RESEARCH ARTICLE

Transient Accumulation of NO$_2^-$ and N$_2$O during Denitrification Explained by Assuming Cell Diversification by Stochastic Transcription of Denitrification Genes

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Abstract

Denitrifying bacteria accumulate NO$_2^-$, NO, and N$_2$O, the amounts depending on transcriptional regulation of core denitrification genes in response to O$_2$-limiting conditions. The genes include $nar$, $nir$, $nor$ and $nosZ$, encoding NO$_2^-$-, NO$_2^-$-, NO- and N$_2$O reductase, respectively. We previously constructed a dynamic model to simulate growth and respiration in batch cultures of Paracoccus denitrificans. The observed denitrification kinetics were adequately simulated by assuming a stochastic initiation of $nir$-transcription in each cell with an extremely low probability (0.5% h$^{-1}$), leading to product- and substrate-induced transcription of $nir$ and $nor$, respectively, via NO. Thus, the model predicted cell diversification: after O$_2$ depletion, only a small fraction was able to grow by reducing NO$_2^-$. Here we have extended the model to simulate batch cultivation with NO$_3^-$, i.e., NO$_2^-$, NO, N$_2$O, and N$_2$ kinetics, measured in a novel experiment including frequent measurements of NO$_3^-$. Pa. denitrificans reduced practically all NO$_3^-$ to NO$_2^-$ before initiating gas production. The NO$_2^-$ production is adequately simulated by assuming stochastic $nar$-transcription, as that for $nir$, but with a higher probability (0.035 h$^{-1}$) and initiating at a higher O$_2$ concentration. Our model assumes that all cells express $nosZ$, thus predicting that a majority of cells have only N$_2$O-reductase (A), while a minority (B) has NO$_2^-$-, NO- and N$_2$O-reductase. Population B has a higher cell-specific respiration rate than A because the latter can only use N$_2$O produced by B. Thus, the ratio $\frac{B}{A}$ is low immediately after O$_2$ depletion, but increases throughout the anoxic phase because B grows faster than A. As a result, the model predicts initially low but gradually increasing N$_2$O concentration throughout the anoxic phase, as observed. The modelled cell diversification neatly explains the observed denitrification kinetics and transient intermediate accumulations. The result has major implications for understanding the relationship between genotype and phenotype in denitrification research.
Author Summary

Denitrifiers generally respire O₂, but if O₂ becomes limiting, they may switch to anaerobic respiration (denitrification) by producing NO⁻³, NO⁻², NO⁻ and/or N₂O reductase, encoded by nar, nir, nor, and nosZ genes, respectively. Denitrification causes transient accumulation of NO⁻² and NO/N₂O emissions, depending on the activity of the four reductases. Denitrifiers lacking nosZ produce ~100% N₂O, whereas organisms with only nosZ are net consumers of N₂O. Full-fledged denitrifiers are equipped with all four reductases, genetic regulation of which determines NO⁻² accumulation and NO/N₂O emissions.

*Paracoccus denitrificans* is a full-fledged denitrifying bacterium, and here we present a modelling approach to understand its gene regulation. We found that the observed transient accumulation of NO⁻² and N₂O can be neatly explained by assuming cell diversification: all cells expressing nosZ, while a minority expressing nar and nir+nor. Thus, the model predicts that in a batch culture of this organism, only a minor sub-population is full-fledged denitrifier. The cell diversification is a plausible outcome of stochastic initiation of nar- and nir transcription, which then becomes autocatalytic by NO⁻² and NO, respectively. The findings are important for understanding the regulation of denitrification in bacteria: product-induced transcription of denitrification genes is common, and we surmise that diversification in response to anoxia is widespread.

Introduction

The dissimilative reduction of nitrate (NO⁻³) to nitrite (NO⁻²), nitric oxide (NO), nitrous oxide (N₂O), and finally to N₂ (denitrification) is an indispensable process in the nitrogen cycle, returning N to the atmosphere as N₂. However, denitrification significantly leaks the gaseous intermediates NO and N₂O, both with serious consequences for the environment. N₂O catalyses depletion of the stratospheric ozone [1] and causes global warming, contributing ~10% to the anthropogenic climate forcing [2]. Data suggests that since the 1950s, the atmospheric N₂O has been increasing, and before being photolysed in the stratosphere, the gas persists for an average ~120 years in the troposphere [3]. ~70% of global N₂O emissions are tentatively attributed to microbial nitrification and denitrification in soils [4], where denitrification, generally, is considered a more dominant source [5].

To mitigate N₂O emissions, we need to understand the physiology of denitrifiers

To devise robust strategies for mitigating global N₂O emissions, a good understanding of its primary source is imperative, i.e., genetics, physiology, and regulatory biology of denitrifiers. Any knowledge of the environmental controllers of N₂O is incomplete without understanding the causal relationships of such controllers at the physiological level [6].

The biogeochemical models developed for understanding the ecosystem controls of denitrification and N₂O emissions treat the denitrifying community of soils and sediments as a single homogenous unit with certain characteristic responses to O₂ and NO⁻³ concentrations [6,7]. Natural denitrifying communities, however, are mixtures of organisms with widely different denitrification regulatory phenotypes [8]. The regulatory response of such mixtures is not necessarily equal to the ‘sum of its components’ because there will be interactions, not the least, via the intermediates NO and NO⁻³. Hence, it is probably a mission impossible to predict the regulatory responses of complex communities based on their phenotypic composition.
Nevertheless, investigations of the regulation in model organisms like *Pa. denitrificans* provide us with essential concepts, enhancing our ability to understand the regulatory responses of mixed communities and to generate meaningful hypotheses. Thus, future biogeochemical models of N2O and NO emissions are expected to have more explicit simulations of the regulatory networks involved, and a first attempt has recently been published [9].

**Simulating the cell diversification in response to impending anoxia to analyse its implications for NO2, N2, and N2O kinetics**

Dynamic modelling has been used to a limited extent to analyse various denitrification phenotypes; for example, to analyse NO3− and NO2− reduction and gas-kinetic data for individual strains [10] and mixtures of selected phenotypes [11]; to model the consequence of competition for electrons between denitrification reductases [12,13]; to investigate the control of O2 on denitrification enzymes and inhibition of cytochrome c oxidase by NO in Agrobacterium tumefaciens [14]; and to examine the effect of copper availability on N2O reduction in *Paracoccus denitrificans* [15]. In our previous model [16], we simulated O2 and N2 kinetics from batch incubations of *Pa. denitrificans* [8,17] to test if a postulated cell diversification, driven by stochastic initiation of *nirS*, could explain the N2 production kinetics in NO2-supplemented media. The available data also contained NO3−-supplemented treatments but NO3− and NO2− were not monitored, and the experiment provided no information about the N2O kinetics, except that the concentrations were extremely low (below the detection limit of the thermal conductivity detector used). Recently, a neat dataset was generated from batch incubations supplemented with NO3−, with frequent measurements of NO2− and a more sensitive detection of N2O by an electron capture detector [18]. That encouraged us to extend our previous model and simulate the cell diversification during transition from oxic to anoxic conditions, targeting the regulation of Nar and cNor/NosZ (N2O emissions) in *Pa. denitrificans*.

**Regulatory network of denitrification in *Paracoccus denitrificans***

*Pa. denitrificans* is a facultative anaerobe capable of reducing NO3− all the way to N2:

\[
\text{NO}_3^− \xrightarrow{\text{Nar}} \text{NO}_2^− \xrightarrow{\text{NirS}} \text{NO} \xrightarrow{\text{cNor}} \text{N}_2 \xrightarrow{\text{NosZ}} \text{N}_2O
\]

In response to impending anoxic conditions, the organism sustains respiratory metabolism by producing the membrane-bound cytoplasmic nitrate reductase (Nar), cytochrome cd1 nitrite reductase (NirS), cytochrome c dependent nitric oxide reductase (cNor), and nitrous oxide reductase (NosZ). Transcription of the genes encoding these reductases (*narG*, *nirS*, *norBC*, and *nosZ*, respectively) are regulated by the FNR-type proteins FnrP, NarR, and NNR. FnrP contains a 4Fe-4S cluster for sensing O2, and NNR harbours a NO-sensing haem; NarR, however, is poorly characterised and is most likely a NO2-sensor [19–21]. All these sensors remain inactive during aerobic growth conditions [19].

**Transcription of denitrification genes in *Pa. denitrificans***. FnrP and NarR facilitate a product-induced transcription of the *nar* genes, and NNR facilitates a product-induced transcription of the *nirS* genes (Fig 1, see P1 and P2): Low oxygen concentration ([O2]) activates the self-regulating FnrP, which induces *nar* transcription in coaction with NarR. The self-regulating NarR was previously assumed to be activated by either NO3− or NO2− [21], but a recent proteomics study indicates that NO3− is the activator [19]. Thus once a cell starts producing traces of NO3−, nar expression becomes autocatalytic. Transcription of *nirS* is induced by NNR, which is apparently inactivated by O2 [22,23], but under anoxic/micro-oxic conditions, NNR is activated by NO. Thus, once traces of NO are produced, the expression of *nirS* also becomes
autocatalytic [19,20]. In contrast, nor transcription is substrate (NO) induced via NNR while nosZ is equally induced by NNR or FnrP [24]. High concentrations of NO may inactivate both FnrP [25] and NosZ [26]. These observations, however, are ignored for our modelling because *Pa. denitrificans* restricts NO to nanomolar levels.

