In chronic infections, bacterial pathogens typically grow as small dense cell aggregates embedded in a matrix consisting of, e.g., wound bed sludge or lung mucus. Such biofilm growth mode exhibits extreme tolerance towards antibiotics and the immune defence system. The bacterial aggregates are exposed to physiological heterogeneity and O₂ limitation due to steep chemical gradients through the matrix, which is hypothesised to contribute to antibiotic tolerance. To study this phenomenon, we investigated growth patterns and chemical dynamics of the opportunistic pathogenic bacterium Pseudomonas aeruginosa. The standard method of confocal laser scanning microscopy (CLSM) of stained or fluorescently tagged P. aeruginosa provides very localised information on biomass distribution (at µm scale). In combination with quantitative peptide nucleic acid (PNA)-FISH, CLSM can also be applied to quantify the growth potential of P. aeruginosa 16S rRNA. While providing high-resolution data on bacterial growth, this method is based on fixed samples and is therefore an invasive technique. When aiming to unravel structural biofilm properties at mesoscopic to macroscopic levels (10 µm—mm length scale), optical coherence tomography (OCT) is a suitable alternative imaging technique. OCT employs near-infrared radiation (NIR) and provides a non-invasive alternative to light microscopy, enabling high-resolution 3D scanning of larger (mm³ to cm³) biofilm volumes in near-real time. As previously mentioned, bacterial aggregates are exposed to steep O₂ gradients. To further elucidate this aspect, microsensors can be used to investigate the chemical environment in a minimal invasive fashion. Microsensors are available for several analytes including O₂ and nitrous oxide (N₂O), a key intermediate product of denitrification. Another approach is to use chemical imaging techniques.
with optical sensors (either immobilised in sensor films or particles) to visualise the chemical microenvironments in biofilms.31–34

Biofilms are often studied in vitro using continuous flow cell systems, wherein biofilms are grown attached to a surface and can exhibit a variety of structural morphologies including mushroom-shaped structures.35 However, in CF lungs and chronic wounds P. aeruginosa grows in dense suspended aggregates separated by a secondary matrix and with no attachment to a solid substrate or surface.36 The typical growth mode and biofilm shapes observed in flow chambers are thus not representative of the observed in vivo growth patterns of biofilms associated with chronic infections. To better mimic the in vivo conditions of P. aeruginosa in chronic infections, we recently employed an alginate bead model,37 wherein the bacteria form dense, spatially segregated microcolonies similar in size and structure to P. aeruginosa aggregates observed ex vivo in lungs from CF patients38 and chronic wounds.4

P. aeruginosa can grow anaerobically by performing arginine fermentation or using alternative electron acceptors36–38 and there is increasing evidence that P. aeruginosa can utilise the high physiological NO3− and NO2− levels in the CF lungs to grow under O2 limitation by performing denitrification.39,40 In this study, we investigated P. aeruginosa aggregate growth in alginate beads with different O2 and NO3− availability mimicking physiological conditions encountered in the chronic infections.41,42 The bacterial biomass, growth rate, and chemical microenvironment in the beads was characterised using a novel combination of OCT, CLSM, biomass, growth rate, and chemical microenvironment in the

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RESULTS
Optical coherence tomography
OCT facilitated non-invasive macroscopic imaging of the alginate beads (Supplementary Fig. S1A, B), wherein light scattering bacterial aggregates were identified by their high OCT signal. Due to dense growth of bacterial aggregates in the bead periphery, the vertical OCT signal attenuation was rapid and at about 200 μm below the bead surface the OCT signal approached that of the OCT signal of pure alginate (Supplementary Fig. S2). The OCT dB signal from the outermost peripheral 150 μm of the beads could thus be used as a proxy for bacterial biomass distribution. The OCT dB signal of anoxic beads (without NO3−) was similar to the blank control (without bacteria), thus suggesting no or very minor growth (Fig. 1). In contrast, the OCT signal from anoxic beads with NO3− was significantly higher relative to the control after 48 h (p = 0.035). Normoxic beads and normoxic beads supplemented with NO3− gave rise to the strongest OCT signal, indicative of intense growth and biomass accumulation in the bead periphery (Fig. 1, S1).

