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Hyperbaric oxygen sensitizes anoxic Pseudomonas aeruginosa biofilm to ciprofloxacin

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1 **Title:**

2 **Hyperbaric oxygen sensitizes anoxic *Pseudomonas aeruginosa* biofilm to ciprofloxacin**

3 **Running title:**

4 **HBOT sensitizes *P. aeruginosa* biofilm to ciprofloxacin**

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42 ## Abstract

43 Chronic *Pseudomonas aeruginosa* lung infection is characterized by the presence of endobronchial
44 antibiotic-tolerant biofilm subject to strong oxygen (O_2) depletion due to the activity of surrounding
45 polymorphonuclear leukocytes. The exact mechanisms affecting the antibiotic susceptibility of
46 biofilms remain unclear, but accumulating evidence suggests that the efficacy of several
47 bactericidal antibiotics is enhanced by stimulation of aerobic respiration of pathogens, while lack of
48 O_2 increases their tolerance. In fact, the bactericidal effect of several antibiotics depends on active
49 aerobic metabolism activity and the endogenous formation of reactive O_2 radicals (ROS). In this
50 study we aimed to apply hyperbaric oxygen treatment (HBOT) in order to sensitize anoxic *P.*
51 *aeruginosa* agarose-biofilms established to mimic situations with intense O_2 consumption by the
52 host response in the cystic fibrosis (CF) lung. Application of HBOT resulted in enhanced
53 bactericidal activity of ciprofloxacin at clinically relevant durations and was accompanied by
54 indications of restored aerobic respiration, involvement of endogenous lethal oxidative stress and
55 increased bacterial growth. The findings highlight that oxygenation by HBOT improves the
56 bactericidal activity of ciprofloxacin on *P. aeruginosa* biofilm and suggest that bacterial biofilms is
57 sensitized to antibiotics by supplying hyperbaric O_2.

58

59 ## Introduction

60 Chronic pulmonary infection with *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients is the
61 first biofilm infection described in humans (1). In CF patients, the chronic lung infection with *P.*
62 *aeruginosa* constitutes the major cause of increased morbidity and mortality (2). Therefore, the
63 dramatically increased tolerance of *P. aeruginosa* biofilms to antibiotics is a critical challenge for
64 improving the antibiotic treatment of chronic lung infections in CF patients (3). Increased tolerance
65 of *P. aeruginosa* biofilms to antibiotics is multi-factorial (4) and may to some extent depend on

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66 restriction of molecular oxygen (O_2) (5, 6), which is distributed at low levels reaching anoxia in
67 parts of the endobronchial secretions of chronically infected CF patients (7-9). Since O_2 is a
68 prerequisite for aerobic respiration, shortage of O_2 may decelerate aerobic respiration leading to
69 increased tolerance to several antibiotics (10-12). This enhanced tolerance possibly relies on
70 decreased expression of antibiotic targets and antibiotic uptake (13) as well as reduced endogenous
71 lethal oxidative stress in response to downstream events resulting from interaction between drugs
72 and targets (11, 12). In accordance, we have previously shown that re-oxygenation of O_2 depleted
73 *P. aeruginosa* biofilms using hyperbaric O_2 treatment (HBOT) increases the susceptibility to
74 ciprofloxacin (14). In that study the O_2 was removed by bacterial aerobic respiration (14). However,
75 this may be in contrast to the consumption of O_2 in the endobronchial secretions of CF patients
76 where the vast majority of O_2 is consumed by the PMNs for production of reactive O_2 species
77 (ROS) and nitric oxide (NO) whereas only a minute part of O_2 was consumed by aerobic respiration
78 (8, 15). In fact, ongoing anaerobic respiration and low *in vivo* growth rates of *P. aeruginosa*
79 biofilms (16) and of several other bacterial pathogens (17-19) suggest limited bacterial aerobic
80 respiration (20). Therefore in order to mimic situations in CF lungs where intense O_2 consumption
81 by activated PMNs prevents engagement of bacterial aerobic respiration we have grown bacterial
82 biofilm without O_2 prior to antibiotic treatment and HBOT. Using this approach, we aimed to
83 examine if absent aerobic respiration may be restored by HBOT for clinically relevant durations
84 leading to increased bactericidal effect of ciprofloxacin.

85

86 ## Results

87 **Effect of HBOT on *P. aeruginosa* biofilm during ciprofloxacin treatment.**

88 Significantly less PAO1 bacteria survived 90 min of treatment with ciprofloxacin when HBOT was
89 applied ($p < 0.0001$, n =13-19) (Fig. 1a). The maximum enhancement of bacterial killing by HBOT

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90 exceeded 2 log units when supplemented with 0.5 mg L^{-1} of ciprofloxacin indicating that HBOT
91 exposed *P. aeruginosa* biofilm can be treated with lower ciprofloxacin concentrations than controls.
92 It is striking that the potentiation of ciprofloxacin is stronger after 90 min of HBOT than for 2 h of
93 HBOT as previously reported (14). However, the present model has been developed to better
94 represent the *in vivo* microenvironment where *P. aeruginosa* is deprived of O_2 due to intense O_2
95 depletion by the surrounding PMNs creating anoxia (8). Furthermore, the depth of the agarose
96 embedded biofilm has been decreased in order for O_2 to penetrate through larger parts of entire
97 biofilm within 90 min.
98 In *P. aeruginosa* a major part of detoxification of ROS is contributed by catalase enzymes encoded
99 by the katA gene (21, 22). Accordingly, the increased susceptibility to antibiotics in mutants with
100 defective katA expression as well as enhanced tolerance to antibiotics in mutants with
101 overexpression of catalase are recognized as direct evidence for a lethal effect of ROS generation
102 during antibiotic treatment (12, 23, 24).
103 Therefore we employed Δ*katA* biofilms to elucidate that ROS play a role in the increased lethality
104 of ciprofloxacin during HBOT. We found significantly less Δ*katA* bacteria surviving 90 min of
105 treatment with ciprofloxacin when HBOT was applied compared with PAO1 biofilms ($p < 0.0024$,
106 $n = 11\text{-}14$), demonstrating a contribution of oxidative stress to decreased bacterial survival (Fig 1b).
107 This indicates that HBOT enabled aerobic respiration allowing ciprofloxacin to induce formation of
108 lethal amounts of ROS (10). However, an increased susceptibility of Δ*katA* was only seen for the
109 higher concentrations of ciprofloxacin suggesting that other anti-oxidative mechanisms protects
110 against the ROS produced during treatment with low amounts of ciprofloxacin (10).
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114 **HBOT expands the bactericidal zone of ciprofloxacin treatment in *P. aeruginosa* biofilm.**