**Entrapment of cells in anoxia: The underlying hypothesis and modelling**

Denitration proteome, once produced in response to an anoxic spell, is likely to linger within the cells under subsequent oxic conditions, ready to be used if anoxia recurs. But the proteome will be diluted by aerobic growth because the transcription of denitrification genes is inactivated under oxic conditions [20]. Hence, a population growing through many generations under fully oxic conditions is expected to undertake de novo synthesis of denitrification enzymes when confronted with anoxia. Batch cultivations of such aerobically raised *Pa. denitrificans* provided indirect evidence for a novel claim that, in response to anoxia, only a small fraction of the incubated population is able to produce denitrification proteome [8,17,27,28]. Our dynamic modelling of
Bergaust et al.'s [17] NO\textsubscript{3}\textsuperscript{-}supplemented incubations corroborated this, suggesting that a probabilistic function (specific probability = 0.005 h\textsuperscript{-1}) resulting in the recruitment of 3.8–16.1% of all cells to denitrification is adequate to explain the measured N\textsubscript{2} kinetics [16].

Our model was based on the hypothesis that the entrapment of a large fraction in anoxia is due to a low probability of initiating nir\textsubscript{S} transcription, which in response to O\textsubscript{2} depletion is possibly mediated through a minute pool of intact NNR, crosstalk with other factors (such as FnrP), unspecific reduction of NO\textsubscript{2} to NO by Nar, and/or through non-biologically formed traces of NO found in a NO\textsubscript{3}\textsuperscript{-}-supplemented medium. Regardless of the exact mechanism(s), once nir\textsubscript{S} transcription is initiated, the positive feedback via NO/NNR (Fig 1, see P\textsubscript{2}) would allow the product of a single transcript of nir\textsubscript{S} to induce a subsequent burst of nir\textsubscript{S} transcription. The activated positive feedback will also help induce nor and nos\textsubscript{Z} transcription via NNR, rapidly transforming a cell into a full-fledged denitrifier. We further hypothesised that recruitment to denitrification will only be possible as long as a minimum of O\textsubscript{2} is available because, since Pa. denitrificans is non-fermentative, the synthesis of first molecules of NirS will depend on energy (ATP) for enzyme synthesis.

The above hypothesis was modelled by segregating the culture into two pools (subpopulations): one for the cells without (ND\textsuperscript{−}) and the other with denitrification enzymes (ND\textsuperscript{+}). Initially, all cells were ND\textsuperscript{−}, growing by consuming O\textsubscript{2}. As [O\textsubscript{2}] fell below a certain threshold, ND\textsuperscript{−} recruited to ND\textsuperscript{+} with a constant probability (h\textsuperscript{-1}), assumed to be that of the nir\textsubscript{S} transcriptional activation, and the recruitment halted as O\textsubscript{2} was completely exhausted, assuming lack of energy (ATP) for enzyme synthesis.

Underlying assumptions and aims of the present modelling
The present model is an extension of that developed in Hassan et al. [16]. Here we have divided the respiring culture into four pools (Fig 2A):

1. Z\textsuperscript{−}: cells without Nar, NirS, and cNor
2. Z\textsuperscript{Na}: cells with Nar
3. Z\textsuperscript{NaNi}: cells with Nar, NirS, and cNor
4. Z\textsuperscript{Ni}: cells with NirS and cNor

All these subpopulations are assumed to scavenge O\textsubscript{2} (if present) and produce Nos\textsubscript{Z} in response to impending anoxia. The latter because the nos\textsubscript{Z} genes are readily induced by the O\textsubscript{2}-sensor FnrP [24].

The Z\textsuperscript{−} pool (Fig 2A) contains the inoculum that grows by aerobic respiration. As [O\textsubscript{2}] falls below a critical threshold [empirically determined, 18], the cells within Z\textsuperscript{−} are assumed to start synthesising Nar with a certain probability and populate the Z\textsuperscript{Na} pool. The aim here is to investigate whether, like for nir\textsubscript{S}, the initiation of nar transcription (by some combined activity of FnrP and NarR) can also be explained as a probabilistic phenomenon, quickly differentiating a cell into a full-fledged NO\textsubscript{2} scavenger through product (NO\textsubscript{3}\textsuperscript{-}) induced transcription via NarR (Fig 1, see P\textsubscript{1}). If so, we were interested to estimate what fraction of the cells is required to adequately simulate the measured data (NO\textsubscript{3}\textsuperscript{-} production), aiming at scrutinising the general assumption that all cells in batch cultures produce Nar in response to impending anoxia.

Next, when [O\textsubscript{2}] is further depleted to another critical threshold [18], the Z\textsuperscript{−} and Z\textsuperscript{Na} cells are assumed to initiate nir\textsubscript{S} transcription with a low per hour probability and, thereby, populate the Z\textsuperscript{Ni} and Z\textsuperscript{NaNi} pools, respectively. As explained above for our previous model, NirS + cNor production is assumed to be a) coordinated because the transcription of both nir\textsubscript{S} and nor is induced by NO via the NO-sensor NNR (Fig 1), and b) stochastic because the initial transcription of nir\textsubscript{S}
(paving the way for the autocatalytic expression of NirS and substrate-induced nor transcription) happens in the absence of NO or at too low [NO] to be sensed by NNR.

Synthesis of denitrification enzymes requires energy, which all the subpopulations can obtain by respiration only. Hence, the initiation of the autocatalytic expression of nar and nirS
(i.e., recruitment to Z$^\text{Na}$ and Z$^{\text{NaNi/ZNi}}$, respectively, Fig 2A) depends on the availability of the relevant terminal e$^-$-acceptor(s) above a critical concentration to sustain a minimum of respiration. For Z$^-\text{Ni}$, the only relevant e$^-$-acceptors are O$_2$ and the traces of N$_2$O produced by Z$^{\text{Ni}}$ and Z$^{\text{NaNi}}$. The same applies for Z$^{\text{NaNi}}$, but in addition, this subpopulation can also obtain energy by reducing NO$_3$ if present. In our previous model [16], we assumed that recruitment to denitrification was sustained by energy from O$_2$-respiration only; not NO$_2^-$ because we simulated NO$_2^-$-supplemented treatments, and not by N$_2$O because we naïvely assumed that the pool of this e$^-$-acceptor was insignificant (N$_2$O concentrations were below the detection limit of the system used for those experiments). However, the present model assumes that the recruitment from Z$^-\text{Na}$ to Z$^{\text{Na}}$ and Z$^-\text{Ni}$ to Z$^{\text{Ni}}$ is sustained by both O$_2$- and N$_2$O-reduction, and the recruitment from Z$^{\text{Na}}$ to Z$^{\text{NaNi}}$ is sustained by O$_2^-$, N$_2$O- and NO$_3^-$-reduction, when above a critical minimum ($v_{\text{min}}$). The default value for $v_{\text{min}}$ was set to an arbitrary low value (= 0.44% of the maximum e$^-$-flow rate to O$_2$), and we have investigated the consequences of increasing, decreasing, and setting $v_{\text{min}} = 0$. The expressions of $\text{nar}$ and $\text{nirS} + \text{nor}$ (recruitments to Z$^{\text{Na}}$ and Z$^{\text{NaNi/ZNi}}$, respectively, Fig 2A) are modelled as instantaneous discrete-events in each cell, thus ignoring the time-lag from the initiation of gene transcription till the cell is fully equipped with the reductase(s) in question. That is because the lag observed between the emergence of denitrification gene transcripts and the subsequent gas products suggests that the synthesis of denitrification enzymes takes less than half an hour [17,18], which is negligible for our purposes here.

The main purpose of the present modelling is to investigate if a full-fledged model, including all four functional denitrification reductases, could adequately simulate the observed kinetics and stoichiometry of denitrification products [18]. These cultures reduced all available NO$_3^-$ to NO$_2^-$ prior to the onset of gas production and accumulated traces of N$_2$O throughout the anoxic phase, as illustrated in S1 Fig. In particular, we were interested to investigate the NO$_2^-$ kinetics, controlled by $\text{nar}$- and $\text{nirS}$ transcription, and to test if the peculiar N$_2$O kinetics (low, but increasing concentrations throughout the anoxic phase) could be explained by our modelled cell diversification.