Microscopy
Bacterial growth and organisation in the alginate beads was visualised microscopically by CLSM using green fluorescent protein (GFP)-tagged P. aeruginosa (Fig. 2). In normoxic beads, P. aeruginosa showed peripheral growth (growth in the outermost ~100 μm of the bead) of bacterial microcolonies after 24 and 48 h (Fig. 2b). Normoxic growth in the presence of NO3− resulted in a very intense growth, with larger aggregates in the periphery than observed under anoxic growth of P. aeruginosa in the presence of NO3−. Moreover, 48 h normoxic growth of P. aeruginosa with NO3− resulted in a heterogeneous growth pattern, with bacterial microcolonies decreasing in size with distance from the surface of the alginate bead (Fig. 2a). Anoxic beads with NO3− showed a homogeneous distribution of aggregate size in the bead after 24 h, but also exhibited signs of a more heterogeneous aggregate size distribution after 48 h growth with largest aggregates closer to the bead surface (Fig. 2c). No growth was observed in anoxic beads without alternative electron acceptors at 48 h (Fig. 2d), which was supported by the OCT imaging results.

Microscopy combined with quantitative PNA-FISH
The mean fluorescence intensity used here as a proxy for the growth potential (the maximum possible growth rate) of P. aeruginosa21 was quantified and plotted for each of the growth conditions. In normoxic beads, we found that NO3− supplementation induced significantly deeper growth of P. aeruginosa aggregates (Fig. 3a). The median growth depth (interquartile range, IQR) was 51.6 (17.4–92.0) μm vs. 20.0 (2.0–45.0) μm (p < 0.0001) below the alginate bead surface. When NO3− was present, the median growth depth (IQR) was comparable under normoxic vs. anoxic conditions, i.e., 51.6 (17.4–92.0) μm vs. 41.6 (14.2–88.6) μm (p = 0.481).

In normoxic beads with NO3−, the growth potential of P. aeruginosa was significantly higher after 24 h than after 48 h (10212 FU ± 3298 FU vs. 7141 FU ± 1470 FU; p < 0.001). Furthermore, we found a significantly higher growth depth at 24 h vs. 48 h, with a median growth depth (IQR) of 62.9 (25.9–98.0) μm vs. 38.8 (11.3–70.3) μm (p = 0.023) below the alginate bead surface (Fig. 3a). Growth depth in normoxic beads without NO3− did not differ between the two time points (p = 0.139), but the growth potential, as estimated by mean fluorescence, was significantly higher at 24 h as compared to 48 h (9243 ± 2433 vs. 6586 ± 1614; p < 0.001) (Fig. 3b). In anoxic beads with NO3−, there was no significant difference in neither growth depth (p = 0.761) or in growth potential (p = 0.124) at 24 h vs. 48 h, and the growth potential remained high (9258 FU ± 1865 FU vs. 8750 FU ± 1780 FU) both after 24 and 48 h (Fig. 3c).

When correlating growth potential to growth depth, we found no statistically significant correlation under any conditions. It was not possible to prepare paraffin slices for anoxic beads without NO3−, but as previously shown P. aeruginosa failed to grow under these conditions (Figs. 2, 4).

Fig. 1 OCT signal intensity (dB) of alginate beads over 48 h of incubation. The OCT signal was averaged for an area covering a vertical depth of 150 μm (from the bead surface into the interior) and a lateral width of 100 μm and was used as a proxy for bacterial growth potential. Bars represent average ± standard errors of the means from three replicates. Significant differences as indicated.

*p < 0.05; **p ≤ 0.01; ***p ≤ 0.001
Fig. 2  400× CLSM images of the growth dynamics of GFP-tagged *P. aeruginosa* grown under different conditions after 24 and 48 h. 

**a** Normoxic growth in the presence of NO$_3^-$ resulted in intense and deep growth with a tendency to form smaller aggregates in the deeper parts over time. 

**b** Normoxic growth resulted in peripheral growth over time. 

**c** Anoxic growth with NO$_3^-$ also supported growth, but with apparently smaller aggregates. 

**d** Anoxic growth without NO$_3^-$ did not support growth. The edge of the alginate bead is oriented to the right in the images. Size of scale bars: 50 μm
leading to more O₂ accumulation in the beads over time. No N₂O production was detected in normoxic grown alginate beads without NO₃⁻ (Fig. 5a). In normoxic alginate beads supplemented with NO₃⁻, the maximal N₂O concentration was detected in the lower, hypoxic zone (Fig. 6b). Contrary to normoxic beads (without NO₃⁻), O₂ profiles in NO₃⁻ supplemented beads were stable between 24 and 48 h, whereas N₂O production decreased (Fig. 6b). N₂O profiles in anoxic beads with NO₃⁻ revealed an increasing N₂O concentration throughout the bead, with a slight decrease in production after 48 h (Fig. 6a).

