

Title: Adaptation of *Pseudomonas aeruginosa* to constant sub-inhibitory concentrations of quaternary ammonium compounds

Running title: Adaptation of *P. aeruginosa* to QACs

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1 **Abstract**

2 Quaternary ammonium compounds (QACs) are widely used in consumer products for
3 disinfection purposes. QACs are frequently detected in aquatic systems at sub-
4 inhibitory concentrations and were found to affect the development of antimicrobial
5 resistance if bacteria are exposed to increasing concentrations. However, the effect of
6 a constant sub-inhibitory concentration on the development of bacterial resistance is
7 unknown. A constant exposure to 88% of the minimum inhibitory concentration (MIC)
8 of benzalkonium chloride (BAC) led to an increase of the MIC of *P. aeruginosa*. It
9 increased from 80 mg l⁻¹ to 150 mg l⁻¹ after 10 cycles of exposure and remained stable
10 after removal of BAC. When exposed to cetyltrimethyl ammonium chloride (CTMA), *P.*
11 *aeruginosa*'s MIC increased from 110 mg l⁻¹ to 160 mg l⁻¹ after 10 cycles of exposure
12 and decreased to 120 mg l⁻¹ after removal of CTMA. Additionally, cross-resistance
13 between the QACs was observed. When exposed to BAC, the MIC for CTMA
14 increased from 110 mg l⁻¹ to 200 mg l⁻¹, and when exposed to CTMA, the MIC for BAC
15 increased from 80 mg l⁻¹ to 160 mg l⁻¹. In contrast, the susceptibility to 16 antibiotics
16 was not significantly affected by exposure to QACs. Finally, analyses of the
17 membranes' nanomechanical properties of *P. aeruginosa* with atomic force
18 microscopy (AFM) showed increases in cell roughness, adhesion and stiffness after
19 treatment with CTMA. Since sub-inhibitory concentrations of QACs can be detected in
20 (technical) aquatic systems including sediments, this may lead to a dissemination of
21 bacteria with higher QAC resistance in the environment.

22 Introduction

23 Quaternary ammonium compounds (QACs) are widely used disinfection agents in
24 industrial, medical and household environments (1,2). Benzalkonium chloride (BAC,
25 Figure 1A), a mixture of alkyl dimethyl benzylammonium chlorides, is among the most
26 common QACs found in many consumer, industrial and medical products (3–5).
27 Another widely used QAC is cetyltrimethyl ammonium chloride (CTMA; also known as
28 cetrimonium chloride, Figure 1B). In the clinical context, high loads of QACs are used
29 for the disinfection and cleaning of surfaces and medical instruments (6). The majority
30 of the QACs is discharged to wastewaters (7–11), where they are further diluted and
31 become micropollutants (12). QACs are known to have a poor biodegradability and to
32 persist in waters (13–18). In hospital wastewaters, their concentrations have been
33 measured in the mg l^{-1} range (19–21), which is in the sub-inhibitory range for several
34 bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) (19,20) In municipal
35 wastewaters, where the dilution effect is bigger, concentrations were measured in the
36 $\mu\text{g l}^{-1}$ range (13,21). Concentrations of QACs in the ng l^{-1} to the $\mu\text{g l}^{-1}$ have been
37 detected in surface water [13], [19]. In soils and sediments, relatively high
38 concentrations of QACs were also found, the sorption of QAC being high and also
39 highly dependent on their structure (22). As desorption was also observed (22,23),
40 local increases of concentrations of QACs are also possible in surface waters.

41 Low concentrations of QACs can lead to a selection pressure on microorganisms'
42 populations, that may yield to more QAC-resistant populations (5,24). Bacterial
43 populations have been known to adapt to sub-inhibitory concentrations by mechanisms
44 such as modification of cell membrane structure, efflux pump expression and
45 enhancement of biofilm formation (10,25). The mode of action of QACs at low
46 concentrations may differ from the one at higher concentrations and may involve

47 multiple processes similar to antibiotics (10,26). Because of these similar mechanisms,
48 cross-resistance towards other antibacterial agents can occur (20,27–29). Previous
49 studies reported different results, either cross-resistance of *P. aeruginosa* to other
50 disinfection agents and antibiotics or an absence of cross-resistance (28,30–35). The
51 different results obtained in these previous studies highlight the need for more
52 information on a potential development of cross-resistance.

53 The level of resistance of bacteria or the potential for resistance development is linked
54 with intrinsic properties of bacterial species (36). Among the different bacterial species,
55 *P. aeruginosa* is known to be particularly resistant to QACs and to adapt easily to the
56 presence of antibacterial agents (31). Moreover, *P. aeruginosa* is an important
57 nosocomial bacterium, which is present in tap, recreational and surface water and has
58 been involved in many infectious outbreaks (37–40). These characteristics make this
59 species particularly suitable for investigating the effect of constant sub-inhibitory
60 concentrations of QACs and other disinfection agents.

61 QACs are part of commercial disinfection agents because of their antibacterial
62 properties. QACs have been used for decades as active substances in commercial
63 disinfection agents. Deconex® 53 PLUS, is a commercial product containing various
64 disinfectants and a QAC (see below). Deconex® 53 PLUS is used for the pre-cleaning
65 and disinfection of medical instruments. Other commercial disinfection agents include
66 recently developed substances other than QACs. Incidin® PLUS, for example contains
67 glucoprotamin as an active substance, which is included in several commonly used
68 surface and instruments disinfection agents in the health care environment (41,42). A
69 tetracycline-resistant strain of *P. aeruginosa* (PAO-LAC ATCC 47085) was found to be
70 more resistant to this product than a non-antibiotic-resistant strain (42). However, no

71 studies were found on the potential of this product to promote resistance at sub-
72 inhibitory concentrations.

73 To investigate the effects of QACs or other disinfection agents on bacteria, classical
74 methods such as cultivation are commonly used. In addition to classical microbiological
75 methods, atomic force microscopy (AFM) has been successfully applied to investigate
76 and characterize the bacterial morphology related to resistance to antimicrobial
77 compounds at a single-cell level (43–46). Measurements of alive bacteria in
78 physiological conditions is possible with AFM, preventing any changes in the structure
79 of the membrane when imaging the cells. The primary target of QACs is the bacterial
80 membrane and changes in the membranes are suspected to be involved in the
81 resistance mechanisms of *P. aeruginosa* to QACs. Therefore, AFM is likely to reveal
82 potential changes in the membrane morphology as well as changes in the properties
83 of the membrane in real-time (47). The aforementioned methods (classical
84 microbiology and AFM) have been used to determine the effect of BAC exposure of
85 bacteria. Other QACs, including CTMA, have been scarcely investigated. Studies on
86 BAC focused on the development of resistance when exposed to increasing
87 concentrations of the QAC, but the effect of a constant sub-inhibitory concentration of
88 a QAC or glucoprotamin has had little attention (48).

89 In this study, the consequences of an exposure of *P. aeruginosa* to BAC, CTMA,
90 Deconex® 53 PLUS and to glucoprotamin were investigated. More specifically, the
91 evolution of the MIC, cross-resistance with QACs and antibiotic susceptibilities
92 following exposure were studied. Culture-based methods combined with AFM
93 investigations were applied to characterize the properties of *P. aeruginosa* and
94 highlight any potential change in the bacterial membrane.

95

96 **Materials and Methods**

97 **Bacterial strains**

98 *Pseudomonas aeruginosa* (ATCC® 27853™) was obtained from the ATCC collection.
99 The strain was stored at -80°C using cryoinstant vials (VWR, Switzerland). Stock
100 cultures were recovered from beads prior to each exposure experiment, by plating the
101 beads on Lysogeny Broth Agar (LBA; Sigma-Aldrich, Switzerland) and on Columbia
102 Blood Agar (Sigma-Aldrich, Switzerland). Colonies from these stock cultures were
103 picked and dissolved in Mueller-Hinton broth (MHB; Sigma-Aldrich, Switzerland) to
104 prepare overnight cultures. Overnight cultures were prepared by incubating the tubes
105 at 37°C and 220 rpm for 12 to 18 hours.