Materials and Methods

An overview of the modelled experiment

**Batch incubation.** Qu [18] incubated Pa. denitrificans (DSM-413) at 20°C using 50 mL Sistrom’s [29] medium in 120 mL gas-tight vials. Either succinate or butyrate (5 mM) was used as the main carbon source, enough to secure consumption of all available e$^-$-acceptors. After distribution of the medium, each vial was loaded with a magnetic stirring bar, sterilised through autoclaving, supplemented with 2 mM KNO$_3$, and was tightly sealed. To remove O$_2$ and N$_2$ from the headspace, the headspace air was evacuated and replaced by helium (He) through several cycles of evacuation and He-filling (He-washing). Some vials were supplemented with oxygen to reach 7 vol.% O$_2$ in headspace (treatment designated 7% O$_2$). The remaining vials received no O$_2$ (designated 0% O$_2$, although there were traces of O$_2$ present despite the He washing). For each treatment (i.e., C source and initial O$_2$), there were three replicates, and each vial was inoculated with $2.2 \times 10^8$ aerobically grown cells.

**NO$_2^-$ and gas measurement.** Gases (CO$_2$, O$_2$, NO, N$_2$O, and N$_2$) were monitored by frequent sampling of the headspace, using an improved version of the robotised incubation system [30]. In short, the system draws gas samples from the headspace (peristaltic pumping) via the septum pierced by a needle, filling three loops used for injecting samples into the two GC columns and the chemiluminescence NO analyser. The sample drawn is replaced by He (reversing the peristaltic pump), thus securing ~1 atm pressure. The primary improvements of the new system are a more sensitive detection of N$_2$O (by an electron capture detector), lower
sampling volumes (~1 mL), and lower leaks of O₂ and N₂ through the sampling system (4 nmol O₂ and 12 nmol N₂ per sampling, which is ~20% of that for the old system).

To extract samples for measuring NO₃⁻ without tampering the original vials, identical (parallel) vials were prepared for each treatment. Using sterile syringes, samples of 0.1 mL were regularly drawn from the liquid-phase of the parallel vials and immediately analysed for NO₂⁻.

Results for one of the treatments are shown in S1 Fig, illustrating the complete reduction of NO₃⁻ to NO₂⁻ prior to the onset of significant N-gas production. In previous experiments [17], N₂O concentrations were below the detection limit of the system, but thanks to the new system, the N₂O kinetics were monitored with a reasonable precision.

The model

The model is constructed in Vensim DSS 6.2 Double Precision (Ventana Systems, Inc., http://vensim.com/) using techniques from the field of system dynamics [31].

**Cell diversification and growth.** The respiring population is divided into four subpopulations, according to their reductases (Fig 2A): 1) Z⁻: cells without Nar, NirS, and cNor; 2) ZNa: cells with Nar; 3) ZNaNi: cells with Nar, NirS, and cNor; and 4) ZNi: cells with NirS and cNor. All the subpopulations are assumed to equally respire O₂ if present, and express nosZ in response to oxygen depletion [24]. Z⁻ contains the inoculum (= 2.2×10⁸ cells) that grows by aerobic respiration. As O₂ is depleted, the Z⁻ cells populate the other pools by producing Nar and/or NirS + cNor.

The recruitment from Z⁻ to ZNa (RNa, Fig 2A) takes place first:

\[
R_{Na} = Z^- \times r_{Na}(O_2, N_2O)
\]

(cells h⁻¹)

where \( r_{Na}(O_2, N_2O) \) is a conditional specific probability (h⁻¹) for any Z⁻ cell to initiate nar transcription (quickly transforming it into a NO₃⁻ scavenger through autocatalytic gene expression, see Fig 1, P1):

\[
r_{Na}(O_2, N_2O) = \begin{cases} 
0 & \text{ELSE} \\
\frac{IF \left[ [O_2]_{aq} < [O_2]_{na} \text{ AND } (ve^{O_2}_{Na} + 0.5 \times ve^{N_2O}_{Na}) > ve^{min}_{Na}\right]}{(h^{-1})} & \text{THEN} \\
0 & \end{cases}
\]

where \( r_{Na} (h^{-1}) \) is a constant specific probability for a cell to initiate nar transcription once O₂ concentration in the aqueous-phase ([O₂]ₐq, mol L⁻¹) falls below a critical concentration ([O₂]ₐq, empirically determined as the [O₂]ₐq (= 4.75×10⁻⁵ mol L⁻¹) at the outset of NO₃⁻ accumulation in the medium [18]. The second condition for a cell to produce first molecules of Nar is a minimum of e⁻ flow to an e⁻ acceptor (veₐ_{min}, mol e⁻ cell⁻¹ h⁻¹), assumed to generate minimum ATP required for protein synthesis. veₐ_{O₂} and veₐ_{N₂O} (mol e⁻ cell⁻¹ h⁻¹) are the cell-specific velocities of e⁻ flow to O₂ and N₂O, respectively. The latter is weighed down by 0.5 because mole ATP per mole e⁻ transferred to NO₃⁻/NO₂⁻ is lower for denitrification than for aerobic respiration [17,20]. For a Z⁻ cell, veₐ_{NO₂} and veₐ_{NO₃} are not considered here, since such a cell is assumed to have no NirS and cNor.

The fraction of the cells that successfully produces Nar (FNa) is calculated based on the integral of the recruitment (Eq 1):

\[
F_{Na} = 1 - e^{-r_{Na} \times t_{Na}}
\]

(dimensionless)
where $t_{Na}$ is the time-window available for the recruitment. In theory, $t_{Na}$ is the time-period when $[O_2]_{aq} < [O_2]_{na}$ AND $(v_{O_2}^- + 0.5 \times v_{NO_2}^-) > v_{min}$ (Eq 2). Since the $e^-$ flow to $N_2O$ started after all $NO_3$ had been reduced to $NO_2^- \ (S1$ Fig), the recruitment based on $v_{N_2O}^-$ would be inconsequential for the simulated (and measured) $NO_3^-$ kinetics. Therefore, to calculate the functional $F_{Na}$ actually responsible for producing $NO_2^-$, we ignored the $N_2O$-sustained recruitment, thus considering $t_{Na}$ to be the time when $[O_2]_{aq} < [O_2]_{na}$ AND $v_{O_2}^- > v_{min}$.

Next, the cells within $Z_{Na}$ and $Z^-$ are recruited to $Z_{NaNi}$ and $Z_{Ni}$ ($R_{NaNi}$ and $R_{Ni}$, respectively, Fig 2A), as they are assumed to stochastically initiate $nirS$ transcription, paving the way for $NO/NNR$ mediated autocatalytic expression of $nirS^+ + nor$ (Fig 1). In principle, the rates of both these recruitments are modelled as that of the recruitment from $Z^-$ to $Z_{Na}$ (Eqs 1 and 2):

\[ R_{NaNi} = Z_{Na} \times r_{Ni}(O_2, NO_3^-, N_2O) \]  

\[(cells \ h^{-1}) \]

\[ r_{Ni}(O_2, NO_3^-, N_2O) = \]

\[
\begin{align*}
IF & \ [O_2]_{aq} < [O_2]_{na} \ AND \ (v_{O_2}^- + 0.5 \times v_{NO_2}^-) > v_{min} \\
THEN & r_{Ni} \\
ELSE & 0
\end{align*}
\]

\[(h^{-1}) \]

where $r_{Ni}$ is a constant specific probability (h$^{-1}$) for the initiation of $nirS$ transcription. $v_{NO_3}^-$ and $v_{N_2O}^-$ are multiplied with 0.5 for the same reasons as described for Eq 2.

The recruitment from $Z^-$ to $Z_{Ni}$ ($R_{Ni}$, Fig 2A) is modelled as a product of $Z^-$ and a conditional specific probability, $r_{Ni}(O_2, N_2O)$, which is different from Eq 5 only in that $v_{NO_3}^-$ is omitted, since $Z^-$ do not possess Nar:

\[ R_{Ni} = Z^- \times r_{Ni}(O_2, N_2O) \]

\[(cells \ h^{-1}) \]

\[ r_{Ni}(O_2, N_2O) = \]

\[
\begin{align*}
IF & \ [O_2]_{aq} < [O_2]_{na} \ AND \ (v_{O_2}^- + 0.5 \times v_{N_2O}^-) > v_{min} \\
THEN & r_{Ni} \\
ELSE & 0
\end{align*}
\]

\[(h^{-1}) \]

The fraction that successfully produced Nir + cNor ($F_{Ni}$) is calculated based on the integral of $R_{NaNi}$ and $R_{Ni}$:

\[ F_{Ni} = (1 - e^{-r_{Na} \times t_{Na}}) \times F_{Na} + (1 - e^{-r_{Ni} \times t_{Ni}}) \times (1 - F_{Na}) \]

\[(dimensionless) \]
where $t_{NaNi}$ is the duration of the recruitment from $Z^{Na}$ to $Z^{NaNi}$, i.e., when $[O_2]_{aq} < [O_2]_{nir}$ AND $(ve^-_{O_2} + 0.5 \times ve^-_{NO_3} + 0.5 \times ve^-_{NO_2}) > ve^-_{min}$ (Eqs 4 and 5), $F_{Na}$ is the fraction recruited to the pool of Nar positive cells ($Z^{Na}$, Eq 3), and $t_{Ni}$ is the duration of the recruitment from $Z^*$ to $Z^N$, i.e., when $[O_2]_{aq} < [O_2]_{nir}$ AND $(ve^-_{O_2} + 0.5 \times ve^-_{NO_2}) > ve^-_{min}$ (Eqs 6 and 7).

