Oxygen restriction induces a viable but non-culturable population in bacteria

Running title:

Oxygen restriction induces VBNC bacteria

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Abstract: 154 words

Paper: 6122 words

Abstract

This study examined whether anoxic conditioning induces viable but non-culturable (VBNC) bacteria during biofilm growth and if reactive oxygen species (ROS) contribute to this loss of culturability. A significant subpopulation of VBNC *Pseudomonas aeruginosa* was induced by anoxic conditioning, ranging from 5 to 90 % of the total population, in both planktonic and biofilm models. Anoxic conditioning also induced VBNC subpopulations of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Resuscitation of the VBNC population was achieved by substituting 10 mM NO_3^- as an alternative electron acceptor or, in the case of *P. aeruginosa*, by adding sodium pyruvate as a ROS scavenger during normoxic incubation. Bacterial detection in clinical samples was improved by supplementing 10 mM NO_3^- to LB plates and incubating under anoxic conditions. These results demonstrate that habituation to an infectious anoxic micro-environment complicates diagnostic culturing of bacteria, especially in the case of chronic infections where oxygen is restricted due to the host immune response.

Keywords:

anoxia / **biofilm** / ***P. aeruginosa*** / **reactive oxygen species** / **viable but non-culturable**

Background

Bacteria cultured in laboratories only represent a small fraction of the bacteria found in nature. It is estimated that less than 1 % of environmental bacteria can grow on standard laboratory media (Davey, 2011). This phenomenon is also present in clinical diagnostics where detection of bacteria by 16S rDNA-PCR often detects a wider variety of bacteria than conventional, growth-depend methods (Costerton, Post *et al*., 2011, Grif, Heller *et al*., 2012, Meyer, Franke *et al*., 2014). Clinical laboratories often culture bacteria with enriched media, developed to support the growth of particular pathogens. However, even these human bacterial pathogens can enter a growth-restricted, transient state resulting in loss of culturability, especially following antibiotic treatment (Pasquaroli, Zandri *et al*., 2013). These bacteria are termed viable but non-culturable (VBNC) and are defined by their lack of growth during conventional plating (Oliver, 2010). They are, nonetheless, viable and may still be virulent (Ramamurthy, Ghosh *et al*., 2014). VBNC bacteria are characterized by low metabolic activity, significant cell dwarfing, altered cell-wall composition, decreased respiration and decreased macromolecule biosynthesis (Li, Mendis *et al*., 2014, Oliver, 2010). VBNC bacteria are apparent by microscopic identification in clinical cases presenting sign of infection, but no positive cultures (Bjarnsholt, Tolker-Nielsen *et al*., 2009, Burmølle, Thomsen *et al*., 2010, Costerton *et al*., 2011, Stewart, 2012). These cases are especially prevalent during chronic infection, where lack of culturability prevents proper diagnosis and treatment *(*Costerton *et al.*, 2011, Høiby, Bjarnsholt *et al*., 2010, Høiby, Bjarnsholt *et al*., 2015).

Several environmental stresses may induce a VBNC state, e.g. change in temperature or pH, nutrient starvation, change in osmotic concentrations and presence of heavy metals or antibiotics (Oliver, 2010). Oxygen (O_2) starvation can also induce a VBNC state in batch cultures of *Pseudomonas aeruginosa*, but supplementation with nitrate (NO_3^-) as an alternative electron acceptor during anoxic plating restores culturability (Binnerup, 1993). This suggests that anoxic

conditioning sensitizes a subpopulation of *P. aeruginosa* to O_2 or its toxic derivatives, such as reactive oxygen species (ROS). Presence of ROS has, in some studies, been shown to generate VBNC bacteria (Noor, 2015, Oliver, 2010), but most research has focused on *Escherichia coli* strains or *Vibrio* spp., while the effects of ROS on *P. aeruginosa* are not well characterized. Evidence of anoxic zones and anaerobic bacterial activity in chronic infections suggests that colonizing bacteria, such as *P. aeruginosa,* experience O_2 starvation (Worlitzsch, Tarran *et al.*, 2002; Hassett, Cuppoletti *et al.*, 2002; Kolpen, Kühl *et al.*, 2014). High rates of O_2 consumption by polymorphonuclear leukocytes generate local, anoxic zones and likely play a major role in O_2 depletion during chronic infection (Høiby *et al.*, 2015, Kolpen, Bjarnsholt *et al.*, 2014, Kolpen, Hansen *et al.*, 2010). Many chronic infections are thought to contain bacteria in the biofilm mode of growth (Costerton, Veeh *et al.*, 2003) and endogenous O_2 depletion inside the biofilm (Sønderholm, Koren *et al.*, 2018), along with intense O_2 consumption by the host immune response (James, Ge Zhao *et al.*, 2016, Jensen, Kolpen *et al.*, 2017, Kragh, Alhede *et al.*, 2014, Trunk, Benkert *et al.*, 2010, Worlitzsch, Tarran *et al.*, 2002), may therefore increase the number of VBNC bacteria. Lack of growth in diagnostic clinical cultures may lead to incorrect diagnosis, thus preventing optimal treatment. In the worst case, samples appear negative despite a true infection, leading to insufficient treatment for the patient.

This study aimed to determine whether anoxic conditioning generates VBNC cells during biofilm growth. Furthermore, we investigated whether ROS are involved in the loss of culturability. Finally, we evaluated the efficiency of two novel, anoxic growth medias to grow bacteria from clinical samples with suspected biofilm infection.

Results

Anoxic conditioning induced oxygen intolerant subpopulations of *P. aeruginosa* in biofilm and planktonic models.

To examine if anoxic conditioning during biofilm growth affects the subsequent normoxic and anoxic plating, *P. aeruginosa* was grown in an alginate bead biofilm model (Sønderholm, Kragh *et al*., 2017). Viable plate counts (CFU/mL) were performed on LB plates supplemented with 10 mM NO_3^-. Plates were incubated under normoxic and anoxic conditions (plating method) and plate counts were compared from the beads (biofilm) and the surrounding media (planktonic) of the same vials over a period of 21 days (Figure 1A and 1B, respectively). The log difference was significantly ($p = 0.003$) higher for biofilm than planktonic cells (Figure 1C). The cells represented by this difference were considered VBNC and ranged from 5 to 54 % of the entire population from day 6 to day 19 (Appendix Figure S1).

P. aeruginosa was also grown anoxically or normoxically on a filter biofilm model using LB plates supplemented with 10 mM NO_3^- over a period of 17 days. CFU/mL was then determined using anoxic and normoxic plating, as above (Figure 2A and 2B, respectively). The log difference between plating methods was significantly ($p = 0.01$) higher for anoxically conditioned biofilms than normoxically conditioned biofilms (Figure 2C). The fraction of VBNC *P. aeruginosa* ranged from 6 to 23 % of the entire population from day 3 to day 17 (Appendix Figure S1).

Similar results were obtained for anoxically and normoxically conditioned planktonic batch cultures of *P. aeruginosa* over a period of 28 days (Figure 3A and 3B, respectively). The log difference between plating methods was significantly ($p < 0.0001$) higher for anoxically conditioned batch cultures (Figure 3C). The fraction of VBNC bacteria ranged from 60 to 90 % of the entire population from day 9 to 21 (Appendix Figure S1).

To further investigate the effect of anoxic conditioning, colonies of *P. aeruginosa* were grown on LB plates supplemented with 10 mM NO_3^- under anoxic and normoxic conditions over a period of 20 days (Figure 4A and 4B, respectively). The log difference between plating methods was significantly higher ($p < 0.0001$) for anoxically conditioned colonies (Figure 4C). The fraction of VBNC *P. aeruginosa* ranged from 12 to 46 % of the whole population from day 6 to 20 (Appendix Figure S1).

10 mM NO_3^- in LB plates did not affect CFU/mL in batch cultures of *P. aeruginosa*.

To ensure that presence of NO_3^- did not affect the number of CFU generated on LB plates, CFU/mL was determined anoxically (LB plates + 10 mM NO_3^-) and normoxically (LB plates +/- 10 mM NO_3^-) from 24-hour-old batch cultures of *P. aeruginosa*. We found no difference between types of incubation ($p = 0.93$): anoxic log CFU/mL + NO_3^- = 9.96 (± 0.22), normoxic log CFU/mL + NO_3^- = 9.91 (± 0.10) and normoxic log CFU/mL - NO_3^- = 9.91 (± 0.14). Furthermore, there was no effect of NO_3^- supplementation on normoxic CFU/mL determinations in the prolonged experiment with 28-day-old batch cultures (Figure 3A and 3B). It was not possible to detect growth of *P. aeruginosa* on LB plates under anoxic conditions without NO_3^-, but growth was observed when these plates were placed under normoxic conditions.

Oxidative stress restricted the growth of anoxically conditioned *P. aeruginosa* when re-grown in a normoxic environment.

To investigate whether formation of ROS contributed to the VBNC state induced by anoxic conditioning, bacteria from 16-day-old anoxically conditioned batch cultures of *P. aeruginosa* were plated on LB plates with and without 0.3 % sodium pyruvate. The presence of sodium pyruvate as a ROS scavenger (Wang, Perez *et al*., 2007) significantly ($p = 0.02$) increased normoxic CFU/mL

compared to incubation without sodium pyruvate (Figure 5A). This was not the case in 24-hour-old batch cultures of *P. aeruginosa* ($p = 0.62$), indicating that the effect was restricted to anoxically conditioned cells (Figure 5B). This experiment was then repeated with a catalase deficient *P. aeruginosa* mutant (Δ*katA* PAO1), which is more susceptible to oxidative stress (Hassett, Ma *et al.*, 1999, Jensen, Briales *et al.*, 2014). The log difference between plating methods for the Δ*katA* mutant and reference strain was significantly ($p = 0.00002$, unpaired t-test) different. These log difference values were 3.02 ± 0.05 and 1.61 ± 0.07 for Δ*katA* PAO1 and the reference strain, respectively (Figure 5C).

Direct viable count with LIVE/DEAD staining revealed a larger population of VBNC *P. aeruginosa*.

Direct viable counts were carried out for 16-day-old, anoxically conditioned batch cultures to estimate the size of the VBNC population. Findings were compared to normoxic and anoxic plate counts performed on LB plates supplemented with 10 mM NO_3^- (Appendix Figure S2-B). Anoxic incubation yielded a significantly ($p = 0.002$) higher CFU/mL than normoxic incubation (0.85 log values ± 0.04), as expected. When determining bacterial counts with direct viable counting, a significant higher number of viable cells could be calculated compared to normoxic incubation (2.31 log values ± 0.4, $p = 0.002$) and anoxic incubation (1.46 log values ± 0.5, $p = 0.008$). Direct viable counts were also performed on a 24-hour-old batch culture to investigate whether findings of VBNC *P. aeruginosa* was restricted to anoxically conditioned cells. No difference of bacterial counts were observed (Appendix Figure S2-A).

