

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/131178/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Rubiano, M.E., Maillard, J.-Y., Rubino, J.R. and Ijaz, M.K. 2020. Use of a small-scale, portable test chamber for determining the bactericidal efficacy of aerosolized glycol formulations. *Letters in Applied Microbiology* 70 (5), pp. 356-364.
10.1111/lam.13289

Publishers page: <http://dx.doi.org/10.1111/lam.13289>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



1 **Use of a small-scale, portable test chamber for determining the**
2 **bactericidal efficacy of aerosolized glycol formulations**

3

4 ¹Rubiano ME and ¹Maillard J-Y*, ²Rubino JR, and ^{2,3}Ijaz MK

5

6 Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University,
7 Cardiff, UK

8 ²Research & Development, RB, Montvale, New Jersey, USA, and

9 ³Department of Biology, Medgar Evers College of the City University of New
10 York (CUNY), Brooklyn, NY, USA

11

12 * Corresponding author

13 Jean-Yves Maillard

14 Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University,
15 Redwood Building, King Edward VII avenue, Cardiff CF10 3NB, UK

16 Tel: +44(0)2920879088

17 Email: maillardj@cardiff.ac.uk

18

19 Running title: Antimicrobial activity of aerosolised glycols

20

21 Keywords: aerosolisation, glycols, bactericidal

22

23

24 **Significance and Impact of the study:** There is an increased interest in
25 developing effective microbicidal aerosolised formulations. The development of
26 a small in-house test chamber allowed the measurement of the microbicidal
27 efficacy of an aerosolised glycol/ethanol formulation at a low cost. We showed
28 that a glycol/ethanol aerosolised formulation caused extensive structural
29 damage in Gram-negative and -positive bacteria resulting in a 3 log₁₀ reduction
30 in viability.

31

32 **Abstract**

33 This study aimed to understand the efficacy and mechanisms of action of an
34 aerosolised glycol-ethanol formulations against bacteria. We validated a small
35 scale in-house test chamber to determine the microbicidal efficacy of four
36 aerosolized formulations combining dipropylene glycol and ethanol against
37 *Staphylococcus aureus* and *Escherichia coli* embedded in alginate. The
38 aerosolised glycol/ethanol formulation decreased bacterial viability by 3 Log₁₀
39 and was more efficacious than an ethanol only control formulation. Electron
40 microscopic examination indicated extensive structural damage in both bacteria,
41 and membrane damage was confirmed with potassium release in *S. aureus*
42 and DNA release in *E. coli*. The development of a small test chamber facilitated
43 the measurement of the microbicidal efficacy and experiments to understand
44 the mechanism of action of an aerosolised microbicidal formulation.

45

46

47 **Introduction**

48 Pathogens discharged into the air may settle on environmental surfaces, which
49 could then become secondary vehicles for the spread of infectious agents
50 indoors occurring at the air-surface-air nexus (Gralton *et al.*, 2011; Ijaz *et al.*,
51 2016). Considering the concern about the potential spread of microorganisms
52 indoors and the limited techniques for testing new formulations, Sattar and
53 colleagues (2016) designed a protocol to study survival and inactivation of
54 human pathogens in indoor air. These authors tested the efficacy of a
55 microbicidal formulation containing 4% dipropylene glycol (DPG) and 35%
56 ethanol distributed within a 24.3 m³ aerobiology chamber using an air purifier
57 with a newly-designed fogger (Sattar *et al.* unpublished data).

58 The study of human pathogens aerosols requires the ability to produce them
59 experimentally in appropriate droplet size and sample them safely for analysis
60 over a predetermined time periods (Sattar and Ijaz, 1987). Since the recovery
61 of aerosolised bacterial inocula from large-scale microbicide experiments
62 presents several technical challenges, the immobilization of bacteria in an
63 alginate support may provide an appropriate alternative (Shackelford *et al.*,
64 2006). The alginate matrix provides sufficiently large pores to allow the easy
65 penetration of microbicide and may enable testing of microbicidal efficacy
66 without the confounding effects of bacterial aerosol-induced cell injury or drying
67 (Tattawasart *et al.*, 2000a; Shackelford *et al.*, 2006) or aerosolising bacteria
68 with the negative effects of aerosolising process on bacterial viability (O'Jeil *et al.*,
69 2013). In addition, the use of an alginate matrix allows recovering bacteria
70 in high number for the study of mechanisms of action (Tattawasart *et al.*, 2000a;
71 Shackelford *et al.*, 2006). Glycols such as propylene glycol (PG) and triethylene

72 glycol (TEG) are greatly used in formulations, generally as solvent, extractant
73 and on occasions as preservatives at concentrations 15-30% (Rowe *et al.*,
74 2009). The microbicidal activity of glycols has however not been widely
75 reported.

76 The aim of this study was to measure the bactericidal efficacy of several
77 aerosolised glycol-based formulations and their mechanisms of microbicidal
78 action using a small scale aerosolisation test chamber.

