Low Probability of Initiating nirS Transcription Explains Observed Gas Kinetics and Growth of Bacteria Switching from Aerobic Respiration to Denitrification

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Abstract

In response to impending anoxic conditions, denitrifying bacteria sustain respiratory metabolism by producing enzymes for reducing nitrogen oxyanions/-oxides (NO₃⁻) to N₂ (denitrification). Since denitrifying bacteria are non-fermentative, the initial production of denitrification proteome depends on energy from aerobic respiration. Thus, if a cell fails to synthesise a minimum of denitrification proteome before O₂ is completely exhausted, it will be unable to produce it later due to energy-limitation. Such entrapment in anoxia is recently claimed to be a major phenomenon in batch cultures of the model organism Paracoccus denitrificans on the basis of measured e⁻ flow rates to O₂ and NO₃⁻. Here we constructed a dynamic model and explicitly simulated actual kinetics of recruitment of the cells to denitrification to directly and more accurately estimate the recruited fraction (F_den). Transcription of nirS is pivotal for denitrification, for it triggers a cascade of events leading to the synthesis of a full-fledged denitrification proteome. The model is based on the hypothesis that nirS has a low probability (F_nirS, h⁻¹) of initial transcription, but once initiated, the transcription is greatly enhanced through positive feedback by NO, resulting in the recruitment of the transcribing cell to denitrification. We assume that the recruitment is initiated as [O₂] falls below a critical threshold and terminates (assuming energy-limitation) as [O₂] exhausts. With F_nirS = 0.005 h⁻¹, the model robustly simulates observed denitrification kinetics for a range of culture conditions. The resulting F_den (fraction of the cells recruited to denitrification) falls within 0.038–0.161. In contrast, if the recruitment of the entire population is assumed, the simulated denitrification kinetics deviate grossly from those observed. The phenomenon can be understood as a ‘bet-hedging strategy’: switching to denitrification is a gain if anoxic spell lasts long but is a waste of energy if anoxia turns out to be a ‘false alarm.’

Introduction

A complete denitrification pathway includes the dissimilatory reduction of nitrate (NO₃⁻) through nitrite (NO₂⁻), nitric oxide (NO), and nitrous oxide (N₂O) to di-nitrogen (N₂). Typically, the genes encoding reductases for these nitrogen oxyanions/-oxides (NOₓ) are not expressed constitutively but only in response to O₂ depletion, making denitrification a facultative trait [1]. Hence, during anoxic spells, the process enables denitrifying bacteria to sustain respiratory metabolism, replacing O₂ by NOₓ as the terminal electron (e⁻) acceptors. Since permanently anoxic environments lack available NOₓ, denitrification is confined to sites where O₂ concentration fluctuates, such as biofilms, surface layers of sediments, and drained soil (which turns anoxic in response to flooding).

From modelling denitrifying communities as a homogenous unit to a model of regulation of denitrification in an individual strain

Denitrification is a key process in the global nitrogen cycle and is also a major source of atmospheric N₂O [2]. A plethora of biogeochemical models have been developed for understanding the ecosystem controls of denitrification and N₂O emissions [3]. A common feature of these models is that the denitrifying community of the system (primarily soils and sediments) in question is treated as one homogenous unit with certain characteristic responses to O₂ and NOₓ concentrations. This simplification is fully legitimate from a pragmatic point of view, but in reality any denitrifying community is composed of a mixture of organisms with widely different denitrification regulatory phenotypes [4]. Modelling has been used to a limited extent to analyse kinetic data for various phenotypes (See [5] and references therein) and for understanding the accumulation of intermediates [6]. To our knowledge, however, no attempts have been made to model the regulation during transition from aerobic to anaerobic respiration in individual strains, despite considerable progress in the understanding of their regulatory networks. It would be well worth the effort, since the regulatory phenomena at the cellular level provide clues as to how denitrification and NO and N₂O emissions therefrom are regulated in intact soils [7]. Explicit modelling of the entire denitrification regulatory network,
Author Summary

In response to oxygen-limiting conditions, denitrifying bacteria produce a set of enzymes to convert NO\textsubscript{3}\^-/NO\textsubscript{2}\^- to N\textsubscript{2} via NO and N\textsubscript{2}O. The process (denitrification) helps generate energy for survival and growth during anoxia. Denitrification is imperative for the nitrogen cycle and has far-reaching consequences including contribution to global warming and destruction of stratospheric ozone. Recent experiments provide circumstantial evidence for a previously unknown phenomenon in the model denitrifying bacterium *Paracoccus denitrificans*: as O\textsubscript{2} depletes, only a marginal fraction of its population appears to switch to denitrification. We hypothesise that the low success rate is due to a) low probability for the cells to initiate the transcription of genes (*nirS*) encoding a key denitrification enzyme (NirS), and b) a limited time-window in which NirS must be produced. Based on this hypothesis, we constructed a dynamic model of denitrification in *Pa. denitrificans*. The simulation results show that, within the limited time available, a probability of 0.005 h\textsuperscript{-1} for each cell to initiate *nirS* transcription (resulting in the recruitment of 3.8–16.1% cells to denitrification) is sufficient to adequately simulate experimental data. The result challenges conventional outlook on the regulation of denitrification in general and that of *Pa. denitrificans* in particular.

However, would take us beyond available experimental evidence, with numerous parameters for which there are no empirical values. Considering this limitation, here we have constructed a simplified model to investigate if a stochastic transcriptional initiation of key denitrification genes (*nirS*) could possibly explain peculiar kinetics of e\textsuperscript{-}/flow as *Paracoccus denitrificans* switch from aerobic to anaerobic respiration [4,8].

Although denitrification is widespread among bacteria, the *ß*-proteobacterium *Pa. denitrificans* is the ‘paradigm’ model organism in denitrification research. Recent studies [4,8,9] have indicated a previously unknown phenomenon in this species that, in response to O\textsubscript{2} depletion, only a marginal fraction of its population appears to switch to denitrification. In these studies, however, F\textsubscript{den} is inferred from rates of consumption and production of gases (O\textsubscript{2}, NO\textsubscript{x}, and N\textsubscript{2}O), and a clear hypothesis as to the underlying cause of the low F\textsubscript{den} is also lacking. To fill these gaps, we formulated a refined hypothesis addressing the underlying regulatory mechanism of the cell differentiation in response to O\textsubscript{2} depletion. On its basis, we constructed a dynamic model and explicitly simulated the actual kinetics of recruitment of the cells from aerobic respiration to denitrification. The model adequately matches batch cultivation data for a range of experimental conditions [4,8] and provides a direct and refined estimation of F\textsubscript{den}. The exercise is important for understanding the physiology of denitrification in general and of *Pa. denitrificans* in particular and carries important implications for correctly interpreting various denitrification experiments.

Regulation of denitrification in terms of relevance to fitness

Generally, the transcription of genes encoding denitrification enzymes is inactivated in the presence of O\textsubscript{2}. A population undertaking denitrification typically responds to full aeration by completely shutting down denitrification and immediately initiating aerobic respiration [10]. Thus, O\textsubscript{2} controls denitrification at transcriptional as well as metabolic level, and both have a plausible fitness value. The transcriptional control minimises the energy cost of producing denitrification enzymes, and the metabolic control maximises ATP (per mole electrons transferred) because the mole ATP per mole electrons transferred to the terminal e\textsuperscript{-}-acceptor is \(|\sim50\%\) higher for aerobic respiration than for denitrification [10].

Denitrification enzymes produced in response to anoxic spell are likely to linger within the cells under subsequent oxic conditions (although, this has not been studied in detail), ready to be used if O\textsubscript{2} should become limiting later on. However, these enzymes will be diluted by aerobic growth, since the transcription of their genes is effectively inactivated by O\textsubscript{2}. Hence, a population growing through many generations under fully oxic conditions will probably be dominated by the cells without intact denitrification proteome. When confronted with O\textsubscript{2} depletion, such a population will have to start from scratch, i.e., transcribe the relevant genes, translate mRNA into peptide chains (protein synthesis by ribosomes) and secure that these chains are correctly folded by the chaperones, transport the enzymes to their correct locations in the cell, and insert necessary co-factors (e.g., Cu, Fe, or Mo). In *E. coli* grown under optimal conditions, the whole process from the transcriptional activation to a functional enzyme takes ≈20 minutes [11] and costs significant amount of energy (ATP).

Synthesis of denitrification enzymes is rewarding if anoxia lasts long and NO\textsubscript{x} remains available, but it is a waste of energy if anoxia is brief. Since the organisms cannot sense how long an impending anoxic spell will last, a ‘bet-hedging strategy’ [12] where one fraction of a population synthesises denitrification enzymes while the other does not may increase overall fitness.

