# Nitrous Oxide Production in Sputum from Cystic Fibrosis Patients with Chronic *Pseudomonas aeruginosa* Lung Infection

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## Abstract

Chronic lung infection by *Pseudomonas aeruginosa* is the major severe complication in cystic fibrosis (CF) patients, where *P. aeruginosa* persists and grows in biofilms in the endobronchial mucus under hypoxic conditions. Numerous polymorphonuclear leukocytes (PMNs) surround the biofilms and create local anoxia by consuming the majority of $O_2$ for production of reactive oxygen species (ROS). We hypothesized that *P. aeruginosa* acquires energy for growth in anaerobic endobronchial mucus by denitrification, which can be demonstrated by production of nitrous oxide ($N_2O$), an intermediate in the denitrification pathway. We measured $N_2O$ and $O_2$ with electrochemical microsensors in 8 freshly expectorated sputum samples from 7 CF patients with chronic *P. aeruginosa* infection. The concentrations of $NO_3^-$ and $NO_2^-$ in sputum were estimated by the Griess reagent. We found a maximum median concentration of 41.8 µM $N_2O$ (range 1.4–157.9 µM $N_2O$). The concentration of $N_2O$ in the sputum was higher below the oxygenated layers. In 4 samples the $N_2O$ concentration increased during the initial 6 h of measurements before decreasing for approximately 6 h. Concomitantly, the concentration of $NO_3^-$ decreased in sputum during 24 hours of incubation. We demonstrate for the first time production of $N_2O$ in clinical material from infected human airways indicating pathogenic metabolism based on denitrification. Therefore, *P. aeruginosa* may acquire energy for growth by denitrification in anoxic endobronchial mucus in CF patients. Such ability for anaerobic growth may be a hitherto ignored key aspect of chronic *P. aeruginosa* infections that can inform new strategies for treatment and prevention.

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## Introduction

Cystic fibrosis (CF) is an autosomal recessive disease. It is caused by mutations in the cystic fibrosis trans-membrane conductance regulator gene [1] affecting apical ion transport. In the lungs, the defective ion transport results in endobronchial accumulation of thick, viscous mucus that prevents mucociliar cleaning of the lungs, and increases susceptibility to chronic respiratory infections [2,3]. *Pseudomonas aeruginosa* is a Gram-negative, gamma proteobacterium, which dominates chronic lung infections in CF patients and is considered the most serious complication of CF [4,5]. The chronic *P. aeruginosa* lung infection in CF patients is characterized by presence of endobronchial biofilm aggregates surrounded by numerous polymorphonuclear leukocytes (PMNs) [6,7]. Despite the bactericidal activity of the PMNs and intensive antibiotic therapy, these biofilms persist and grow in the endobronchial mucus of CF patients over many years [7,8]. *P. aeruginosa* can withstand the bactericidal activity of the PMNs by forming biofilms of the protective mucoid phenotype [9] and by quorum sensing (QS)-regulated production of leukolytic amounts of rhamnolipid [10–13]. The summoned PMNs produce reactive oxygen species (ROS) through a respiratory burst, which leads to intense depletion of molecular oxygen ($O_2$) [14], a common feature of infected endobronchial mucus in CF [6]. Biofilm formation may explain why *P. aeruginosa* survives the attacking PMNs, but it is not known how *P. aeruginosa* acquires the energy required for the observed growth in endobronchial secretions [8] when $O_2$ is absent. However, *P. aeruginosa* can grow anaerobically with alternative electron acceptors or by arginine fermentation [15], and it has been suggested that *P. aeruginosa* can respire by denitrification in anoxic CF mucus utilizing nitrate ($NO_3^-$) and nitrite ($NO_2^-$), which are both present in sufficient amounts [15,16]. Although the ability of *P. aeruginosa* to utilize reduction of NOx for anaerobic respiration is well known [17], denitrification in mucus and persistent biofilms present in the airways of CF patients remains to be demonstrated. Since $N_2O$ is a natural intermediate belonging to the gases defining denitrification [17], we used electrochemical microsensors [18] to measure $O_2$ and

$N_2O$ concentration gradients at high spatio-temporal resolution in freshly expectorated sputum from CF patients with chronic *P. aeruginosa* lung infection.

Further evidence for denitrification was obtained from nitrate ($NO_3^-$) and nitrite ($NO_2^-$) turnover measurements in the sputum samples. These measurements provided important new insights to the micro-environmental conditions and chemical dynamics associated with persistent *P. aeruginosa* lung infections in CF patients and indicate that nitrogen compounds can play an important role in the interaction between pathogenic bacteria and an active immune response.

## Results

### $N_2O$ and $O_2$ in sputum from CF patients with chronic *P. aeruginosa* lung infection

Representative measurements of $O_2$ and $N_2O$ in freshly expectorated sputum were acquired with $O_2$- and $N_2O$ microsensors (Fig 1A). Measurements of $O_2$- and $N_2O$ profiles in expectorated sputum from a CF patient with chronic *P. aeruginosa* lung infection showed the distribution of an upper oxygenated zone and a lower anoxic zone. The $N_2O$ profile reached the maximal concentration of $N_2O$ in the lower anoxic part of the sputum sample, suggesting that denitrification is mainly confined to the anoxic zone. A slow decline of $O_2$ was apparently detected above the sputum surface. This may be because the position of the sputum surface was estimated by visual inspection, which is associated with uncertainty due to small amounts of heterogeneous saliva (Fig 1B).

