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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Abstract

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

Introduction

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

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

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

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

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

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

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

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

Materials and Methods

Bacterial strains

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

Disinfection agents

The disinfection agents used in this study were two QACs, benzalkonium chloride (BAC, CAS 63449-2) and cetyltrimethyl ammonium chloride (CTMA, CAS 112-02-7) and two ~~one~~ commercial products, Deconex® 53 PLUS and Incidin® PLUS. BAC (Sigma-Aldrich, Switzerland) was composed of 70% benzyldimethyldodecyl ammonium chloride and 30% benzyldimethyltetradecyl ammonium chloride. The concentration of the product was ≥ 95% and was of the highest purity available. CTMA (Sigma-Aldrich, Switzerland) had a concentration of 25 wt. % in H_2O with the highest purity available. Deconex® 53 PLUS (Ecolab Helathcare, Switzerland) contains 9.4% of the active substances (3.8 g alkyl propylene diamine guanidinium diacetate and 5.6 g *N,N*-didecyl-*N*-methyl-poly(oxyethyl)ammonium propionate per 100g of product). Incidin® PLUS (Ecolab Healthcare, Switzerland) contained 26% of glucoprotamin as the active substance. Stock solutions of 10,000 mg l^{-1} of the different antibacterial agents were prepared in Mueller Hinton Broth (MHB). The MHB was sterilized by

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

Determination of MICs

MICs were determined with broth microdilutions following the protocol by Wiegand *et al.* (2008) (49). Bacterial suspensions were prepared by overnight culture, two to three colonies were diluted in 10 ml of autoclaved MHB and incubated during 12 to 18 hours at 37°C and 220 rpm. The overnight cultures were diluted to achieve an optical density of 0.12 at 600 nm (OD600) with an optical path length of 1 cm, which was determined to correspond to a concentration of (1-2) x 10^8 CFU ml^{-1} (colony forming units per ml). This solution was then diluted 1:100 to reach a final concentration of (1-2) x 10^6 CFU ml^{-1}.

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

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

Figure 2 summarizes the exposure experiments carried out with *P. aeruginosa*. Tubes containing 10 ml of sub-inhibitory concentrations of BAC, CTMA, Deconex® 53 PLUS or Incidin® PLUS were inoculated with 100 µl of an overnight culture of *P. aeruginosa* diluted to obtain a final concentration of 10^6 CFU ml^{-1} in the tube at the beginning of the cycle. Concentrations of CTMA and BAC were set at 86 and 88% of the MIC, at 87% of the MIC for Deconex® 53 PLUS and at 88% of MIC for Incidin® PLUS, to remain in the sub-inhibitory range while still having a high selective pressure. For BAC, an additional exposure concentration of 50% of the MIC was tested. For the QACs, one exposure cycle consisted of a 48 hours' incubation at 37°C and 220 rpm. After 48 hours, the MIC was determined and the bacterial suspension was used to inoculate a new series of tubes with the same sub-inhibitory concentration. A purity check was added by plating 50 µl of the bacterial suspension on plate count agar (PCA; Sigma-Aldrich, Switzerland). This was repeated to reach 10 cycles. Samples were cryopreserved using cryoinstant vials after 5 and 10 cycles of exposure. Two controls were added, one by cycling the bacteria in absence of the disinfection agent in the growth medium (MHB) and a negative control with the growth medium only. A slightly different protocol was used for the exposure to Deconex® 53 PLUS and to Incidin® PLUS, one exposure cycle was defined as 24 hours and the total experiment lasted 10 cycles of exposure for Deconex® 53 PLUS and 15 for Incidin® PLUS ~~15 cycles of exposure~~ followed by 5 cycles of stability. The MICs were determined every five cycles of exposure and the samples were cryopreserved at the same time.

Stability

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

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

Determination of the antibiotic susceptibility profiles

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

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

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

Cross-resistances among QACs

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

AFM Imaging

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

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

AFM Data Processing

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

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

Statistical analyses

1. MIC evolution upon exposure to disinfection agents

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

2. Stability period and cross-resistance

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

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

3. Antibiotic susceptibilities

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

4. AFM data

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

Results

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

CTMA

The MIC of *P. aeruginosa* for CTMA was determined by broth microdilutions to be 110 mg l^{-1}. Based on this result, the sub-inhibitory concentration to which the bacterial 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 ± 0.8 mg l^{-1} (replicate 1), 3.6 ± 0.9 mg l^{-1} (replicate 3)
295 and 2.7 ± 0.7 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 ± 9.6 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 ± 0.45 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.