A delayed response to O\textsubscript{2} depletion may lead to entrapment in anoxia

Most, if not all, denitrifying bacteria are non-fermentative and completely rely on respiration to generate energy [13,14]. This implies that their metabolic machinery will run out of energy whenever deprived of terminal e\textsuperscript{-}-acceptors. When [O\textsubscript{2}] falls below some critical threshold, the cells will ‘sense’ this and start synthesising denitrification proteome, utilising energy from aerobic respiration [10]. However, if O\textsubscript{2} is suddenly exhausted or removed, the lack of a terminal e\textsuperscript{-}-acceptor will create energy limitation, restraining the cells from enzyme synthesis, hence, entrapping them in anoxia. This was clearly demonstrated by Højberg et al. [15], who used silicone immobilised cells to transfer them from a completely oxic to a completely anoxic environment. Such a rapid transition is unlikely to occur in nature; however, the experiment illustrates one of the apparent perils in the regulation of denitrification: the cells that respond too late to O\textsubscript{2} depletion will be entrapped in anoxia, unable to utilise alternative electron acceptors for energy conservation and growth.

Højberg et al.’s [15] observations have largely been ignored in the research on the regulation of denitrification, and it is implicitly assumed that, in response to O\textsubscript{2} depletion, all cells in cultures of denitrifying bacteria will switch to denitrification. Contrary to this, however, Bergaust et al. [4,8,16] followed by Nadeem et al. [9] proposed that in batch cultures of *Pa. denitrificans*, only a small fraction of all cells is able to switch to denitrification. During transition from oxic to anoxic conditions, they observed a severe depression in the total e\textsuperscript{-}/flow rate (i.e., to O\textsubscript{2}+NO\textsubscript{x}, see Fig. 1), which was estimated on the basis of measured gas kinetics. Had all of the cells switched to denitrification as O\textsubscript{2} exhausted, the total e\textsuperscript{-}/flow rate would have carried on increasing, without such a depression. The depression was followed by an exponential increase in the e\textsuperscript{-}/flow rate, which was tentatively ascribed to anaerobic growth of a small F\textsubscript{den} (fraction recruited to denitrification). It was postulated that this fraction escaped entrapment in
anoxia by synthesising initial denitrification proteins within the time-window when O2 was still present, whereas the majority of the cells (1 − Fdon) failed to do so, thus remained unable to utilise NOx.

The core hypothesis: A low probability of initiating nirS transcription seems to drive the cell differentiation

Autocatalytic transcription of denitrification genes. In *Pa. denitrificans*, denitrification is driven by four core enzymes: Nar (membrane-bound nitrate reductase), NirS (cytochrome cd1 nitrite reductase), cNor (nitric oxide reductase), and NosZ (nitrous oxide reductase, see Fig. 2). The transcriptional regulation of genes encoding these enzymes (*nar, nirS, nor* and *nosZ*, respectively) involves, at least, three FNR-type proteins acting as sensors for O2 (FnRP), NO\textsuperscript{−}/NO\textsuperscript{2−} (NarR), and NO (NNR) [10,17,18]. NarR and NNR facilitate product-induced transcription of the *nar* and *nirS* genes: When anoxia is imminent, the low [O2] is sensed by FnRP, which in interplay with NarR induces *nar* transcription. NarR is activated by NO\textsuperscript{2−} (and/or probably by NO\textsuperscript{−}); thus once a cell starts producing traces of NO\textsuperscript{−}, *nar* expression becomes autocatalytic. The transcription of *nirS* is induced by NNR, which requires NO for activation; thus once traces of NO are produced, the expression of *nirS* also becomes autocatalytic. In contrast, the transcription of *nor* is substrate (NO) induced via NNR, while *nosZ* is equally but independently induced by NNR and FnRP [19]. Here we are concerned with the dynamics that start with the transcription of *nirS*, since the experimental treatments that we simulated were not supplemented with NO\textsuperscript{2−} but various concentrations of NO\textsuperscript{−} only (Table 1).

Low probability of initiating *nirS* transcription. The transcription of *nirS* is known to be suppressed by O2 [4,8], but the exact mechanism remains unclear. Circumstantial evidence suggests that it is due to O2 inactivating NNR [20] (dashed link in Fig. 2), but this is not necessary to explain the repression of NirS. There are several mechanisms through which high O2 concentrations may restrain NirS activity, i.e., through post-transcriptional regulation, direct interaction with the enzyme, or due to competition for electrons. Regardless of the exact mechanism(s),
the ultimate consequence is the elimination of the positive feedback via NO and NNR. When O₂ falls below a critical threshold, facilitating NirS activity, this positive feedback would allow the product of a single transcription of nirS to induce a subsequent burst of nirS transcription in response to NO. Such 'switches' in gene expression by positive-feedback loops are not uncommon in prokaryotes, and they have been found to result in cell differentiation because the initial transcription is stochastic with a relatively low probability [21].

Our model assumes such stochastic recruitment to denitriication, triggered by an initial nirS transcription occurring with a low probability. This initial transcription is possibly mediated by a minute pool of intact NNR and/or through crosstalk with other probability. This initial transcription is possibly mediated by a minute pool of intact NNR and/or through crosstalk with other factors, such as FurP. A NO₃ -supplemented medium contains non-biologically formed traces of NO which, once diffused into the cells while O₂ is low, will activate background levels of NNR and, thereby, may also increase the probability of triggering nirS transcription.

For this modelling exercise, we do not need a full clarification of the mechanisms involved but only to assume that the probability of an autocatalytic transcriptional activation of nirS would be practically zero as long as O₂ concentration is above a certain threshold. This assumption is backed by empirical data indicating that NO is not produced to detectable levels before O₂ concentration falls below a critical threshold [8,22]. For O₂ concentrations below this threshold, the model assumes a low (but unknown) probability for each cell to initiate the autocatalytic transcription of nirS, paving the way for the rest of the denitrification process.

O₂ is required for the initial production of NirS. We further assume that the recruitment to denitriication will only be possible as long as a minimum of O₂ is available because the synthesis of first molecules of NirS will depend on energy from aerobic respiration.

Can NO produced within one cell help activate the autocatalytic transcription of nirS in the neighbouring cells? It is perhaps less obvious that the autocatalytic transcriptional activation of nirS takes place only within the NO-producing cell because NO diffuses easily across membranes [23]. However, the average distance between the cells in a culture with 10⁶ cells mL⁻¹ (roughly the numbers that we are dealing with) is ~10 µm, which is ~10 times the diameter of a cell. This implies that an NO molecule produced by a cell has a much higher probability to react with and activate the NNR inside the same cell than to do so in another one.

Modelling the cell differentiation

To represent the batch cultivation conducted by Bergaust et al. [4,8], the model explicitly simulates growth of two sub-populations, one with denitrification enzymes (ND₋) and the other without (ND₊); both equally consume O₂, but ND₋ cannot reduce NO to N₂. Once oxygen concentration in the liquid (O₂)LP falls below a critical level (O₂trigger) [22], the cells within ND₋ are assumed to initiate nirS transcription (and thereby ensure recruitment to ND₊) with a rate described by a probabilistic function: ND₋rdenO₂ (O₂) (cells h⁻¹), where rdenO₂(O₂) is assumed to be an (O₂)LP dependent probability (h⁻¹) for any cell within ND₋ to initiate nirS transcription (leading to a full denitrification capacity). When O₂LP falls below O₂trigger, rdenO₂(O₂) triggers and holds a constant value as long as O₂LP is above a critical minimum (O₂min). For O₂LP > O₂trigger, rdenO₂(O₂) is zero (assuming the inactivation of NNR by O₂); rdenO₂(O₂) is also zero for [O₂]LP < [O₂]min (assuming the lack of energy for protein synthesis).

The recruitment of ND₋ to ND₊ is simulated as an instantaneous event; thus, the model does not take into account the time-lag between the initiation of nirS transcription and the time when the transcribing cell has become a fully functional denitrifier. This simplification is based on the evidence that this lag is rather short. Experiments with E. coli [11] under optimal conditions suggest lags of ~20 minutes between the onset of transcription and the emergence of a functional enzyme. In Pa. denitrificans [8,22], the lag observed between the emergence of denitrification gene transcripts and the subsequent gas products suggests that the time required for synthesising the enzymes is within the same range.

Employing the model to understand 'diauxic lags' between the aerobic and anaerobic growth-phases

In a series of experiments with denitrifying bacteria (Pseudomonas denitrificans, Pseudomonas fluorescens, Alcaligenes eutrophus and Paracoccus pantotrophus) [24–26], oxic cultures were sparged with N₂ to remove O₂ and were monitored by measuring optical density (OD₅₅₀). All the strains except Ps. fluorescens went through a conspicuous 'diauxic lag: a period of little or no growth' [26]; the OD remained practically constant during the lag period, lasting 4–30 hours, which was eventually followed by anaerobic growth.

