Title:

Hyperbaric oxygen sensitizes anoxic *Pseudomonas aeruginosa* biofilm to ciprofloxacin

Running title:

HBOT sensitizes *P. aeruginosa* biofilm to ciprofloxacin

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Abstract

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

Introduction

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

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

Results

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

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

exceeded 2 log units when supplemented with 0.5 mg L^{-1} of ciprofloxacin indicating that HBOT exposed *P. aeruginosa* biofilm can be treated with lower ciprofloxacin concentrations than controls. It is striking that the potentiation of ciprofloxacin is stronger after 90 min of HBOT than for 2 h of HBOT as previously reported (14). However, the present model has been developed to better represent the *in vivo* microenvironment where *P. aeruginosa* is deprived of O_2 due to intense O_2 depletion by the surrounding PMNs creating anoxia (8). Furthermore, the depth of the agarose embedded biofilm has been decreased in order for O_2 to penetrate through larger parts of entire biofilm within 90 min.

In *P. aeruginosa* a major part of detoxification of ROS is contributed by catalase enzymes encoded by the katA gene (21, 22). Accordingly, the increased susceptibility to antibiotics in mutants with defective katA expression as well as enhanced tolerance to antibiotics in mutants with overexpression of catalase are recognized as direct evidence for a lethal effect of ROS generation during antibiotic treatment (12, 23, 24).

Therefore we employed Δ*katA* biofilms to elucidate that ROS play a role in the increased lethality of ciprofloxacin during HBOT. We found significantly less Δ*katA* bacteria surviving 90 min of treatment with ciprofloxacin when HBOT was applied compared with PAO1 biofilms ($p < 0.0024$, $n = 11\text{-}14$), demonstrating a contribution of oxidative stress to decreased bacterial survival (Fig 1b). This indicates that HBOT enabled aerobic respiration allowing ciprofloxacin to induce formation of lethal amounts of ROS (10). However, an increased susceptibility of Δ*katA* was only seen for the higher concentrations of ciprofloxacin suggesting that other anti-oxidative mechanisms protects against the ROS produced during treatment with low amounts of ciprofloxacin (10).

HBOT expands the bactericidal zone of ciprofloxacin treatment in *P. aeruginosa* biofilm.

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

HBOT stimulates growth in *P. aeruginosa* biofilm.

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

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

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

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

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

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

Discussion

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

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

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

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

In addition to a growth-related enhancement of ciprofloxacin treatment during HBOT, it was speculated that HBOT induced re-oxygenation of the biofilm leads to accumulation of cytotoxic ROS in response to ciprofloxacin. Induction of endogenous production of cytotoxic ROS has been shown to contribute to the aerobic killing of planktonic bacteria by several major classes of antibiotics (11, 12, 43) including aerobic *P. aeruginosa* biofilms (44) although the significance of this has been challenged (11, 45, 46). However, increased susceptibility to antibiotics of mutants with deficient anti-oxidative defence is regarded as solid indication for a contribution of ROS to the bactericidal effect of antibiotics (23). Thus, the increased killing of the Δ*katA*-mutant in our study supports that endogenous generation of ROS can contribute to an enhanced bactericidal effect of ciprofloxacin on biofilm during adjuvant HBOT. Growth of Δ*katA* was not impaired with HBOT in the absence of ciprofloxacin treatment as compared to the wild-type, indicating a lack of cytotoxic ROS generation by HBOT alone (data not shown).

Biofilm infections are notoriously difficult to eradicate with antimicrobial treatment, as frequently higher concentrations of antibiotics are required for killing of biofilms compared to planktonic bacteria, with these concentrations being difficult to match *in vivo* (47). Our finding of a significantly increased bacterial killing during HBOT with only 2 x MIC and 4 x MIC of ciprofloxacin indicates that by using HBOT, *P. aeruginosa* biofilms can be effectively treated with lower ciprofloxacin levels, that are attainable *in vivo*.

Although still controversial, there is an increasing acceptance of the advantages of HBOT with a small number of studies focusing on the use of HBOT on biofilm infections e.g. associated with periodontal disease, osteomyelitis and chronic wounds (48-50). The effect of HBOT on biofilm infections in the pulmonary system remain largely unknown, though some studies have demonstrated the beneficial effect of HBOT in patients with acute abscesses and in experimental pulmonary infection models with *P. aeruginosa* (51, 52). The feasibility of HBOT to sensitize

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

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

Materials and methods

Bacterial strains, media and antibiotics

Wild-type *P. aeruginosa* strain PAO1 was obtained from the *Pseudomonas* Genetic Stock Centre (http://www.pseudomonas.med.ecu.edu). Both the wild-type and a catalase A negative PAO1 (*ΔkatA*) mutant (22) were tested for susceptibility to the bactericidal antibiotic ciprofloxacin (Bayer GmbH, Leverkusen, Germany). *katA* encodes the catalase enzyme responsible for the major part of detoxification of ROS in *P. aeruginosa* and accordingly the *ΔkatA* mutant was chosen to demonstrate ROS contribution to ciprofloxacin activity. The minimum inhibitory concentration (MIC) of PAO1 was 0.125 mg L^{-1} as determined by Etest (BioMérieux, Ballerup, Denmark). Growth was in Lysogeny broth (LB) [5 g L^{-1} yeast extract (Oxoid, Basingstoke, UK), 10 g L^{-1} tryptone (Oxoid) and 10 g L^{-1} NaCl (Merck, Rahway, NJ), pH 7.5], incubated overnight at 37°C and 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 $Na_2S_2O_3 \cdot H_2O$, 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

