**Pharmacodynamics of ciprofloxacin against *Pseudomonas aeruginosa* planktonic and biofilm-derived cells.**

Cláudia N. H. Marques1,2# and Shona M. Nelson3

1Department of Biological Sciences, Binghamton University, Binghamton, NY, 13902

2Binghamton Biofilm Research Center (BBRC), Binghamton University, Binghamton, NY, 13902

3Department of Applied Sciences, Faculty of Health and Applied Sciences, University of the West of England, Bristol, Coldharbour Lane, Bristol BS16 1QY, England, United Kingdom

# Corresponding author:

Mailing address:

Cláudia N. H. Marques

Binghamton University, State University of New York at Binghamton

Department of Biological Sciences

Biotechnology Building, BI2403

65 Murray Hill Road

Binghamton, NY 13902

Phone (607) 777-5755

Fax: (607) 777-6521

E-mail: cmarques@binghamton.edu

**Running title**

Susceptibility of *P. aeruginosa* biofilm-derived cells

**Topic:** Biofilms, antibiotics, Pseudomonads, resistance, microbial physiology

**SIGNIFICANCE AND IMPACT OF THE STUDY**

Removal of biofilms from surfaces and infection sites via disaggregation and induction of dispersion may reverse their antibiotic tolerant state. However, little is known of the recovery of the cells upon disaggregation from biofilms. Driven by this gap in knowledge we quantified the effect of ciprofloxacin on disaggregated biofilms of *Pseudomonas aeruginosa*, including those previously exposed to ciprofloxacin. Our results provide further insight into bacterial resilience, regrowth, and antimicrobial efficacy, as reduction of cell viability does not directly correlate with the metabolic activity of bacteria at the time of the exposure to antimicrobials. Thus, despite a perceived reduction in viability, the potential for cell persistence and regrowth remains and recovery is quicker upon subsequent exposure to antimicrobial, supporting the increase of resilience and recurrence of infections.

ABSTRACT

The influence of growth phase and state on survival and recovery of *Pseudomonas aeruginosa* exposed to ciprofloxacin was investigated using batch culture grown planktonic cells and disaggregated biofilm populations. Biofilms were either non-antibiotic exposed or previously exposed to ciprofloxacin before disaggregation and subsequent challenge with ciprofloxacin. Viable counts showed that late stationary phase cells were tolerant to ciprofloxacin over 24 h exposure, whilst all other populations presented a biphasic killing pattern. In contrast, the metabolic activity of planktonic and biofilm-derived cells remained similar to controls during the initial 6 h of ciprofloxacin exposure, despite a significant reduction in viable cell numbers. A similar effect was observed when assessing the PAE of 1 h ciprofloxacin exposure. Thus, although cell reduction occured, the metabolic status of the cells remained unchanged. The recovery of disaggregated biofilm cells previously exposed to ciprofloxacin was significantly quicker than naïve biofilm cells, and this latter population’s recovery was significantly slower than all planktonic populations. Results from this work have implications for our understanding of biofilm-related infections and their resilience to antimicrobial treatment.

**INTRODUCTION**

Biofilms are bacterial communities with a distinct phenotype and are considered to be responsible for most chronic infections. The main biofilm characteristics include differential gene expression, presence of a self-produced extracellular polymeric matrix (EPS) surrounding the cells, decreased susceptibility to antimicrobials (10-1000 times), and the ability to escape the host immune response (Gilbert *et al.* 2002; Stoodley *et al.* 2002; Southey-Pillig *et al.* 2005; Hurley *et al*. 2012; Bjarnsholt *et al.* 2013; Kostakioti *et al*. 2013). Thus, biofilm growth results in increased resistance to environmental stresses and an ability to persist within harsh environments (Stoodley *et al.* 2002). This resistance is based on multifactorial interactions of different physico-chemical, pharmacological and phenotypic properties (Prosser *et al.* 1987).

 Mechanisms thought to be responsible for the decrease of biofilm susceptibility include penetration failure due to the protection provided by EPS (Hoyle *et al*. 1990; Yassien *et al.* 1995; Stewart 1998; Schierholz *et al.* 1999; Allison 2003), the presence of a heterogeneous environment where cells experience slightly different micro-gradients of metabolic substrates and products (Kinniment and Wimpenny 1992), slow growth rate leading to physiological changes (Prosser *et al.* 1987; Brown *et al*. 1988; Gilbert *et al*. 1990; Brown and Barker 1999), the biofilm phenotype where a subpopulation of the community upregulates active mechanisms that combat the detrimental effects of antimicrobial agents (Kuchma and O’Toole 2000), and the presence of persisters (Spoering and Lewis 2001; Keren *et al.* 2004). For biofilm eradication to occur, antimicrobials must overcome all of these mechanisms simultaneously (Schierholz *et al.* 1999), a process difficult to accomplish (Anwar *et al.* 1989a; Anwar *et al.* 1989b; Anwar *et al*. 1990). Delaying of antimicrobial therapy implementation in patients decreases the possibility of biofilm eradication (Anwar *et al.* 1990); ideally, when using biofilm eradicating concentrations, bacteria can be killed before cells express antibiotic resistance genes (Anwar *et al.* 1992) and before biofilms are fully developed (Gupta *et al.* 2013).