Anoxic conditioning affects *Staphylococcus aureus* and *Staphylococcus epidermidis* but not *Escherichia coli* and *Enterococcus faecalis*

178 The effect of anoxic conditioning was then tested on a selected group of pathogens to determine if
179 this phenomenon was restricted to *P. aeruginosa*. *S. aureus* (methicillin susceptible) was tested as
180 described in the *P. aeruginosa* filter biofilm setup. Viable plate counts for anoxic and normoxic
181 conditioned filters was determined (Figure 6A and 6B, respectively). The log difference between
182 plating method was significantly higher ($p < 0.001$) when viable plate counts were performed from
183 anoxically conditioned filter biofilms than from normoxically conditioned filter biofilms (Figure
184 6C). The fraction of VBNC methicillin susceptible *S. aureus* ranged from 3 to 89 % of the entire
185 population from day 1 to day 17 (Appendix Figure S1). Since both *P. aeruginosa* and *S. aureus*
186 demonstrated effects of anoxic conditioning, a smaller experiment was initiated to test other
187 pathogens. The effect of anoxic conditioning was determined on LB plates supplemented with 10
188 mM NO_3^- with and without sodium pyruvate to investigate if ROS were involved in the lack of
189 growth. Both *S. aureus* (MRSA) and *S. epidermidis* showed effects of anoxic conditioning,
190 resulting in an significant ($p = 0.01$ and $p = 0.03$, respectively) increase in CFU/mL during anoxic
191 incubation compared to normoxic incubation (Appendix Figure S3). There was no observed effect
192 of sodium pyruvate during normoxic incubation for these two strains. In the case of *E. coli* and *E.*
193 *faecalis*, there was no observed effect of anoxic conditioning (Appendix Figure S4).
194
195 **Implementation of a supplementary diagnostic media increased findings from samples where**
196 **biofilm infections could be expected.**
197 To determine the effect of VBNC on the outcome of culturing from patient samples, LB and ABTG
198 plates supplemented with 10 mM NO_3^- were introduced as an anoxic growth media in addition to
199 standard culturing practices at the Department of Clinical Microbiology, Rigshospitalet,
200 Copenhagen, Denmark. A total of 135 samples were consecutively collected from 73 patients. Only
201 samples from sterile parts of the body, e.g. soft tissues and bones, were included. Appendix Figure

S5 A and B show the distribution of bacterial findings from the conventional method compared with LB and ABTG plates supplemented with 10 mM NO_3^-. When comparing LB plates supplemented with 10 mM NO_3^- and the conventional method, an equally number of culture positive samples (45 vs. 50) were identified, resulting in 27 and 28 patients with culture positive findings, respectively (Appendix Figure S5 B). Interestingly, 19 microorganisms from 16 patients were found exclusively on LB plates supplemented with 10 mM NO_3^- and in 5 cases (6.9 %), only NO_3^- supplemented LB plates were culture positive (Table 1). The clinical significance of these observations was not possible to investigate due to Danish legislation regarding access to patient files. The use of ABTG plates supplemented with 10 mM NO_3^- did not provide significant benefit for growing microorganisms from clinical samples (Appendix Figure S5 A).

Discussion

VBNC *P. aeruginosa*

In the current study, we demonstrate that viable plate counting with *P. aeruginosa* is significantly affected by anoxic conditioning when cultured in the presence of atmospheric oxygen levels. Oxygen intolerant subpopulations are created after only a few days of anoxic conditioning in both biofilm and planktonic models.

VBNC *P. aeruginosa* was observed in the beads from the bead biofilm model, but not in the surrounding suspension, despite that the cultures had full access to atmospheric oxygen. This indicates that oxygen restriction within a biofilm induces a VBNC subpopulation. To our knowledge, it has not previously been reported that a VBNC state can be induced in a *P. aeruginosa* biofilm. However, this has been demonstrated in *S. epidermidis* biofilms (Cerca, Trigo *et al*., 2011). The VBNC state, detected as increased growth during anoxic incubation with NO_3^-, has previously been generated in planktonic *P. aeruginosa* by energy starvation after cultivation without O_2 as

electron acceptor for aerobic respiration (Binnerup, 1993). *In vitro* biofilms of *P. aeruginosa* may contain internal anoxic zones (Kolpen, Mousavi *et al*., 2016, Walters, Roe *et al*., 2003), so we hypothesized that anoxia inside the biofilm contributes to the induction of VBNC *P. aeruginosa*. Accordingly, VBNC *P. aeruginosa* was only observed in the filter biofilm model and the colony model when the model was anoxically conditioned. Furthermore, VBNC *P. aeruginosa* was also induced in planktonic cultures by anoxic conditioning.

In the present study, it appears that approximately 50 % of the population in the alginate beads was VBNC even though they were kept in normoxically-conditioned vials. In comparison, the fraction of VBNC organisms was approximately 90 % in the anoxically conditioned batch cultures. The difference (90 % vs. 50 %) may be explained by the fact that the majority of the bacteria in the beads are peripherally located where they have increased access to oxygen compared to the center of the beads (Sønderholm *et al*., 2017). In comparison, bacteria from the anoxically conditioned batch cultures were fully deprived of O_2. The observed difference in viable plate counts between normoxic and anoxic incubation in the bead model could be of crucial importance in a clinical setting, as it affects the number of organisms that can be cultured.

In the present study, the effect of anoxic conditioning was restricted to an intermediate period. This is probably an artefact due to the static methodological setup. Dynamic processes, such as entry of nutrients and removal of waste, occur during infection and are not modeled here (Brown, Palmer *et al*., 2008). Nevertheless, this is not the first time a "resuscitation window" has been described for VBNC bacteria. Similar studies have shown that VBNC bacteria can only be detected in an intermediate period of culturing (Pinto, Santos *et al*., 2015).

ROS restricts growth of anoxic conditioned *P. aeruginosa*

The VBNC fraction of bacteria in this study appeared to be O_2 intolerant given that growth only was achievable when NO_3^- served as an alternative electron acceptor during anaerobic respiration.

This led us to investigate whether this phenomena was due to oxidizing properties of ROS created by incomplete reduction of O_2 during aerobic respiration (Fenchel & Finlay, 2008). Sodium pyruvate increased viable counts under normoxic incubation (roughly 43 % of the VBNC population) and we believe that it was due to its properties as a ROS scavenger (Wang *et al*., 2007). Accordingly, 0.3 % sodium pyruvate has been used to resuscitate VBNC populations of *S. aureus* (Pasquaroli *et al*., 2013). It has been suggested that VBNC cells cannot be resuscitated by addition of ROS scavengers and that "revived" cells in the presence of ROS scavengers are only injured cells and not VBNC cells (Pinto *et al*., 2015). It was not possible to determine whether bacteria were in an injured state in this study. Instead, the effect of ROS was further confirmed with a catalase A deficient *P. aeruginosa* mutant (Δ*katA* PAO1). The difference between normoxic and anoxic CFU counts were significantly greater than the difference observed with the reference strain *P. aeruginosa* grown under same conditions. In contrary, anoxic CFU determination of Δ*katA* PAO1 yielded almost as many CFU as it did with the reference strain. These results indicate that accumulation of H_2O_2 during aerobic respiration has an impact on viable plate counting when *P. aeruginosa* has been anoxically conditioned.

Direct viable counting reveals a larger VBNC population

To account for the limitations of plate counting, direct viable counts were conducted to investigate whether the viable population was larger than observed during plating with NO_3^- supplementation. When comparing plate counts from 16-day-old anoxic conditioned batch cultures, we were able to show that anoxic incubation increased the number of viable cells with 0.85 log values compared to normoxic plate counting, thus 86 % of the population was VBNC. When comparing direct viable counting with normoxic viable plate counts, we observed a difference of 2.31 log values, thus the "real" fraction of VBNC *P. aeruginosa* was 99.68 %. As all methods, direct viable counting has its limitations. LIVE/DEAD staining is based upon membrane permeability and is only an

approximation of true viability. Cells that were stained both red and green (resulting in yellow) were counted as dead cells, but may still be viable.

S. epidermidis and _S. aureus_ also become VBNC after anoxic conditioning

Additional experiments were conducted to elucidate whether anoxic conditioning induces VBNC cells in other facultative pathogens. Filter biofilms of methicillin susceptible *S. aureus* showed the same effects of anoxic conditioning as *P. aeruginosa*. The difference between normoxic and anoxic plating for anoxically conditioned filters was significantly ($p < 0.001$) higher than the difference observed for normoxically conditioned filters. These experiments were also repeated for *S. epidermidis*, *S. aureus* (MRSA), *E. coli* and *E. faecalis*. Additionally, CFU was determined on LB plates supplemented with NO_3^- with and without sodium pyruvate to see if ROS was involved in a loss of cultivability. Both *S. epidermidis* and *S. aureus* were significantly ($p = 0.01$ and $p = 0.03$, respectively) affected by anoxic conditioning, whereas *E. coli* and *E. faecalis* were not. No effect of sodium pyruvate was found during normoxic incubation for any of these tested organisms, suggesting that ROS is not involved in the loss of culturability for these pathogens, but additional ROS scavengers should be tested.

Efficacy of LB plates supplemented with NO_3^- in a clinical setting

LB plates supplemented with NO_3^- were tested in a clinical setting and enabled detection of bacteria in almost as many patients as the conventional method (27 vs. 28, respectively). Findings of microorganisms (n=19 in 16 patients) exclusively detected on LB plates supplemented with NO_3^- were primarily “low-virulent” bacteria. These samples often also contained "highly virulent” bacteria. "Highly virulent” bacteria grew equally well on both LB plates supplemented with NO_3^- and with the conventional method. With the exception of *Helcococcus kunzii*, all identified bacteria detected exclusively by LB plates supplemented with NO_3^- were known to harbor nitrate reductase, enabling reduction of NO_3^-. Furthermore, according to the literature, all identified bacteria are able

to produce biofilm and have been associated with chronic infections (Table 1). LB plates supplemented with NO_3^- detected growth in 6.9 % of patients (n=5, mostly Staphylococcal species), whereas the conventional method did not. These organisms are often associated with chronic infections, although they are considered "low virulent" (Rogers, Fey *et al*., 2009). These findings indicate that anoxic LB agar plates supplemented with NO_3^- may be useful as a supplementary medium for the cultivation of "low-virulent" bacteria from chronic infections.

The VBNC state is thought to be a stress response to harsh environments. This is supported by findings that changes in pH, temperature, nutrient starvation, oxygen depletion combined with low redox potential and antimicrobial substances can induce the VBNC state (Mascher, Hase *et al*., 2000, Oliver, 2010, Pasquaroli *et al*., 2013). Recently, Li *et al*. (2014) listed 51 human pathogens that were able to enter the VBNC state, including *P. aeruginosa*. Only 26 of these pathogens were resuscitated, possibly due to inadequate culture methods (Li *et al*., 2014). The list was later extended to 68 human pathogens (Pinto *et al*., 2015). As interest within the phenomena of VBNC cells grows, it becomes more apparent that it may be a universal trait for bacteria.

