



# INDISIM-Paracoccus, an individual-based and thermodynamic model for a denitrifying bacterium



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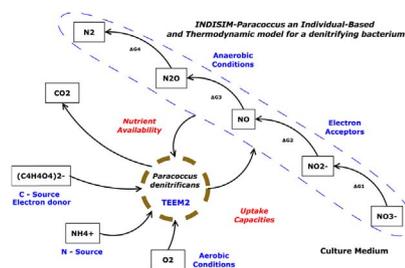
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## HIGHLIGHTS

- An IBM to study denitrification that uses thermodynamics for the cellular activity.
- The simulator facilitates interaction between modelers and experts in denitrification.
- The thermodynamic properties embedded into individual cells for modeling.

## GRAPHICAL ABSTRACT

The individual-based model approach with the thermodynamics embedded as an intracellular model defines the behavior-rule of the individual cell for maintenance and biomass generation to study the denitrification products dynamics, especially the greenhouse gas  $N_2O$ , carried out by denitrifying bacterium *Paracoccus denitrificans*.



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## ABSTRACT

We have developed an individual-based model for denitrifying bacteria. The model, called INDISIM-Paracoccus, embeds a thermodynamic model for bacterial yield prediction inside the individual-based model INDISIM, and is designed to simulate the bacterial cell population behavior and the product dynamics within the culture. The INDISIM-Paracoccus model assumes a culture medium containing succinate as a carbon source, ammonium as a nitrogen source and various electron acceptors such as oxygen, nitrate, nitrite, nitric oxide and nitrous oxide to simulate in continuous or batch culture the different nutrient-dependent cell growth kinetics of the bacterium *Paracoccus denitrificans*. The individuals in the model represent microbes and the individual-based model INDISIM gives the behavior-rules that they use for their nutrient uptake and reproduction cycle. Three previously described metabolic pathways for *P. denitrificans* were selected and translated into balanced chemical equations using a thermodynamic model. These stoichiometric reactions are an intracellular model for the individual behavior-rules for metabolic maintenance and biomass synthesis and result in the release of different nitrogen oxides to the medium. The model was implemented using the NetLogo platform and it provides an interactive tool to investigate the different steps of denitrification carried out by a denitrifying bacterium. The simulator can be obtained from the authors on request.

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## 1. Introduction

Denitrification is one of the key processes of the global nitrogen cycle driven by bacteria (Blackburn, 1990; Zumft, 1997). One of the reasons for studying denitrification is that it contributes to nitrous oxide ( $\text{N}_2\text{O}$ ) emissions when denitrifying bacteria do not complete the metabolic pathway implicated (Davidson et al., 1991; Snyder et al., 2009). The global warming potential of  $\text{N}_2\text{O}$  is 296 times greater than a unit of  $\text{CO}_2$  (Richardson et al., 2009). In agricultural soils,  $\text{N}_2\text{O}$  emissions are of great importance due to the large amount of N-fertilizer in crops and soil organic matter mineralization which depends on the conditions the microorganism encounters in its surrounding environment (Snyder et al., 2009; Woolfenden et al., 2013).

In conditions of low oxygen ( $\text{O}_2$ ) availability, such as waterlogged soils, certain bacteria are able to use nitrate ( $\text{NO}_3^-$ ) as a final electron acceptor and carry out respiratory metabolism in anaerobic conditions (denitrification). These bacteria are known as heterotrophic denitrifying bacteria and are widespread in agricultural soils (Felgate et al., 2012; Richardson et al., 2009). The bacterium *Paracoccus denitrificans* is one of the best-characterized prokaryotes and one of the paradigm species for studies of the biochemistry and regulatory biology of denitrification (Bergaust et al., 2010; Caspi et al., 2012).

To model the dynamics of a bacterial denitrification system with *P. denitrificans* at least three metabolic pathways must be considered as follows. In the aerobic phase it can execute “Aerobic respiration” with the oxygen ( $\text{O}_2$ ) as the electron acceptor, and “Nitrate reduction - Dissimilatory” with nitrate ( $\text{NO}_3^-$ ) as the electron acceptor (Baker et al., 1998; Beijerinck, 1910; Caspi et al., 2012), and in anoxic conditions it executes the “Nitrate reduction - Denitrification process” because it is capable of anaerobic growth in the presence of  $\text{NO}_3^-$ , nitrite ( $\text{NO}_2^-$ ), nitric oxide (NO) or  $\text{N}_2\text{O}$  as electron acceptors (Baumann et al., 1996; Bergaust et al., 2010, 2012; van Verseveld et al., 1983).

The choice of a modeling approach to study a bacterial system, either population-level (top-down, usually continuous with differential equations) or individual-based (bottom-up, discrete and computational model) is an important decision depending on the project’s specific aspects, the characteristics of the system and the questions to be answered (Ferrer et al., 2008). A number of denitrification models have been reviewed by Heinen (2006). Most of them incorporate a large number of parameters including  $\text{NO}_3^-$ , soil moisture, soil temperature and pH. The simplest models are obtained by adjusting empirical functions to the experimental results used for their studies. More recently, Kampschreur et al. (2012) and Woolfenden et al. (2013) published specific denitrification models describing the process carried out by microbes in terms of a set of differential equations according to Monod and Michaelis–Menten kinetics. Therefore, the population-level models deal with population variables and fix a set of governing laws (equations) which are based on, or at least consistent with, an assemblage of assumptions about the individual behavior of microbes.

Alternatively, it is possible to simulate the interactions of autonomous agents (individual and collective entities) and their environment, using agent-based models or, more specifically, Individual-Based Models (IBMs) that are defined by agents which model living entities (Grimm, 1999). IBMs have the ability to simulate variability among individuals, local interactions, complete life cycles and individual behavior according to the changing individual internal and external conditions, linking mechanisms at the individual level to behavior at the population level (Grimm, 1999; Mantzaris, 2007). IBMs consider individuals as discrete entities that follow behavior-rules that drive how the individuals

interact with their surrounding environment and other individuals, so that the individual and the environment can change and adapt their characteristics over time. This makes it possible to explore connections between micro-level behaviors of individuals to macro-level patterns that emerge from their interactions (Prats et al., 2008; Wilensky, 1999).

The two approaches, the continuous-macroscopic and the discrete-microscopic approaches, are not incompatible or exclusive, but are complementary. Population-level approaches are mostly used for predictive purposes, due to their simplicity and computational efficiency. Moreover, they have been widely tested and, nowadays, many modeling frameworks exist. IBMs have had their own place in microbial research and have also been used for some predictive purposes, but their strength lies in the means they offer to disentangle and understand the dynamics of bio-systems (Hellweger and Bucci, 2009; Kreft et al., 2013).

In summary, in addition to the characteristics just described, IBMs are useful to study the relations between experimental data and theoretical proposals, allowing testing of the consistency of different microbial models, and supplying holistic knowledge of the systems under study (Ferrer et al., 2008).

Ginovart et al. (2002) developed a discrete simulation model to study bacterial cultures called INDISIM. This model has been used as the core for other models such as INDISIM-SOM (Ginovart et al., 2005), INDISIM-YEAST (Ginovart and Cañadas, 2008), INDISIM-COMP (Prats et al., 2010) and INDISIM-Saccha (Portell et al., 2014) to deal with soil organic matter dynamics, to study yeast fermentations and multi-species composting, and to analyze the dynamics of *Saccharomyces cerevisiae* anaerobic cultures, respectively. For a review of some microbial system evolutions using the IBM methodology see, for instance, (Bley, 2011; Ferrer et al., 2008; Hellweger and Bucci, 2009; Kreft et al., 2013; Lee et al., 2009; Resat et al., 2012).

Several approaches have been reported to develop a rigorous thermodynamic description for biomass yield prediction (Christensen and McCarty, 1975; Heijnen and Van Dijken, 1992; Liu et al., 2007; Maskow and von Stockar, 2005; McCarty, 1971; Rittmann and McCarty, 2001; Tjihuis et al., 1993; von Stockar and van der Wielen, 1997; Xiao and VanBriessen, 2006). These approaches consider the Gibbs energy for cell synthesis from C-sources and N-sources, the energy available from substrate transformation, the specific Gibbs energy consumption for cellular maintenance, and the energy efficiency transfer to the overall process to describe growth of micro-organisms in a standard mathematical and thermodynamic model.

To tackle and understand the environmental factors that control the denitrification process it is convenient to investigate the bacterial denitrification dynamics in a controlled environment such as a bioreactor (Baker et al., 1998; Baumann et al., 1996; Felgate et al., 2012; Richardson et al., 2009). In this paper we will: (i) Design, implement, and parameterize thermodynamic behavior-rules for a *P. denitrificans* model in the INDISIM methodology context; (ii) Simulate a bioreactor containing a culture medium where *P. denitrificans* develop and grow in order to mimic the experimental protocols presented by Felgate et al. (2012); and (iii) Investigate the effects of the priority in the use of different electron acceptors at the microbial level formulating two hypotheses about the order in which the reactions are followed by the bacteria *P. denitrificans* while the denitrification process occurs, and test these hypotheses with the simulator developed, comparing the simulation outputs with experimental data reported in Felgate et al. (2012).

