

Mass degradation to reduce cytotoxic products as an individual behavior-rule embedded in a microbial model for the study of the denitrification process

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Denitrification has a wide range of factors that control its complex regulatory network, one of which is the low concentration of electron donors. In this case the absence of available electrons causes high concentrations of cytotoxic gases (NO and/or N₂O) for denitrifying bacteria. The individual adaptive behavior to environmental conditions through different behavior-rules is one of the most powerful aspects of microbial individual-based models. INDISIM-Paracoccus is a model to study *P. denitrificans* in batch and continuous cultures under both aerobic and anaerobic conditions. The aim of this study was to improve the metabolism sub-model with the introduction of a new individual behavior-rule for this bacterium in order to reduce cytotoxic products with individual mass degradation. A sensitivity analysis for two parameters involved in this behavior-rule, individual cellular maintenance energy and individual mass degradation coefficient, was performed. With this new version of the INDISIM-Paracoccus, implemented in the NetLogo platform, and with a specific combination of the values for these two parameters, the simulation outputs of this computational model, more realistic and mechanistic than other approaches, were in closer agreement with the published experimental data.

Keywords: Denitrification, *Paracoccus denitrificans*, microbial individual-based model, mass degradation, INDISIM.

1. Introduction

Denitrification is an important process driven by bacteria. It has been known for more than one hundred years and is widely recognized as a key process in the biogeochemical nitrogen cycle which has been investigated at many levels, ranging from gene expression to global nitrogen flux [1, 2]. These denitrifying bacteria are facultative aerobes and the most common genera are *Pseudomonas*, *Achromobacter* (*Alcaligenes*), *Paracoccus*, *Thiobacillus*, *Bacillus*, *Halobacterium*, *Chromobacterium*, *Hyphomicrobium* and some species of *Moraxella*. They are commonly found in soils, sediments, surface and ground waters, and wastewater treatment plants [3–5]. Denitrifying bacteria are able to use N-oxides as electron acceptors in place of O₂, which means that they use some nitrogen oxide as respiration substrate when oxygen (O₂) becomes limited. For instance, nitrate (NO₃⁻), nitrite (NO₂⁻), nitric oxide (NO) and nitrous oxide (N₂O) all become electron acceptors in a similar pathway when O₂ is used in aerobic respiration [4, 6]. Therefore, denitrification is the dissimilatory reduction of NO₃⁻ to (mainly) dinitrogen gas (N₂) by bacteria [5, 7]. A complete denitrification pathway is defined as the assemblage of four subsequent reactions (NO₃⁻ → NO₂⁻ → NO → N₂O → N₂).

A wide range of factors control this complex regulatory network carried out by denitrifying bacteria, one of which is the low concentration of electron donors (usually C-sources), which limits the supply of electrons to drive the reductive reactions [8]. Therefore, the electron fluxes over the successive denitrification steps are lopsided, and this can cause accumulation of the denitrification intermediates, NO₂⁻, NO and/or N₂O. An excessive NO emission from soil into the atmosphere is undesirable because it promotes an increase in tropospheric ozone, a greenhouse gas. Additionally, N₂O is a potent greenhouse gas and air pollutant with a large global warming potential. It dominates the decrease of the ozone layer [9]. Furthermore, these gases show bacterial cytotoxic properties such as free radicals and oxidants manifested by the inactivation of an essential cellular cofactor in B12-dependent enzymes, provoking loss of cell division and cell viability [10].

To study the effects of low concentration of C-source on the denitrification driven by bacteria, computational models can be helpful tools. The modeling approach used is called "individual-based modeling" (Individual-based Model, IBM) which, by joining different levels of observation, cell and population, is able to predict the changes at the population and system levels as an emergent property from individual (cellular) activity [11–13]. The IBMs of microbes are called "microbial individual-based models" (μIBMs) [13] and they act as follows: (a) they describe system behavior as a whole by establishing procedural rules for the individuals (bacteria) and their

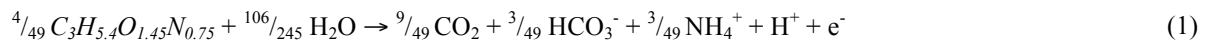
interactions; (b) they reproduce real system diversity because they allow the introduction of randomness and individual variability, and (c) they take into account the individual adaptive behavior to environmental conditions. With all of this the evolution of the whole system arises from the individual dynamics that govern the system [11, 14].