106 **Disinfection agents**

107 The disinfection agents used in this study were two QACs, benzalkonium chloride
108 (BAC, CAS 63449-2) and cetyltrimethyl ammonium chloride (CTMA, CAS 112-02-7)
109 and two other commercial products, Deconex® 53 PLUS and Incidin® PLUS. BAC
110 (Sigma-Aldrich, Switzerland) was composed of 70% benzyldimethyldodecyl
111 ammonium chloride and 30% benzyldimethyltetradecyl ammonium chloride. The
112 concentration of the product was ≥ 95% and was of the highest purity available. CTMA
113 (Sigma-Aldrich, Switzerland) had a concentration of 25 wt. % in H₂O with the highest
114 purity available. Deconex® 53 PLUS (Ecolab Healthcare, Switzerland) contains 9.4%
115 of the active substances (3.8 g alkyl propylene diamine guanidinium diacetate and 5.6
116 g *N,N*-didecyl-*N*-methyl-poly(oxyethyl)ammonium propionate per 100g of product).
117 Incidin® PLUS (Ecolab Healthcare, Switzerland) contained 26% of glucoprotamin as
118 the active substance. Stock solutions of 10,000 mg l⁻¹ of the different antibacterial
119 agents were prepared in Mueller Hinton Broth (MHB). The MHB was sterilized by

120 autoclaving before the addition of the QACs or the disinfection agents and the stock
121 solution (MHB and disinfection agent) was filtered by 0.2 µm filters (Filtropur S 0.2S;
122 Sarstedt, Switzerland). The filter-sterilized solutions were then further diluted with in
123 autoclaved MHB to the concentrations of interest for the experiments (MIC
124 determination and repeated exposure). The stock solutions were kept at room
125 temperature and used within a week.

126 **Determination of MICs**

127 MICs were determined with broth microdilutions following the protocol by Wiegand *et*
128 *al.* (2008) (49). Bacterial suspensions were prepared by overnight culture, two to three
129 colonies were diluted in 10 ml of autoclaved MHB and incubated during 12 to 18 hours
130 at 37°C and 220 rpm. The overnight cultures were diluted to achieve an optical density
131 of 0.12 at 600 nm (OD₆₀₀) with an optical path length of 1 cm, which was determined
132 to correspond to a concentration of (1-2) x 10⁸ CFU ml⁻¹ (colony forming units per ml).
133 This solution was then diluted 1:100 to reach a final concentration of (1-2) x 10⁶ CFU
134 ml⁻¹.

135 The 10 first rows of a sterile 96-well microplate (Nunclon™ Delta Surface, Thermo
136 Scientific, Switzerland) were filled with 50 µl of increasing concentrations of
137 antibacterial solutions. The eleventh column was filled with 50 µl of MHB and 50 µl of
138 bacterial suspension (growth control) and the twelfth with 100 µl of a sterile control.
139 Each well of the antibacterial testing and the growth control were inoculated with 50 µl
140 of the bacterial suspension. The microplate was incubated at 37°C for 16-20 hours.
141 The concentrations used for the MIC determination are available in the Supporting
142 Information (Table S1, ESI). After incubation, growth was assessed by turbidity or
143 sediments in the wells. The MIC is defined as the lowest concentration of the
144 antibacterial agent that inhibits visible growth (49,50).

145 **Repeated exposure to a sub-inhibitory concentration of a disinfection agent**

146 Figure 2 summarizes the exposure experiments carried out with *P. aeruginosa*. Tubes
147 containing 10 ml of sub-inhibitory concentrations of BAC, CTMA, Deconex® 53 PLUS
148 or Incidin® PLUS were inoculated with 100 µl of an overnight culture of *P. aeruginosa*
149 diluted to obtain a final concentration of 10⁶ CFU ml⁻¹ in the tube at the beginning of
150 the cycle. Concentrations of CTMA and BAC were set at 86 and 88% of the MIC, at
151 87% of the MIC for Deconex® 53 PLUS and at 88% of MIC for Incidin® PLUS, to remain
152 in the sub-inhibitory range while still having a high selective pressure. For BAC, an
153 additional exposure concentration of 50% of the MIC was tested. For the QACs, one
154 exposure cycle consisted of a 48 hours' incubation at 37°C and 220 rpm. After 48
155 hours, the MIC was determined and the bacterial suspension was used to inoculate a
156 new series of tubes with the same sub-inhibitory concentration. A purity check was
157 added by plating 50 µl of the bacterial suspension on plate count agar (PCA; Sigma-
158 Aldrich, Switzerland). This was repeated to reach 10 cycles. Samples were
159 cryopreserved using cryoinstant vials after 5 and 10 cycles of exposure. Two controls
160 were added, one by cycling the bacteria in absence of the disinfection agent in the
161 growth medium (MHB) and a negative control with the growth medium only. A slightly
162 different protocol was used for the exposure to Deconex® 53 PLUS and to Incidin®
163 PLUS, one exposure cycle was defined as 24 hours and the total experiment lasted 10
164 cycles of exposure for Deconex® 53 PLUS and 15 for Incidin® PLUS ~~15 cycles of~~
165 ~~exposure~~ followed by 5 cycles of stability. The MICs were determined every five cycles
166 of exposure and the samples were cryopreserved at the same time.

167 **Stability**

168 To investigate if the effects of the exposure to disinfection agents remains stable in
169 absence of the disinfection agent from the medium, five cycles in disinfection agent-

170 free MHB were added at the end of the total exposure (10 cycles). As in the exposure
171 experiments, cycles of 48 h were carried out and a purity check was added after each
172 cycle. The MIC was determined after each cycle, the antibiotic susceptibility profile and
173 the cross-resistance was assessed at the end of the three cycles. For Deconex® 53
174 PLUS and Incidin® PLUS, cycles of 24 h were added for the stability experiments.

175 **Determination of the antibiotic susceptibility profiles**

176 10 antibiotics, which are known to be effective against *P. aeruginosa*, were selected
177 according to the European Committee on Antimicrobial Susceptibility Testing
178 (EUCAST) recommendations (51). The selected antibiotics belong to the classes of
179 aminoglycosides (tobramycin 10 µg; amikacin 30 µg; gentamycin 10 µg),
180 cephalosporin (cefepime 30 µg; ceftazidime 10 µg), fluoroquinolones (ciprofloxacin 5
181 µg; levofloxacin 5 µg); penicillin (piperacillin-tazobactam 36 µg) and carbapenem
182 (imipenem 10 µg; meropenem 10 µg). In addition, 6 antibiotics, to which *P. aeruginosa*
183 is naturally resistant, were also tested. These antibiotics belong to the classes of
184 cephalosporin (cefoxitine 30 µg; cefuroxime 30 µg), penicillin (ampicillin 10 µg),
185 sulfonamide (co-trimoxazole 25 µg), nitrofurantoin (nitrofurantoin 100 µg) and tetracycline
186 (minocycline 10 µg). They were used in the form of antimicrobial susceptibility disks
187 (Thermo Scientific™ Oxoid™ Gentamycin Antimicrobial Susceptibility Disks, Thermo
188 Scientific, Switzerland). The disks were stored at -20°C, according to the
189 manufacturer's instructions.

190 The antibiotic susceptibility profiles were determined using the disc diffusion method
191 after 5 and 10 cycles of exposure, following the EUCAST methodology (51). The
192 bacterial growth was diluted in MHB to reach an OD₆₀₀ of 0.12, as in the determination
193 of the MIC. This solution was swabbed on Mueller-Hinton agar plates (MHA plates).
194 Antibiotic discs were then applied to the agar surface using a disc dispenser (Oxoid™

195 Antimicrobial Susceptibility Disk Dispenser) and the plates were incubated for 24 hours
196 at 37°C. The inhibition zone diameters (IZD) were recorded using calipers after the
197 incubation period. The IZDs were then compared to the EUCAST database for *P.*
198 *aeruginosa* (51).