115 *P. aeruginosa* embedded in agarose that grows in discrete aggregates was detected by confocal
116 microscopy (Fig 2) (25). Variations in aggregate size may depend on whether initiation is from
117 single or multiple cells. Aggregate diameter was significantly larger after 90 min of HBOT (100 %
118 O_2, 2.8 bar) than after anoxia (median diameter (range) (µm)): 37 µm (9-193) vs 23 (7-66); $p <$
119 0.0001, $n = 139$) estimated from live/dead staining of samples without ciprofloxacin treatment in
120 the upper 100 µm of the agarose embedded biofilm. Aggregate volume was 4.2 fold larger after 90
121 min of HBOT than after anoxia (median volume (μm^3): 27 vs 6.4, $n = 139$), indicative of 4.2 fold
122 more bacterial cells and an additional 2 divisions compared to anoxic treatment. Furthermore, the PI
123 experiments were intended to confirm the statistically significant difference found with CFU
124 counting and to visualize the increased zone of bactericidal activity caused by HBOT during
125 ciprofloxacin treatment.

126

127 **HBOT stimulates growth in *P. aeruginosa* biofilm.**

128 Untreated PAO1 biofilms embedded in agarose were exposed to HBOT with a significantly
129 increased bacterial growth demonstrated during the 90 min of incubation ($p < 0.0001$, $n =19$).
130 Compared with growth under anoxic conditions, HBOT increased the density of PAO1 biofilms
131 without antibiotic treatment indicating that aerobic respiration increases bacterial growth (Fig 3). In
132 fact, 90 min of HBOT increased the bacterial growth by ½ log as compared to anaerobic growth.

133

134 **Distribution of O_2 in *P. aeruginosa* biofilm after HBOT**

135 Vertical profiling of O_2 concentration in the agarose-embedded biofilm immediately after
136 termination of 90 minutes of HBOT, demonstrated O_2 concentrations exceeding 1000 µmol L^{-1} in
137 the media above the biofilm surface (Fig 4). Serial profiling revealed both rapid depletion of O_2 in

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138 the upper part of the biofilm and O_2 diffusion from the supernatant to the normobaric atmosphere.
139 However, within 20 min post HBOT, the zone of O_2 depletion inside the biofilm was expanded and
140 the O_2 concentration of the supernatant decreased below atmospheric saturation, indicating that
141 PAO1 was utilising the available O_2 for aerobic respiration until O_2-depletion in the biofilm would
142 necessitate conversion to anaerobic respiration (Fig 4).

143 O_2 diffusion through the agarose gel alone was detected at agarose concentrations from 0.125% to
144 2%. As expected (26), no significant concentration dependence or deviation from free diffusion was
145 observed and accordingly the assumption made that O_2 diffusion is not hindered by agarose or
146 water in the biofilm model (data not shown).

147 Ciprofloxacin efficacy is known to be linked to growth in view of the quinolone target's increased
148 activity during DNA replication both planktonically and in biofilms (27, 28). However, the inability
149 to respire during aerobic respiration allows bacteria to arrest growth in a manner that increases
150 tolerance. This study shows that addition of O_2 sensitizes bacteria by stimulating growth in areas
151 deprived of O_2. It has been shown previously that quinolones also have a bactericidal effect on
152 flow-cell biofilms, but that subpopulations remained tolerant to treatment. Similarly, our results on
153 non-attached biofilm reflecting a more accurate representation of chronic lung infection show a
154 bactericidal effect of ciprofloxacin improved with HBOT.

155

156 **Discussion**

157 *P. aeruginosa* is clinically a very important respiratory pathogen that causes the most severe
158 complication of chronic lung infection in CF patients (2). Throughout the chronic infection state,
159 microbial biofilms form as cell aggregates and become trapped in the endobronchial mucus (29)
160 with the host response creating chemical microenvironments favouring bacterial physiology
161 associated with tolerance against multiple antibiotics (20). Therefore, new treatment strategies are

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162 required to overcome these resilient bacterial infections HBOT has beneficial effects on the
163 treatment of a number of infectious diseases, clinically, experimentally and *in vitro* (14, 20, 30)
164 although whether these can be expanded to biofilm infections has not been extensively examined.
165 The present study utilised a model in which anoxic *P. aeruginosa* was embedded in an agarose gel,
166 trapping bacteria as aggregates throughout the gel in order to mimic biofilm infection *in vivo* (14,
167 30-32).

168 Few studies have shown that HBOT can be used as an adjuvant to ciprofloxacin treatment on *P.*
169 *aeruginosa* (33, 34) and to our knowledge our recently published proof-of-concept study provided
170 the first demonstration that HBOT can enhance the bactericidal activity of ciprofloxacin on biofilms
171 (14). In the present study it has been substantiated that bactericidal activity of ciprofloxacin is
172 enhanced after only 90 minutes of HBOT, representing a typical time frame used clinically for
173 HBOT (35, 36). The Undersea and Hyperbaric Medical Society recommends 90 to 120 min of
174 HBOT per session (37). Prior to HBOT, bacterial growth supported by aerobic respiration in the
175 biofilm model was prevented by O_2 exclusion while addition of NO_3^- enabled anaerobic respiration
176 by denitrification (38, 39). The rapid decrease from hyperoxia to hypoxia demonstrated by serial
177 measurements of O_2 concentration profiles in the biofilm immediately after HBOT indicated
178 engagement of aerobic bacterial respiration during HBOT with this metabolic shift likely explaining
179 the observation of faster growth of PAO1 under HBOT (40). Induction of increased metabolic
180 activity by HBOT was further indicated by increased SYTO9 fluorescence intensity and bacterial
181 aggregate size after HBOT resembling colonies in metabolically active zones in similar biofilm
182 models (31, 41).

183 Consequentially, activation of aerobic respiration by HBOT may contribute to the enhanced
184 bactericidal activity of ciprofloxacin by accelerating bacterial growth, as the susceptibility to
185 ciprofloxacin of *P. aeruginosa* biofilm is correlated to growth rate (42).