## 2. Materials and methods

### 2.1. INDISIM

The IBM approach to studying bacterial cultures called INDISIM was proposed by [Ginovart et al. \(2002\)](#) in order to simulate the growth and behavior of bacterial populations. Using this as a core, we establish the INDISIM-Paracoccus model in which each simulated individual represents a single bacterial cell of *P. denitrificans*, each of them follows the individual behavior-rules concerning their motion, nutrient uptake and reproduction in the INDISIM framework.

### 2.2. Thermodynamic approach

With the word metabolism we could summarize all the biochemical and physiochemical processes that happen within a microbial cell. In brief, the microbial metabolism is comprised of two general sub-processes, catabolism and anabolism. Catabolism is all the processes involved in the substrates oxidation or in the use of sunlight in order to gain metabolic energy, and anabolism is the synthesis of cellular components from C-sources and N-sources through the catabolic energy coupling. Therefore, the energy required by the anabolic processes could come from catabolic processes ([Heijnen, 1999](#)). The micro-organisms obtain this energy for maintenance and cell growth from biochemical reactions that involve several chemical species, usually an electron donor and an electron acceptor.

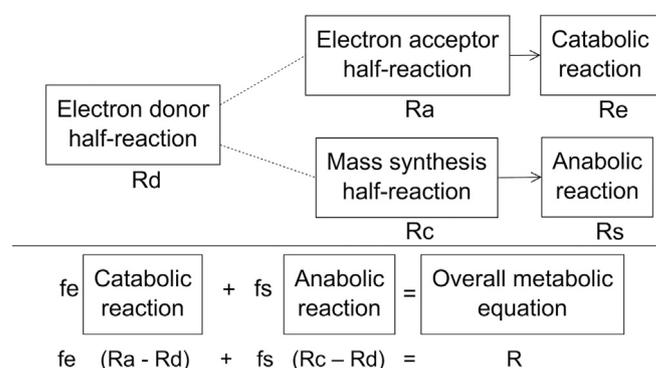
The second version of the Thermodynamic Electron Equivalents Model (TEEM2) ([McCarty, 2007](#)) can make an adjustment between cell synthesis reaction ( $R_s$ ), which represents the microbial anabolism, and the energy reaction ( $R_e$ ), which represents the microbial catabolism to predict bacterial yield ( $Y_{c/c}$ ) with the associated Gibbs free energies for these reactions.

$R_e$  is the combination of the reduction-half-reaction for the electron donor ( $R_d$ ) with the reduction-half-reaction for the electron acceptor ( $R_a$ ).  $R_s$  is the combination of  $R_d$  with the half-reaction for the biomass synthesis ( $R_c$ ) which considers ammonium or other nitrogen sources for new biomass generation ([Rittmann and McCarty, 2001](#)).

TEEM2 is based on terms from the Gibbs free energy involved in how the energy between catabolism and anabolism is coupled using a term of energy-transfer-efficiency ( $\epsilon$ ) and the relation with the electrons involved in both processes. The electrons that come from the electron-donor will be divided into two portions. The first portion ( $fe^o$ ) is transferred to the electron acceptor to generate energy (catabolism) and the other portion of electrons ( $fs^o$ ) is transferred to the N-source for cell synthesis (anabolism). TEEM2 calculates the relationship between  $fe^o$  and  $fs^o$  using: (i) Gibbs standard free energy of  $R_d$ ,  $R_a$  and  $R_c$ , (ii) other Gibbs energy potential terms, and (iii) a term for energy-transfer efficiency ( $\epsilon$ ). This term is included because TEEM2 considers that a fraction of the thermodynamic free energy involved is lost at each energy transfer between catabolism and anabolism. The  $\epsilon$  value is considered by [McCarty \(1971\)](#) to be in the range between 0.2 and 0.8. [Christensen and McCarty \(1975\)](#) and [VanBriesen \(2002\)](#) suggested a value of 0.2–0.3 for aerobic heterotroph micro-organisms, and [McCarty \(2007\)](#) and [Xiao and VanBriesen \(2006, 2008\)](#) proposed a value between 0.4 and 0.7 for anaerobic heterotroph micro-organisms. [Fig. 1](#) shows the TEEM schematic diagram.

### 2.3. Experimental data

To examine the denitrification process, [Felgate et al. \(2012\)](#) cultured *P. denitrificans* in a bioreactor growing under batch conditions in an oxygen-saturated medium for 24 h. The aeration was then shut down and the system switched to continuous culture



**Fig. 1.** Schematic representation of TEEM, adapted from ([Rittmann and McCarty, 2001](#)).

with a dilution rate of  $0.05 \text{ h}^{-1}$ . Under these conditions two experiments took place, one in which the reservoir medium feed contained  $20 \text{ mM NO}_3^-$ ,  $5 \text{ mM succinate}$  and  $10 \text{ mM NH}_4^+$  which was designed to achieve an electron donor-limited/electron acceptor-sufficient steady state (succinate-limited/ $\text{NO}_3^-$ -sufficient). In the other experiment the reservoir medium feed contained  $5 \text{ mM NO}_3^-$ ,  $20 \text{ mM succinate}$  and  $10 \text{ mM NH}_4^+$  to achieve an electron donor-sufficient/electron acceptor-limited steady state (succinate-sufficient/ $\text{NO}_3^-$ -limited). The data was collected from 0 to 120 h ([Felgate et al., 2012](#)).

Therefore, the bacteria in our model will grow and develop in two different conditions: the first during the aerobic phase (from 0 to 24 h) in a batch culture, and the second one during the anaerobic phase (from 24 to 120 h) in a continuous culture. Also the bacteria will be handled in two different experiments: (i) in conditions of succinate-sufficient/ $\text{NO}_3^-$ -limited (experiment E1), and (ii) in conditions succinate-limited/ $\text{NO}_3^-$ -sufficient (experiment E2). The two hypotheses proposed (the metabolic hypothesis and the Gibbs' hypothesis) will be tested using the model and taking into account all these laboratory conditions.

### 2.4. Programming environment and model analysis

The model is implemented in the widely used, free and open source IBM software platform NetLogo, a multi-agent programming language and modeling environment for simulating natural phenomena ([Wilensky, 1999](#)).

Given that the main purposes of the virtual experiments with the computational model obtained are principally exploratory as to the nature and dynamics of the bio-system, the model is not designed for predictive purposes; a best-fit calibration (i.e., a calibration aiming for one unique set of parameter values giving model results best matched to some exact criteria) was avoided. Instead a categorical calibration, which searches for parameter values producing results within a category or range defined as acceptable was performed ([Railsback and Grimm, 2012](#)).

The multiple outputs used to test models is one of the main features of the pattern-oriented modeling strategy and very valuable for IBMs. Once a system representation is built, a depth exploration of how well the model really explains observed phenomena can be carried out with a quantitative analysis ([Thiele et al., 2014](#)). To assess the two hypotheses (the metabolic hypothesis and the Gibbs' hypothesis) and to facilitate parameter estimation, we established multiple fitting criteria using the parameter uptake-rate for all nutrients involved with the experimental data of [Felgate et al. \(2012\)](#). The basic idea is to find ranges of these uptake-rate values that make it possible to roughly reproduce the evolution of a set of focus variables or patterns observed in the two experiments.

Taking into account that the bacteria grow in aerobic and anaerobic conditions, to calibrate the model in aerobic conditions we combined the uptake-rates for succinate (uSuccinate) and nitrate in aerobic phase (uNitrate-a). To calibrate the model in anaerobic conditions we first combined the uptake-rates for succinate and nitrate in anaerobic phase (uNitrate-x). After that we combined the uptake-rate of nitrate-x with the uptake-rates of nitrite, nitric oxide and nitrous oxide. Then we combined the uptake-rate of nitrite with the uptake-rates of nitric oxide and nitrous oxide. And finally we combined the uptake-rate of nitric oxide with the uptake-rate of nitrous oxide. Therefore, the parameters are combined in pairs, and in all cases two parameters change and the others remain constant.

To assess whether a certain combination of parameter values leads to acceptable model output, we calculate a score based on the evaluation of the seven patterns controlled for each of the experiments E1 and E2. Therefore, to appraise: (i) in each of the temporal evolutions of microbial biomass, nitrate, nitrite and nitrous oxide, if the simulation result agrees well with the experimental data 1 point is assigned, if agreement is fair 0.5 points are assigned and if agreement is poor 0 points are assigned, (ii) the NO concentration in the culture medium, 1 point is assigned if the maximum NO value is under  $10^{-3}$  mM, 0.5 points if the value is in the range  $[10^{-3}, 0.5]$  mM and 0 points in all other cases, (iii) the  $N_2$  production, 1 point is assigned if the  $N_2$  production is in the range reported by Felgate et al. (2012) and 0 points in other cases, and (iv) the role of succinate or nitrate as a limited-nutrient during the steady state, if the simulation result agrees well with the experimental data 1 point is assigned, if agreement is fair 0.5 points are assigned and if agreement is poor 0 points are assigned.