The fact that bacteria can return to a viable state supports the hypothesis that VBNC is a survival strategy. Another hypothesis suggests that the VBNC state is a transition state of a degenerating bacterial population leading to cell death, but there is shortage of evidence supporting this hypothesis, why the first hypothesis is generally more accepted (Li *et al*., 2014).

Our results suggest that habituation to the environment prior to regrowth should be considered in the case of pathogens such as *P. aeruginosa*, *S. aureus* and *S. epidermidis* and possibly other facultative organisms.

Conclusion

The VBNC state can be induced by several physiological stresses and our knowledge in this area is expanding, though far from fully resolved. We demonstrate that a VBNC subpopulation is induced by anoxic conditioning during biofilm growth in *P. aeruginosa*, *S. aureus* and *S. epidermidis*. The VBNC population was only able to grow under anoxic conditions in the presence of NO_3^- as an alternative electron acceptor. In the case of *P. aeruginosa*, this phenomenon was, in part, explained by creation of lethal amounts of ROS during aerobic respiration and the bacteria's inability to neutralize it. LB plates supplemented with 10 mM NO_3^- were an effective, anoxic growth medium for resuscitation of anoxically conditioned *P. aeruginosa*, and improved bacterial growth in clinical samples.

Materials and Method

Bacterial strains

Pseudomonas aeruginosa (PAO1, ATCC 15692), a catalase A deficient *Pseudomonas aeruginosa* strain (Δ*katA* PAO1) (Hassett *et al*., 1999), *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* NCTC 8325-4 (methicillin susceptible) (Frees, Chastanet *et al*., 2004), *Staphylococcus aureus* USA300 JE2 (MRSA) (Fey, Endres *et al*., 2013), E*scherichia coli* CFT073 (Welch, Burland *et al*., 2002) and a clinical strain of *Enterococcus faecalis* from the Department of Clinical Microbiology, Copenhagen University Hospital – Rigshospitalet, Denmark were used in this study.

Agar plates and media

Primarily lysogeny broth (LB) agar plates were applied in this study. Lysogeny broth (pH 7.5) consisted of 5 g/L yeast extract (Oxoid, Roskilde, Denmark), 10 g/L tryptone (Oxoid), 10 g/L NaCl (Merck, USA). Both LB and minimum media plates were tested as supplementary anoxic growth media for clinical samples. Minimum media consisted of Btrace media buffered with 10% A-10

phosphate buffer and supplemented with 0.5 % (w/v) glucose (Panum Institute Substrate Department, DK), referred to as ABTG media throughout the paper. All plates contained 2 % agarose. Plates used for anoxic growth were supplemented with 10 mM KNO_3 (Sigma-Aldrich, USA) to serve as alternative electron acceptor, referred to as NO_3^- throughout the paper. All agar plates and media in this study were supplied by the Panum Institute Substrate Department (Copenhagen, DK).

Anoxic growth

Experiments investigating growth under anoxic conditions were performed in an anaerobic chamber (Concept 400 Anaerobic Workstation, Ruskinn Technology Ltd, UK). The gas atmosphere consisted of $N_2/H_2/CO_2$ (ratio - 80:10:10). Anoxic chamber environment was confirmed with an oxygen sensor (HQ40d multi, HACH Company, USA). All media and chemical solutions used in anaerobic experiments were equilibrated in the anaerobic chamber 3 days prior to experiment. In the case of solutions requiring refrigeration, a minimal volume was applied (< 1 mL), sealed with Parafilm M, and thoroughly shaken upon entry into anaerobic chamber for quick gas equilibration.

Bead-embedded inoculum of *P. aeruginosa*

Preparation of alginate beads with *P. aeruginosa* was carried out according to a method described by Sønderholm *et al.* (2017). Subsequently, beads were divided (10 beads per vial) into vials (Oximate Vial, PerkinElmer Inc., USA) containing 15 mL LB medium. Vials were sealed with Parafilm M incubated at 37°C on an orbital shaker at 180 rpm. Anoxic and normoxic CFU counts were determined from beads and from the surrounding suspension from the same vials on each sampling day (day 3, 6, 8, 12, 19 and 21). Two beads were sampled per biological replicate (4 biological replicates in total). Before determination of CFU, the beads were washed twice with 0.9 % NaCl to remove non-attached cells and transferred to 1.5 mL microcentrifuge tubes (Sigma-Aldrich, USA). One-hundred microliters of 0.1 M sodium carbonate (Na_2CO_3) followed by 100 µL

of 0.04 M citric acid was added to the tubes to dissolve the beads. The suspensions were then sonicated (5 min degas + 5 minutes sonication; Bransonic ultrasonic cleaner 2510, Emerson Electric, USA) before ten-fold dilution series were performed in 0.9 % NaCl. CFU was determined by plating three, 10 µL-drops per dilution per replicate. Anoxic CFU determinations were performed inside an anaerobic chamber on LB plates supplemented with 10 mM NO_3^-. The same dilution method was applied to normoxic CFU determination outside the anaerobic chamber on LB plates with 10 mM NO_3^-. Plates were incubated 2 days before counting CFU.

Filter biofilms with *P. aeruginosa*

This protocol was adapted to grow reproducible biofilms under anoxic and normoxic conditions. The method has previously been described by Bjarnsholt *et al.* (2015). The filter biofilms were kept on the same LB plates throughout the experiment. *P. aeruginosa* was propagated from frozen stock and grown overnight in 20 mL LB medium at 37˚C on an orbital shaker at180 rpm. Cultures were adjusted to an optical density of 0.05 (OD_{600}; UV spectrophotometer UV-1800 UV-VIS, Shimadzu corporation, JP) and 10 µL was transferred to the cellulose nitrate membrane filters (25 mm in diameter, GE Healthcare Life Sciences, UK). Plates were incubated under normoxic and anoxic conditions and kept in plastic bags with wet paper to avoid dehydration. Two filters were sampled per biological replicate (4 biological replicates in total) on each sampling day (day 1, 3, 7, 15 and 17). Filters were removed, placed in 10 mL tubes containing 5 mL 0.9 % NaCl, vortexed thoroughly (1 min) and sonicated (5 min degas + 5 minutes sonication). A ten-fold dilution series was performed in 0.9 % NaCl and normoxic and anoxic CFU were determined by plating three 10 µL-drops per filter on LB plates supplemented with 10 mM NO_3^-. Plates were incubated for 2 days before counting CFU. The anoxic setup was conducted in an anaerobic chamber and CFU determination was carried out in the same way as the normoxic setup.

Liquid batch cultures of *P. aeruginosa*

P. aeruginosa was propagated from frozen stock and grown overnight in 20 mL LB media at 37ºC and orbitally shaken at 180 rpm. Cultures were adjusted to an optical density (OD_{600}) of 0.1 in glass vials (Oximate Vial) with a final volume of 20 mL LB. Vials were left to incubate at 37°C and orbitally shaken at 180 rpm. To create a normoxic environment, half of the vials were incubated with Parafilm M on top (normoxic conditioning), while the rest were incubated with a lid on top creating an anoxic environment (anoxic conditioning). Anoxic and normoxic determination of CFU was carried out from normoxic and anoxic conditioned liquid batch cultures (referred to as batch cultures throughout the paper) on each sampling day (day 1, 3, 5, 9, 11, 14, 16, 18, 21 and 28). Normoxic CFU determination was carried out on LB plates with and without 10 mM NO_3^- to test whether presence of NO_3^- affected the number of CFU. Two mL (2 x 1 mL = 2 technical replicates) were sampled per biological replicate (4 biological replicates in total) on each sampling day. CFU/mL was determined as described in previous sections.

Colonies of *P. aeruginosa*

P. aeruginosa was propagated from frozen stock and grown overnight in 20 mL LB media at 37ºC on an orbital shaker at 180 rpm. The cultures were then streaked onto LB plates supplemented with 10 mM NO_3^-. Plates were incubated under normoxic and anoxic conditions and kept in plastic bags with wet paper to avoid dehydration. A 1 µL loop was used to sample colony material from each biological replicate (3 biological replicates in total) on each sampling day (day 6, 9, 13, 15 and 20). Colonies were transferred to 1.5mL microcentrifuge tubes (Sigma-Aldrich, Denmark) with 0.5 mL 0.9 % NaCl, vortexed thoroughly (1 min) and sonicated (5 min degas + 5 minutes sonication). CFU/mL was determined as described earlier.

Reactive oxygen species (ROS)

To elucidate whether growth of anoxic conditioned bacteria was restricted by creation of ROS during aerobic respiration, sodium pyruvate (Sigma-Aldrich, USA) was tested as ROS scavenger in

LB plates with 10 mM NO_3^-. Anoxic and normoxic CFU/mL was determined from 24-hour-old batch cultures and anoxic conditioned 16-day-old batch cultures of *P. aeruginosa*. Only LB plates used for normoxic determination of CFU were casted with 0.3 % sodium pyruvate. Furthermore, CFU/mL was also determined from 16-day-old anoxic conditioned batch cultures of *P. aeruginosa* and a catalase A deficient *P. aeruginosa* (Δ*katA* PAO1) to test the influence of ROS. All experiments were performed in biological triplicates.

Filter biofilms with other pathogens

To determine if other bacteria behaved in the same way as *P. aeruginosa*, we investigated the effect of anoxic conditioning on a selected group of pathogens (*Staphylococcus epidermidis*, *Staphylococcus aureus* (methicillin susceptible), *Staphylococcus aureus* USA300 JE2 (MRSA), E*scherichia coli* CFT073 and a clinical strain of *Enterococcus faecalis*). We used the filter biofilm method as described earlier. For the methicillin susceptible strain, CFU was determined in the same way as described for the filters with *P. aeruginosa*. For the remaining strains, CFU was determined at day 1 and 9 on LB plates supplemented with 10 mM NO_3^- +/- O_2. Furthermore, CFU was determined on LB plates supplemented with 10 mM NO_3^- and 0.3 % sodium pyruvate to evaluate if the effect of ROS was likewise causing the lack of growth for these pathogens. A minimum of 3 biological replicates was performed and CFU was carried out in the same way as described earlier.

Direct viable count with LIVE/DEAD staining

LIVE/DEAD staining was applied to estimate the proportion of viable and non-viable cells in 24-hour-old batch cultures and in 16-day-old anoxic conditioned batch cultures of *P. aeruginosa*. The dyes consisted of two fluorescent nucleic acid stains; the green fluorescent stain (live cells) SYTO9 (Invitrogen, USA) and the red fluorescent stain (dead cells) propidium iodide (PI, Sigma-Aldrich, USA). SYTO9 penetrates both intact and damaged membranes while PI only stains damaged cells, thereby creating an opportunity to discriminate between live and dead cells (Li *et al*., 2014).