199 **Cross-resistances among QACs**

200 Susceptibility to the QAC to which the bacterial populations were not exposed was
201 determined to investigate if exposure to one QAC leads to a higher resistance to the
202 other one. The MIC for CTMA was determined after 5 and 10 cycles of exposure to
203 BAC and after the stability period using the broth microdilution method. The MIC for
204 BAC was determined after 5 and 10 cycles of exposure to CTMA and after the stability
205 period using the broth microdilution method.

206 **AFM Imaging**

207 AFM measurement were performed on the treated cells with approximately 90% of the
208 MIC during 10 cycles (for CTMA and BAC only). The measurements were performed
209 before the stability period but in absence of the QACs. The bio-mechanical properties
210 of *P. aeruginosa* strains were measured using a JPK NanoWizard 3 AFM (Bruker Nano
211 GmbH, Germany), equipped with a Zeiss TE-100 inverted microscope (Bruker,
212 Germany) in PBS buffer at room temperature (25 °C). The so called “quantitative
213 imaging” mode was used to image the cells to gather both high quality 3D topology
214 and determine mechanical properties, adhesion and stiffness. The scan size of the
215 AFM images was adapted to fit an area with at least 20 cells and 10 areas per samples
216 have been imaged. Typically, a square ranging from 15x15 to 25x25 µm was chosen.
217 Every image consisted of either a 64x64 or a 128x128 force-distance (F/d) curve
218 recorded with an indentation dwell-time of 80 milliseconds and a set-point force of 1200

219 pN. Specifically, each pixel in the AFM adhesion image has a value of force that is
220 basically the registered minimum in the retrace function of the F/d curve. The adhesion
221 force is a minimum value because it is considered as a pulling force from the cell
222 towards the cantilever, and thus generated from the cell. For these experiments we
223 used 200 μm -long triangular silicon nitride probes, supplied by pyramidal tips with a
224 nominal radius of 20 nm (DNP-10 Bruker, Bruker Nano Inc., CA, USA) and a nominal
225 spring constant of 0.06 N/m. The scanned height was set to 1 μm , to be able to
226 completely cover the cell height. The bottom glass-modified petri dishes, employed for
227 the AFM experiments, allowed us to strongly improve the stiffness image contrast, due
228 to few orders of magnitude differences between cells (hundreds of kPa) and glass
229 (hundreds of MPa) Young's modulus.

230 **AFM Data Processing**

231 The AFM data were processed using the "JPK Data Processing" software to obtain the
232 values for the adhesion parameters and by applying the Hertz-Sneddon fit it calculates
233 the Young's modulus. A Matlab (MathWorks Inc., CA, USA) script, developed in our
234 laboratory allowed to sharply discriminate and extract the points belonging to the
235 bacteria cells from the background and then use them to plot the final graphs. Briefly,
236 the script analyses each stiffness, adhesion and height image files and it creates a new
237 matrix text file (64 \times 64 or 128 \times 128 points, based on the original image size) made of 0
238 and 1, where 0 means background and 1 means cell. The selection process consists
239 of applying a threshold on the height AFM image. The so generated matrix files are
240 then plotted as black and white images to check whether the script worked correctly.
241 Afterwards the matrix files are used as a mask to extract the positive matching points
242 from the stiffness, adhesion and height files. The selected points are finally plotted and
243 averaged using the software OriginPro 2018 (OriginLab Corporation, MA, USA).

244 The average roughness was calculated by the “JPK Data Processing” software, which
245 can calculate the average roughness (here named also Ra) of a specifically selected
246 cross section from the raw files.

247 **Statistical analyses**

248 1. MIC evolution upon exposure to disinfection agents

249 To determine the statistical significance of the results for the evolution of the MIC
250 during exposure to sub-inhibitory QAC concentrations, linear regression analysis was
251 used. The analyses were performed in R using the R package lme4 (52). To test if the
252 MIC significantly changes over the cycles when exposed to a disinfection agent,
253 measurements taken during the stability period were excluded and a linear regression
254 was performed. The variables of the mixed model were selected using a backward
255 approach; a reduced model missing the variable of interest was compared to the full
256 model by an ANOVA. The p-value was extracted and the variable was considered as
257 having a significant effect on the MIC for p-values < 0.01.

258 2. Stability period and cross-resistance

259 To determine the statistical significance of the stability period, dependent t-tests were
260 performed. MIC at the end of the exposure period (after 10 cycles) were compared to
261 values after the three cycles of stability. If significant differences were observed (p-
262 value < 0.01), the MIC after stabilization was compared to the MIC before exposure to
263 assess if the MIC was reversible.

264 The significance of the cross-resistance values was investigated using dependent and
265 independent t-tests. MICs for one disinfection agent were compared at similar time
266 points after exposure to both disinfection agents and to the control. Differences were
267 considered significant for p-values < 0.01.

268 3. Antibiotic susceptibilities

269 The effects of the exposure to disinfection agents on antibiotic susceptibilities were
270 analyzed using ANOVA on three populations (exposed to CTMA, exposed to BAC and
271 not exposed) after 5 and 10 cycles of exposure and after the stability period. False
272 discovery rate adjustment (Bonferroni) (53) was applied to correct the p-values. For
273 the antibiotics for which the difference was still significant after correction (p-value <
274 0.01), Tukey's HSD was applied to determine for which pairs the difference was
275 significant. The results were then compared to the EUCAST threshold values for
276 resistance (51).

277 4. AFM data

278 The effect of the exposure to disinfection agents on the bacterial mechanical properties
279 was analyzed using a one-tail ANOVA on the three populations (exposed to CTMA,
280 exposed to BAC and not exposed). The p-value was considered significant if < 0.05.
281 To assess the roughness property difference, a non-parametric Mann-Whitney test
282 was applied with a p-value < 0.05.

283

284 **Results**

285 **1.1. Evolution of the MIC of *P. aeruginosa* for CTMA and BAC with exposure** 286 **cycles and stability after removal of the disinfection agent**

287 *CTMA*

288 The MIC of *P. aeruginosa* for CTMA was determined by broth microdilutions to be 110
289 mg l⁻¹. Based on this result, the sub-inhibitory concentration to which the bacterial
290 populations were exposed was set at 95 mg l⁻¹ (86% of MIC). This concentration is in

291 the sub-inhibitory range but will also exert a mild selective pressure (24). For *P.*
292 *aeruginosa* exposed to this sub-inhibitory concentration of CTMA a significant increase
293 of the MIC was observed. Each cycle contributed significantly ($p < 0.001$) to an average
294 increase of the MIC between $5.0 \pm 0.8 \text{ mg l}^{-1}$ (replicate 1), $3.6 \pm 0.9 \text{ mg l}^{-1}$ (replicate 3)
295 and $2.7 \pm 0.7 \text{ mg l}^{-1}$ (replicate 2). After 10 cycles, the MIC reached a value of 150 ± 10
296 mg l^{-1} (Figure 3A). Overall the MIC increased by a factor of 1.3 - 1.45. In the control
297 experiment (cycling without exposure), no significant changes in the MICs were found
298 (Table S1, ESI).

299 The stability of the adaptation to CTMA was tested by five additional cycles in absence
300 of CTMA. The MIC was again tested at the end of these cycles and a significantly lower
301 value was found. However, the MIC after this procedure was still significantly higher
302 ($127.5 \pm 9.6 \text{ mg l}^{-1}$) than the initial value. The values were at an intermediate level
303 between samples that were non-exposed and exposed to CTMA for 10 cycles (Figure
304 3A, Table S2, ESI).

305 *BAC*

306 The MIC of *P. aeruginosa* for BAC was determined to be 80 mg l^{-1} .

307 *P. aeruginosa* was exposed to 40 mg l^{-1} BAC, which corresponds to 50% of the MIC
308 (Figure S1, ESI). The effect of exposure on the MIC of the strains was significant but
309 small for replicate 1, where an average increase of the MIC of $1.36 \pm 0.45 \text{ mg l}^{-1}$ per
310 cycle was observed. The effect of the cycles was not significant and no increase was
311 observed for replicates 2 and 3. After removal of the BAC from the medium, the small
312 increase observed after 10 cycles is no more present and the MIC is back towards the
313 initial value.