Taking into account the sum of points achieved for each combination and each experiment (7 patterns x 2 experiments = 14 items assessed), we use a percentage to represent a global model adequacy for the experimental data. This percentage is represented using bubble graphs, where the bubble radius is proportional to the percentage. The tool “Behavior-Space” incorporated in NetLogo was used for running simulation experiments with varying parameters and writing model outputs to files that were used for the fitting criteria.

Additionally, in order to compare the simulation results with the experimental data we also used the geometric reliability index (GRI) values, a statistical method to determine the reliability of a model (Jachner et al., 2007). This coefficient can deal with precise notions of model accuracy. For models with simulation results reasonably close to experimental observations this GRI shows a resulting factor of 1 to 3, with 1 corresponding to 100% accuracy (Leggett and Williams, 1981).

### 3. INDISIM-Paracoccus modeling

#### 3.1. Metabolic reactions

To develop the individual behavior-rule for cell maintenance it is necessary to write the energy reactions for aerobic and anaerobic conditions. For the aerobic phase we consider the reaction between succinate (which is always the electron donor) and oxygen (as electron acceptor), while for the anaerobic phase the electron acceptors are nitrogen oxides. To formulate maintenance reactions and calculate the corresponding stoichiometric coefficients, we used the reduction-half-reactions for *Rd* and different *Ra* shown in Table 1 to write the energy reactions (Table 2). With these energy reactions and appropriate maintenance requirements, we then designed the individual rule for cell maintenance (see Supplementary material for details).

To develop the individual behavior-rule for biomass synthesis it is necessary to write the metabolic pathways for aerobic and

**Table 1**

Inorganic and organic half-reactions and their Gibbs standard free energy according to Rittmann and McCarty (2001).

Reduction-half-reaction	$\Delta G^\circ$ (kJ/eeq)
Rd $1/7 \text{ CO}_2 + 1/7 \text{ HCO}_3^- + \text{H}^+ + \text{e}^- \rightarrow 1/14 (\text{C}_4\text{H}_4\text{O}_4)^{2-} + 3/7 \text{ H}_2\text{O}$	29.090
Ra(1) $1/4 \text{ O}_2 + \text{H}^+ + \text{e}^- \rightarrow 1/2 \text{ H}_2\text{O}$	- 78.719
Ra(2) $1/8 \text{ NO}_3^- + 5/4 \text{ H}^+ + \text{e}^- \rightarrow 1/8 \text{ NH}_4^+ + 3/8 \text{ H}_2\text{O}$	- 35.11
Ra(3) $1/2 \text{ NO}_3^- + \text{H}^+ + \text{e}^- \rightarrow 1/2 \text{ NO}_2^- + 1/2 \text{ H}_2\text{O}$	- 41.650
Ra(4) $2\text{H}^+ + \text{NO}_2^- + \text{e}^- \rightarrow \text{NO} + \text{H}_2\text{O}$	- 33.718
Ra(5) $\text{H}^+ + \text{NO} + \text{e}^- \rightarrow 1/2 \text{ N}_2\text{O} + 1/2 \text{ H}_2\text{O}$	- 115.829
Ra(6) $\text{H}^+ + 1/2 \text{ N}_2\text{O} + \text{e}^- \rightarrow 1/2 \text{ N}_2 + 1/2 \text{ H}_2\text{O}$	- 133.469
Rc $9/49 \text{ CO}_2 + 3/49 \text{ NH}_4^+ + 3/49 \text{ HCO}_3^- + \text{H}^+ + \text{e}^- \rightarrow 4/49 \text{ C}_3\text{H}_{5.4}\text{O}_{1.45}\text{N}_{0.75} + 106/245 \text{ H}_2\text{O}$	20.398 <sup>a</sup>

<sup>a</sup> This value was estimated from reported value of 18.8 kJ/eeq for an assumed cell relative composition of  $\text{C}_5\text{H}_7\text{O}_2\text{N}$  (Rittmann and McCarty, 2001) and considering  $\text{NH}_4^+$  as source for cell synthesis of  $\text{C}_3\text{H}_{5.4}\text{O}_{1.45}\text{N}_{0.75}$  (van Verseveld et al., 1983).

**Table 2**

Balanced energy reactions (*Re*) for cellular maintenance in aerobic and anaerobic phase.  $Re = Ra - Rd$  according to (Rittmann and McCarty, 2001).

Aerobic maintenance with succinate and oxygen: $(\text{C}_4\text{H}_4\text{O}_4)^{2-} + 3.5 \text{ O}_2 \rightarrow 2 \text{ CO}_2 + 2 \text{ HCO}_3^- + \text{H}_2\text{O}$
Anaerobic maintenance with succinate and nitrate: $(\text{C}_4\text{H}_4\text{O}_4)^{2-} + 7 \text{ NO}_3^- \rightarrow 2 \text{ CO}_2 + 2 \text{ HCO}_3^- + 7 \text{ NO}_2^- + \text{H}_2\text{O}$
Anaerobic maintenance with succinate and nitrite: $(\text{C}_4\text{H}_4\text{O}_4)^{2-} + 14 \text{ NO}_2^- + 14 \text{ H}^+ \rightarrow 14 \text{ NO} + 2 \text{ CO}_2 + 2 \text{ HCO}_3^- + 2 \text{ H}_2\text{O}$
Anaerobic maintenance with succinate and nitric oxide: $(\text{C}_4\text{H}_4\text{O}_4)^{2-} + 14 \text{ NO} \rightarrow 7 \text{ N}_2\text{O} + 2 \text{ CO}_2 + 2 \text{ HCO}_3^- + \text{H}_2\text{O}$
Anaerobic maintenance with succinate and nitrous oxide: $(\text{C}_4\text{H}_4\text{O}_4)^{2-} + 7 \text{ N}_2\text{O} \rightarrow 7 \text{ N}_2 + 2 \text{ CO}_2 + 2 \text{ HCO}_3^- + \text{H}_2\text{O}$

anaerobic conditions. To formulate these reactions (R1 to R6) and calculate the corresponding stoichiometric coefficients we used the TEEM2 methodology. In all reactions succinate is the universal electron donor (*Rd*) and C-source, and ammonia is the universal N-source to the cell synthesis (*Rc*), and the nutrients used as electron acceptors (*Ra*) are different, in aerobic conditions they are  $\text{O}_2$  and  $\text{NO}_3^-$  and in anaerobic conditions they are  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}$  and  $\text{N}_2\text{O}$  (Table 1). The stoichiometric coefficients for each metabolic pathway were obtained (Table 3) from Gibbs free energy for each half-reaction with a different assigned  $\epsilon$  value for each reaction in the range proposed for McCarty (1971, 2007) and Rittmann and McCarty (2001). With TEEM2 methodology each metabolic pathway accomplishes balances for carbon, nitrogen, electron and energy (see supplementary material for details).

#### 3.2. INDISIM-Paracoccus model description

To describe our model we use the ODD protocol (“Overview, Design concepts, and Details”) which helps to ensure that the model explanation is complete (Grimm, 1999; Grimm et al., 2010; Railsback and Grimm, 2012).

##### 3.2.1. Purpose

To develop a computational model for the denitrification process carried out by the bacteria *P. denitrificans* growing in batch and continuous culture, in aerobic and anaerobic growing conditions, in order to reproduce a bioreactor experimental protocol and explore the consequence of different priorities in the individual use of nutrients on the system dynamics. The first hypothesis (the Gibbs hypothesis) is that the denitrification reactions succeed sequentially according to their standard Gibbs energy, assuming that the bacterium goes first for the more spontaneous reactions. Reactions with lower Gibbs energy are expected to occur

**Table 3**

Balanced chemical equations (R) for biomass synthesis in aerobic and anaerobic phase. ( $R = f_e^a Ra + f_s^a Rc - Rd$ ) according to TEEM2 (McCarty, 2007).

R1	Aerobic conversion of succinate: $(C_4H_4O_4)^{2-} + 0.60 NH_4^+ + 1.04 O_2 \rightarrow 0.81 C_3H_{5.4}O_{1.45}N_{0.75} + 0.19 CO_2 + 1.40 HCO_3^- + 0.34 H_2O$
R2	$NO_3^-$ conversion in aerobic phase: $(C_4H_4O_4)^{2-} + 0.08 NH_4^+ + 0.52 NO_3^- + 1.05 H^+ + 0.18 H_2O \rightarrow 0.80 C_3H_{5.4}O_{1.45}N_{0.75} + 0.20 CO_2 + 1.40 HCO_3^-$
R3	$NO_3^-$ reduction with succinate (anaerobic phase): $(C_4H_4O_4)^{2-} + 0.30 NH_4^+ + 4.55 NO_3^- \rightarrow 0.40 C_3H_{5.4}O_{1.45}N_{0.75} + 4.55 NO_2^- + 1.10 CO_2 + 1.70 HCO_3^- + 0.67 H_2O$
R4	$NO_2^-$ reduction with succinate (anaerobic phase): $(C_4H_4O_4)^{2-} + 0.58 NH_4^+ + 4.55 NO_2^- + 4.55 H^+ \rightarrow 0.77 C_3H_{5.4}O_{1.45}N_{0.75} + 4.55 NO + 0.26 CO_2 + 1.42 HCO_3^- + 2.64 H_2O$
R5	NO reduction with succinate (anaerobic phase): $(C_4H_4O_4)^{2-} + 0.58 NH_4^+ + 4.55 NO \rightarrow 0.77 C_3H_{5.4}O_{1.45}N_{0.75} + 2.28 N_2O + 0.26 CO_2 + 1.42 HCO_3^- + 0.36 H_2O$
R6	$N_2O$ reduction with succinate (anaerobic phase): $(C_4H_4O_4)^{2-} + 0.58 NH_4^+ + 2.28 N_2O \rightarrow 0.77 C_3H_{5.4}O_{1.45}N_{0.75} + 2.28 N_2 + 0.26 CO_2 + 1.42 HCO_3^- + 0.36 H_2O$

first. In this case the order is: R3, R6, R5 and R4 (see [supplementary material](#) for details). The second hypothesis (the metabolic hypothesis) is that the bacterial cell prioritizes the use of those nitrogen oxides with a higher degree of oxidation over others, which is the common order established in the denitrification pathway (Caspi et al., 2012). In this case the order is: R3, R4, R5 and R6.