Bacterial suspensions were vortexed thoroughly (1 min) and sonicated (5 min degas + 5 minutes sonication) before staining. To stain the cells, 1 µL of PI and SYTO9 was added to 1 mL of bacterial suspension and incubated for 15 minutes at room temperature. After staining, the suspensions were filtered through a 0.2 µm black Whatman, Nuclepore Trach-Etch Membrane (Sigma-Aldrich, Denmark). Bacteria on filters were visualized with confocal laser scanning microscopy using a Zeiss LSM 710 with a 63×/1.4 (numerical aperture) objective (Zeiss, Germany). Fifteen random fields (135µm x 135 µm) were examined for each filter. Enumeration of live (green) and dead (red) bacteria were done with the IMARIS software package (Bitplane AG, Schwitzerland). Cells that were stained both with PI and SYTO9 were considered non-viable and thus counted as dead cells. Normoxic and anoxic CFU/mL was carried out simultaneously to estimate the proportion of VBNC cells. CFU/mL was determined in the same way as described for liquid batch cultures. Conversion of enumerated viable and dead cells to bacterial counts per milliliter was performed as described by Boulos, Prevost *et al.*, 1999 to compare them with CFU/mL.

Implementation of supplementary plates

LB and ABTG plates, supplemented with 10 mM NO_3^-, were tested as a supplementary anoxic growth media at the Department of Clinical Microbiology, Rigshospitalet, Denmark. A total of 135 plates of each were applied to test if NO_3^- would improve microbiological findings from clinical samples where potential biofilm infections could be suspected. The plates were implemented in the daily routine on tissue and bone samples from sterile sites of the body. Plates were incubated up to 7 days in an anaerobic chamber. Microbiological findings were performed by inspection of plates at day 3, 5 and 7, and confirmation of species was performed with Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Microflex LT instrument (Bruker Daltonik GmbH, Germany). Protein profile was obtained by the software FlexControl 3.3

(Bruker Daltonik GmbH), analyzed with FlexAnalysis 3.3 (Bruker Daltonik GmbH). The database used to match spectra was Bruker Taxonomy (7311 MSPs). Results were estimated per patient and compared with findings from conventional methods applied at the Department of Clinical Microbiology, Rigshospitalet. The conventional method consisted of determination of species from solid agar plates under normoxic and anoxic incubation. Normoxic incubation was performed on solid agar plates ['Blue plates', a modified Conradi-Drigalski medium containing 10 g/L detergent, 1 g/L $Na_2S_2O_3 \cdot H_2O$, 0.1 g/L bromothymol blue, 9 g/L lactose, 0.4 g/L glucose, pH 8.0], [Blood agar plates, 5% horse blood, pH 7.4] and in serum bouillons [Basal culture medium supplemented with defibrinated horse blood and horse serum]. Anoxic incubation was performed on solid agar plates ['Chocolate plates', a modified Reyn and Bentzon medium containing defibrinated horse blood, ascitic fluid in a broth-agar base consisting of 2.4 % of Danish AKI agar in beefheart broth with 1 % of peptone ("Orthana" special), 0.3% of NaCl and 0.2% of $Na_2HPO_4 \cdot 12H_2O$] (Møller & Reyn, 1965), ['anaerobic plates', prepared as chocolate medium and supplemented with vitamin K and cysteine] and in thioglycollate broths [anaerobic culture medium for sterility]. All media applied at the clinic were produced and delivered by Statens Serum Institut (Copenhagen, Denmark). Microbiological findings by the conventional method were also confirmed with MALDI-TOF MS.

Ethics

A comparison of culture-methods for quality assurance is according to Danish legislation not a health research project, as defined by the 'Danish Act on Research Ethics Review of Health Research Projects'. Hence, comparison of culture methods on clinical samples could be initiated without approval from The Committees on Health Research Ethics in the Capital Region of Denmark.

Statistics

To evaluate the difference in bacterial growth between incubation conditions (normoxic and anoxic conditioning), a linear regression was used with the difference between the logarithmically transformed values for normoxic and anoxic colony counts as outcome and with day (categorical) and interaction between day and incubation condition (binary) as explanatory variables in SAS Genmod Procedure. The p-value of the interaction term was used as the p-value for the difference in bacterial growth. Log difference was calculated as ($\log_{10}[\text{CFU/mL}]_{\text{anoxic}}$ - $\log_{10}[\text{CFU/mL}]_{\text{normoxic}}$). The mean and standard error of the mean (SEM) were calculated for recovering bacteria and plotted using GraphPad Prism 6.1 (GraphPad Software, La Jolla, USA). Fractions of VBNC bacteria were estimated in growth experiments when difference in plate counts was noted between plating methods. From the ratio between anoxic (CFU $-O_2$) and normoxic (CFU $+O_2$) colony counts it was possible to calculate the fraction of VBNC bacteria [% VBNC bacteria = $\frac{(\text{CFU} -O_2)-(\text{CFU} +O_2)}{(\text{CFU} -O_2)}$ x 100]. Data that were not part of the long-term experiments were instead analyzed with a 1-way ANOVA followed by Tukey's multiple comparison tests, Fisher's exact test or an unpaired t-test. A p-value $\leq$ 0.05 was considered statistically significant. The tests were performed with either Prism 6.1 (GraphPad Software, La Jolla, USA) or SAS v.9.4 (SAS Institute Inc., Cary, NC, USA)

Funding information

This study was supported by grants from The Lundbeck foundation to TB and by a UC-CARE (University of Copenhagen–Center for Antimicrobial Research) grant to MK. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication

Acknowledgments

We would like to thank the laboratory technicians at the Department of Clinical Microbiology, Rigshospitalet, for helping us with the collection of samples.

Author contributions

LK performed the majority of the experiments. MK, MS, BFG, SC and KNK also performed experiments. TB, PØJ, KNK AK, and LK conceived and designed experiments. LK wrote the manuscript. MA and PØJ performed statistics. All authors analyzed data. All authors contributed to and corrected the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in the bead biofilm model

A) Normoxic and anoxic CFU/mL of PAO1 over 21 days from the beads. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

B) Normoxic and anoxic CFU/mL of PAO1 over 21 days from the suspension surrounding the beads. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

C) The log difference was calculated as the difference in mean log CFU/mL between plating methods from the suspension and the beads, respectively. The log difference was significantly higher ($p = 0.003$, linear regression) in the beads than the surrounding suspension. Symbols with error bars indicate the mean + confidence intervals.

Figure 2 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in the filter biofilm model

A) Normoxic and anoxic CFU/mL of PAO1 over 17 days from anoxically conditioned filters. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

B) Normoxic and anoxic CFU/mL of PAO1 over 17 days from normoxically conditioned filters. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

C) The log difference was calculated as the difference in mean log CFU/mL between plating methods from the normoxic and anoxic conditioned filters, respectively. The log difference was

725 significantly higher ($p = 0.01$, linear regression) in the anoxic conditioned filters than the
726 normoxic conditioned. Symbols with error bars indicate the mean + confidence intervals.
727
728 **Figure 3 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was**
729 **induced in the planktonic batch cultures**
730 A) Normoxic and anoxic CFU/mL of PAO1 over 28 days from anoxic conditioned batch cultures.
731 Symbols with error bars indicate the mean ± SEM ($n = 4$). $+NO_3^-$ refers to the addition of 10 mM
732 KNO_3 to LB agar plates.
733 B) Normoxic and anoxic CFU/mL of PAO1 over 28 days from normoxic conditioned batch
734 cultures. Symbols with error bars indicate the mean ± SEM ($n = 4$). $+NO_3^-$ refers to the addition
735 of 10 mM KNO_3 to LB agar plates.
736 C) The log difference was calculated as the difference in mean log CFU/mL between plating
737 methods from the normoxic and anoxiccally conditioned batch cultures, respectively. The log
738 difference was significantly higher ($p < 0.0001$, linear regression) in the anoxically conditioned
739 batch cultures than the normoxically conditioned. Symbols with error bars indicate the mean +
740 confidence intervals.
741
742 **Figure 4 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was**
743 **induced in colonies**
744 A) Normoxic and anoxic CFU/mL of PAO1 over 20 days from anoxically conditioned colonies.
745 Symbols with error bars indicate the mean ± SEM ($n = 3$). $+NO_3^-$ refers to the addition of 10 mM
746 KNO_3 to LB agar plates.

747 B) Normoxic and anoxic CFU/mL of PAO1 over 20 days from normoxic conditioned colonies.
748 Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM
749 KNO_3 to LB agar plates.
750 C) The log difference was calculated as the difference in mean log CFU/mL between plating
751 methods from the normoxic and anoxically conditioned colonies, respectively. The log
752 difference was significantly higher ($p < 0.0001$, linear regression) in the anoxic conditioned
753 colonies than the normoxic conditioned. Symbols with error bars indicate the mean + confidence
754 intervals.
755
756 **Figure 5 – Reactive oxygen species partly explains creation of the VBNC subpopulation of**
757 ***Pseudomonas aeruginosa* (PAO1)**
758 A) Normoxic and anoxic determination of CFU/mL were determined for 16-day-old anoxically
759 conditioned batch cultures of PAO1. Normoxic CFU was determined with and without addition
760 of 0.3% sodium pyruvate. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$
761 refers to the addition of 10 mM KNO_3 to LB agar plates. Significant difference between
762 normoxic incubation +/- 0.3 % sodium pyruvate ($p = 0.01$, one-way ANOVA test). Significant
763 difference ($p = 0.0075$, one-way ANOVA test) between anoxic and normoxic incubation + 0.3%
764 sodium pyruvate.
765 B) Normoxic and anoxic determination of CFU/mL were determined for 24-hour-old batch cultures
766 of PAO1. Normoxic CFU was determined with and without addition of 0.3% sodium pyruvate.
767 Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM
768 KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).
769 C) Normoxic and anoxic determination of CFU/mL were determined for 16-day-old anoxically
770 conditioned batch cultures of PAO1 and *ΔkatA* PAO1. Symbols with error bars indicate the mean

± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. Significant difference between anoxic and normoxic incubation with both PAO1 and *ΔkatA* PAO1 ($p = 0.001$ and $p < 0.0001$, respectively, one-way ANOVA test)

Figure 6 - An oxygen intolerant subpopulation of *Staphylococcus aureus* (methicillin susceptible) was induced in the filter biofilm model

A) Normoxic and anoxic CFU/mL) of *Staphylococcus aureus* over 17 days from anoxic conditioned filters. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

B) Normoxic and anoxic CFU/mL of *S. aureus* over 17 days from normoxic conditioned filters. Symbols with error bars indicate the mean ± SEM (n = 4). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

C) The log difference was calculated as the difference in mean log CFU/mL between plating methods from the normoxic and anoxically conditioned filters, respectively. The log difference was significantly higher ($p < 0.001$, linear regression) in the anoxically conditioned filters than the normoxic conditioned. Symbols with error bars indicate the mean + confidence intervals.