314 To induce a potentially larger effect, *P. aeruginosa* was exposed to a concentration
315 equal to 88% of the MIC (70 mg l⁻¹). When exposed to this concentration of BAC, the
316 MIC increased first to 125 mg l⁻¹ and then to 150 mg l⁻¹ (replicates 1 and 2). For
317 replicate 3, the MIC increased to 100 mg l⁻¹. Thus, the final observed increase factors
318 were 1.9 (replicates 1 and 2) and 1.25 times the initial MIC (replicate 3, Figure 3B,
319 Table S2, ESI).

320 The stability of the adaptation to BAC was tested by five additional cycles in absence
321 of BAC. The MIC was again measured after these cycles and the value obtained was
322 similar to the one obtained at the end of the exposure period. Therefore, the MIC values
323 remained at 150 mg l⁻¹ (replicates 1 and 2) and a small decrease was observed for
324 replicate 3, with a MIC at 90 mg l⁻¹ after the stability period.

325 **1.2. Cross-resistance between the QACs**

326 Cross-resistance between the QACs was assessed by testing the populations for 5
327 and 10 cycles of exposure to BAC followed by 5 cycles of stability against CTMA and
328 the CTMA-exposed population against BAC. The results are provided in Table S3 (ESI)
329 and in Figures 5A and B. A weak positive correlation was also found between the
330 increase of the MIC for BAC and the increase of the MIC for CTMA when exposed to
331 BAC (Spearman correlation factor of 0.6) (Figure 5A). A stronger positive correlation
332 between the increase of the MIC for exposure to CTMA and the increase of the MIC
333 for BAC when pre-exposed to CTMA was found (Spearman correlation factor of 0.8)
334 (Figure 5B).

335 **1.3. Antibiotic susceptibilities after exposure to QACs**

336 The effects of the exposure of *P. aeruginosa* to sub-inhibitory QAC concentrations after
337 5 and 10 cycles on the antibiotic susceptibilities was tested using an analysis of

338 variance between the control and the exposed populations followed by a Dunnett post-
339 hoc test (Table S4, ESI). From the results of the statistical analyses, after 10 cycles of
340 exposure, significant differences ($p < 0.01$) were observed for amikacin, tobramycin,
341 gentamycin (higher resistance of *P. aeruginosa*), and piperacillin-tazobactam (lower
342 resistance of *P. aeruginosa*) for the strain exposed to CTMA versus the control.
343 Bacteria exposed to BAC exhibited statistical differences for levofloxacin, imipenem
344 and minocycline (lower resistance of *P. aeruginosa*) after 10 cycles of exposure.
345 However, the difference in resistance observed with these antibiotics was insufficient
346 to modify the categorical interpretation (Susceptible /Intermediate /Resistant)
347 according to EUCAST breakpoints. Among the 16 antibiotics tested, *P. aeruginosa* is
348 intrinsically resistant to six of them and being exposed to either BAC or CTMA did not
349 affect this intrinsic resistance.

350 **1.4. Evolution of the MIC of *P. aeruginosa* for Deconex[®] 53 PLUS with** 351 **exposure cycles and stability after removal of the disinfection agent**

352 Figure 4 shows the evolution of the MIC of *P. aeruginosa* for exposure to 140 mg l⁻¹ of
353 Deconex[®] 53 PLUS (13 mg l⁻¹ active substance), which corresponds to 87% of the MIC
354 (160 mg l⁻¹, 15 mg l⁻¹ of active substance). The MIC increased to 300 mg l⁻¹ (28 mg l⁻¹
355 of active substance) for one replicate (replicate 1) and to 266 mg l⁻¹ (25 mg l⁻¹) for two
356 replicates (replicates 2 and 3). Each cycle contributed to an increase of the MIC by an
357 average of 10 mg l⁻¹, independently of the replicate. The control remained at 168±20
358 mg l⁻¹ (16±1.8 mg l⁻¹ active substances). After a stability period of 5 cycles in absence
359 of the disinfection agents, the MIC either stayed at the higher value (replicates 1 and
360 3) or decreased to 212 mg l⁻¹ (replicate 2).

361 An exposure to Deconex[®] 53 PLUS also led to a decreased susceptibility to both CTMA
362 and BAC (Figures 5C and D). When exposed to Deconex[®] 53 PLUS, the MIC for CTMA

363 increased to 250 mg l⁻¹, 2.25 times the initial MIC, for replicate 1, and to 200 mg l⁻¹ ,
364 1.8 times the initial MIC, for replicates 2 and 3 (Figure 5C). The MIC for BAC increased
365 to 140 mg l⁻¹, 1.75 times the initial MIC for replicate 1 and to 100 mg l⁻¹, 1.25 times the
366 initial MIC, for replicates 2 and 3 (Figure 5D).

367 The increase in the MIC was strongly correlated with increases in the MIC for BAC and
368 CTMA with Spearman correlation factors of 0.94 (for CTMA) and 0.84 (for BAC).

369 **1.5. Evolution of the MIC of *P. aeruginosa* for Incidin® PLUS with exposure** 370 **cycles and stability after removal of the disinfection agent**

371 Exposure to Incidin® PLUS during 15 cycles of 24 hours led to a significant increase of
372 the MIC of *P. aeruginosa*. The MIC was determined to be 40 mg l⁻¹ (10 mg l⁻¹ of
373 glucoprotamin) prior to exposure. The concentration of exposure was set at 35 mg l⁻¹
374 (9 mg l⁻¹ glucoprotamin; 88% of the MIC). The MIC reached a maximum of 100 mg l⁻¹
375 (26 mg l⁻¹ glucoprotamin) after 15 cycles of exposure (Figure 4B, replicate 3), which
376 correspond to an increase of the initial MIC by a factor of 2.5 and each cycle increased
377 the MIC by an average of 3.2 ± 1.2 mg l⁻¹. An increase to 70 mg l⁻¹ (18 mg l⁻¹
378 glucoprotamin) was observed for replicate 3, which corresponds to an increase of the
379 initial MIC by a factor 1.75 and an increase per cycle of 1.73 ± 0.5 mg l⁻¹. The control
380 (not exposed to Incidin® PLUS) remained at 40 mg l⁻¹ (10 mg l⁻¹ glucoprotamin) during
381 the cycling period (Figure 4B).

382 The stability of the adaptation to Incidin® PLUS was investigated by removing the
383 disinfection agent from the growth media during 5 cycles (in this case, of 24 hours).
384 The increases in the MICs were stable and irreversible, remaining at the values
385 obtained at the end of the exposure period.

386 **1.6. Antibiotic susceptibilities following exposure to Deconex® 53 PLUS and**
387 **Incidin® PLUS**

388 The effects of exposure to sub-inhibitory concentrations of Deconex® 53 PLUS and of
389 Incidin® PLUS concentrations after 5, 10 and 15 cycles on the antibiotic susceptibilities
390 was tested using an analysis of variance between the control and the exposed
391 populations followed by a Dunnett post-hoc test, similarly to QACs. No differences
392 were observed between the Incidin® PLUS exposed strains and the controls (data not
393 shown).

394 **1.7. AFM**

395 The AFM measurements allowed to investigate the mechanical properties of single *P.*
396 *aeruginosa* cells at the nanometric scale. The main properties that were elucidated by
397 this method are: (1) The roughness of the bacterial outer membrane, (2) the stiffness
398 of the whole bacteria cell and (3) its adhesion properties. The AFM probe (also named
399 cantilever tip) having a diameter of roughly 40 nm, is able to detect and scan very small
400 details of the bacterial surface. The *P. aeruginosa* cells showed an average roughness
401 (Ra) of 30nm (Figure 6A - left) when no QAC exposure occurred. CTMA treatment
402 strongly affected the outer membrane characteristics of *P. aeruginosa*. The measured
403 Ra after exposure to CTMA was around 95nm, on average, with peaks of 200 nm
404 (Figure 6A – right). When BAC was administered, the average measured Ra was
405 around 75 nm (Figure 6A – middle). Hence, for both tested QACs the outer membrane
406 roughness significantly changed as a consequence of the positive charge interactions
407 with the membrane components.