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186 In addition to a growth-related enhancement of ciprofloxacin treatment during HBOT, it was
187 speculated that HBOT induced re-oxygenation of the biofilm leads to accumulation of cytotoxic
188 ROS in response to ciprofloxacin. Induction of endogenous production of cytotoxic ROS has been
189 shown to contribute to the aerobic killing of planktonic bacteria by several major classes of
190 antibiotics (11, 12, 43) including aerobic *P. aeruginosa* biofilms (44) although the significance of
191 this has been challenged (11, 45, 46). However, increased susceptibility to antibiotics of mutants
192 with deficient anti-oxidative defence is regarded as solid indication for a contribution of ROS to the
193 bactericidal effect of antibiotics (23). Thus, the increased killing of the Δ*katA*-mutant in our study
194 supports that endogenous generation of ROS can contribute to an enhanced bactericidal effect of
195 ciprofloxacin on biofilm during adjuvant HBOT. Growth of Δ*katA* was not impaired with HBOT in
196 the absence of ciprofloxacin treatment as compared to the wild-type, indicating a lack of cytotoxic
197 ROS generation by HBOT alone (data not shown).
198 Biofilm infections are notoriously difficult to eradicate with antimicrobial treatment, as frequently
199 higher concentrations of antibiotics are required for killing of biofilms compared to planktonic
200 bacteria, with these concentrations being difficult to match *in vivo* (47). Our finding of a
201 significantly increased bacterial killing during HBOT with only 2 x MIC and 4 x MIC of
202 ciprofloxacin indicates that by using HBOT, *P. aeruginosa* biofilms can be effectively treated with
203 lower ciprofloxacin levels, that are attainable *in vivo*.
204 Although still controversial, there is an increasing acceptance of the advantages of HBOT with a
205 small number of studies focusing on the use of HBOT on biofilm infections e.g. associated with
206 periodontal disease, osteomyelitis and chronic wounds (48-50). The effect of HBOT on biofilm
207 infections in the pulmonary system remain largely unknown, though some studies have
208 demonstrated the beneficial effect of HBOT in patients with acute abscesses and in experimental
209 pulmonary infection models with *P. aeruginosa* (51, 52). The feasibility of HBOT to sensitize

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210 infectious biofilm to antibiotics in patients is indicated by the fact of PAO1 being a clinical isolate
211 from a burn wound (53, 54). In addition, we have recently demonstrated potentiation of tobramycin
212 by HBOT on both *in vitro* and *in vivo* biofilms of clinical isolate of *Staphylococcus aureus* (55).
213 However, a better understanding of the usefulness of HBOT in CF patients awaits further
214 experiments with pathogens isolated longitudinal as well as isolates with known resistance
215 including highly resistant strains. The risk of development of barotrauma in the lungs, however,
216 should raise concerns when applying HBOT to patients with severely damaged lung tissue.

217 In summary, the findings of this study point to a new treatment strategy for biofilm infections by
218 providing HBOT as an adjuvant to ciprofloxacin treatment, where the increased availability of O_2
219 leads to an increased susceptibility of *P. aeruginosa* biofilms to clinically relevant concentrations of
220 antibiotic.

221

222 Materials and methods

223 *Bacterial strains, media and antibiotics*

224 Wild-type *P. aeruginosa* strain PAO1 was obtained from the *Pseudomonas* Genetic Stock Centre
225 (http://www.pseudomonas.med.ecu.edu). Both the wild-type and a catalase A negative PAO1
226 (*ΔkatA*) mutant (22) were tested for susceptibility to the bactericidal antibiotic ciprofloxacin (Bayer
227 GmbH, Leverkusen, Germany). *katA* encodes the catalase enzyme responsible for the major part of
228 detoxification of ROS in *P. aeruginosa* and accordingly the *ΔkatA* mutant was chosen to
229 demonstrate ROS contribution to ciprofloxacin activity. The minimum inhibitory concentration
230 (MIC) of PAO1 was 0.125 mg L^{-1} as determined by Etest (BioMérieux, Ballerup, Denmark).
231 Growth was in Lysogeny broth (LB) [5 g L^{-1} yeast extract (Oxoid, Basingstoke, UK), 10 g L^{-1}
232 tryptone (Oxoid) and 10 g L^{-1} NaCl (Merck, Rahway, NJ), pH 7.5], incubated overnight at 37°C and
233 shaken at 150 rpm. For determination of bacterial CFU counts, solid lactose agar plates ('Blue

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234 plates' based on a modified Conradi–Drigalski medium containing 10 g L^{-1} detergent, 1 g L^{-1}
235 $Na_2S_2O_3 \cdot H_2O$, 0.1 g L^{-1} bromothymolblue, 9 g L^{-1} lactose and 0.4 g L^{-1} glucose, pH 8.0; Statens
236 Serum Institut, Copenhagen, Denmark) were used to select for Gram-negative bacteria. All plates
237 were incubated overnight at 37°C.
238
239 ***Anaerobic growth***
240 *P. aeruginosa* biofilms were grown and treated under anoxic conditions in an anaerobic growth
241 chamber (Concept 400 Anaerobic Workstation, Ruskinn Technology Ltd, UK). The gas atmosphere
242 consisted of $N_2/H_2/CO_2$ (ratio - 80:10:10). Anoxia was confirmed with an optical O_2 sensor (HQ40d
243 multi, HACH Company, CO, US) placed in the growth chamber. To remove traces of O_2, all media
244 and chemical solutions applied for anaerobic work were equilibrated in the anaerobic chamber 3
245 days prior to experiment.
246
247 ***Susceptibility testing of mature biofilms***
248 Survival curves were assayed to investigate the effect of HBOT on *P. aeruginosa* biofilms treated
249 with ciprofloxacin during 90 min. Overnight cultures of PAO1 or *ΔkatA* optical density at 600 nm
250 (OD_{600}) was adjusted to 0.4 before 100-fold dilution in LB medium supplemented with 2 % 2-
251 hydroxyethyl-agarose (Sigma–Aldrich, Brøndby, Denmark) and 50 µL loaded into 96-well
252 microtiter plates (Nucleon Delta Surface; Thermo Fisher Scientific, Waltham, MA, USA) to
253 achieve a cell loading of ≈ 10^6 cells mL^{-1}. The medium was supplemented with $NaNO_3$ (1 mM)
254 (Sigma–Aldrich) to enable anaerobic respiration. The supernatant was replaced daily with 50 µL of
255 LB medium supplemented with 1 mM $NaNO_3$. Microtiter plates were covered with Parafilm
256 (Bemis, Neenah, WI, USA) and lid and were incubated under anoxic conditions at 37◦C for 3 days
257 to establish mature biofilms. The density of mature untreated PAO1 and *ΔkatA* biofilms was 7.7 x