Tables and their legends

Table 1 – Bacterial finding in a clinical setting on LB plates supplemented with 10 mM KNO_3^-

Bacteria exclusively detected on lysogeny broth (LB) agar plates supplemented with 10 mM KNO_3^- (n = 19 in 16 patients). In 5 cases (5 patients) growth was only detected with LB + NO_3^-. Bacteria marked with * were not possible to identify with MALDI-TOF MS to the genus or species level. NA means not applicable, this is due to the failure of proper identification. Ability to reduce nitrate is elucidated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database under the nitrogen metabolism - reference pathway (Laboratories, 2015). +/- refers to the ability of the organism to perform or not perform either nitrate reductase, biofilm or be associated with chronic infections.

Bacteria found exclusively on LB supplemented with NO_3^-.	Numbers detected	Growth only detected with LB + NO_3^-.	Nitrate reductase	Biofilm formation	Possible infection (chronic)	Literature
Staphylococcus epidermidis	4	2	+	+	+	(Lentino, 2004, Rogers *et al*., 2009)
Rhizobacterium (Agrobacterium) radiobacter	1	-	+	+	+	(Müller & Gabriel, 1999, Rodby & Glick, 1991, Yu, Wang et al., 1997)
Helcococcus kunzii	1	-	-	+	+	(Perez-Jorge, Cordero et al., 2012, Stanger, Albert *et al*., 2013)
Staphylococcus capitis	1	-	+	+	+	(Cui, Smooker et al., 2015, Frid, Tornes *et al*., 2009)
Propionebacterium acnes	2	-	+	+	+	(Achermann, Goldstein *et al*., 2014, Frid *et al*., 2009)
Finegoldia magna	4	-	+	+	+	(Donelli, Vuotto *et al*., 2012, Seng, Bayle *et al*., 2015)
Staphylococcus warneri	2	2	+	+	+	(Al-Shuneigat, Cox *et al*., 2005, Announ, Mattei *et al*., 2004)
Peptostreptococcus anaerobius	1	-	+	+	+	(Fukuda, 2004, Weckwerth, de Mattias Franco *et al*., 2014)

Lactobacillus rhamnosus	1	-	+	+	+	(Ambalam, Kondepudi *et al*., 2012)
*Propionebacterium sp.**	1	-	NA	NA	NA	-
*Cocci**	1	1	NA	NA	NA	-

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Appendix figure legends

Appendix Figure S1 – Percentage distribution of VBNC bacteria over time in different growth models

A) The percentage of VBNC *Pseudomonas aeruginosa* after anoxic conditioning in the bead biofilm model.

B) The percentage of VBNC *Pseudomonas aeruginosa* after anoxic conditioning in the filter biofilm model.

C) The percentage of VBNC *Pseudomonas aeruginosa* after anoxic conditioning in batch cultures.

D) The percentage of VBNC *Pseudomonas aeruginosa* after anoxic conditioning as colonies.

E) The percentage of VBNC *Staphylococcus aureus* after anoxic conditioning in the filter biofilm model.

Appendix Figure S2 – Direct viable counting reveals a larger viable but non-culturable population of *Pseudomonas aeruginosa* (PAO1) after anoxic conditioning.

A) Bacterial counts per milliliter were determined with plate counting and direct viable counting from 24-hour-old batch cultures of PAO1. Bacteria were stained with LIVE/DEAD staining to estimate the proportion of viable ("live") and non-viable ("dead") cells. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates.

B) Bacterial counts per milliliter were determined with plate counting and direct viable counting from 16-day-old anoxically conditioned batch cultures of PAO1. Bacteria were stained with LIVE/DEAD staining to estimate the proportion of viable ("live") and non-viable ("dead") cells. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. There was significantly more bacterial counts ($p = 0.0497$, one-way

ANOVA test) when applying direct viable counting in comparison to normoxic grown colony forming units per milliliter (CFU/mL). CFU/mL was significantly higher ($p = 0.001$, one-way ANOVA test) when PAO1 was grown under anoxic conditions in comparison to normoxic conditions

Appendix Figure S3 - An oxygen intolerant subpopulation of S*taphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* was induced in the filter biofilm model.

A) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old anoxically conditioned filters with *Staphylococcus aureus* (MRSA). Symbols with error bars indicate the mean ± SEM ($n = 3$). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. Significant difference between anoxic and normoxic incubation with and without addition of sodium pyruvate at day 9 ($p = 0.01$ and $p < 0.01$, respectively, one-way ANOVA test)

B) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old normoxic conditioned filters with *Staphylococcus aureus* (MRSA). Symbols with error bars indicate the mean ± SEM ($n = 3$). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

C) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old anoxically conditioned filters with *Staphylococcus epidermidis*. Symbols with error bars indicate the mean ± SEM ($n = 3$). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. Significant difference between anoxic and normoxic incubation at day 9 ($p = 0.003$, one-way ANOVA test)

D) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old normoxic conditioned filters with *Staphylococcus epidermidis*. Symbols with

error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

Appendix Figure S4 – Anoxic conditioning did not affect E*scherichia coli* or *Enterococcus faecalis* during growth in the filter biofilm model.

A) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old anoxically conditioned filters with *E. coli*. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

B) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old normoxic conditioned filters with *E. coli*. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

C) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old anoxically conditioned filters with *E. faecalis*. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

D) Normoxic and anoxic (+/- sodium pyruvate) determination of CFU/mL were determined for 1 and 9 days old normoxically conditioned filters with *E. faecalis*. Symbols with error bars indicate the mean ± SEM (n = 3). $+NO_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. No difference between types of incubation (one-way ANOVA test).

Appendix Figure S5 – Implementation of a supplementary diagnostic media at the Department of Clinical Microbiology, Rigshospitalet.

873 A) Percentage distribution of culture positive findings in patient samples between the conventional
874 method, LB agar plates and ABTG plates supplemented with 10 mM NO_3^-. LB plates
875 supplemented with 10 mM NO_3^- and the conventional method found significantly more positive
876 samples in comparison to ABTG plates supplemented with 10 mM NO_3^- ($p = 0.0003$ and $p =$
877 0.0001, respectively, Fisher's exact test).
878 B) Number of patients with culture positive findings by conventional method and on anoxic LB
879 plates supplemented with 10 mM NO_3^-.
880

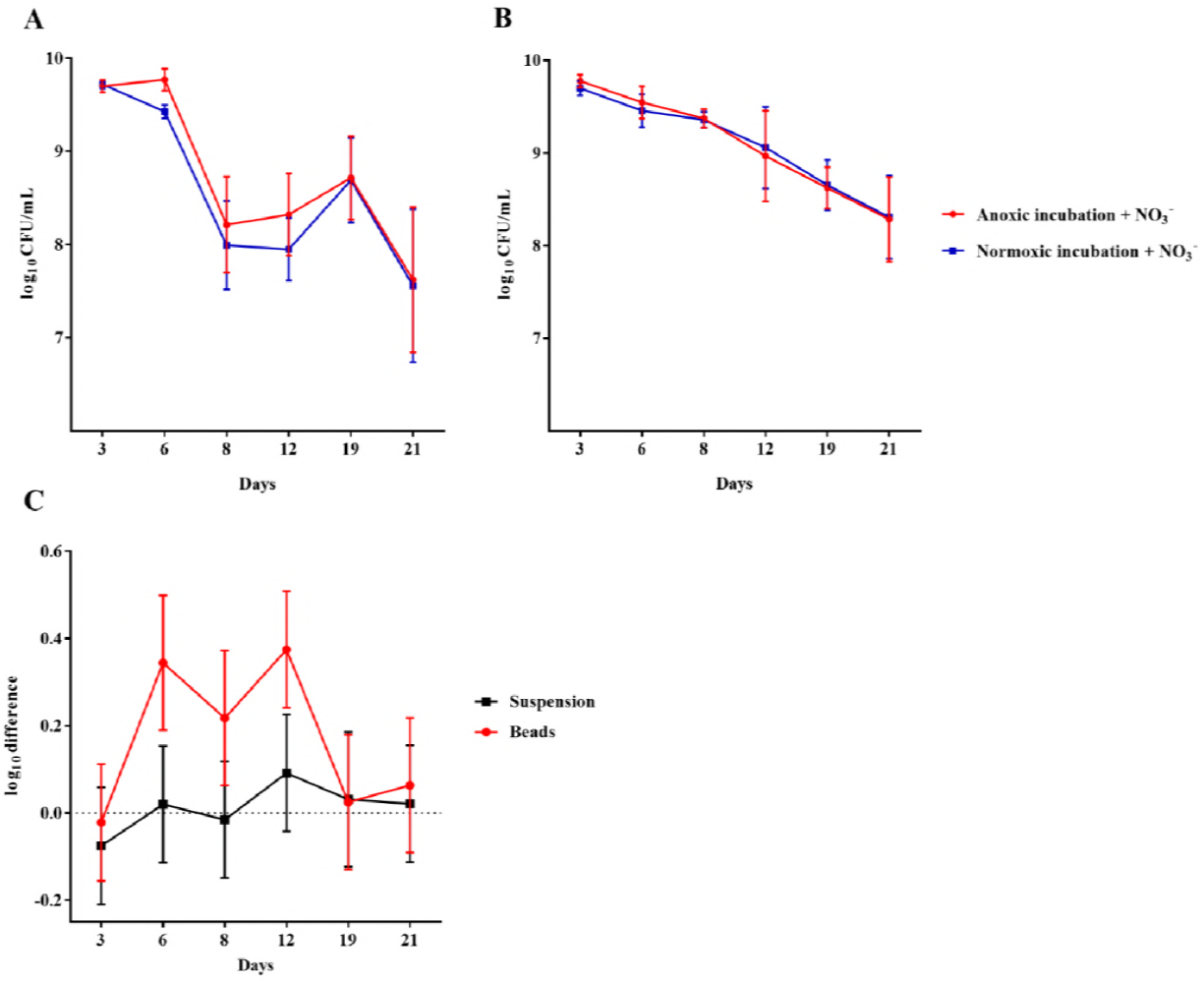


Figure 1 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in the bead biofilm model

