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

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Running title

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32

33 SIGNIFICANCE AND IMPACT OF THE STUDY

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

44

45 ABSTRACT

46 The influence of growth phase and state on survival and recovery of *Pseudomonas aeruginosa*
47 exposed to ciprofloxacin was investigated using batch culture grown planktonic cells and
48 disaggregated biofilm populations. Biofilms were either non-antibiotic exposed or previously
49 exposed to ciprofloxacin before disaggregation and subsequent challenge with ciprofloxacin. Viable
50 counts showed that late stationary phase cells were tolerant to ciprofloxacin over 24 h exposure,
51 whilst all other populations presented a biphasic killing pattern. In contrast, the metabolic activity of
52 planktonic and biofilm-derived cells remained similar to controls during the initial 6 h of
53 ciprofloxacin exposure, despite a significant reduction in viable cell numbers. A similar effect was
54 observed when assessing the PAE of 1 h ciprofloxacin exposure. Thus, although cell reduction
55 occured, the metabolic status of the cells remained unchanged. The recovery of disaggregated biofilm
56 cells previously exposed to ciprofloxacin was significantly quicker than naïve biofilm cells, and this

57 latter population's recovery was significantly slower than all planktonic populations. Results from
58 this work have implications for our understanding of biofilm-related infections and their resilience to
59 antimicrobial treatment.

60

61 **INTRODUCTION**

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

71 Mechanisms thought to be responsible for the decrease of biofilm susceptibility include
72 penetration failure due to the protection provided by EPS (Hoyle *et al*. 1990; Yassien *et al.* 1995;
73 Stewart 1998; Schierholz *et al.* 1999; Allison 2003), the presence of a heterogeneous environment
74 where cells experience slightly different micro-gradients of metabolic substrates and products
75 (Kinniment and Wimpenny 1992), slow growth rate leading to physiological changes (Prosser *et al.*
76 1987; Brown *et al*. 1988; Gilbert *et al*. 1990; Brown and Barker 1999), the biofilm phenotype where
77 a subpopulation of the community upregulates active mechanisms that combat the detrimental effects
78 of antimicrobial agents (Kuchma and O'Toole 2000), and the presence of persisters (Spoering and
79 Lewis 2001; Keren *et al.* 2004). For biofilm eradication to occur, antimicrobials must overcome all
80 of these mechanisms simultaneously (Schierholz *et al.* 1999), a process difficult to accomplish
81 (Anwar *et al.* 1989a; Anwar *et al.* 1989b; Anwar *et al*. 1990). Delaying of antimicrobial therapy

82 implementation in patients decreases the possibility of biofilm eradication (Anwar *et al.* 1990);
83 ideally, when using biofilm eradicating concentrations, bacteria can be killed before cells express
84 antibiotic resistance genes (Anwar *et al.* 1992) and before biofilms are fully developed (Gupta *et al.*
85 2013).

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

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

107 reduced thus, possibly allowing for the increase in cell number to be undetected by the immune
108 system when in an infection, similarly to what has been recently found to occur for persister cells
109 (Mina and Marques 2016).
110
111 **RESULTS AND DISCUSSION**

112 Resilience of biofilms to antimicrobials has been widely described and usually is considered to be
113 similar to that of stationary phase cells (Spoering and Lewis 2001). Previously, we found that repeated
114 exposure of perfused biofilms to ciprofloxacin resulted in a decrease in post antibiotic effect (PAE)
115 for both biofilm and eluted cells, indicating increased resilience to antimicrobials (Nelson *et al.* 2003).
116 Recently, inducing dispersion and disaggregation of biofilms has been suggested as a means to
117 remove biofouling and to treat and clear infections (Davies 2003; Barraud *et al.* 2006, 2009; Marques
118 *et al*. 2015). It is known that upon biofilm dispersion cells become more susceptible to antimicrobials,
119 although they are not considered identical to mid-exponential phase planktonic cells (Anderl *et al.*
120 2003; Fux *et al*. 2004; Chambers *et al*. 2017). However, little is known on the recovery rate of these
121 newly planktonic cells. In this work, biofilms which were either non-antibiotic exposed (QSS_0),
122 immediately post-exposed to ciprofloxacin (PE) or which had recovered from exposure to
123 ciprofloxacin (QSS_1) were disaggregated and challenged with ciprofloxacin. Time kill curve (TKC)
124 experiments investigated 24 h ciprofloxacin exposure whilst PAE was assessed via exposure for 1 h
125 then recovery following drug removal. For comparison, mid-exponential, early stationary and late
126 stationary phase planktonic cells were similarly exposed to ciprofloxacin. Cell survival in all
127 experiments was monitored by viable counting and bioluminescence measurements. Prior to
128 performing time kill curves and post antibiotic effect determinations, the ciprofloxacin MIC of the
129 various populations to be studied was determined and found to be: 0.25 mg l^{-1} for all planktonically-
130 derived cells, 0.5 mg l^{-1} for QSS_0, and 1 mg l^{-1} for QSS_1 and PE.
131