Sputum is composed of heterogeneously distributed bacterial aggregates surrounded by PMNs consuming $O_2$, and this respiratory burst creates local anoxic microenvironments in the sputum [14]. The metabolic mechanisms are thus compartmentalized according to the availability of $O_2$ with an oxygenated zone, wherein the majority of $O_2$ is reduced to superoxide by the summoned PMNs, and an anoxic zone, where *P. aeruginosa* can utilize nitrate as electron acceptor during oxidative phosphorylation (Fig. 1C).

### $NO_3^-$ and $NO_2^-$ in sputum from CF patients with chronic *P. aeruginosa* lung infection

$NO_3^-$ and $NO_2^-$ concentrations in sputum samples were measured before $N_2O$ profiling and 1 day later (Fig 2). The concentration of $NO_3^-$ was significantly higher immediately before $N_2O$ profiling as compared to 1 and 2 days after incubation indicating $NO_3^-$ depletion due to ongoing denitrification (Fig 2A, B). The $NO_2^-$ concentration was not changed significantly after one day (Fig 2C), but by including additional measurements of the $NO_2^-$ concentration in 7 sputum samples a significantly decreased $NO_2^-$ concentration was detected (Fig 2D).

### Distribution of $N_2O$ in sputum from CF patients with chronic *P. aeruginosa* lung infection

Vertical profiles of $O_2$ in sputum samples showed depletion of $O_2$, indicating the formation of anoxic zones below a mean depth of 3.1 mm (SD = 3.0 mm) from the sputum surface (Fig 3A) suggesting that the average depth of $O_2$ penetration of ~3 mm. A higher concentration of $N_2O$ was observed in the anoxic zone as compared to the oxic zone ($p<0.026$, $n=8$) (Fig 3B). To verify that $N_2O$ is related to *P. aeruginosa* we found significantly less $N_2O$ in three control sputum samples from 1 CF patient and from 2

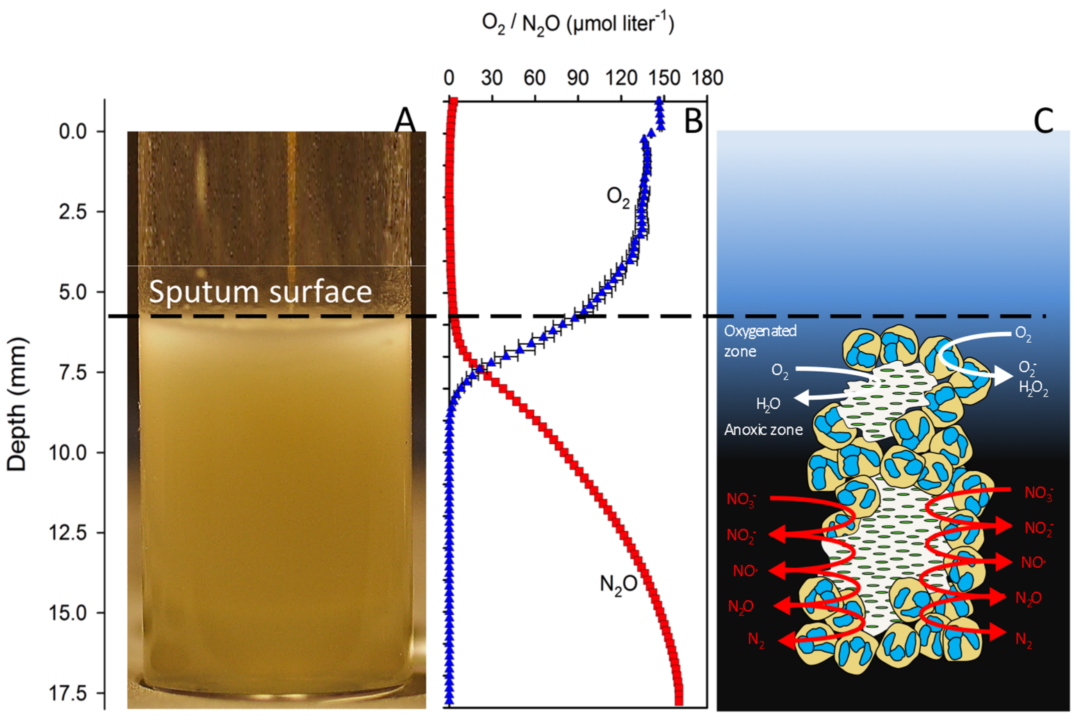


**Figure 1. Microsensor measurements of chemical gradients in sputum.** (A) Close up of a sputum sample from a cystic fibrosis patient with chronic *P. aeruginosa* lung infection with an inserted microsensor. (B) Representative microprofiles of $N_2O$ and $O_2$ in a CF sputum sample. $O_2$ profiles are shown as the mean and SD of three microprofiles recorded in the beginning of the experiment and did not change significantly throughout the experimental period, while the $N_2O$ profile represents the maximal $N_2O$ levels measured about 6–7 h after beginning. (C) A schematic model of the involved PMN and biofilm processes in CF sputum explaining the microprofiles.
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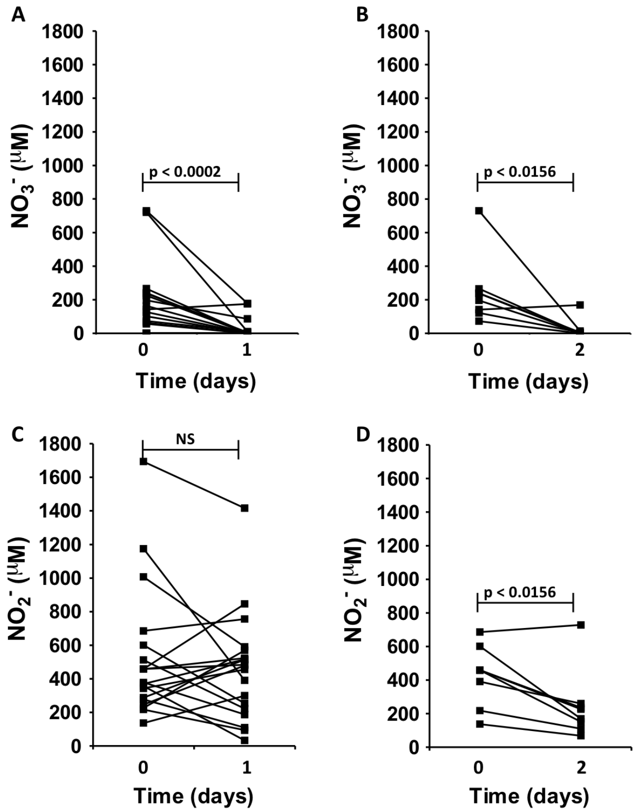


