Modelling denitrification as an electric circuit accurately captures electron competition between individual reductive steps: the ASM-Electron Competition model

Domingo-Felez, Carlos; Smets, Barth F.

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1 **Title**

2 Modelling Denitrification as an Electric Circuit Accurately Captures
3 Electron Competition Between Individual Reductive Steps: The ASM-
4 Electron Competition Model.

5
6 **Author list**

7 Carlos Domingo-Félez*, Barth F. Smets*
8
9

10 **ABSTRACT**

11 Heterotrophic denitrification consists of the four-step sequential reduction of nitrate to dinitrogen gas over
12 nitrite, nitric oxide and nitrous oxide. Oxidation processes, commonly of organic compounds, provide the
13 electrons needed for the sequential reaction steps. The intracellular electron distribution is a competitive
14 process among the four reduction steps. In this study, a model describing organic carbon oxidation and four-
15 step denitrification through electron competition is proposed (Activated Sludge Model – Electron
16 Competition, ASM-EC). The model describes denitrification rates as an analogy to how current intensity
17 varies through a parallel set of resistors in electric circuits.¹ The ASM-EC model was calibrated with data
18 from batch experiments with heterotrophic denitrifying communities where reduction of mixtures of nitrogen
19 oxides was monitored while different carbon sources were supplied in excess. The carbon sources included
20 methanol, ethanol, acetate, and their ternary mixture. The electron distribution preference and electron
21 uptake rates varied between the carbon sources and were captured by the model structure for most of the
22 experiments. The ASM-EC model uses fewer parameters compared to existing state-of-the-art denitrification
23 models and performed equally well in the tested scenarios. We advocate the use of this model for
24 denitrification in the activated sludge model, which can easily be integrated in existing model structures, as it
25 provides a parsimonious description of electron competition during denitrification.

1. INTRODUCTION

In residual water treatment operations nitrogen oxides (NO_x^-) can be biologically reduced to dinitrogen gas (N_2) in the presence of electron donors, by heterotrophic denitrifying bacteria, which can also respire oxygen.²

Denitrification in biological wastewater treatment

Nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) are obligate intermediates during nitrate (NO_3^-) reduction to N_2 ; NO_2^- and NO can be toxic for cell metabolism.³ The four-step reduction is carried out by enzymes encoded by the *nar* (and *nap*), *nir*, *nor* and *nos* genes; NAR is a membrane-bound enzyme while NOR, NIR and NOS are located in the periplasm.^{4,5} Although inorganic compounds such as hydrogen can be used as electron donors by chemolithoautotrophs, the majority of electron donors in treatment processes are organic compounds, used by chemoorganoheterotrophs.

Electrons are made available by the oxidative transformations of the organic compounds and are captured by electron carriers like FAD, FMN, or NAD. They are subsequently delivered to the respiratory electron transport chain in the ubiquinone/ubiquinol pool and branched to: nitrate reductases (NAR, NAP), cytochrome ba_3 oxidase, and cytochrome bc_1 complex.² Similarly, electrons are branched from the reduced cytochrome *c* to, among others, the nitrite, nitric oxide, and nitrous oxide reductases.⁶ These branches compete for a limited flow of electrons supplied by electron-transfer flavoproteins.⁷ Thus, the rate of individual nitrogen oxide reductions (e.g. nitrate) can be influenced by the presence of other terminal acceptors (i.e. nitrite, nitric oxide, nitrous oxide).⁷

The microbial composition, denitrification rates and biomass growth yields can vary significantly with the addition of different external carbon sources.⁸ For example, methanol oxidizing bacteria typically represent a small fraction of the denitrifying guild in wastewater treatment communities but increase after an adaptation to methanol dosage.^{9,10} Hence, competition for electrons from the rate limiting carbon oxidation also depends on the carbon source and the denitrifying gene pool composition.

50 **Modelling heterotrophic denitrification**

51 Early kinetic models describing heterotrophic denitrification were informed by pure culture studies and
52 described each denitrification step with Michaelis-Menten equations.¹¹ This approach considers the rate of
53 every reduction step as independent from others; and is the basis of the Activated Sludge Model for Nitrogen
54 (ASMN), widely used to model wastewater treatment processes.¹² Substrate inhibition by NO_2^- or NO were
55 later added.^{12,13} Experimental studies have observed that maximum reduction rates for any NO_x were higher
56 than in the presence of other NO_x .^{14,15} Even under non rate limiting carbon concentrations the carbon
57 oxidation rate can limit the four reduction rates which compete for electrons. However, the ASMN model
58 structure assumes that carbon oxidation supplies all the electrons necessary for the four denitrification steps
59 and models denitrification with non-competitive kinetics.

60 Grant and Pattey, (1999) developed a more accurate model where a maximum electron supply is distributed
61 among electron acceptors, improving the predictions of ASMN with a similar set of equations.¹⁶ Preference
62 in reduction rate is given to the most oxidized nitrogen species in a feed-back redox control, but assumes no
63 competition of NO_3^- reduction with NO_x .

64 Thomsen et al., (1994) described electron competition first via use of common electron mobile carriers as
65 additional state variables, but did not validate their model.¹⁷ Almeida et al., (1997) proposed an analogy
66 between electron flow through an electric circuit and the electron flow through the respiratory chain for 3-
67 step denitrification, not including NO reduction.¹ The model was fitted to experimental results from studies
68 with *Pseudomonas fluorescens* fed with NO_3^- and acetate where one intermediate, NO_2^- , accumulated. The
69 model predicted NO_2^- and N_2O accumulation from *Paracoccus denitrificans* fed with NO_3^- and acetate, but
70 simulations used arbitrary values for the model parameters. Overall, Almeida et al. (1997) validated the
71 capability of the model structure to describe electron competition during denitrification, but the
72 identifiability of the estimated parameters was not assessed. Also, the model was not evaluated with a mixed
73 denitrifying community or for the effect of different carbon sources.