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

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

1.2. Cross-resistance between the QACs

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

1.3. Antibiotic susceptibilities after exposure to QACs

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

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

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

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

An exposure to Deconex® 53 PLUS also led to a decreased susceptibility to both CTMA 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.

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

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

1.7. AFM

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

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

event, of either weak or strong value in between the tip and the protein/lipid matrix of the outer membrane, allowed to obtain roughness and adhesion parameters. Even though, the cantilever tip was not functionalized with any biological molecule (for instance: fibronectin, collagen, concanavalin-A, antibodies, etc.) and the indentation contact time was less than 100ms, a measurable interaction still remained with ~~an~~ adhesion peaks plotted in Figure 6B. In Figures 6B and C, each point corresponds to a pixel belonging to the whole bacteria population that has been imaged with the AFM (almost 100 cells per treatment in at least 5 different images of 20 μm × 20 μm, with a resolution of 128x128 pixels). For *P. aeruginosa,* the registered average value of adhesion was almost 1.0 nN, whereas after the treatment with BAC it dropped to less than the half, specifically 0.4 nN (Figure 6B). Interestingly, the treatment of *P. aeruginosa* with CTMA increased the adhesion properties between the outer membrane and the cantilever tip by almost 50% compared to the original strain, yielding an average value of 1.5 nN. When the adhesion properties after BAC treatment is compared with the CTMA treatment a statistically significant difference (one-tail ANOVA, p-value < 0.05) of more than 3-fold was found, 0.4 versus 1.5 nN, respectively.

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

Discussion

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

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

The results showed that an increase was still present at the end of the 10 cycles investigated (Figures 3 and 5). In previous studies it has been shown that an increasing 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

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

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

2.2. Morphological evolution of the bacteria

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

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

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

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

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

2.3. Practical implications: exposure to commercial disinfection agents

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

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

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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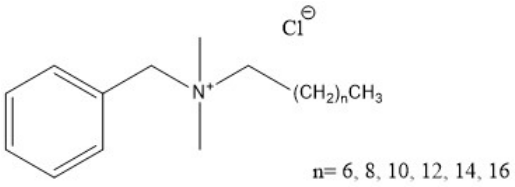

A

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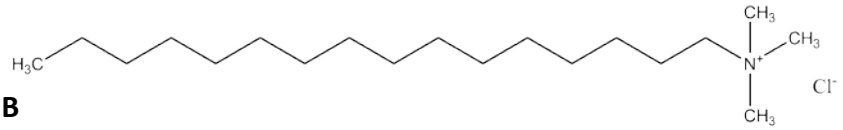


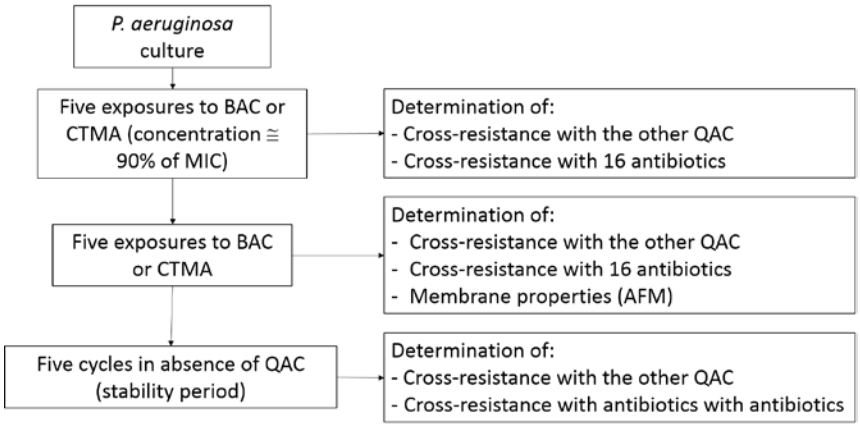
B

820

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

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

823



824

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.