**Figure 2. Consumption of NO3⁻ and NO2⁻ in sputum.** (A, B) $NO_3^-$ concentration in sputum samples from cystic fibrosis patients with chronic *P. aeruginosa* lung infection. (C, D) $NO_2^-$ concentration in sputum samples from cystic fibrosis patients with chronic *P. aeruginosa* lung infection. Samples were collected immediately after expectoration and after 1 (n = 20) and 2 days (n = 7) of incubation. Data were analyzed by Wilcoxon signed rank test.
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primary ciliary dyskinesia (PCD) patients without detectable *P. aeruginosa* ($p<0.030$).

## Dynamics of $N_2O$ in sputum from a CF patient with chronic *P. aeruginosa* lung infection

Figure 4 displays time series of representative $N_2O$ profiles measured vertically through a sputum sample. The distribution of $O_2$ is displayed at 0 hr. During the initial measuring period, $N_2O$ accumulated in the anoxic zone reaching a maximum concentration of 160 µM after 6.5 h incubation, which indicates ongoing production of $N_2O$. Within the subsequent 4 hours the accumulated $N_2O$ decreased indicating consumption through reduction to $N_2$.

## Rates of $N_2O$ production and consumption in sputum samples

Measurements of the $N_2O$ concentration dynamics over time in particular depths of a sputum sample showed an initial build-up of $N_2O$ in layers below 7 mm (Fig. 5). In each layer, the slope of the net production curves was quasi-linear after ~180 min indicating a constant production of $N_2O$ related to the particular layer and therefore that $N_2O$ originates from immobile sources such as biofilm. The production ceased about 6–7 h after start of the sample incubation, and was then followed by a net consumption of $N_2O$ over the following 4–5 h leading to $N_2O$ depletion in the sputum sample after ~10–12 hours. In 4 sputum samples it was possible to estimate $N_2O$ production and consumption rates (Table 1) and $N_2O$ flux rates and cumulated emission (Figure 6) from measurements of such dynamic $N_2O$ concentration microgradients. A substantial initial $N_2O$ concentration was observed in the anaerobic zone of the remaining 4 assayed sputum samples. In these samples the $N_2O$ concentration decreased steadily during incubation.

# Discussion

The ability of microorganisms to exploit a wide range of electron acceptors for ATP generation by oxidative phosphorylation provides metabolic flexibility in transient environments as these organisms inhabit a variety of habitats ranging from soils, sediments to aquatic environments [19]. Even though several human pathogens, including *P. aeruginosa*, are equipped with the genetic setup for denitrification [20–22] including nitric oxide reductase (NOR) [22], we present the very first observations of $N_2O$ production in clinical material from infected human airways demonstrating pathogenic metabolism based on denitrification. These data indicate that denitrification may serve as an alternative metabolic pathway allowing *P. aeruginosa* to thrive in $O_2$ depleted micro niches in the airways of CF patients. Besides our study, denitrification in humans has previously been demonstrated in human dental plaque [23] and has been related to infections of the gastrointestinal tract by the increased concentration of $N_2O$ in exhaled breath from patients after oral intake of $NO_3^-$ [24].

Seminal observations of $O_2$ depletion and the presence of OprF porin, which is involved in $NO_3^-$ and $NO_2^-$ diffusion, in habitats of *P. aeruginosa* during chronic lung infection of CF patients provided initial evidence for anaerobic respiration by denitrification [6,16]. To demonstrate denitrification we have included CF patients, who suffered from chronic *P. aeruginosa* infection in the endobronchial mucus as detected by routine culturing. We revealed a depletion of $O_2$ in CF sputum samples, which is in accordance with the steep $O_2$ gradients in endobronchial CF mucus [6] and due to $O_2$ consumption by activated PMNs for generation of ROS [14]. Our $O_2$ measurements in sputum confirmed the presence of $O_2$ concentration gradients reaching anoxia ~3 mm below the sputum surface.

The depletion of $O_2$ for microbial respiration in infected endobronchial CF mucus has motivated the present and several other studies of anaerobic metabolism by *P. aeruginosa* based on denitrification during chronic lung infection in CF. We demonstrated $N_2O$ production and consumption in the sputum samples indicating the presence of active NOR and nitrous oxide reductase ($N_2OR$) for the reduction of nitric oxide (NO•) and $N_2O$ [17]. Previously, NOR has been isolated from *P. aeruginosa* [25], the genes (*norCB*) have been sequenced [26] and functional NOR has been observed in clinical strains of *P. aeruginosa* by consumption of NO• [27].