Fluxes of O₂ and N₂O
We found a stable O₂ flux into the normoxic beads supplemented with NO₃⁻ (3.18 ± 0.66–3.19 ± 0.84 nmol O₂ cm⁻² min⁻¹), and a tendency towards a decreasing O₂ flux into normoxic beads (without NO₃⁻) between 24 and 48 h (3.07 ± 1.33–1.84 ± 0.32 nmol O₂ cm⁻² min⁻¹). Calculations of the efflux of N₂O from the alginate beads revealed a stable N₂O efflux in anoxic beads with NO₃⁻ between 24 and 48 h (0.10 ± 0.03–0.13 ± 0.1 nmol N₂O cm⁻² min⁻¹), whereas the normoxic beads with NO₃⁻ showed a tendency towards a decrease in N₂O efflux between 24 and 48 h (0.21 ± 0.06–0.09 ± 0.03 nmol N₂O cm⁻² min⁻¹). None of the changes in flux over time were statistically significant.

NO₃⁻, NO₂⁻, and N₂O dynamics in growth medium
Measurements of NO₃⁻ and NO₂⁻ concentrations in the R2A growth medium supplemented with NO₃⁻ showed that the initial concentration (t = 0) of NO₃⁻ in the medium was 10.450 µM ± 148 µM (Fig. 6a), while no NO₂⁻ could be detected (Fig. 6b). After 24 h, the NO₃⁻ concentration had declined significantly to 4.119 ± 0.149 and 3.319 ± 0.191 µM (p < 0.0001) in the anoxic and normoxic cultures, respectively, and was completely depleted within 48 h (Fig. 6a). NO₂⁻ could be detected in the normoxic cultures, but in the anoxic cultures there was a significant accumulation of NO₂⁻ after 24 h (p < 0.0001), which was depleted again after 48 h (Fig. 6b). When measuring the N₂O concentrations directly in the growth medium with electrochemical sensors, we found no N₂O in the growth medium without added NO₃⁻ (data not shown). In the anoxic culture flasks with NO₃⁻ addition (sealed airtight), denitrification led to an accumulation of N₂O over time (Fig. 6c). After 24 h of incubation, a N₂O concentration of 140 µM was measured in the medium, increasing to 225 µM after 48 h. In the normoxic culture with NO₃⁻, N₂O was measured at a concentration of 120 µM after 24 h, but only 1.5 µM after 48 h.

**Fig. 3 Growth potential of P. aeruginosa grown in alginate beads expressed as mean fluorescence intensity.** a) Normoxic growth conditions supplemented with NO₃⁻, b) normoxic growth conditions without NO₃⁻, and c) anoxic growth conditions in the presence of NO₃⁻. P. aeruginosa was fluorescently labelled with Texas-Red-conjugated PNA-FISH probe tagging ribosomes, imaged by CLSM and quantification of intensity was performed by Imaris.
The incorporation of O₂-sensitive nanoparticles in the alginate beads enabled visualisation of the O₂ distribution relative to the bacterial aggregates. The O₂ images revealed steep O₂ gradients in the periphery of the beads (Fig. 7c) forming a heterogeneous landscape of O₂ concentration within the O₂ provisioned part of the beads that roughly followed the aggregate distribution, where aggregates exhibited complete O₂ depletion and hypoxia in the surrounding alginate matrix. Atmospheric O₂ saturation was only observed at the immediate bead surface.

**DISCUSSION**

 Cultures of *P. aeruginosa* in alginate beads exhibit several characteristics of aggregated bacteria found in the lungs of CF patients and in chronic wounds, in regard to aggregate size, growth, O₂ limitation, physiological heterogeneity, and antibiotic tolerance. This enabled our study of in vivo-like growth patterns and the chemical microenvironment of *P. aeruginosa* with and without electron acceptors via a variety of invasive and non-invasive methods. The most intense growth of *P. aeruginosa* was observed in the presence of the two electron acceptors O₂ and NO₃⁻, enabling both aerobic respiration and denitriﬁcation in the alginate beads, while complete absence of electron acceptors resulted in an arrested growth state.