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258 10^6 CFU mL^{-1} and 7.6 x 10^6 CFU mL^{-1} under anaerobic growth conditions. Treatment with
259 ciprofloxacin was initiated by replacing the supernatant with 50 µL of a ciprofloxacin solution in
260 LB medium (supplemented with 1 mM NO_3^-) in two-fold dilutions from 0 to 2 mg L^{-1}. The plates
261 were then further incubated for 90 min under anoxic or HBO conditions. At the termination of
262 experiments, the supernatant was discarded and the agarose-embedded PAO1 biofilms were placed
263 in 2.95 mL of phosphate-buffered saline (PBS) (Substrate Department, Panum Institute,
264 Copenhagen, Denmark) before re-suspension for 15–20s in a homogenizer (SilentCrusher M;
265 Heidolph, Schwabach, Germany). Quantitative bacteriology was performed by standard
266 microbiological methods incubated overnight at 37◦C.
267
268 ***Hyperbaric oxygen treatment***
269 Agarose-embedded bacteria were exposed to HBOT (100% O_2) at a pressure of 280 kPa (2.8 bar) at
270 37^oC in a hyperbaric oxygen chamber (OXYCOM 250 ARC; Hypcom Oy, Tampere, Finland). The
271 HBOT sequence consisted of pressurization over 5 min to a pressure of 280 kPa. The pressure was
272 then applied for 90 min followed by 5 min of decompression. A constant temperature at 37^oC in the
273 biofilm samples was established by a circulating water system heater (FL300, Julabo, Seelbach,
274 Germany) placed underneath the microtiter plates in the hyperbaric oxygen chamber.
275
276 ***Sectioning and microscopy of agarose embedded biofilm samples***
277 Larger amounts of agarose-embedded biofilms were grown anaerobically with NO_3^- for 3 days in
278 24-well microtiter plates as described above before subjection to similar treatment with
279 ciprofloxacin and HBOT as the 96 well plate biofilm assays.
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282 ***Microscopy and image analysis***

283 With the use of a sterile 5 mm biopsy punch a cylindrical sample was taken from the central part of
284 the wells in the 24-well microtiter plates. The cylindrical gel samples were cut in two halves each
285 with a flat cut side. The cut samples were stained by applying 100 µL of a live/dead-stain mix of
286 Syto9 (5 µM; Molecular Probes, USA) and propidium iodide (PI) (20 µM; Thermo Fisher, USA) in
287 MiliQ water. The stained samples were incubated in the dark for 15 min at room temperature,
288 before being placed flat-cut side down on coverslips.

289 Samples were evaluated by confocal laser scanning microscopy (CLSM) on an LSM 880 Zeiss
290 inverted microscope running Zen 2012 (Zeiss, Germany). The samples were imaged at 100x
291 magnification by parallel tracks running 488 nm and 561 nm Lasers exciting Syto9 and PI,
292 respectively. Samples were imaged with a 1 x 6 tile scan (1416 µm x 7091 µm) and over a depth of
293 136 µm in z-direction. Obtained z-stacks were rendered into 3D projections and created in Imaris
294 8.3 (Bitplane, Switzerland).

295 Size and biomass of aggregates in CLSM image was measured with the use of the Measure Pro
296 Expansion to Imaris 8.3. An iso-surface was applied over the Syto9 stained biomass as well as
297 biomass stained with PI. Iso-surface particles larger than 100 um^3 were consisted. All aggregates
298 within a depth of 100 µm from the surface of the gel were measured, and returned as a measured
299 volume. The radius of aggregates was calculated based on the assumption that aggregates were
300 spherical. For fractionation of live and dead cells, the sum of biomass between Syto9 and PI was
301 used as total biomass. A fraction of both Syto9 and PI of the total biomass was then used as an
302 estimate of live and dead cells.

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306 ***Oxygen measurements***

307 A 3 day old untreated biofilm in a 24-well microtiter plate was treated for 90 min with HBOT.
308 Within 1 min of ending the experiment the microtiter plate was positioned on a heated metal rack,
309 kept at 37°C and vertical micro-profiles of O_2 concentration were recorded using a computer-
310 controlled micromanipulator (Pyro Science GmbH, Germany) equipped with a fiber-optic O_2
311 microsensor (50 µm tip diameter; Pyro Science GmbH, Germany) that was connected to a fiber-
312 optic O_2 meter (FireSting2, Pyro Science GmbH, Germany). The microsensor was calibrated
313 according to the manufacturer's recommendations (air saturated and O_2 free water). As the sample
314 was kept at 37ºC this temperature was set as measurement temperature in the software. The
315 microsensor was positioned manually at the base of the biofilm sample and profile measurements
316 were taken by moving the sensor in vertical steps of 100 µm through the biofilm sample.
317 Positioning and data acquisition were controlled by dedicated software (Profix version 4.51, Pyro
318 Science).

319

320 ***Oxygen diffusion (control)***

321 Diffusion of oxygen in gels without cells was compared between agarose concentrations 0.125% -
322 2% with NaCl concentration 0.9 g L^{-1}. The gels were placed in test tubes of 65 mm height and inner
323 diameter 12 mm and left to congeal. Heights of the agarose gels ranged from 21 - 41 mm. Hereafter
324 100 µL saline water (0.9 g L^{-1}) was added on top of the gel to avoid drying and the tubes were
325 sealed with parafilm. The test tubes were placed in an anaerobic chamber (Concept 400 from Baker
326 Ruskinn) at 37 ^{o}C for at least 8 days to deoxygenate. The tip of the fiber-optic O_2 micro sensor
327 (OXR50-UHS from Pyroscience) was then positioned at 6 mm depth and the oxygen level was
328 recorded under normoxic conditions as the gel re-oxygenated.