To understand the diauxic lag, Liu et al. [24] used the common assumption that all cells would eventually switch to denitriication. They constructed a simulation model based on the assumption that all the cells contained a minimum of denitrification proteome (even after many generations under oxic conditions). This minimum would allow them to produce more denitrification enzymes when deprived of O₂, albeit very slowly due to energy limitation. The time taken to effectively produce adequate amounts of denitrification enzymes (= the diauxic lag) was taken to be a function of the initial amounts of these enzymes per cell. Although their model may possibly explain short time-lags, it appears unrealistic for lag phases as long as 10–30 hours [25] because to produce such long lags, conceivably, the initial enzyme concentration would be less than one enzyme molecule per cell, which is mathematically possible but biologically meaningless.

| Table 1. The simulated experiment of Bergaust et al [4,8]. |
|-----------------|-----------------|-----------------|
| Batch No.       | O₂HS (t₀) (vol. %)* | NO₂ (t₀) (mM)  |
| 1               | ~0              | 0.2             |
| 2               | ~0              | 1               |
| 3               | ~0              | 2               |
| 4               | 1               | 0.2             |
| 5               | 1               | 1               |
| 6               | 1               | 2               |
| 7               | 7               | 0.2             |
| 8               | 7               | 1               |
| 9               | 7               | 2               |

*Targeted values for initial O₂ in the headspace (where the headspace vol.= 70 mL). The actual initial O₂ measured in the 0, 1, and 7% treatments was 0.012–0.19, 1.2–1.66, 6.6–6.8 vol.% respectively. The O₂ present in the ~0% treatments was due to traces of O₂ left behind despite various cycles of evacuation of the headspace air and subsequent flushing of the vials with helium (He-washing). doi:10.1371/journal.pcbi.1003933.t001
The model presented in this paper provides an alternative explanation for the apparent diauxic lags: a sudden shift from fully oxic to near anoxic conditions (by sparging with N₂) would leave the medium with only traces of O₂, which would be quickly depleted due to aerobic respiration. As a consequence, the available time for initiating the synthesis of denitrification proteome would be marginal, allowing only a tiny fraction (F_{den}) of the cells to switch to denitrification. This marginal fraction would grow exponentially from the very onset of anoxic conditions, but it would remain practically undetectable as measured (OD) for a long time, creating the apparent 4–30 h lag. The length of the lag depends on the fraction of the cells switching to denitrification. To demonstrate this alternative explanation, we adjusted our model to the reported conditions and simulated the experiment of Liu et al [24]. The model produced qualitatively similar ‘diauxic lags’ in the simulated cell density (OD), although the time length of the lag could be anything (depending on assumptions regarding the residual O₂ after sparging, which was not measured).

Materials and Methods

An overview of the modelled experiment: Batch incubations in gas-tight vials

Bergaust et al. [4,8] studied aerobic and anaerobic respiration rates in *Paracoccus denitrificans* (DSM413). The cells were incubated (at 20°C) as stirred batches in 120 mL gastight vials, containing 50 mL Sistrom’s medium [27] (Fig. 3). The medium was supplemented with various concentrations of KNO₃ or KNO₂. Prior to inoculation, air in the headspace was replaced with He to remove O₂ and N₂ (He-washing), followed by the injection of no, 1, or 7 headspace-vol.% O₂. Finally, each vial was inoculated with ~3×10⁸ aerobically grown cells.

**Figure 3. An overview of the modelled system: batch incubation in a gas-tight vial.** The experiment: The stirred Sistrom’s medium [27] was inoculated with aerobically grown *Pa. denitrificans* cells, which were provided with different concentrations of O₂ and NO₃⁻ (g or aq with a chemical species-name represents gaseous or aqueous, respectively). O₂ is consumed by respiration, driving its transport from the headspace to the liquid. Once the aerobic respiration becomes limited, the cells may switch to denitrification (recruitment), reducing NO₃⁻ to N₂ via the intermediates NO and N₂O (not shown). For monitoring O₂, CO₂, NO and N₂O, a robotised incubation system [28] was used, which automatically takes samples from the headspace by piercing the rubber septum. Each sampling removes a fraction (3–3.4%) of all gases in the headspace, but it also involves a marginal leakage of O₂ and N₂ into the vial (as indicated by the two-way arrows at the top of the figure). The model: The model operates with two sub-populations: one without and the other with denitrification enzymes (ND₋ and ND₊, respectively). Both consume O₂ if present, but ND₋ cannot reduce NO₃⁻. The ND₋ cells may be recruited to the ND₊ pool as [O₂]_{aq} falls below a critical threshold. The rate of recruitment (R_{rec}) is modelled as a probabilistic function: R_{rec} = N_{D₋} × r_{den}(O₂) (cells h⁻¹), where r_{den}(O₂) represents an O₂ dependent specific-probability (h⁻¹) for any ND₋ cell to initiate *nirS* transcription (leading to the synthesis of a full-fledged denitrification proteome).

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Treatments selected for simulation. Only NO$\textsubscript{3}^-$-supplemented treatments (Table 1) were selected for this modelling exercise for two reasons. First, NO$\textsubscript{3}^-$ was not monitored; hence, results of the NO$\textsubscript{3}^-$-supplemented treatments could not provide exact estimates of anaerobic respiration rates (due to an unknown transient accumulation of NO$\textsubscript{2}^-$). Second, by excluding the treatments requiring Nar, we could single out and focus on the regulation of the other key enzyme NirS.

Aerobic respiration followed by denitrification. O$_2$ diffused from the headspace to the liquid (Fig. 3), where the cells consumed it before switching to denitrification; the stepwise reduction of NO$_2^-$ to N$_2$ via the intermediates NO and N$_2$O (not shown). Headspace concentrations of gases were monitored by frequent sampling (every 3 hours). A typical result is shown in Fig. 1A, illustrating the increasing rate of O$_2$ consumption until depletion, followed by transition to denitrification. The denitrification rate increased exponentially till all the NO$_2^-$ present in the medium was recovered as N$_2$. The medium contained ample amounts of carbon substrate (34 mM sucinate) to support the consumption of all available electron acceptors.

Sampling procedure. To monitor O$_2$, CO$_2$, NO, N$_2$O, and N$_2$ in the headspace for respiring cultures, Bergaust et al. [4,8] used a robotised incubation system, which automatically takes samples from the headspace by piercing the rubber septum (Fig. 3). The auto-sampler is connected to a gas chromatograph (GC) and an NO analyser (For details, see [28]). The system uses peristaltic pumping, which removes a fraction (3–3.4%) of all the gases in the headspace and then reverses the pumping to inject an equal amount of He into the headspace, thus maintaining ~1 atmosphere pressure inside the vial. Sampling also involves a marginal leakage of O$_2$ and N$_2$ into the headspace (~22 and ~60 nmol per sampling, respectively) through tubing and membranes of the injection system.

Calculation of gases in the liquid. Concentrations of gases in the liquid were calculated using the solubility of each gas at the given temperature (20°C), assuming equilibrium between the headspace and the liquid. However, the O$_2$ consumption rate was so high that to calculate [O$_2$] in the liquid, its transport rate (from the headspace to the liquid) had to be taken into account.

An overview of the model

The model effectively represents the physical phenomena mentioned above, so as to ensure that the simulation results match the measured data for the right reasons. Net effect of sampling (dilution and leakage) is included in the simulation of O$_2$ kinetics at the reported sampling times. Transport of O$_2$ between the headspace and the liquid is modelled using an empirically determined transport coefficient and the solubility of O$_2$ in water at 20°C. To simulate the metabolic activity (O$_2$ consumption and N$_2$ production) and growth, the model divides the cells into two sub-populations: one without and the other with denitrification enzymes (ND$^{-}$ and ND$^{+}$ pools, respectively, see Fig. 3). Both equally consume O$_2$ if present, but ND$^{-}$ cannot reduce NO$_2^-$ to N$_2$. Those ND$^{-}$ cells that, in response to O$_2$ depletion, are able to initiate nirS transcription (see Fig. 2) are recruited to the ND$^{+}$ pool, where ND$^{-}$ = 0 prior to the recruitment. The recruitment rate ($r_{rec}$) is modelled according to a probabilistic function described below (Eqs. 7–8).

The model ignores sampling effect on N$_2$ (leakage and loss), thus calculating the cumulative N$_2$ production as if no sampling took place. That is because the experimentally determined N$_2$ accumulation (which is to be compared with the model predictions) was already corrected for the net sampling effect.

The model is developed in Vensim DSS 6.2 Double Precision (Ventana Systems, Inc. http://vensim.com/) using techniques from the field of system dynamics [29]. The model is divided into three sectors: I. O$_2$ kinetics, II. Population dynamics of ND$^{-}$ and ND$^{+}$, and III. Denitrification kinetics (Fig. 4).

Sector I: O$_2$ kinetics

Structural-basis for the O$_2$ kinetics is mapped in Fig. 4A: the squares represent the state variables, the circles the rate of change of the state variables, and the arrows mutual dependencies between the variables, and the edges represent flows into or out of the state variables. Briefly, Fig. 4A (left to right) shows that O$_2$ in the vial’s headspace ($O_{2HS}$) is transported ($Tr_{O2}$) to the liquid-phase ($O_{2LP}$), where it is consumed ($Cr_{O2}$) by both the ND$^{-}$ and ND$^{+}$ populations (lacking and carrying denitrification enzymes, respectively) in proportion to an identical cell-specific velocity of O$_2$ consumption ($v_{O2}$). $\Delta O_{2/S}$ represents net marginal changes in $O_{2HS}$ due to sampling. Below we present equations and a detailed explanation of the structural components shown for this sector.