In our study, the initial phase of $N_2O$ production in the sputum samples was followed by a period of net $N_2O$ consumption suggesting a depletion of NO• and a concomitant reduction of $N_2O$ to $N_2$ by $N_2OR$. The $N_2O$ consumption is in agreement with the demonstration of $N_2OR$ activity and the identification of the *nos* genes in *P. aeruginosa* [28] as well as the induced genes for a $N_2OR$ precursor in clinical isolates [29].

Our demonstration of significant $N_2O$ production in sputum indicates ample presence of $NO_3^-$ and $NO_2^-$ that serve as electron acceptors for the denitrification pathway. We found high levels of $NO_3^-$ and $NO_2^-$ in the sputum, which are in agreement with previous findings [30–32]. It has been proposed that $NO_3^-$

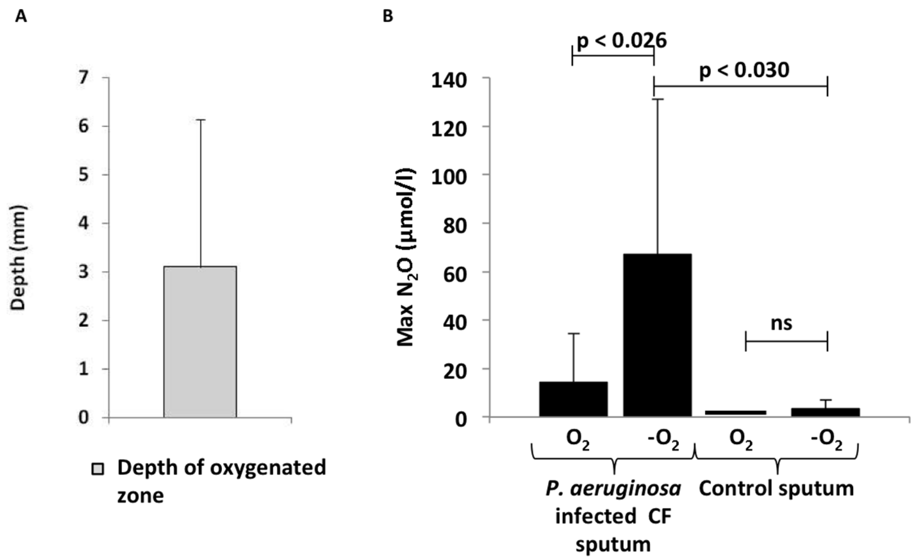


**Figure 3. Distribution of $O_2$ and $N_2O$ in sputum.** (A) Depth of oxygenated zone in sputum samples from cystic fibrosis patients with chronic *P. eruginosa* lung infection (n = 8). (B) Maximal $N_2O$ concentration in the oxic zone and anoxic zone in sputum samples from cystic fibrosis patients with chronic *P. aeruginosa* lung infection (n = 8). Control sputum without detectable *P. aeruginosa* was obtained from two PCD patients and one CF patient. Statistical analysis was performed by Student t-test.
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and $NO_2^-$ in CF sputum originates from the rapid reaction between superoxide ($O_2^-$) and NO• [15]. In this regard, we suggest the summoned activated PMNs [14] as a major source of $O_2^-$, while NO•, which is present in CF exhaled breath [33,34], may be produced by a variety of cells in the lungs. In fact, inhalation of NO• or incubation of sputum samples with NO• resulted in elevated levels of $NO_3^-$ and $NO_2^-$ in sputum from CF patients [35]. In addition, ongoing activity of the patients nitric oxide synthases was evidenced by the increased exhaled NO• from infected CF patients following supplementation with the substrate L-arginine [36,37].

As a consequence of our demonstration of $N_2O$ production, we expected a consumption of the precursors $NO_3^-$ and $NO_2^-$. Accordingly, $NO_3^-$ was depleted in the sputum after incubation for 1 day, which likely is due to the membrane-bound nitrate reductase of *P. aeruginosa* [29]. $NO_3^-$ consumption may also accompany assimilatory denitrification and ammonification resulting in the formation of ammonia ($NH_4^+$) [17], which has been detected in CF sputum [27]. However, assimilatory denitrification and ammonification does not involve production of $N_2O$ [17,38,39] and $NH_4^+$ is also produced by several human cell types [40]. The concentration of $NO_2^-$ was not changed during 1 day of incubation, but after 2 days of incubation the concentration of $NO_2^-$ in the sputum was decreased significantly. This indicates that the production of NO• from $NO_2^-$ is slower than the generation of $NO_2^-$ resulting from reduction of $NO_3^-$. Indeed, during reduction of $NO_3^-$ transient accumulation of $NO_2^-$ is known from anaerobic cultures of *P. aeruginosa* growing by denitrification [16,41,42].

A further verification of ongoing dissimilatory denitrification in sputum is evident from the calculated rate of $N_2O$ production (Fig. 6A), which easily can explain the depletion of $NO_3^-$ during incubation (Fig. 2A). The depletion of $NO_3^-$ in the sputum samples indicates that the $NO_3^-$ in sputum samples is not replaced by the reaction between $O_2^-$ and NO. This is possibly due to lack of contributions from immigrating PMNs and the epithelia as opposed to the conditions in the endobronchial mucus.

Since we calculated the rates of $N_2O$ production by assuming linear changes between subsequent measurements in the beginning of incubation, the estimates are likely to reflect the situation in the endobronchial mucus, where reduced $NO_3^-$ and $NO_2^-$ is continuously being replaced as indicated by the high $NO_3^-$ and $NO_2^-$ content in fresh sputum. The estimated $N_2O$ production, however, is calculated from the actual $N_2O$ content and does not include the reduction of $N_2O$ to $N_2$. Therefore, the actual rate of denitrification may be higher than our estimates.