74 Recently, the ASM-ICE (Indirect Coupling of Electrons) model was introduced to describe electron
75 competition of a methanol-enriched denitrifying community,¹⁸ in line with the theoretical approach by

Thomsen et al., (1994). The ASM-ICE model explicitly calculates the concentration of internal electron carriers as done by Thomsen et al., (1994), uncoupling the carbon oxidation and denitrification processes over one branch. The ASM-ICE model captures the electron competition at a cost of higher model complexity with additional state variables and model parameters compared to the ASMN model (17 and 16 respectively). While the ASM-ICE fit was reported as more accurate than ASMN, possible overparameterization, or the marginal improvement in model fit with increasing model complexity (i.e. number of parameters) was not examined.¹⁹

In this study, we (1) examine whether the model structure developed by Almeida et al., (1997) can be expanded to describe the 4-step denitrification and aerobic carbon oxidation processes – as an analogy to electric circuits; (2) and can be calibrated with experimental results from mixed denitrifying microbial communities as well as pure cultures; and we (3) compare the electron distribution rates for four different carbon sources across the four denitrification enzymes; and (4) contrast the performance of the model developed with other state-of-the-art denitrification models.

2. MATERIALS AND METHODS

2.1. MODEL DEVELOPMENT - ASM-EC

A model structure describing 4-step denitrification, aerobic respiration, and organic carbon oxidation is derived from the analogy between electron competition during respiration and electron distribution in a multi-resistor electric circuit (**Figure 1**).¹

A potential ($E_{X,HB}$, gVSS/m³) is created by the presence of heterotrophic denitrifying bacteria and an electron donor/electron acceptor pair. Reaction rates ($Rate_i$, $i=OrgC, NAR, NIR, NOR, NOS, O_2r$, g/(m³·h)) are analogous to the current intensity (i_i) through a resistor (Ohm's Law). The resistance of the resistor i (r_i , gVSS·h/g) is considered to depend inversely on the concentration of the corresponding substrate (S_i), which we model using a saturation function ($K_{S,i}$) and a minimum resistance (R_i) (**Eq. 1**). As the substrate is depleted the resistance increases, towards infinite, and no current flows, while at excess substrate concentrations ($S_i \gg K_{S,i}$) the resistance attains the minimal value (R_i), which corresponds to the inverse of the maximum specific reaction rate ($Rate_i$).

$$r_i = R_i \cdot \frac{(S_i + K_{S,i})}{S_i} \cdot \frac{(S_{j,\text{inhib}} + K_{S,j,\text{inhib}})}{K_{S,j,\text{inhib}}} \left(\frac{gVSS \cdot h}{g} \right) \quad (\text{Eq. 1})$$

For example, in the presence of organic carbon but absence of all electron acceptors, the overall circuit resistance is very large and negligible current flows ($r_{\text{Donor}} \downarrow$, $r_{\text{Acceptor}} \uparrow$) (i.e. negligible reaction rates). Addition of an electron acceptor such as oxygen increases the electron flow, the exact value of which depends on the carbon and oxygen concentrations and their respective maximum rates ($r_{\text{Donor}} \downarrow$, $r_{\text{Acceptor}} \downarrow$). When a substrate reaches limiting concentrations ($S_i = K_{S,i}$), or when an inhibitor is present ($S_j = K_{S,j,\text{inhib}}$), its resistance will increase ($r_i = 2R_i$), halving the maximum specific reaction rate.

The competition for electrons between two denitrifying enzymes (e.g. *nar*, *nir*) is analogous to electron flow through parallel resistors. The individual resistances vary with the substrate concentrations (NO_3^- and NO_2^-) (**Eq. 1**), and the electron current released in carbon oxidation (i_{OrgC}) will be distributed between the NO_3^- vis-a-vis NO_2^- reduction rates (i_{NO_3} , i_{NO_2}). Following conservation of potential (**Eq. 2**) and conservation of charge (**Eq. 3**), the current through any resistor can be calculated.

$$E_{\text{circuit}} = E_D + E_A = i_{\text{circuit}} \cdot r_{\text{circuit}} = i_{\text{circuit}} \cdot (r_D + r_A) \quad (\text{Eq. 2})$$

$$i_{\text{circuit}} = i_D = i_{\text{OrgC}} = \sum i_A = i_{\text{O}_2} + \sum i_{\text{NOX}} \quad (\text{Eq. 3})$$

Almeida et al., (1997) assumed that the oxidation of the primary electron source was not rate limiting and the electron transport chain acted as a resistor of constant value ($r_D = R_{\text{Quinones}}$). The ASM-EC accounts for rate limitations by both electron donors and electron acceptors, making this assumption unnecessary. The circuit potential corresponds to the concentration of biomass, and the distribution of potential in the circuit between e-donors (E_D) and e-acceptor (E_A) will be determined by the relative value of the two in-series resistors ($r_{\text{circuit}} = r_D + r_A$).

The model describes four-step denitrification where NO_3^- , NO_2^- , NO and N_2O are sequentially reduced and oxygen acts as inhibitor. Under aerobic conditions oxygen reduction enhances while denitrification slows. Compared to the structure proposed by Almeida et al., (1997) the model is extended to include NO reduction and aerobic organic carbon removal. The values of all circuit resistors are calculated, from which the current through each electron acceptor (i.e. the process rate), can be derived (**Eq. 4**). Based on the conservation of charge the oxidation rate is equivalent to the sum of the reduction rates (**Eq. 3, 5**).

$$\text{Rate}_i = E_{X,HB} / \left(r_{\text{OrgC}} \cdot \left(\sum_{j=1}^k \frac{r_i}{r_j} \right) + r_i \right) \quad \left(\frac{\text{g}}{\text{m}^3 \cdot \text{h}} \right) \quad (\text{Eq. 4})$$

$$\text{Rate}_{\text{OrgC}} = \sum_i \text{Rate}_i \quad \left(\frac{\text{g}}{\text{m}^3 \cdot \text{h}} \right) \quad (\text{Eq. 5})$$

Where i corresponds to each electron acceptor (O_2 , NO_3^- , NO_2^- , NO , N_2O), k is the number of electron acceptors, $E_{X,HB}$ is the biomass concentration, and r_i the individual substrate-limited resistances (**Eq. 1**).