For the study of the denitrification process driven by denitrifying bacteria, we are developing a μ IBM called INDISIM-Paracoccus [15, 16], part of the INDISIM family [14, 17], which has been implemented in the NetLogo platform [18]. The model takes into account the cell actions of the bacterium *Paracoccus denitrificans* which grows and develops in batch or continuous cultures containing a C-source, N-source and various electron acceptors such as O_2 , NO_3^- , NO_2^- , NO and N_2O . Some preliminary simulated results obtained from this computational model, the concentrations of two cytotoxic gases, NO and N_2O , were inconsistent with the experimental data [19] used to test the model. The main objective of this contribution is to improve the INDISIM-Paracoccus metabolism sub-model by means of the introduction of a new behavior-rule for the bacterium, with individual mass degradation in order to reduce intermediate denitrification products. A sensitivity analysis with the two model parameters involved in this sub-model, the individual mass degradation coefficient and the cellular maintenance energy, was performed to help in the parameterization process for this computational model.

2. Thermodynamic approach – balanced biochemical equations for mass degradation

The IBM approach to the study of bacterial cultures called INDISIM was projected to simulate the growth and behavior of bacterial populations [17]. Using this as a core, the INDISIM-Paracoccus model used in this study considers each simulated individual as a single bacterium of *P. denitrificans*, where each of them follows the individual behavior-rules concerning their motion, nutrient uptake and reproduction according to the INDISIM framework. The behavior-rules concerning individual cellular maintenance and individual mass synthesis were formulated with a thermodynamic base [16, 17]. Our model is based on the explanation of the cause that provokes the start of the denitrification process and considers as the key factor involved low oxygen concentration and nutrient availability (sources of electron donors and electron acceptors). Some authors consider that microbial mass can be used to complete the denitrification process [4] when it is used as an electron donor.

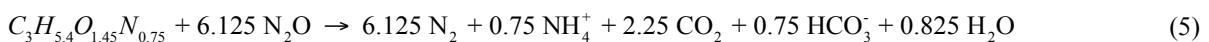
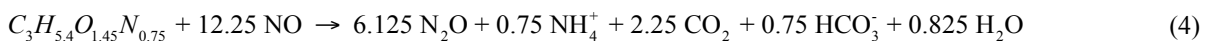
The cellular maintenance and mass synthesis in the INDISIM-Paracoccus model were based on balanced biochemical equations which have been calculated using thermodynamics [16]. It seems necessary to include a new individual behavior-rule to reduce the concentration of cytotoxic gases (NO and/or N_2O), so individual mass degradation will be used by the bacterium when the C-source is a limiting substrate in the media. Following the methodology established in the development of INDISIM-Paracoccus model, a balanced biochemical equation for individual mass degradation is required. To obtain this, the biomass half-reaction acts as electron donor and combined with the electron acceptor half-reaction, the individual mass degradation reaction can be written. Therefore, considering the elementary cell composition for *P. denitrificans* ($C_3H_{5.4}N_{0.75}O_{1.45}$) [20, 21], the general biomass half-reaction equation [4] may be written as:



The equation (1) is the electron donor half-reaction which considers the individual mass as electron source, breaking it down into carbon dioxide, bicarbonate and ammonium. These electrons will be transferred to the acceptor, so it is necessary write the half-reactions for the two electron acceptors considered, as follows:



By adding the equations (1) and (2) the balanced reaction for NO reduction is obtained (4), and for the reduction of the N_2O (5) the equations (1) and (3) must be combined.