132 **Bioluminescence does not directly correlate with a reduction of survival**

133 To compare the ciprofloxacin activity against cells in the three different planktonic physiological 134 states, no dilution or transfer of cells to new media or PBS was carried out (Zeiler 1985; Spoering 135 and Lewis 2001; Sufya *et al*. 2003). Instead, the cultures were maintained under the same conditions 136 after addition of ciprofloxacin (5 mg l^{-1}) to CDM, without any interference in the culture growth. 137 Control, non-ciprofloxacin challenged, planktonic cultures of *P. aeruginosa* generally behaved as 138 would be expected for cultures at each planktonic growth stage (Fig. 1). Upon resuspension in CDM, 139 QSS_0 and QSS_1 disaggregated biofilm populations were identical in terms of cell numbers (Fig. 2 A 140 and B) and metabolic activity (Fig. 2 C and D). QSS_0 controls (Fig. 2A) had an initial lag phase of 141 135 min followed by exponential growth rate (μ) of 0.808 h^{-1} and duplication time (Td) of 0.858 h, 142 whilst QSS_1 controls (Fig. 2B) showed a shorter lag period (45 min) but a slower exponential μ (0.433 143 h^{-1}) and Td (1.6 h).

144 When challenged with ciprofloxacin for 24 h, a biphasic killing pattern was observed where the 145 cell viability decreased in the first 6 hours, followed by a plateau. The viability reduction consisted 146 of 5.2 Log for mid-exponential phase (Fig. 1A), 4.2 Log for early stationary phase (Fig. 1B), 5.5 Log 147 for QQS_0 (Fig. 2A) and 4.2 Log for QSS_1 (Fig. 2B). Thus, QQS_0 disaggregated biofilm cell survival 148 was similar to mid-exponential phase cells, while QQS_1 were similar to early stationary phase, as 149 anticipated when correlating their growth rates, the latter 2 populations being slower growing. These 150 observations contradict previous findings where viable counts of *P. aeruginosa* were reduced more 151 effectively and rapidly in slowly growing bacteria (Dalhoff *et al*. 1995). The observed biphasic killing 152 is a typical death curve supporting the presence of a persister cell sub-population (Sufya *et al*. 2003; 153 Keren *et al.* 2004; Marques *et al.* 2014). In contrast to viable count measurements, bioluminescence 154 remained relatively constant and similar to controls during the initial 6 h. Late stationary phase 155 cultures (Fig. 1 C and F) displayed no change in cell viability and a more erratic bioluminescence 156 measurements although, again, challenged cells appeared to be as metabolically active as non-

157 challenged cells. The discrepancy between viable counts and bioluminescence has been previously
158 described (Nelson *et al.* 2003; Marques *et al.* 2005) and could be occurring due to persister cell
159 formation and an active SOS response (Keren *et al.* 2004; Niepa *et al*. 2012) as, the SOS response
160 induces DNA repair which can be correlated with an increase of bioluminescence (Cutter *et al*. 2007;
161 Alloush *et al.* 2010). Furthermore, these findings could also be due to the occurrence of cell
162 elongation, as exposure to ciprofloxacin results in cell elongation (Zahller and Stewart 2002), and
163 one elongated cell would only give rise to one CFU on agar, whilst the light output might correspond
164 to the number of cells that make up the long, non-divided filament. Similarly, biomass would
165 correspond to these undivided cells and even if, in the absence of ciprofloxacin, a filament eventually
166 divided and individual cells separated, the overall biomass would still remain the same.
167

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

170 When determining viable cell recovery following exposure to ciprofloxacin, we found that the time
171 to recover to T= 0 h (Q T log) in mid-exponential phase cells (Fig. 3A) and late stationary phase cells
172 (Fig. 3B) was longer than for early stationary phase cells (Fig. 3C) being 13.2, 15.8 and 9.6 h,
173 respectively. As control cultures of stationary phase populations did not increase by one Log
174 throughout the time of the experiments (Fig. 3B and C), calculation of Q PAE duration was only
175 possible for mid-exponential phase cells, consisting of 5 h (Fig. 3A). This was anticipated as
176 stationary phase cells are not in an active dividing state. Disaggregation of *P. aeruginosa* QSS_0
177 biofilms and exposure to ciprofloxacin (5 mg l^{-1}) as batch cultures resulted in an initial > 4 Log
178 decline with a Q T log of 40.1 h and QPAE duration of 28.3 h (Fig. 4A). By contrast, the disaggregated
179 QSS_1 population presented an initial 2.7 Log decline followed by rapid increase of viable counts with
180 a Q T Log of 10.6 h and a QPAE duration of 3.0 h (Fig. 4D). Thus, a quick recovery occurred in
181 populations previously exposed to ciprofloxacin. This is in agreement with previous findings where