O$_2$ in the headspace. ($O_{2HS}$, mol vial$^{-1}$) is initialised by measured initial concentrations (Table 1) and modelled as a function of transport ($Tr_{O2}$) between the headspace and the liquid [28]:

$$Tr_{O2} = k_0 \times \left(\frac{k_{H(O2)} \times P_{O2}}{O_{2LP}}\right)$$

Units: mol vial$^{-1}$ h$^{-1}$

where $k_0$ (L vial$^{-1}$ h$^{-1}$) is the empirically determined coefficient for the transport of O$_2$ between the headspace and the liquid (See Table 2 for parametric values and their sources), $k_{H(O2)}$ (mol L$^{-1}$ atm$^{-1}$) is the solubility of O$_2$ in water at 20°C, $P_{O2}$ (atm) is the partial pressure of O$_2$ in the headspace, and $O_{2LP}$ (mol L$^{-1}$) is the O$_2$ concentration in the liquid-phase ($O_{2LP} = \frac{O_{2LP}}{V_{VolLP}}$).

In addition, changes in O$_{2HS}$ due to sampling are included at the reported sampling times. The robotised incubation system [28] used in the experiment monitors gas concentrations by sampling the headspace, where each sampling alters the concentrations in a predictable manner: a fraction of O$_2$ is removed and replaced by He (dilution), but the sampling also results in a marginal leakage of O$_2$ through the tubing and membranes of the injection system. Eq. 2 shows how the model calculates the net change in O$_{2HS}$ ($\Delta O_{2/S}$) as a result of each sampling:

$$\Delta O_{2/S} = \frac{(O_{2/leak} - O_{2HS} \times D)}{t_s}$$

mol vial$^{-1}$ h$^{-1}$

where $O_{2/leak}$ (mol vial$^{-1}$) is the O$_2$ leakage into the headspace, D (dilution) is the fraction of O$_{2HS}$ replaced by He, and $t_s$ (h) is the time taken to complete each sampling. $\Delta O_{2/S}$ is negative if O$_{2HS}$ is greater than 0.38 µmol vial$^{-1}$ and marginally positive if it is less than that.

O$_2$ in the liquid-phase. ($O_{2LP}$, mol vial$^{-1}$, see Fig. 4A) is initialised by assuming equilibrium with O$_{2HS}$ at the time of inoculation ($O_{2LP}(t_0) = P_{O2} \times k_{H(O2)} \times V_{VolLP}$). O$_{2LP}$ is modelled as a function of its transport into the liquid ($Tr_{O2}$, Eq. 1) and consumption rate ($Cr_{O2}$, mol vial$^{-1}$ h$^{-1}$), where the latter is...
modelled as a function of total cell numbers and the cell-specific velocity of O₂ consumption:

$$\frac{d(O_{2}\text{LP})}{dt} = TrO_2 - CrO_2 = TrO_2 - (N_{D-} + N_{D+}) \times v_{O2}$$ (3)

where $N_{D-}$ and $N_{D+}$ (cells vial⁻¹, see Sector II for details) are the cells without and with denitrification enzymes, respectively, and $v_{O2}$ (mol cell⁻¹ h⁻¹) is the cell-specific velocity of O₂ consumption. Thus, we assume that the $N_{D-}$ and $N_{D+}$ cells have the same potential to consume O₂.

$N_{D-}$ is modelled as a Michaelis-Menten function of O₂ concentration:

$$v_{O2} = \frac{v_{max(O_2)} \times [O_2]_{LP}}{K_m(O_2) + [O_2]_{LP}}$$ (4)

where $v_{max(O_2)}$ (mol cell⁻¹ h⁻¹) is the maximum cell-specific velocity of O₂ consumption (determined under the actual experimental conditions), $[O_2]_{LP}$ (mol L⁻¹) is the O₂ concentration in the liquid-phase, and $K_m(O_2)$ (mol L⁻¹) is the half saturation constant for O₂ reduction.

Sector II: Population dynamics of the cells without ($N_{D-}$) and with ($N_{D+}$) denitrification proteome

Fig. 4B represents the structure governing the population dynamics of the cells without ($N_{D-}$) and with ($N_{D+}$) denitrification enzymes. Briefly, it shows that both the populations are able to grow by aerobic respiration ($Gr_{D-}$ and $Gr_{AE}$, respectively). The growth rate of $N_{D+}$, however, is primarily based on denitrification ($Gr_{DE}$). Initially, $N_{D+} = 0$ and is populated through recruitment ($R_{rec}$) of the cells from $N_{D-}$, where the recruitment is a product of $N_{D-}$ and an $[O_2]$ dependent specific-probability ($r_{den(O_2)}$), for any $N_{D-}$ cell.

C. The panel represents the structural basis for the NO₂⁻/N₂ kinetics. Briefly, it illustrates that $N_{D+}$ control the consumption rate of NO₂⁻ ($Cr_{NO_2}$), recovered as N₂, in proportion to a cell-specific velocity of NO₂⁻ consumption ($v_{NO_2}$).
Table 2. Model parameters.

| Description | Value | Units | Reference |
|-------------|-------|-------|-----------|
| \(D\) | 0.035 | Unitless | [28] |
| \(k_{H(\text{O}_2)}\) | 0.00139 | mol L\(^{-1}\) atm\(^{-1}\) | [37] |
| \(k_t\) | 1.62 | L vial\(^{-1}\) h\(^{-1}\) | [28] |
| \(O_{\text{leak}}\) | 2.04 \times 10^{-8} | mol vial\(^{-1}\) | [28] |
| \(v_s\) | 0.017 | h | [28] |
| \(K_m(\text{O}_2)\) | The half saturation constant for \(\text{O}_2\) consumption | 2.5 \times 10^{-7} | mol L\(^{-1}\) | Model-based estimation |
| \(r_{\text{max}}(\text{O}_2)\) | The maximum cell-specific velocity of \(\text{O}_2\) consumption | 1.33 \times 10^{-15} | mol cell\(^{-1}\) h\(^{-1}\) | [4,8] |
| \(\text{O}_2\) \(_{\text{min}}\) | [\(\text{O}_2\)] in the liquid below which the recruitment to \(\text{N}_{\text{D}_+}\) halts | 1 \times 10^{-9} | mol L\(^{-1}\) | Assumption |
| \(\text{O}_2\) \(_{\text{trigger}}\) | [\(\text{O}_2\)] below which the recruitment to \(\text{N}_{\text{D}_+}\) triggers | 9.75 \times 10^{-6} | mol L\(^{-1}\) | [22] |
| \(r_{\text{den}}\) | The specific-probability of recruitment of a cell to \(\text{N}_{\text{D}_+}\) | 0.0052 | h\(^{-1}\) | Model-based estimation |
| \(Y_{\text{NO}_2^-}\) | The growth yield per molN \(\text{NO}_2^-\) | 5.79 \times 10^{13} | cells molN\(^{-1}\) | [4,8] |
| \(Y_O\) | The growth yield per mol \(\text{O}_2\) | 15 \times 10^{13} | cells mol\(^{-1}\) | [4,8] |
| \(K_m(\text{NO}_2^-)\) | The half saturation constant for \(\text{NO}_2^-\) reduction | 4 \times 10^{-6} | molN L\(^{-1}\) | [33,34] |
| \(r_{\text{max}}(\text{NO}_2^-)\) | The maximum cell-specific velocity of \(\text{NO}_2^-\) reduction | 1.83 \times 10^{-15} | molN cell\(^{-1}\) h\(^{-1}\) | [4,8] |
| \(R\) | Universal gas constant | 0.083 | L atm K\(^{-1}\) mol\(^{-1}\) | – |
| \(T\) | Temperature | 293.1 | K | [4,8] |
| \(\text{Vol}_{\text{HS}}\) | Headspace volume | 0.07 | L vial\(^{-1}\) | [4,8] |
| \(\text{Vol}_{\text{LP}}\) | Liquid-phase volume | 0.05 | L vial\(^{-1}\) | [4,8] |

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The pool of the cells lacking denitrification proteome. The pool of the cells lacking denitrification proteome (\(\text{N}_{\text{D}_-}\)) is initialised with \(3 \times 10^6\) cells vial\(^{-1}\). The population dynamics of \(\text{N}_{\text{D}_-}\) are modelled as:

\[
\frac{d(\text{N}_{\text{D}_-})}{dt} = \text{Gr}_{\text{D}_-} - R_{\text{rec}}
\]

(5)

where \(\text{Gr}_{\text{D}_-}\) (cells vial\(^{-1}\) h\(^{-1}\)) is the (aerobic) growth rate, and \(R_{\text{rec}}\) (cells vial\(^{-1}\) h\(^{-1}\), Eq. 7) is the rate of recruitment of \(\text{N}_{\text{D}_-}\) to the \(\text{N}_{\text{D}_+}\) pool.