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330 ***Statistical methods***

331 Statistical significance was evaluated by ordinary one or two-way analysis of variance (ANOVA)
332 followed by Dunnett's or Bonferroni's multiple comparison test respectively and by Students T-test.
333 A P-value of ≤ 0.05 was considered statistically significant. Data from at least 3 independent
334 experiments were compared. Tests were performed with GraphPad Prism 6.1 (GraphPad Software
335 Inc., La Jolla, CA) and Microsoft Excel (Microsoft Corp., Redmond, WA).

336

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340

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353 **References**

354 1. Høiby N, Bjarnsholt T, Moser C, Jensen PØ, Kolpen M, Qvist T, Aanaes K, Pressler T,
355 Skov M, Ciofu O. 2017. Diagnosis of biofilm infections in cystic fibrosis patients. APMIS
356 125:339-343.

357 2. Høiby N, Ciofu O, Bjarnsholt T. 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis.
358 Future Microbiol 5:1663-74.

359 3. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of
360 bacterial biofilms. Int J Antimicrob Agents 35:322-32.

361 4. Ciofu O, Rojo-Molinero E, Macia MD, Oliver A. 2017. Antibiotic treatment of biofilm
362 infections. APMIS 125:304-319.

363 5. Walters MC, 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of
364 antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of
365 *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents
366 Chemother 47:317-23.

367 6. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. 2004. Oxygen limitation
368 contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrob
369 Agents Chemother 48:2659-64.

370 7. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G,
371 Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G. 2002.
372 Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic
373 fibrosis patients. J Clin Invest 109:317-25.

374 8. Kolpen M, Hansen CR, Bjarnsholt T, Moser C, Christensen LD, van Gennip M, Ciofu O,
375 Mandsberg L, Kharazmi A, Dõring G, Givskov M, Høiby N, Jensen PØ. 2010.
376 Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas*
377 *aeruginosa* pneumonia in cystic fibrosis. Thorax 65:57-62.

Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy
Downloaded from http://aac.asm.org/ on September 11, 2017 by DTU Library

Accepted Manuscript Posted Online

378 9. Cowley ES, Kopf SH, LaRiviere A, Ziebis W, Newman DK. 2015. Pediatric cystic fibrosis
379 sputum can be chemically dynamic, anoxic, and extremely reduced due to hydrogen sulfide
380 formation. MBio 6:e00767.

381 10. Lobritz MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil
382 AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl
383 Acad Sci U S A 112:8173-80.

384 11. Brochmann RP, Toft A, Ciofu O, Briales A, Kolpen M, Hempel C, Bjarnsholt T, Høiby N,
385 Jensen PØ. 2014. Bactericidal effect of colistin on planktonic *Pseudomonas aeruginosa* is
386 independent of hydroxyl radical formation. Int J Antimicrob Agents 43:140-7.

387 12. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT,
388 Lobritz MA, Braff D, Schwarz EG, Ye JD, Pati M, Vercruysse M, Ralifo PS, Allison KR,
389 Khalil AS, Ting AY, Walker GC, Collins JJ. 2014. Antibiotics induce redox-related
390 physiological alterations as part of their lethality. Proc Natl Acad Sci U S A 111:E2100-9.

391 13. Van Acker H, Coenye T. 2017. The Role of Reactive Oxygen Species in Antibiotic-
392 Mediated Killing of Bacteria. Trends Microbiol doi:10.1016/j.tim.2016.12.008.

393 14. Kolpen M, Mousavi N, Sams T, Bjarnsholt T, Ciofu O, Moser C, Kühl M, Høiby N, Jensen
394 PØ. 2016. Reinforcement of the bactericidal effect of ciprofloxacin on *Pseudomonas*
395 *aeruginosa* biofilm by hyperbaric oxygen treatment. Int J Antimicrob Agents 47:163-7.

396 15. Kolpen M, Bjarnsholt T, Moser C, Hansen CR, Rickelt LF, Kühl M, Hempel C, Pressler T,
397 Høiby N, Jensen PØ. 2014. Nitric oxide production by polymorphonuclear leucocytes in
398 infected cystic fibrosis sputum consumes oxygen. Clin Exp Immunol 177:310-9.

399 16. Kragh KN, Alhede M, Jensen PØ, Moser C, Scheike T, Jacobsen CS, Seier Poulsen S,
400 Eickhardt-Sørensen SR, Trøstrup H, Christoffersen L, Hougen HP, Rickelt LF, Kühl M,
401 Høiby N, Bjarnsholt T. 2014. Polymorphonuclear leukocytes restrict growth of
402 *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. Infect Immun 82:4477-86.

403 17. Kolpen M, Kragh KN, Bjarnsholt T, Line L, Hansen CR, Dalbøge CS, Hansen N, Kühl M,
404 Høiby N, Jensen PØ. 2015. Denitrification by cystic fibrosis pathogens - *Stenotrophomonas*
405 *maltophilia* is dormant in sputum. Int J Med Microbiol 305:1-10.

Downloaded from http://aac.asm.org/ on September 11, 2017 by DTU Library

AAC
Antimicrobial Agents and Chemotherapy

Accepted Manuscript Posted Online

Downloaded from http://aac.asm.org/ on September 11, 2017 by DTU Library

406 18. Kopf SH, Sessions AL, Cowley ES, Reyes C, Van Sambeek L, Hu Y, Orphan VJ, Kato R,
407 Newman DK. 2016. Trace incorporation of heavy water reveals slow and heterogeneous
408 pathogen growth rates in cystic fibrosis sputum. Proc Natl Acad Sci U S A 113:E110-6.

409 19. DePas WH, Starwalt-Lee R, Van Sambeek L, Ravindra Kumar S, Gradinaru V, Newman
410 DK. 2016. Exposing the Three-Dimensional Biogeography and Metabolic States of
411 Pathogens in Cystic Fibrosis Sputum via Hydrogel Embedding, Clearing, and rRNA
412 Labeling. MBio 7.

413 20. Jensen PØ, Kolpen, M., Kragh, K. N., Kuhl, M. 2017. Micro-environmental characteristics
414 and physiology of biofilms in chronic infections of CF patients are strongly affected by the
415 host immune response. APMIS 125:276-288.