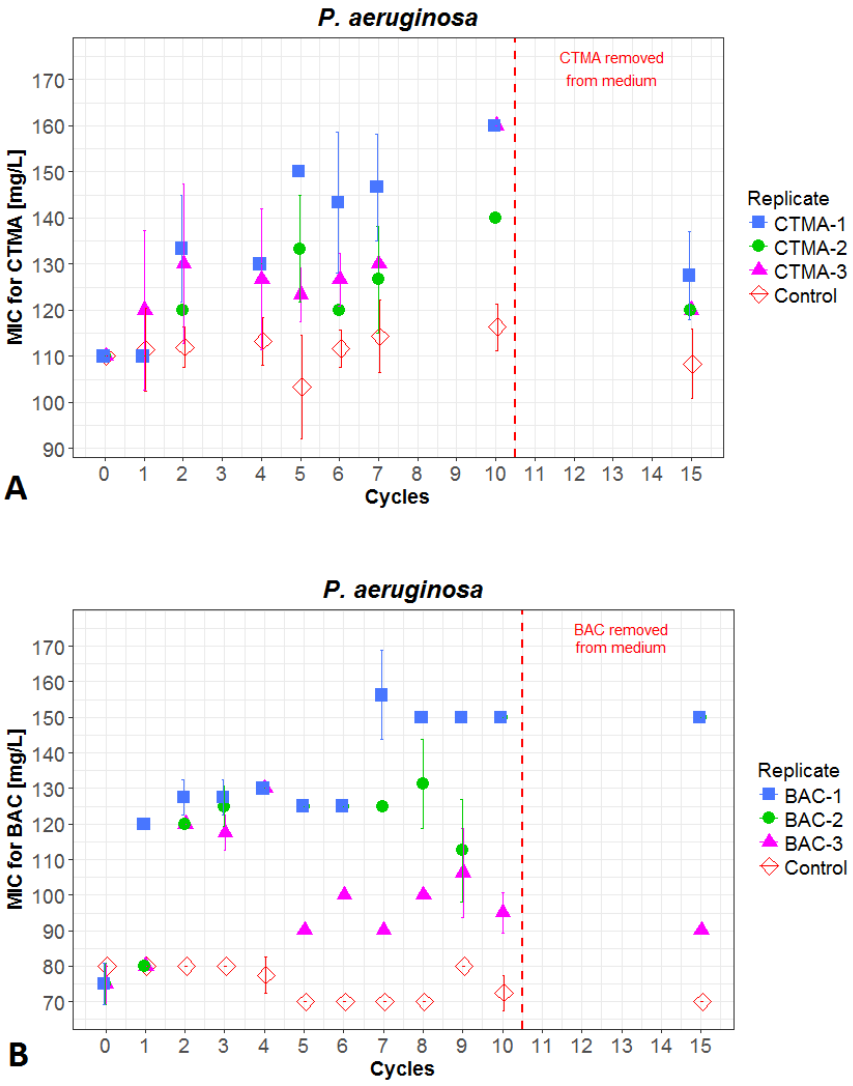
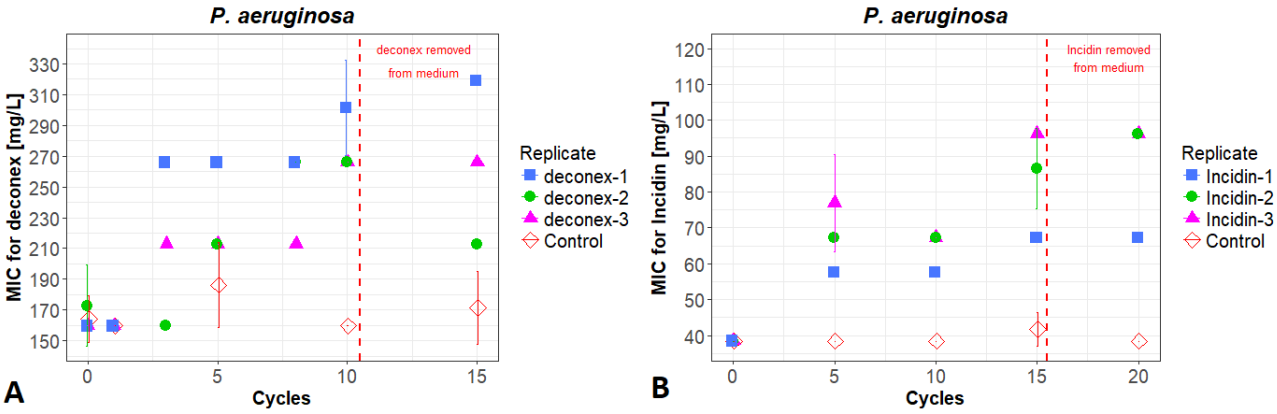


Figure 3. Evolution of the MICs of *P. aeruginosa* when exposed to sub-inhibitory concentrations of (A) CTMA (95 mg l^{-1}) or (B) BAC (70 mg l^{-1}). The effect of absence of the QACs after 10 cycles on the MICs is also shown. Each exposure cycle lasted 48 hours. Each point is the average of at least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



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.