79

80 **Results and discussion**

81

82 There is a great interest in gaseous and air decontamination technologies
83 notably in healthcare settings (Davies *et al.*, 2011). The use of highly reactive
84 chemistry such as vaporized hydrogen peroxide has been successful in
85 controlling pathogen outbreaks in healthcare environments (Ray *et al.*, 2010;
86 Goyal *et al.*, 2014). Aerosolised chlorine-based microbicides have also been
87 explored against *Staphylococcus aureus* with various degrees of efficacy
88 (Thorn *et al.*, 2015). However, these highly reactive chemistries are toxic and
89 the room to be decontaminated needs to be vacated and sealed during the
90 gaseous process. In addition, testing the efficacy of aerosolised formulations is
91 expensive and require the use of specifically designed aerobiology test
92 chambers (Sattar *et al.*, 2016). Here, we successfully developed a small scale
93 test chamber to study the efficacy and mechanisms of action of aerosolised
94 formulations. The combination of the chamber with an immobilised bacterial
95 inoculum allowed the recovery of a high bacterial inoculum facilitating the study
96 of the formulations' mechanisms of action.

97

98 *Effect of liquid formulations on bacterial growth*

99 We are not aware of any scientific publications describing of the microbicidal
100 activity of dipropylene glycol. Our results show that the test formulations,
101 including dipropylene glycol only (6.67%) inhibited the growth of the test
102 bacteria. All liquid formulations affected bacterial growth in comparison to the
103 TSB positive growth control (Figures 2 and 3). The formulations containing
104 glycol, or ethanol or a combination of glycol and ethanol had a more
105 pronounced effect inhibiting completely the growth of both bacteria comparing
106 to the blank formulation that affected bacterial growth rate and final OD value
107 (Figures 2 and 3).

108

109 *Bactericidal efficacy of aerosolised formulations*

110 Percentage recoveries of bacteria embedded in the alginate were 90.8% and
111 91.63% for *E. coli* and *S. aureus*, respectively. The use of the fogger
112 formulations containing glycol in our test chamber (10 sec aerosol exposure,
113 40-60% RH) produced a reduction in bacterial viability. However, the complete
114 formulation (Glycol + Ethanol) was significantly (ANOVA; $P < 0.001$) more
115 efficacious with a reduction of $3.20 \pm 0.13 \text{ Log}_{10}$ for *S. aureus aureus* and 3.19
116 $\pm 0.39 \text{ Log}_{10} \text{ cfu ml}^{-1}$ for *E. coli* compared to 1.54 ± 0.31 and 1.41 ± 0.15 with
117 each bacterium respectively (Table 3). There were no statistically significant
118 differences (ANOVA; $P > 0.1$) between the efficacy of the glycol only, the ethanol
119 only formulations and the blank formulation (Table 3). The use of chlorine-
120 based aerosols (with 20 min aerosol exposure, 10 min resting time, and 50%RH)
121 produced 1-5 \log_{10} reduction in *S. aureus* concentration on stainless steel

122 surfaces depending on the initial chlorine solution (sodium hypochlorite,
123 chlorine dioxide or electrochemically activated solution) (Thorn et al., 2013).
124 Gaseous chlorine (250 mg l⁻¹) has been used *in situ* to decontaminate indoor
125 air pathogens and contributed to a reduction <1500 cfu m³ in bacteria and of
126 <1000 cfu m³ in fungi (Hsu et al., 2015).

127 The bactericidal activity of other glycols has been reported. Berry (1944)
128 reported MIC ethylene glycol monophenyl ether of 0.8% v/v against *S. aureus*
129 and 0.5 % v/v against *E. coli* while a number of chloro-and methyl-substituted
130 aryl ethers of glycerol, propylene glycol and trimethylene glycol were shown to
131 have some activity against bacteria, fungi and yeast (Berger et al., 1953).
132 Propylene glycol has been used as a solvent in combination with phenol with
133 report of some bactericidal activity (Baker and Twort, 1941). It is however clear
134 that RH played an important role in the activity of the formulation (Baker and
135 Twort, 1941). Chirife and colleagues (1983) studied the microbicidal activity of
136 polyethylene glycol 400 and suggested that the bactericidal activity observed
137 at 35°C was a combination of lowering water activity and a direct effect of
138 bacterial cells demonstrated by cell clumping. Recently ethylene glycol
139 bactericidal activity was reported against *E. coli* with MIC and MBC values of
140 18 and 24 % v/v (Moghayedi et al. 2017).

141

142 *Mechanisms of bactericidal action of aerosolised glycol formulations*

143 The mechanisms of action of the aerosolised formulations were explored. It was
144 hypothesized that the bactericidal activity observed resulted from membrane
145 damage. Using the FEI Quanta 200F software (Eindhoven, The Netherlands),
146 direct SEM examination showed evidence of structural damage for both *S.*

147 *aureus* and *E. coli* with all the formulations tested (Figures 4 and 5). Ninety
148 percent of the bacterial population was damaged following exposure to the
149 control formulations (Table 3). Such level of inactivation contributes to create a
150 substantial artefact as demonstrated from the SEM images and OMP analysis.
151 Loss of bacterial viability during sample preparation prior to testing has been
152 well reported. The decrease in bacterial concentration depends on the material
153 used (Best et al., 1988; Thorn et al., 2013) and the type of bacteria; some recent
154 European efficacy test protocol such as the EN14776 recommends the use of
155 glycerol with the Gram-negative bacterial inoculum to protect test inocula from
156 dehydration. Here, the use of the alginate limited bacterial loss and contributed
157 to the use of a highly reproducible test inoculum concentration, as described by
158 Shackelford and colleagues (2006).