Each of the populations will grow depending on the rates of $e^-$-flow to the various $e^-$-acceptors they are able to use:

$$G_{Z^-} = Z^- \times (Ye^-_{O_2} \times ve^-_{O_2} + Ye^-_{NO_3} \times ve^-_{NO_3})$$

$$G_{Z^{Na}} = Z^{Na} \times [Ye^-_{O_2} \times ve^-_{O_2} + Ye^-_{NO_3} (ve^-_{NO_3} + ve^-_{NO_2} + ve^-_{NO_2})]$$

$$G_{Z^{NaNi}} = Z^{NaNi} \times [Ye^-_{O_2} \times ve^-_{O_2} + Ye^-_{NO_3} (ve^-_{NO_3} + ve^-_{NO_2} + ve^-_{NO_2} + ve^-_{NO_2})]$$

$$G_{Z^{Ni}} = Z^{Ni} \times [Ye^-_{O_2} \times ve^-_{O_2} + Ye^-_{NO_3} (ve^-_{NO_3} + ve^-_{NO_2} + ve^-_{NO_2})]$$

(cells h$^{-1}$)

where $Ye^-_{X}$ (cells mol$^{-1}$ e$^-$ to X = O$_2$ or NO$_x$/NO$_x$) is the growth yield determined under the actual experimental conditions, and $ve^-_{X}$ (mol e$^-$ cell$^{-1}$ h$^{-1}$) is the cell-specific velocity of $e^-$-flow to X (O$_2$ or NO$_x$/NO$_x$), which depends on the concentration of the $e^-$-acceptor (see Eqs 17, 20 and 28). For NO$_3$ and NO$_2$, a restricted velocity ($ve^-_{NO_2}$) is used so that when electrons flow to O$_2$, NO$_3$, and NO$_2$ simultaneously, the total $ve^-$ per cell does not exceed the maximum electrons that the TCA cycle ($ve^-_{maxTCA}$) can deliver per hour (see Eqs 21 and 22).

**O$_2$ kinetics.** O$_2$ is initially present in the headspace (O$_2$(h), mol, initialised according to the experiment, see Table 1) but is transported to the liquid-phase (O$_2$(l)) due to its consumption therein (Fig 2B). The transport rate ($Tr_{O_2}$) is modelled according to Molstad et al. [30]:

$$Tr_{O_2} = k (k_{T(O_2)} \times P_{O_2} - [O_2]_{lP})$$

(mol h$^{-1}$)

where $k_t$ (L h$^{-1}$) is the empirically determined coefficient for the transport of gas between the headspace and the liquid, $k_{T(O_2)}$ (mol L$^{-1}$ atm$^{-1}$) is the solubility of O$_2$ in water at 20°C, $P_{O_2}$ (= [O$_2$]$_{h} \times R \times T$, atm) is the partial pressure of O$_2$ in the headspace, and $[O_2]_{aq}$ (mol L$^{-1}$) is the O$_2$ concentration in the liquid ($[O_2]_{aq} = \frac{O_2}{Vol_{aq}}$).

In addition, the model simulates the changes in O$_2$(h) due to sampling. The robotised incubation system used monitors gas concentrations by sampling the headspace, where each sampling alters the concentrations in a predictable manner: a fraction of O$_2$(h) is removed and replaced by

| Batch | C-source | $O_2$(h) (t$_0$) (vol.%) | NO$_2$(t$_0$) (mM) | Replicates |
|-------|----------|----------------------|-----------------|------------|
| 1     | Butyrate | 0                    | 2               | 3          |
| 2     | Butyrate | 7                    | 3               | 3          |
| 3     | Succinate| 0                    | 2               | 3          |
| 4     | Succinate| 7                    | 3               | 3          |

*Target values for initial O$_2$ concentrations in the headspace (vol.%). −0 means that the intended concentration should be zero, but there were detectable traces of O$_2$, despite several cycles of evacuation and He-flushing of the headspace.

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He (dilution), but the sampling also results in a marginal leakage of O$_2$ through the tubing and membranes in the injection system. The net change in O$_2$ ($\Delta O_2(S)$) as a result of each sampling is calculated as:

$$\Delta O_2(S) = \frac{O_{2\text{leak}}}{C_0O_2} \times D \times t_s$$  \hspace{1cm} (14)

where $O_{2\text{leak}}$ (mol vial$^{-1}$) is the O$_2$-leakage into the headspace, D (dilution) is the fraction of each headspace gas removed and replaced by equal amount of He, and $t_s$ (h) is the time taken to complete each sampling. $\Delta O_2(S)$ is negative if O$_2$ is high and marginally positive at very low oxygen concentrations.

O$_2$ in the liquid-phase (O$_2$$_{aq}$, mol, Fig 2B) is initialised by assuming equilibrium with O$_2$ at the time of inoculation ($O_{2\text{aq}}(t_0) = P_{O2} \times k_{H(O2)} \times V_{O2a}$). The dynamics of O$_2$$_{aq}$ are modelled as a function of transport between the headspace and the liquid ($Tr_{O2}$, Eq 13) and its reduction rate ($Rr_{O2}$, mol h$^{-1}$):

$$\frac{d(O_{2\text{aq}})}{dt} = Tr_{O2} - Rr_{O2}$$  \hspace{1cm} (15)

$$Rr_{O2} = (Z^- + Z^{Na} + Z^{NaNi} + Z^{Ni}) \times \nu_{O2}$$  \hspace{1cm} (16)

where $Z^-$, $Z^{Na}$, $Z^{NaNi}$, and $Z^{Ni}$ (cells) are all the sub-populations present (described above); thus, we assume that all cells have the same potential to consume O$_2$. $\nu_{O2}$ (mol cell$^{-1}$ h$^{-1}$) is the cell-specific velocity of O$_2$ consumption, obtained by the velocity of e$^-$-flow to O$_2$

$$\nu_{O2} = \frac{v_{e_{maxO2}}}{K_{mO2} + [O_2]_{aq}}$$  \hspace{1cm} (17)

Denitrification kinetics. The NO$_3^-$ and NO$_2^-$ pools (mol, Fig 2C) are initialised according to the experiment (Table 1; NO$_2^-$ = 0). The kinetics of these nitrogen oxyanions (NO$_x^-$) are modelled as:

$$\frac{d(\text{NO}_3^-)}{dt} = -Rr_{NO3} = -(Z^{Na} + Z^{NaNi}) \times \nu_{NO3}$$  \hspace{1cm} (18)

$$\frac{d(\text{NO}_2^-)}{dt} = Rr_{NO2} - Rr_{NO3} = Rr_{NO2} - (Z^{NaNi} + Z^{Ni}) \times \nu_{NO2}$$  \hspace{1cm} (19)

Denitrification kinetics. The NO$_3^-$ and NO$_2^-$ pools (mol, Fig 2C) are initialised according to the experiment (Table 1; NO$_2^-$ = 0). The kinetics of these nitrogen oxyanions (NO$_x^-$) are modelled as:

$$\frac{d(\text{NO}_3^-)}{dt} = -Rr_{NO3} = -(Z^{Na} + Z^{NaNi}) \times \nu_{NO3}$$  \hspace{1cm} (18)

$$\frac{d(\text{NO}_2^-)}{dt} = Rr_{NO2} - Rr_{NO3} = Rr_{NO2} - (Z^{NaNi} + Z^{Ni}) \times \nu_{NO2}$$  \hspace{1cm} (19)
cell-specific velocity of NO\textsuperscript{3-} consumption, obtained by the velocity of e\textsuperscript{-}-flow to NO\textsubscript{2} (\(\frac{1}{2}\) mol NO\textsuperscript{3-} and \(\frac{1}{2}\) mol e\textsuperscript{-}). The latter is modelled as a Michaelis-Menten function of NO\textsubscript{x} concentration:

\[
ve^\text{NO}_x = \frac{ve^\text{maxNO}_x \times [NO_x]_{aq}}{K_{mNO_x} + [NO_x]_{aq}}
\]  

(mol e\textsuperscript{-} cell\textsuperscript{-1} h\textsuperscript{-1})

where \(ve^\text{maxNO}_x\) (mol e\textsuperscript{-} cell\textsuperscript{-1} h\textsuperscript{-1}) is the maximum velocity of e\textsuperscript{-}-flow to NO\textsuperscript{3-} per cell (determined under the actual experimental conditions), [NO\textsubscript{x}]\textsubscript{aq} (mol L\textsuperscript{-1}) is the NO\textsubscript{x} concentration in the aqueous-phase, and \(K_{mNO_x}\) (mol L\textsuperscript{-1}) is the half-saturation constant for NO\textsubscript{x} reduction.