The previous INDISIM-Paracoccus model considered two parameters for cellular maintenance, one for the aerobic phase and one for the anaerobic phase. The current version of the model will consider a single parameter for cellular maintenance in both growth phases ($g_{C_{donor}} \cdot g_{C_{mic}}^{-1} \cdot h^{-1}$). To include the mass degradation behavior-rule inside the model it is necessary to include a mass degradation coefficient (h^{-1}), which indicates the

maximum amount of individual mass per time unit and mass unit that a bacterium can oxidize to reduce the cytotoxic gases (N₂O and/or NO). To investigate the effect of including this new parameter in the INDISIM-Paracoccus a categorical exploration is performed, which searches for parameter values producing results of the model within a range defined as acceptable. The sensitivity analysis of the cellular maintenance and the mass degradation parameters for different values are chosen, while the other parameters are kept constant. A set of simulations for both parameters and their interactions are carried out to check the model response regarding the temporal evolutions of biomass, NO₃⁻, NO₂⁻, NO and N₂O. The parameter values for the cellular maintenance and mass degradation are in Table 1.

Table 1. Values for sensitivity analysis parameters for cellular maintenance and mass degradation.

Cellular maintenance (gC _{donor} ·gC _{mic} ⁻¹ ·h ⁻¹)	2.0x10 ⁻⁵	2.0x10 ⁻⁴	2.0x10 ⁻³ (a,b)	3.0x10 ⁻³ (b)	4.0x10 ⁻³ (b)	2.0x10 ⁻²	2.0x10 ⁻¹	2.0x10 ⁰	2.0x10 ¹
Mass degradation (h ⁻¹)	1.8x10 ⁻⁵	1.8x10 ⁻⁴	1.8x10 ⁻³ (b)	1.8x10 ⁻² (a,b)	3.0x10 ⁻² (b)	4.0x10 ⁻²	1.8x10 ⁻¹	1.8x10 ⁰	1.8x10 ¹

(a) Reference value obtained from initial model calibration. (b) Values to study the parameter interaction.

3. New version of INDISIM-Paracoccus

INDISIM-Paracoccus is a μ IBM for the denitrification process carried out by the bacterium *P. denitrificans* growing in batch and continuous culture in aerobic and anaerobic conditions. It has two entities: individuals and square patches of culture medium. An individual represents a unique bacterium of *P. denitrificans* and has the following variables: a unique identification number, location, mass, reproduction mass, degradation mass, internal product amounts and counters for each metabolic pathway and reproduction cycle. The smallest microorganism has a diameter of $\sim 0.5 \mu\text{m}$ and the largest has a diameter of $\sim 0.9 \mu\text{m}$. A two-dimensional lattice of 25x25 grid cells represents the bioreactor that contains the culture medium; one spatial cell represents 1 nl, so the total bioreactor volume is 625 nl. Their variables are: position identifier in XY coordinates, total amount of each nutrient, succinate, NH₄⁺, O₂, NO₃⁻, and metabolic products, NO₂⁻, NO, N₂O, N₂, HCO₃⁻ and CO₂. All microbial and culture medium actions are discretized in time steps. One time step represents 5 minutes. At each time step all the individuals are controlled by a set of time-dependent variables, and they perform the following processes: nutrient uptake, cellular maintenance, mass synthesis, metabolic product generation, mass degradation and bipartition. Culture medium processes are different depending on the bioreactor management protocol. At the beginning of the simulation the bioreactor works as a batch culture with oxygen saturated conditions, and the user decides at what time this phase ends and switches to continuous culture in anoxic conditions. A microbe in INDISIM-Paracoccus checks the local oxygen-dissolved level, and if it is lower than a threshold value (O_{2-MIN}) the microbe follows the anaerobic metabolism; otherwise it carries out aerobic metabolism. This change is discrete for each bacterium in the time step; therefore there is a gradual modification of the population medium variables. When the microbe has selected the metabolism pathway, it carries out its maintenance according to its own requirements, and after that, if the electron donor intake and the quantity of some electron acceptors are greater than zero, the bacterium performs mass synthesis. After this, mass degradation takes place when one of the cytotoxic gases NO or N₂O, or both, are present due to the denitrification not being completed and the C-source being limited. For each individual behavior-rule (cellular maintenance, mass synthesis and mass degradation), the bacterium selects the limiting nutrient and with this information provides the quantities of the other nutrients according to the corresponding balanced biochemical equations [15, 16]. To reduce the internal quantities of the cytotoxic gases NO and N₂O, the microbe executes a mass degradation behavior-rule, so first it establishes the maximum amount of its mass that can be catabolized, which is proportional to its mass by the mass degradation coefficient (h⁻¹). The cytotoxic product and the individual mass are reduced following the reaction coefficients (Eqs. 4 and 5), and the remaining unused intakes are expelled to the medium. The bipartition process is an INDISIM sub-model [17]. The sub-models related to the bioreactor's procedure are: i) Agitation: nutrients and metabolic products are redistributed in the culture medium and microorganism positions change randomly, ii) Input flow: The bioreactor is refilled with fresh culture medium, and iii) Output flow: A fraction of individuals and culture medium are randomly removed. The model designed has been implemented in the NetLogo [18], and the simulator may be obtained from the authors on request.