Detailed derivation of model equations and examples of model circuits can be found elsewhere (**S1**).

A second cell structure, which more accurately represents the electron flow inside the cell, was investigated (**Figure 1B**)¹. The oxidation of organic carbon provides electrons from the cytoplasm in parallel to three resistors: oxidase, nitrate reductase, and cytochrome c . Electrons are distributed from the cytochrome c in parallel to the periplasmic nitrite, nitric oxide, and nitrous oxide reductases. Hence, compared to ASM-EC, Model_{per} adds a resistor, R_{per} , that precedes the reduction of NO_2^- , NO and N_2O (**S1**).^{6,7} Model simulations were performed in the Matlab environment (The Mathworks Inc., Natick, USA).

2.2. EXPERIMENTAL CONDITIONS FOR MODEL CALIBRATION

First, model simulations were performed to validate the capability of the ASM-EC model structure to describe electron competition. In a batch reactor, electron acceptors were spiked consecutively (10 mgN/L, mgCOD/L) individually or in combinations under non-limiting soluble organic carbon concentrations consisting of NO_3^- , NO_2^- , N_2O , $\text{NO}_3^- + \text{NO}_2^-$, $\text{NO}_3^- + \text{NO}_2^- + \text{N}_2\text{O}$, $\text{NO}_3^- + \text{O}_2$. Parameters values for minimum resistances (R_i) were user-defined. Model simulated substrate and electron consumption rates were calculated (**S2**).

Previously published batch test results from three different enriched denitrifying cultures were considered for model calibration (Summary in **Table 1**). Detailed information about the cultures can be found elsewhere.^{14,15,20}

The first set of denitrification rates experiments considered from a methanol-fed denitrifying culture.¹⁵ Carbon and nitrogenous concentrations were non-limiting throughout the experiments. Initial batch conditions for nitrogen species were defined as: three individually (NO_3^- , NO_2^- , N_2O), three pairwise ($\text{NO}_3^- +$

152 N_2O , $\text{NO}_2^- + \text{N}_2\text{O}$, $\text{NO}_3^- + \text{NO}_2^-$) and one with all three ($\text{NO}_3^- + \text{NO}_2^- + \text{N}_2\text{O}$). From reported rates and initial
153 batch conditions we generated in-silico time series for NO_3^- , NO_2^- and N_2O (S2).

154 The second set of experiments consisted of the same experimental design (nitrogen oxide additions under
155 non-limiting carbon concentrations) for a denitrifying culture fed with a mixture of acetate, ethanol, and
156 methanol.¹⁴ Four different carbon sources were considered (acetate, ethanol, methanol, carbon mix [equal
157 acetate, ethanol, methanol]), and NO_3^- , NO_2^- and N_2O were added (individually, pairwise, or all three). Batch
158 tests were performed at least in duplicates. N_2O liquid concentration was monitored online and NO_3^- , NO_2^- ,
159 and VSS via grab samples.

160 The third set of experiments consisted of batch tests with a pure culture of *Paracoccus pantotrophus* (ATCC
161 35512).²⁰ Experiments were performed at room temperature, with acetate as an electron donor and carbon
162 source, and NO_3^- or NO_3^- plus N_2O as electron acceptors. NO_3^- , NO_2^- , acetate and biomass concentrations
163 were measured via grab samples, while N_2O was always non-limiting and not measured.

164 The first and second set of experiments represent extant biodegradation kinetics with low substrate-to-
165 biomass conditions ($\text{S/X} \approx 0.04\text{-}0.1$), while the third experiment describes growth curves with high substrate-
166 to-biomass conditions ($\text{S/X} \approx 5\text{-}50$).²¹

167 2.3. PARAMETER ESTIMATION

168 The observed nitrogen oxide reduction rates in the mixed culture datasets^{14,15} for each carbon source
169 (Methanol, Ethanol, Acetate, C-mix) were significantly different. Hence, datasets from each combination
170 (experiment, carbon source) were used to estimate a set of unique parameters ($\theta_{\text{EI.Methanol}}$, $\theta_{\text{EII.Acetate}}$, $\theta_{\text{EIII.Ethanol}}$,
171 $\theta_{\text{EIV.Methanol}}$, $\theta_{\text{EII.C-Mix}}$, $\theta_{\text{EIII.Acetate}}$). The sensitivity of the model variables measured (NO_3^- , NO_2^- , N_2O) was
172 largest to the minimum resistance parameters, R_i , which were estimated for each dataset: R_{NAR} , R_{NIR} , and
173 R_{NOS} . Because bulk concentrations of NO are typically low we assumed that the NO reduction (R_{NOR}) was
174 faster than NO_2^- reduction ($R_{\text{NOR}} \ll R_{\text{NIR}}$).²² Parameter values for substrate affinity, substrate inhibition, and
175 growth yield were considered from literature.¹² The resistance imposed by the carbon source oxidation,

176 R_{OrgC} , was fixed for all experiments as no carbon datasets were available to allow the direct comparison of
 177 each parameter set (for more details see section 3.3.). The error function for the problem minimization was
 178 defined as:

$$179 \quad RMNSE = \sum_k^m \sum_j^n \frac{RMSE_j}{\bar{y}_{obs,j}}; \quad RMSE_j = \sqrt{\frac{\sum_i^p (y_{sim,i} - y_{obs,i})^2}{p}}$$

180 Where m is the number of experiments in one scenario (e.g. 3 biological replicates in scenario E, Table 1), n
 181 the number of data series in one experiment (e.g. NO_2^- and N_2O), p experimental points of each data series,
 182 $y_{sim,i}$ the model predicted data and $y_{obs,i}$ the experimental data at time i . Parameters relating to the elemental
 183 biomass composition (e.g. i_{NXB}) and temperature coefficients were fixed at default values and not subject to
 184 estimation. A global search algorithm (Global Search) was initiated over a wide parameter space (2 orders of
 185 magnitude) from user-defined values. The approximate confidence regions and pairwise correlations were
 186 calculated from multiple local minimizations (Pattern Search) following Beale (1960), and results from at
 187 least 50 local solutions within the 95% critical value that defines the surface of the parameter uncertainty
 188 region were approximated to normal distributions (S3).