We studied the growth patterns of *P. aeruginosa* in the presence and absence of O₂ and NO₃⁻ at different spatial scales using OCT for mesoscopic and macroscopic scale, CLSM to unravel structures at a microscopic scale, and quantitative PNA-FISH for information on the growth potential. OCT has previously been used to study the formation and growth dynamics of bioﬁlms in ﬂow chambers and various carrier materials and of clinical bioﬁlms in nasal polyps, but to our knowledge OCT has not previously been used to investigate bacteria growing in alginate beads. The presence of bacteria increased local backscatter in the alginate matrix resulting in a stronger OCT signal. However, the alginate matrix also scattered light, and a quantiﬁcation of bacterial growth thus relies on a clear separation of the OCT signals originating from the bacteria and the alginate. We found that bacterial growth led to an enhancement of the OCT signal relative to the alginate bead, but only within the ﬁrst 200 µm from the bead surface (see Supplementary Fig. S2). While OCT allows for a potential operational depth of view of several mm’s, the high density of bacteria in the outermost bead layers leads to intense multiple

**Fig. 5** O₂ and N₂O proﬁles from alginate-encapsulated *P. aeruginosa*. a *P. aeruginosa* cultured under normoxic conditions (circles) and anoxic conditions with 10 mM NO₃⁻ (triangles). Furthermore, a control measurement of N₂O on alginate-encapsulated *P. aeruginosa* grown without NO₃⁻ (asterisk). b *P. aeruginosa* cultured under normoxic conditions supplemented with 10 mM NO₃⁻. O₂ proﬁles (circles) and N₂O proﬁles (triangles). Bars represent average ± standard deviation from 3 to 4 replicates. All measurements were performed at 37 °C in growth medium.

**Fig. 6** Dynamics of a NO₃⁻, b NO₂⁻, and c N₂O (µM) in growth medium quantiﬁed by Griess colometric reaction and microsensor measurements directly in growth medium. NO₃⁻ and NO₂⁻ measurements are based on duplicate sampling from medium (error bars too small to be visualised), and N₂O concentration represents on a single measurement directly in medium. Signiﬁcant differences as indicated. n.s. not signiﬁcant; ****p ≤ 0.0001

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scattering of the NIR probing light causing a rapid vertical attenuation and thus loss of image contrast of the collected OCT signal resulting in an operational depth of view that was comparable to CLSM. Evidently, such application of OCT can be further optimised, e.g., by decreasing scatter in the alginate matrix using washed less optically opaque alginate, but such technical optimisation was beyond the scope of this study. Still, the OCT data in our study complemented higher-resolution microscopy techniques and provided a non-invasive macroscopic overview of the growth dynamics and growth zones in intact alginate beads. With OCT, it was thus possible to monitor the overall growth zone of P. aeruginosa in alginate beads (Fig. S1), while high-resolution CLSM enabled (i) visualisation and quantification of microcolony size and distribution in the alginate beads (Fig. 2) and (ii) local growth potential measurements when used in combination with a quantitative PNA-FISH protocol (Fig. 3).

Supplementing P. aeruginosa with two electron acceptors (O2 and NO3−) resulted in higher growth potentials (Figs. 2, 3) at 24 h as compared to 48 h incubation. Moreover, a heterogeneous growth pattern of P. aeruginosa was observed in the alginate beads after 48 h incubation with larger aggregates situated near the oxygenated bead surface. Physiological heterogeneity within biofilms is largely driven by the activity and biomass distribution of the bacteria in combination with mass transfer limitation by diffusion of electron acceptors, substrate and products of bacterial metabolism in the biofilm matrix and in the diffusive boundary layer between the mixed water phase and the biofilm. Typically, bacterial depletion of O2 in the biofilm periphery limits diffusion to deeper parts and we found that such development of O2 concentration gradients in the alginate beads lead to a heterogeneous growth pattern over time, where only the bacteria positioned near the bead surface had sufficient access to the preferred electron acceptor O2. While anoxic grown P. aeruginosa with access to NO3− displayed a very homogeneous growth pattern after 24 h, these beads also showed signs of a heterogeneous growth pattern after 48 h (Fig. 2). This shift was probably due to depletion of bead-incorporated NO3−, where after NO3− was only available by diffusion from the growth medium. These results are in accordance with previous studies showing a heterogeneous distribution of bacterial aggregate sizes, with significantly larger aggregates in the periphery as compared to deeper in the beads after 48 h in normoxic grown alginate-encapsulated P. aeruginosa with and without 100 mM NO3− supplement.