416 21. Hassett DJ, Cohen MS. 1989. Bacterial adaptation to oxidative stress: implications for
417 pathogenesis and interaction with phagocytic cells. FASEB J 3:2574-82.

418 22. Hassett DJ, Elkins JG, Ma JF, McDermott TR. 1999. *Pseudomonas aeruginosa* biofilm
419 sensitivity to biocides: use of hydrogen peroxide as model antimicrobial agent for examining
420 resistance mechanisms. Methods Enzymol 310:599-608.

421 23. Hassett DJ, Imlay JA. 2007. Bactericidal antibiotics and oxidative stress: a radical proposal.
422 ACS Chem Biol 2:708-10.

423 24. Van Acker H, Sass A, Bazzini S, De Roy K, Udine C, Messiaen T, Riccardi G, Boon N,
424 Nelis HJ, Mahenthiralingam E, Coenye T. 2013. Biofilm-grown *Burkholderia cepacia*
425 complex cells survive antibiotic treatment by avoiding production of reactive oxygen
426 species. PLoS One 8:e58943.

427 25. Sønderholm M, Kragh KN, Koren K, Jakobsen TH, Darch SE, Alhede M, Jensen PØ,
428 Whiteley M, Kühl M, Bjarnsholt T. 2017. *Pseudomonas aeruginosa* Aggregate Formation in
429 an Alginate Bead Model System Exhibits In Vivo-Like Characteristics. Appl Environ
430 Microbiol 83.

431 26. Van der Meeren P, De Vleeschauwer, D., Debergh, P. 2001. Determination of oxygen
432 profiles in agar-based gelled in vitro plant tissue culture media. Plant cell, tissue and organ
433 culture 65:239-245.

AAC
Antimicrobial Agents and Chemotherapy

Accepted Manuscript Posted Online

434 27. Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R,
435 Schafhauser J, Wang Y, Britigan BE, Singh PK. 2011. Active starvation responses mediate
436 antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982-6.

437 28. Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa*
438 have similar resistance to killing by antimicrobials. J Bacteriol 183:6746-51.

439 29. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T,
440 Givskov M, Høiby N. 2009. *Pseudomonas aeruginosa* biofilms in the respiratory tract of
441 cystic fibrosis patients. Pediatr Pulmonol 44:547-58.

442 30. Sjöberg F, Singer M. 2013. The medical use of oxygen: a time for critical reappraisal. J
443 Intern Med 274:505-28.

444 31. Pabst B, Pitts B, Lauchnor E, Stewart PS. 2016. Gel-entrapped *Staphylococcus aureus*
445 bacteria as models of biofilm infection exhibit growth in dense aggregates, oxygen
446 limitation, antibiotic tolerance, and heterogeneous gene expression. Antimicrob Agents
447 Chemother 60:6294-301.

448 32. Crone S, Garde C, Bjarnsholt T, Alhede M. 2015. A novel in vitro wound biofilm model
449 used to evaluate low-frequency ultrasonic-assisted wound debridement. J Wound Care
450 24:64, 66-9, 72.

451 33. Pakman LM. 1971. Inhibition of *Pseudomonas aeruginosa* by hyperbaric oxygen. I.
452 Sulfonamide activity enhancement and reversal. Infect Immun 4:479-87.

453 34. Lima FL, Joazeiro PP, Lancellotti M, de Hollanda LM, de Araujo Lima B, Linares E,
454 Augusto O, Brocchi M, Giorgio S. 2015. Effects of hyperbaric oxygen on *Pseudomonas*
455 *aeruginosa* susceptibility to imipenem and macrophages. Future Microbiol 10:179-89.

456 35. Neuman TS, Thom, S.R. 2008. Physiology and Medicine of Hyperbaric Oxygen Therapy, 1
457 st ed. Saunders Elsevier, Philadelphia, PA.

458 36. Brummelkamp WH, Hogendijk, W,T. Boerema, I. 1961. Treatment of anaerobic infections
459 (clostridial myositis) by drenching the tissues with oxygen under high pressure. Surgery
460 49:299-302.

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Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy

Accepted Manuscript Posted Online

461 37. Gill AL, Bell CN. 2004. Hyperbaric oxygen: its uses, mechanisms of action and outcomes.
462 QJM 97:385-95.

463 38. Kolpen M, Kühl M, Bjarnsholt T, Moser C, Hansen CR, Liengaard L, Kharazmi A, Pressler
464 T, Høiby N, Jensen PØ. 2014. Nitrous oxide production in sputum from cystic fibrosis
465 patients with chronic *Pseudomonas aeruginosa* lung infection. PLoS One 9:e84353.

466 39. Line L, Alhede M, Kolpen M, Kühl M, Ciofu O, Bjarnsholt T, Moser C, Toyofuku M,
467 Nomura N, Høiby N, Jensen PØ. 2014. Physiological levels of nitrate support anoxic growth
468 by denitrification of *Pseudomonas aeruginosa* at growth rates reported in cystic fibrosis
469 lungs and sputum. Front Microbiol 5:554.

470 40. Alvarez-Ortega C, Harwood CS. 2007. Responses of *Pseudomonas aeruginosa* to low
471 oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol
472 Microbiol 65:153-65.

473 41. Sønderholm M, Kragh, K. N., Koren, K., Jakobsen, T., Darch, S., Alhede, M., Jensen, P. Ø.,
474 Whiteley, M., Kühl, M., Bjarnsholt, T. 2017. Aggregate formation of *Pseudomonas*
475 *aeruginosa* in an alginate bead model system exhibits in vivo like characteristics. Applied
476 and Environmental Microbiology.

477 42. Evans DJ, Allison DG, Brown MR, Gilbert P. 1991. Susceptibility of *Pseudomonas*
478 *aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth
479 rate. J Antimicrob Chemother 27:177-84.

480 43. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common
481 mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797-810.

482 44. Jensen PØ, Briales A, Brochmann RP, Wang H, Kragh KN, Kolpen M, Hempel C,
483 Bjarnsholt T, Høiby N, Ciofu O. 2014. Formation of hydroxyl radicals contributes to the
484 bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms. Pathog Dis
485 70:440-3.

486 45. Liu Y, Imlay JA. 2013. Cell death from antibiotics without the involvement of reactive
487 oxygen species. Science 339:1210-3.

