



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**5 **Authors:**

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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 (O₂) 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 O₂ increases their tolerance. In fact, the bactericidal effect of several antibiotics depends on active
49 aerobic metabolism activity and the endogenous formation of reactive O₂ 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 O₂ 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 O₂.

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

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

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 O₂ due to intense O₂
95 depletion by the surrounding PMNs creating anoxia (8). Furthermore, the depth of the agarose
96 embedded biofilm has been decreased in order for O₂ 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 $\Delta katA$ biofilms to elucidate that ROS play a role in the increased lethality
104 of ciprofloxacin during HBOT. We found significantly less $\Delta katA$ bacteria surviving 90 min of
105 treatment with ciprofloxacin when HBOT was applied compared with PAO1 biofilms ($p < 0.0024$,
106 $n = 11-14$), 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 $\Delta katA$ 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).

111

112

113

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 O₂, 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 (μm³): 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 O₂ in *P. aeruginosa* biofilm after HBOT**

135 Vertical profiling of O₂ concentration in the agarose-embedded biofilm immediately after
136 termination of 90 minutes of HBOT, demonstrated O₂ concentrations exceeding 1000 μmol L⁻¹ in
137 the media above the biofilm surface (Fig 4). Serial profiling revealed both rapid depletion of O₂ in

138 the upper part of the biofilm and O₂ diffusion from the supernatant to the normobaric atmosphere.
139 However, within 20 min post HBOT, the zone of O₂ depletion inside the biofilm was expanded and
140 the O₂ concentration of the supernatant decreased below atmospheric saturation, indicating that
141 PAO1 was utilising the available O₂ for aerobic respiration until O₂-depletion in the biofilm would
142 necessitate conversion to anaerobic respiration (Fig 4).

143 O₂ 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 O₂ 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 O₂ sensitizes bacteria by stimulating growth in areas
151 deprived of O₂. 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

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 O₂ exclusion while addition of NO₃⁻ enabled anaerobic respiration
176 by denitrification (38, 39). The rapid decrease from hyperoxia to hypoxia demonstrated by serial
177 measurements of O₂ 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).

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 $\Delta katA$ -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 $\Delta katA$ 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

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 O₂
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 (Δ *kata*) 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 Δ *kata* 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

234 plates' based on a modified Conradi–Drigalski medium containing 10 g L⁻¹ detergent, 1 g L⁻¹
235 Na₂S₂O₃·H₂O, 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 N₂/H₂/CO₂ (ratio - 80:10:10). Anoxia was confirmed with an optical O₂ sensor (HQ40d
243 multi, HACH Company, CO, US) placed in the growth chamber. To remove traces of O₂, 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 $\Delta katA$ optical density at 600 nm
250 (OD₆₀₀) 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 $\approx 10^6$ cells mL⁻¹. The medium was supplemented with NaNO₃ (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 NaNO₃. 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 $\Delta katA$ biofilms was 7.7 x

258 10^6 CFU mL⁻¹ and 7.6×10^6 CFU mL⁻¹ under anaerobic growth conditions. Treatment with
259 ciprofloxacin was initiated by replacing the supernatant with 50 μ L of a ciprofloxacin solution in
260 LB medium (supplemented with 1 mM NO₃⁻) 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% O₂) at a pressure of 280 kPa (2.8 bar) at
270 37°C 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°C 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 NO₃⁻ 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.

280

281

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 μm^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.

303

304

305

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 O₂ concentration were recorded using a computer-
310 controlled micromanipulator (Pyro Science GmbH, Germany) equipped with a fiber-optic O₂
311 microsensor (50 µm tip diameter; Pyro Science GmbH, Germany) that was connected to a fiber-
312 optic O₂ meter (FireSting2, Pyro Science GmbH, Germany). The microsensor was calibrated
313 according to the manufacturer's recommendations (air saturated and O₂ free water). As the sample
314 was kept at 37°C 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 Ruskin) at 37 °C for at least 8 days to deoxygenate. The tip of the fiber-optic O₂ 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.