\(\text{Gr}_{\text{D}_-}\) is modelled as:

\[
\text{Gr}_{\text{D}_-} = \text{N}_{\text{D}_-} \times v_{O_2} \times Y_O
\]

(6)

where \(v_{O_2}\) (mol cell\(^{-1}\) h\(^{-1}\), Eq. 4) is the cell-specific velocity of \(\text{O}_2\) consumption, and \(Y_O\) (cells mol\(^{-1}\)) is the cell yield per mole of \(\text{O}_2\) (determined under the actual experimental conditions).

The rate of recruitment. The rate of recruitment \(R_{\text{rec}},\) see Fig. 4B) of the cells from \(\text{N}_{\text{D}_-}\) to \(\text{N}_{\text{D}_+}\) is modelled as:

\[
R_{\text{rec}} = \text{N}_{\text{D}_-} \times r_{\text{den}}(\text{O}_2)
\]

(7)

where \(r_{\text{den}}(\text{O}_2)\) (h\(^{-1}\)) represents the conditional specific-probability for any \(\text{N}_{\text{D}_-}\) cell to be recruited to denitrification, modelled as a function of \(\text{O}_2\) concentration in the liquid-phase ([\(\text{O}_2\)]\(_{\text{LP}}\), see Fig. 5):

\[
r_{\text{den}}(\text{O}_2) = \begin{cases} 
0 & \text{for} [\text{O}_2]_{\text{LP}} > [\text{O}_2]_{\text{trigger}} \\
[\text{r}_{\text{den}}]_{\text{LP}} & \text{for} [\text{O}_2]_{\text{min}} < [\text{O}_2]_{\text{LP}} < [\text{O}_2]_{\text{trigger}} \\
0 & \text{for} [\text{O}_2]_{\text{LP}} < [\text{O}_2]_{\text{min}}
\end{cases}
\]

(8)

where \(r_{\text{den}}\) (h\(^{-1}\)) is a constant representing the specific-probability of the recruitment, \([\text{O}_2]_{\text{trigger}}\) is the \(\text{O}_2\) concentration above which the transcription of \(\text{nirS}\) is effectively suppressed by \(\text{O}_2\), and \([\text{O}_2]_{\text{min}}\) is the \(\text{O}_2\) concentration assumed to provide minimum energy for the initial transcription to result in functional \(\text{NirS}\). Once the first molecules of \(\text{NirS}\) are produced while \([\text{O}_2]_{\text{min}} < [\text{O}_2]_{\text{LP}} < [\text{O}_2]_{\text{trigger}},\) the transcription of \(\text{nirS}\) will be greatly enhanced through positive feedback by \(\text{NO}\), paving the way for a full-scale production of denitrification proteome [10] (See Introduction and Fig. 2 for details).

\([\text{O}_2]_{\text{trigger}} = 9.75 \times 10^{-6}\) mol L\(^{-1}\) is the empirically determined \([\text{O}_2]_{\text{LP}}\) at the outset of \(\text{NO}\) accumulation: Bergaust et al. [8] estimated \([\text{O}_2]_{\text{trigger}}\) between 0.1-12 \(\mu\)M, but recent *Pa. denitrificans* batch incubation data have provided a more precise estimate between 8.8-10.7 \(\mu\)M (average = 9.75 \(\mu\)M) [22].
As for $O_2^{1/2}$, we lack empirical basis for determining the parameter value, but sensitivity of the model to this parameter was tested (See Results/Discussion). Our simulations were run with $O_2^{1/2} = 1.6 	imes 10^{-2}$ mol L$^{-2}$, which would sustain an aerobic respiration rate equivalent to 0.4% of the empirically determined $v_{max}(O_2)$ (assuming our estimated $K_{m}(O_2) = 2.5 	imes 10^{-7}$ mol L$^{-1}$, Table 2).

As modelled, the time-window for the recruitment to denitrification depends on the time taken to deplete $O_2^{1/2}$LP from $O_2^{1/2}$ trigger to $O_2^{1/2}$ min (Fig. 5); for obvious reasons, the length of this time-window depends on the cell density.

The lag observed between the emergence of denitrification gene transcripts and the subsequent gas products is as short as 20 minutes [8,22], which is insignificant in the sense that the estimations of $r_{den}$ and $F_{den}$ will not be affected by including it in the model. Therefore, the recruitment (Eq. 7) is modelled as an instantaneous event.

**Calculation of $F_{den}$:** The fraction of the cells recruited to denitrification (Eq. 7):

$$ F_{den} = 1 - e^{-r_{den} \times (t_m - t)} $$

**Dimensionless**

where $r_{den}$ (h$^{-1}$, see Eqs. 7-8 and Fig. 5) is the specific-probability for the recruitment of a cell to denitrification, $t_i$ is the time when $[O_2]$ in the liquid falls below $[O_2]_{trigger}$ (the concentration below which $r_{den}$ triggers), and $t_m$ is the time when $[O_2]$ in the liquid falls below $[O_2]_{min}$ (the concentration below which $r_{den}$ is assumed to be zero). Hence, effectively, $F_{den}$ expresses the probability for any cell to switch to denitrification within the time-frame $t_m - t_i$.

**The pool of the cells carrying denitrification proteome.** The pool of the cells carrying denitrification proteome ($N_{D+}$, see Fig. 4B) is initialised with zero cells, and its population dynamics are modelled as:

$$ \frac{d(N_{D+})}{dt} = R_{rec} + G_{DE} + G_{AE} $$

where $R_{rec}$ (cells vial$^{-1}$ h$^{-1}$, Eq. 7) is the recruitment rate, $G_{DE}$ (cells vial$^{-1}$ h$^{-1}$) the denitrification-based growth and $G_{AE}$ (cells vial$^{-1}$ h$^{-1}$) the aerobic growth rate.
\( \text{Gr}_{DE} \) is modelled as:

\[
\text{Gr}_{DE} = N_D^+ \times \nu_{\text{NO}_2^-} \times Y_{\text{NO}_2^-}
\]

\( \text{cells mol}^{-1} \text{ h}^{-1} \)

where \( \nu_{\text{NO}_2^-} \) (molN cell\(^{-1}\) h\(^{-1}\), see Eq. 15) is the cell-specific velocity of \( \text{NO}_2^- \) reduction, and \( Y_{\text{NO}_2^-} \) (cells molN\(^{-1}\)) is the growth yield per molN of \( \text{NO}_2^- \) as the electron-acceptor (determined under the actual experimental conditions).

The \( N_{D^+} \) cells are assumed to have the same ability as \( N_D \) to grow by aerobic respiration; their aerobic growth rate is formulated as:

\[
\text{Gr}_{AE} = N_D^+ \times \nu_{O_2} \times Y_{O_2}
\]

\( \text{cells mol}^{-1} \text{ h}^{-1} \)

where \( \nu_{O_2} \) (mol cell\(^{-1}\) h\(^{-1}\), see Eq. 4) is the cell-specific velocity of \( O_2 \) consumption, and \( Y_{O_2} \) (cells mol\(^{-1}\)) is the growth yield per mole of \( O_2 \) as the electron-acceptor.

### Sector III: Denitrification kinetics

The structure controlling the denitrification kinetics is mapped in Fig. 4C. Briefly, the figure shows that the cells with a denitrification proteome (\( N_{D^+} \)) control the consumption rate of \( \text{NO}_2^- \) (\( \text{Cr}_{\text{NO}_2^-} \)), recovered as \( N_D \), in proportion to a cell-specific growth rate (\( \text{Gr}_{AE} \)), in proportion to a cell-specific growth rate (\( \text{Gr}_{AE} \)).

The structure controlling the denitrification kinetics is mapped in Fig. 4C. Briefly, the figure shows that the cells with a denitrification proteome (\( N_{D^+} \)) control the consumption rate of \( \text{NO}_2^- \) (\( \text{Cr}_{\text{NO}_2^-} \)), recovered as \( N_D \), in proportion to a cell-specific growth rate (\( \text{Gr}_{AE} \)).

\[
\frac{d(\text{NO}_2^-)}{dt} = -\text{Cr}_{\text{NO}_2^-}
\]

\( \text{molN mol}^{-1} \text{ h}^{-1} \)

where \( \text{Cr}_{\text{NO}_2^-} \) is the consumption rate of \( \text{NO}_2^- \):

\[
\text{Cr}_{\text{NO}_2^-} = N_{D^+} \times \nu_{\text{NO}_2^-}
\]

\( \text{molN cell}^{-1} \text{ h}^{-1} \)

where \( N_{D^+} \) (cells vial\(^{-1}\)) is the number of denitrifying cells, and \( \nu_{\text{NO}_2^-} \) (molN cell\(^{-1}\) h\(^{-1}\)) is the cell-specific velocity of \( \text{NO}_2^- \) reduction, which is modelled as a function of \( \text{NO}_2^- \) using the Michaelis-Menten equation:

\[
\nu_{\text{NO}_2^-} = \frac{v_{\text{max}}(\text{NO}_2^-) \times [\text{NO}_2^-]}{K_m(\text{NO}_2^-) + [\text{NO}_2^-]}
\]

\( \text{molN cell}^{-1} \text{ h}^{-1} \)

where \( v_{\text{max}}(\text{NO}_2^-) \) (molN cell\(^{-1}\) h\(^{-1}\)) is the maximum cell-specific velocity of \( \text{NO}_2^- \) consumption (determined under the actual experimental conditions), \( [\text{NO}_2^-] \) (molN L\(^{-1}\)) is the \( \text{NO}_2^- \) concentration in the liquid-phase, and \( K_m(\text{NO}_2^-) \) (molN L\(^{-1}\)) is the half saturation constant for \( \text{NO}_2^- \) reduction.