When bacteria disperse from the biofilm, antibiotic sensitivity is restored, suggesting an adaptive resistance mechanism rather than a genetic alteration (Stewart 2002). Thus, an alternative means of biofilm eradication is by inducing biofilm dispersion. Upon dispersion from biofilms, cells become more susceptible to antimicrobials, albeit not completely identical to mid-exponential phase planktonic cells (Anderl *et al.* 2003; Fux *et al*. 2004). The reversion of dispersed cells to a susceptible state occurs gradually for a period of up to two hours (Chambers *et al*. 2017).

 In this work we sought to better understand the resilience of biofilms by quantifying the pharmacodynamics of *P. aeruginosa* biofilms disaggregated prior to and post-exposure to antibiotics and comparing it to planktonic cells populations. The antibiotic of choice was ciprofloxacin, a quinolone antibiotic that targets DNA gyrase, interfering with DNA supercoiling and activation of the SOS response, resulting in bacterial filamentation, vacuole formation and sometimes lysis (Gellert *et al.* 1977; Sugino *et al.* 1977; Elliot *et al*. 1987; Mason *et al.* 1995). To achieve this a bioluminescent reporter strain, *P. aeruginosa* PAO1 MCS5-lite, and monitored bioluminescence (light output) and viable counts during antibiotic exposure and recovery post-antibiotic. Each measurement provided different information as to the effect of the antimicrobial challenge (Beard *et al.* 2002). Bioluminescence has previously been shown to be a valuable reporter of bacterial metabolism and cell death in real-time (Nelson *et al.* 2003; Marques *et al.* 2005). Overall, we found that during post antibiotic exposure, contrary to viable counts, the metabolism did not initially decrease, indicating that although cells are not able to divide on an agar medium, they are still active. In addition, the recovery of disaggregated biofilm cells previously exposed to ciprofloxacin was significantly quicker than naïve biofilm cells. Furthermore, upon viable cell recovery, the metabolism of the cells seemed reduced thus, possibly allowing for the increase in cell number to be undetected by the immune system when in an infection, similarly to what has been recently found to occur for persister cells (Mina and Marques 2016).

RESULTS AND DISCUSSION

Resilience of biofilms to antimicrobials has been widely described and usually is considered to be similar to that of stationary phase cells (Spoering and Lewis 2001). Previously, we found that repeated exposure of perfused biofilms to ciprofloxacin resulted in a decrease in post antibiotic effect (PAE) for both biofilm and eluted cells, indicating increased resilience to antimicrobials (Nelson *et al.* 2003). Recently, inducing dispersion and disaggregation of biofilms has been suggested as a means to remove biofouling and to treat and clear infections (Davies 2003; Barraud *et al.* 2006, 2009; Marques *et al*. 2015). It is known that upon biofilm dispersion cells become more susceptible to antimicrobials, although they are not considered identical to mid-exponential phase planktonic cells (Anderl *et al.* 2003; Fux *et al*. 2004; Chambers *et al*. 2017). However, little is known on the recovery rate of these newly planktonic cells. In this work, biofilms which were either non-antibiotic exposed (QSS0), immediately post-exposed to ciprofloxacin (PE) or which had recovered from exposure to ciprofloxacin (QSS1) were disaggregated and challenged with ciprofloxacin. Time kill curve (TKC) experiments investigated 24 h ciprofloxacin exposure whilst PAE was assessed via exposure for 1 h then recovery following drug removal. For comparison, mid-exponential, early stationary and late stationary phase planktonic cells were similarly exposed to ciprofloxacin. Cell survival in all experiments was monitored by viable counting and bioluminescence measurements. Prior to performing time kill curves and post antibiotic effect determinations, the ciprofloxacin MIC of the various populations to be studied was determined and found to be: 0.25 mg l-1 for all planktonically-derived cells, 0.5 mg l-1 for QSS0, and 1 mg l-1 for QSS1 and PE.

Bioluminescence does not directly correlate with a reduction of survival

To compare the ciprofloxacin activity against cells in the three different planktonic physiological states, no dilution or transfer of cells to new media or PBS was carried out (Zeiler 1985; Spoering and Lewis 2001; Sufya *et al*. 2003). Instead, the cultures were maintained under the same conditions after addition of ciprofloxacin (5 mg l-1) to CDM, without any interference in the culture growth. Control, non-ciprofloxacin challenged, planktonic cultures of *P. aeruginosa* generally behaved as would be expected for cultures at each planktonic growth stage (Fig. 1). Upon resuspension in CDM, QSS0 and QSS1 disaggregated biofilm populations were identical in terms of cell numbers (Fig. 2 A and B) and metabolic activity (Fig. 2 C and D). QSS0 controls (Fig. 2A) had an initial lag phase of 135 min followed by exponential growth rate (µ) of 0.808 h-1 and duplication time (Td) of 0.858 h, whilst QSS1 controls (Fig. 2B) showed a shorter lag period (45 min) but a slower exponential µ (0.433 h-1) and Td (1.6 h).