159 It is clear that the aerosol of the blank formulation by itself caused membrane
160 damage, with indication that the Gram-negative was more affected with release
161 of DNA, although not to the OMP. Although the extent of damage could not be
162 quantified, the combined formulations seems to disrupt structural integrity of *S.*
163 *aureus* more extensively when compared to the other formulations (Figure 4).
164 The severity of damage caused by the combined formulation was more
165 apparent in *E. coli* (Figure 5). Exposure to the glycol only or ethanol only
166 formulation seems to 'smooth' the surface of *E. coli* while the blank formulation
167 affected the bacterial surface but not to same extent as the combined
168 formulation (Figure 5). SEM images indicated structural changes when bacteria
169 were exposed to the formulations with the different formulations. Damage
170 caused by the combined glycol and ethanol formulation seemed more severe
171 in both Gram-negative and Gram-positive bacteria. A recent study showed

172 severe bacterial structural damage in *E. coli* exposed to 25% ethylene glycol
173 (Moghayedi et al. 2017).

174 Potassium leakage measurements indicated that the full formulation may
175 interact with the cytoplasmic membrane of the Gram-positive bacterium,
176 releasing some potassium, but not larger cell components. Potassium release
177 is the first indicator of membrane damage followed by larger cytoplasmic
178 constituents (Maillard, 2002). Here, a small but statistically significant increase
179 (ANOVA; $P < 0.001$) in potassium leakage was observed when testing the glycol
180 and ethanol (A), and glycol only formulations (B) against *S. aureus* compared
181 to the blank formulation (D) (Table 4). No potassium was released from *E. coli*
182 exposed to the aerosolised formulations (Table 4).

183 The release of DNA from bacteria following exposure to the aerosolised
184 formulations was measured spectrophotometrically following exposure. The
185 OD₂₆₀ reading allows for the calculation of the concentration of nucleic acid in
186 the sample. An OD of 1 corresponds to ~50 µg ml⁻¹ for DNA. The OD₂₆₀/OD₂₈₀
187 ratio reading provides an estimate of the purity of the nucleic acid. Pure
188 preparations of DNA have OD₂₆₀/OD₂₈₀ values of ≥1.8. There were more DNA
189 in the *E. coli* samples than in the *S. aureus* ones (Table 5). Exposure to the
190 aerosolised formulation contained glycols and ethanol or to the nebulised blank
191 formulation resulted in very little DNA release, 4% and 6% of total estimated
192 DNA in *S. aureus* and *E. coli* respectively.

193

194 The results of SDS-PAGE indicated no changes in OMP in *E. coli* after
195 exposure to the blank and the formulation containing combined glycols and
196 ethanol were detected by the technique. There was no apparent change in the

197 OMP profile following exposure to the blank or the combined glycol and ethanol
198 formulation, although an increase in the band concentration of three specific
199 OMP of 35 kD, 27 kD and 16 kD approximately was observed when bacteria
200 were exposed to the aerosolised formulations (Figure 6).

201 In conclusion, the test chamber described in our study allowed the microbicidal
202 evaluation of foggers containing glycol-based formulations and also
203 nebulization of blank formulation against bacteria embedded in alginate matrix.
204 The advantage of the small size chamber is the rapidity of the experiment and
205 cost to set up (compared to the use of an aerobiology chamber). One limitation
206 is that the aerosolised formulations need to be adapted to the small volume of
207 our test chamber. The bactericidal efficacy of the combined aerosolised glycol
208 and ethanol formulation could be partly attributed to damaging the cytoplasmic
209 membrane of Gram-positive bacteria and to damaging the outer membrane of
210 Gram-negative bacteria.

211

212 **Material and methods**

213

214 *Aerosolized Formulations*

215 Four formulations (Table 1) combining DPG and anhydrous ethanol provided
216 by Reckitt Benckiser (One Philips Parkway, Montvale, NJ 07645. USA) were
217 studied. Samples were stored at room temperature prior to fogging.

218

219 *Bacterial strains*

220 *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 11229)
221 commonly used in standard efficacy test protocols were used as test bacteria.

222 Both strains were stored on Nutriprotect beads (Fisher Scientific,
223 Loughborough, UK) at $-80 \pm 1^{\circ}\text{C}$ and restricted to a maximum of 2 subcultures
224 from the original freezer stock prior to any testing. Both strains were grown in
225 tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at $37 \pm 1^{\circ}\text{C}$ for 16-24 hours.
226 Test inoculum were prepared from harvesting an overnight TSB culture
227 centrifuged at 5,000 g for 10 minutes and re-suspended in phosphate buffer
228 saline (PBS) (Fisher Scientific, Loughborough, UK).

229

230 *Sodium alginate test inoculum preparation*

231 The effects of aerosolised formulations were analysed against vegetative
232 bacteria embedded in sodium alginate. The test protocol was adapted from the
233 procedure described in Shackelford *et al.* (2006). A 1 ml sample of bacterial
234 suspension was added to a 1 ml cooled 3% (w/v) sodium alginate solution in
235 deionised water and mixed by pipetting (BDH Chemicals Ltd, Poole, UK). The
236 final concentration in the bacteria/alginate mixture was $1-10 \times 10^9$ cfu ml⁻¹.
237 Aliquot of alginate/bacteria (0.2 ml) was dispensed onto the centre of a moulds
238 holder system of M8 flat stainless still washers placed on top of stainless still
239 coupons grade 2B of 0.22 mm X 0.22 mm (Goodfellows Cambridge Ltd.
240 Huntington, UK). Both, flat washers and coupons were soaked with 5 %
241 Decon90 (Decon Laboratories Limited, Hove, UK) in deionised water for 60 min,
242 rinsed, dried and then autoclaved before use.