We found the highest concentration of $N_2O$ in the anoxic zone of the confined sputum samples indicating higher rate of denitrification without $O_2$ as previously demonstrated [43]. Accordingly, we suggest that the low concentration of $N_2O$ found in the oxygenated zone is mainly due to diffusion from the active anoxic zone. Additionally, our estimate of the depth of the oxygenated zone implies that the bronchi, with diameters ranging from 0.8 to 13 mm [44,45], allow for numerous anoxic zones in the endobronchial mucus of the lungs and confirms the *in vivo* demonstration of $O_2$ depletion in the endobronchial mucus [6]. Consequently, our results propose the existence of several zones with $N_2O$ production in the anoxic endobronchial mucus of the lungs of CF patients with chronic *P. aeruginosa* lung infection. However, such *in vivo* production of $N_2O$ in CF patients still awaits direct experimental confirmation.

The involvement of denitrification enzymes as terminal oxidases that reduce nitrogen oxides in the highly branched respiratory chain of *P. aeruginosa* may enable anaerobic growth in the presence of nitrate or nitrite [19,46]. But the engagement of denitrification in *P. aeruginosa* may also contribute to virulence as evidenced by the finding of antibodies directed against components of denitrification in CF patients with *P. aeruginosa* lung infection [16,47] and the dependence on nitrite reductase for type III secretion [48]. In anaerobic cultures, denitrification promotes growth of *P. aeruginosa* [49], increases antibiotic tolerance of *P. aeruginosa* [50] and favors maintenance of the virulent mucoid phenotype [30].

A particular contribution to the pathogenesis of chronic lung infection in CF by NOR activity, is suggested by the induced *in vivo*