 When challenged with ciprofloxacin for 24 h, a biphasic killing pattern was observed where the cell viability decreased in the first 6 hours, followed by a plateau. The viability reduction consisted of 5.2 Log for mid-exponential phase (Fig. 1A), 4.2 Log for early stationary phase (Fig. 1B), 5.5 Log for QQS0 (Fig. 2A) and 4.2 Log for QSS1 (Fig. 2B). Thus, QQS0 disaggregated biofilm cell survival was similar to mid-exponential phase cells, while QQS1 were similar to early stationary phase, as anticipated when correlating their growth rates, the latter 2 populations being slower growing. These observations contradict previous findings where viable counts of *P. aeruginosa* were reduced more effectively and rapidly in slowly growing bacteria (Dalhoff *et al*. 1995). The observed biphasic killing is a typical death curve supporting the presence of a persister cell sub-population (Sufya *et al*. 2003; Keren *et al.* 2004; Marques *et al.* 2014). In contrast to viable count measurements, bioluminescence remained relatively constant and similar to controls during the initial 6 h. Late stationary phase cultures (Fig. 1 C and F) displayed no change in cell viability and a more erratic bioluminescence measurements although, again, challenged cells appeared to be as metabolically active as non-challenged cells. The discrepancy between viable counts and bioluminescence has been previously described (Nelson *et al.* 2003; Marques *et al.* 2005) and could be occurring due to persister cell formation and an active SOS response (Keren *et al.* 2004; Niepa *et al*. 2012) as, the SOS response induces DNA repair which can be correlated with an increase of bioluminescence (Cutter *et al*. 2007; Alloush *et al.* 2010). Furthermore, these findings could also be due to the occurrence of cell elongation, as exposure to ciprofloxacin results in cell elongation (Zahller and Stewart 2002), and one elongated cell would only give rise to one CFU on agar, whilst the light output might correspond to the number of cells that make up the long, non-divided filament. Similarly, biomass would correspond to these undivided cells and even if, in the absence of ciprofloxacin, a filament eventually divided and individual cells separated, the overall biomass would still remain the same.

Disaggregated biofilms exposed to ciprofloxacin for the first time require longer time to recover.

When determining viable cell recovery following exposure to ciprofloxacin, we found that the time to recover to T= 0 h (Q T log) in mid-exponential phase cells (Fig. 3A) and late stationary phase cells (Fig. 3B) was longer than for early stationary phase cells (Fig. 3C) being 13.2, 15.8 and 9.6 h, respectively. As control cultures of stationary phase populations did not increase by one Log throughout the time of the experiments (Fig. 3B and C), calculation of Q PAE duration was only possible for mid-exponential phase cells, consisting of 5 h (Fig. 3A). This was anticipated as stationary phase cells are not in an active dividing state. Disaggregation of *P. aeruginosa*QSS0 biofilms and exposure to ciprofloxacin (5 mg l-1) as batch cultures resulted in an initial > 4 Log decline with a Q T log of 40.1 h and QPAE duration of 28.3 h (Fig. 4A). By contrast, the disaggregated QSS1 population presented an initial 2.7 Log decline followed by rapid increase of viable counts with a Q T Log of 10.6 h and a QPAE duration of 3.0 h (Fig. 4D). Thus, a quick recovery occurred in populations previously exposed to ciprofloxacin. This is in agreement with previous findings where following the first ciprofloxacin exposure, the number of cells shed on the eluate took longer to recover (Q T log of 24 h) than the one following subsequent ciprofloxacin exposures (Q T log of 8.2 h) (Nelson *et al.* 2003). Thus, disaggregation of biofilms prior to exposure to ciprofloxacin results in a PAE behaviour similar to eluate cells.

 When assessing bioluminescence, Q T log or QPAE calculations were not determined (ND) when ciprofloxacin exposed samples values were similar to controls (Fig. 3E and 3F). The Q T log of mid-exponential phase populations was 52.2 h and the bioluminescence levels indicated that cell metabolism was slower, although the metabolic activity began to increase 24 h post antibiotic (Fig. 3D). Bioluminescence measurements for both QSS0 and QSS1 resuspended biofilm cells (Fig. 4), differed significantly from controls. The bioluminescence of ciprofloxacin-treated QSS0 declined whilst it increased in controls for up to 30 h, followed by a sharp decrease (Fig. 4B). Interestingly, this decline only began 5 h after ciprofloxacin removal (Fig. 4B). QSS1 culture, previously exposed to ciprofloxacin as intact biofilms, did not present this marked decline in bioluminescence, and at 19 h post-ciprofloxacin challenge bioluminescence levels began to increase significantly (Fig. 4E), further confirming the resilience of previously exposed biofilms.The ciprofloxacin effect on the biofilm-derived cells (Fig. 4) was still present in the early stages of the experiment, particularly in bioluminescence, where despite its initial increase it remained relatively constant. This effect was in contrast to that observed in planktonic grown mid-exponential, early-stationary and late-stationary cultures (Fig. 3). A possible result of exposing biofilm cells to ciprofloxacin and enabling them to recover may have been the selection of a sub-population less susceptible to ciprofloxacin. However, the surviving cells were less metabolically active with a slow growth rate (Fig. 2). These finding were supported by the emergence of smaller, slower growing colonies, on agar plates where QSS1 (Fig. 4F) contained a high number of smaller morphotypes (Td: 1.375 h, µ=0.504 h-1), and QSS0 (Fig. 4E) contained a high number of large morphotypes (Td: 1 h, µ=0.693 h-1). Emergence of phenotypic diversity in *P. aeruginosa* biofilms has previously been reported for both *in vitro* and *in vivo* cultures and it is thought to lead to certain insurance benefits (Speert *et al.* 1990; Deziel *et al*. 2001; Haussler *et al.* 2003; Boles *et al.* 2004; Kirisits *et al.* 2005; Yarwood *et al.* 2007).