243 Loaded mould holder system was placed into individual wells of sterile 6 well
244 plates (Corning® Costar®, Sigma-Aldrich UK) containing 10 ml of 2% calcium
245 chloride solution (BDH chemicals, Poole, UK) and left for 5 min to form a gel as

246 the sodium ions are exchanged with calcium ions and the polymers become
247 cross-linked (Waldman *et al.*, 1998).

248 To recover the bacteria from the alginate matrix, both control (no formulation
249 added) and post formulations exposure, the gels were rinsed in five changes of
250 10 ml of sterile distilled water and dissolved in 10 ml of McIlvaine's buffer (0.1
251 mol l⁻¹ citric acid and 0.2 mol l⁻¹ disodium phosphate at pH 7.4; Fisher Scientific).
252 Samples were taken from the dissolved gels, serially diluted in sterile PBS (pH
253 7.4; Fisher Scientific) and CFU counts were performed using the Miles and
254 Misra drop count method. The bacterial recovery and microbicidal efficacy (BE)
255 was calculated as follows: $BE = \log N_c - \log N_b$ where N_c and N_b represent the
256 numbers of CFU ml⁻¹ in the control and biocide fogger formulations, respectively.
257

258 *In-house test chamber conditions*

259 A small portable aerosolisation test chamber was modified from the test
260 chamber described by O'Jeil and colleagues (2013). Fogger release conditions
261 were adapted to the size of the chamber consisted of a 23 cm stainless steel
262 tube connected at the other end to an Andersen cascade impactor (Westech
263 Instrument Services Ltd, Henlow, UK). A constant low flow rate measured by a
264 Copley Scientific DFM2000 (Nottingham, UK) flow meter was generated
265 through a vacuum pump (Fisherbrand, Loughborough, UK) connected to the
266 Andersen cascade impactor (Figure 1). Temperature and relative humidity
267 conditions on surfaces were determined using a S154TH temperature and
268 relative humidity probe. Testing parameters were established according to the
269 chamber dimension size. Test operation conditions (Table 2) were set up to
270 mimic a previous study performed with the aerobiology chamber (Sattar *et al.*,

271 2016). The procedure was carried out in a class-2 microbiological safety
272 cabinet facility.

273 The blank formulation (Table 1) could not be delivered by fogger due to
274 excessive foam release. Instead the blank formulation was nebulised using a
275 nebuliser (Philips Respronic, Best, The Netherlands) connected to the 23 cm
276 stainless steel tube of the rig. All test parameters were the same for all tested
277 formulations.

278

279 *Bactericidal activity of formulations in suspension*

280 Bacterial growth kinetics was determined using the Bioscreen C Microbial
281 Analyser (Labsystems, Helsinki, Finland) for both microorganisms using the
282 four formulations as solutions: glycol+ethanol (A), glycol (B), ethanol (C) and
283 blank (D). Controls consisted of each bacterium growing in TSB. The Bioscreen
284 was run for 24 h at 25°C and readings were taken using a wideband filter (420-
285 580nm) every 15 min preceded by 10 s shaking.

286

287 *Microbicide mechanisms of action*

288 Bacterial gross structural damage following exposure to aerosolised
289 formulations was explored by scanning electron microscopic examination
290 (SEM imaging) (Walkera *et al.*, 2003) using the FEI Quanta 200F (Eindhoven,
291 The Netherlands). Loss of (cytoplasmic) membrane integrity was measured
292 with potassium leakage according to Walsh *et al.* (2003). A number of controls
293 were performed including boiled bacteria at 80°C for 20 min (maximum
294 potassium release; positive control 1), boiled bacteria embedded in the matrix
295 at 80°C for 20 min (effect of alginate on potassium release; positive control 2),

296 effect of nebulisation on the release of K⁺ from embedded bacteria in the
297 alginate (nebulisation control). Potassium release was measured by inductively
298 coupled plasma mass spectrometry (Agilent 7900 ICP-MS). A five-point
299 calibration (1, 0.1, 0.01 and 0.001 mg l⁻¹, (Tune) and calibration blank (CAL))
300 was run and an internal standard (IS) was used throughout the analysis. All
301 standards (Tune/Calibration and IS) are certified reference standards from
302 Agilent. Samples were run in duplicate with blanks in between different samples
303 to ensure there was no carryover of K⁺.

304 To evidence gross membrane damage, DNA release was measured by UV
305 spectrometry following exposure to the four formulations. Following exposure
306 to aerosolised formulation and dissolution of the alginate, samples were added
307 to a 1 cm path-length cuvette and OD recorded 260 nm and at 280 nm. Samples
308 with an OD of 1 at 260 nm contain approximately 50 µg ml⁻¹ double-stranded
309 DNA (Nicklas and Buel, 2003).

310 Positive controls consisted in DNA measured spectrophotometrically of boiled
311 bacteria at 80°C during 20 min.

312 Damage to the Gram-negative outer membrane proteins (OMP) after exposure
313 to the different formulations were examined using SDS-PAGE according to
314 Tattawasart and colleagues (2000b) with the silver staining performed
315 according to Hitchcock and Brown (1983). ImageJ software (Schindelin *et al.*,
316 2012) was used as a semi-quantitative tool to determine the amount of OMP
317 observed.