10^6 CFU mL^{-1} and 7.6 x 10^6 CFU mL^{-1} under anaerobic growth conditions. Treatment with ciprofloxacin was initiated by replacing the supernatant with 50 µL of a ciprofloxacin solution in LB medium (supplemented with 1 mM NO_3^-) in two-fold dilutions from 0 to 2 mg L^{-1}. The plates were then further incubated for 90 min under anoxic or HBO conditions. At the termination of experiments, the supernatant was discarded and the agarose-embedded PAO1 biofilms were placed in 2.95 mL of phosphate-buffered saline (PBS) (Substrate Department, Panum Institute, Copenhagen, Denmark) before re-suspension for 15–20s in a homogenizer (SilentCrusher M; Heidolph, Schwabach, Germany). Quantitative bacteriology was performed by standard microbiological methods incubated overnight at 37◦C.

Hyperbaric oxygen treatment

Agarose-embedded bacteria were exposed to HBOT (100% O_2) at a pressure of 280 kPa (2.8 bar) at 37°C in a hyperbaric oxygen chamber (OXYCOM 250 ARC; Hypcom Oy, Tampere, Finland). The HBOT sequence consisted of pressurization over 5 min to a pressure of 280 kPa. The pressure was then applied for 90 min followed by 5 min of decompression. A constant temperature at 37°C in the biofilm samples was established by a circulating water system heater (FL300, Julabo, Seelbach, Germany) placed underneath the microtiter plates in the hyperbaric oxygen chamber.

Sectioning and microscopy of agarose embedded biofilm samples

Larger amounts of agarose-embedded biofilms were grown anaerobically with NO_3^- for 3 days in 24-well microtiter plates as described above before subjection to similar treatment with ciprofloxacin and HBOT as the 96 well plate biofilm assays.

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 °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.

329

Statistical methods

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

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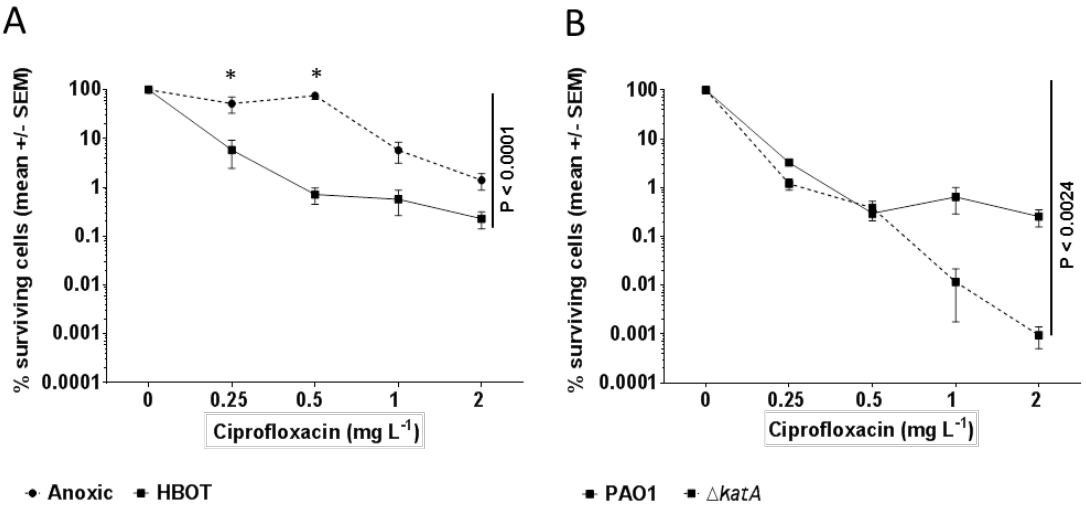
538 **Figures**