The velocity of NO\textsubscript{3-} and NO\textsubscript{2} consumption had to be restricted (\(ve^\text{NO}_x\)res) to ensure that when electrons flow to O\textsubscript{2}, NO\textsubscript{3-}, and NO\textsubscript{2} simultaneously, the total e\textsuperscript{-} per cell does not exceed an estimated maximum delivery of electrons from the TCA cycle (\(ve^\text{maxTCA}\)). In competition for electrons, O\textsubscript{2} is prioritised \([20]\), followed by NO\textsubscript{3-} and NO\textsubscript{2}, respectively \([18]\):

\[
ve^\text{NO}_x\text{res} = \text{Min} (ve^\text{NO}_x, (ve^\text{maxTCA} - ve^\text{O}_2))
\]  

\[
ve^\text{NO}_x\text{res} = \text{Min} (ve^\text{NO}_x, (ve^\text{maxTCA} - ve^\text{O}_2 - ve^\text{NO}_2\text{res}))
\]  

(mol e\textsuperscript{-} cell\textsuperscript{-1} h\textsuperscript{-1})

where \(ve^\text{NO}_x\)res is the realised e\textsuperscript{-}-flow to NO\textsubscript{3-}, limited either by available NO\textsubscript{x} or the availability of electrons (due to competition with O\textsubscript{2}); \(ve^\text{NO}_x\)res is the realised e\textsuperscript{-}-flow to NO\textsubscript{2}. Such competition for electrons was not implemented for ve\textsubscript{NO} and ve\textsubscript{N\textsubscript{2}O} because at the onset of NO\textsubscript{2}, N\textsubscript{2}O- and N\textsubscript{2} production, the total velocity of e\textsuperscript{-}-flow to all available e\textsuperscript{-}-acceptors (as predicted by the enzyme kinetics alone) never exceeded \(ve^\text{maxTCA}\).

Gas consumption and production takes place in the aqueous phase, but the gases are transported between aqua and the headspace depending on their concentrations in the two phases. Each gas in aqua, X\textsubscript{aq} (mol N, Fig 2C), is modelled as a function of production, consumption (not applicable to N\textsubscript{2}), and the net transport, where N\textsubscript{2}O\textsubscript{aq} and N\textsubscript{2}aq are initialised with zero, and NO\textsubscript{aq} is initialised with a negligible 1×10\textsuperscript{-25} mol to avoid division by zero (in Eq 28).

\[
\frac{d([NO_x]_{aq})}{dt} = R_{NO} - R_{NO} + Tr_{NO}
\]  

\[
\frac{d([N_2O]_{aq})}{dt} = R_{NO} - R_{N_2O} + Tr_{N_2O}
\]  

\[
\frac{d([N_2]_{aq})}{dt} = R_{N_2O} + Tr_{N_2}
\]  

(mol N h\textsuperscript{-1})

where \(R_{NO}\) (mol N h\textsuperscript{-1}) is the relevant NO\textsubscript{2}/NO\textsubscript{x} reduction rate, and Tr\textsubscript{N\textsubscript{2}} represents the gas transport rate between aqua and the headspace (Eq 29; N.B. Tr\textsubscript{N\textsubscript{2}} < 0 for the net transport from aqua to the headspace).

The reduction of NO to N\textsubscript{2}O (R\textsubscript{N\textsubscript{2}O}) and N\textsubscript{2}O to N\textsubscript{2} (R\textsubscript{N\textsubscript{2}O}) is modelled likewise as a function of the number of relevant cells and the velocity of e\textsuperscript{-}-flow to NO and N\textsubscript{2}O (mol e\textsuperscript{-} cell\textsuperscript{-1} h\textsuperscript{-1}),
respectively:

\[ R_{rNO} = (Z^{Ni} + Z^{Ni}) \times v_{NO} \]  (26)

\[ R_{rN_2O} = (Z^{-} + Z^{Ni} + Z^{Ni} + Z^{Ni}) \times v_{N_2O} \]  (27)

\[(\text{molN h}^{-1})\]

where \( v_{NO} \) and \( v_{N_2O} \) are obtained by the velocity of e^{-} flow to NO and N_{2}O, respectively

\[ 1 \text{ mol N} \text{ e^{-}} \]. \( v_{e^{\text{NO}}} \) is modelled as a Michaelis-Menten function of \([N_{2}O]_{aq}\), similarly as that of O_{2}, NO_{3}^{-}, and NO_{2}^{-} (Eqs 17 and 20), but \( v_{e^{\text{NO}}} \) is modelled assuming a cooperative binding of two NO molecules with cNor to form N_{2}O [32]:

\[ \frac{v_{e^{\text{NO}}}}{1 + K_{1}^{\text{NO}} \frac{[N_{2}O]_{aq}}{[N_{2}O]_{aq}} + K_{2}^{\text{NO}}}} \]  (28)

\[(\text{mol cell}^{-1} \text{ h}^{-1})\]

where \( v_{e^{\text{maxNO}}} \) (mol e^{-} cell^{-1} h^{-1}) is the empirically determined maximum velocity of e^{-} flow to NO per cell, \([NO]_{aq} \) (mol L^{-1}) is the NO concentration in the liquid-phase, and \( K_{1}^{\text{NO}} \) & \( K_{2}^{\text{NO}} \) (mol L^{-1}) are the equilibrium dissociation constants for the cNor/NO- and cNor/(NO)_{2} complex, respectively.

The transport of NO, N_{2}O, and N_{2} between the liquid and the headspace (Eqs 23–25) is modelled as:

\[ T_{rN} = k_{i} \times (k_{H(N)} \times P_{N} - [N]_{aq}) \]  (29)

\[(\text{molN h}^{-1})\]

where \( k_{i} \) is the empirically determined coefficient for the transport of each gas between the headspace and the liquid, \( k_{H(N)} \) (molN L^{-1} atm^{-1}) is the solubility of NO, N_{2}O, or N_{2} in water at 20°C, \( P_{N} \) (= \([N]_{aq} \times {R \times T}, \text{atm}) \) is the partial pressure of each gas in the headspace, and \([N]_{aq} \) (mol L^{-1}) represents the concentration of each gas in the liquid-phase.

The amount of NO and N_{2}O in the headspace (NO_{xg}, molN, Fig 2C) is a function of transport (Eq 29) and the disturbance by gas sampling. The latter is simulated as discrete events at time-points given as input to the model (equivalent to the sampling times in the experiment):

\[ \Delta N_{O_{xg}(S)} = \frac{N_{O_{xg}} \times D}{t_{s}} \]  (30)

\[(\text{molN h}^{-1})\]

where \( \Delta N_{O_{xg}(S)} \) is the net change in the amount of NO_{xg} (molN), D (dilution) is the fraction of each gas removed and replaced by equal amount of He, and \( t_{s} \) (h) is the time taken to complete each sampling. For N_{2}, the model ignores the sampling loss because the N_{2} production data to be compared with the model output are already corrected for the sampling disturbance [30]. Thus, the model estimates somewhat higher N_{2} concentrations than that experienced by the organisms, which is acceptable, since the concentration of N_{2} is unlikely to have consequences for the metabolism.

Parameterisation

Most of the parameter values used in the model are well established in the literature (see Table 2); however, uncertain parameters include \( K_{mO_{2}}, K_{mN_{2}O}, v_{e^{\text{maxO}_{2}}}, \) and \( v_{e^{-}} \).
Table 2. Model parameters.