182 following the first ciprofloxacin exposure, the number of cells shed on the eluate took longer to
183 recover (Q T log of 24 h) than the one following subsequent ciprofloxacin exposures (Q T log of 8.2
184 h) (Nelson *et al.* 2003). Thus, disaggregation of biofilms prior to exposure to ciprofloxacin results in
185 a PAE behaviour similar to eluate cells.

186 When assessing bioluminescence, Q T log or QPAE calculations were not determined (ND)
187 when ciprofloxacin exposed samples values were similar to controls (Fig. 3E and 3F). The Q T log
188 of mid-exponential phase populations was 52.2 h and the bioluminescence levels indicated that cell
189 metabolism was slower, although the metabolic activity began to increase 24 h post antibiotic (Fig.
190 3D). Bioluminescence measurements for both QSS_0 and QSS_1 resuspended biofilm cells (Fig. 4),
191 differed significantly from controls. The bioluminescence of ciprofloxacin-treated QSS_0 declined
192 whilst it increased in controls for up to 30 h, followed by a sharp decrease (Fig. 4B). Interestingly,
193 this decline only began 5 h after ciprofloxacin removal (Fig. 4B). QSS_1 culture, previously exposed
194 to ciprofloxacin as intact biofilms, did not present this marked decline in bioluminescence, and at 19
195 h post-ciprofloxacin challenge bioluminescence levels began to increase significantly (Fig. 4E),
196 further confirming the resilience of previously exposed biofilms. The ciprofloxacin effect on the
197 biofilm-derived cells (Fig. 4) was still present in the early stages of the experiment, particularly in
198 bioluminescence, where despite its initial increase it remained relatively constant. This effect was in
199 contrast to that observed in planktonic grown mid-exponential, early-stationary and late-stationary
200 cultures (Fig. 3). A possible result of exposing biofilm cells to ciprofloxacin and enabling them to
201 recover may have been the selection of a sub-population less susceptible to ciprofloxacin. However,
202 the surviving cells were less metabolically active with a slow growth rate (Fig. 2). These finding were
203 supported by the emergence of smaller, slower growing colonies, on agar plates where QSS_1 (Fig.
204 4F) contained a high number of smaller morphotypes (Td: 1.375 h, μ=0.504 h^{-1}), and QSS_0 (Fig. 4E)
205 contained a high number of large morphotypes (Td: 1 h, μ=0.693 h^{-1}). Emergence of phenotypic
206 diversity in *P. aeruginosa* biofilms has previously been reported for both *in vitro* and *in vivo* cultures

207 and it is thought to lead to certain insurance benefits (Speert *et al.* 1990; Deziel *et al*. 2001; Haussler
208 *et al.* 2003; Boles *et al.* 2004; Kirisits *et al.* 2005; Yarwood *et al.* 2007).
209
210 **Exposure to ciprofloxacin as a biofilm followed by biofilm disaggregation results in a reduced**
211 **cell recovery time similar to the biofilm recovery time.**
212 To further investigate the effect of ciprofloxacin on biofilms, QSS_0 cells were exposed to
213 ciprofloxacin for a period of one hour, disaggregated, and immediately resuspended in CDM (PE).
214 Upon resuspension, the PE population presented similar metabolic activity (Fig. 5B) and a 2.1 Log
215 decrease in viable counts, compared to non-pre-exposed controls (Fig. 5A). When determining viable
216 counts, PE had a lag phase of 3 h followed by exponential growth with QT Log and Q PAE duration
217 of 6.5 h and 1.1 h, respectively (Fig. 5A). In contrast, bioluminescence decreased from 2 to 7 h, with
218 14.7 h Q T Log and 9.8 h of Q PAE duration (Fig. 5B). The Q T log determined for viable counts
219 was shorter than the one found for all other populations (Fig. 3 and 4) in this study; however, it was
220 similar to the one previously found for *P. aeruginosa* biofilms once exposed or re-exposed to
221 ciprofloxacin (Nelson *et al.* 2003). Thus, confirming that cells exposed to ciprofloxacin while in
222 biofilms are more resilient, possible due to the adsorption of ciprofloxacin by the EPS (Zhang *et al.*
223 2018), and that disaggregation of biofilms following exposure does not result in a change of their
224 recovery time.
225
226 Overall our study demonstrated that repeated exposure of biofilms to ciprofloxacin results in a
227 population with a reduced recovery time thus more resilience to antimicrobials. Disaggregation of
228 biofilms immediately following the first ciprofloxacin exposure results in a recovery time similar to
229 the one found in intact biofilms (Nelson *et al.* 2003), albeit with a higher metabolic activity.
230 Disruption of biofilms prior to exposure to ciprofloxacin presents a pattern of recovery similar to the
231 one found for the eluate shed from biofilms and for planktonic cells (Nelson *et al.* 2003), indicating

