chattopadhyay et al., 2017) and Estrada-Perez et al. (Estrada-Perez et al.,
408 2018) in term of aerosol samplers, Gram-negative bacillus targets, and detection methods. Table
409 4 shows that the concentrations of *Legionella* aerosols generated by the vibrating-mesh
410 nebulizer (8.9×10^3 CFU.m³) or by the jet nebulizer (1.4×10^3 CFU.m³.min⁻¹) in our experimental

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

413
 414 **Table 4** Comparison of the experimental conditions and results between our study and 2 recent,
 415 close and complementary studies.

	Target organisms	C _A : Air bacteria concentration (CFU.m ⁻³)	MMAD of bioaerosols
Chattopadhyay et al. 2017	<i>Brevundimonas diminuta</i> (10 ⁹ CFU.L ⁻¹)	Cold: 5.2 x 10 ³ to 1.8 x 10 ⁴ Cool: 3.8 x 10 ³ to 1.9 x 10 ⁴ Hot: 7.1 x 10 ² to 5.7 x 10 ³	[2.6-4.3 μm] Measured by MLI and ACI, respectively
	<i>Pseudomonas aeruginosa</i> (10 ⁹ CFU.L ⁻¹)	Cold: 68 to 9.5 x 10 ³ Cool: 5.4 x 10 ² to 6.6 x 10 ⁴ Hot: 2.2 x 10 ² to 4.4 x 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 x 10 ⁴ and 5.7 x 10 ⁵ during experiment)	6.2 and 3.2 x 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 x 10 ⁴ CFU.L ⁻¹)	2.9 x 10 ³	Not evaluated
	<i>Legionella pneumophila</i> (3.3 x 10 ⁴ CFU.L ⁻¹)	(jet nebulizer) 1.4 x 10 ³ (vibrating-mesh nebulizer) 8.9 x 10 ³	1.2 ± 0.4 μm 3.4 ± 0.4 μm

416
 417 Studies using animal models or manikins calculate the dose of inhaled *Legionella* from
 418 mathematical models. Here, bioaerosols were inhaled by a well-validated 3D replica of the upper
 419 airways (Le Guellec et al., 2014; Pourchez et al., 2017). A respiratory pump was used, to fit the
 420 breathing parameters corresponding to adult male physiology at rest: a breathing rate of 15
 421 breaths per minute and a tidal volume of 500 mL. The number of viable bacteria reaching the
 422 thoracic region represents the dose of inhaled *Legionella* that can result in LD. As observed in
 423 Table 1, 4.3 ± 4.0% of the viable (VC + VBNC) *Legionella* aerosolized during the experimentation
 424 (experimental setup combined with the vibrating-mesh nebulizer) reached the thoracic region. In
 425 the context of the shower used as a reference (water network contaminated by 3.3 x 10⁴
 426 *Legionella*.L⁻¹), 22 viable and potentially pathogenic *Legionella* may reach the pulmonary region
 427 after a one-minute shower. Depending on the exposure scenario, we now have several
 428 experimental setups to mimic a large range of shower systems, by adjusting the concentration of

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

431

432 **Conclusion**

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

446

447 **Conflict of interest**

448 The authors declare no conflict of interest.

449

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460

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