### 3.2.2. Entities, state variables, and scales

The INDISIM-Paracoccus model has two types of entities: individuals and square patches of culture medium. Each individual represents a unique bacterium of *P. denitrificans* and has the variables: unique identification number, location (XY grid cell coordinates of where it is), mass, reproduction mass, and counters for each metabolic pathway and reproduction cycle. Therefore, the model assumes that the smallest individual represents a bacterium with a diameter of  $\sim 0.5 \mu m$  and the largest one a bacterium with a diameter of  $\sim 0.9 \mu m$  (Holt et al., 1994). All bacteria have spherical shape and their individual mass is deduced from cell volume by assuming the microbial mass density equal to  $1.1 g cm^{-3}$ , which has been used in previous INDISIM models (Gras et al., 2011). In order to characterize the composition of the microbial cells, the model uses the empirical formula  $C_3H_{5.4}N_{0.75}O_{1.45}$  (van Verseveld et al., 1979, 1983) so that each bacterium is assumed to have this elementary cell composition.

A two-dimensional lattice of  $25 \times 25$  grid cells represents the bioreactor that contains the culture medium; each cell represents 1 pl, so that the total bioreactor volume is 625 pl. The spatial cell variables are: unique position identifier in XY coordinates, total amount of each nutrient: succinate,  $NH_4^+$ ,  $O_2$ ,  $NO_3^-$ , and metabolic products,  $NO_2^-$ , NO,  $N_2O$ ,  $N_2$  and  $CO_2$ . All microbial and culture medium processes are discretized in time steps. One time step represents 5 min; for the current work the simulations were run for 1440 time steps (120 h). With these units, graphical and numerical model outputs are the molar concentration of nutrients and metabolic products expressed in millimolar (mM) or micromolar ( $\mu M$ ) and dry mass in  $mg ml^{-1}$ .

### 3.2.3. Process overview and scheduling

The initial configuration of our model has two parts: the first one for the system and the second one for the entities (culture medium and bacteria). The initial system setup sets the world size and topology, and the time scaled factor (time step). The topology of the world is programmed using the torus mode; therefore, rectangular periodic boundary conditions were used. The initial

culture medium concentrations and the initial bacterial population are established using random variables, normal probability distributions with mean values that are determined by the experimental procedure (Felgate et al., 2012).

At each time step a group of individuals are controlled using a set of time-dependent variables for each bacterium. All individuals perform the following processes: nutrient uptake, cellular maintenance, biomass synthesis, metabolic products generation and bipartition. Culture medium processes are different depending on the management bioreactor protocol, but in any case the culture medium is randomly homogenized to simulate chemostat agitation. At the beginning of the simulation the bioreactor works as a batch culture with oxygen saturated conditions ( $236 \mu M$ ), and the user can choose at what time to end this phase, and switch to continuous culture in anoxic conditions, with input-output culture media (with nutrients, metabolic products and micro-organisms) according to the dilution rate fixed. For each time step the time-dependent variables of microorganisms and culture medium are calculated, updating the graphics and digital outputs according to the time scale proposed. The model also controls the whole carbon and nitrogen mass inside and outside of the system to ensure the carbon and nitrogen are balanced. During the simulation processes the bacteria are called in a different random order in each time step and the state variables changes are immediately assigned generating an asynchronous update. Fig. 2 shows the INDISIM-Paracoccus schematic diagram.

### 3.2.4. Design concepts

**3.2.4.1. Basic principles.** The model has two kinds of behavior-rules, rules for the individuals (bacteria) and different rules for the environment (culture medium). The set of individuals and the environment is called the system (bioreactor). All of the rules are used at the level of the sub-models and they are explained in the corresponding section. The individual rules are: (i) nutrient uptake, (ii) cellular maintenance (Table 2), (iii) cellular growth when a micro-organism executes any of the metabolic reactions adjusted by TEEM2 (Table 3), and (iv) cellular division following binary fission. The system rules are those that mimic the general bioreactor procedures when it works as: (i) a batch culture with constant oxygenation, with  $O_2$  input flow, (ii) a continuous culture with a dilution rate, with the entrance of fresh medium, and output of medium and bacteria, and (iii) the stirring culture, with redistribution of compounds, which permits the exclusion of local diffusion limitations.

**3.2.4.2. Emergence.** The system dynamics emerge as the result of the interaction between bacteria and the culture medium that they find inside of the bioreactor. The model outputs are: the biomass evolution, nutrient consumption, metabolic and/or denitrification products generation, or other parameters that appear at the system level as a consequence, and from, the individual bacterial activity.

**3.2.4.3. Adaptation.** All the individuals (bacteria) are programmed with the same rules, some of these rules will be executed and others not, depending on the internal changes of the individuals and/or the characteristics of their local environments. Individuals act one after another, not in parallel. Hence, after one individual carries out all of its actions the composition of the spatial cell where it lives changes and the next individual meets a different medium composition in relation to the previous acting or post-acting individuals. In particular, the metabolic pathway that it might employ could be different. The first individual rule is how to respond to the  $O_2$  dissolved level in the culture medium: if the  $O_2$  dissolved level in the spatial cell is lower than a threshold value ( $O_{2-MIN}$ ) the bacterium uses the anaerobic metabolism and

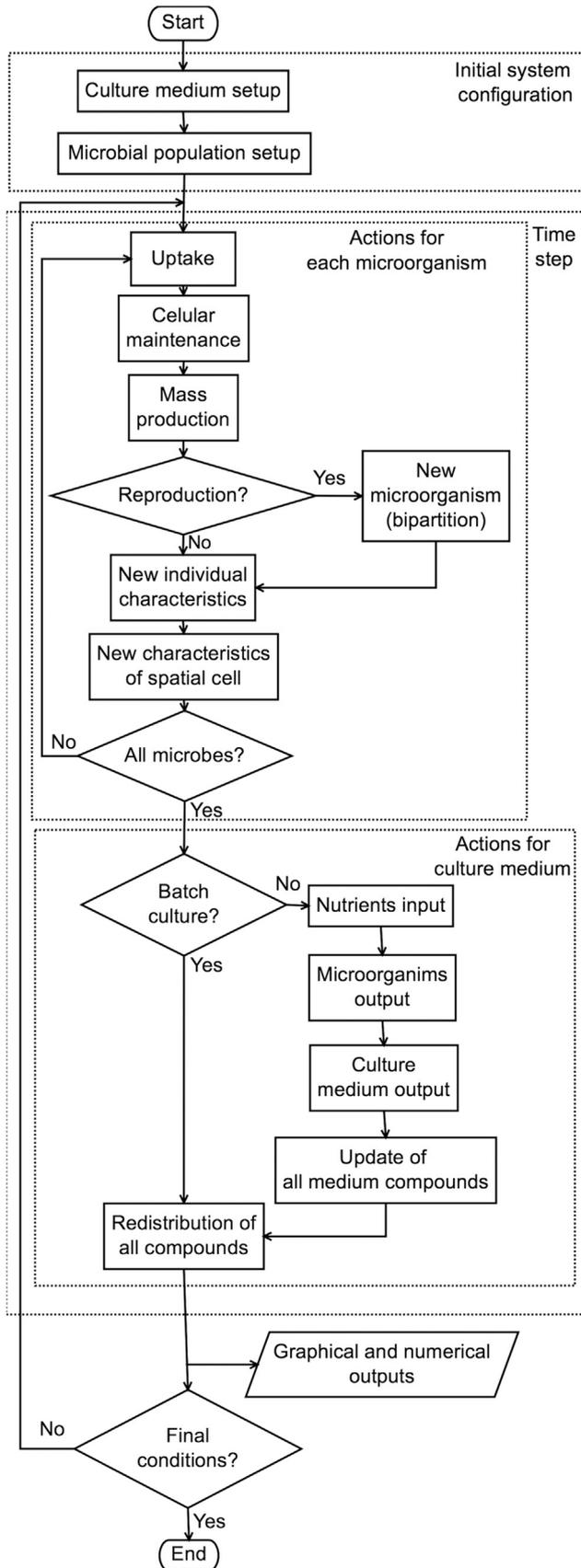


Fig. 2. Flow chart of the INDISIM-Paracoccus model.

otherwise it uses the aerobic metabolism. The second individual rule is performing biomass synthesis (Table 3) to growth and metabolic product generation. This rule is executed only when the amounts of nutrients taken in the uptake are enough to accomplish the maintenance requirement (Table 2) and after updated amounts also allow execution of the corresponding synthesis reaction (Table 3) in the aerobic or anaerobic phase. The third individual rule is whether to divide or not, depending on whether or not it has reached the minimum reproduction mass. The reproduction mass ( $m_R$ ) is the mass the bacterium must reach to start the bipartion ( $m_R$  is obtained from a normal random distribution with a mean value of 75% of the larger bacterium size) (Table 4).