408 Furthermore, the probe indentation on the bacterial cell allowed to obtain a precise
409 measurement of cell stiffness. In addition, the recording of any occurring interaction

410 event, of either weak or strong value in between the tip and the protein/lipid matrix of
411 the outer membrane, allowed to obtain roughness and adhesion parameters. Even
412 though, the cantilever tip was not functionalized with any biological molecule (for
413 instance: fibronectin, collagen, concanavalin-A, antibodies, etc.) and the indentation
414 contact time was less than 100ms, a measurable interaction still remained with an
415 adhesion peaks plotted in Figure 6B. In Figures 6B and C, each point corresponds to
416 a pixel belonging to the whole bacteria population that has been imaged with the AFM
417 (almost 100 cells per treatment in at least 5 different images of $20\ \mu\text{m} \times 20\ \mu\text{m}$, with a
418 resolution of 128×128 pixels). For *P. aeruginosa*, the registered average value of
419 adhesion was almost 1.0 nN, whereas after the treatment with BAC it dropped to less
420 than the half, specifically 0.4 nN (Figure 6B). Interestingly, the treatment of *P.*
421 *aeruginosa* with CTMA increased the adhesion properties between the outer
422 membrane and the cantilever tip by almost 50% compared to the original strain,
423 yielding an average value of 1.5 nN. When the adhesion properties after BAC treatment
424 is compared with the CTMA treatment a statistically significant difference (one-tail
425 ANOVA, p-value < 0.05) of more than 3-fold was found, 0.4 versus 1.5 nN, respectively.

426 In Figure 6C the Young's modulus values distribution is plotted as a parameter to
427 represent the bacterial stiffness. The measured stiffness from the three bacteria
428 populations differed significantly only for the CTMA treated case. The determined value
429 for the investigate *P. aeruginosa* strain (ATCC 27853) is around 210 kPa, which is the
430 same as for the BAC-treated case (almost 210 kPa). Remarkably, CTMA acted
431 differently again from the BAC counterpart by significantly increasing the bacteria
432 stiffness value by almost a factor of three, up to 610 kPa (one-tail ANOVA, p-value <
433 0.01).

434

435 Discussion

436 2.1. Evolution of the MIC of *P. aeruginosa* for CTMA and BAC with exposure 437 cycles, stability after removal of the disinfection agent and cross- 438 resistances between the QACs and with antibiotics

439 Populations of *P. aeruginosa* were exposed to constant sub-inhibitory concentrations
440 of CTMA and BAC, and adaptation occurred (increase in MIC) when the bacteria were
441 exposed to a concentration of the selected QACs corresponding to approximately 90%
442 of the MIC. A small increase in the MIC was observed for one replicate (out of three)
443 when *P. aeruginosa* was exposed to 50% of the MIC. The strength of the selection
444 pressure was important, as in the case of BAC, the adaptation was much stronger with
445 an exposure concentration at 90% of the MIC compared to an exposure concentration
446 of 50% of the MIC. However, it cannot be excluded that stronger adaptation may occur
447 for longer exposure periods with < 90% of the MIC. When exposed to approximately
448 90% of the MIC of the selected QACs, the general trend was an increase of the MIC,
449 however, the individual replicates showed different patterns. Previous studies revealed
450 that a long-term exposure to BAC led to a reduction in the community diversity and an
451 increase in the resistant bacteria (25,54). Variability in the adaptation was also
452 observed here, resulting from a low selective pressure. This variability can be the result
453 of either different mechanisms or differences in the speed of adaptation. Exposure of
454 *E. coli* strains to sub-inhibitory concentrations of BAC led to different phenotypical
455 variants (25) and it is possible that the resulting exposed populations in this study are
456 also comprised of phenotypic variants.

457 The results showed that an increase was still present at the end of the 10 cycles
458 investigated (Figures 3 and 5). In previous studies it has been shown that an increasing
459 concentration of QACs ultimately reaches a plateau of the MIC (35). It can be

460 hypothesized that an exposure to a constant sub-inhibitory concentration of QACs also
461 ultimately leads to a plateau of the MIC.

462 After removal of the QAC from the growth medium, the MIC for CTMA decreased to
463 similar values prior to the exposure. However, when exposed to BAC, the MIC reached
464 during the exposure period remained stable at the higher level. If the mechanisms are
465 of similar nature, as shown with the experiments on cross-resistance, exposure to
466 CTMA seems to trigger a transient mechanism of adaptation, which is different from
467 BAC. Mechanisms of adaptation or resistance to QACs includes modification of the
468 membrane or expression of efflux pumps (29,33,54). These mechanisms are
469 consistent with a transient change either in the structure of the membrane or in the
470 level of expression of other resistance mechanisms. The differences observed
471 between the two selected QACs might be linked to the composition of the QACs, BAC
472 being a mixture of several quaternary ammonium compounds with different aliphatic
473 chains ranging from 8 to 16 carbon atoms with an aromatic ring (Figure 1A), while
474 CTMA is a single quaternary ammonium compound with one aliphatic chain length
475 (Figure 1B). However, this would need further investigations.

476 When exposed to CTMA first, *P. aeruginosa* became more resistant to BAC and when
477 exposed to BAC, it became more resistant to CTMA (Figures 5 A and B,). This result
478 was demonstrated by a positive correlation score obtained in both cases. This might
479 mean that the mechanisms involved are shared or similar for the two QACs. The nature
480 of the mechanism might rather be the result of phenotypical changes for CTMA,
481 because the increase of the MIC after removal of the QAC was reversible. For the other
482 tested compounds, genotypic changes cannot be excluded. The increased resistance
483 might be linked to morphological changes of the membrane observed by AFM or to the
484 expression of other mechanisms, such as efflux pumps as shown in previous studies

485 (29). When exposed to a commercial disinfection agent containing QACs, cross-
486 resistance was also observed with BAC and CTMA. Strains exposed to Deconex® 53
487 PLUS demonstrated a higher tolerance for CTMA and BAC compared to their control.
488 Similarly, to BAC and CTMA only, the degree of tolerance was strongly correlated with
489 the level of adaptation. The decrease in the susceptibility, when exposed to Deconex®
490 53 PLUS, was stronger for CTMA, for which a maximum increase in the MIC of up to
491 a factor 1.25 was observed (Figure 5C). For BAC, it reached a maximum increase of
492 the MIC by a factor 1.8 (Figure 5D).

493 Cross-resistance with antibiotics was less evident than for QACs. Statistical
494 differences were observed with either increased or decreased resistance to antibiotics.
495 However, the categorical interpretation (S/I/R) according to EUCAST breakpoints were
496 not modified. These results are in accordance with previous studies, in which exposure
497 to BAC or CTMA did not lead to any significant difference in the resistance profile to
498 antibiotics (30–32).

499 **2.2. Morphological evolution of the bacteria**

500 AFM investigations revealed some differences in the treated compared the non-treated
501 bacterial cells. The roughness of the cells increased with the treatment, similarly to the
502 results of other studies investigating antibiotics or antimicrobial agents (46,55,56). As
503 QACs are agents targeting the outer membrane of bacteria, this result indicates that
504 even at subinhibitory concentrations, bacterial membranes are affected by QACs. The
505 mechanism of action of QACs is known to be an association of the positively charged
506 quaternary nitrogen with the head groups of acidic-phospholipids of the membrane
507 (57). This interaction decreases the fluidity of the membrane at concentrations close
508 to the MIC (57). Adaptation of *P. aeruginosa* to one QAC was observed to result in
509 changes in the cell surface hydrophobicity and biofilm formation, but also to be the

510 cause of changes in the outer membrane proteins and the permeability (31,33). When
511 exposed to CTMA, the stiffness of the cells increased by a factor of 3 (Figure 6C),
512 leading to more rigid cells compared to the control, which is consistent with the
513 expected mode of action of QACs. This was not the case for the cells exposed to BAC,
514 as such an increase in the Young modulus was not observed.