| Description                                                                 | Value                        | Units             | Reference |
|-----------------------------------------------------------------------------|------------------------------|-------------------|-----------|
| **Butyrate treatments**                                                     |                              |                   |           |
| \( \nu_{\max\text{TCA}} \) Max. cell-specific rate of e-\( \rightarrow \) delivery from the TCA cycle | \( 1 \times 10^{14} \)      | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{O}_2} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{O}_2 \) | \( 4.22 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{NO}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{NO} \) | \( 1 \times 10^{-14} \)      | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{N}_2\text{O}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{N}_2\text{O} \) | \( 2.65 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\min} \) The minimum velocity of e-\( \rightarrow \) flow to \( \text{NO}_2/\text{NO}_x \) required for protein synthesis (ATP) | \( 1.87 \times 10^{-17} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | Assumption|
| \( Y_{\text{e}^-/\text{O}_2} \) The growth yield per mole of electrons transferred to \( \text{O}_2 \) | \( 2.74 \times 10^{13} \)    | cells (mol e\(^{-1} \)) | [18]      |
| \( Y_{\text{e}^-/\text{NO}} \) The growth yield per mole of electrons transferred to \( \text{NO} \) | \( 1.12 \times 10^{13} \)    | cells (mol e\(^{-1} \)) | [18]      |
| **Succinate treatments**                                                    |                              |                   |           |
| \( \nu_{\max\text{TCA}} \) Max. cell-specific rate of e-\( \rightarrow \) delivery from the TCA cycle | \( 9.34 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{O}_2} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{O}_2 \) | \( 4.42 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{NO}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{NO} \) | \( 9.34 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\max\text{N}_2\text{O}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{N}_2\text{O} \) | \( 2.01 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [18]      |
| \( \nu_{\min} \) The minimum velocity of e-\( \rightarrow \) flow to \( \text{NO}_2/\text{NO}_x \) required for protein synthesis (ATP) | \( 1.95 \times 10^{-17} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | Assumption|
| \( Y_{\text{e}^-/\text{O}_2} \) The growth yield per mole of electrons transferred to \( \text{O}_2 \) | \( 4.97 \times 10^{13} \)    | cells (mol e\(^{-1} \)) | [18]      |
| \( Y_{\text{e}^-/\text{NO}} \) The growth yield per mole of electrons transferred to \( \text{NO} \) | \( 1.52 \times 10^{13} \)    | cells (mol e\(^{-1} \)) | [18]      |
| **Parameters common to both succinate and butyrate treatments**             |                              |                   |           |
| \([\text{O}_2]\)_{\text{sat}} \) The \([\text{O}_2]\) in aqua below which Nar production triggers | \( 5.95 \times 10^{-5} \)    | mol L\(^{-1} \)  | [18]      |
| \([\text{O}_2]\)_{\text{nr}} \) The \([\text{O}_2]\) in aqua below which NirS production triggers | \( 9.75 \times 10^{-6} \)    | mol L\(^{-1} \)  | [18]      |
| \( f_{\text{Nar}} \) The specific-probability for Nar production            | 0.035                        | h\(^{-1} \)       | Optimisation|
| \( f_{\text{NirS}} \) The specific-probability for NirS production         | 0.004                        | h\(^{-1} \)       | Optimisation|
| \( \nu_{\max\text{NO}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{NO} \) | \( 3.56 \times 10^{-15} \)  | mol e- cell\(^{-1} \) h\(^{-1} \) | [33]      |
| \( \nu_{\max\text{N}_2\text{O}} \) The maximum cell-specific velocity of e-\( \rightarrow \) flow to \( \text{N}_2\text{O} \) | \( 5.5 \times 10^{-15} \)    | mol e- cell\(^{-1} \) h\(^{-1} \) | [24]      |
| \( K_{\text{NO}_2} \) The half-saturation constant for \( \text{O}_2 \) reduction | \( 2.25 \times 10^{-7} \)    | mol L\(^{-1} \)  | Optimisation|
| \( K_{\text{NO}_x} \) The half-saturation constant for \( \text{NO}_x \) reduction | \( 5 \times 10^{-6} \)       | mol L\(^{-1} \)  | [34,35]   |
| \( K_{\text{N}_2\text{O}} \) The half-saturation constant for \( \text{N}_2\text{O} \) reduction | \( 4.13 \times 10^{-6} \)    | mol L\(^{-1} \)  | [36,37]   |
| \( K_{\text{NO}} \) The equilibrium dissociation constant for cNor/NO complex | \( 8 \times 10^{-14} \)     | mol L\(^{-1} \)  | [33]      |
| \( K_{\text{N}_2\text{O}} \) The equilibrium dissociation constant for cNor/(NO)\(_2\) complex | \( 34 \times 10^{-9} \)    | mol L\(^{-1} \)  | [33]      |
| \( K_{\text{N}_2\text{O}} \) The half-saturation constant for \( \text{N}_2\text{O} \) reduction | \( 5.93 \times 10^{-7} \)    | mol N\(_2\)O-N L\(^{-1} \) | Optimisation|
| \( D \) Dilution (due to sampling): fraction of gas replaced by He         | 0.013–0.016                  | –                 | [18]      |
| \( k_{\text{H}_2\text{O}(1)} \) Solubility of \( \text{O}_2 \) in water at 20°C | 0.0014                       | mol L\(^{-1} \) atm\(^{-1} \) | [38]      |
| \( k_{\text{NO}(1)} \) Solubility of NO at 20°C                            | 0.0021                       | mol L\(^{-1} \) atm\(^{-1} \) | [39]      |
| \( k_{\text{N}_2\text{O}(1)} \) Solubility of \( \text{N}_2\text{O} \) at 20°C | 0.056                        | mol N\(_2\)O-N L\(^{-1} \) atm\(^{-1} \) | [38]      |
| \( k_{\text{N}_2\text{O}_3(1)} \) Solubility of \( \text{N}_2\text{O}_3 \) at 20°C | 0.00035                      | mol N\(_2\)O-N L\(^{-1} \) atm\(^{-1} \) | [38]      |
| \( k_{\text{H}_2\text{O}} \) The coeff. for gas transport between headspace and liquid | 3.6                          | L vial\(^{-1} \) h\(^{-1} \) | Measured  |
| \( O_{\text{leak}} \) O\(_2\) leakage into the vial during each sampling | \( 2.92 \times 10^{-9} \)   | mol               | Measured  |
| \( R \) Universal gas constant                                             | 0.083                        | L atm K\(^{-1} \) mol\(^{-1} \) | –         |
| \( T \) Temperature                                                        | 293.15                       | K                 | [18]      |
| \( t_{\text{d}} \) The time taken to complete each sampling               | 0.017                        | h                 | [30]      |
| \( \text{Vol}_{\text{g}} \) Headspace volume                              | 0.07                         | L                 | [18]      |
| \( \text{Vol}_{\text{aq}} \) Aqueous-phase volume                          | 0.05                         | L                 | [18]      |

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with $K_{mO_2}$ (Eq 17). *Pa. denitrificans* has three haem-copper terminal oxidoreductases [39] with $K_{mO_2}$ ranging from nM to µM [40,41], so we decided to estimate the parameter value by optimising $K_{mO_2}$ for the low [O$_2$] treatments data. Vensim was used for the optimisation, where $K_{mO_2} = 2.25 \times 10^{-7}$ neatly simulated the O$_2$ depletion for both the succinate- and butyrate-supplemented treatments.

$K_{mN_2O_5}$. *In vitro* studies of NosZ from *Pa. denitrificans* estimate the values for $K_{mN_2O_5} = 5 \mu M$ at 22°C and pH 7.1 [42] and 6.7 µM at 25°C and pH 7.1 [43]. When our model was simulated with $K_{mN_2O_5}$ in this range, given our empirically estimated $v_e^{-maxN_2O_5}$ [24], the simulated N$_2$O reached concentrations much higher than that measured (see Results/Discussion). A more adequate parameter value (= 0.6 µM) was found by optimising $K_{mN_2O_5}$ in Vensim. The value is within the range determined for soil bacterial communities [44].

$v_e^{-maxO_2}$ (Eq 17) could be estimated using the empirically determined cell yield per mole of electrons to O$_2$ ($Y_e^{-O_2}$, cells per mol e$^-$) and the maximum specific growth rate ($\mu$, h$^{-1}$):

$$v_e^{-maxO_2} = \frac{\mu}{Y_e^{-O_2}}.$$  

We are confident about the yields for the two C-substrates used, but the empirically determined $\mu$ for the butyrate treatments is suspiciously low ($= 0.067$ h$^{-1}$), providing $v_e^{-maxO_2} = 2.45 \times 10^{-15}$ mol e$^-$ cell$^{-1}$ h$^{-1}$. Simulations with this value grossly underestimated the rate of O$_2$ depletion compared to measured, which forced us to estimate the parameter value by optimisation: $v_e^{-maxO_2} = 4.42 \times 10^{-15}$ and $4.22 \times 10^{-15}$ mol e$^-$ cell$^{-1}$ h$^{-1}$ for the succinate- and butyrate treatments, respectively. These values give $\mu = 0.22$ and 0.12 h$^{-1}$, respectively; for the succinate treatments, the value is very close to that empirically determined ($= 0.2$ h$^{-1}$); for the butyrate treatments, the value seems more realistic than 0.067 h$^{-1}$.

$v_e^{-min}$ (Eqs 2, 5 and 7) is the per cell velocity of e$^-$ flow to O$_2$ ($Y_e^{-O_2}$) assumed to generate minimum ATP required for synthesising the initial molecules of denitrification enzymes. Since we lack any empirical or other estimations for this parameter, it is arbitrarily assumed to be the $v_e^{-O_2}$ when [O$_2$]$_{aq}$ reaches 1 nM. At this concentration, $v_e^{-min}$ is determined by the Michaelis-Menten equation:

$$v_e^{-min} = \frac{v_e^{-maxO_2} \cdot [O_2]_{aq}}{K_{mO_2} + [O_2]_{aq}}.$$  

The values obtained for the succinate- and butyrate-supplemented treatments = $1.96 \times 10^{-12}$ and $1.87 \times 10^{-12}$ mol e$^-$ cell$^{-1}$ h$^{-1}$, respectively, which for both the cases is 0.44% of $v_e^{-maxO_2}$. To investigate the impact of $v_e^{-min}$ on the model behaviour ($r_{Na}$ and $r_{Ni}$, Eqs 1, 2, 4, 5, 6 and 7), sensitivity analyses were performed by simulating the model with $v_e^{-min}$ corresponding to [O$_2$]$_{aq} = 5 \times 10^{-9}, 5 \times 10^{-9}$, and 0 mol L$^{-1}$ (see Results/Discussion).