3.2.4.4. *Interaction.* *P. denitrificans* is the only bacteria species in the virtual bioreactor. The micro-organisms interact with the culture medium; therefore there is an indirect interaction in which nutrient competition takes place among the bacteria that share the same spatial cell.

3.2.4.5. *Collective.* The simulated bacteria do not form aggregates; each individual acts uniquely.

3.2.4.6. *Stochasticity.* Several processes are modeled on criteria of randomness: (i) the reproduction sub-model, (ii) a portion of the uptake sub-model, (iii) some parameters involved in the bioreactor management or operating protocol, and (iv) a part of the initial system configuration. Inside of the reproduction sub-model we consider that the reproduction threshold biomass for each bacterium is determined using a value from a normal random distribution (Table 4). For the physical separation of the two bacteria the original mass is separated into two new bacteria with masses according to a value from the normal random distribution with mean value 0.5 and standard deviation 0.075 (Table 4). Thus, the mass of the original bacterium does not divide exactly in the proportion 50–50.

Inside the uptake sub-model we consider that at each time step, each individual nutrient uptake-rate ( $u_i$ ) for each nutrient is established from a normal random distribution with the mean value shown in Table 4 and a standard deviation of 5% of this value. Regarding the bioreactor management: (i) the dilution rate parameter, for each input–output, is obtained by using the normal random distribution with mean value  $0.05 \text{ h}^{-1}$  and standard deviation 0.0025, in order to represent experimental error, and (ii) each bacterium position randomly changes at some time steps in order to represent the mixing effects from the stirred tank. For the initial system configuration we consider that the initial culture medium composition, the initial population biomasses and  $\text{O}_2\text{-MIN}$  threshold value are established from normal distributions with mean values determined by the experimental procedure (Table 4) and standard deviations of 5% of these values. To represent the small reactor with constant agitation, we introduce a redistribution of nutrients and metabolic products in random time steps. When the simulation starts each bacterium has a position randomly assigned in the culture medium.

3.2.4.7. *Observation.* The graphical and numerical outputs of the model are the concentration ( $\text{mmol l}^{-1}$  or  $\text{umol l}^{-1}$ ) of each culture medium component (succinate,  $\text{NH}_4^+$ ,  $\text{O}_2$ ,  $\text{NO}_3^-$ ,  $\text{CO}_2$ ,  $\text{HCO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$ ), microbial biomass ( $\text{mg ml}^{-1}$ ) and the population biomass distribution at each time step (the user can obtain all simulated data in the output file with the extension “.txt”).

**Table 4**  
INDISIM-Paracoccus model parameters values.

Nutrient	Culture medium initial concentration [mM] according to Felgate et al. (2012)	Availability coefficient – $a_i$ – ( $h^{-1}$ ) fixed according to Dab	Uptake-rate – $u_i$ – ( $mol_{nutrient} \cdot mol_{mass}^{-1} h^{-1}$ )			
			Testing values			Calibrated values
			Low (L)	Medium (M)	High (H)	
Succinate	5 <sup>c</sup> –20 <sup>d</sup>	0.28 <sup>a,b,e,f</sup>	0.065	0.13	0.52 <sup>g</sup>	0.52 <sup>a,b,e,f</sup>
Ammonium	10 <sup>c,d</sup>	0.84 <sup>a,b,e,f</sup>	–	–	0.31 <sup>g</sup>	0.31 <sup>a,b,e,f</sup>
Oxygen	0.236 <sup>c,d</sup>	0.79 <sup>a,b,e,f</sup>	–	–	0.54 <sup>g</sup>	0.54 <sup>a,e,f</sup>
Nitrate-a (aerobic)	4.9983 <sup>d</sup> –21.6095 <sup>c</sup>	0.63 <sup>a,b,e,f</sup>	0.034	0.068	0.27 <sup>g</sup>	0.27 <sup>a,e,f</sup>
Nitrate-x (anaerobic)			0.019	0.119	1.19 <sup>g,h</sup>	0.119 <sup>b,e,f</sup>
Nitrite	0.0255 <sup>c</sup> –0.0112 <sup>d</sup>	0.79 <sup>a,b,e,f</sup>	0.0062	0.062	0.62 <sup>g,h</sup>	0.062 <sup>b,e</sup> – 0.62 <sup>b,f</sup>
Nitric Oxide	–	1.00 <sup>a,b,e,f</sup>	0.0000062	0.00062	0.62 <sup>g,h</sup>	0.62 <sup>b,e,f</sup>
Nitrous Oxide	0.003 <sup>c</sup> –0.000028 <sup>d</sup>	0.50 <sup>a,b,e,f</sup>	0.0031	0.031	0.31 <sup>g,h</sup>	0.31 <sup>b,e,f</sup>
<b>Other bacterial parameters</b>						
Parameter	Testing range	Calibrated value	Reference			
Cellular maintenance ( $gC_{donor} gC_{mic}^{-1} h^{-1}$ )	–	0.0020 <sup>a</sup> –0.0040 <sup>b</sup>	Gras et al. (2011) and van Verseveld et al. (1983)			
Mass split	–	0.50 (15% coefficient of variation)	Derived from (Ginovart et al., 2002)			
Small bacterium size ( $\mu m$ )	0.4–0.6 <sup>a,b</sup>	0.5 <sup>a,b</sup>	Holt et al. (1994)			
Big bacterium size ( $\mu m$ )	0.8–1.0 <sup>a,b</sup>	0.9 <sup>a,b</sup>				
Minimum bacterium size at reproduction	–	75% of big bacterium size (15% coefficient of variation)	Derived from (Gras et al., 2011) and (Ginovart et al., 2002)			

Phase: (a) Aerobic, (b) Anaerobic. Experiment: (c) Succinate-limited/ $NO_3^-$ -sufficient, (d) Succinate-sufficient/ $NO_3^-$ -limited. Hypothesis: (e) Metabolic, (f) Gibbs. The values (g) are the result of perform calculations between the maximum growth rate ( $\mu_{max}=0.418 h^{-1}$ , van Verseveld et al., 1983) and the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table 3). The values (h) are the result of divide each high uptake-rate by 4 due to the maximum growth rate is achieved when the four reactions are carried out by the bacterium.

### 3.2.5. Initialization

The user can adjust: (i) the culture medium composition ( $mmol l^{-1}$ ) of succinate,  $NH_4^+$ ,  $O_2$  and  $NO_3^-$ , (ii)  $O_{2-MIN}$  value which is in the range of 0.01–0.31 mM  $O_2$ , (iii) dilution rate ( $h^{-1}$ ), (iv) initial amount of viable micro-organisms (bacteria), (v) total simulation time (h), (vi) step time (min), (vii) time (h) for shut-down  $O_2$  input flow, and (viii) the maintenance energy requirement for aerobic and anaerobic phases ( $gC_{donor} gC_{mic}^{-1} h^{-1}$ ).

### 3.2.6. Sub models

The bipartition reproduction process is a sub model that is taken from INDISIM, the generic and core bacterial model (Ginovart et al., 2002). Thus, we only describe the individual sub-models that we designed particularly for the *P. denitrificans*.

**3.2.6.1. Uptake.** Each nutrient uptake depends on the individual capacity to capture nutrients through the cell membrane-associated proteins (Button, 1998) and on the nutrient availability in the medium (Gras et al., 2011). In our model, to determine the amount of each nutrient captured (absorbed) by each bacterium at each time step, two values are compared, the maximum uptake capacities ( $U_i$ ,  $mol_{nutrient} h^{-1}$ ) of the bacterium and the nutrient available in the culture medium ( $A_i$ ,  $mol_{nutrient} h^{-1}$ ), and the lowest value is chosen.

$U_i$  is assumed to be proportional to the individual mass and to the uptake-rate ( $u_i$ ) being  $i$  the nutrient, so:

$$U_i = u_i^* \text{ individual-mass} \quad (1)$$

$u_i$  is a model parameter which represents the amount of nutrient that could be absorbed per unit of time and mass, its units are  $mol_{nutrient} mol_{mass}^{-1} h^{-1}$ , where  $mol_{mass}$  denotes the moles of microbial mass (the microbial mass equals  $C_3H_{5.4}N_{0.75}O_{1.45}$ ).