515 Interestingly, the bacterial surface adhesion properties, measured as the pulling force
516 exerted by the membrane matrix towards an uncoated-silicon probe, revealed a strong
517 decrease when the bacteria were treated with BAC, whereas in the case of CTMA an
518 increase in the unspecific adhesion properties was registered (Figure 6B). Adhesion
519 properties of a bacterial cell are also linked with their ability to create biofilms (58). *P.*
520 *aeruginosa* is known to form biofilms and to use biofilms as a defense against different
521 chemicals. It was previously observed that BAC inhibits the biofilm formation of *P.*
522 *aeruginosa*, which could explain the observed decrease in the adhesion properties
523 when exposed to BAC (59). In contrast, in this study, the adaptation of *P. aeruginosa*
524 to CTMA was associated with modifications of the bacterial membrane and an increase
525 in adhesion properties, which could enhance the biofilm formation. These two
526 mechanisms are consistent with previous observations when increasing
527 concentrations of QACs were used, either with biofilm as a resistance mechanism (60)
528 or with an exposure to BAC preventing the biofilm formation (59).

529 Even though CTMA and BAC modified the biomechanical properties of *P. aeruginosa*
530 in a significantly different way, they both strongly affect the cells by modifying their
531 morphological properties and hence the bacterial interaction with the external
532 environment. Besides, the outer membrane roughness significantly changed by
533 increasing the numbers and the height of “hills” and “valleys” of the external lipid bilayer
534 and the lipo-poly-saccharides matrix. Potentially, the measured roughness could be a

535 consequence of a deeper and stronger modification at a lower membrane level, mainly
536 affecting the inner membrane.

537 **2.3. Practical implications: exposure to commercial disinfection agents**

538 Two commercial disinfection agents, currently used in the health care context for
539 medical instrument pre-cleaning/disinfection and surface cleaning detergent were
540 tested to compare their effects with single compounds (BAC and CTMA). Exposure to
541 both disinfection agents, in their commercial forms, led to an adaptation of the exposed
542 populations. The increase of the MIC, after 10 cycles, was similar for both products,
543 an average factor of 1.7 for Deconex[®] 53 PLUS and 1.75 for Incidin[®] PLUS. Both
544 commercial products led to adapted populations of *P. aeruginosa* similarly to QACs.
545 The adaptation is stable during the 5 cycles in absence of the product, which is similar
546 to BAC but different to CTMA. Exposure to QAC-containing commercial products also
547 yielded an increased tolerance to the two pure QACs tested, but no changes in the
548 antibiotic resistances.

549 This result is relevant for cases where bacterial populations are in contact with residual
550 concentrations of the product. Due to a widespread application of QACs in hospitals,
551 some uses in cleaning and discharge of wastewaters or other point sources may
552 contain relatively high sub-inhibitory concentrations of disinfection agents, which may
553 lead to development of increased MICs in the bacterial communities. Depending on
554 the applied QAC-containing disinfection agents, it may also lead to morphological
555 changes and adhesion and biofilm formation potentials. In contrast to these high sub-
556 inhibitory concentration scenarios, the exposure to the µg/L levels of QACs present in
557 municipal wastewater treatment plants may not lead to changes in the MICs.

558 **Conclusion**

559 This study showed for two selected QACs and two commercial disinfection agents that
560 *P. aeruginosa* cells can adapt to these treatments, when exposed to approximately
561 90% of the MIC. This was exemplified by an increase of the MICs by a maximum factor
562 of 1.45 for CTMA, 1.9 for BAC, 1.7 for Deconex[®] 53 PLUS and 1.75 for Incidin[®] PLUS.
563 When exposed to a lower concentration (approximately 50% of MIC), strains exposed
564 to BAC showed a slight adaptation to the product in one replicate. The slight increase
565 observed upon exposure to 50% of the MIC, may suggest that adaptation is also
566 possible at smaller concentrations, but longer exposure times are necessary.
567 Moreover, not only, the MIC for the QAC to which the bacteria were exposed was
568 increased but also the MIC for the other selected QAC was increased, which means
569 that there is cross-resistance between the two selected QACs. The increase in the
570 resistance to the compounds can be a result of an increase of the stiffness as shown
571 by the AFM results for CTMA. However, the increase in resistance observed when
572 exposed to BAC, is likely caused by a different mechanism, as no increase in the
573 stiffness was observed. Differences were also observed in the adhesion properties
574 following exposure and would require further investigation especially on the biofilm
575 formation potential after exposure to sub-inhibitory concentrations of QACs. The
576 roughness, was increased when exposed to both QACs, confirming that QACs are
577 affecting the membrane and the observed increase might be the consequence of
578 changes at the inner membrane level. The differences observed in the
579 nanomechanical properties of the membrane are also consistent with the differences
580 observed in the resistance evolution following exposure. This may indicate that
581 different or slightly different mechanisms are involved with the different QACs. In
582 contrast, no significant change of the QAC pre-treated bacteria was observed
583 regarding the resistance to antibiotics in this experimental setup based on the EUCAST

584 interpretational scheme. This result seems to exclude the spread of antibiotic resistant
585 *P. aeruginosa* in the environment following exposure to constant sub-inhibitory
586 concentrations of QACs. The effects obtained with two commercial disinfections
587 agents (containing a QAC or glucoprotamin) were similar to results obtained with
588 QACs.

589

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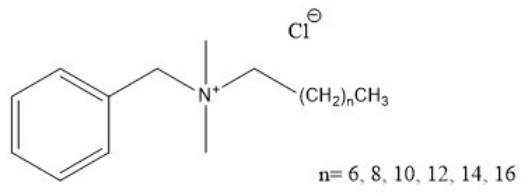
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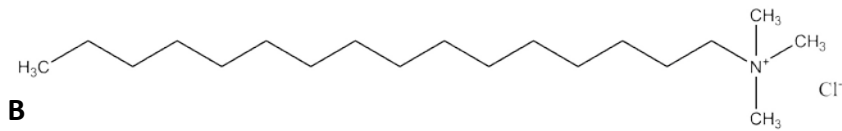
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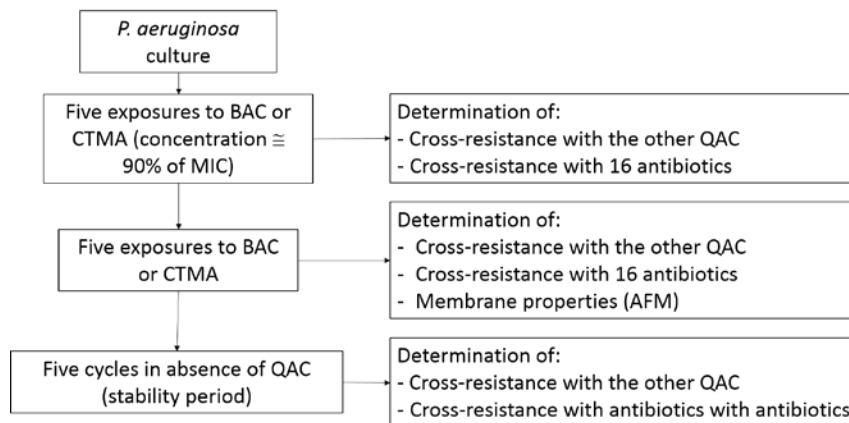


B

821 **Figure 1.** Chemical structures of quaternary ammonium compounds. A. Benzalkonium