See Table 2 for a summary of the parametric values and their sources and Table 3 for the initial values assigned to the state variables.

### Table 3. Initial values for the state variables.

| Sector 1: O2 Kinetics | Symbol | Value | Units | Reference |
|-----------------------|--------|-------|-------|-----------|
| Initial \( O_2 \) in the headspace | \( O_{2HS}(t_0) \) | See Table 5 | mol vial\(^{-1}\) | [4,8] |
| Initial \( O_2 \) in the liquid-phase | \( O_{2LP}(t_0) \) | Equilibrium with \( O_{2HS}(t_0) \) | mol vial\(^{-1}\) | Assumption |

### Sector II: Population dynamics of the cells without \( (N_{D^-}) \) and with \( (N_{D^+}) \) denitrification proteome

| The initial number of cells | \( N_d \) \((t_0)\) | \( 3 \times 10^8 \) | cells vial\(^{-1}\) | [4,8] |
| The initial number of denitrifying cells | \( N_{D^+} \) \((t_0)\) | 0 | cells vial\(^{-1}\) | Assumption |

### Sector III: Denitrification Kinetics

| Initial \( \text{NO}_2^- \) in the liquid-phase | \( \text{NO}_2^- (t_0) \) | See Table 5 | molN vial\(^{-1}\) | [4,8] |
| Initial \( \text{N}_2 \) in the headspace | \( \text{N}_2(t_0) \) | 0 | molN vial\(^{-1}\) | [4,8] |

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estimated \([O_2]_{\text{trigger}}\) between 0.1–12 \(\mu\text{M}\), but recent batch incubation data from \textit{Pa. denitrificans} have provided a more precise estimate in the range 8.8–10.7 \(\mu\text{M}\) (average = 9.75 \(\mu\text{M}\)) [22]. The model, however, is not sensitive to \([O_2]_{\text{trigger}}\) within the latter range because of a high velocity of \(O_2\) depletion. 

\([O_2]_{\text{min}}\) (=1 \times 10^{-3} \text{ mol L}^{-1}) is assigned an arbitrary low value, since we lack any empirical estimation/data to support it. To compensate for the uncertainty, we conducted a sensitivity analysis exploring the consequences of increasing or decreasing \([O_2]_{\text{min}}\) by one order of magnitude (See Results/Discussion).

Results/Discussion

The specific-probability \((r_{\text{den}}, \text{h}^{-1})\) of recruitment of a cell to denitrification

To test the assumption of a single homogeneous population, we forced our model to achieve 100% recruitment to denitrification by setting \(r_{\text{den}} = 1 \text{ h}^{-1}\). In consequence, the simulated \(N_2\) accumulation (molN vial\(^{-1}\)) showed gross overestimation as compared to the measured for all the treatments (as illustrated for some randomly selected ones in Fig. 6).

To find a more adequate value, \(r_{\text{den}}\) was calibrated to produce the best possible match between the simulated and measured \(N_2\) through optimisation. (The optimisation was carried out in Vensim DSS 6.2 Double Precision, http://vensim.com/). Table 4 presents the optimal \(r_{\text{den}}\) for each treatment; no consistent effect of initial \([O_2]\) and \([NO_2]^-\) was found on the optimal results. The average for all the treatments = 0.0052, which appears to give reasonable fit between the simulated and measured \(N_2\) (See Figs. 7, 8, and 9). This indicates that the simulations with \(r_{\text{den}} = 0.0052\) should provide a reasonable approximation of \(F_{\text{den}}\) (the fraction recruited to denitrification) during the actual experiment.

Sensitivity analysis. \([O_2]_{\text{min}}\) (the \(O_2\) concentration below which the recruitment is arrested) was arbitrarily chosen to be 1 \times 10^{-9} \text{ mol L}^{-1}. In order to evaluate the sensitivity of the model to this parameter, we tested the model performance by increasing and decreasing \([O_2]_{\text{min}}\) by one order of magnitude. For each parameter value, we estimated \(r_{\text{den}}\) for the individual vials by optimisation (as outlined in the foregoing paragraph). A good fit was obtained for both the \([O_2]_{\text{min}}\) values, but the optimisation resulted in slightly different \(r_{\text{den}}\) values. Increasing \([O_2]_{\text{min}}\) by a

![Figure 6. Comparison of the measured [4,8] and simulated data assuming \(r_{\text{den}} = 1 \text{ h}^{-1}\). Assuming a single homogeneous population, as we forced our model to achieve 100% recruitment to denitrification by setting the specific-probability of recruitment \((r_{\text{den}})\) to 1 h\(^{-1}\), the simulated \(N_2\) accumulation (molN vial\(^{-1}\)) showed considerable overestimation as compared to that measured. To illustrate this, the simulated and measured data are compared here for some randomly chosen treatments. Initial vol.% \(O_2\) in the headspace and initial \(NO_2^-\) is shown above each panel. doi:10.1371/journal.pcbi.1003933.g006](#)
factor of 10 (to $1 \times 10^{-8} \text{ mol L}^{-1}$) resulted in 18–38% higher $r_{\text{den}}$ estimates (average $= 28\% \pm \text{stdev } 10$). Decreasing $[O_2]_{\text{min}}$ by a factor of 0.1 (to $1 \times 10^{-10} \text{ mol L}^{-1}$) resulted in 5–17% lower $r_{\text{den}}$ estimates (average $= 11\% \pm \text{stdev } 6$).

The fraction recruited to denitrification ($F_{\text{den}}$)

A refined estimation with the presented model. Bergaust et al. [8,16] and Nadeem et al. [9] used data from batch cultivations of Pa. denitrificans, as illustrated in Fig. 1, to assess $F_{\text{den}}$. Their estimation was effectively $F_{\text{den}} = \frac{N_{\text{NDz}}(t)}{N_{\text{tex}}}$, where $t_{\text{ex}}$ is the time when $O_2$ is exhausted, $N_{\text{NDz}}$ (cells vial$^{-1}$) is the number of actively denitrifying cells estimated by the measured rate of denitrification (molN h$^{-1}$) divided by the cell-specific denitrification (molN cell$^{-1}$ h$^{-1}$), and $N$ is the total number of cells estimated on the basis of $O_2$ consumption. Although this equation indisputably estimates the fraction of the cells that was actively denitrifying at the time $t_{\text{ex}}$, it is a biased estimate of the ‘true’ $F_{\text{den}}$ because the number of cells does not remain constant through the recruitment phase: $N_{\text{ND}}$ (the cells without denitrification enzymes) and $N_{\text{NDz}}$ will both grow until $O_2$ is depleted, but $N_{\text{NDz}}$ will grow faster because their growth is supported by both $O_2$ and NOx. As a result, the estimation of $F_{\text{den}}$ by this equation might be too high.

### Table 4. Specific-probability of recruitment of a cell to denitrification ($r_{\text{den}}$) estimated for each batch culture by optimisation (best match between the simulated and measured N$_2$ kinetics).

| Batch No. | Treatment*: $O_{2\text{HS}}(t_0)$ (vol.%) | NO$_2^{-}(t_0)$ (mM) | Optimal $r_{\text{tex}}$ (h$^{-1}$) |
|-----------|----------------------------------|----------------|-----------------|
| 1         | $\sim 0, 0.2$                   |                 | 0.0066          |
| 2         | $\sim 0, 1$                     |                 | 0.0059          |
| 3         | $\sim 0, 2$                     |                 | 0.0029          |
| 4         | 1, 0.2                          |                 | 0.0033          |
| 5         | 1, 1                            |                 | 0.0062          |
| 6         | 1, 2                            |                 | 0.0020          |
| 7         | 7, 0.2                          |                 | 0.0018          |
| 8         | 7, 1                            |                 | 0.0117          |
| 9         | 7, 2                            |                 | 0.0066          |
| Avg.      |                                 |                 | $0.0052$        |

*Treatment refers to the initial concentration of $O_2$ in the headspace (measured as headspace vol.%) and the initial concentration of NO$_2^{-}$ in the medium (mM).
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### Figure 7. Simulations of the treatments with $\sim 0$ vol.% $O_{2\text{HS}}$ using $r_{\text{tex}} = 0.0052$ h$^{-1}$.