Antimicrobial Agents and Chemotherapy

AAC

Antimicrobial Agents and Chemotherapy

Downloaded from http://aac.asm.org/ on September 11, 2017 by DTU Library

Accepted Manuscript Posted Online

488 46. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. 2013. Killing by bactericidal antibiotics
489 does not depend on reactive oxygen species. Science 339:1213-6.

490 47. Høiby N, Bjarnsholt T, Moser C, Bassi GL, Coenye T, Donelli G, Hall-Stoodley L, Hola V,
491 Imbert C, Kirketerp-Møller K, Lebeaux D, Oliver A, Ullmann AJ, Williams C, Biofilms
492 ESGf, Consulting External Expert Werner Z. 2015. ESCMID guideline for the diagnosis and
493 treatment of biofilm infections 2014. Clin Microbiol Infect 21 Suppl 1:S1-25.

494 48. Coulson A, Peek A, Haugen D. 2016. Femoral Vein Cannulation in the Treatment of
495 Osteomyelitis. Wounds 28:194-9.

496 49. Fang RC, Galiano RD. 2009. Adjunctive therapies in the treatment of osteomyelitis. Semin
497 Plast Surg 23:141-7.

498 50. Signoretto C, Bianchi F, Burlacchini G, Canepari P. 2007. Microbiological evaluation of the
499 effects of hyperbaric oxygen on periodontal disease. New Microbiol 30:431-7.

500 51. Luongo C, Imperatore F, Matera MG, Mangoni G, Marmo M, Baroni A, Catalanotti P,
501 Rossi F, Filippelli A. 1999. Effect of hyperbaric oxygen therapy in experimental
502 subcutaneous and pulmonary infections due to *Pseudomonas aeruginosa*. Undersea Hyperb
503 Med 26:21-5.

504 52. Marmo M, Contaldi G, Luongo C, Imperatore F, Tufano MA, Catalanotti P, Baroni A,
505 Mangoni G, Stefano S, Rossi F. 1996. [Effects of hyperbaric oxygenation in skin and
506 pulmonary infections caused by *Pseudomonas aeruginosa*]. Minerva Anestesiol 62:281-7.

507 53. Holloway BW. 1955. Genetic recombination in *Pseudomonas aeruginosa*. J Gen Microbiol
508 13:572-81.

509 54. Holloway BW. 1975. Genetic organization of *Pseudomonas*, p 133-161. *In* Clarke PHR,
510 M.H. (ed), Genetics and Biochemistry of *Pseudomonas*. John Wiley & Sons Ldt, London,
511 United Kingdom.

512 55. Lerche CJ, Christophersen LJ, Kolpen M, Nielsen PR, Trøstrup H, Thomsen K, Hyldegaard
513 O, Bundgaard H, Jensen PØ, Høiby N, Moser C. 2017. Hyperbaric oxygen therapy

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514 augments tobramycin efficacy in experimental *Staphylococcus aureus* endocarditis. Int J
515 Antimicrob Agents doi:10.1016/j.ijantimicag.2017.04.025.

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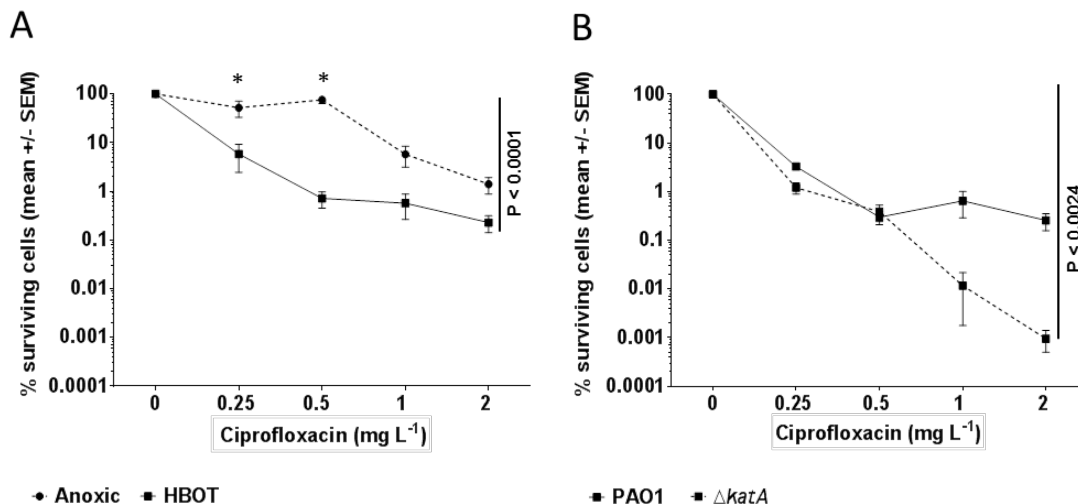
538 **Figures**



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540 **Figure 1: Effect of simultaneous hyperbaric oxygen treatment (HBOT) on ciprofloxacin**
541 **(0.25–2 mg L^{-1}) treatment of anaerobic *Pseudomonas aeruginosa* biofilms** a, Effect of anoxic
542 (dotted line) and HBOT (filled line) conditions on % surviving cells on agarose embedded PAO1
543 biofilms to ciprofloxacin (calculated as Δlog_{10} cell numbers) after treatment for 90 min. Bars
544 indicate the mean ± standard error of the mean (n = 13-19). b, Effect of ciprofloxacin- and HBO-
545 treatment on 3-day-old agarose embedded biofilms of PAO1 (filled line) and *ΔkatA* (dotted line)
546 (calculated as Δlog_{10} cell numbers) after treatment for 90 min. Bars indicate the mean ± standard
547 error of the mean (n = 11-14). Significant changes ($p \leq 0.05$) by particular ciprofloxacin
548 concentrations are indicated by asterisks (*). Statistical significance was evaluated by a two-way
549 ANOVA test followed by Bonferroni's multiple comparison tests.