851

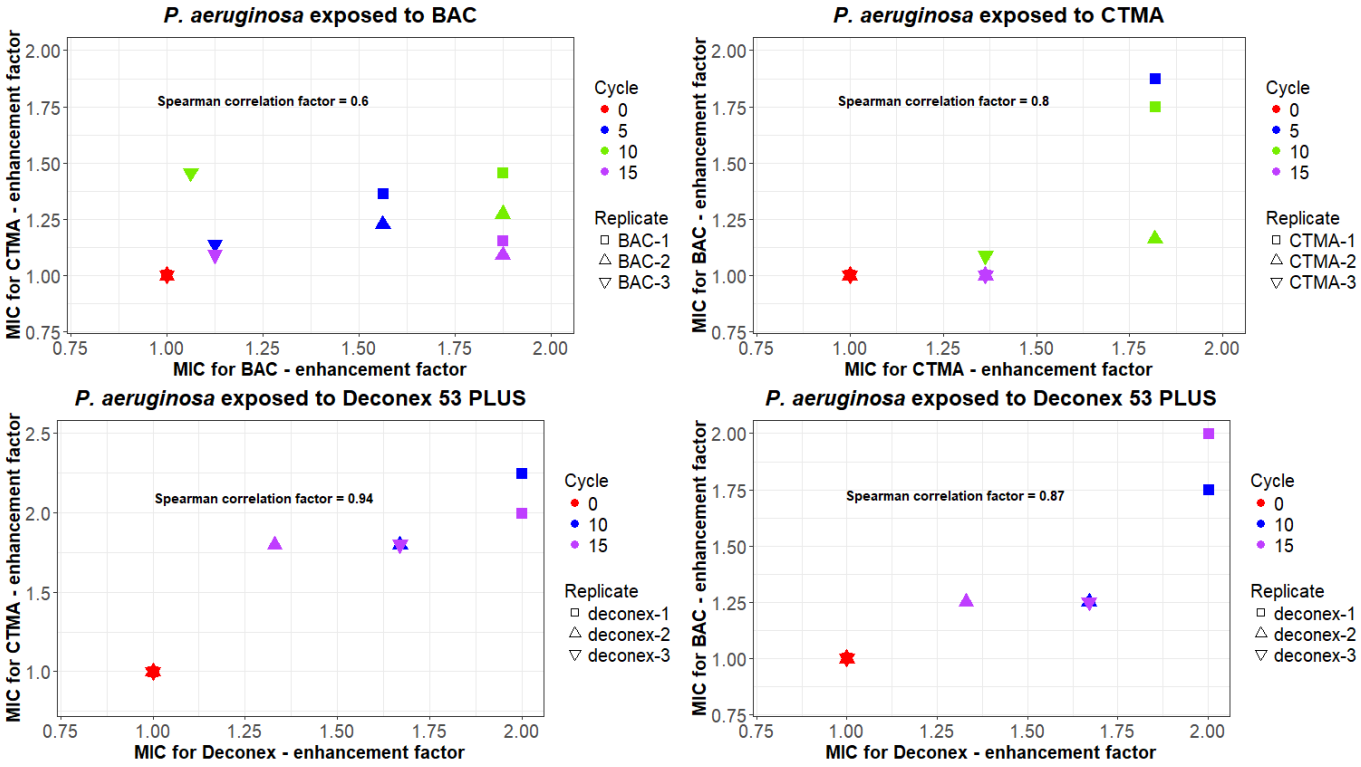


Figure 5. Correlation factors for the cross-resistance experiment. A. Correlation between the enhancement factor of the MIC for CTMA and the MIC for BAC when exposed to CTMA. **B.** Correlation between the enhancement factor of the MIC for BAC and the MIC for CTMA when exposed to CTMA. **C.** Correlation between the enhancement factor of the MIC for Deconex® 53 PLUS and the MIC for BAC when exposed to Deconex® 53 PLUS. **D.** Correlation between the enhancement factor of the MIC for Deconex® 53 PLUS and the MIC for CTMA when exposed to Deconex® 53 PLUS.