318

319 *Statistical analysis*

320 All experiments were performed in triplicate unless otherwise stated. Bacterial
321 reduction and effects of the possible mechanism of action of the formulations
322 tested were compared by one-way ANOVA and Tukey tests with a 95%
323 confidence level using Rstudio software (Version 1.1.383). Results were
324 considered significant when $P < 0.001$.

325

326 **Acknowledgement**

327 None

328

329 **Funding**

330 A grant was provided to Cardiff University by Reckitt Benckiser to conduct this
331 short study.

332

333 **Conflict of interest**

334 JR Rubino and Ijaz MK are employees of Reckitt Benckiser.

335

336 **References**

337 Baker, A.H. and Twort, C.C. (1941) The effect of humidity of air on the
338 disinfection capacity of mechanically atomized and heat-volatilized
339 germicidal aerosols. *J Hyg* **41**, 117-130.

340 Berger, F.M., Hubbard, C.V. and Ludwig, B.J. (1953) The antimicrobial action
341 of certain glycerol ethers and related compounds. *Appl Microbiol* **1**, 146-149.

342 Berry, H. (1944) Antibacterial values of ethylene glycol monophenyl ether. *The*
343 *Lancet* **247**, 175-176

344 Best, M., Sattar, S. A., Springthorpe, V.S., Kennedy, M.E. (1988) Comparative
345 mycobactericidal efficacy of chemical disinfectants in suspension and
346 carrier tests. *Appl Environ Microbiol* **54**, 2856-2858.

347 Chirife, J., Herszage, L., Joseph, A., Bozzini, J.P., Leardini, N. and Kohn, E.S.
348 (1983) In vitro antibacterial activity of concentrated polyethylene glycol 400
349 solutions. *Antimicrob Agents Chemother* **24**, 409-412.

350 Davies, A., Pottage, T., Bennett, A. and Walker, J. (2011) Gaseous and air
351 decontamination technologies for *Clostridium difficile* in the healthcare
352 environment. *J Hosp Infect* **77**,199-203.

353 Galton, J., Tovey, E., McLaws, M.L., Rawlinson, W.D. (2011) The role of
354 particle size in pathogen transmission: a review. *J Infect* **62**, 1-13.

355 Goyal, S.M., Chander, Y., Yezli, S. and Otter, J.A. (2014) Evaluating the
356 virucidal efficacy of hydrogen peroxide vapour. *J Hosp Infect* **86**, 255-259.

357 Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among
358 *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide
359 gels. *J Bacteriol* **154**, 269-277.

360 Hsu, C.-S., Lu, M.-C. and Huang, D.-J. (2015) Disinfection of indoor air
361 microorganisms in stack room of university library using gaseous chlorine
362 dioxide. *Environ Monitor Assess* **187**, DOI: 10.1007/s10661-014-4235-2

363 Ijaz MK, Zargar B, Wright KE, Rubino, J.R., and Sattar, S.A. Genetic aspects
364 of the airborne spread of human pathogens indoors and emerging air
365 decontamination technologies. *Am J Infect Control* 2016;**44**:S109-S120.

366 Maillard, J.-Y. (2002). Bacterial target sites for biocide action. *Symp Ser Soc*
367 *Appl Microbiol Symposium Supplement* **92**, 16S–27S.

368 Moghayedi, M., Ahmadzadeh, H., Ghazvini, K. and Goharshadi, E.K. (2017)
369 Neglected antibacterial activity of ethylene glycol as a common solvent.
370 *Microb Pathogen* **107**, 457-46.

371 Nicklas, J.A. and Buel, E. (2003). Quantification of DNA in forensic samples.
372 *Anal Bioanal Chem* **376**, 1160-1167.

373 Ojeil, M., Jermann, C., Holah, J., Denyer, S., and Maillard, J.-Y. (2013)
374 Evaluation of antimicrobial surface activity with a newly developed in vitro
375 efficacy test reflective of conditions found in UK hospitals. *J Hosp Infect* **85**,
376 274-281.

377 Ray, A., Perez, F., Beltramini, A.M., Jakubowycz, M., Dimick, P., Jacobs, M.R.,
378 Roman, K., Bonomo, R.A. and Salata, R.A. (2010) Use of vaporized
379 hydrogen peroxide decontamination during an outbreak of multidrug-
380 resistant *Acinetobacter baumannii* infection at a long-term acute care
381 hospital. *Infect Control Hosp Epidemiol* **31**,1236–1241

382 Rowe, R.C., Sheskey, P.J. and Quinn, M.E. (eds.)(2009). Handbook of
383 Pharmaceutical Excipients, 6th edn. Pp. 592-594. Pharmaceutical Press:
384 London

385 Sattar, S.A. and Ijaz, M.K. (1987). Spread of viral infections by aerosols. *Crit*
386 *Rev Environ Control* **17**, 89-131.

387 Sattar, S.A., Kibbee, R.J., Zargar, B., Wright, K.E., Rubino, J.R., Ijaz, M.K.
388 (2016) Decontamination of indoor air to reduce the risk of airborne
389 infections: studies on survival and inactivation of airborne pathogens
390 using an aerobiology chamber. *Am J Infect Control* **44**, e177-e182.

391 Schindelin, J., Arganda-Carreras, I., Frise, E. et al. (2012), Fiji: an open-source
392 platform for biological-image analysis. *Nature Meth* **9**, 676-682.

