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Hyperbaric oxygen sensitizes anoxic *Pseudomonas aeruginosa* biofilm to ciprofloxacin

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 (O2) (5, 6), which is distributed at low levels reaching anoxia in parts of the endobronchial secretions of chronically infected CF patients (7-9). Since O2 is a prerequisite for aerobic respiration, shortage of O2 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 O2 depleted *P. aeruginosa* biofilms using hyperbaric O2 treatment (HBOT) increases the susceptibility to ciprofloxacin (14). In that study the O2 was removed by bacterial aerobic respiration (14). However, this may be in contrast to the consumption of O2 in the endobronchial secretions of CF patients where the vast majority of O2 is consumed by the PMNs for production of reactive O2 species (ROS) and nitric oxide (NO) whereas only a minute part of O2 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 O2 consumption by activated PMNs prevents engagement of bacterial aerobic respiration we have grown bacterial biofilm without O2 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 \textit{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 \(\Delta\katA\) biofilms to elucidate that ROS play a role in the increased lethality of ciprofloxacin during HBOT. We found significantly less \(\Delta\katA\) bacteria surviving 90 min of treatment with ciprofloxacin when HBOT was applied compared with PAO1 biofilms (\(p < 0.0024, n = 11-14\)), 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 \(\Delta\katA\) 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 % O₂, 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 (µm³): 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 O₂ in *P. aeruginosa* biofilm after HBOT

Vertical profiling of O₂ concentration in the agarose-embedded biofilm immediately after termination of 90 minutes of HBOT, demonstrated O₂ concentrations exceeding 1000 µmol L⁻¹ in the media above the biofilm surface (Fig 4). Serial profiling revealed both rapid depletion of O₂ in
the upper part of the biofilm and O₂ diffusion from the supernatant to the normobaric atmosphere. However, within 20 min post HBOT, the zone of O₂ depletion inside the biofilm was expanded and the O₂ concentration of the supernatant decreased below atmospheric saturation, indicating that PAO1 was utilising the available O₂ for aerobic respiration until O₂-depletion in the biofilm would necessitate conversion to anaerobic respiration (Fig 4).

O₂ 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 O₂ 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 O₂ sensitizes bacteria by stimulating growth in areas deprived of O₂. 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 O₂ exclusion while addition of NO₃⁻ enabled anaerobic respiration
by denitrification (38, 39). The rapid decrease from hyperoxia to hypoxia demonstrated by serial
measurements of O₂ 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 ΔkatA-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 ΔkatA 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 O2 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
plates’ based on a modified Conradi–Drigalski medium containing 10 g L\(^{-1}\) detergent, 1 g L\(^{-1}\) Na\(_2\)S\(_2\)O\(_3\)·H\(_2\)O, 0.1 g L\(^{-1}\) bromothymolblue, 9 g L\(^{-1}\) lactose and 0.4 g L\(^{-1}\) glucose, pH 8.0; Statens Serum Institut, Copenhagen, Denmark) were used to select for Gram-negative bacteria. All plates were incubated overnight at 37°C.

**Anaerobic growth**

*P. aeruginosa* biofilms were grown and treated under anoxic conditions in an anaerobic growth chamber (Concept 400 Anaerobic Workstation, Ruskinn Technology Ltd, UK). The gas atmosphere consisted of N\(_2\)/H\(_2\)/CO\(_2\) (ratio - 80:10:10). Anoxia was confirmed with an optical O\(_2\) sensor (HQ40d multi, HACH Company, CO, US) placed in the growth chamber. To remove traces of O\(_2\), all media and chemical solutions applied for anaerobic work were equilibrated in the anaerobic chamber 3 days prior to experiment.

**Susceptibility testing of mature biofilms**

Survival curves were assayed to investigate the effect of HBOT on *P. aeruginosa* biofilms treated with ciprofloxacin during 90 min. Overnight cultures of PAO1 or ΔkatA optical density at 600 nm (OD\(_{600}\)) was adjusted to 0.4 before 100-fold dilution in LB medium supplemented with 2 % 2-hydroxyethyl-agarose (Sigma–Aldrich, Brøndby, Denmark) and 50 µL loaded into 96-well microtiter plates (Nucleon Delta Surface; Thermo Fisher Scientific, Waltham, MA, USA) to achieve a cell loading of \(\approx 10^6\) cells mL\(^{-1}\). The medium was supplemented with NaNO\(_3\) (1 mM) (Sigma–Aldrich) to enable anaerobic respiration. The supernatant was replaced daily with 50 µL of LB medium supplemented with 1 mM NaNO\(_3\). Microtiter plates were covered with Parafilm (Bemis, Neenah, WI, USA) and lid and were incubated under anoxic conditions at 37°C for 3 days 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 \times 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 15–20 s 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.
**Microscopy and image analysis**