The figure compares the measured and simulated $O_2$ depletion (mol vial$^{-1}$) and N$_2$ accumulation (molN vial$^{-1}$) for the $\sim 0$ vol.% $O_2$ treatments of Bergaust et al. [4,8], i.e., the vials with near-zero $O_2$ in the headspace ($O_{2\text{HS}}$) at the time of inoculation. Separate plots are shown for each initial concentration of NO$_2^{-}$ (0.2, 1, and 2 mM). The measured initial $O_2$ was somewhat erratic due to episodes of needle clogging and/or high $O_2$ leakage during sampling, so the initial $O_{2\text{HS}}$ used in the simulations is chosen somewhat ad lib so that the simulated $O_2$ depletion coincides with that measured. The discrepancy compared to the measured $O_2$ seems to be significant for 2 mM NO$_2^{-}$ treatment. That is most likely due to the inhibitory effect of nitrite on aerobic respiration, which is not taken into account; all simulations are run with an identical $K_{m}(O_2)$. Near exhaustion, the simulated $O_2$ increases slightly at each sampling time; that is due to the leakage of $O_2$ via the injection system exceeding dilution of the headspace (with He) during each sampling.
doi:10.1371/journal.pcbi.1003933.g007
Besides, the experimental estimation is prone to error because of infrequent sampling, since the sampling time does not necessarily coincide with $t_{\text{mix}}$.

In contrast, our model directly and more precisely calculates $F_{\text{den}}$ (Eq. 9) by a) explicitly simulating the actual kinetics of the recruitment of the cells to denitrification (in contrast to estimating total and denitrifying cell numbers from gas kinetics) and b) avoiding aerobic and anaerobic growth of the cells. Table 5 shows the model’s estimations of $F_{\text{den}}$ and the time-span of the recruitment ($t_m - t_l$) along with the $F_{\text{den}}$ estimations of Bergaust et al. [8,16].

**In the $0\% O_2$ treatments, $F_{\text{den}}$ is supported by the sampling leaks of $O_2$.** Due to low cell density in the $0\% O_2$ treatments (initial $O_2 = 1.5–2 \mu$mol), the $O_2$ leakage into the vial during sampling (every 3 hours) caused oxygen concentrations to exceed $[O_2]_{\text{min}}$ for 0.1–2.4 hours. This resulted in various spikes of recruitment after the initial $O_2$ was depleted. The recruitment through these spikes amounted to, on average, $\sim 19\%$ of $F_{\text{den}}$ in the $0\% O_2$ treatments.

$F_{\text{den}} < 100\%$. The model’s estimations of $F_{\text{den}}$ (Table 5) corroborate the suggestion of Bergaust et al. [8,16] and Nadeem et al. [9] that in batch cultures of *P. denitrificans* $F_{\text{den}}$ remains far below 100%. According to Bergaust et al. [8,16], $F_{\text{den}}$ was 2–21% (average = 10%), whereas the model estimated it between 3.8–16.1% (average = 8.2%).

**$F_{\text{den}}$ is inversely related to cell density.** Bergaust et al. [16] argued that as the velocity of $O_2$ depletion is proportional to cell density, the time-frame available for the cells to produce (necessary initial) denitrification proteome would be inversely related to the cell density at the time of $O_2$ depletion. Simulation results (Table 5) support this: high initial $O_2$ concentrations resulted in high cell densities at the time of $O_2$ depletion, shortening the time-span for the recruitment to denitrification, hence resulting in the low $F_{\text{den}}$.

**Underlying cause of the low $F_{\text{den}}$.** $F_{\text{den}}$ remains low because of a) the limited time-window available to the cells for the recruitment and b) the low $r_{\text{den}}$ (specific-probability of the recruitment), presumably due to a low probability of initiating *nirS* transcription (subsequently reinforced through positive feedback by NO).

**Simulation of the ‘diauxic lag’**

To investigate whether the recruitment of a small fraction of the cells to denitrification could explain the ‘diauxic lag’ observed by Liu et al. [24], we used our model to simulate the conditions they reported for their experiment. In short, Liu et al. [24] incubated *P. denitrificans* (ATCC 13867) in oxic batch cultures, which were sparged with $N_2$ as the cultures had reached different cell densities ($OD_{550} = 0.05–0.17$). The sparging resulted in apparent diauxic lags, i.e., periods with little or no detectable growth. The length of...
Figure 9. Simulations of the treatments with 7 vol.% O\textsubscript{2}HS using $r_{\text{den}} = 0.0052 \text{ h}^{-1}$. The figure compares the measured and simulated O\textsubscript{2} depletion (mol vial$^{-1}$) and N\textsubscript{2} production (molN vial$^{-1}$) for the treatments with 7 vol.% O\textsubscript{2} in the headspace (O\textsubscript{2}HS) at the time of inoculation; separate plots are shown for each initial concentration of nitrite (0.2, 1, and 2 mM). At each sampling time, the simulated O\textsubscript{2} is visibly reduced because of sampling, which results in 3.4% dilution of the headspace (with He).

Table 5. The model’s and Bergaust et al.’s [16] estimations of the fraction recruited to denitrification ($F_{\text{den}}$).

| Batch No. | O\textsubscript{2}HS ($t_0$) (vol.%) NO\textsubscript{2} ($t_0$) (mM) | O\textsubscript{2}HS ($t_0$) (mol) | Model-based Estimations | Estimations of [16] |
|-----------|-----------------|-----------------|-----------------|-----------------|
|           |                 |                 | $t_\text{on} - t_\text{off}$ | $F_{\text{den}}$ | $F_{\text{den}}$ |
| 1         | 0, 0.2          | 2               | 25.8            | 0.141           | 0.19           |
| 2         | 0, 1            | 1.5             | 29.2            | 0.161           | 0.21           |
| 3         | 0, 2            | 1.7             | 27.2            | 0.156           | 0.19           |
| 4         | 1, 0.2          | 50.1            | 10.1            | 0.052           | 0.03           |
| 5         | 1, 1            | 37.8            | 11.1            | 0.056           | 0.07           |
| 6         | 1, 2            | 38.4            | 11.3            | 0.057           | 0.04           |
| 7         | 7, 0.2          | 199             | 7.4             | 0.038           | 0.02           |
| 8         | 7, 1            | 200             | 7.4             | 0.038           | 0.07           |
| 9         | 7, 2            | 200             | 7.4             | 0.038           | 0.08           |

*Refers to the initial values of O\textsubscript{2} in the headspace (O\textsubscript{2}HS) used in the simulations. The values show some inconsistency for the treatments corresponding to the same vol.% because of traces of O\textsubscript{2} left behind after He-washing.

**$t_\text{on}$ is the time when [O\textsubscript{2}] in the liquid falls below $[O\textsubscript{2}]_{\text{trigger}} (= 9.75 \text{ mM} [22], the concentration below which recruitment of the cells to denitrification is assumed to trigger), and $t_\text{off}$ is the time when [O\textsubscript{2}] in the liquid falls below $[O\textsubscript{2}]_{\text{min}} (= 1 \text{ mM}, a practically zero concentration below which the recruitment is assumed to terminate). Due to low cell density in the 0% O\textsubscript{2} treatments, the O\textsubscript{2} leakage into the vial during sampling (every 3 hours) caused oxygen concentration to exceed $[O\textsubscript{2}]_{\text{min}}$ for 0.1–2.4 hours. This resulted in various recruitment spikes after the initial O\textsubscript{2} was depleted. If such recruitment is omitted, $F_{\text{den}} = 0.126, 0.142,$ and 0.133 for the treatments 1, 2, and 3, respectively.

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such lags increased with the cell density present at the time of sparging.

Structural amendments and parameterisation of the model. To tentatively simulate their experiment, two changes were made in the O$_2$ kinetics sector (Fig. 4A). Firstly, the net sampling loss of O$_{2\text{HS}}$ (ΔO$_{2\text{HS}}$) was omitted, since it was specifically set up for the robotised incubation system [28] used by Bergaust et al [4,8]. Secondly, a sparging event was introduced, which immediately takes O$_{2\text{HS}}$ down to very low levels ($=1 \times 10^{-3}$ mol vial$^{-1}$). Since we lack information about the exact concentration of O$_2$ immediately after the sparging, the present exercise is only qualitative.