393 Shackelford, J.C.N., Hanlon, G. and Maillard, J.-Y. (2006) Use of a new alginate
394 film test to study the bactericidal efficacy of the high-level disinfectant ortho-
395 phthalaldehyde. *J Antimicrob Chemother* **57**, 335-338.

396 Tattawasart, U., Hannb, A.C., Maillard, J.-Y., Furra, J.R. and Russell, A.D.
397 (2000a) Cytological changes in chlorhexidine-resistant isolates of
398 *Pseudomonas stutzeri*. *J Antimicrob Chemother* **45**, 145-152.

399 Tattawasart, U., Maillard, J.-Y., Furr, J.R. and Russell, A.D. (2000b) Outer
400 membrane changes in *Pseudomonas stutzeri* resistant to chlorhexidine
401 diacetate and cetylpyridinium chloride. *Int J Antimicrob Agents* **16**, 233–238.

402 Thorn, R.M.S., Robinson G.M. and Reynolds, D.M. (2013) Comparative
403 antimicrobial activities of aerosolized sodium hypochlorite, chlorine dioxide,
404 and electrochemically activated solutions evaluated using a novel
405 standardized assay. *Antimicrob Agents Chemother* **57**,2216-2225.

406 Walkera, M., Hobotb, J.A., Newmanb, G.R. and Bowlera, P.G. (2003).
407 Scanning electron microscopic examination of bacterial immobilization in a
408 carboxymethyl cellulose (AQUACELs) and alginate dressings. *Biomaterials*
409 **24**, 883–890.

410 Walsh, S.E., Maillard, J-Y., Russell, A.D., Catrenich, C.E., Charbonneau, C.E.
411 and Bartolo, R.G. (2003) Activity and mechanisms of action of selected
412 biocidal agents on Gram-positive and Gram-negative bacteria. *J Appl*
413 *Microbiol* **94**, 240-247.

414 Waldman, A.S., Schechinger, L., Govindarajoo, G., Nowick, J.S. and Pignolet,
415 L.H. (1998) The alginate demonstration: polymers, food science, and ion
416 exchange. *J Chem Educ* **75**, 1430-1431.

417
418

419

420

421 **Table 1** Aerosolized formulations tested

Sample	Active	Alcohol	Concentration (%)
Full formulation (A)	Dipropylene glycol	Anhydrous ethanol	6.67% / 46.67%
Glycol formulation (B)	Dipropylene glycol	-	6.67%
Ethanol formulation (C)	-	Anhydrous ethanol	46.67%
Blank formulation (D)	-	-	0

422

423 **Table 2** In-house chamber testing conditions

Parameter	Specification	Condition tested
Chamber dimension	Size	230 cm ³
Test operation	Air rate (flow)	2 l min ⁻¹
	Soil load	-
	Fogger release	10 sec
	Relative humidity	40-60%
	Temperature	24°C
Glycol release	Concentration	0.18 g
Microorganisms tested	Bacterial concentration	1 x 10 ⁹ CFU ml ⁻¹

424

425 **Table 3** Bactericidal efficacy of aerosolised formulations against *S. aureus* and *E. coli* with a flow of 2.0 l min⁻¹.

Formulation	<i>S. aureus</i> (Log ₁₀ ± SD)			<i>E. coli</i> (Log ₁₀ ± SD)		
	T=0	T=10 sec		T=0	T=10 sec	
		Recovery	Reduction		Recovery	Reduction
Complete formulation- Glycol and Ethanol	9.93 ± 0.15	6.73 ± 0.04	3.20 ± 0.13	9.93 ± 0.05	6.74 ± 0.33	3.19 ± 0.39
Glycol only formulation	9.68 ± 0.00	8.12 ± 0.20	1.56 ± 0.20	9.68 ± 0.11	7.81 ± 0.18	1.87 ± 0.26
Ethanol only formulation	9.68 ± 0.00	7.90 ± 0.20	1.78 ± 0.20	9.68 ± 0.11	7.61 ± 0.44	2.07 ± 0.52
Blank formulation	9.38 ± 0.00	7.84 ± 0.31	1.54 ± 0.31	9.07 ± 0.00	7.66 ± 0.15	1.41 ± 0.15

426

427 **Table 4** Potassium release from *S. aureus* and *E. coli* after exposure with
 428 aerosolised or nebulised formulations.

429

430 Potassium Release (ppm)

431

SAMPLE (n=2)	<i>S. aureus</i>	<i>E. coli</i>
Positive control: bacteria only	100.99	26.75
Positive control: bacteria + matrix	48.51	12.36
Nebulisation control	0.57	0.14
Blank formulation	0.69	0.19
Complete formulation-Glycol and Ethanol	0.91	0.26
Glycol only formulation	0.87	0.19
Ethanol only formulation	0.49	0.18

432 **Table 5** DNA release from *S. aureus* and *E. coli* following exposure to the aerosolised formulations.