189 The Akaike's Information Criterion (AIC)²⁴ was calculated to elucidate whether more complex model
 190 structures such as ASM-ICE ($p_{ICE} = 17$) improve the predicting capabilities significantly compared to a
 191 simpler model structure as ASM-EC ($p_{EC} = 12$). For a given dataset with n number of points, a likelihood
 192 function (SS_i) for two models, A and B , with different number of parameters ($p_A < p_B$) can be calculated. The
 193 AIC concludes that a simpler model A , can perform equally well than a more complex model B for a larger
 194 likelihood function of model A compared to model B (Eq. 6). This relationship indicates how much worse
 195 the likelihood function of A can be compared to that of B ($SS_A > SS_B$).

$$196 \quad \frac{SS_A}{SS_B} = \left(\frac{n-p_A}{n-p_B} \right) \cdot \exp \left(\frac{2(p_B-p_A)}{n} \right) \quad (\text{Eq. 6})$$

197 Because high frequency data acquisition can lead to autocorrelated residuals ($y_{sim} - y_{obs}$), which violate the
 198 assumption of residual independency,²⁵ autocorrelation of N_2O data points in Experiment II was minimized

199 by lowering the data acquisition frequency from 0.2 to 0.017 Hz. Uncertainty from best-fit parameters
200 estimates was propagated to model outputs.²⁶ Latin hypercube sampling was used to cover the parameter
201 space (n = 500). The effect of directly estimated parameter uncertainty was evaluated by Monte Carlo
202 simulations. For a list of abbreviations, see **S1**.

203 **3. RESULTS AND DISCUSSION**

204 **3.1. THE ANALOGY OF ELECTRIC CIRCUITS TO DENITRIFICATION CAPTURES** 205 **ELECTRON COMPETITION**

206 Simulations of the ASM-EC model were performed to assess its capability to predict electron competition
207 during denitrification. Under non-limiting concentrations of organic carbon addition of individual
208 nitrogenous spikes (NO_3^- , NO_2^- , N_2O) leads to maximum substrate consumption rates and different electron
209 consumption rates (**Figure 2**). The ASM-EC does not describe denitrification rates as additive processes but
210 as rates competing for a maximum electron supply rate. Hence, when combinations of substrates are added
211 ($\text{NO}_3^- + \text{NO}_2^-$, $\text{NO}_3^- + \text{NO}_2^- + \text{N}_2\text{O}$) the specific denitrification rates are lower than the obtained for all
212 individual denitrification steps. The effect of oxygen inhibition on denitrification is also described, where a
213 spike of dissolved oxygen during nitrate reduction leads to preferential electron supply to the oxidase, and
214 lower denitrification rates. As the organic carbon concentration becomes limiting the denitrification rates are
215 gradually reduced down due to a lower electron supply.

216 **3.2. VALIDATION OF THE ASM-EC MODEL STRUCTURE TO DESCRIBE** 217 **DENITRIFICATION (EXPERIMENT I)**

218 The results from Pan et al., (2013a) were used to generate in-silico data for batch experiments with single
219 pulses of NO_3^- , NO_2^- , N_2O , and their combinations (**Table 1, S2**). The model was calibrated with datasets
220 from batches A through E, and validated with F and G. Calibration results show a good fit between model
221 predictions and experimental denitrification rates for the entire Methanol dataset ($R^2 > 0.99$, F-test = 1 for
222 NO_3^- , NO_2^- and N_2O) (**Figure 3, S3**).

223 The estimated values for the minimum resistances were $R_{\text{NAR}} = 3.52 \pm 0.18$, $R_{\text{NIR}} = 1.85 \pm 0.11$, and $R_{\text{NOS}} =$
 224 0.26 ± 0.01 gCOD·h/gN (**Table 2**). The lower value of R_{NOS} compared to R_{NAR} and R_{NIR} indicates a much
 225 faster N_2O compared to NO_3^- or NO_2^- reduction. Simulation of individual substrate spikes (A, B, C) led to
 226 higher reduction rates compared to mixtures of nitrogen oxides (D, E, F, G). For example, the NO_3^- reduction
 227 rate was largest in batch A compared to D, F or G, where NO_3^- was added together with N_2O , NO_2^- or N_2O -
 228 NO_2^- . Under excess electron donor concentrations the maximum NO_3^- reduction rate decreased to 39% (D),
 229 72% (F), and 35% (G) due to the competition for electrons from NO_2^- -, NO -, and N_2O -reductions steps. To
 230 elucidate the fate of electrons released during organic carbon oxidation the electron consumption rate of each
 231 denitrification step was calculated from the simulated true denitrification rates (calculation details in **S2**)
 232 (**Figure 3**). The maximum electron consumption rate of each reduction step (Nar, Nir, Nor, Nos) occurred
 233 for individual substrate spikes (A, B, C) compared to the mixture of nitrogen oxides (D, E, F, G). However,
 234 the total electron consumption rate for each batch did not follow the same pattern as denitrification rates. The
 235 combined addition of NO_3^- and N_2O resulted in an overall higher electron consumption rate compared to
 236 NO_3^- or N_2O individually (D vs. A, C), but not in an additive manner, indicating competition for electrons.
 237 When two nitrogen oxides are available at excess concentrations the value of the equivalent circuit resistance
 238 is always lower than the minimum value R_i . The lower circuit resistance yields higher total but lower
 239 individual electron consumption rates. Hence, the organic carbon oxidation rate depends on the composition
 240 of the electron acceptor pool.