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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 ± 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 $\Delta\log_{10}$ 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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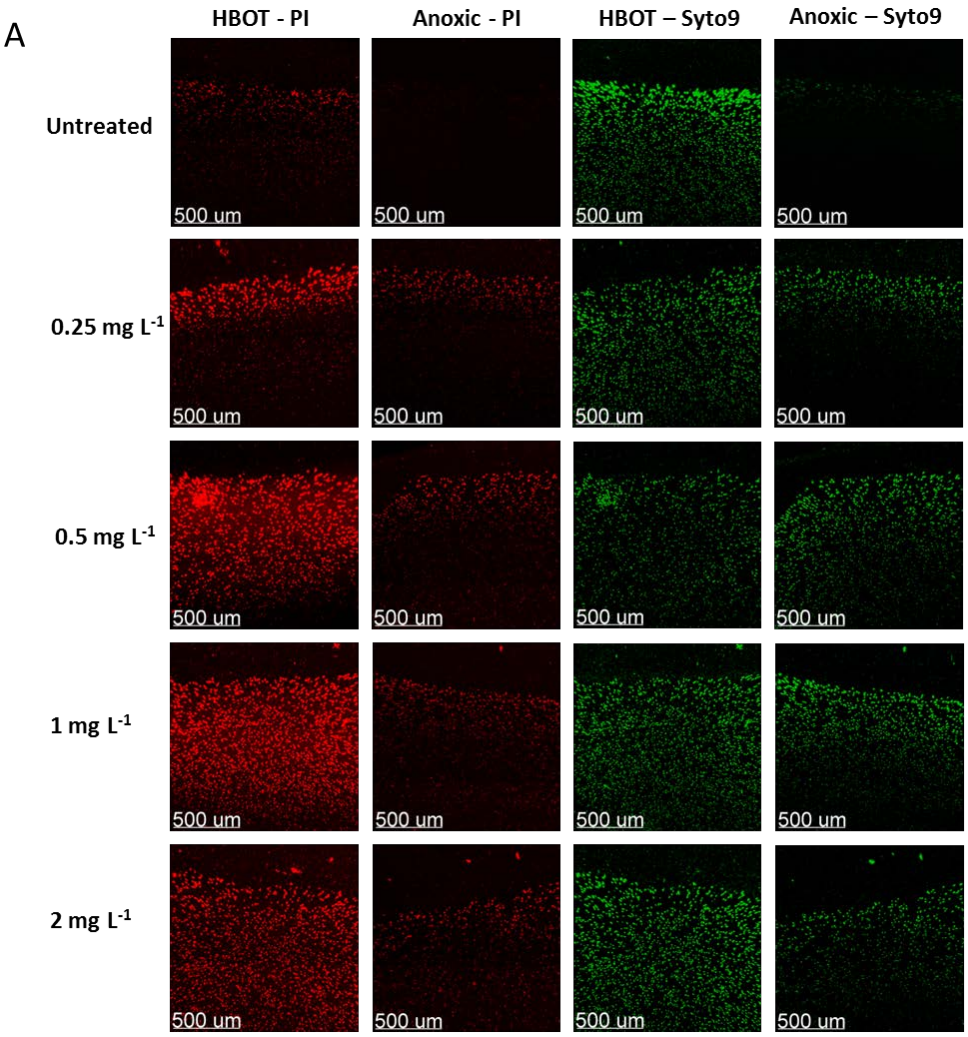
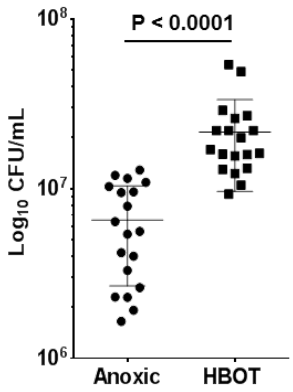


Figure 2: Lethality of ciprofloxacin-treated agarose-embedded *Pseudomonas aeruginosa* biofilms during anoxic or HBOT conditions.ciprofloxacin- and HBO-treated 3-day-old agarose imbedded biofilms of PAO1. Ciprofloxacin (0.25–2 mg L^{-1}) treatment in anoxic agarose embedded biofilms of PAO1 and in HBOT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

green bacteria are alive, since they have intact membranes that are not permeable to PI. The bar in 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 $\Delta\log_{10}$ 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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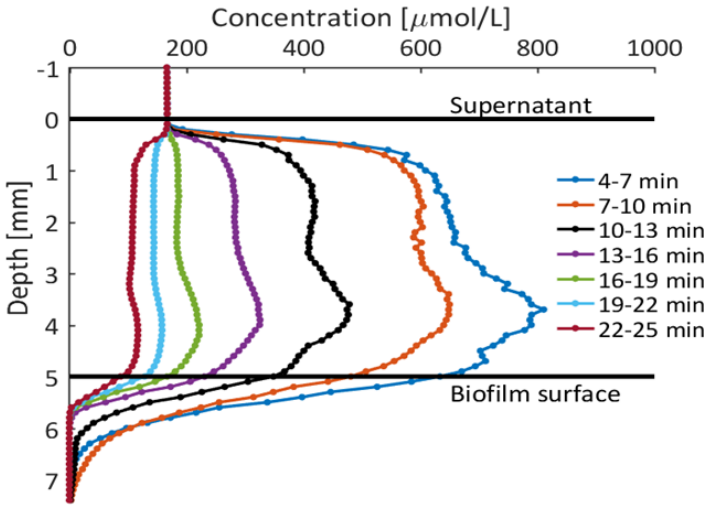

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596 **Figure 4: Optical microsensor measurement of the chemical gradient of O_2 in ciprofloxacin-**
597 **treated agarose-embedded *Pseudomonas aeruginosa* biofilm.** Representative micro-profiling of
598 the spatio-temporal dynamics of O_2 in an agarose 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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