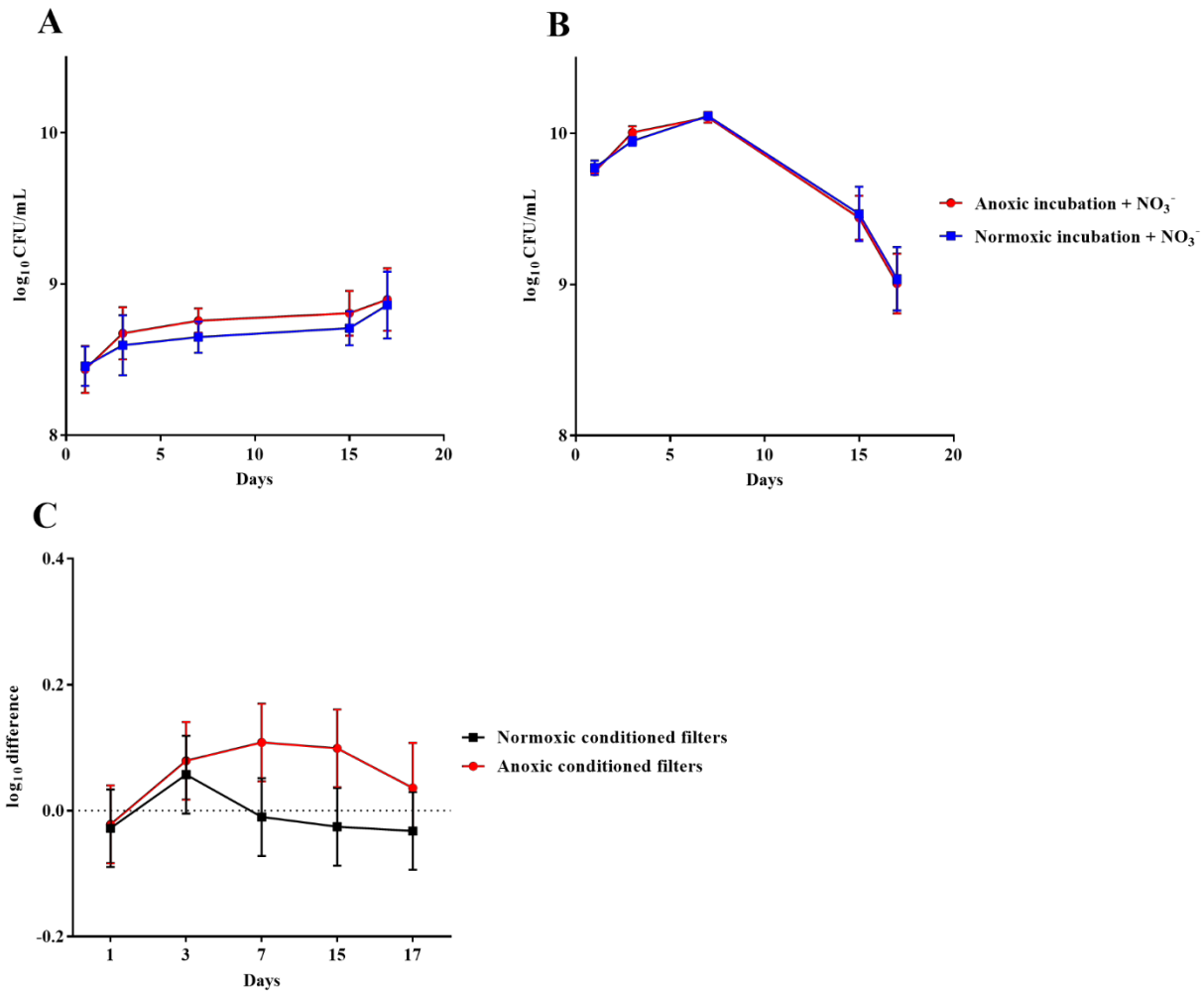


Figure 2 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in the filter biofilm model

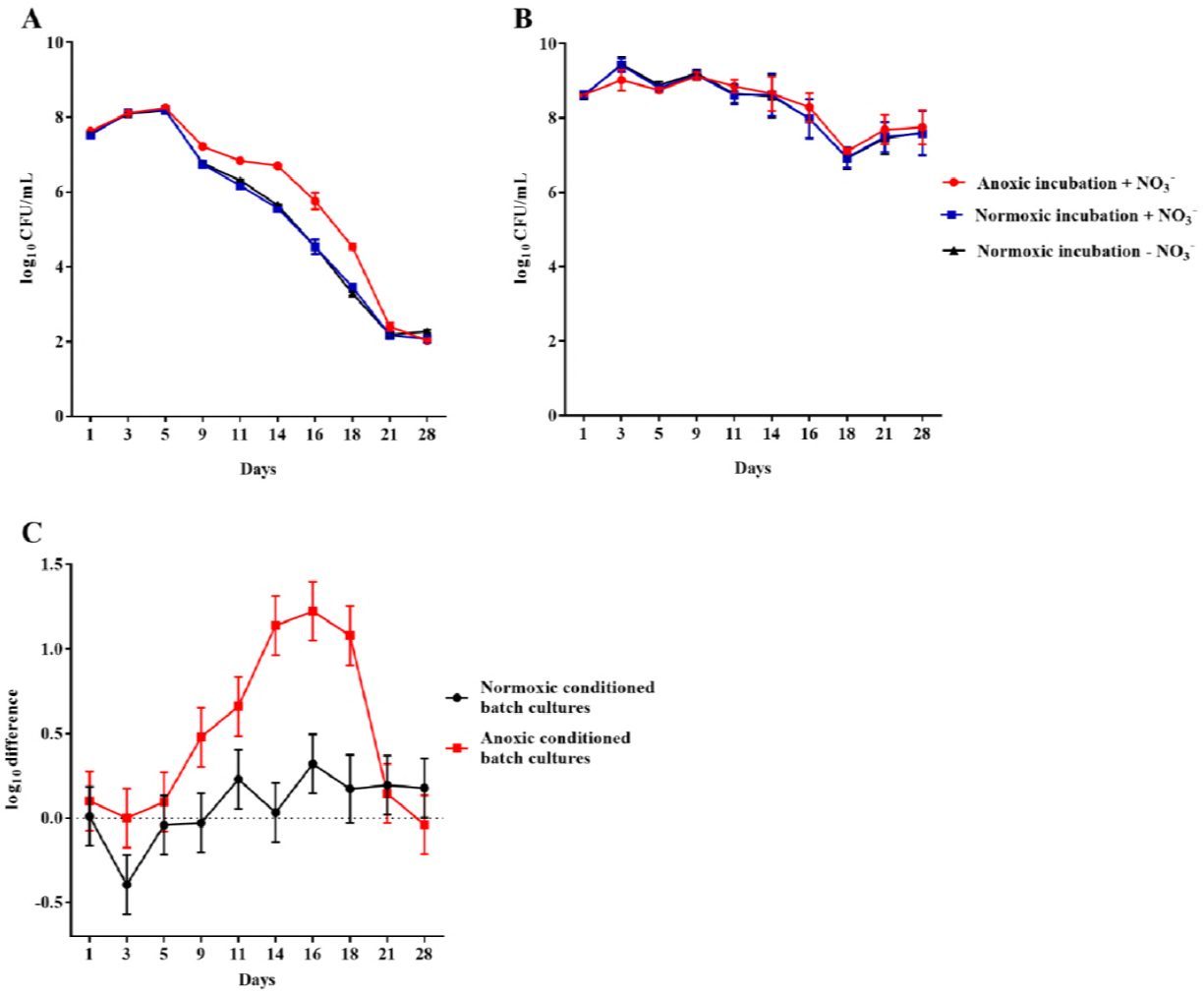


Figure 3 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in the planktonic batch cultures

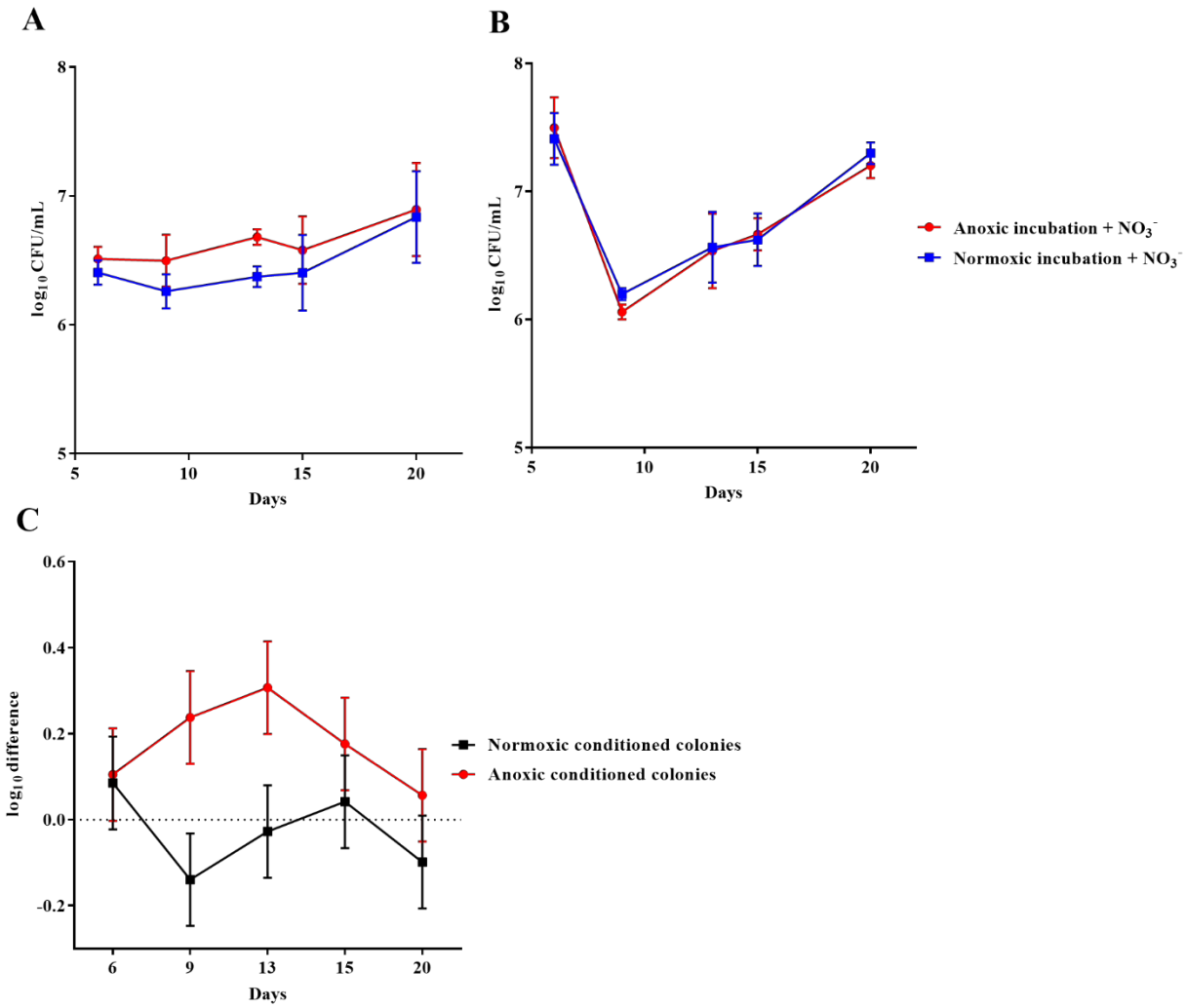


Figure 4 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was induced in colonies