The results from OCT and CLSM corresponded well with the quantitative PNA-FISH results in terms of showing increased growth depth in the presence of NO3−, while the availability of O2 did apparently not affect growth depth into the alginate beads. The latter finding confirms that O2 is strongly affected by diffusion limitation. In addition, quantitative PNA-FISH provided an estimate on the growth potential, expressed as fluorescence intensity, which has been shown to correlate with growth rate in P. aeruginosa. Contrary to growth depth, the growth potential was affected by the presence of NO3− and we found a significantly higher growth potential with O2 at 24 h as compared to 48 h. This suggests that the contribution of O2 to the overall growth may be limited after 48 h, and that the preferred electron acceptor O2 initially facilitates a more intense growth burst than with NO3−, which correlates well with the low growth rate of P. aeruginosa observed in vivo in the mucus of chronically infected CF lungs. Furthermore, this is in accordance with a higher energy yield by O2 respiration as compared to denitrification. Evidently, such application of OCT can be further confirmed a more intense growth in the presence of both O2 and NO3− (Fig. 4).

Interestingly, bacterial growth in beads with no electron acceptors did not result in a decrease in CFU but rather lead to a steady state in cell counts throughout the study, which is in support of P. aeruginosa’s remarkable ability for long-time survival. Previous studies have thus demonstrated that P. aeruginosa is capable of long-term anaerobic survival via fermentation of amino acids, which were present in the growth medium.

To gain further insight into factors governing the growth dynamics of P. aeruginosa in the beads as observed with OCT, CLSM, and quantitative PNA-FISH, we used microsensors to quantify chemical gradients in the alginate beads. Based on such measurements, we estimated oxic respiration from the net consumption of O2 and denitrification from the net production of N2O in the beads, respectively. The observed decline in O2 in the water just above the alginate bead surface showed the presence of a diffusive boundary layer around the bead, and microsensor measurements demonstrated the presence of steep O2 concentration gradients and thus high O2 consumption in beads incubated under normoxic conditions. However, the O2 uptake decreased with time (Fig. 5a) in line with our observation of a decreasing growth rate over time as determined by quantitative PNA-FISH (Fig. 3). This may indicate onset of substrate limitation for bacterial growth in the alginate beads.

Beads incubated in normoxic medium with NO3− showed steep O2 concentration gradients, but contrary to beads grown without NO3−, the O2 uptake did not decrease with time (Fig. 5b). However, such sustained high O2 consumption was not reflected in higher growth potentials, which showed a decrease between 24 and 48 h of incubation (Fig. 3a). Some bacteria can perform so-called aerobic denitrification during hypoxic conditions in the presence of NO3−. Initially described by Robertson and Kuenen, aerobic denitrification can explain how NO3− respiration may proceed in the presence of O2. In P. aeruginosa this ability is
evidenced by the expression of overlapping gene sets depending on O₂ concentration, where hypoxia triggers denitrification genes. Moreover, the ability to perform intra-aerobic respiration has been proposed, where NO₃⁻ is reduced to NO, which is dismutated into N (nitrogen) and O₂. Such formed O₂ would in theory proceed in the aerobic respiration chain, which would then support high O₂ consumption rates. However, we note that the presence of aerobic denitrification or intra-aerobic respiration remains to be demonstrated in P. aeruginosa. Maximal N₂O concentration was detected in the hypoxic zone of the alginate beads, indicating that denitrification mainly occurred here (Fig. 5).

Production of N₂O decreased with time (Fig. 5b), which may reflect NO₃⁻ depletion in the beads and the surrounding medium. This was supported by the complete exhaustion of NO₃⁻ after 48 h (Fig. 6a).

In beads incubated in anoxic medium with NO₃⁻, P. aeruginosa solely relied on denitrification for anaerobic respiration as indicated by the observed N₂O production, which decreased over time (Fig. 5b), leading to the appearance of a more heterogeneous size distribution of bacterial aggregates in the beads (Fig. 2) resembling the growth pattern observed in normoxic beads. As N₂O is a key intermediate in the denitrification metabolic pathway, a decreasing N₂O flux could indicate the onset of NO₃⁻ limitation within the beads after 48 h of growth, which was supported by our finding of complete consumption of NO₃⁻ after 48 h (Fig. 6a). We also followed the fate of NO₂⁻ in the growth

Fig. 8  Schematic presentation of growth dynamics and chemical dynamics in alginate beads under different growth conditions. a Normoxic growth, b normoxic growth with NO₃⁻, and c anoxic growth with NO₃⁻. Green circles represent aggregates of P. aeruginosa within the alginate bead. O₂: blue/light blue. NO₃⁻: red/light red. Sizes not to scale.
medium (Fig. 6b), which showed a peak in NO$_2^-$ for anoxic grown beads after 24 h, and complete depletion after 48 h. In normoxic incubations (with NO$_3^-$), no increase in NO$_2^-$ was observed, but we speculate that such a peak may have occurred before the 24 h measurement, as NO$_3^-$ in the presence of O$_2$ was shown to facilitate a more intense growth initially. The N$_2$O measurements in the growth medium showed that N$_2$O accumulated to very high levels in the sealed system after 48 h (Fig. 6c) further supporting a strong NO$_3^-$ consumption during the incubations.