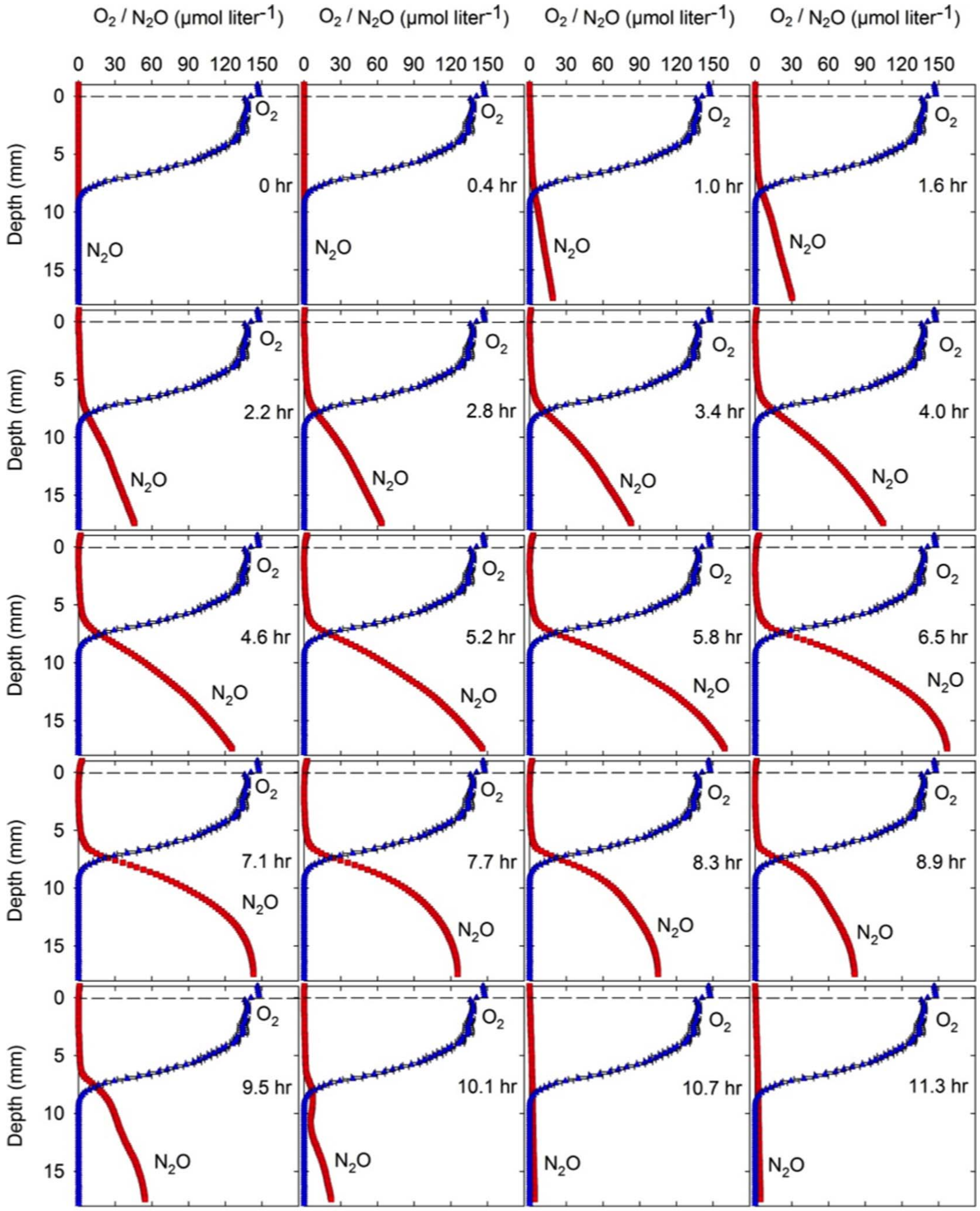


**Figure 4. Generation and depletion of $N_2O$ in sputum.** Spatio-temporal dynamics of $N_2O$ concentration profiles in a representative sputum sample from a cystic fibrosis patient with chronic *P. aeruginosa* lung infection showing initial accumulation of $N_2O$ in the anoxic zone followed by total depletion. The $O_2$ concentration profile is shown as the mean and SD of three microprofiles recorded at the beginning of the experiment.
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gene expression in clinical isolates [29] including the highly virulent mucoid isolates [51]. In this respect, the reduction of NO• to $N_2O$ by active NOR may actually protect *P. aeruginosa* from the bactericidal action of NO• generated by the immune system. In fact, NOR-deficient *P. aeruginosa* is more susceptible to NO• generated by macrophages [52] and less virulent during infection of silkworm [53]. In addition, NOR activity increases the virulence of several pathogens [54–56].

In conclusion, this study points to the presence of anoxic microenvironments with strong spatio-temporal heterogeneity as well as a possible stratification of metabolic processes in the biofilm aggregates characteristic of chronic *P. aeruginosa* infections in the airways of CF patients. Such structural and metabolic heterogeneity may be a characteristic trait ensuring persistent infection. Indeed, spatio-temporal resolved measurements enabled the demonstrated of $N_2O$ production in the anaerobic zones of freshly expectorated sputum samples from CF patients with chronic *P. aeruginosa* lung infection for the first time. Analysis of the $N_2O$ production rates suggests ongoing generation of $N_2O$ in the lungs of CF patients with chronic *P. aeruginosa* infection. $N_2O$ production

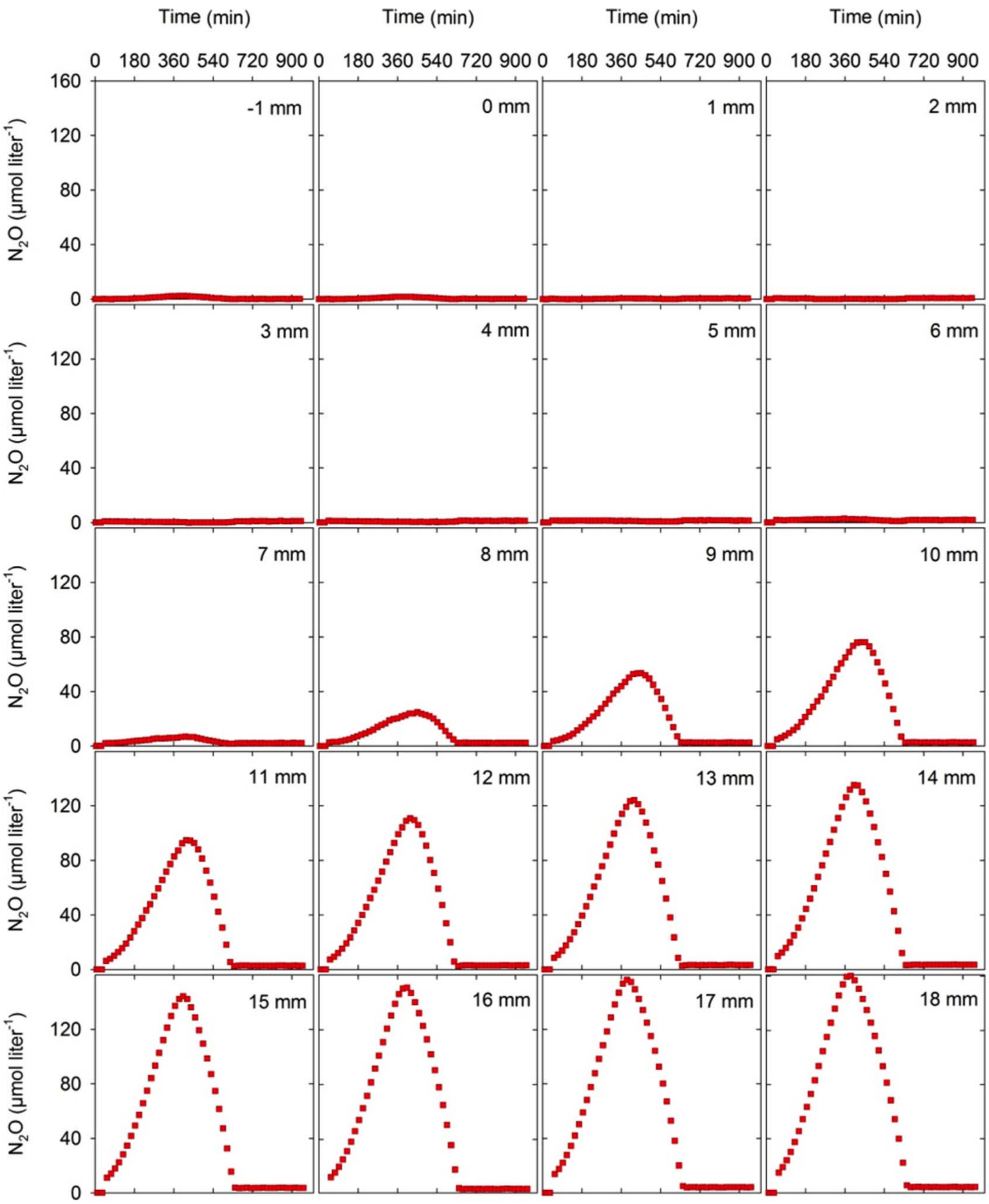


**Figure 5. Rates of N2O production and consumption in sputum.** Depth specific plots of $N_2O$ concentration vs. time at particular measuring depths in the same sputum sample as displayed in Fig 4. Accumulation and thus net production of $N_2O$ in all depths was observed until approximately 6 h, followed by net consumption of $N_2O$ presumably due to depletion of nitrate around 6 h.
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**Table 1.** $N_2O$ production, consumption, max emission, and cumulated emission in 4 CF sputum samples.