232 that disruption decreases the tolerance of the cells to ciprofloxacin. Furthermore, in all populations
233 studied, the initial metabolic activity of ciprofloxacin exposed cultures was identical to controls.
234 Bioluminescence provided a means to monitor changes at the point in time when samples were taken.
235 Cells were still alive, but not dividing, thus giving lower viable counts on agar medium. Previous
236 work monitoring biofilm responses to ciprofloxacin with viable counts gave the impression that
237 bacteria are rapidly killed. However, monitoring light output in real-time, indicates that cells continue
238 metabolising for many hours after antibiotic addition, even though they may not multiply (Salisbury
239 *et al.* 1999). Metabolising cells will continue to produce reactive oxygen species (Britigan *et al*. 1999)
240 and extracellular proteins such as toxin A, proteases and elastases (Bjorn *et al*. 1979) leading to tissue
241 damage, cleavage of transferrin and triggering further immune responses. Results from this work
242 have implications in the implementation of antimicrobial treatment of infections, as they confirm that
243 re-exposure of biofilms to antimicrobials results in a population further able to the withstand
244 antimicrobial treatment and thus different treatment strategies will need to be implemented.

245

246 **MATERIALS AND METHODS**

247 **Bacterial strains and culture media.** *Pseudomonas aeruginosa* PAO1 MCS5-lite (Marques *et al.*,
248 2005) was maintained on nutrient agar (NA) containing gentamicin (10 mg l^{-1}) and was cultured on
249 iron-deplete chemically defined medium (CDM) (Hodgson *et al.* 1995). Ciprofloxacin solution (200
250 mg l^{-1}), included in the CDM for drug perfusion experiments, was the gift of Southmead Hospital
251 (Bristol, UK).

252
253 **Determination of minimum inhibitory concentration (MIC).** MICs of ciprofloxacin for *P.*
254 *aeruginosa* PAO1 MCS5-lite were determined in CDM, using standard methods (Andrews 2001).
255 Carrier controls consisted of CDM alone. Growth of bacteria was determined based on visually
256 observed turbidity following 24 hr incubation at 37°C.

257

258 **Biofilm cultures.** Biofilms were grown using the Sorbarod *in vitro* continuous perfusion biofilm
259 culture system described previously (Hodgson *et al.* 1995; Maira-Litrán *et al*. 2000; Marques *et al.*
260 2005) using the conditions described by Marques *et al.* (2005). Briefly, Sorbarods were inoculated
261 with 1 ml of an exponential phase culture of *P. aeruginosa* PAO1 MCS5-lite. CDM with gentamicin
262 (10 mg l^{-1}), to maintain the plasmid, was delivered into the system at a controlled flow rate of 15 ml
263 h^{-1}. Once cultures reached quasi-steady-state (QSS_0) at 24 h of culture (where the culture was
264 shedding a constant number of cells in the eluted medium) ciprofloxacin (5 mg l^{-1}) was perfused in
265 the CDM for 1 hour. Subsequently this was replaced with fresh CDM and gentamicin (10 mg l^{-1}) and
266 the culture was once again allowed to reach a new steady state (QSS_1). Thus, 3 biofilm time points
267 were used in this study: QSS_0, following antimicrobial exposure (PE) of QSS_0, and QSS_1. Each
268 biofilm was processed by homogenization, as described previously (Hodgson *et al.* 1995; Marques
269 *et al.* 2005) and resuspended in 50 ml of CDM (in an Erlenmeyer flask) with and without
270 ciprofloxacin to be used for analysis of the cell survival (time kill curves) and post antibiotic recovery.
271 PE samples were washed in 0.85% saline and then resuspended in 50 ml of fresh CDM.