While our microsensor measurements revealed insight to the overall dynamics of O$_2$ and N$_2$O gradients in the alginate beads when incubated under different electron acceptor availability, we note that such measurements cannot provide detailed insight to the heterogeneous chemical landscape of individual microcolonies in the alginate beads. In a first attempt to link microcolony heterogeneity to the distribution in O$_2$, we used ratiometric O$_2$ imaging in a 24 h old alginate bead supplemented with O$_2$-sensitive nanoparticles and grown under normoxic conditions (Fig. 7c). Ratiometric O$_2$ imaging showed pronounced local variation in the outermost 100 µm of the alginate bead. However, the used camera system for O$_2$ imaging did not have enough spatial resolution to investigate co-localisation and enable correlations between microcolony size and O$_2$ distribution. However, such combined biomass and O$_2$ imaging in the alginate beads has a strong potential to further resolve links between the chemical microenvironment and growth of *P. aeruginosa* in the alginate bead. Such approach would require more measurements of O$_2$ distribution with nanoparticles in the beads using higher-resolution microscopic imaging.

In conclusion, growth in alginate beads represents a useful in vitro model for the in vivo growth of *P. aeruginosa* in chronic infections. This model system is suitable for testing responses in bacterial metabolism and growth patterns to the availability of different electron acceptors and donors during chronic infections, and the effect of the different treatments are summarised in Fig. 8. The alginate bead model may also prove very suitable for testing antimicrobial susceptibility and tolerance of *P. aeruginosa* and other pathogens involved in mono-species and multi-species infections. It is well known that O$_2$ limitation contributes to antibiotic tolerance of bacteria in biofilms,

### METHODS

#### Bacterial strains

A wild-type *P. aeruginosa* strain PAO1 was obtained from the *Pseudomonas* Genetic Stock Center (http://www.pseudomonas.med.ecu.edu). To enable visualisation of PAO1 cells, GFP constitutively expressed on plasmid pMRP92 was used.

#### Growth conditions

Cultures were propagated from –80 °C freeze culture stocks and grown overnight (ON) in lysogeny broth (LB) for approximately 18 h at 37 °C under continuous shaking at 180 RPM. Subsequently, the LB ON culture was used for inoculation in low nutritional R2A broth (Lab M Limited, UK) supplemented with 0.05 M Tris-HCl buffer (pH 7.6) and 0.5% glucose (abbreviated R2A), and left to acclimatise ON until further use. The medium was flushed with nitrogen gas (N$_2$) for 5 min, where after the culture flask was immediately sealed airtight.

The bacterial growth of *P. aeruginosa* in the alginate beads was studied after 24 and 48 h in response to four permutations of the growth conditions: (i) Normoxic culture without NO$_3^-$, (ii) normoxic culture with NO$_3^-$, (iii) anoxic culture without NO$_3^-$, and (iv) anoxic culture with NO$_3^-$.

#### Optical coherence tomography

We used a commercially available spectral-domain OCT system (Ganymede II, Thorlabs GmbH, Germany) equipped with an objective lens with an effective focal length of 36 mm, and a working distance of 25.1 mm (LSM03; Thorlabs GmbH, Germany). The operating principle and components of the OCT system are described in detail elsewhere and in Supplementary. In order to compare bacterial growth between experimental treatments, OCT measurements were performed under well-defined optical conditions in terms of OCT system settings. A single alginate bead was placed in a black screw cap filled with 800 µL of distilled water. Using z-axis OCT scans (so-called A-scans), the image was brought into focus via the manual focusing stage and by adjusting the reference light intensity as well as the position of the reference length.

After acquisition of image acquisition settings, the configuration was not changed, and subsequent measurements on alginate beads were performed under identical conditions. Each alginate bead was first scanned at high resolution in three (technical) replicate cross-sectional scans (so-called B-scans) followed by one full 3D scan (so-called C-scan) and rendering of the entire bead. OCT imaging was done in (biological) triplicates for each treatment after 24 and 48 h of incubation, respectively.