### Results/Discussion

**Low probabilistic initiation of nar transcription, resulting in the fraction of the population with Nar < 100%**

To test the assumption of a single homogeneous population with all cells producing Nar in response to O$_2$ depletion, we simulated the model with the specific probability for a Z$^-$ cell to initiate nar transcription ($r_{Na}$) = 4 h$^{-1}$. This resulted in 98% of the cells possessing Nar within an hour (see Eqs 1–3). Evidence suggests that less than half an hour is required to synthesise denitrification enzymes [17,18], but an hour’s time is assumed here to allow margin for error. The results show that, for all the treatments, the simulated NO$_2^-$ production (mol vial$^{-1}$) grossly overestimates that measured (Fig 3).

To find a reasonable parameter value, we optimised $r_{Na}$ for the 0% O$_2$ treatments, so that the simulated NO$_2^-$ production matches that measured. The results (Table 3) suggest that a low probabilistic initiation of nar transcription (average $r_{Na} = 0.035$ h$^{-1}$) is adequate to simulate the
measured NO\textsubscript{2}^- kinetics (Fig 3). In the Butyrate, 7% O\textsubscript{2} treatment (Fig 3B), the simulated NO\textsubscript{2}^- starts earlier, but the rate of accumulation is similar to that measured.

Once O\textsubscript{2} falls below a certain threshold, the production of Nar is assumed to trigger with \( r_{Na} = 0.035 \, \text{h}^{-1} \) and last until a minimum of respiration is sustained by the \( e^- \)-flow to O\textsubscript{2} and N\textsubscript{2}O (\( ve_{O_{2}}^- \) and \( ve_{N_{2}O}^- \)), assumed to fulfil the ATP needs for Nar production (Eqs 1 and 2). But
the production of Nar sustained by $v_{e^{-}}/C_{0}$N$_{2}$O was inconsequential for simulating the measured NO$_{3}^{-}$ production, since NO$_{3}^{-}$ was already exhausted when N$_{2}$O started accumulating (i.e., when $v_{e^{-}}/C_{0}$N$_{2}$O $> 0$). For this reason, the fraction that produced Nar (F$_{Na}$, Eq 3 and Table 4) is calculated as functional ($= 0.23$–$0.43$) and theoretical ($= 0.56$–$0.81$), where the first is the fraction actually responsible for NO$_{3}^{-}$ production (sustained by $v_{e^{-}}/C_{0}$O$_{2}$), but the latter also incorporates the fraction that produced Nar after the exhaustion of NO$_{3}^{-}$ (sustained by $v_{e^{-}}/C_{0}$O$_{2}$ as well as $v_{e^{-}}/C_{0}$N$_{2}$O). The rationale behind calculating the theoretical F$_{Na}$ is the empirical data indicating that Nar transcription is not turned off in response to NO$_{3}^{-}$ depletion [18]. Although our model cannot test the theoretical F$_{Na}$, but the functional F$_{Na}$ suggests that, contrary to the common assumption, the measured NO$_{3}^{-}$ kinetics can be neatly explained by only 23–43.3% of the population producing Nar in response to O$_{2}$ depletion.

**Very low probabilistic initiation of nirS transcription**

When we optimised the specific probability of nirS transcriptional activation ($r_{Ni}$, see Eqs 4, 5, 6 and 7) to fit the measured data, the average $r_{Ni} = 0.004$ h$^{-1}$ (Table 3) adequately simulated the measured NO$_{3}^{-}$ depletion and N$_{2}$ accumulation (Fig 4). The recruitment to denitrification lasted for 19.5–47.3 h, i.e., the time when [O$_{2}$] was below a critical concentration and the velocity of $e^{-}$-flow to O$_{2}$ and the relevant NO$_{3}^{-}$/NO$_{2}$ remained above a critical minimum (Eqs 4, 5, 6 and 7). The resulting fraction recruited to denitrification (F$_{Ni}$, see Eq 8 and Table 4) was 0.08–0.18, the bulk of which depended on the $e^{-}$-flow to NO$_{3}^{-}$ and N$_{2}$O (instead of aerobic respiration).

To test whether the measured data could be explained without the recruitment sustained by NO$_{3}^{-}$ and N$_{2}$O respiration, we also simulated the model with the recruitment as a function of

**Table 3. Specific-probability of nar and nirS transcriptional initiation ($r_{Na}$ and $r_{Ni}$, respectively) estimated for each treatment by optimisation (best match between the simulated and measured data).**

| Batch | C-source | Treatment*: O$_{2}$ (vol.%), NO$_{3}^{-}$ (mM) | Optimal $r_{Na}$ (h$^{-1}$) | Optimal $r_{Ni}$ (h$^{-1}$) |
|-------|----------|---------------------------------------------|-----------------------------|-----------------------------|
| 1     | Butyrate | $-0, 2$                                     | 0.041                       | 0.005                       |
| 2     | Butyrate | $7, 2$                                      | $-$                         | 0.004                       |
| 3     | Succinate| $-0, 2$                                     | 0.030                       | 0.005                       |
| 4     | Succinate| $7, 2$                                      | $-$                         | 0.003                       |
|       |          |                                             | Avg. = 0.035                | Avg. = 0.004                |

*Treatment refers to the C-source, initial oxygen concentration in the headspace (measured as headspace-vol.%), and initial NO$_{3}^{-}$ concentration in the medium (mM). doi:10.1371/journal.pcbi.1004621.t003

**Table 4. The fraction of the population with Nar (F$_{Na}$) and NirS (F$_{Ni}$) estimated based on the optimal specific-probability of nar and nirS transcriptional initiation ($r_{Na}$ and $r_{Ni}$, respectively).**

| Batch | C-source | O$_{2}$ (vol.%), NO$_{3}^{-}$ (mM) | Functional F$_{Na}$* (unitless) | Theoretical F$_{Na}$* (unitless) | F$_{Ni}$ (unitless) |
|-------|----------|----------------------------------|----------------------------------|----------------------------------|---------------------|
| 1     | Butyrate | $-0, 2$                          | 0.433                            | 0.813                            | 0.221               |
| 2     | Butyrate | $7, 2$                           | 0.343                            | 0.656                            | 0.088               |
| 3     | Succinate| $-0, 2$                          | 0.357                            | 0.803                            | 0.206               |
| 4     | Succinate| $7, 2$                           | 0.230                            | 0.564                            | 0.077               |

*Functional F$_{Na}$ is the fraction of cells expressing Nar while NO$_{3}^{-}$ is still present, while Theoretical F$_{Na}$ is the fraction expressing Nar when including the theoretical recruitment after NO$_{3}^{-}$ depletion (supported by energy from N$_{2}$O reduction). doi:10.1371/journal.pcbi.1004621.t004
O2 alone and re-optimised rNi, which on average increased to 0.012 h⁻¹ (providing FNi = 0.083 – 0.35). This was expected since O2 is exhausted rather quickly, shrinking the time-window available for the recruitment. Comparatively, these simulations were less satisfactory: using the average rNi = 0.012 h⁻¹ generally resulted in larger deviations than for the default simulations (S2 Fig), and the optimal rNi for individual treatments varied grossly (50% higher values for the

**Fig 4. Comparison of measured and simulated data assuming stochastic initiation of nirS transcription.** Each panel compares the measured NO₃⁻ depletion (sub-panel) and N₂ accumulation (main panel; n = 3–4) with simulations. The simulations are carried out with an optimised specific-probability of nirS transcriptional initiation (average rNi = 0.004 h⁻¹, Eqs 4, 5, 6 and 7), allowing 7.7–22.1% of the population to produce NirS + cNor (Eq 8) during the available time-window (= 19.5–47.3 h).

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~0% O₂ treatments than for the 7% O₂ treatments). This contrasts the default simulations, where the optimal rNi values for individual treatments were quite similar.

### Sensitivity of r_{Na} and r_{Ni} to \( \frac{v_{e}}{C_{0}} \)_{min}

Recruitment to denitrification (both nar and nirS transcription) is assumed to continue only as long as the combined e⁻-flow to O₂, NO₃ and N₂O is greater than \( \frac{v_{e}}{C_{0}} \)_{min} (Eqs 1, 2, 4, 5, 6 and 7). To test the model’s sensitivity to this parameter, we estimated r_{Na} and r_{Ni} by optimisation for different values of \( \frac{v_{e}}{C_{0}} \)_{min}, relative to the default value = 1.95×10⁻¹⁷ mol e⁻·cell⁻¹·h⁻¹. For all cases, the model was able to adequately simulate the measured N₂ kinetics by moderate adjustments of r_{Na} and r_{Ni}. Table 5 shows the average optimal values of r_{Na} and r_{Ni}, obtained by fitting the simulated N₂ kinetics to the data, for different values of \( \frac{v_{e}}{C_{0}} \)_{min}. S3 Fig shows adequate simulations of the measured N₂ kinetics assuming \( \frac{v_{e}}{C_{0}} \)_{min} = 0, with optimised r_{Na} = 0.033 h⁻¹ and r_{Ni} = 0.0033 h⁻¹. Thus, although assuming \( \frac{v_{e}}{C_{0}} \)_{min} > 0 appears logical, it is not necessary to explain the measured data.