Exposure to ciprofloxacin as a biofilm followed by biofilm disaggregation results in a reduced cell recovery time similar to the biofilm recovery time.

To further investigate the effect of ciprofloxacin on biofilms, QSS0 cells were exposed to ciprofloxacin for a period of one hour, disaggregated, and immediately resuspended in CDM (PE). Upon resuspension, the PE population presented similar metabolic activity (Fig. 5B) and a 2.1 Log decrease in viable counts, compared to non-pre-exposed controls (Fig. 5A). When determining viable counts, PE had a lag phase of 3 h followed by exponential growth with QT Log and Q PAE duration of 6.5 h and 1.1 h, respectively (Fig. 5A). In contrast, bioluminescence decreased from 2 to 7 h, with 14.7 h Q T Log and 9.8 h of Q PAE duration (Fig. 5B). The Q T log determined for viable counts was shorter than the one found for all other populations (Fig. 3 and 4) in this study; however, it was similar to the one previously found for *P. aeruginosa* biofilms once exposed or re-exposed to ciprofloxacin (Nelson *et al.* 2003). Thus, confirming that cells exposed to ciprofloxacin while in biofilms are more resilient, possible due to the adsorption of ciprofloxacin by the EPS (Zhang *et al.* 2018), and that disaggregation of biofilms following exposure does not result in a change of their recovery time.

Overall our study demonstrated that repeated exposure of biofilms to ciprofloxacin results in a population with a reduced recovery time thus more resilience to antimicrobials. Disaggregation of biofilms immediately following the first ciprofloxacin exposure results in a recovery time similar to the one found in intact biofilms (Nelson *et al.* 2003), albeit with a higher metabolic activity. Disruption of biofilms prior to exposure to ciprofloxacin presents a pattern of recovery similar to the one found for the eluate shed from biofilms and for planktonic cells (Nelson *et al.* 2003), indicating that disruption decreases the tolerance of the cells to ciprofloxacin. Furthermore, in all populations studied, the initial metabolic activity of ciprofloxacin exposed cultures was identical to controls. Bioluminescence provided a means to monitor changes at the point in time when samples were taken. Cells were still alive, but not dividing, thus giving lower viable counts on agar medium. Previous work monitoring biofilm responses to ciprofloxacin with viable counts gave the impression that bacteria are rapidly killed. However, monitoring light output in real-time, indicates that cells continue metabolising for many hours after antibiotic addition, even though they may not multiply (Salisbury *et al.* 1999). Metabolising cells will continue to produce reactive oxygen species (Britigan *et al*. 1999) and extracellular proteins such as toxin A, proteases and elastases (Bjorn *et al*. 1979) leading to tissue damage, cleavage of transferrin and triggering further immune responses. Results from this work have implications in the implementation of antimicrobial treatment of infections, as they confirm that re-exposure of biofilms to antimicrobials results in a population further able to the withstand antimicrobial treatment and thus different treatment strategies will need to be implemented.

MATERIALS AND METHODS

Bacterial strains and culture media. *Pseudomonas aeruginosa* PAO1 MCS5-lite (Marques *et al.*, 2005) was maintained on nutrient agar (NA) containing gentamicin (10 mg l-1) and was cultured on iron-deplete chemically defined medium (CDM) (Hodgson *et al.* 1995). Ciprofloxacinsolution (200 mg l-1), included in the CDM for drug perfusionexperiments, was the gift of Southmead Hospital (Bristol, UK).

**Determination of minimum inhibitory concentration (MIC).** MICs of ciprofloxacin for *P. aeruginosa* PAO1 MCS5-lite were determined in CDM, using standard methods (Andrews 2001). Carrier controls consisted of CDM alone. Growth of bacteria was determined based on visually observed turbidity following 24 hr incubation at 37oC.