SAMPLE (n=2)	OD ₂₆₀	OD ₂₈₀	OD ₂₆₀ :O D ₂₈₀	Estimated DNA concentration (µg ml ⁻¹)
<i>S. aureus</i>				
Positive control (boiled bacteria)	0.50	0.20	2.5	25.0
Complete formulation-Glycol and Ethanol	0.02	0.02	1.00	1.0
Blank formulation	0.02	0.01	1.67	1.0
<i>E. coli</i>				
Positive control (boiled bacteria)	3.00	2.02	1.49	150
Complete formulation-Glycol and Ethanol	0.19	0.09	2.01	9.5
Blank formulation	0.13	0.07	1.87	6.5

433

434 **Figure 1.** In-house aerosolised test chamber

435

436

437

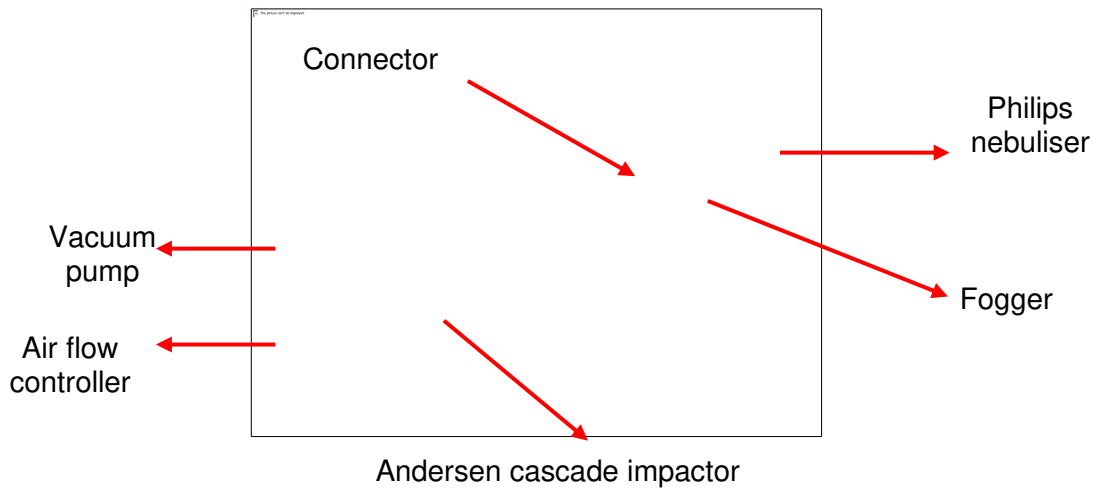
438

439

440

441

442



443 **Figure 2** Effect of the tested formulations on the growth of *S. aureus*. (■)

444 Complete formulation-Glycol and Ethanol; (■): Glycol only formulation; (■)

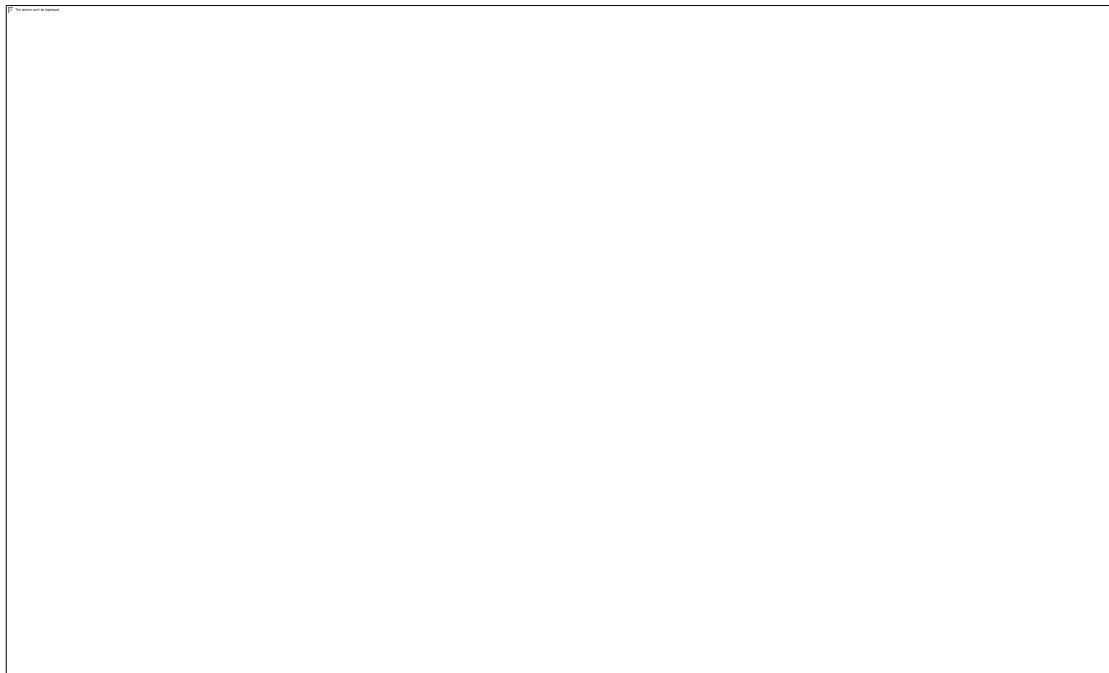
445 Ethanol only formulation; (■): Blank formulation and (■): growth control (TSB)

446

447

448

449



450 **Figure 3** Effect of the tested formulations on the growth of *E. coli*. (■) Complete
451 formulation-Glycol and Ethanol; (■): Glycol only formulation; (■) Ethanol only
452 formulation; (■): Blank formulation and (■): growth control (TSB). (n=32)