### N₂O kinetics

To simulate the N₂O kinetics, we used \( v_{e_{max_{N₂O}}} = 5.5\times10^{-15} \) mol e⁻·cell⁻¹·h⁻¹, empirically determined under similar experimental conditions as simulated here [24], and adopted the literature values for K_{m_{N₂O}} = 5 and 7 μM [42,43, respectively]. But with K_{m_{N₂O}} = 5 μM, the model predicted N₂O accumulation ~10–20 times higher than measured for the ~0% and ~2–3 times higher for the 7% O₂ treatments (Fig 5). This forced us to simulate the model with the parameter value estimated by optimisation, providing the average K_{m_{N₂O}} = 0.6 μM.

The measured N₂O shows a conspicuous increase throughout the entire active denitrification period, and this phenomenon is neatly captured by the model. The reason for this model prediction is that the number of N₂O producing cells (Z_{NaNi} + Z_{Ni}, Fig 2A) is low to begin with compared to the number of N₂O consuming cells (Z⁻ + Z_{Na} + Z_{NaNi} + Z_{Ni}), but the fraction of N₂O producers will increase during the anoxic phase for two reasons: one is the recruitment to Z_{NaNi} & Z_{Ni}, another is the fact that the model predicts approximately three times faster cell-specific growth rate for Z_{NaNi} & Z_{Ni} than for Z⁻ & Z_{Na} (\( v_{e_{max_{N₂O}}} \)) is identical for all groups, while \( v_{e_{NO}} \) and \( v_{e_{NO}} \) are both zero for Z⁻ & Z_{Na} but for Z_{NaNi} & Z_{Ni}, it holds that \( v_{e_{NO₂}} \approx v_{e_{NO}} > v_{e_{N₂O}} \). To illustrate this phenomenon, we ran the model, assuming that the Z⁻ & Z_{Na} cells had no N₂O reductase, resulting in a) constant N₂O concentration throughout the entire anoxic phase and b) much higher N₂O concentrations than measured (Fig 5). The overestimation is a trivial result, easily avoidable by increasing \( v_{e_{max_{N₂O}}} \) or decreasing K_{m_{N₂O}} moderately. However, the prediction of a constant N₂O concentration is clearly in conflict with the experimental data.

| \( \frac{v_{e}}{C_{0}} \)_{min} (mol e⁻·cell·h⁻¹) | Optimal r_{Na} (h⁻¹) | Optimal r_{Ni} (h⁻¹) |
|---|---|---|
| 5 x Default* | 0.041 | 0.0062 |
| Default | 0.035 | 0.0041 |
| 0.5 x Default | 0.034 | 0.0035 |
| 0 | 0.033 | 0.0033 |

*Refers to the default value = 1.95×10⁻¹⁷ mol e⁻·cell⁻¹·h⁻¹.

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data, and no parameterisation could force the model to reproduce this phenomenon other than the differential expression of denitrification genes.

Hence, although there is room for further refinements, our default assumption regarding differential expression of NirS and NosZ explains the observed N\textsubscript{2}O kinetics: 1) abrupt initial accumulation to very low levels due to recruitment of relatively small numbers to the N\textsubscript{2}O
producing pools ($Z^{NaNi}$ & $Z^{Ni}$), and 2) increasing N$_2$O concentration due to recruitment and faster cell-specific growth of $Z^{NaNi}$ & $Z^{Ni}$ than that of the cells only consuming N$_2$O ($Z^- + Z^{NaNi}$).

This modelling exercise sheds some light on the possible role of regulatory biology of denitrification in controlling N$_2$O emissions from soils. If all cells in soils had the same regulatory phenotype as $Pa$. denitrificans, their emission of N$_2$O would probably be miniscule, and soils could easily become strong net sinks for N$_2$O because the majority of cells would be ‘truncated denitrifiers’ with only N$_2$O reductase expressed. It remains to be tested, however, if the regulatory phenotype of $Pa$. denitrificans is a rare or a common phenomenon among full-fledged denitrifiers. We foresee that further exploration of denitrification phenotypes will unravel a plethora of response patterns.

**Conclusion**

Using dynamic modelling, we have demonstrated that the denitrification kinetics in $Pa$. denitrificans can be adequately explained by assuming low probabilistic transcriptional activation of the nar and nirS genes and a subsequent autocatalytic expression of the enzymes. Such autocatalytic gene expressions are common in prokaryotes, rendering a population heterogeneous because of the stochastic initiation of gene transcription, with a low probability [45]. For N$_2$O kinetics, our hypothesis was that a) the gas is produced by a fraction of the incubated population that is able to initiate nirS transcription with a certain probability, leading to a coordinated expression of nirS + nor via NO [16], and b) N$_2$O is consumed by the entire population because, in response to anoxia, nosZ is readily induced by FnrP [24]. Our model corroborated this hypothesis by reasonably simulating the N$_2$O kinetics with the specific-probability of nirS transcriptional activation = 0.004 h$^{-1}$, resulting in 7.7–22.1% of the population producing NirS + cNor (hence N$_2$O), but all cells producing NosZ (hence equally consuming N$_2$O).

**Supporting Information**

**S1 Dynamic Model.** The folder contains the dynamic model used in this study ‘Hassan_et_al_2015_Pa_.denitrificans.mdl’. The model requires Vensim (Double Precision), which is available at [http://vensim.com/free-download/](http://vensim.com/free-download/). The zip folder also contains files with the empirical data; these files are automatically loaded into the model when it is run. (ZIP)

**S1 Fig. $Pa$. denitrificans gas and NO$_2^-$ kinetics.** Typical gas kinetics (O$_2$, NO, N$_2$O, N$_2$) and NO$_2^-$ accumulation in $Pa$. denitrificans during the transition from aerobic respiration to denitrification; batch cultures, n = 3; 20°C; Sistrom’s medium; 2 mM KNO$_3$ and 7 vol% initial O$_2$ in the headspace. All the available NO$_3^-$ (100 μmol vial$^{-1}$) was recovered as NO$_2^-$ before the onset of N-gas production. In previous experiments [17], N$_2$O concentrations were below the detection limit of the system, but thanks to a new system with electron capture detector, the N$_2$O kinetics were monitored with reasonable precision. Adapted from [18]. (TIF)

**S2 Fig.** Comparison of measured and simulated data assuming stochastic initiation of nirS transcription with aerobic respiration being the only energy source for producing NirS + cNor. In each panel, the measured NO$_2^-$ depletion (sub-panel) and N$_2$ accumulation (main panel; n = 3–4) are compared with simulations. The simulations here are to be compared with the default simulations (Fig 4), which were run assuming that the coordinated NirS + cNor production (via nirS transcriptional activation) is sustained by the energy generated by O$_2$ as well as NO$_3^-$ and/or N$_2$O reduction. The default simulations provided an average specific-probability of nirS transcriptional activation ($r_{Ni}$) = 0.004 h$^{-1}$ (Eqs 4, 5, 6 and 7) by optimisation,
allowing 7.7–22.1% of the population to produce NirS + cNor (Eq 8) in 19.5–47.3 h. To match the measured data here, the average \( r_{\text{Ni}} \) had to be raised to 0.012 h\(^{-1}\), since the time available for the enzyme synthesis shrank (= 3.5–16 h) due to a rapid exhaustion of O\(_2\). Comparatively, the assumption that the ATP from NO\(_3\) and/or N\(_2\)O reduction should help cells produce denitrification enzymes seems more plausible and provide better agreement with the measured data.

(TIF)

S3 Fig. Measured vs. simulated N\(_2\) kinetics assuming \( v e_{\text{min}} = 0 \). The default simulations are carried out assuming that for a cell to produce first molecules of Nar and NirS, a minimum of e\(^+\)-flow to an available e\(^+\)-acceptor (\( ve_{\text{min}} \), mol e\(^+\) cell\(^{-1}\) h\(^{-1}\)) is necessary to generate a minimum of ATP required for protein synthesis (Eqs 1, 2, 4, 5, 6 and 7). Although assuming \( ve_{\text{min}} > 0 \) seems logical, the measured N\(_2\) kinetics are adequately simulated here with \( ve_{\text{min}} = 0 \). This shows that the assumption is not necessary to explain the measured data.

(TIF)

Author Contributions
Conceived and designed the experiments: LLB LRB. Performed the experiments: ZQ LLB. Analyzed the data: JH LRB. Contributed reagents/materials/analysis tools: JH. Wrote the paper: JH LRB.

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