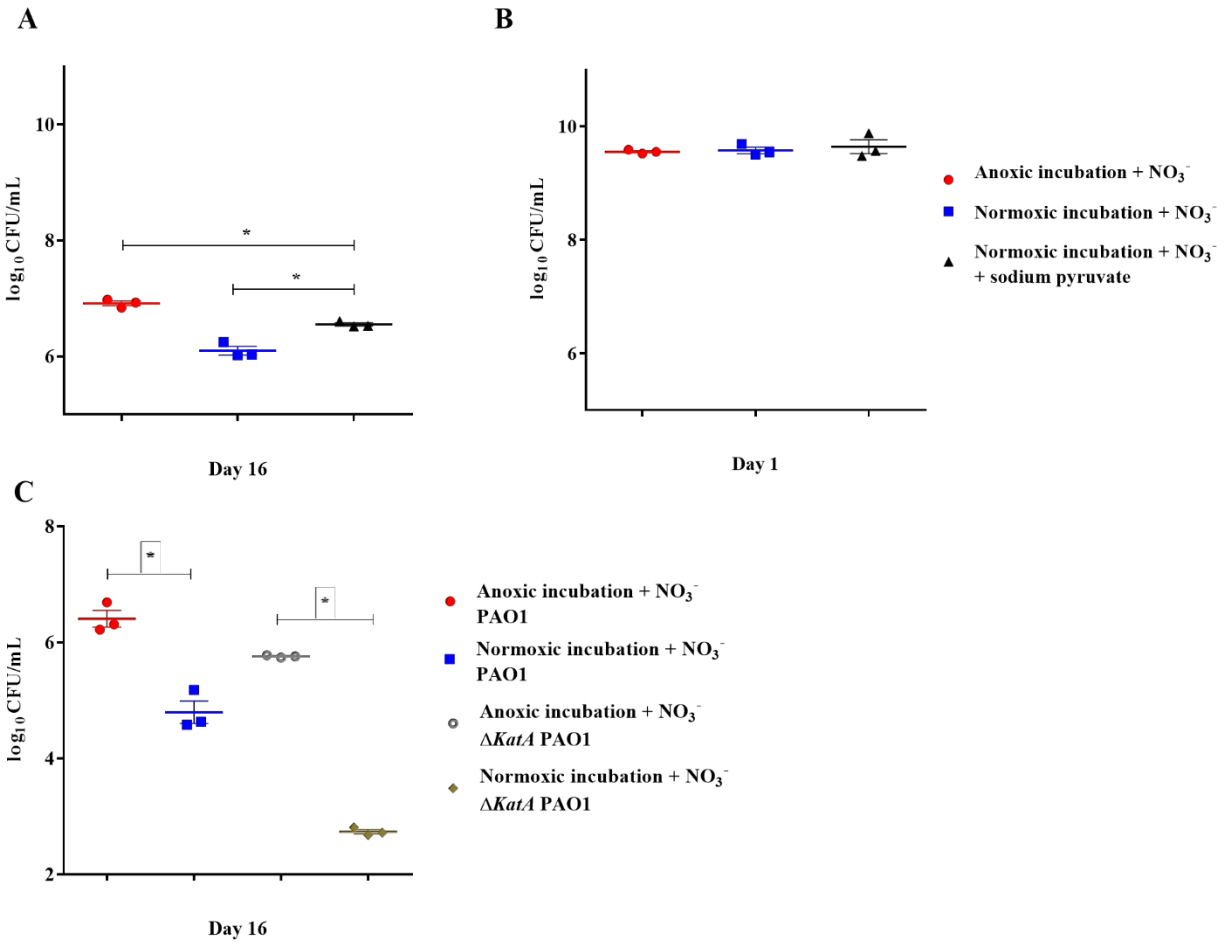


Figure 5 – Reactive oxygen species partly explains creation of the VBNC subpopulation of *Pseudomonas aeruginosa* (PAO1)

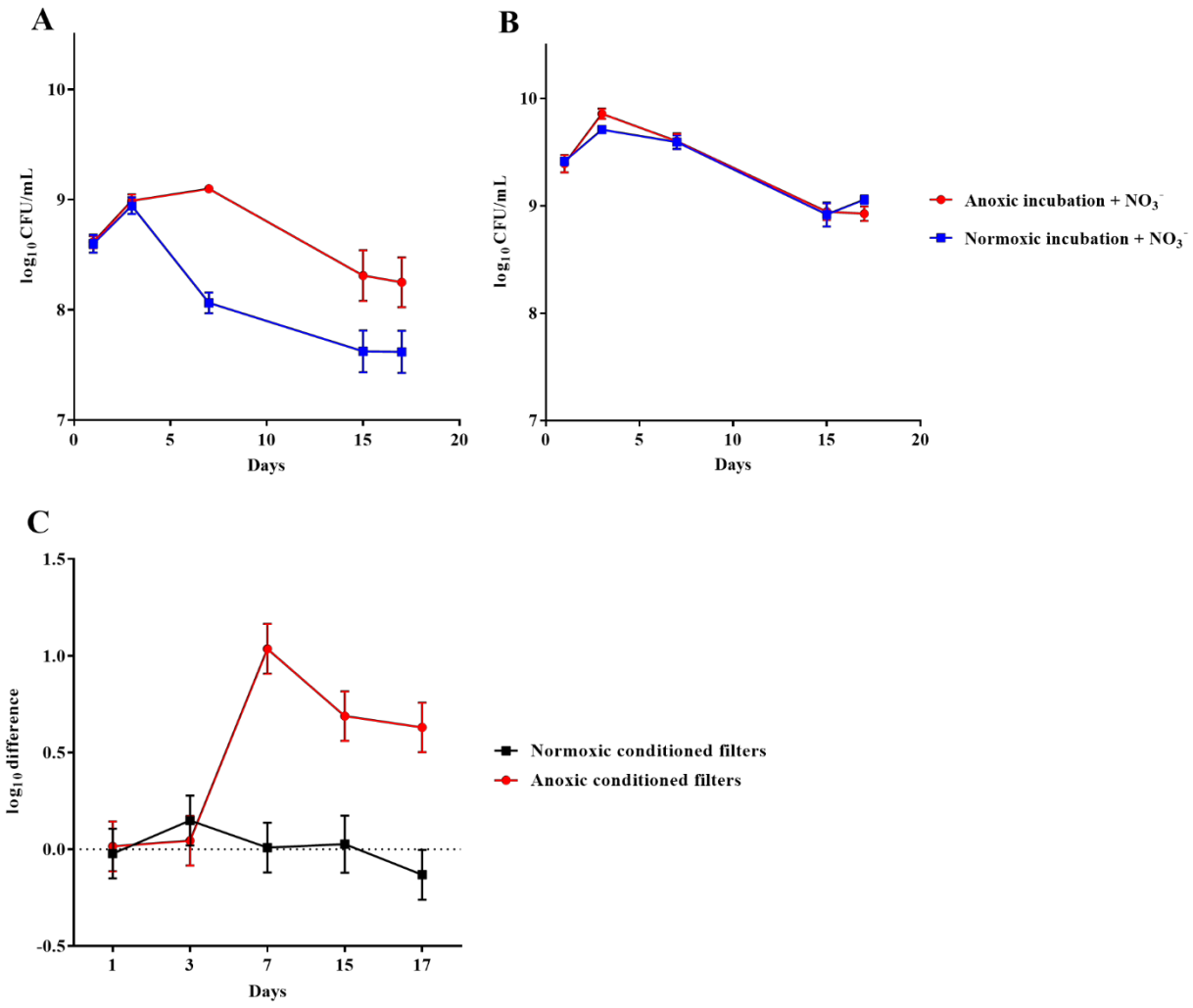
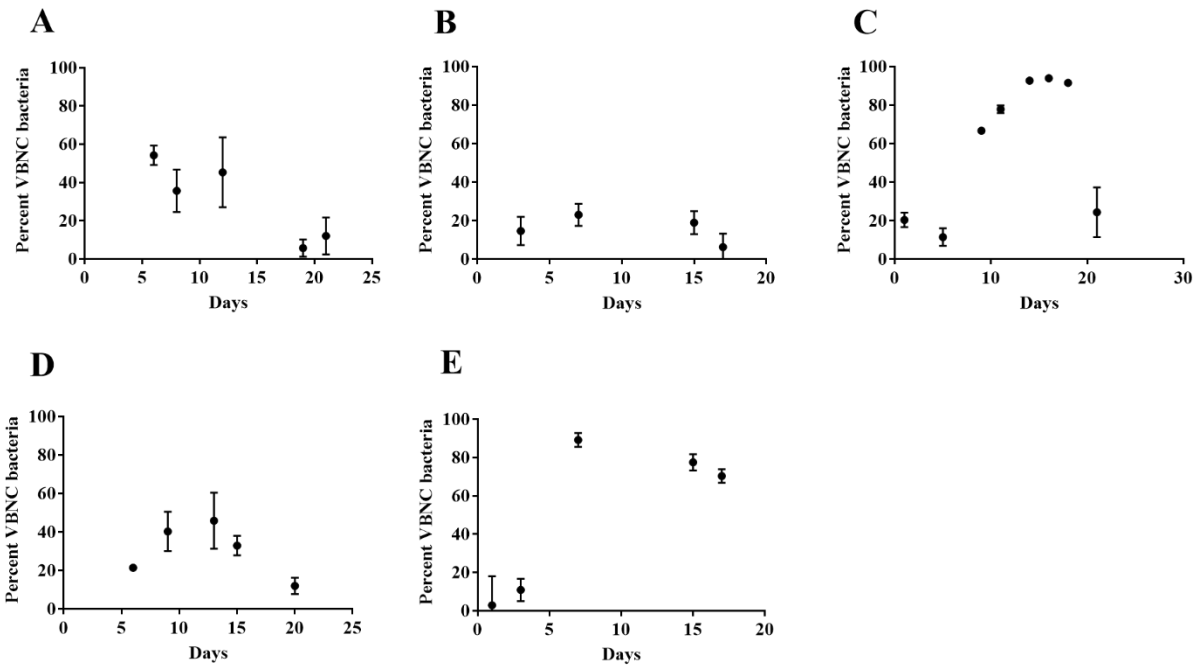
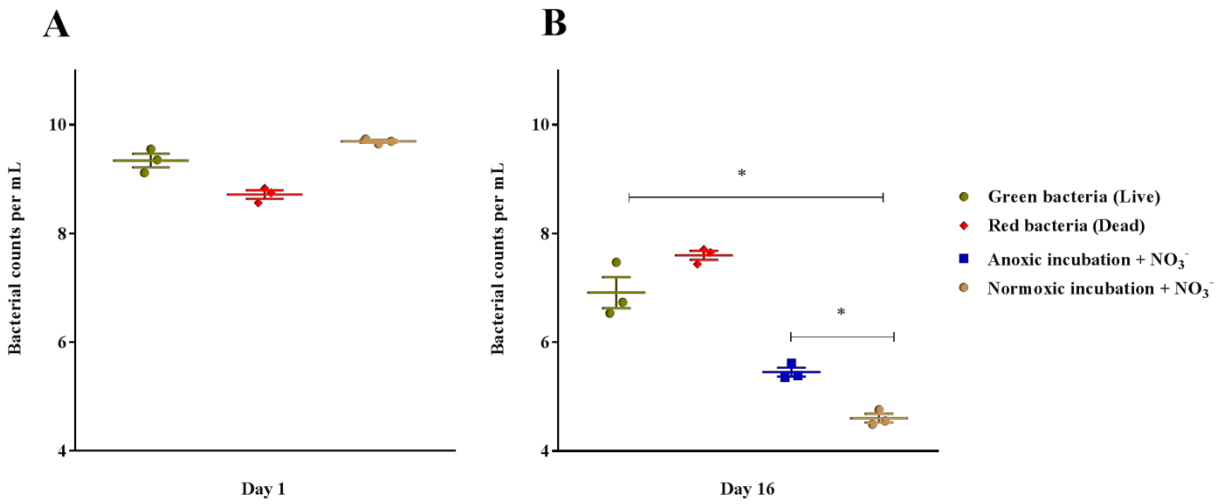