Biofilm cultures. Biofilms were grown using the Sorbarod *in vitro* continuous perfusion biofilm culture system described previously (Hodgson *et al.* 1995; Maira-Litrán *et al*. 2000; Marques *et al.* 2005) using the conditions described by Marques *et al.* (2005). Briefly, Sorbarods were inoculated with 1 ml of an exponential phase culture of *P. aeruginosa* PAO1 MCS5-lite. CDM with gentamicin (10 mg l-1), to maintain the plasmid, was delivered into the system at a controlled flow rate of 15 ml h-1. Once cultures reached quasi-steady-state (QSS0) at 24 h of culture (where the culture was shedding a constant number of cells in the eluted medium) ciprofloxacin (5 mg l-1) was perfused in the CDM for 1 hour. Subsequently thiswas replaced with fresh CDM and gentamicin (10 mg l-1) and the culture was once again allowed to reach a new steady state (QSS1). Thus, 3 biofilm time points were used in this study: QSS0, following antimicrobial exposure (PE) of QSS0, and QSS1. Each biofilm was processed by homogenization, as described previously (Hodgson *et al.* 1995; Marques *et al.* 2005) and resuspended in 50 ml of CDM (in an Erlenmeyer flask) with and without ciprofloxacin to be used for analysis of the cell survival (time kill curves) and post antibiotic recovery. PE samples were washed in 0.85% saline and then resuspended in 50 ml of fresh CDM.

Time-kill curves (TKC).Killing kinetics in the presence of ciprofloxacin (5 mg l-1) were monitored and established for *P. aeruginosa* PAO1 MCS5-lite at different growth stages of both planktonic and biofilm-derived cells. In planktonically-derived cultures, three growth stages were analysed: mid-exponential phase, early stationary phase and late stationary phase, all cultures were standardized to approximately 108 CFU ml-1. For biofilm-derived cultures, QSS0 and QSS1 populations (approximately 107 CFU ml-1) were disaggregated and resuspended into 50 ml of CDM, incubated at 37oC with shaking (200 rpm) and exposed to ciprofloxacin (5 mg l-1) for 24 h. Samples were taken every 45 min over 6 h, and at 24 h and quantified for viable counts (spiral plating onto nutrient agar; spiral plater Autoplate model 3000, Spiral Biotech, Bethesda, MD, USA) and bioluminescence (using a 1250–001 luminometer, BioOrbit, Finland), as described previously (Marques *et al.* 2005). Control cultures consisted of samples incubated in the absence of ciprofloxacin.

Post antibiotic effect (PAE).As with TKC, PAE analysis was carried out on *P. aeruginosa* PAO1 MCS5-lite mid-exponential phase, early stationary phase and late stationary phase planktonic cultures and on disaggregated QSS0, PE and QSS1 biofilm cultures. The planktonic and biofilm-derived cultures (approximately 108 CFU ml-1) were exposed to ciprofloxacin (5 mg l-1) for one hour at 37oC with shaking (200 rpm) in an Erlenmeyer flask. Bacterial cells were harvested by centrifugation (8500 x *g* for 20 min) and resuspended in fresh CDM. Samples were taken every hour for a period of at least 24 h and recovery was quantified by viable counts and bioluminescence. Controls consisted of samples non-exposed to ciprofloxacin. Experiments were carried out in triplicate. Q T log and the Q PAE duration PAE variables were calculated. Q T log was equal to the x value (h) on the second degree polynomial fitting curve that corresponded to the y value that was one log greater than the y value on the polynomial at T = 0 h (Smith *et al.* 2003). Q PAE duration was equal to Q T log treated – Q T log control (Smith *et al.* 2003).

**Statistical analysis.** All data was analysed using GraphPad Prism 7.0e. One-way ANOVA was performed for multivariant analysis followed by Tukey’s or Dunnett’s multiple comparison tests. In PAE determinations, second degree polynomial fitting was performed using the method of least squares.

**ACKNOWLEDGEMENTS**

We are grateful to Roger Lewis, Prof. Vyvyan Salisbury, Dr. Karen Bowker and Prof. John Greenman for many insightful discussions. CNHM was supported by HEFCE QR and by a BSAC project grant. Parts of this study were presented at the 103rd ASM General Meeting (C. Marques *et al.*).

**CONFLICT OF INTEREST:**

No conflict of interest declared.

**REFERENCES**

Allison, D.G. (2003) The biofilm matrix. *Biofouling* **19**, 139–150.

Alloush, H.M., Anderson, E., Martin, A.D., Ruddock, M.W., Angell, J.E., Hill, P.J., Mehta, P., Smith, M.A., Smith, J.G. and Salisbury, V.C. (2010) A bioluminescent microbial biosensor for in vitro pretreatment assessment of cytarabine efficacy in leukemia. *Clin Chem* **56**, 1862–1870.

Anderl, J.N., Zahller, J., Roe, F. and Stewart, P.S. (2003) Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* **47**, 1251–1256.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* **48**(suppl 1), 5–16.

Anwar, H., van Biesen, T., Dasgupta, M., Lam, K. and Costerton, J.W. (1989)a Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system. *Antimicrob Agents Chemother* **33**, 1824–1826.

Anwar, H., Dasgupta, M.K. and Costerton, J.W. (1990) Testing the susceptibility of bacteria in biofilms to antibacterial agents. *Antimicrob Agents Chemother* **34**, 2043–2046.