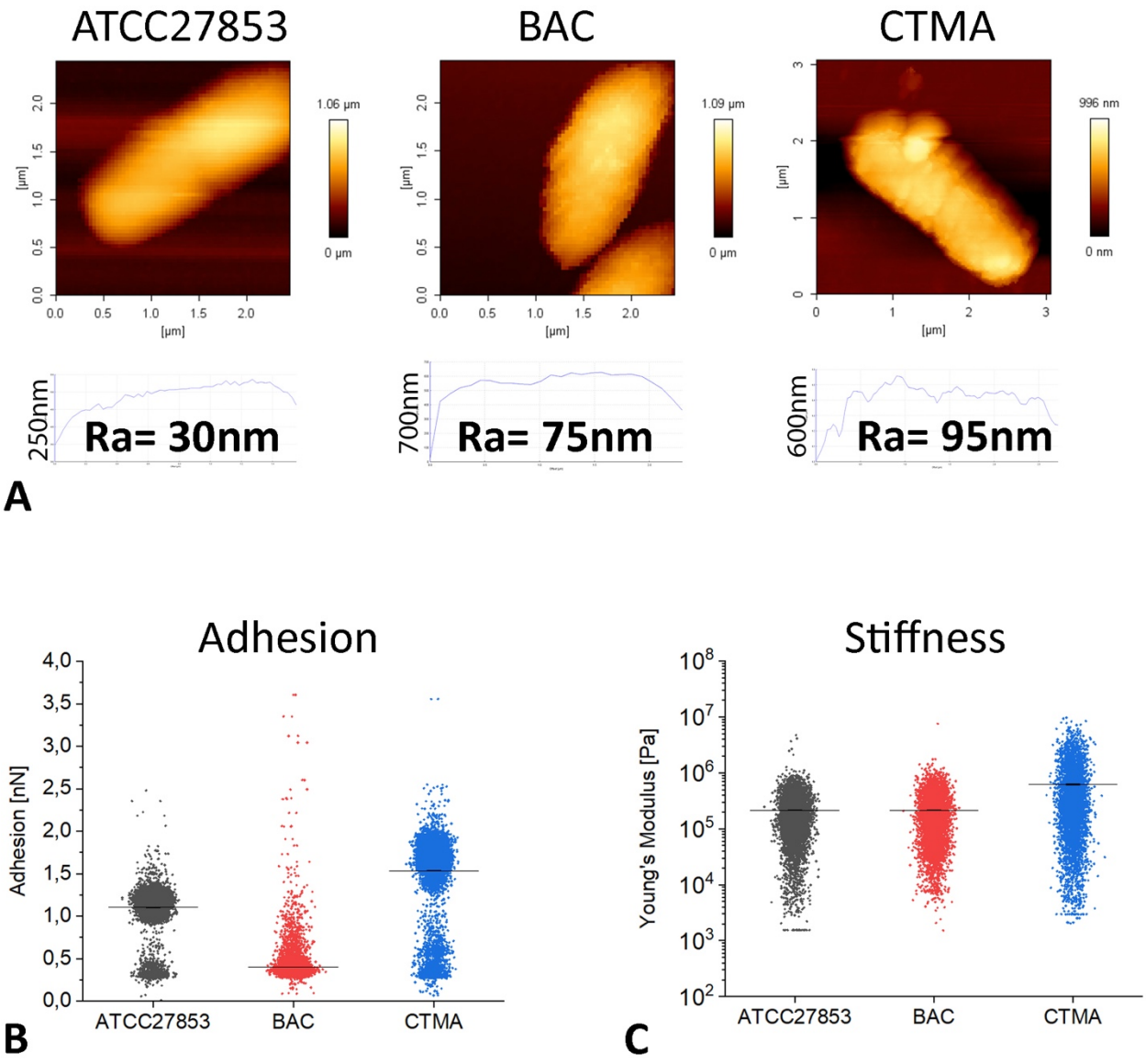


Figure 6. AFM investigation of the mechanical properties of *P. aeruginosa*. A: Two representative images are shown for each tested QAC (BAC, middle and CTMA, right) and one without treatment (ATCC 27583). Below, a typical profile for the outer membrane roughness (Ra) is plotted as its average value. The Y-axes scales differ significantly and from left to right are as follows: 250nm, 700nm and 600nm, respectively. Both treatments with QACs significantly changed the membrane average roughness as reported in numbers in each plot: 30, 75 and 95 nm, respectively. Statistical significance was assessed by the Mann-Whitney test and a p-value < 0.05 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.