Figure 6 - An oxygen intolerant subpopulation of *Staphylococcus aureus* (methicillin susceptible) was induced in the filter biofilm model

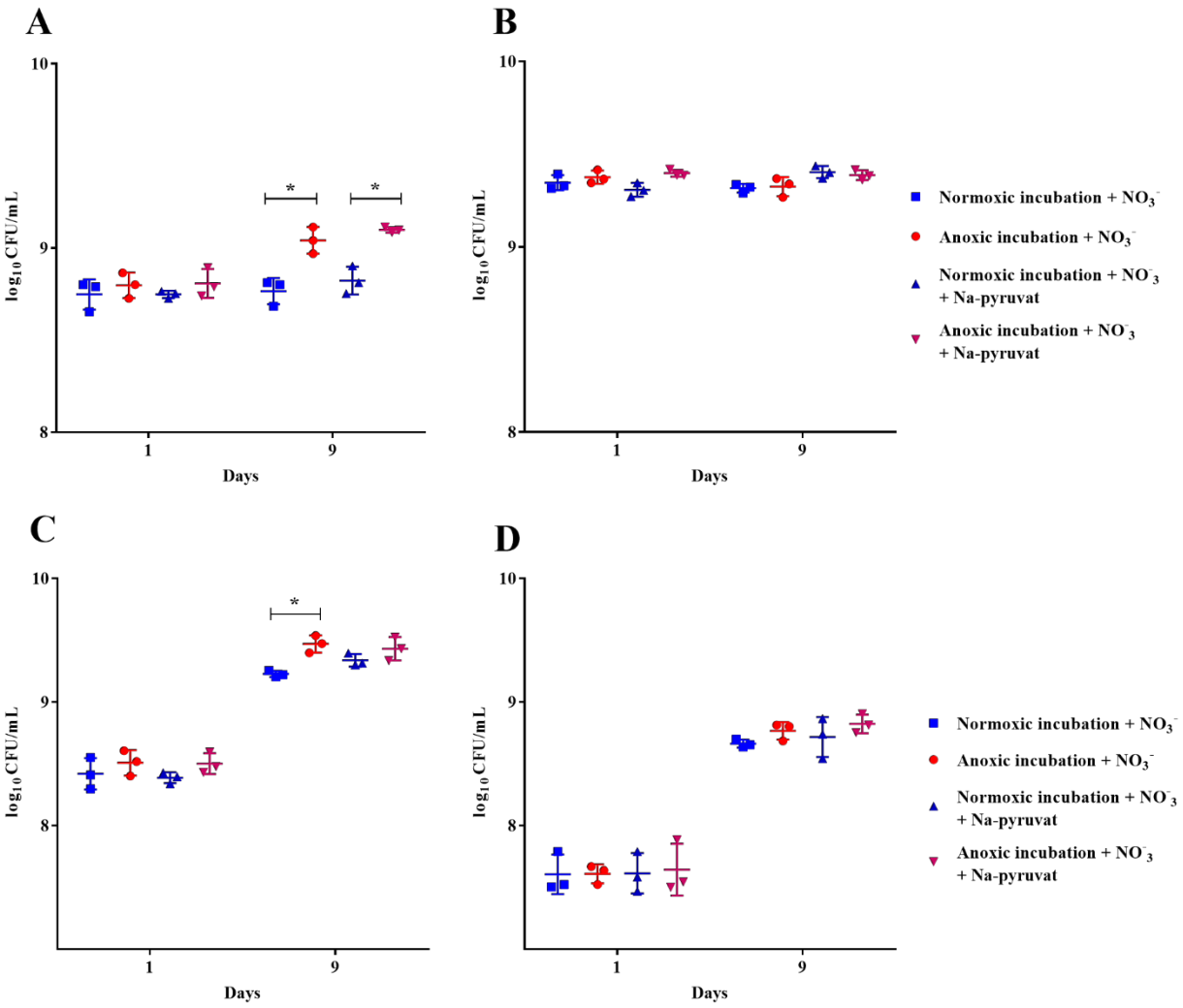
Appendix figure legends



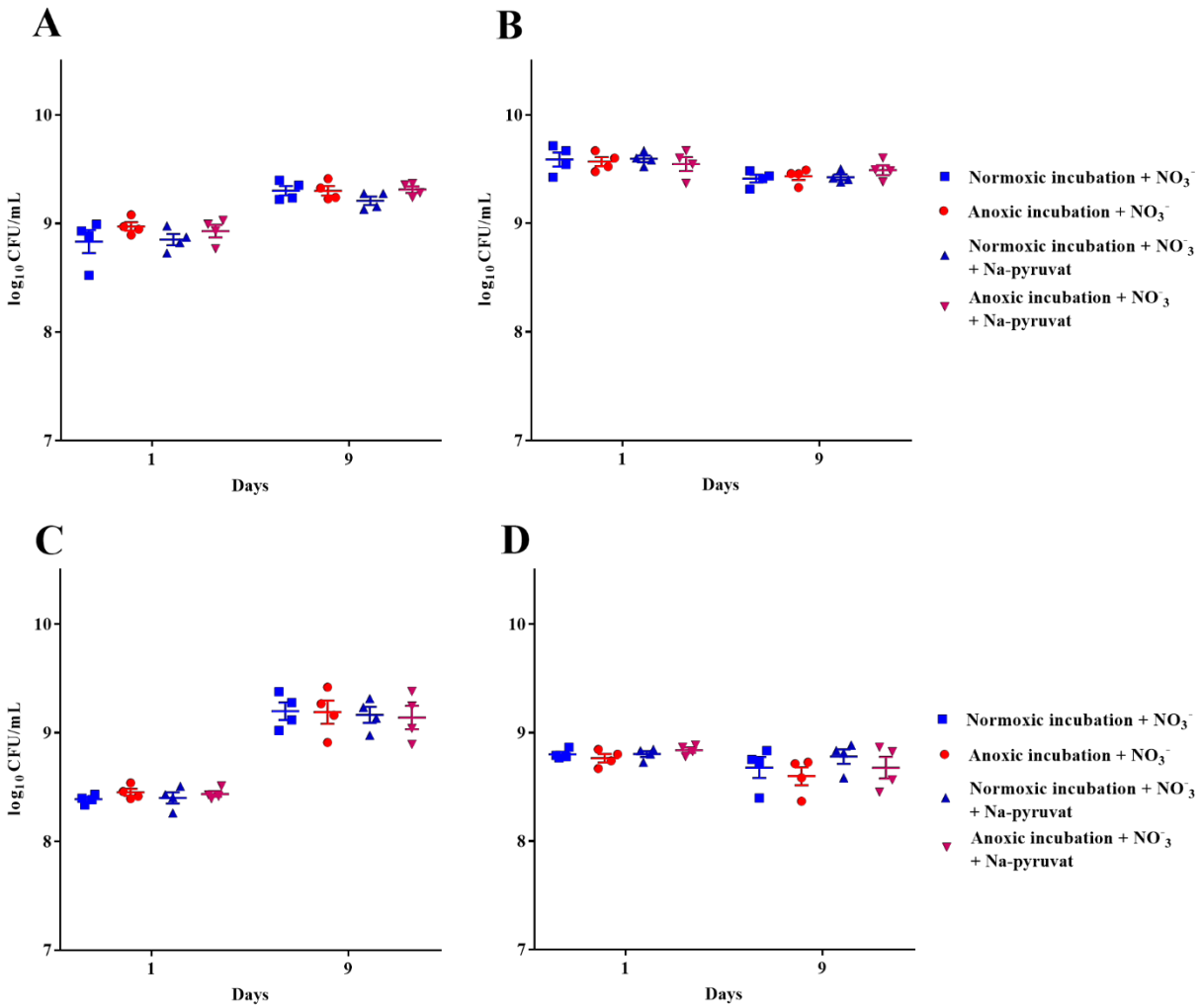
Appendix Figure S1 – Percentage distribution of VBNC bacteria over time in different growth models



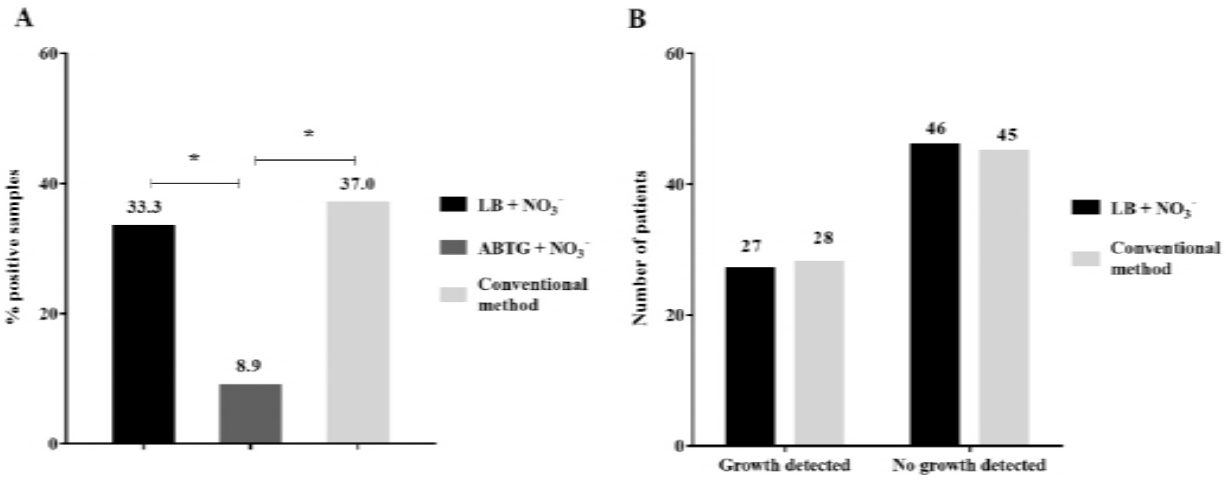
Appendix Figure S2 – Direct viable counting reveals a larger viable but non-culturable population of *Pseudomonas aeruginosa* (PAO1) after anoxic conditioning.



Appendix Figure S3 - An oxygen intolerant subpopulation of *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* was induced in the filter biofilm model.



Appendix Figure S4 – Anoxic conditioning did not affect *Escherichia coli* or *Enterococcus faecalis* during growth in the filter biofilm model.



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