| | Net production rate (nmol cm$^{-3}$min$^{-1}$) | Net consumption rate (nmol cm$^{-3}$min$^{-1}$) | Max emission (nmol cm$^{-2}$ min$^{-1}$) | Cumulated emission (nmol cm$^{-2}$) |
|---|---|---|---|---|
| Median | 0.47 | −0.39 | $5.06\times10^{-5}$ | $1.05\times10^{-2}$ |
| Range | 0.40–0.70 | −0.77–−0.10 | $1.8\times10^{-5}$–$6.78\times10^{-5}$ | $3.94\times10^{-3}$–$1.46\times10^{-2}$ |

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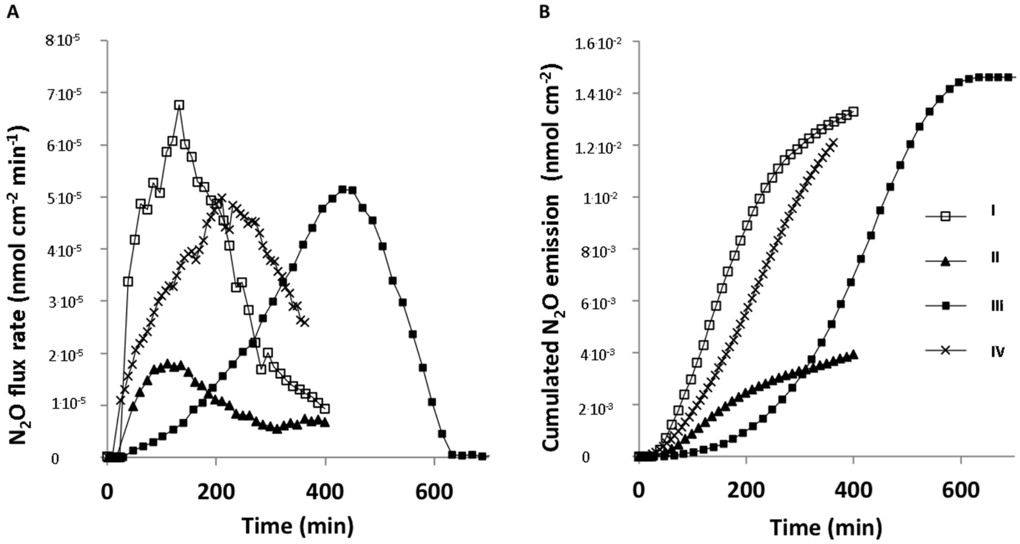


**Figure 6. Efflux and cumulated emission of $N_2O$ from sputum samples.** (A) Estimated $N_2O$ efflux rates in sputum samples from cystic fibrosis patients with chronic *P. aeruginosa* lung infection as calculated from $N_2O$ microprofiles (n = 4). (B) Cumulated $N_2O$ emission as calculated from $N_2O$ microprofiles (n = 4). I, II, III and IV represents 4 different sputum samples.
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by *P. aeruginosa* in this environment is associated with anaerobic growth, which can promote increased virulence and tolerance to antibiotic, as well as contribute to evasion of the host response. The chronic infected CF lung is in many ways a black box. By using the presented approach to elucidate the essential metabolites we may now open the black box and start mapping the micro-environment of infection which may inspire new strategies for prevention and treatment of chronic lung infections in CF.

## Materials and Methods

### Sputum Samples

As defined by the "Danish Act on Research Ethics Review of Health Research Projects" Section 2 the project does not constitute a health research project and was thus initiated without approval from The Committees on Health Research Ethics in the Capital Region of Denmark. Therefore, verbal informed consent was obtained using waiver of documentation of consent. The study was carried out on 21 anonymized samples of surplus expectorated sputum from 21 CF patients and 2 PCD patients (Table 2). Chronic *P. aeruginosa* infection was defined as the presence of *P. aeruginosa* in the lower respiratory tract at each monthly culture for >6 months, or for a shorter time in the presence of increased antibody response to *P. aeruginosa* (>2 precipitating antibodies, normal: 0–1) [57].

**Microsensor Measurements of $O_2$ and $N_2O$.** Each of 8 different sputum samples (1–2 ml) was added to a glass vial (35×12 mm) (Schuett Biotec, Germany) and allowed to settle for about 10 min. The glass vials were positioned in a heated metal rack, kept at 37°C. Vertical $O_2$-concentration profiles were recorded in the sputum with an amperometric $O_2$ microsensor (OX25, Unisense A/S, Århus, Denmark) mounted in a motorized PC-controlled profiling setup (MM33 and MC-232, Unisense A/S). Subsequently, vertical $N_2O$ concentration profiles were recorded at defined time intervals for up to 12 hours with an amperometric $N_2O$ microsensor [18] ($N_2O$-25, Unisense A/S) mounted in the micromanipulator.

The microsensors (tip diameter 25 µm) were connected to a picoammeter (PA2000, Unisense A/S) and positioned manually onto the upper surface of the sputum sample. Profile measurements were taken by movement of the sensor in vertical steps of 100 or 200 µm through the sputum sample. Positioning and data acquisition were controlled by dedicated software (Sensortrace Pro 2.0, Unisense A/S). The software was set to wait 3 seconds for the $O_2$-microprofile and 5 seconds for the $N_2O$-microprofile, before actual measurement and subsequent movement of the sensors to the next measuring depth. The interval between each cycle of profile measurements was 10 seconds.