241 **3.3.EFFECT OF ORGANIC CARBON SOURCE ON ELECTRON COMPETITION IN A** 242 **MIXED COMMUNITY (EXPERIMENT II)**

243 Reduction rates of individual nitrogen oxides and their mixtures were measured for four different carbon
 244 sources with a denitrifying biomass fed enriched on a mixture of acetate, ethanol, and methanol.¹⁴ The model
 245 was calibrated for each carbon source with dataset from experiments A-G. For the four carbon sources best-
 246 fit simulations described both the single and combined NO_x^- reduction rates ($R^2 = 0.79 - 0.95$) (**S3**).
 247 Residuals from 10 out of the 12 datasets did not pass the F-test even though the regression between

experimental and modelling results was not poor ($R^2 \geq 0.79$). The model predicted the different specific denitrification rates for each carbon source during NO_3^- reduction, with faster reduction rates with ethanol compared to acetate, the carbon mixture, or methanol ($R_{\text{NAR.EtOH}} < R_{\text{NAR.Acet}} < R_{\text{NAR.CMix}} < R_{\text{NAR.MeOH}}$). NO_3^- and NO_2^- were simultaneously reduced with acetate and methanol, but NO_2^- accumulated with ethanol and the carbon mixture (**Figure 4**).

For the case of acetate as the sole carbon source the estimated values of the three minimum resistances are of similar magnitude ($R_{\text{NAR}} \approx R_{\text{NIR}} \approx R_{\text{NOS}}$). The slightly faster NO_3^- or NO_2^- reduction rate compared to N_2O predicts N_2O accumulation when acetate is the sole carbon source (**Figure 4, 5**). When only ethanol or methanol are fed the predicted NO_2^- reduction rate is significantly lower than the NO_3^- or N_2O reduction rate ($[R_{\text{NAR}}, R_{\text{NOS}}] < R_{\text{NIR}}$). Under ethanol excess, any condition of electron competition would yield a higher NO_2^- accumulation compared to NO_3^- or N_2O (**Figure 4**). For the case of methanol, N_2O reduction has the highest affinity for electrons compared to NO_3^- and NO_2^- ($R_{\text{NOS}} < R_{\text{NAR}} < R_{\text{NIR}}$). When the mixture of carbon sources was in excess the estimated values of the minimum resistances are similar to the case of ethanol, because of the higher R_{NIR} value, NO_2^- would accumulate in the presence of NO_3^- ($R_{\text{NIR}} > [R_{\text{NAR}}, R_{\text{NOS}}]$) (**Figure 4, 5**). Overall, the model captures the effect of carbon source on denitrifying rates, with ethanol and methanol favoring NO_3^- and N_2O reduction and acetate, favoring NO_3^- and NO_2^- reduction.

The predicted electron consumption rates associated with the oxidation of ethanol and acetate occurred, on average, at similar rates, while methanol was oxidized at significantly lower rates (**S3**). The three minimum resistances of methanol are significantly higher than for any other carbon source, corresponding to the lowest specific denitrification rates.

3.4. GROWTH CURVES OF *PARACOCCLUS PANTOTROPHUS* (EXPERIMENT III)

Datasets from Experiment I and II did not include soluble or particulate organic carbon dynamics, and experiments were carried under low substrate-to-biomass conditions (0.01-0.15 gN/gVSS). To test the ASM-EC response towards electron donor (acetate) and biomass concentration the model was fitted to growth

curves from *P. pantotrophus* from Experiment III ($R^2 > 0.8$), with high substrate-to-biomass conditions (70-710 gN/gVSS).²⁰ In the presence of excess acetate, NO_3^- and N_2O , the model describes NO_2^- accumulation during NO_3^- depletion (**Figure 6**). Following the NO_3^- and NO_2^- sequential depletion acetate oxidation is only driven by N_2O reduction, which remains under excess. The estimated minimum resistance values of *P. pantotrophus* indicate a preference for NO_3^- reduction over N_2O and NO_2^- reduction, with NO_2^- accumulating during NO_3^- reduction ($R_{\text{NAR}} < R_{\text{NOS}} < R_{\text{NIR}}$) (**Figure 6**). To describe the acetate and biomass concentration curves (sCOD, $X_{P.pantotrophus}$) the anoxic growth yield was estimated ($Y_{P.pantotrophus} = 0.37 \pm 0.02$ mgCOD/mgN) and showed a good fit between experimental and predicted organic carbon compounds ($R^2 > 0.98$). The ASM-EC model describes concentration dynamics of electron donor, acceptor, and biomass species.

3.5. PARAMETER ESTIMATION RESULTS

Three parameters, R_{NAR} , R_{NIR} , and R_{NOS} , were estimated for every analysed carbon source analysed (**Table 2**). In the electric circuit analogy these parameters represent the minimum resistance to current flow. The average model fit for all datasets was good ($R^2_{\text{avg}} = 0.92$), but the F-test detected a bias or slope error between the 1:1 line for a perfect fit between data and model in 13 out of the 19 individual datasets, which regression or visual analysis does not detect.²⁷ The approximated 95% confidence regions are narrow (CV < 10%) and pairwise correlation coefficients of the estimated parameters below 0.7 for all datasets indicates good identifiability, reported in-depth in **S3**.²⁸

The denitrification pathway is highly modular, with some community structures being very different from the canonical 4-step denitrification.^{29,30} The presence of members with truncated pathways (only able to perform some of the 4-steps) in mixed communities I and II could explain the different maximum rates for NO_3^- , NO_2^- and N_2O reduction (**Table 2**). Under acetate dosing NO_3^- reduction did not lead to NO_2^- build-up by the mixed community in Experiment II ($R_{\text{NAR}} \approx R_{\text{NIR}}$), but did for the pure cultures, *P. pantotrophus* and *Pseudomonas fluorescens*¹ ($R_{\text{NAR}} < R_{\text{NIR}}$).