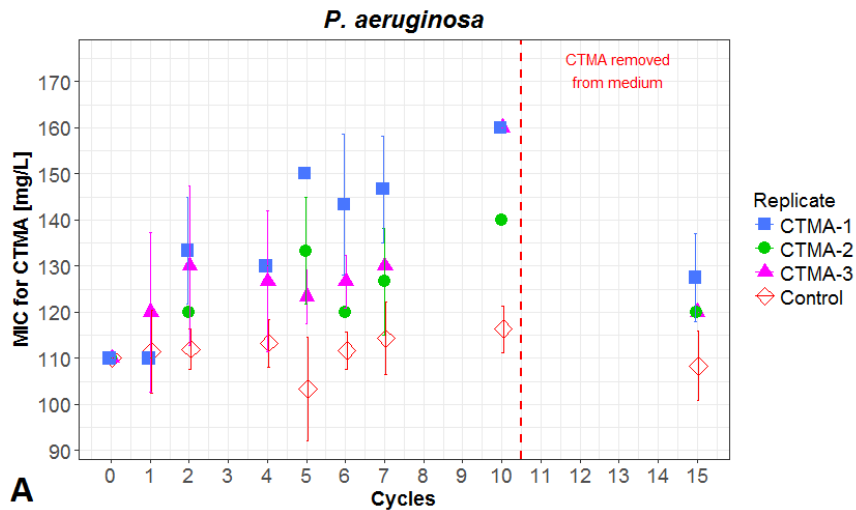
822 chloride (BAC); B. Cetyltrimethylammonium chloride (CTMA)

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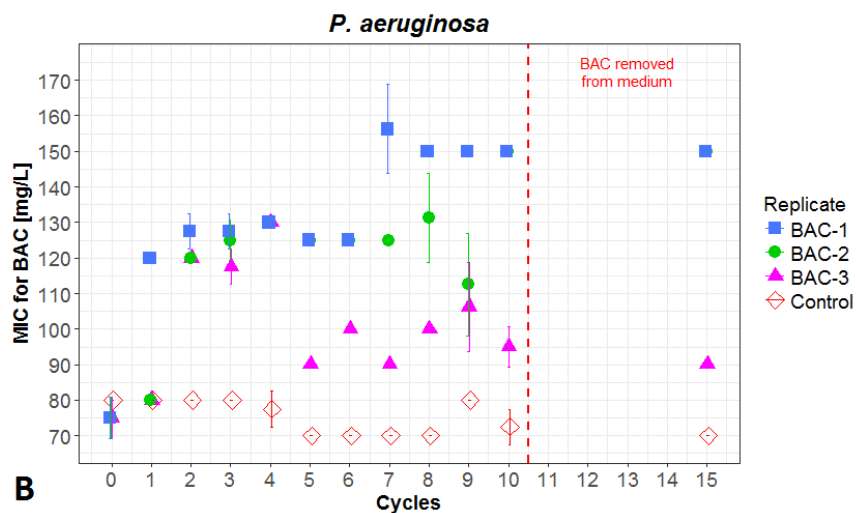


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825 **Figure 2.** Summary of the experiments performed with *P. aeruginosa* and the two
 826 QACs. Tubes of MHB with a sub-inhibitory concentration of QAC (either BAC or CTMA)
 827 were inoculated with a pure *P. aeruginosa* culture. After five and ten exposure cycles,
 828 cross-resistance to the other QAC and to antibiotics was determined. Additionally, after
 829 10 cycles, populations were analyzed by AFM. After exposure to QACs, populations
 830 were sub-cultured for five additional cycles in QAC-free MHB and cross-resistance to
 831 the other QAC and antibiotics determined.



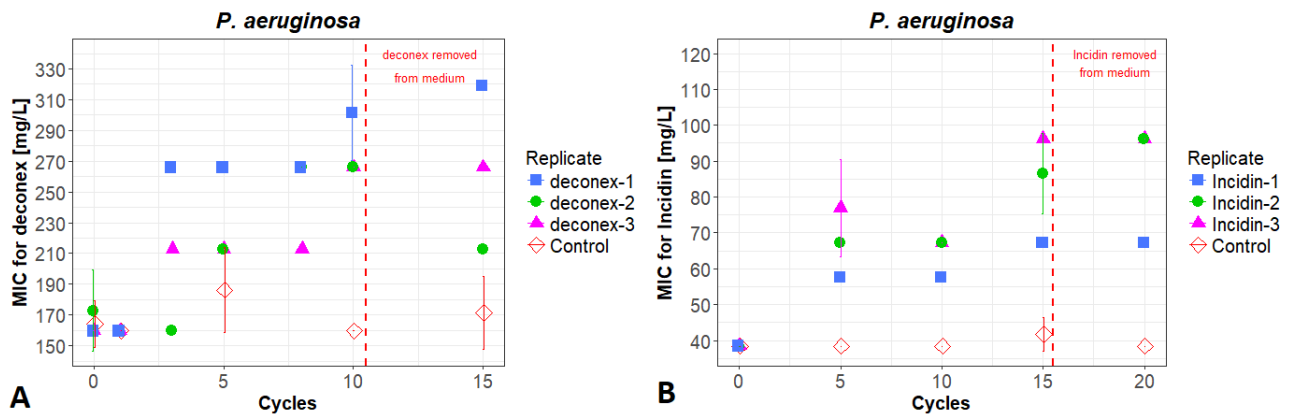
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834 **Figure 3. Evolution of the MICs of *P. aeruginosa* when exposed to sub-inhibitory**
 835 **concentrations of (A) CTMA (95 mg l⁻¹) or (B) BAC (70 mg l⁻¹).** The effect of absence
 836 of the QACs after 10 cycles on the MICs is also shown. Each exposure cycle lasted 48
 837 hours. Each point is the average of at least 4 technical measurements, error bars
 838 represent the standard deviations. **A.** MIC evolution of three *P. aeruginosa* populations
 839 exposed to CTMA (CTMA-1/2/3) and one control (not exposed to CTMA) as a function
 840 of the exposure cycles. **B.** MIC evolution of three *P. aeruginosa* populations exposed
 841 to BAC (BAC-1/2/3) and one control (not exposed to BAC) as a function of the
 842 exposure cycles.

843



844

845 **Figure 4. Evolution of the *P. aeruginosa*'s MIC when exposed to (A) Deconex® 53**

846 **PLUS or (B) Incidin® PLUS.** Three *P. aeruginosa* populations (exposed to Deconex®