Anwar, H., Dasgupta, M., Lam, K. and Costerton, J.W. (1989)b Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. *J Antimicrob Chemother* **24**, 647–655.

Anwar, H., Strap, J.L., Chen, K. and Costerton, J.W. (1992) Dynamic interactions of biofilms of mucoid *Pseudomonas aeruginosa* with tobramycin and piperacillin. *Antimicrob Agents Chemother* **36**, 1208–1214.

Barraud, N., Hassett, D.J., Hwang, S.H., Rice, S.A., Kjelleberg, S. and Webb, J.S. (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 7344–7353.

Barraud, N., Schleheck, D., Klebensberger, J., Webb, J.S., Hassett, D.J., Rice, S.A. and Kjelleberg, S. (2009) Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic di-GMP levels, and enhanced dispersal. *J. Bacteriol* **191**, 7333–7342.

Beard, S.J., Salisbury, V., Lewis, R.J., Sharpe, J.A. and MacGowan, A.P. (2002) Expression of *lux* genes in a clinical isolate of *Streptococcus pneumoniae*: using bioluminescence to monitor gemifloxacin activity. *Antimicrob Agents Chemother* **46**, 538–542.

Bjarnsholt, T., Alhede, M., Alhede, M., Eickhardt-Sørensen, S.R., Moser, C., Kühl, M., Jensen, P.Ø. and Høiby, N. (2013) The *in vivo* biofilm. *Trends Microbiol* **21**, 466–474.

Bjorn, M.J., Sokol, P.A. and Iglewski, B.H. (1979) Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. *J Bacteriol* **138**, 193–200.

Boles, B.R., Thoendel, M. and Singh, P.K. (2004) Self-generated diversity produces “insurance effects” in biofilm communities. *Proc Natl Acad Sci USA* **101**, 16630–16635.

Britigan, B.E., Railsback, M.A. and Cox, C.D. (1999) The *Pseudomonas aeruginosa* secretory product pyocyanin inactivates alpha1 protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease. *Infect Immun* **67**, 1207–1212.

Brown, M.R. and Barker, J. (1999) Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends Microbiol* **7**, 46–50.

Brown, M.R.W., Allison, D.G. and Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother* **22**, 777–780.

Chambers, J.R., Cherny, K.E. and Sauer, K. (2017) Susceptibility of *Pseudomonas aeruginosa* dispersed cells to antimicrobial agents is dependent on the dispersion cue and class of the antimicrobial agent used. *Antimicrob Agents Chemother* **61**, e00846-17.

Cutter, K.L., Alloush, H.M. and Salisbury, V.C. (2007) Stimulation of DNA repair and increased light output in response to UV irradiation in *Escherichia coli* expressing *lux* genes. *Luminescence*, **22**, 177–181.

Dalhoff, A., Matutat, S. and Ullmann, U. (1995) Effect of quinolones against slowly growing bacteria. *Chemotherapy* **41**, 92–99.

Davies, D. (2003) Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* **2**, 114–122.

Deziel, E., Comeau, Y. and Villemur, R. (2001) Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* **183**, 1195–1204.

Elliot, T.S.J., Shelton, A. and Greenwood, D. (1987) The response of *Escherichia coli* to ciprofloxacin and norfloxacin. *J Med Microbiol* **23**, 83–88.

Fux, C.A., Wilson, S. and Stoodley, P. (2004) Detachment characteristics and oxacillin resistance of *Staphylococcus aureus* biofilm emboli in an *in vitro* cathether infection model. *J Bacteriol* **186**, 4486–4491.

Gellert, M., Mizuuchi, K., O’Dea, M.H., Itoh, T. and Tomizawa, J.I. (1977) Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc Natl Acad Sci USA* **74**, 4772–4776.

Gilbert, P., Collier, P.J. and Brown, M.R. (1990) Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrob Agents Chemother* **34**, 1865–1868.

Gilbert, P., McBain, A.J. and Bloomfield, S.F. (2002) Biocide abuse and antimicrobial resistance: being clear about the issues. *J Antimicrob Chemother* **50**, 137–139.

Gupta, K., Marques, C.N.H., Petrova, O.E. and Sauer, K. (2013) Antimicrobial tolerance of *Pseudomonas aeruginosa* biofilms is activated during an early developmental stage and requires the two-component hybrid SagS. *J Bacteriol* **195**, 4975–4987.

Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B. and Steinmetz, I. (2003) Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol* **52**, 295–301.

Hodgson, A.E., Nelson, S.M., Brown, M.R. and Gilbert, P. (1995) A simple *in vitro* model for growth control of bacterial biofilms. *J Appl Bacteriol* **79**, 87–93.

Hoyle, B.D., Jass, J. and Costerton, J.W. (1990) The biofilm glycocalyx as a resistance factor. *J Antimicrob Chemother* **26**, 1–5.

Hurley, M.N., Cámara, M. and Smyth, A.R. (2012) Novel approaches to the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. *Eur Respir J* **40**,1014–23.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y. and Lewis, K. (2004) Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**, 13–18.

Keren, I., Shah, D., Spoering, A., Kaldalu, N. and Lewis, K. (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J. Bacteriol* **186**, 8172–8180.