$A_i$  is assumed to be proportional to the nutrient amount in each spatial cell and to the availability coefficient ( $a_i$ ) being  $i$  the nutrient, so:

$$A_i = a_i^* \text{ nutrient-amount} \quad (2)$$

$a_i$  is a model parameter directly related to the nutrient characteristics and not to the types of micro-organisms involved, which represents the fraction of each nutrient in a spatial cell that is accessible per unit of time and for the individual, its units are  $h^{-1}$ .

Following the INDISIM framework (Gras et al., 2011) the maximum population growth rate ( $\mu_{max}$ ) has been used to estimate the individual maximum uptake-rates ( $u_i$ ). vanVerseveld et al. (1983) reported for *P. denitrificans* a growth rate value equal to  $0.418 h^{-1}$  which was obtained in the change from a culture growing in anaerobic nitrate-limited conditions to aerobic succinate-limited conditions. Using this value and performing calculations with the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2, we obtained the maximum uptake-rate for each nutrient (see Supplementary material for details). In order to give values to the availability coefficient ( $a_i$ ), and only as a macro reference to sort and represent numerically the availabilities of the nutrients in the culture medium, the Fick's law binary diffusion coefficients (Dab) in water was used. Therefore we assumed in the modeling process that the nutrient with maximum Dab has the highest availability; the other availability values are assigned proportionally (Table 4).

**3.2.6.2. Maintenance.** Before biomass synthesis, it is necessary that each bacterium achieve some energetic requirements to ensure its viability. The cellular maintenance sub-model has two main components, the maintenance requirement and the energy reactions (Re) written with TEEM2 (Table 2). The maintenance requirements are proportional to individual's mass. The coefficients determine an amount of nutrients per time step for cellular maintenance. Gras et al. (2011) consider an appropriate maintenance requirement for soil heterotrophic microorganisms of  $0.002 gC_{donor} gC_{mic}^{-1} h^{-1}$ , which was assumed in the model for aerobic phase. For *P. denitrificans* in anaerobic phase growth and taking succinate as electron donor and  $NO_3^-$  as electron acceptor, van Verseveld et al. (1977) give a maintenance coefficient of  $0.004 gC_{donor} gC_{mic}^{-1} h^{-1}$  which was assumed for anaerobic phase. The energy reactions (Table 2) indicate the stoichiometry

that the nutrients follow when each bacterium executes this action or rule. Each bacterium achieves its maintenance when the amount of nutrients taken in is enough to accomplish the maintenance requirement and these amounts also allow it to execute the corresponding energy reaction. Performing calculations with the energy reactions (Table 2), we establish the maintenance requirements for aerobic and anaerobic phases (see [Supplementary material](#) for details). When the individual carries out its maintenance, the  $\text{CO}_2$  and the reduced electron acceptors are expelled to the culture medium except for the  $\text{NO}_2^-$ , which is added to its corresponding intake. In anaerobic phase the first individual option is to accomplish the maintenance requirement carrying out the energy reaction with succinate and nitrate, if the bacterium cannot reach its maintenance requirements, it can try it with succinate and another electron acceptor following other reactions according to the hypothesis test. After the maintenance, if the remaining succinate uptaken and the quantity of electron acceptors are higher than zero, the individual can perform biomass synthesis.

**3.2.6.3. Biomass synthesis and metabolic products.** With the nutrient intakes updated and using the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2, each bacterium divides the amount of each nutrient uptaken by its respective stoichiometric coefficient and selects the smallest value (the limiting nutrient). This information provides the demands of each one of the nutrients and drives the creation of new mass and metabolic products generation. After executing any metabolic reaction the  $\text{CO}_2$  produced is released to the culture medium. When the bacterium executes the reactions of denitrification, nitrogen oxides are produced, and they are not expelled into the culture medium; only the nitrogen gas is expelled, and the amounts of nitrogen oxides generated are added to its corresponding intakes. The execution of each metabolic reaction is limited to the existence of sufficient quantities of electron donors and acceptors. After this, if there are any intakes, the microbe can perform the next metabolic reaction. When this condition is not fulfilled the syntheses finish and the remaining intakes are returned to the culture medium.

The sub models related to the bioreactor's procedure are: (i) *Agitation*. To represent the agitation inside of the small experimental reactor, which causes homogeneity in the culture medium, nutrients and metabolic products are redistributed in various time steps. In the culture medium the micro-organism positions change randomly, (ii) *Input flow*. The bioreactor is refilled with fresh culture medium (succinate,  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ) with a composition equal to the initial one, and (iii) *Output flow*. A fraction of the individuals and culture medium are randomly removed. The input and output flows are performed according to the dilution rate parameter.

#### 4. Results and discussion

INDISIM-Paracoccus was implemented in the NetLogo platform. It is straightforward to change parameter values, to modify the source code of the model and to investigate alternative mechanisms or add additional processes relevant to a particular study. It is hoped that this NetLogo simulator will facilitate new fruitful interactions between modelers and experts in the field of denitrification.

We used a variety of measures and basic techniques in order to verify that our implementation was in accordance with the conceptual model and its quantification. For instance, to ensure that the stoichiometry and the bioreactor inputs/outputs are accurately implemented, one of the main tasks was to control the differences between carbon and nitrogen levels, to ensure that the simulator

accomplished balances for C and N. For each time step the following is controlled: (i) the entrance and exit of the all chemical species involved, (ii) the product generations and nutrient consumptions from each balanced chemical equation executed, and (iii) the bacteria inside and outside of the system.

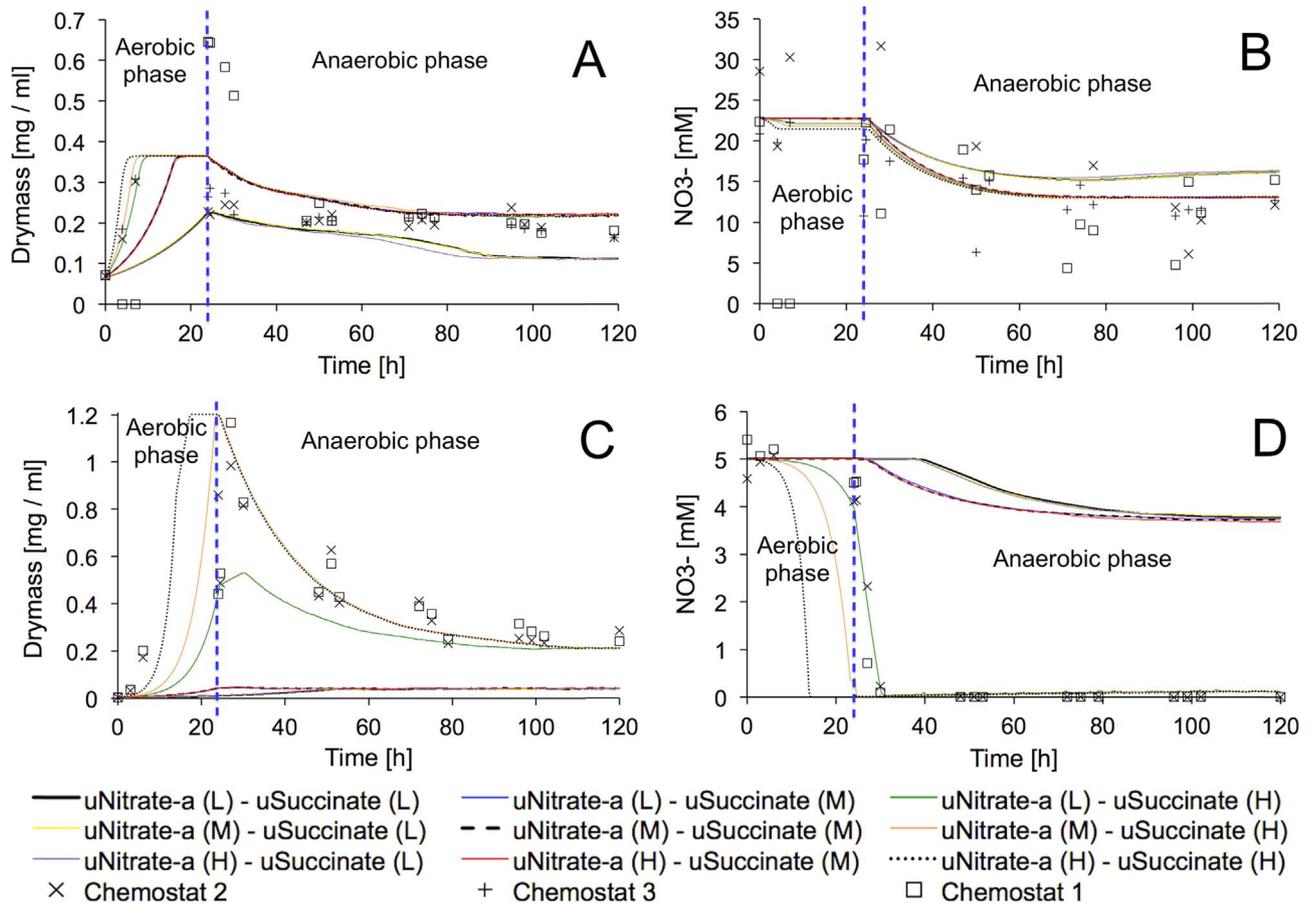
At the end of every time step, from each one of the patches, the simulator obtains the amount of each nutrient and metabolic product, and for each one of the bacteria the value of its mass. To ensure that the carbon and nitrogen are balanced, the model implementation summarizes all of the carbon and nitrogen inside and outside of the system and compares this value with the same calculation obtained in the previous time step. We expected that these two values should be the same, but there exist small differences (not higher than 0.05%). This procedure is repeated and the simulator registers these numbers and presents them as graphical and numerical outputs (in [Supplementary material](#), Fig. S1).