**Visualisation of OCT B-scan and C-scan** was done with the manufacturers imaging software (ThorImage 4.2; Thorlabs GmbH, Germany) using the built-in brightness and contrast functions. The images were visualised assuming a constant refractive index of water ($n = 1.33$). The attenuation of the A-scan signal can be used to understand changes in the structure of the alginate bead. We extracted three vertical A-scans from the B-scans over the area surrounding the highest point of the alginate bead (Fig. S1). OCT images were extracted in B-scan mode with manually optimised brightness and contrast adjustment and assuming a refractive index of 1.33 (for water). These adjustments separated the background noise from the OCT signal generated from the alginate beads.

#### Microscopy

A confocal laser scanning microscope (Zeiss Imager.Z2, LSM710 CLSM; Zeiss) operated with the manufacturers software (Zen2010, version 6.0; Zeiss, Germany) was used for imaging alginate-encapsulated GFP-tagged PAO1. Samples were prepared by cutting the beads in half with a sterile scalpel. Cut beads were mounted in the dents of a flow-cell with the cut surface facing upwards. 10 µL milliQ water was applied to the cut bead surface, and a cover glass was fixed to the flow-cell with silicone sealant, making sure the cut surface was in close contact with the cover glass. The flow-cell was mounted on the microscope, and images were taken from the cut surface of the bead enabling visualisation of the distribution of bacterial aggregates from the periphery of the beads and towards the bead interior. Imaging of GFP-tagged *P. aeruginosa* was done with a 40x/NA 1.3 oil objective, using laser excitation at 488 nm, and an emission filter range from 495 to 605 nm with a peak at 510 nm. The resulting images were processed with Imaris image processing software (v83.1.1; Bitplane, Switzerland).

**Microscopy combined with quantitative PNA-FISH**

Alginate beads from the four permutations, sampled after 24 and 48 h, were stored for at least 24 h in 4% formaldehyde (Hounisen, Denmark) kept at 4 °C and supplemented with 0.25 CaCl$_2$ for stabilisation. Subsequently, the beads were embedded in paraffin, cut in 4-µm thick sections with a standard microtome, fixed on cover slides and kept dark at 4 °C until further use; we note that this procedure did not work with the anoxic beads incubated without NO$_3^-$ due to bead disruption. Staining of bead sections was conducted with a Texas Red-conjugated PNA-FISH probe specific for *P. aeruginosa* 16S rRNA (AdvanDx, USA) by previously described methods. A microscope slide with the fixed and PNA-FISH stained 4-µm sections of alginate beads was mounted on the microscope. Imaging of the alginate bead sections for quantitative PNA-FISH was done
with identical image acquisition settings for all pictures: Fluorescence images were recorded as 1-μm step size z-stacks at an image resolution of 4096 × 4096 pixels, with an averaging of two scans, and 16-bit colour depth using a 63×/1.4NA oil immersion objective, laser excitation at 594 nm, and emission range from 600 to 695 nm with a peak of 615 nm. Quantification of microcolony fluorescence (mean intensity) was performed using Imaris image processing software (v8.3.1; Bitplane, Switzerland). Image thresholding was applied, which discriminated background and foreground fluorescence with the use of the Measuring Pro expansion pack for the Imaris software (Bitplane, Switzerland). A minimum colony size was set to 10 μm² to avoid inclusion of planktonic bacteria in the analysis of bacterial aggregates. The mean fluorescence intensity of each microcolony was measured on a 16-bit scale from 0–65535 fluorescence intensity units (FU). Koepf et al.31 previously described a linear correlation between growth rate and fluorescence intensity of P. aeruginosa stained rRNA molecules in P. aeruginosa. Based on this relationship, the fluorescence intensity of PNA-FISH stained samples could be used as a proxy for apparent growth rate. Colony distance from the discernible periphery of alginate beads was measured manually for individual colonies using the measuring tool in the Imaris software (Bitplane, Switzerland) and was plotted against fluorescence intensity.

Colony-forming units

For quantification of CFU, beads were dissolved using a solution of Na₂CO₃ (0.05 M) and citric acid (0.02 M). The dissolved bead slurry was serially diluted before plating on LB plates for enumeration of cells via colony formation. Colony-forming units (CFUs) were determined in biological triplicates.