Competition for electrons occurs in the reduction steps in all the modelled experiments; the rate-limiting step is the oxidation of organic carbon, as seen by the non-additive oxidation rates (**S3**). The oxidation of each electron donor by varying fractions of the community would explain the different rates among carbon sources modelled in Experiment II. The acetate and ethanol oxidation rates were, in average, 7 and 6 fold higher than for methanol, predicted by high resistances for methanol oxidation ($\theta_{EII,Acetate} \approx \theta_{EII,Ethanol} < \theta_{EII,Methanol}$). A study describing the oxidation of biodegradable COD in anaerobic digestion liquor included an additional carbon oxidation process rate to model ethanol dosage, as the reaction rates were significantly different.³¹ Experiments I and II employed mixed denitrifying cultures enriched on methanol (SRT = 10 days) and a carbon mixture (SRT = 20 days, 33% methanol), respectively. A higher relative abundance of specific methanol-degraders in Experiment I compared to Experiment II would explain the lower R_i values ($\theta_{EI,Methanol} < \theta_{EII,Methanol}$) (**Table 2**).

3.6. PARAMETER IDENTIFIABILITY IN ELECTRIC CIRCUIT-BASED DENITRIFICATION MODELS

The proposed ASM-EC model includes six new parameters: the minimum resistor value for an electron donor and five electron acceptors (R_{OrgC} , R_{O_2r} , R_{NAR} , R_{NIR} , R_{NOR} , and R_{NOS}). Following the conservations of potential and charge, the oxidation rate of the electron donor equals the sum of the reduction rates of the electron acceptors. $R_{Acceptor}$ and R_{Donor} (R_{OrgC}) are two resistors in-series, linearly dependent, and cannot be uniquely identified for the case of a single electron donor regardless of data availability (**Eq. 2**). If multiple electron donors are oxidized, the individual oxidation rates are described by parallel resistors (**S1**) and the $R_{Donor,i}$ values are identifiable. Among $R_{Acceptor,i}$ neither R_{NOR} nor R_{O_2r} could be identified from any experiment as no dissolved oxygen or nitric oxide information was available. Hence, for model calibrations only R_{NAR} , R_{NIR} , and R_{NOS} were estimated, and the rest user-defined.

To explore a more accurate description of the electron flow during denitrification compared to ASM-EC, competition between NO_3^- and cytochrome b was included in model $Model_{per}$, described by R_{per} (**Figure 1**).¹ In the study by Almeida *et al.*, (1997) R_{per} was estimated from a dataset where NO_3^- , NO_2^- and N_2O were the

only measurements,¹⁷ yet no identifiability or confidence metrics for the best-fit parameter estimates were reported. The parameter R_{per} is not identifiable for low values ($R_{\text{per}} < \min(R_{\text{NIR}}, R_{\text{NOR}}, R_{\text{NOS}})$) as the circuits in models $\text{Model}_{\text{per}}$ and ASM-EC are equivalent. Calibration results of $\text{Model}_{\text{per}}$ with Experiment I and II showed marginal fitting benefits (0.60% lower error function value). The parameter values for R_{per} , R_{NIR} , and R_{NOS} were highly correlated ($R^2_{\text{avg}} > 0.96$) and uncertain ($\text{CV}_{\text{avg}} = 53\%$), indicating poor identifiability of the R_{per} , R_{NIR} , and R_{NOS} parameter subset. Hence, the $\text{Model}_{\text{per}}$ structure does not improve the prediction capabilities of ASM-EC. Details of the quantitative comparison between ASM-EC and $\text{Model}_{\text{per}}$ are found in **S3.4**.

3.7. NON-COMPETITIVE AND COMPETITIVE HETEROTROPHIC DENITRIFICATION MODELS.

Experimental studies have identified the competitive nature of the reductive denitrification steps, but most models consider non-competitive structures.^{12,32} In non-competitive approaches to model denitrification the oxidation rate of organic carbon is proportional to the presence of nitrogenous substrates.¹¹ Non-competitive models successfully describe single-substrate experiments; and in presence of multiple nitrogen oxides only at limiting concentrations, when the electron demand limits denitrification. As electron demand increases, the reducing power and not the oxidizing power limits denitrification, and non-competitive models overestimate denitrification rates. Hence, for systems where combinations of nitrogen oxides are present at non rate limiting concentrations models require a competitive electron distribution approach.¹⁹

The ASM-ICE model describes electron competition during heterotrophic denitrification with the novelty of modelling artificial internal electron carriers with additional parameters and two state variables.¹⁸ Based on the AIC of Experiment I (42 datapoints, **S2**), the performance of two models can be considered equivalent if the error of simpler (ASM-EC, 12 parameters) was 52% larger than that of the complex model (ASM-ICE, 17 parameters) (**S3**). Direct comparison cannot be made as no statistical metric was reported,¹⁸ but visual inspection of reported simulated denitrification rates by the ASM-ICE model show similar fit to ASM-EC in **Figure 2** ($R^2 > 0.99$, F-test = 1). Hence, in the scenarios evaluated in this study (excess electron donor and

346 electron acceptor) the electric circuit approach performs better than the internal electron carriers. Further
347 evaluation under a wider range of operation conditions (e.g. different carbon loadings) will benefit model
348 discrimination, as the fewer parameters lead to better possibilities for model calibration.

349 The ASM-EC model can substitute the process rates describing denitrification in N-removing models (S1).
350 Incorporating the effects of electron competition to water treatment models will allow us, among others, to
351 predict more accurately the concentration of denitrification intermediates, and to identify optimal carbon
352 dosing based on maximum carbon oxidation rates .

353 **SUPPORTING INFORMATION**

354 Sections 1, 2, 3, 4.

355 **AUTHOR'S INFORMATION**

356 Corresponding Author

357 *Phone: +45 4525 1600; e-mail: b fsm@ env.dtu.dk.