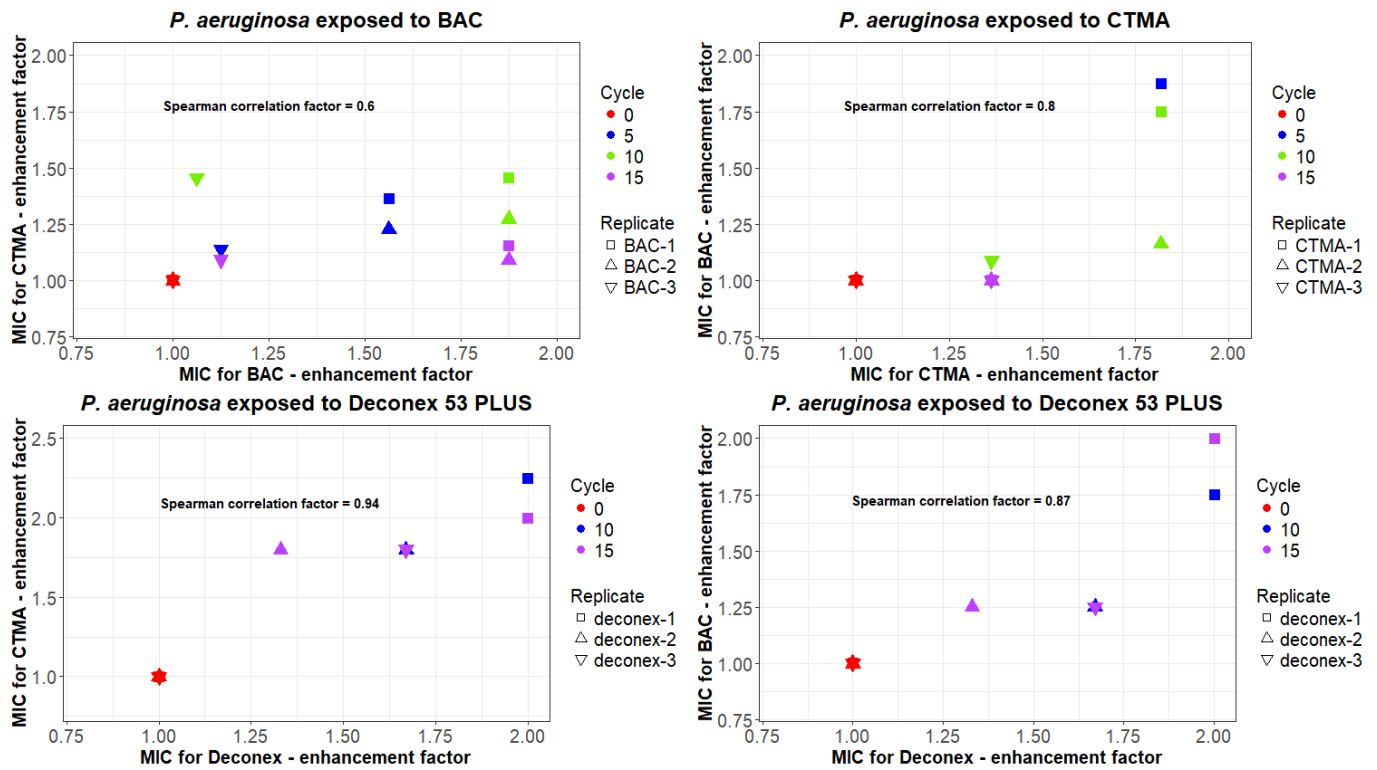
847 53 PLUS (A) or Incidin® PLUS (B)) and one control (not exposed to Deconex® 53 PLUS

848 or Incidin® PLUS) as a function of the exposure cycles. Each cycle lasted 24 hours.

849 Each point is the average of at least 4 technical measurements and error bars

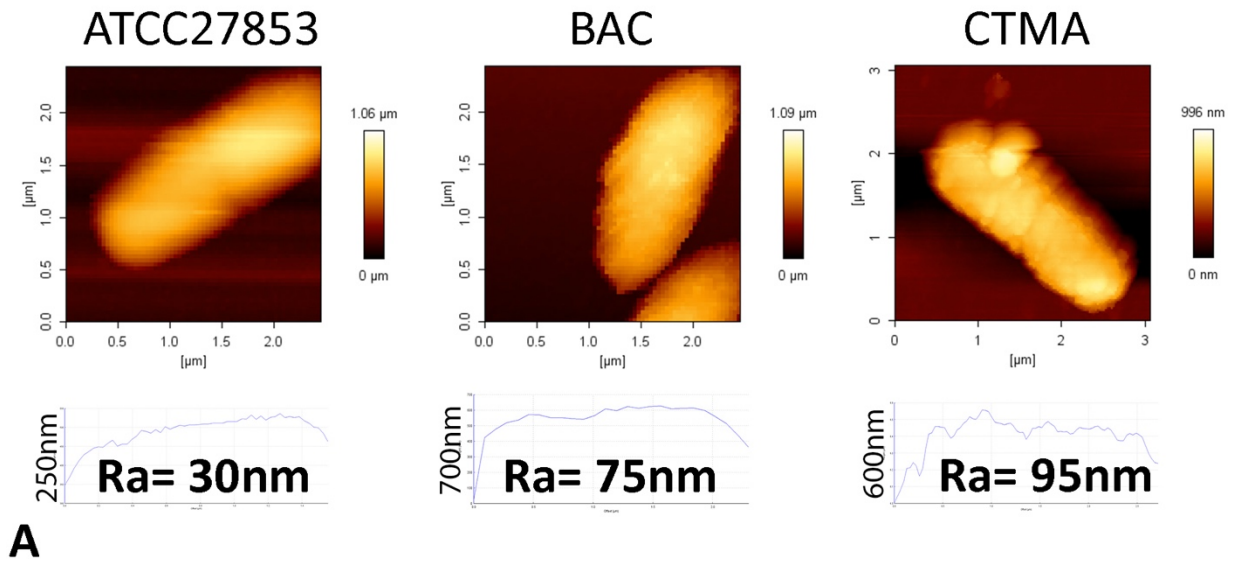
850 represent the standard deviations.

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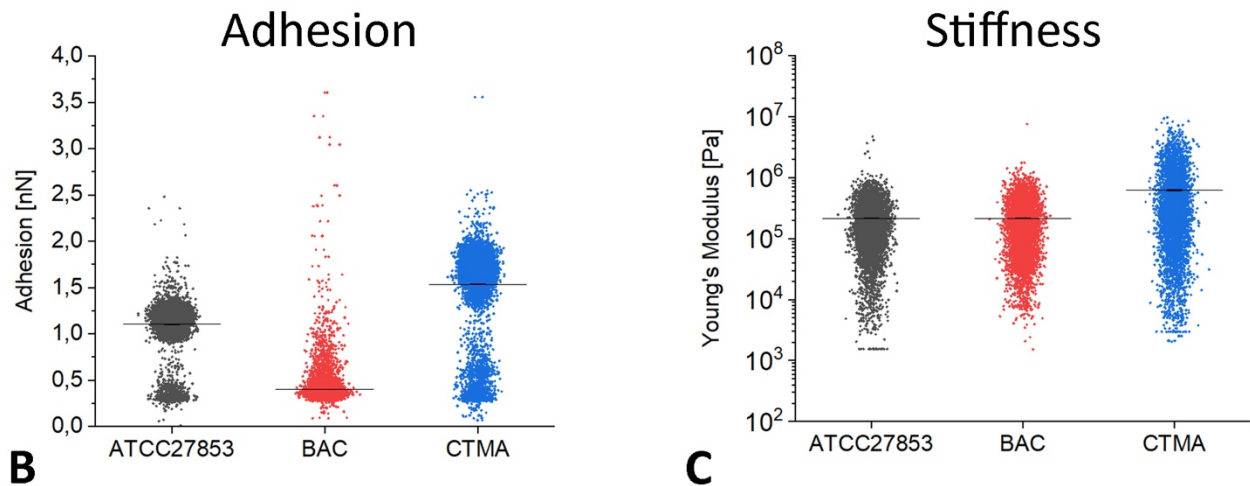


852

853 **Figure 5. Correlation factors for the cross-resistance experiment. A.** Correlation
 854 between the enhancement factor of the MIC for CTMA and the MIC for BAC when
 855 exposed to CTMA. **B.** Correlation between the enhancement factor of the MIC for BAC
 856 and the MIC for CTMA when exposed to CTMA. **C.** Correlation between the
 857 enhancement factor of the MIC for Deconex[®] 53 PLUS and the MIC for BAC when
 858 exposed to Deconex[®] 53 PLUS. **D.** Correlation between the enhancement factor of the
 859 MIC for Deconex[®] 53 PLUS and the MIC for CTMA when exposed to Deconex[®] 53
 860 PLUS.



A



B

C

861

862 **Figure 6. AFM investigation of the mechanical properties of *P. aeruginosa*. A:**

863 Two representative images are shown for each tested QAC (BAC, middle and CTMA,

864 right) and one without treatment (ATCC 27583). Below, a typical profile for the outer

865 membrane roughness (Ra) is plotted as its average value. The Y-axes scales differ

866 significantly and from left to right are as follows: 250nm, 700nm and 600nm,

867 respectively. Both treatments with QACs significantly changed the membrane average

868 roughness as reported in numbers in each plot: 30, 75 and 95 nm, respectively.

869 Statistical significance was assessed by the Mann-Whitney test and a p-value < 0.05

870 was considered significant (n=15). **B:** Adhesion plot distribution. Each dot represents

871 a single measured pixel from a bacteria cell. **C**: Stiffness plot distribution. Each dot
872 represents a single measured pixel from a bacteria cell.