Kinniment, S.L. and Wimpenny, J.W. (1992) Measurements of the distribution of adenylate concentrations and adenylate energy charge across *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **58**, 1629–1635.

Kirisits, M.J., Prost, L., Starkey, M. and Parsek, M.R. (2005) Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **71**, 4809–4821.

Kostakioti, M., Hadjifrangiskou, M. and Hultgren, S.J. (2013) Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* **3**, 1–24.

Kuchma, S.L. and O’Toole, G.A. (2000) Surface-induced and biofilm-induced changes in gene expression. *Curr Opinion Biotechnol* **11**, 429–433.

Maira-Litrán, T., Allison, D.G. and Gilbert, P. (2000) An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump *acrAB* to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms. *J Antimicrob Chemother* **45**, 789–795.

Marques, C., Davies, D. and Sauer, K. (2015) Control of biofilms with the fatty acid signaling molecule *cis*-2-decenoic acid. *Pharmaceuticals* **8**, 816–835.

Marques, C.N.H., Morozov, A., Planzos, P. and Zelaya, H.M. (2014) The fatty acid signaling molecule *cis*-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial susceptible state. *Appl Environ Microbiol* **80**, 6976–6991.

Marques, C.N.H., Salisbury, V.C., Greenman, J., Bowker, K.E. and Nelson, S.M. (2005) Discrepancy between viable counts and light output as viability measurements, following ciprofloxacin challenge of self-bioluminescent *Pseudomonas aeruginosa* biofilms’, *J Antimicrob Chemother* **56**, 665–671.

Mason, D.J., Power, E.G., Talsania, H., Phillips, I. and Gant, V.A. (1995) Antibacterial action of ciprofloxacin. *Antimicrob Agents Chemother* **39**, 2752–2758.

Mina, E.G. and Marques, C.N.H. (2016) Interaction of *Staphylococcus aureus* persister cells with the host when in a persister state and following awakening. *Sci Rep* **6**, 31342.

Nelson, S.M., Marques, C.N.H., Greenman, J., Lewis, R.J., Bowker, K.E. and Salisbury, V.C. (2003) Real-time monitoring of metabolic activity in biofilms. In *Biofilm Communities: Order from Chaos?* Edited by . A. McBain, D. Allison, M. Brading, A. Rickard, J. Verran, and J. Walker. Cardiff: Bioline.

Niepa, T.H.R., Gilbert, J.L. and Ren, D. (2012) Controlling *Pseudomonas aeruginosa* persister cells by weak electrochemical currents and synergistic effects with tobramycin. *Biomaterials* **33**, 7356–7365.

Prosser, B.L., Taylor, D., Dix, B.A. and Cleeland, R. (1987) Method of evaluating effects of antibiotics on bacterial biofilm. *Antimicrob Agents Chemother* **31**, 1502–1506.

Salisbury, V., Pfoestl, A., Wiesinger-Mayr, H., Lewis, R., Bowker, K.E. and MacGowan, A.P. (1999) Use of a clinical *Escherichia coli* isolate expressing *lux* genes to study the antimicrobial pharmacodynamics of moxifloxacin. *J Antimicrob Chemother* **43**, 829–832.

Schierholz, J.M., Beuth, J., Konig, D., Nurnberger, A. and Pulverer, G. (1999) Antimicrobial substances and effects on sessile bacteria. *Zentralbl Bakteriol* **289**, 165–177.

Smith, R.P., Baltch, A.L., Michelsen, P.B., Ritz, W.J. and Alteri, R. (2003) Use of the microbial growth curve in postantibiotic effect studies of *Legionella pneumophila*. *Antimicrob Agents Chemother* **47**, 1081–1087.

Southey-Pillig, C.J., Davies, D.G. and Sauer, K. (2005) Characterization of temporal protein production in *Pseudomonas aeruginosa* biofilms. *J Bacteriol* **187**, 8114–8126.

Speert, D.P., Farmer, S.W., Campbell, M.E., Musser, J.M., Selander, R.K. and Kuo, S. (1990) Conversion of *Pseudomonas aeruginosa* to the phenotype characteristic of strains from patients with cystic fibrosis. *J Clin Microbiol* **28**, 188–194.

Spoering, A. and Lewis, K. (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**, 6746–6751.

Stewart, P.S. (1998) A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. *Biotechnol Bioeng* **59**, 261–272.

Stewart, P.S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol*  **292**, 107–113.

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002) Biofilms as complex differentiates communities. *Annu Rev Microbiol* **56**, 187–209.

Sufya, N., Allison, D.G. and Gilbert, P. (2003) Clonal variation in maximum specific growth rate and susceptibility towards antimicrobials. *J Appl Microbiol* **95**, 1261–1267.

Sugino, A., Peebles, C.L., Kreuzer, K.N. and Cozzarelli, N.R. (1977) Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc Natl Acad Sci USA*, **74**, 4767–4771.

Yarwood, J.M., Paquette, K.M., Tikh, I.B., Volper, E.M. and Greenberg, E.P. (2007) Generation of virulence factor variants in *Staphylococcus aureus* biofilms. *J Bacteriol* **189**, 7961–7967.