4. Some simulation results

The *Behavior-space* tool included in NetLogo was used for running simulation experiments, varying parameters and writing model outputs to files that were used for categorical exploration. For each parameter 27 simulations

were run, taking into account 9 values of the parameter (Table 1) and 3 replicates. After that, the 3 best values for each parameter (cellular maintenance and mass degradation coefficient) were combined and 3 replicates were performed for each combination. When environmental conditions are modified, the individual metabolism of the bacteria changes from aerobic to anaerobic; the production of denitrification intermediates increases their concentration to achieve a maximum value and then decreases so that they achieve a steady state. When the mass degradation behavior-rule is introduced in the model to reduce NO and N₂O gases, the temporal evolutions of biomass, NO₃⁻, NO₂⁻ and N₂O obtained with the simulator INDISIM-Paracoccus are in closer agreement with the data corresponding to experiments performed in a bioreactor with *P. denitrificans* [19]. The experiment used to test the model is designed to keep the electron-donor limited, and for this reason the individual mass degradation takes place and its effects are noteworthy (Fig. 1).

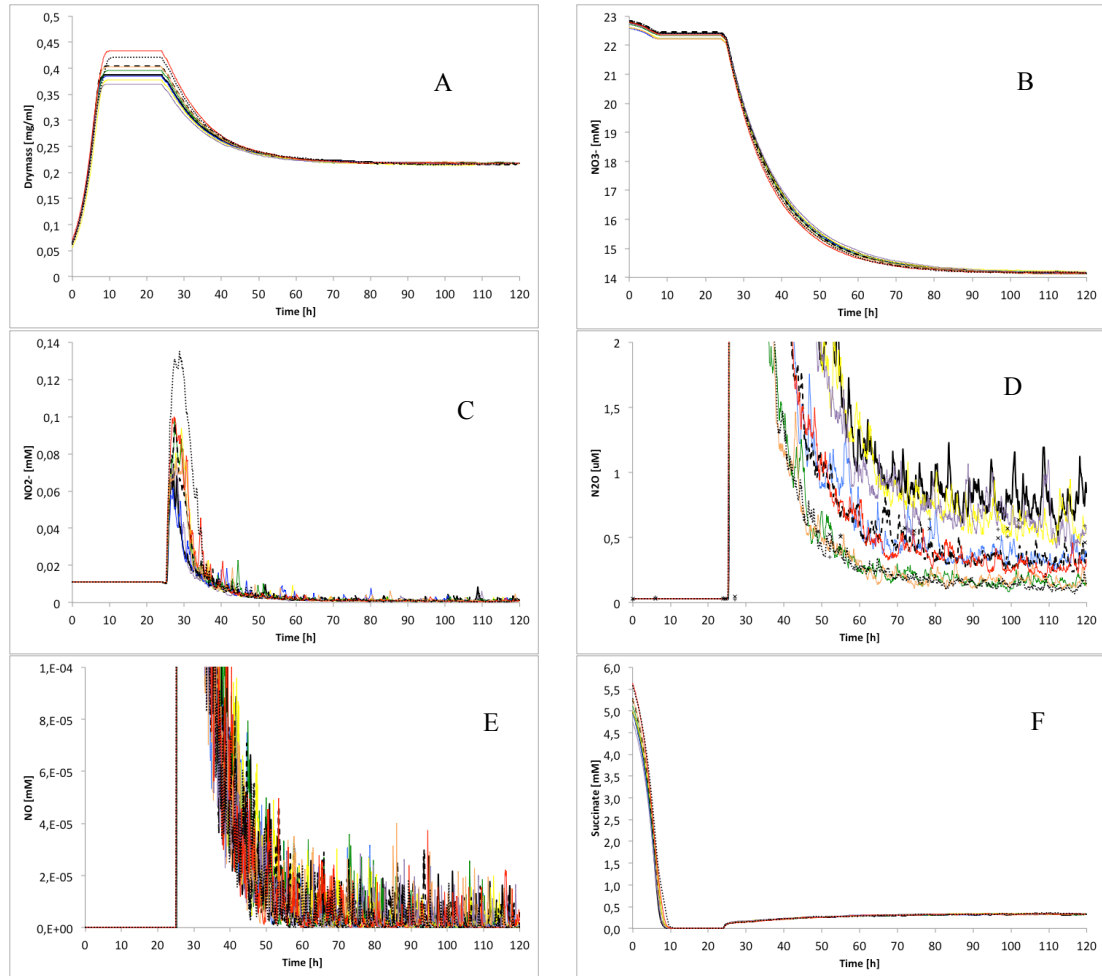


Fig. 1. INDISIM-Paracoccus simulation results in experiment that is electron-donor limited/electron-acceptor sufficient. Temporal evolutions corresponding to the nine combination of the parameters (Table 1) of: A) Biomass, B) NO₃⁻, C) NO₂⁻, D) N₂O, E) NO and F) Succinate.

4. Discussion