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

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

Size and biomass of aggregates in CLSM image was measured with the use of the Measure Pro Expansion to Imaris 8.3. An iso-surface was applied over the Syto9 stained biomass as well as biomass stained with PI. Iso-surface particles larger than 100 μm³ were consisted. All aggregates within a depth of 100 μm from the surface of the gel were measured, and returned as a measured volume. The radius of aggregates was calculated based on the assumption that aggregates were spherical. For fractionation of live and dead cells, the sum of biomass between Syto9 and PI was used as total biomass. A fraction of both Syto9 and PI of the total biomass was then used as an estimate of live and dead cells.
**Oxygen measurements**

A 3 day old untreated biofilm in a 24-well microtiter plate was treated for 90 min with HBOT. Within 1 min of ending the experiment the microtiter plate was positioned on a heated metal rack, kept at 37°C and vertical micro-profiles of O₂ concentration were recorded using a computer-controlled micromanipulator (Pyro Science GmbH, Germany) equipped with a fiber-optic O₂ microsensor (50 μm tip diameter; Pyro Science GmbH, Germany) that was connected to a fiber-optic O₂ meter (FireSting2, Pyro Science GmbH, Germany). The microsensor was calibrated according to the manufacturer’s recommendations (air saturated and O₂ free water). As the sample was kept at 37°C this temperature was set as measurement temperature in the software. The microsensor was positioned manually at the base of the biofilm sample and profile measurements were taken by moving the sensor in vertical steps of 100 μm through the biofilm sample. Positioning and data acquisition were controlled by dedicated software (Profix version 4.51, Pyro Science).

**Oxygen diffusion (control)**

Diffusion of oxygen in gels without cells was compared between agarose concentrations 0.125% - 2% with NaCl concentration 0.9 g L⁻¹. The gels were placed in test tubes of 65 mm height and inner diameter 12 mm and left to congeal. Heights of the agarose gels ranged from 21 - 41 mm. Hereafter 100 μL saline water (0.9 g L⁻¹) was added on top of the gel to avoid drying and the tubes were sealed with parafilm. The test tubes were placed in an anaerobic chamber (Concept 400 from Baker Ruskinn) at 37 °C for at least 8 days to deoxygenate. The tip of the fiber-optic O₂ micro sensor (OXR50-UHS from Pyroscience) was then positioned at 6 mm depth and the oxygen level was recorded under normoxic conditions as the gel re-oxygenated.
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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Figure 1: Effect of simultaneous hyperbaric oxygen treatment (HBOT) on ciprofloxacin (0.25–2 mg L⁻¹) treatment of anaerobic *Pseudomonas aeruginosa* biofilms. 

**A**. Effect of anoxic (dotted line) and HBOT (filled line) conditions on % surviving cells on agarose embedded PAO1 biofilms to ciprofloxacin (calculated as Δlog₁₀ cell numbers) after treatment for 90 min. Bars indicate the mean ± standard error of the mean (n = 13-19). 

**B**. Effect of ciprofloxacin- and HBO-treated 3-day-old agarose embedded biofilms of PAO1 (filled line) and ΔkatA (dotted line) (calculated as Δlog₁₀ cell numbers) after treatment for 90 min. Bars indicate the mean ± standard error of the mean (n = 11-14). Significant changes (p ≤ 0.05) by particular ciprofloxacin concentrations are indicated by asterisks (*). Statistical significance was evaluated by a two-way ANOVA test followed by Bonferroni’s multiple comparison tests.
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 have 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).
Figure 3: Hyperbaric oxygen treatment (HBOT) effect on bacterial growth in *Pseudomonas aeruginosa* biofilms. Effect of anoxic (circles) and HBOT (squares) conditions on bacterial growth (calculated as Δlog_{10} cell numbers) after treatment for 90 min on agarose embedded PAO1 biofilms. Bars indicate the mean ± standard error of the mean (n = 19). Statistical significance (p ≤ 0.05) was evaluated by the Student t-test.
Figure 4: Optical microsensor measurement of the chemical gradient of O\textsubscript{2} in ciprofloxacin-treated agarose-embedded \textit{Pseudomonas aeruginosa} biofilm. Representative micro-profiling of the spatio-temporal dynamics of O\textsubscript{2} in an agarose embedded PAO1 biofilm receiving HBOT for 90 min showing initial accumulation of O\textsubscript{2} in the media above the biofilm surface and inside the biofilm followed by depletion. The measurement of the O\textsubscript{2} concentration profile was initiated 4 min after termination of HBOT with following profiling.