329

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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350

351

352

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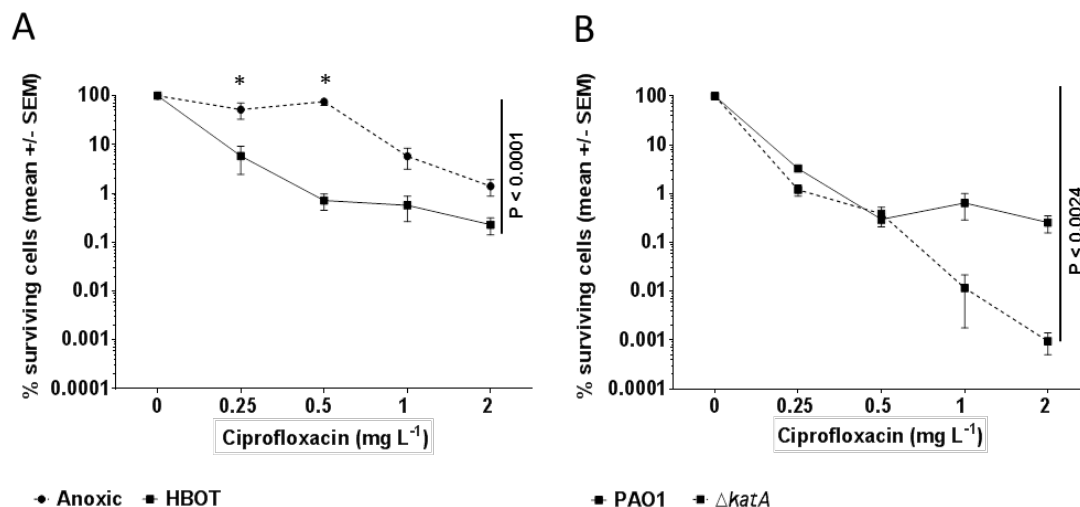
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538 **Figures**

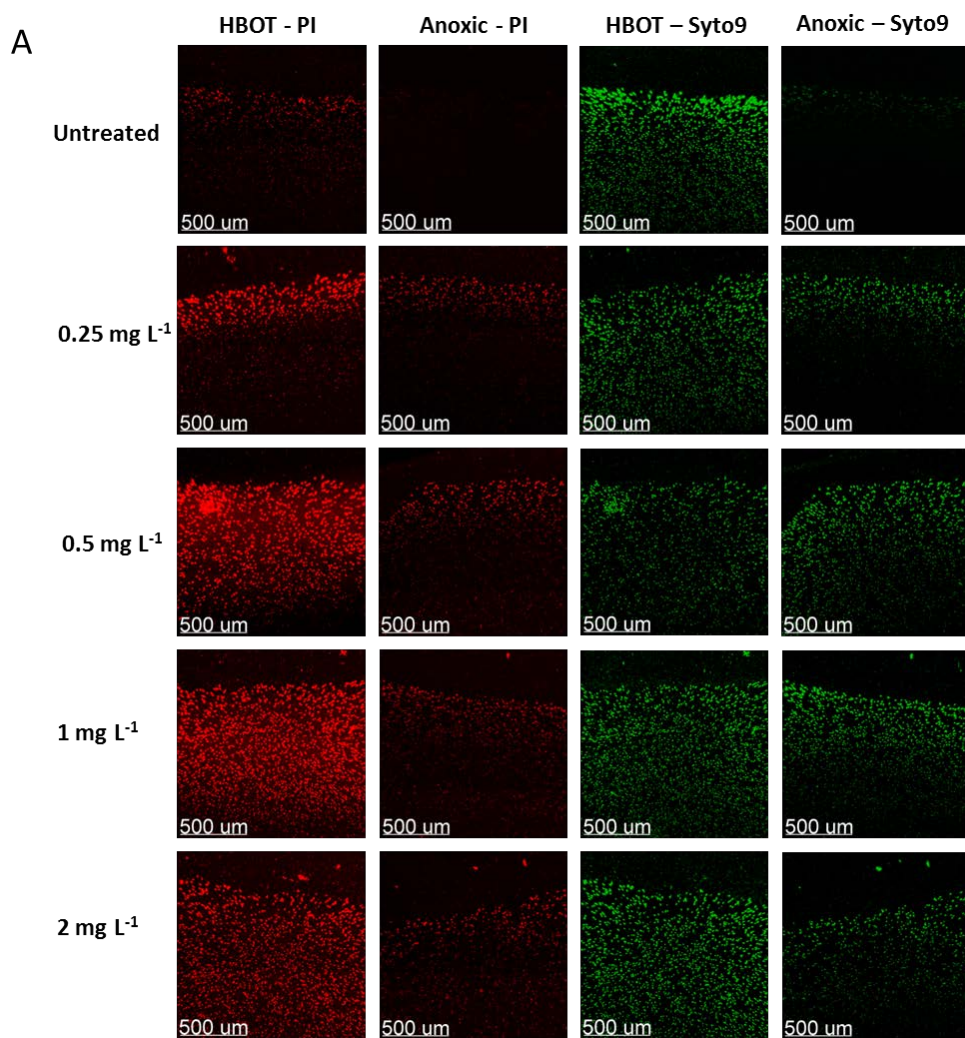
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540 **Figure 1: Effect of simultaneous hyperbaric oxygen treatment (HBOT) on ciprofloxacin**
 541 **(0.25–2 mg L⁻¹) treatment of anaerobic *Pseudomonas aeruginosa* biofilms** a, Effect of anoxic
 542 (dotted line) and HBOT (filled line) conditions on % surviving cells on agarose embedded PAO1
 543 biofilms to ciprofloxacin (calculated as $\Delta\log_{10}$ cell numbers) after treatment for 90 min. Bars
 544 indicate the mean \pm 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 $\Delta katA$ (dotted line)
 546 (calculated as $\Delta\log_{10}$ cell numbers) after treatment for 90 min. Bars indicate the mean \pm 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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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);