Yassien, M., Khardori, N., Ahmedy, A. and Toama, M. (1995) Modulation of biofilms of *Pseudomonas aeruginosa* by quinolones. *Antimicrob Agents Chemother* **39**, 2262–2268.

Zahller, J. and Stewart, P.S. (2002) Transmission electron microscopic study of antibiotic action on *Klebsiella pneumoniae* biofilm. *Antimicrob Agents Chemother* **46**, 2679–2683.

Zeiler, H.J. (1985) Evaluation of the *in vitro* bactericidal action of ciprofloxacin on cells of Escherichia coli in the logarithmic and stationary phases of growth. *Antimicrob Agents Chemother* **28**, 524–527.

Zhang, H., Jia, Y., Khanal, S.K., Lu, H., Fang, H. and Zhao, Q. (2018) Understanding the role of extracellular polymeric substances on ciprofloxacin adsorption in aerobic sludge, anaerobic sludge, and sulfate-reducing bacteria sludge systems. *Environ Sci Technol* **52**, 6476–6486.

**Figure 1. Time kill curves of planktonic cells.** *P. aeruginosa* cultures were grown to mid-exponential-phase (■/□), early-stationary-phase (●/○) and late-stationary-phase (♦/◊) stages and subsequently exposed to ciprofloxacin (5 mg l-1) for a period of 24 h. Bacterial killing was quantified by viable counts (CFU ml-1) (A, B, and C) and bioluminescence (mV ml-1) (D, E, and F). Open symbols represent control cultures (medium alone) and closed symbols represent ciprofloxacin exposed cultures. Values consist of the average of triplicate experiments and error bars represent SD. \* *P*<0.0001 compared to control.

**Figure 2.** **Time kill curves of biofilm-derived cells.** *P. aeruginosa* biofilms were allowed to reach QSS0 (■/□) (initial steady state), and QSS1 (●/○) (steady state reached following one h exposure to ciprofloxacin) and were subsequently disaggregated, resuspended in media with and without ciprofloxacin (5 mg l-1), and monitored for a period of 24 h. Bacterial killing was quantified by viable counts (CFU ml-1) (A and B) and bioluminescence (mV ml-1) (C and D). Open symbols represent control cultures (medium alone) and closed symbols represent ciprofloxacin exposed cultures. Values consist of the average of triplicate experiments and error bars represent SD. \* *P*<0.0001 compared to control.

**Figure 3.** **Post antibiotic effect (PAE) of planktonic cells.** *P. aeruginosa* cultures were grown to mid-exponential-phase (■/□), early-stationary-phase (●/○) and late-stationary-phase (♦/◊) stages, exposed to ciprofloxacin (5 mg l-1) for a period of one hour, and subsequently resuspended in fresh medium. Regrowth was monitored by viable counts (CFU ml-1) (A, B, and C) and bioluminescence (mV ml-1) (D, E, and F). Q T log and QPAE duration were determined. Open symbols represent control cultures (never exposed to ciprofloxacin) and closed symbols represent ciprofloxacin exposed cultures. Values consist of the average of triplicate experiments and error bars represent SD. \*\**P*<0.0001, \* *P*<0.001 compared to control. N.D. indicates that Q T log or QPAE were not able to be determined as no increase of one Log occurred for medium or ciprofloxacin exposed cultures.

**Figure 4.** Post antibiotic effect (PAE) of biofilm-derived cells. *P. aeruginosa* biofilms were allowed to reach QSS0 (■/□) (initial steady state), and QSS1 (●/○) (steady state reached following one-hour exposure to ciprofloxacin) and were subsequently disaggregated and resuspended in media with and without ciprofloxacin (5 mg l-1) for a period of one hour. Following this, the cultures were resuspended in fresh medium. Regrowth was monitored by viable counts (CFU ml-1) (A and B) and bioluminescence (mV ml-1) (C and D). Q T log and QPAE duration were determined. Open symbols represent control cultures (never exposed to ciprofloxacin) and closed symbols represent ciprofloxacin exposed cultures. Colony morphology was also monitored at the end of the experiment for QSS0 (E) and QSS1 (F) derived cells. Values consist of the average of triplicate experiments and error bars represent SD. \* *P*<0.001 compared to control. ND indicates that Q T Log or QPAE were not able to be determined as no increase of one Log occurred for medium or ciprofloxacin exposed cultures.

**Figure 5.** **Disaggregation of biofilms post ciprofloxacin exposure results in reduced PAE.** *P. aeruginosa* biofilms were exposed to ciprofloxacin (5 mg l-1) for a period of one hour once in QSS0 and subsequently disaggregated and resuspended in fresh medium. Regrowth was monitored by viable counts (CFU ml-1) (A) and bioluminescence (mV ml-1) (B). Q T log and QPAE duration were determined. Open symbols represent control cultures (never exposed to ciprofloxacin) and closed symbols represent ciprofloxacin exposed cultures. Values consist of the average of triplicate experiments and error bars represent SD. \* *P*<0.001 compared to control.