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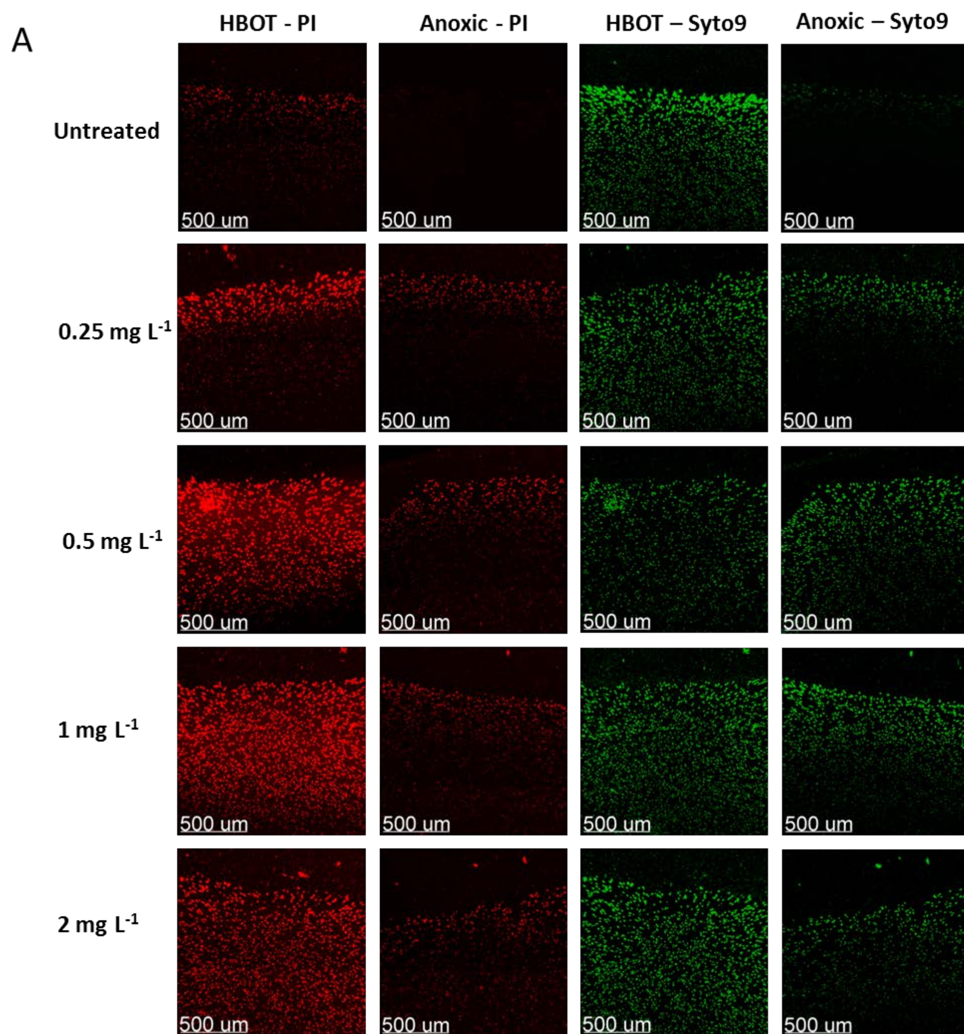

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555 **Figure 2: Lethality of ciprofloxacin-treated agarose-embedded *Pseudomonas aeruginosa***
556 **biofilms during anoxic or HBOT conditions.**ciprofloxacin- and HBO-treated 3-day-old agarose
557 imbedded biofilms of PAO1. Ciprofloxacin (0.25–2 mg L^{-1}) treatment in anoxic agarose embedded
558 biofilms of PAO1 and in HBOT agarose embedded biofilms of PAO1. The samples has been
559 stained with Syto9 and propidium iodide (PI) and obtained by using a 63 x 1.4 NA Zeiss objective
560 on a Zeiss 710 CLSM. Red denotes bacterial membranes that are permeable to PI (dead bacteria);

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561 green bacteria are alive, since they have intact membranes that are not permeable to PI. The bar in

562 the photograph represents 500 µm. (n = 1).

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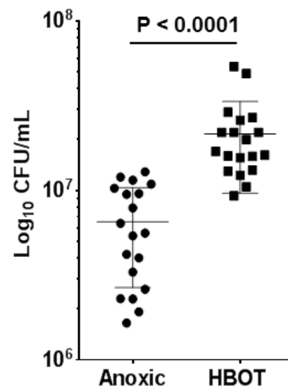

580 **Figure 3: Hyperbaric oxygen treatment (HBOT) effect on bacterial growth in *Pseudomonas***
581 ***aeruginosa* biofilms.** Effect of anoxic (circles) and HBOT (squares) conditions on bacterial growth
582 (calculated as Δlog_{10} cell numbers) after treatment for 90 min on agarose embedded PAO1 biofilms.
583 Bars indicate the mean ± standard error of the mean (n = 19). Statistical significance ($p \leq 0.05$) was
584 evaluated by the Student t-test.

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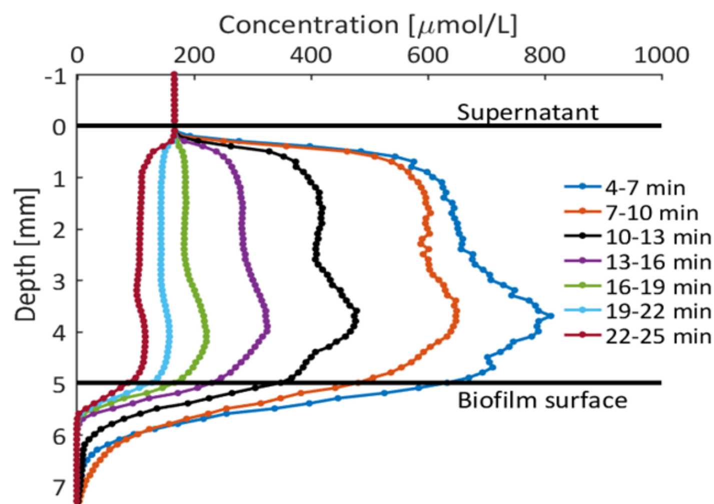

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596 **Figure 4: Optical microsensor measurement of the chemical gradient of O_2 in ciprofloxacin-**
597 **treated agarose-embedded *Pseudomonas aeruginosa* biofilm.** Representative micro-profiling of
598 the spatio-temporal dynamics of O_2 in an agarose embedded PAO1 biofilm receiving HBOT for 90
599 min showing initial accumulation of O_2 in the media above the biofilm surface and inside the
600 biofilm followed by depletion. The measurement of the O_2 concentration profile was initiated 4 min
601 after termination of HBOT with following profiling.

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