Liu et al. [24] inoculated the culture to have an initial OD$_{550}$ = 0.07, which would correspond to $\sim 6.5 \times 10^9$ cells vial$^{-1}$ [4,8]. We used this number to initialise the N$_{D_0}$ pool (shown in Fig. 4B). They used NO$_3^-$ ($= 157$ μmolN vial$^{-1}$) instead of NO$_2^-$, so we replaced the NO$_2^-$ pool (Fig 4C) by the NO$_3^-$ pool, initialised it accordingly, and adjusted Eqs. 11 and 15: In Eq. 11, $v_{\text{max}}$ was replaced with the maximum cell-specific velocity of NO$_3^-$ consumption ($v_{\text{max}(\text{NO}_3^-)} = 2 \times 10^{-15}$ molN cell$^{-1}$ h$^{-1}$), calculated using the maximum specific NO$_3^-$ based growth rate ($= 0.322$ h$^{-1}$) reported for their experiment. Finally, in Eq. 15, $v_{\text{max}(\text{O}_2)}$ was calibrated ($= 2.28 \times 10^{-13}$ mol cell$^{-1}$ h$^{-1}$) with the reported maximum specific aerobic growth rate ($= 0.342$ h$^{-1}$).

The ‘diauxic lag’ is plausibly the initial growth phase of a minute F$_{\text{den}}$ (fraction recruited to denitrification). As the experiment of Liu et al. [24] was simulated with the model’s estimated $t_{\text{den}} = 0.0052$ h$^{-1}$ (specific-probability of recruitment), F$_{\text{den}}$ turned out to be 1.1% for the treatment sparged at h = 1.1 and 0.2% for the one sparged at h = 2.55. Simulations of the total cell density (N$_{D_0}$ + N$_{D_+}$) for these cases (Fig. 10A) showed long apparent lags comparable to 10–30 h lag phases observed in their later experiments [25]. However, lags in the range that Liu et al. [24] observed ($= 3$ and 6 h for sparging at h = 1.1 and 2.55, respectively) could be achieved by our model by assuming higher residual O$_2$ concentrations after sparging (resulting in a higher F$_{\text{den}}$). Fig. 10B isolates the OD of N$_{D_+}$ for the simulated treatments and shows them on a logarithmic scale so that their exponential growth, right from the onset of anoxic conditions, becomes apparent. The figure initially shows a quick recruitment of the cells from the N$_{D_-}$ to the N$_{D_+}$ pool, followed by the exponential growth-phase of N$_{D_+}$.

This exercise serves to illustrate that the ‘diauxic lags’ observed [24–26] may simply be a result of low recruitment to denitrification in response to sudden removal of O$_2$. This is possibly a more plausible explanation than suggested by the authors and further elaborated by Hamilton et al. [35], claiming that there is a true lag caused by extremely slow production of denitrification enzymes due to energy limitation. Our explanation of the apparent diauxic lag is corroborated by a chemostat culturing experiment conducted by Bauman et al. [36]: A steady state carbon (acetate) limited continuous culture with Pa. denitrificans was made anaoxic and monitored for denitrification gene transcription, N-gas production, and acetate concentrations. A transient (3–10 h) peak of acetate accumulation after O$_2$ depletion suggested an apparent diauxic lag in the metabolic activity, but denitrification started immediately and increased gradually throughout the entire ‘lag’ period. They further observed that the number of denitrification gene transcripts peaked sharply during the first 1–2 hours. These observations are in good agreement with our model.

The aforesaid observation of Liu et al. [24] that the length of the apparent lags increased with the aeration period (or the cell density at the time of sparging) is also in agreement with our model demonstrating that the time available for the cells to switch to denitrification is inversely related to the cell density at the time of O$_2$ depletion.

Model-based hypothesis: Initial O$_2$ determines the timespan to denitrify all NO$_3^-$ to N$_2$ in a batch

Two sensitivity analyses were run to investigate the system’s response to initial O$_2$ in the headspace, O$_{2\text{HS}}$(t$_0$); one corresponding...
to a range of initial $[O_2]$ in the liquid-phase ($[O_2]_{LP}(t_0)$) below $[O_2]_{trigger}$ (see Eqs. 7-8) and the other for a range much higher than $[O_2]_{trigger}$. All other model parameters and initial values remained as listed in Tables 2 and 3, respectively. The exercise helps illustrate the relative importance of aerobic growth versus the recruitment ($F_{den}$) in determining the time taken to deplete the NO$_2^-$ pool.

**Sensitivity analysis (1).** Sensitivity analysis (1) was run with three $[O_2]_{LP}(t_0)$ within a very low range, starting from a concentration marginally below $[O_2]_{trigger}$:

1) $O_{2HS}(t_0) = 2.02 \times 10^{-5}$ mol vial$^{-1}$ ($[O_2]_{LP} = 9.75$ μM),
2) $O_{2HS}(t_0) = 1.01 \times 10^{-5}$ mol vial$^{-1}$ ($[O_2]_{LP} = 4.88$ μM),
3) $O_{2HS}(t_0) = 5.04 \times 10^{-6}$ mol vial$^{-1}$ ($[O_2]_{LP} = 2.44$ μM)

This is rather a simple case demonstrating that increasing $[O_2]_{LP}(t_0)$ within this low range (Fig. 11A) will result in increasing rates of denitrification (Fig. 11D) by increasing the number of aerobically grown cells ($N_D$, Fig. 11B) and, thus, the rate of recruitment ($R_{rec}$, Fig. 11C).

**Sensitivity analysis (2).** Sensitivity analysis (2) was run with three initial $O_2$ concentrations much higher than $[O_2]_{trigger}$:

1) $O_{2HS}(t_0) = 2 \times 10^{-4}$ mol vial$^{-1}$ ($[O_2]_{LP} = 93$ μM),
2) $O_{2HS}(t_0) = 1.19 \times 10^{-4}$ mol vial$^{-1}$ ($[O_2]_{LP} = 55$ μM),
3) $O_{2HS}(t_0) = 3.84 \times 10^{-5}$ mol vial$^{-1}$ ($[O_2]_{LP} = 18$ μM)

In this case, the cumulated $N_2$ reached stable plateaus at nearly the same time for all the runs (Fig. 12E), despite that the time taken to deplete $O_2$ below $[O_2]_{trigger}$ decreased with increasing

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**Figure 11. Sensitivity analysis (1): Varying initial $O_2$ in the headspace ($O_{2HS}(t_0)$) within a low range.** The figure shows the impact of varying $O_{2HS}(t_0)$ within a low range on: A. $O_2$ concentration in the liquid-phase ($[O_2]_{LP}$), B. The number of aerobically growing cells ($N_D$), which do not possess denitrification enzymes, C. The rate of recruitment of $N_D$ to denitrification ($R_{rec}$), and D. $N_2$ accumulation. Marked in Panel A, $[O_2]_{trigger}$ is the $[O_2]_{LP}$ below which $R_{rec}$ triggers, and $[O_2]_{min}$ is the $[O_2]_{LP}$ below which $R_{rec}$ terminates. In Panel C, the spikes of recruitment (following the initial recruitment) are due to spikes of $O_2$ by sampling, causing $[O_2]_{LP}$ to transiently exceed $[O_2]_{min}$. The model predicts that reducing $[O_2]_{LP}(t_0)$ within a low range (Panel A) will lower the number of aerobically grown cells (Panel B) and, thereby, the recruitment rate (Panel C), thus increasing the time taken to deplete NO$_2^-$ (slower $N_2$ accumulation, Panel D).

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Figure 12. Sensitivity analysis (2): Varying initial O₂ in the headspace (O₂HS(t₀)) within a high range. The figure shows the impact of varying O₂HS(t₀) within a range much higher than O₂trigger (the [O₂] below which recruitment of the cells to denitrification is assumed to trigger) on:

A. O₂ concentration in the liquid-phase ([O₂]LP)
B. The number of aerobically growing cells (ND), which do not possess denitrification enzymes,
C. The rate of recruitment of ND to denitrification (Rrec),
D. The number of cells as a result of the recruitment alone (NDrec)، i.e., the denitrifying cells (ND) but without aerobic and NOx-based growth, and
E. Cumulated N₂. The cumulated N₂ reached stable plateaus at nearly the same time for all the runs (Panel E), despite the fact that the time taken to deplete O₂ below O₂trigger decreased with increasing O₂HS(t₀) (Panel A). Thus, once denitrification was initiated, the rates increased with increasing initial [O₂]LP due to an increasing population of oxygen-grown cells (Panels B–D). The fraction of the cells recruited to denitrification (Fden) declined with increasing initial O₂ concentration (not shown), but this was not sufficient to compensate for the increasing number of oxygen-raised cells.

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During the transition of the cells from aerobic to anaerobic respiration, the fraction of cells that switch to denitrification (\(F_{\text{den}}\)) increases with decreasing oxygen availability. The model suggests that for a cell to switch to denitrification, the oxygen concentration must be sufficiently low to overcome the energy cost of transcription and translation of the denitrification genes. This behavior is reflected in the model's parameters, such as the energy \(E_{\text{trans}}\) and \(E_{\text{tra}}\), which determine the cost of transcription and translation, respectively.

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**Author Contributions**

Conceived and designed the experiments: LLB LRB. Performed the experiments: LLB. Analyzed the data: JH LRB. Contributed reagents/materials/analysis tools: IDW. Wrote the paper: JH LLB LRB. Constructed the model: JH IDW LRB. Analyzed the simulation results: JH LRB. Edited and revised the text: JH LLB IDW LRB.
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