358 *Phone: +45 4525 1617; e-mail: cadf@ env.dtu.dk

359 **Notes**

360 The authors declare no competing financial interest.

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368 **REFERENCES**

- 369 (1) Almeida, J. S.; Reis, M. A. M.; Carrondo, M. J. T. A Unifying Kinetic Model of Denitrification. *J.*
370 *Theor. Biol.* **1997**, *186*, 241–249.
- 371 (2) Richardson, D. J.; Felgate, H.; Watmough, N.; Thomson, A.; Baggs, E. Mitigating release of the
372 potent greenhouse gas N(2)O from the nitrogen cycle - could enzymic regulation hold the key?
373 *Trends Biotechnol.* **2009**, *27*, 388–397.
- 374 (3) Madigan, M. T.; Martinko, J. M.; Stahl, D.; Clark, D. P. *Brock Biology of Microorganisms*; 13th ed.;
375 Pearson, 2010.
- 376 (4) Berks, B. C.; Ferguson, S. J.; Moir, J. W. B.; Richardson, D. J. Enzymes and associated electron
377 transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim.*
378 *Biophys. Acta - Bioenerg.* **1995**, *1232*, 97–173.
- 379 (5) Domingo-Félez, C.; Smets, B. F. Regulation of key N₂O production mechanisms during biological
380 water treatment. *Curr. Opin. Biotechnol.* **2019**, *57*, 119–126.
- 381 (6) Alefounder, P. R.; Greenfield, A. J.; McCarthy, J. E. G.; Ferguson, S. J. Selection and organisation of
382 denitrifying electron-transfer pathways in *Paracoccus Denitrificans*. *Biochim. Biophys. Acta* **1983**,
383 *724*, 20–39.
- 384 (7) Kucera, I.; Dadak, V.; Dobry, R. The distribution of redox equivalents in the anaerobic respiratory
385 chain of *Paracoccus denitrificans*. *Eur. J. Biochem.* **1983**, *130*, 359–364.
- 386 (8) Mokhayeri, Y.; Riffat, R.; Murthy, S.; Bailey, W.; Takacs, I.; Bott, C. Balancing yield, kinetics and
387 cost for three external carbon sources used for suspended growth post-denitrification. *Water Sci.*
388 *Technol.* **2009**, *60*, 2485.
- 389 (9) Ginige, M. P.; Hugenholtz, P.; Daims, H.; Wagner, M.; Keller, J.; Blackall, L. L. Use of Stable-
390 Isotope Probing, Full-Cycle rRNA Analysis, and Fluorescence In Situ Hybridization-

- 391 Microautoradiography To Study a Methanol-Fed Denitrifying Microbial Community. *Appl. Environ.*
392 *Microbiol.* **2004**, 70, 588–596.
- 393 (10) Lu, H.; Chandran, K.; Stensel, D. Microbial ecology of denitrification in biological wastewater
394 treatment. *Water Res.* **2014**, 64, 237–254.
- 395 (11) Betlach, M. R.; Tiedje, J. M. Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous
396 oxide during bacterial denitrification. *Appl. Environ. Microbiol.* **1981**, 42, 1074–1084.
- 397 (12) Hiatt, W. C.; Grady, C. P. L. An updated process model for carbon oxidation, nitrification, and
398 denitrification. *Water Environ. Res.* **2008**, 80, 2145–2156.
- 399 (13) Schulthess, R.; Kühni, M.; Gujer, W. Release of nitric and nitrous oxide from denitrifying activated
400 sludge. *Water Res.* **1995**, 29, 215–226.
- 401 (14) Ribera-Guardia, A.; Kassotaki, E.; Gutierrez, O.; Pijuan, M. Effect of carbon source and competition
402 for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. *Process*
403 *Biochem.* **2014**, 49, 2228–2234.
- 404 (15) Pan, Y.; Ni, B.-J.; Bond, P. L.; Ye, L.; Yuan, Z. Electron competition among nitrogen oxides
405 reduction during methanol-utilizing denitrification in wastewater treatment. *Water Res.* **2013**, 47,
406 3273–3281.
- 407 (16) Grant, R. F.; Pattey, E. Mathematical modeling of nitrous oxide emissions from an agricultural field
408 during spring thaw. *Global Biogeochem. Cycles* **1999**, 13, 679–694.
- 409 (17) Thomsen, J. K.; Geest, T.; Cox, R. P. Mass spectrometric studies of the effect of pH on the
410 accumulation of intermediates in denitrification by *Paracoccus denitrificans*. *Appl. Environ.*
411 *Microbiol.* **1994**, 60, 536–541.
- 412 (18) Pan, Y.; Ni, B.; Yuan, Z. Modeling electron competition among nitrogen oxides reduction and N₂O
413 accumulation in denitrification. *Environ. Sci. Technol.* **2013**, 47, 11083–11091.

- 414 (19) Pan, Y.; Ni, B.-J.; Lu, H.; Chandran, K.; Richardson, D. J.; Yuan, Z. Evaluating two concepts for the
415 modelling of intermediates accumulation during biological denitrification in wastewater treatment.
416 *Water Res.* **2015**, *71*, 21–31.
- 417 (20) Read-Daily, B. L.; Sabba, F.; Pavissich, J. P.; Nerenberg, R. Kinetics of nitrous oxide (N₂O)
418 formation and reduction by *Paracoccus pantotrophus*. *AMB Express* **2016**, *6*, 85.
- 419 (21) Ellis, T. G.; Barbeau, D. S.; Smets, B. F.; Grady, C. P. L. Respirometric technique for determination
420 of extant kinetic parameters describing biodegradation. *Water Environ. Res.* **1996**, *68*, 917–926.
- 421 (22) Schulthess, R. Von; Wild, D.; Gujer, W. Nitric and nitrous oxides from denitrifying activated sludge
422 at low oxygen concentration. *Water Sci. Technol.* **1994**, *30*, 123–132.
- 423 (23) Beale, E. M. L. Confidence Regions in Non-Linear Estimation. *J. R. Stat. Soc. Ser. B-statistical*
424 *Methodol.* **1960**, *22*, 41–88.
- 425 (24) Akaike, H. A new look at the statistical model identification. *IEEE Trans. Automat. Contr.* **1974**, *19*,
426 716–723.
- 427 (25) Cierkens, K.; Plano, S.; Benedetti, L.; Weijers, S.; de Jonge, J.; Nopens, I. Impact of influent data
428 frequency and model structure on the quality of WWTP model calibration and uncertainty. *Water Sci.*
429 *Technol.* **2012**, *65*, 233–242.
- 430 (26) Sin, G.; Gernaey, K. V.; Neumann, M. B.; van Loosdrecht, M. C. M.; Gujer, W. Uncertainty analysis
431 in WWTP model applications: a critical discussion using an example from design. *Water Res.* **2009**,
432 *43*, 2894–2906.
- 433 (27) Haefner, J. W. *Modeling Biological Systems: Principles and Applications*; Second.; Springer, 2005.
- 434 (28) Domingo-Félez, C.; Calderó-Pascual, M.; Sin, G.; Plósz, B. G.; Smets, B. F. Calibration of the
435 comprehensive NDHA-N₂O dynamics model for nitrifier-enriched biomass using targeted
436 respirometric assays. *Water Res.* **2017**, *126*, 29–39.