When adding the mass degradation behavior-rule into the model, the simulation results are in better accordance with the experimental data reported for *P. denitrificans* [19] than in the previous study [15]. This individual behavior-rule seems to be a plausible strategy to reduce the cytotoxic products N₂O and NO, which is in line with Rittmann & McCarty work [4]. In the case of the succinate consumption during the steady state, the simulation results show values greater than the 10 uM reported. Nevertheless, in the long term the simulation outputs achieved the steady state and the concentration of N₂O is around 0.5 uM and for NO it is around 10⁻⁵ mM, both of which are in the range of the experimental results [19]. This indicates that the use of microbial individual-based models offers a noteworthy strategy to study and understand the denitrifying bacterial activity evolving under different bioreactor protocols. The simulations achieved are very encouraging, offering the possibility to interpret, comprehend and investigate the dynamics of *P. denitrificans* growing in controlled

conditions. Moreover, this microbial model opens the possibility to deal with the intrinsic variability and heterogeneity of denitrifying bacteria; each of these has particular characteristics and acts according to specific behavior-rules related to its biological guidelines. The INDISIM-Paracoccus model is implemented in the widely used, free and open source IBM software platform NetLogo that facilitates interaction among researchers, modelers and academics. Further work will be needed to make adjustments to better deal with the denitrification process according to denitrification enzyme expression as a response to environmental conditions.

Acknowledgements: The financial support of the Ecuador National Secretary of Science, Technology and Innovation (SENESCYT) is acknowledged.

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