We also tested that the individuals were able to carry out all of the reactions in a variety of culture media compositions. In addition, we systematically investigated internal model logic and behaviors by collecting global and individual data through the simulation, which were numerically and visually tested (Fig. S1). The control of the different metabolic pathways used in each time step and for each bacterium is programmed in the computer code, and the simulator facilitates which pathways are in use and which not. The main metabolic differences are present when the model runs with different conditions from experiments (E1 or E2), corresponding to the experimental condition of succinate limited or nitrate limited. Also, in the anaerobic conditions, it is possible to follow (control) the number of bacteria, which do not complete the denitrification pathway or follow (control) the metabolic pathway (synthesis or maintenance) that is the most used by the bacteria. This control is a graphical output in the model's implementation on NetLogo that appears in the user interface of the simulator (Fig. S1). Such tests are essential for increasing the reliability of the computational model, and for contributing to the understanding of the virtual system and the consequences of the modeling assumptions (Scheller et al., 2010).

We have carried out simulations varying the size of the system, from  $25 \times 25 = 625$  patches of culture medium to  $100 \times 100 = 10,000$  patches. The number of individuals in these simulations was increased as well, from 3000 (in the smallest system) to around 50,000 individuals (in the biggest system). The model's outputs for these sets of simulations were very similar (Figs. S2 and S3). With the increase of the system sizes the time spent in the simulations increased along with computational requirements. Taking into account these results we decided to establish a system size that was not too large, but was sufficient to allow us to obtain simulation results that we could compare with the experimental data available. Also, we investigated the time step before fixing it in our simulations, and finally, this was established at 5 min. We tested the model using values from 1 to 10 min (Figs. S4 and S5). This is one of the parameters that can be changed in the initialization of the system before starting the simulation.

During the development of the model some parameters values were obtained from biological constraints or references and were used in all simulations performed (Table 4), but others were not fixed due to the uncertainty in, or complete lack of, observational data.

We used the uptake-rate parameters with a set of simulation series during the categorical calibration process (Table 4). The simulation outputs were compared with the experimental data under the two different scenarios corresponding to the two experimental conditions of Felgate et al. (2012): succinate-sufficient/ $\text{NO}_3^-$  limited (Experiment E1) and succinate-limited/ $\text{NO}_3^-$ -sufficient (Experiment E2) and for each of the hypotheses considered (the metabolic hypothesis and the Gibbs' hypothesis). The simulated cultures were



**Fig. 3.** INDISIM-Paracoccus model calibration outputs for the aerobic phase. Each color line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate ( $u_i$ ) for succinate and nitrate in aerobic phase. The  $u_i$  values are reported in Table 4 (being L=low, M=medium and H=high). Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). For the experiment E1, succinate-limited/ $\text{NO}_3^-$ -sufficient (A and B) and for the experiment E2, succinate-sufficient/ $\text{NO}_3^-$ -limited (C and D). The aerobic phase (from 0 to 24 h) in batch culture, and the anaerobic phase (from 24 to 120 h) in continuous culture. The metabolic hypothesis has been assumed in the simulator to generate these results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

initially grown under batch aerobic conditions (from 0 to 24 h) following the switch to continuous culture where the populations shift to anaerobic metabolism (from 24 to 120 h). It is not possible to perform model calibration separately for these unknown parameters (uptake-rates) because the individual processes in which they are involved are highly dependent on one another.

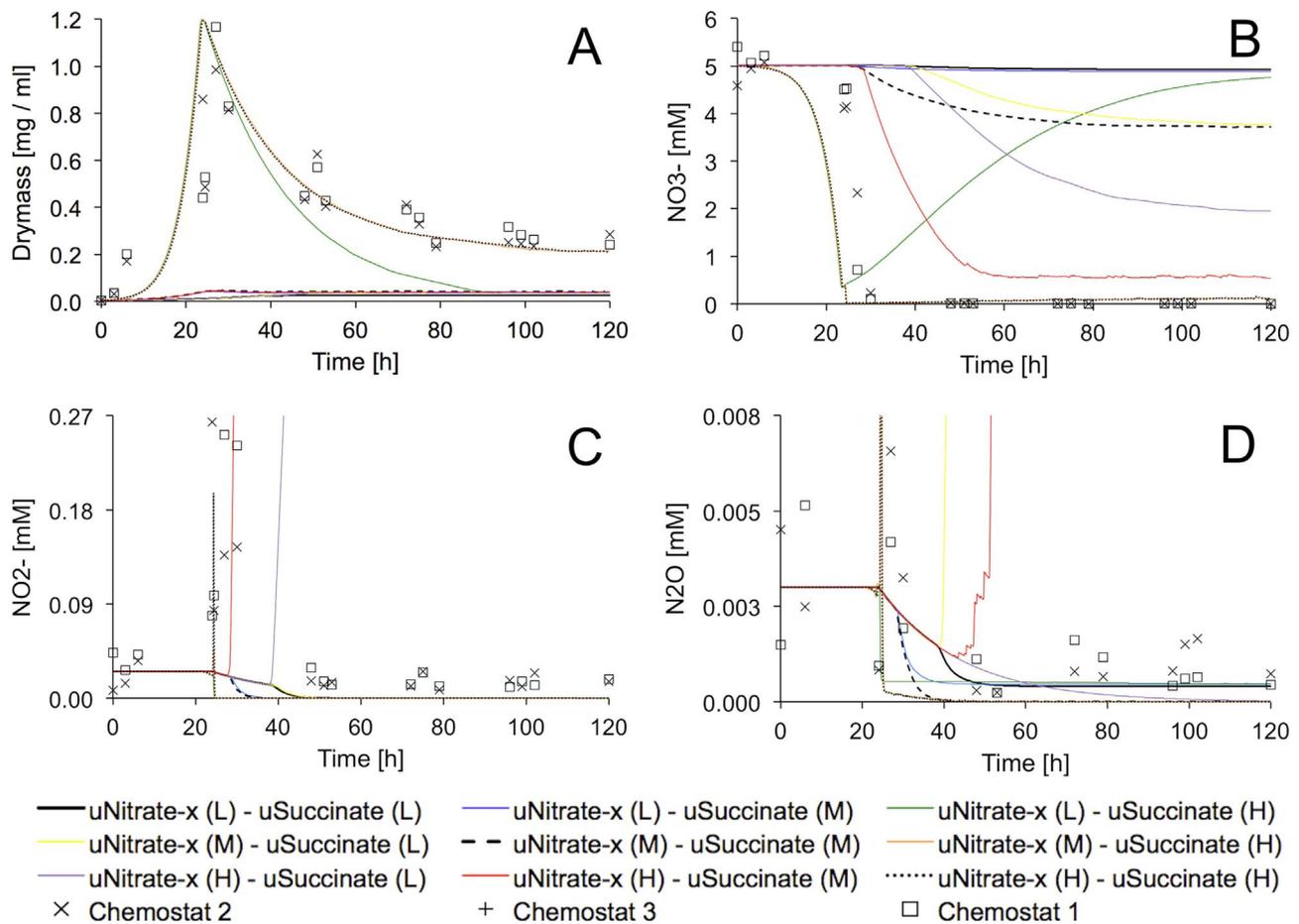
Each curve in Figs. 3–5 represents the simulation result for one combination of values of the parameters “uptake-rate” for the different nutrients. The bacteria in our model grow and develop in two very different conditions: the first condition occurs in aerobic phase (from 0 to 24 h) in batch culture, and the second one occurs in anaerobic phase (from 24 to 120 h) in continuous culture.

The first series of simulations (Fig. 3) were carried out to explore the succinate and nitrate uptakes-rates values during the aerobic phase (Table 4). In all cases, some simulation results shown in Figs. 3A, B and C reproduce in a better way the experimental trend (Felgate et al. 2012) than other simulation results shown in Fig. 3D, but in any case the state achieved in the aerobic phase determined the subsequent denitrification. As the two hypotheses we tested (metabolic and Gibbs) do not play any role in the aerobic phase, the results obtained with a different combination of uptake-rates values for succinate and nitrate in the aerobic phase are a consequence of nutrient usage. When the uptake-rate for nitrate in aerobic phase takes the value of the maximum nutrient uptake-rate ( $u_{\text{Nitrate-a}} = 0.27 \text{ mol}_{\text{Nitrate-a}} \text{ mol}_{\text{mass}}^{-1} \text{ h}^{-1}$ , the high value in Table 4) (see Supplementary material for details) the fit of the model is acceptable (Fig. 6A).