The $O_2$-microsensor was linearly calibrated by measuring the sensor signal in an alkaline sodium ascorbate solution (zero $O_2$) and in air saturated free phosphate buffered saline (PBS) at experimental temperature and salinity. The $O_2$ concentration in air saturated water was determined from the known temperature and salinity according to [58]. The $N_2O$ -microsensor was linearly calibrated according to [18] by measuring sensor signals in $N_2O$ free PBS at experimental temperature and salinity and in PBS with sequential addition of a known volume of $N_2O$ saturated PBS up

**Table 2.** Demographic data of the patients.

| | CF patients | | PCD patients |
|---|---|---|---|
| | Infectious status | | |
| | *P. aeruginosa* | *S. aureus* | *H. influenzae* |
| Number (male) | 20 (10) | 1 (1) | 2 (0) |
| Age (years)* | 39 (24–50) | 17 | 32 (27–36) |
| Duration of chronic infection (years)*, ** | 19 (4–38) | | |
| $FEV_1$ (%)* | 56 (23–96) | 75 | 89 (70–109) |
| FVC (%)* | 88 (46–139) | 82 | 110 (95–125) |

*Values are medians (range).
*Duration of chronic infection is only recorded for *P. aeruginosa* infections.
$FEV_1$, forced expiratory volume in 1 s.
FVC, forced vital capacity.
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to a final concentration of 100 μM $N_2O$. The $N_2O$ concentration in saturated PBS was determined according to [59].

**$NO_3^-$ and $NO_2^-$ quantification.** The concentration of $NO_3^-$ and $NO_2^-$ in sputum was measured in 20 samples. From each sputum sample, 0.1 ml was aspired with a syringe and was immediately diluted 10x in PBS and stored at -20°C for later analysis. The remaining sample was incubated in a glass vial at 37°C for 24 h before dilution 10x in PBS and storage at −20°C. The $NO_3^-$ and $NO_2^-$ levels in the sputum were measured using the Griess colorimetric reaction (no. 780001, Cayman Chemicals, USA) according to the manufacturer's recommendations. For this, sputum samples were transferred to a 96 well microtiter plate. $NO_2^-$ concentration was estimated by addition of the Griess Reagent for 10 minutes, whereby $NO_2^-$ was converted into a purple azo-compound, which was quantitated by the optical density at 540–550 nm measured with an ELISA plate reader (Thermo Scientific Multiskan EX, Thermo Fisher Scientific Inc, BioImage, Denmark). Total $NO_3^-$ and $NO_2^-$ levels were estimated by a two-step analysis process: The first step converted $NO_3^-$ to $NO_2^-$ utilizing $NO_3^-$ reductase. After incubation for 2 hours, the next step involved the addition of the Griess Reagent, whereby $NO_2^-$ was converted into a purple azo-compound. After incubation with Griess Reagent for 10 minutes, the optical density at 540–550 nm was measured with an ELISA plate reader (Thermo Scientific Multiskan EX, Thermo Fisher Scientific Inc, BioImage, Denmark). A $NO_3^-$ standard curve was used for determination of total $NO_3^-$ and $NO_2^-$ concentration, while a $NO_2^-$ standard curve was used for determination of $NO_2^-$ alone. The concentration of $NO_3^-$ was calculated as the difference between the $NO_3^-$ concentration and the total $NO_3^-$ and $NO_2^-$ concentration.

**Calculations of $N_2O$ production rates.** The local $N_2O$ fluxes in sputum samples were calculated from the measured $N_2O$ concentration gradient in the uppermost oxic sputum layer. It was assumed that no production or consumption of $N_2O$ occurred in the presence of $O_2$. The flux was calculated using a modified version of Fick's 1st law of diffusion [60], where the slope of the profile in the sputum surface layer was calculated from the three uppermost measured concentrations (measurement a, b and c):

$$J = 0.5\left[-D\frac{C_a - C_b}{x_a - x_b}\right] + 0.5\left[-D\frac{C_b - C_c}{x_b - x_c}\right]$$

where $\mathcal{J}$ is the flux of $N_2O$ (nmol $N_2O$ $cm^{-2}$ $min^{-1}$), D is the molecular diffusion coefficient of $N_2O$ in water at 37°C ($2.76\times10^{-5}$ $cm^2$ $s^{-1}$) [61] and C is the concentration of $N_2O$ (μmol $liter^{-1}$) at depth $x_n$, where n = a, b or c denote 3 subsequent depths of measurement. The cumulated $N_2O$ emission was calculated by assuming linear changes between subsequent measurements. Net production and net consumption rates of $N_2O$ in particular sputum layers were calculated from the slopes of linear increase and decrease of $N_2O$ concentration at particular measuring depths in the sputum samples [62,63].

**Statistical Analyses.** Statistical significance was evaluated by Wilcoxon Signed Rank Test and by Students T-test. A p value $<0.05$ was considered statistically significant. The tests were performed with Prism 4.0c (GraphPad Software, La Jolla, California, USA).

## Author Contributions

Conceived and designed the experiments: PØJ M. Kolpen M. Kühl. Performed the experiments: PØJ M. Kolpen M. Kühl CRH. Analyzed the data: PØJ M. Kolpen M. Kühl LL. Contributed reagents/materials/analysis tools: PØJ M. Kolpen M. Kühl CRH TP NH. Wrote the paper: PØJ M. Kolpen M. Kühl TB CM AK NH.

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