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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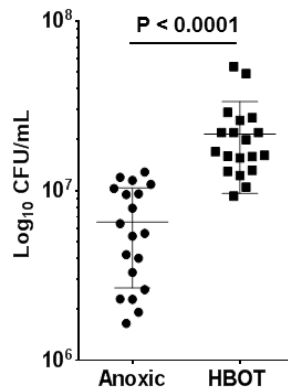
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580 **Figure 3: Hyperbaric oxygen treatment (HBOT) effect on bacterial growth in *Pseudomonas***
581 ***aeruginosa* biofilms.** Effect of anoxic (circles) and HBOT (squares) conditions on bacterial growth
582 (calculated as $\Delta\log_{10}$ cell numbers) after treatment for 90 min on agarose embedded PAO1 biofilms.
583 Bars indicate the mean \pm 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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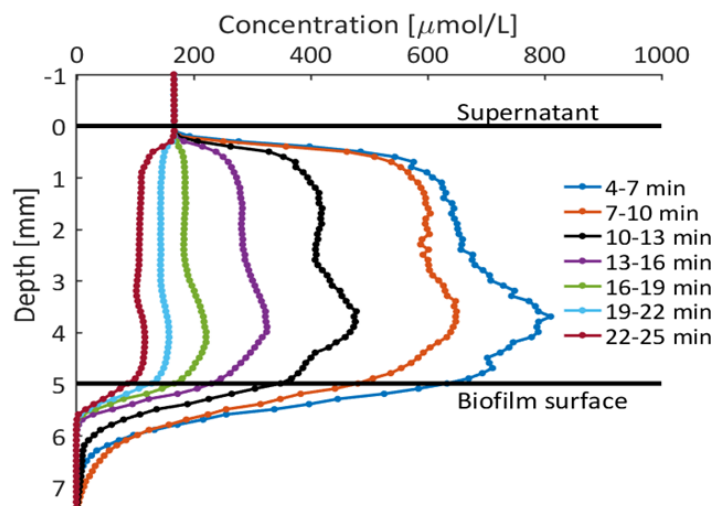
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596 **Figure 4: Optical microsensor measurement of the chemical gradient of O₂ in ciprofloxacin-**
597 **treated agarose-embedded *Pseudomonas aeruginosa* biofilm.** Representative micro-profiling of
598 the spatio-temporal dynamics of O₂ in an agarose embedded PAO1 biofilm receiving HBOT for 90
599 min showing initial accumulation of O₂ in the media above the biofilm surface and inside the
600 biofilm followed by depletion. The measurement of the O₂ concentration profile was initiated 4 min
601 after termination of HBOT with following profiling.

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