Microsensor measurements

Beads were submerged in a Petri dish filled with pre-warmed R2A medium (with or without NO₃⁻) that was kept at 37 °C under gentle ventilation by a fine air or nitrogen stream directed towards the surface via a Pasteur pipette connected to an air pump or N₂ gas cylinder. Probes of O₂ concentration vs. depth in the alginate bead were measured with an amperometric O₂ microsensor (25 μm tip diameter; OX25, Unisense A/S, Denmark) mounted on a motorised micromanipulator (MU1 Pyro-Science GmbH, Germany). Similarly, NO concentration profiles were measured with an O₂-insensitive amperometric NO microsensor (25 μm tip diameter; NO2025, Unisense A/S, Denmark). All measurements were performed in 3–4 biological replicates. Both microsensors were connected to a PA metre (Unisense A/S, Denmark) that was interfaced to a PC via an A/D converter (Profix; PyroScience GmbH, Germany). Linear calibrations of the microsensors were performed as specified by the manufacturer via measurements with the O₂ microsensor in air saturated and O₂-free water, and measurements with the NO microsensor in N,O-free water, followed by measurement upon addition of defined aliquots of N,O saturated water.

Concentration profiles were recorded in beads incubated for 24 and 48 h after encapsulation of P. aeruginosa in alginate, respectively. The position, where the microsensor tip touched the bead surface (depth = 0), was determined visually with the aid of a USB microscope (model AM7515MZTL, dino-lite.eu) aiming after the centre of the uppermost bead. Fluctuations in N,O concentration in fresh and spent NO₃⁻-supplemented R2A medium during the time course of the experiments was quantified by the Griess colorimetric reaction (no. 780001, Cayman Chemicals, USA), in technical duplicates, as previously described.

Imaging of O₂

In order to image the O₂ distribution within alginate beads harbouring P. aeruginosa, O₂-sensitive sensor nanoparticles were incorporated into the beads. The sensor nanoparticles contained an O₂-sensitive indicator (PtTFPP) and an insensitive reference dye (MY) and were prepared as described elsewhere. The sensor nanoparticles were added to the alginate-bacterial solution prior to bead preparation (sensor particle to alginate ratio: 1:50 vol/vol). After 24 h of growth, ratiometric O₂ imaging of the bead was performed as described below.

For imaging, a bead was cut in half and placed on a cover glass with the cut surface facing the glass surface. The cut bead was covered with fresh medium and left to acclimate for a few minutes. A USB RGB microscope with built-in UV (405 nm) LED illumination (model AM4113-FVT, dino-lite.eu) was placed below the cover glass and was used to obtain images of the cut bead surface. After image acquisition, the obtained RGB images were split into the three colour channels (red, green, and blue) using the free image analysis software ImageJ (imagej.net). As the O₂-sensitive indicator emits in the red channel and the reference indicator in the green channel, the ratio between these two channels could be used to obtain an O₂ image. Ratios were calculated using the plugin Ratio Plus (https://imagej.nih.gov/ij/plugins/ratio-plus.html) and were linked to O₂ concentration by a previously obtained calibration using the curve fitting function of ImageJ. For the calibration, alginate beads with sensor nanoparticles but without bacteria were submerged and imaged in water containing known O₂ levels. The O₂ levels were adjusted by flushing the water with an air/N₂ mixture using a PC-controlled gas mixer (Sensorsense, The Netherlands). Detailed additional information on ratiometric O₂ imaging using sensor nanoparticles can be found in recent publications.

Statistics

Data were analysed for statistical significance with SPSS version 24 (IBM, USA) and GraphPad Prism version 6 (GraphPad Software, USA). Data were illustrated with GraphPad Prism. Group comparisons for quantitative PNA-FISH data (Fig. 3) were made using either Mann–Whitney U test (for growth depth calculated as IQR) or independent samples t-test on log transformed values (apparent growth rate, expressed as mean fluorescence intensity). Log growth rate and depth were correlated using Pearson correlation. Flux rates (Fig. 5) were compared by Mann–Whitney U test. Changes in CFU (Fig. 4), concentrations of NO₃⁻ and NO₂⁻ (Fig. 6), as well as differences in OCT d½ signal over time (Fig. 1) were analysed by two-way ANOVA. Based on our experience with the effect of treatment and the standard deviation of the methods rarely exceeding 40% of the effect we expect that three replicates will allow us to detect significant differences with p < 0.05 and a power of 0.80. A two-sided α of 0.05 was considered significant.

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Author Contributions

M.S., M.Ku., K.K., and T.B. conceptualised the study, M.S. and M.Ku. wrote the main manuscript text, K.K. performed data acquisition for Figs. 5 and 7 and prepared Fig. 7, D.W. performed OCT data acquisition and analysis (Fig. 1, and Supplementary Figs. S1, S2), K.N.X. analysed data for Fig. 3, P.O.J. analysed the results and revised the manuscript, M.Ko. assisted with data collection for Fig. 6. All authors reviewed the manuscript.
ADDITIONAL INFORMATION
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