272
273 **Time-kill curves (TKC).** Killing kinetics in the presence of ciprofloxacin (5 mg l^{-1}) were monitored
274 and established for *P. aeruginosa* PAO1 MCS5-lite at different growth stages of both planktonic and
275 biofilm-derived cells. In planktonically-derived cultures, three growth stages were analysed: mid-
276 exponential phase, early stationary phase and late stationary phase, all cultures were standardized to
277 approximately 10^8 CFU ml^{-1}. For biofilm-derived cultures, QSS_0 and QSS_1 populations
278 (approximately 10^7 CFU ml^{-1}) were disaggregated and resuspended into 50 ml of CDM, incubated at
279 37ºC with shaking (200 rpm) and exposed to ciprofloxacin (5 mg l^{-1}) for 24 h. Samples were taken
280 every 45 min over 6 h, and at 24 h and quantified for viable counts (spiral plating onto nutrient agar;
281 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 QSS_0, PE and QSS_1 biofilm cultures. The planktonic and biofilm-derived cultures (approximately 10^8 CFU ml^{-1}) were exposed to ciprofloxacin (5 mg l^{-1}) for one hour at 37°C 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.

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307

308 **CONFLICT OF INTEREST:**

309 No conflict of interest declared.

310

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466

467 **Figure 1. Time kill curves of planktonic cells.** *P. aeruginosa* cultures were grown to mid-
468 exponential-phase (■/□), early-stationary-phase (●/○) and late-stationary-phase (♦/◊) stages and

469 subsequently exposed to ciprofloxacin (5 mg l^{-1}) for a period of 24 h. Bacterial killing was quantified
470 by viable counts (CFU ml^{-1}) (A, B, and C) and bioluminescence (mV ml^{-1}) (D, E, and F). Open
471 symbols represent control cultures (medium alone) and closed symbols represent ciprofloxacin
472 exposed cultures. Values consist of the average of triplicate experiments and error bars represent SD.
473 * $P<0.0001$ compared to control.

474

475 **Figure 2. Time kill curves of biofilm-derived cells.** *P. aeruginosa* biofilms were allowed to reach
476 QSS_0 (■/□) (initial steady state), and QSS_1 (●/○) (steady state reached following one h exposure to
477 ciprofloxacin) and were subsequently disaggregated, resuspended in media with and without
478 ciprofloxacin (5 mg l^{-1}), and monitored for a period of 24 h. Bacterial killing was quantified by viable
479 counts (CFU ml^{-1}) (A and B) and bioluminescence (mV ml^{-1}) (C and D). Open symbols represent
480 control cultures (medium alone) and closed symbols represent ciprofloxacin exposed cultures.
481 Values consist of the average of triplicate experiments and error bars represent SD. * $P<0.0001$
482 compared to control.

483

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

493

494 **Figure 4.** Post antibiotic effect (PAE) of biofilm-derived cells. *P. aeruginosa* biofilms were allowed
495 to reach QSS_0 (■/□) (initial steady state), and QSS_1 (●/○) (steady state reached following one-hour
496 exposure to ciprofloxacin) and were subsequently disaggregated and resuspended in media with and
497 without ciprofloxacin (5 mg l^{-1}) for a period of one hour. Following this, the cultures were
498 resuspended in fresh medium. Regrowth was monitored by viable counts (CFU ml^{-1}) (A and B) and
499 bioluminescence (mV ml^{-1}) (C and D). Q T log and QPAE duration were determined. Open symbols
500 represent control cultures (never exposed to ciprofloxacin) and closed symbols represent
501 ciprofloxacin exposed cultures. Colony morphology was also monitored at the end of the experiment
502 for QSS_0 (E) and QSS_1 (F) derived cells. Values consist of the average of triplicate experiments and
503 error bars represent SD. * $P<0.001$ compared to control. ND indicates that Q T Log or QPAE were
504 not able to be determined as no increase of one Log occurred for medium or ciprofloxacin exposed
505 cultures.

506

507 **Figure 5. Disaggregation of biofilms post ciprofloxacin exposure results in reduced PAE.** *P.*
508 *aeruginosa* biofilms were exposed to ciprofloxacin (5 mg l^{-1}) for a period of one hour once in QSS_0
509 and subsequently disaggregated and resuspended in fresh medium. Regrowth was monitored by
510 viable counts (CFU ml^{-1}) (A) and bioluminescence (mV ml^{-1}) (B). Q T log and QPAE duration were
511 determined. Open symbols represent control cultures (never exposed to ciprofloxacin) and closed
512 symbols represent ciprofloxacin exposed cultures. Values consist of the average of triplicate
513 experiments and error bars represent SD. * $P<0.001$ compared to control.