453

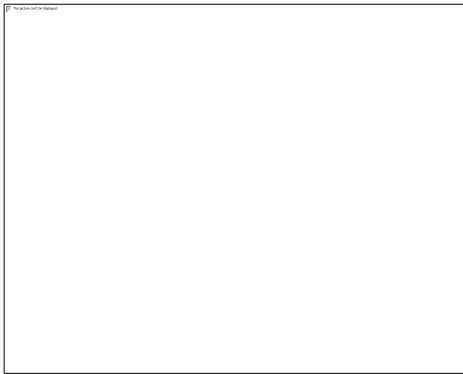
454

455

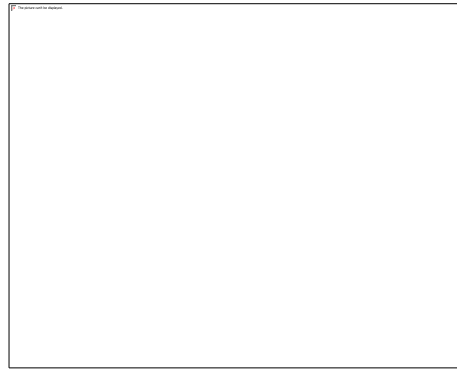
456 **Figure 4** SEM images of *S. aureus* after exposure to aerosolised
457 formulations. (A) Complete formulation-Glycol and Ethanol; (B): Glycol only
458 formulation; (C) Ethanol only formulation; (D): Blank formulation. Bacteria
459 were coloured using the GNU Image Manipulation Program (GIMP) version
460 2.8.22.

461

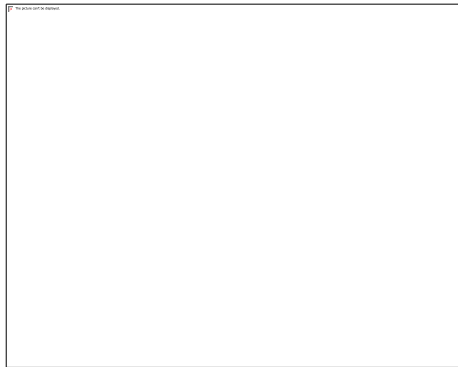
462 (A)



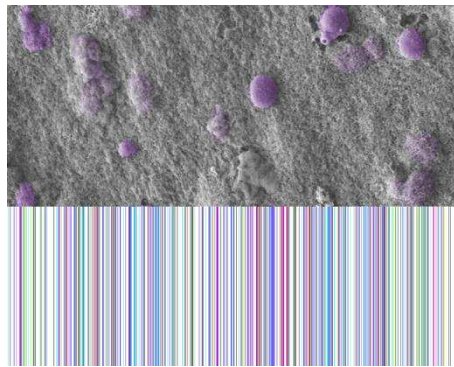
(B)



468 (C)



(D)



469

470

471

472

473

474

475

476

477

478

479 **Figure 5** SEM images of *E. coli* after exposure to aerosolised formulations.

480 (A) Complete formulation-Glycol and Ethanol; (B): Glycol only formulation; (C)

481 Ethanol only formulation; (D): Blank formulation. Bacteria were coloured using

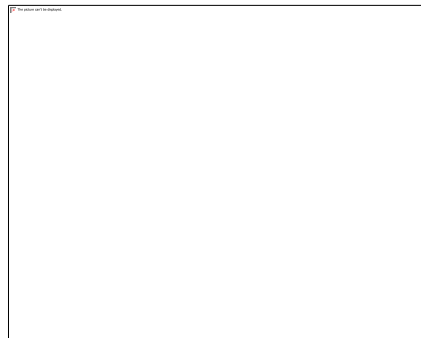
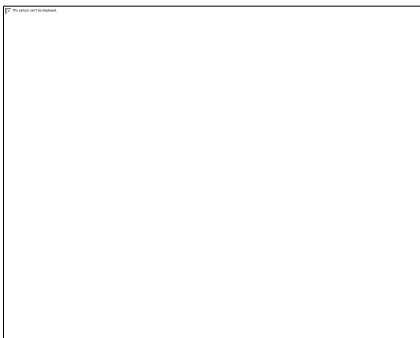
482 the GNU Image Manipulation Program (GIMP) version 2.8.22.

483

484 (A)

(B)

485



486

487

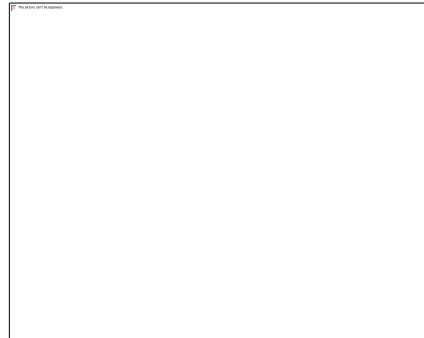
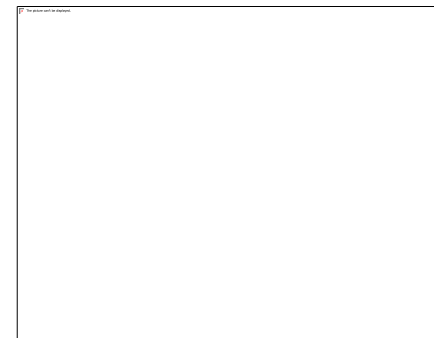
488

489

490 (C)

(D)

491



492

493

494

495

496

497

498

499 **Figure 6** SDS-PAGE analysis of outer membrane proteins. Line 1: Standard
500 molecular weight; line 2: control *E. coli* without aerosolized
501 formulation; line 3. *E. coli* treated with the blank formulation (D) and
502 line 4: *E. coli* treated with the glycol and ethanol formulation (A).

503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522

523

524

525

526

527