- 437 (29) Graf, D. R. H.; Jones, C. M.; Hallin, S. Intergenomic Comparisons Highlight Modularity of the
438 Denitrification Pathway and Underpin the Importance of Community Structure for N₂O Emissions.
439 *PLoS One* **2014**, *9*, e114118.
- 440 (30) Roco, C. A.; Bergaust, L. L.; Bakken, L. R.; Yavitt, J. B.; Shapleigh, J. P. Modularity of nitrogen-
441 oxide reducing soil bacteria: linking phenotype to genotype. *Environ. Microbiol.* **2017**, *19*, 2507–
442 2519.
- 443 (31) Wang, Q.; Ni, B.-J.; Lemaire, R.; Hao, X.; Yuan, Z. Modeling of Nitrous Oxide Production from
444 Nitritation Reactors Treating Real Anaerobic Digestion Liquor. *Sci. Rep.* **2016**, *6*, 25336.
- 445 (32) Ni, B.-J.; Rusalleda, M.; Pellicer-Nàcher, C.; Smets, B. F. Modeling nitrous oxide production during
446 biological nitrogen removal via nitrification and denitrification: extensions to the general ASM
447 models. *Environ. Sci. Technol.* **2011**, *45*, 7768–7776.

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Figure 1. Model of respiratory canonical heterotrophic denitrifying bacteria (A). Electric circuit analogy with parallel electron transport to the oxidase, nitrate reductase, and cytochrome c (B). Simplified electron competition model considered in ASM-EC with parallel electron transport to the oxidase and four nitrogen reductases (C).

Table 1. Experimental datasets considered during calibration and approximate initial conditions. Experiment I - Denitrifying culture fed on MeOH, Experiment II - Denitrifying culture fed on Acetate, EtOH, MeOH, Experiment III - *Paracoccus pantotrophus*.

Experiment ID	A	B	C	D	E	F	G	Initial conditions				
N Substrate	NO ₃ ⁻			NO ₃ ⁻		NO ₃ ⁻	NO ₃ ⁻	NO ₃ ⁻ / NO ₂ ⁻ / N ₂ O	sCOD	VSS	Substrate / Biomass	
C Substrate		NO ₂ ⁻			NO ₂ ⁻	NO ₂ ⁻	NO ₂ ⁻					
			N ₂ O	N ₂ O	N ₂ O		N ₂ O	gN/m ³	gCOD/m ³	g/L		
I	Methanol	▲	▲	▲	▲	●	●	40-60*	300*	0.4-1.0*	≪ 1	[1]
	Acetate	▲	▲	▲	▲		▲				≪ 1	[2]
II	Ethanol	▲	▲	▲	▲	▲	▲	20 / 20 / 20	300	0.7-2.0	≪ 1	[2]
	Methanol	▲	▲	▲	▲	▲	▲				≪ 1	[2]
	Ac + Et +	▲	▲	▲	▲	▲					≪ 1	[2]

Me

III	Acetate	▲	▲	50 / - / 700	650	0.002	>> 1	[3]
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▲ Model Calibration, ● Model Validation, *Generated in-silico
[1] Pan et al. 2013 *WR*, [2] Ribera-Guardia et al. 2014 *PB*, [3] Read-Daily et al. 2016 *AMB*.

Figure 2. Model simulations of substrate spike additions in a batch reactor. Liquid concentrations for individual and combined substrate spikes (top), corresponding substrate consumption rates (middle, arbitrary y-axis) and electron consumption rates (bottom, arbitrary y-axis). $R_{OrgC} = 1.7$, $R_{NAR} = 1.7$, $R_{NIR} = 3.3$, $R_{NOS} = 2.5$ (massX·time/massS).

Figure 3. Model predictions for Experiment I: NO_2^- pulse (left) and NO_3^- - NO_2^- - N_2O pulses (middle). Markers represent experimental data (NO_3^- diamonds, NO_2^- squares, N_2O circles), lines model predictions (black – best fit, red – 95% CI). Electron consumption rates (right) by NO_3^- reduction (Nar – green), NO_2^- reduction (Nir – orange), NO reduction (Nor

– grey) and N₂O reduction (Nos – blue). Experimental and modelling results (left and right bars) (* datasets not used during calibration).

Table 2. Best-fit parameter values of minimum resistances for denitrification batches of Experiment I, II, and III.

Experiment	I	II				III
Carbon Source	Methanol	Ethanol	C-mix	Acetate		
R_{NAR} (gCOD·h/gN)	3.5	117.8	8.0	12.9	10.0	9.2
R_{NIR} (gCOD·h/gN)	1.9	139.1	50.9	39.1	11.1	41.9
R_{NOS} (gCOD·h/gN)	0.3	83.3	8.9	12.0	18.0	16.6

Figure 4. NO₃⁻ reduction experiments from Experiment II: experimental data (marker), lines model predictions (black – best fit, red – 95% confidence intervals).

Figure 5. Experimental and model-estimated true denitrification rates: Experiment I Methanol (left), Experiment II Acetate (middle), Experiment II C-mix (right). Positive and negative values correspond to consumption and accumulation rates respectively.

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482 **Figure 6.** Experiment III: Growth rate curve of *P. pantotrophus* with NO_3^- , N_2O (in excess, data not shown), and acetate
 483 as substrates (top); and NO_3^- and acetate (bottom). Experimental data (marker), lines model predictions (solid – best fit,
 484 dashed – 95% confidence intervals).

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