With the uptake-rate value of nitrate (aerobic phase) fixed, our second series of simulations explored combinations of uptake-rates for succinate ( $u_{\text{Succinate}}$ ) and nitrate in the anaerobic phase ( $u_{\text{Nitrate-x}}$ ) (Figs. 4 and 5). In Fig. 4 we present the simulation results with the experimental data corresponding to experiment E1 and in Fig. 5 to experiment E2. These results are generated using the metabolic hypothesis and compared with the experimental temporal evolutions of biomass,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and  $\text{N}_2\text{O}$ . Therefore, we can see the model behavior during the denitrification process. Taking into account these series of simulations when the uptake-rate for succinate takes the value of the maximum nutrient uptake-rate ( $u_{\text{Succinate}} = 0.52 \text{ mol}_{\text{Succinate}} \text{ mol}_{\text{mass}}^{-1} \text{ h}^{-1}$ ) the model accuracy is good (Figs. 6A and B).

When the electron donor (succinate) is not a limiting nutrient (experiment E1), the model results regarding the time evolutions of nitrite and nitrous oxide shows values equal or close to zero (Figs. 4C and D). This is due to the fact that there exist electron donor, and the electron acceptors can be reduced. When the electron donor (succinate) is limited (experiment E2) the model shows low sensitivity in the time evolution of nitrite (Fig. 5C) and in the nitrous oxide (Fig. 5D). For both experiments (E1 and E2) and for the two hypotheses (Gibbs hypothesis and metabolic hypothesis) the model shows a good response and sensitivity to the time evolutions of dry mass (Figs. 4A and 5A) and nitrate (Figs. 4B and 5B). This is because nitrate is the nutrient by which the denitrification process begins when bacteria meet in its environment anoxic conditions.

In the bubble charts of Fig. 6, each circle radius represents the



**Fig. 4.** INDISIM-Paracoccus model calibration output for the experiment succinate-sufficient/ $\text{NO}_3^-$  limited (E1). Time evolutions of: (A) Biomass, (B) Nitrate, (C) Nitrite and (D) Nitrous oxide. Each color line represents the mean of three simulations obtained with different combinations of the parameters uptake-rate ( $u_i$ ) for succinate and nitrate in anaerobic phase. The  $u_i$  values are reported in Table 4 (being L=low, M=medium and H=high). Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

percentage of the global adequacy of the model compared with the experimental data presented by Felgate et al. (2012). By examining the bubble charts corresponding to Figs. 6C, D and E, the value of Unitrate-x that best fits the simulation results with the experimental points, for both experiments and hypotheses, is  $0.119 \text{ mol}_{\text{nitrate-x}} \text{ mol}_{\text{mass}}^{-1} \text{ h}^{-1}$ . Looking at the bubble charts of Figs. 6F, G and H, it is possible to say that the model's sensitivity is low with the changes of the values corresponding to the uptake-rates of nitrite, nitric oxide and nitrous oxide, since the radius of the circles are similar to each other. Taking into account this calibration process, the calibrated values for all the uptake-rate nutrients, and for both hypothesis, are presented in Table 4. None of the tested parameter combinations met 100% of the calibration criteria defined with the 14 assessment criteria, but some are considerably better than others. The multiple fitting criteria results used to explore these values and to contrast the two hypotheses are presented in bubble charts, which offer some hints as to the delimited range of values and help to discern between the two hypotheses. In all of the cases the metabolic hypothesis shows a bigger radius of the circle than the Gibbs hypothesis (Fig. 6).

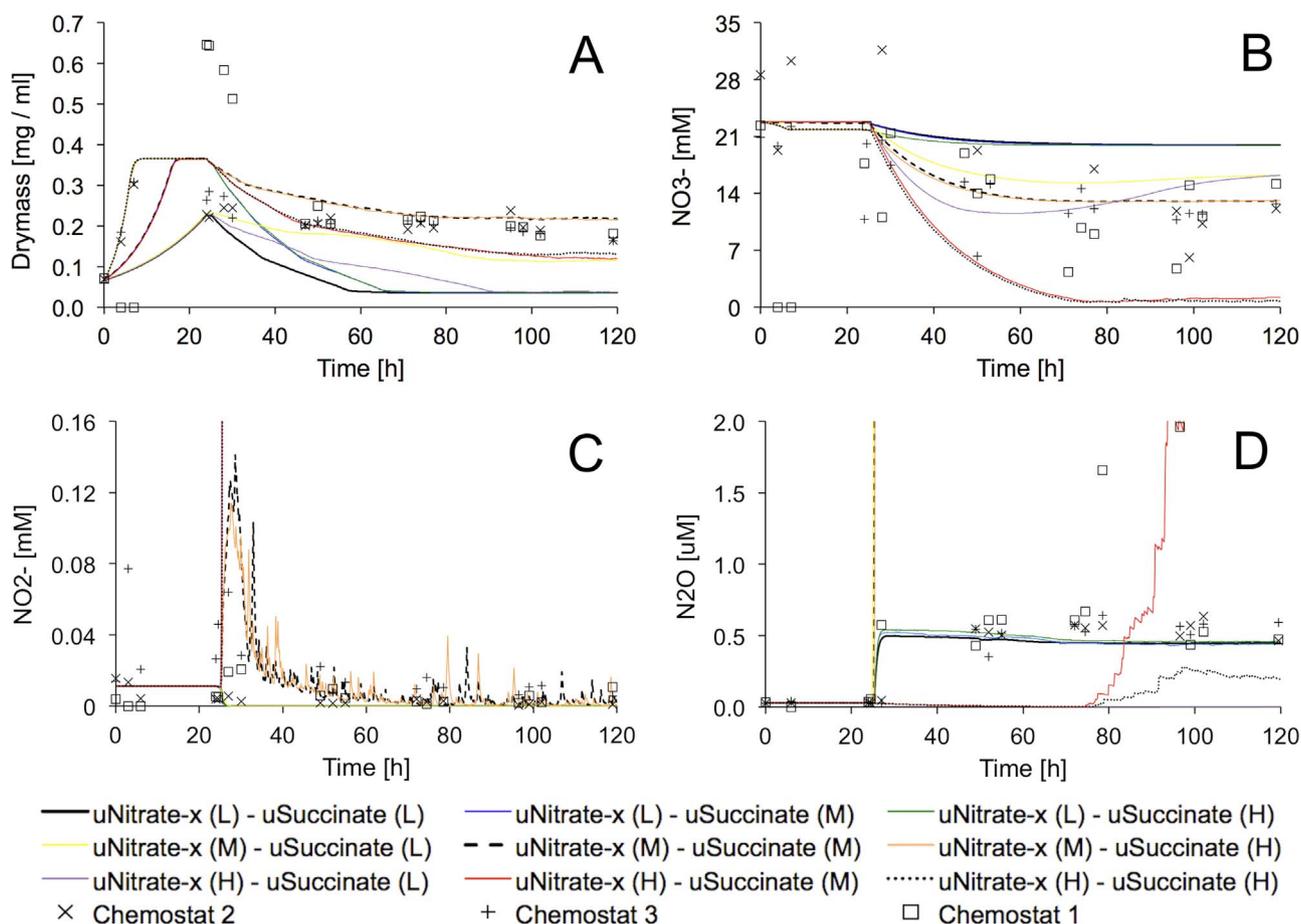
In general, fitting a single response variable is straight-forward, but a global fitting for the whole system is much more demanding and challenging to achieve, and even more so if there are different experimental medium conditions jointly with aerobic and anaerobic metabolisms (Woolfenden et al., 2013). We prioritized the diversity of the results because we are convinced that the use of multiple outputs (patterns) to test models is one of the main and

most relevant features of the pattern-oriented modeling strategy used in the framework of IBMs (Grimm et al., 2005).

The GRI values for both hypotheses, for the four-temporal evolutions studied (biomass, nitrate, nitrite and nitrous oxide) and for the two experiments (E1 and E2), are shown in Table 5. For the Gibbs hypothesis the GRI values are higher than the GRI values of the metabolic hypothesis. It is noteworthy that the temporal evolutions of nitrite and nitrous oxide are outside of the adequate GRI range for both hypotheses and for the two experiments, which suggests it is necessary to include new elements in the individual rules for this denitrifying bacteria which must be relevant in the dynamic of this oxide. Further developments of this model will need to take into account the role of the nitrous oxide in the metabolic reactions and specific experimentation could help to identify the key factors, which control the amount of this product.

## 5. Conclusions

The Gibbs hypothesis seemed a plausible and attractive strategy at individual level because it represents the spontaneity of a reaction carried out by a micro-organism, but the adequacy of the model outputs is slightly better for the metabolic hypothesis. In addition, the metabolic hypothesis links better with the idea of the sequential use of the synthesis and consumption of electron donors, which is probably linked to individual mass degradation to reduce cytotoxic products to complete the denitrification pathway,



**Fig. 5.** INDISIM-Paracoccus model calibration output for the experiment succinate-limited/ $\text{NO}_3^-$  sufficient (E2). Time evolutions of: (A) Biomass, (B) Nitrate, (C) Nitrite and (D) Nitrous oxide. Each color line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate ( $u_i$ ) for succinate and nitrate in anaerobic phase. The  $u_i$  values are reported in Table 4 (being L=low, M=medium and H=high). Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the expression of denitrifying enzymes and the consequent individual activity.

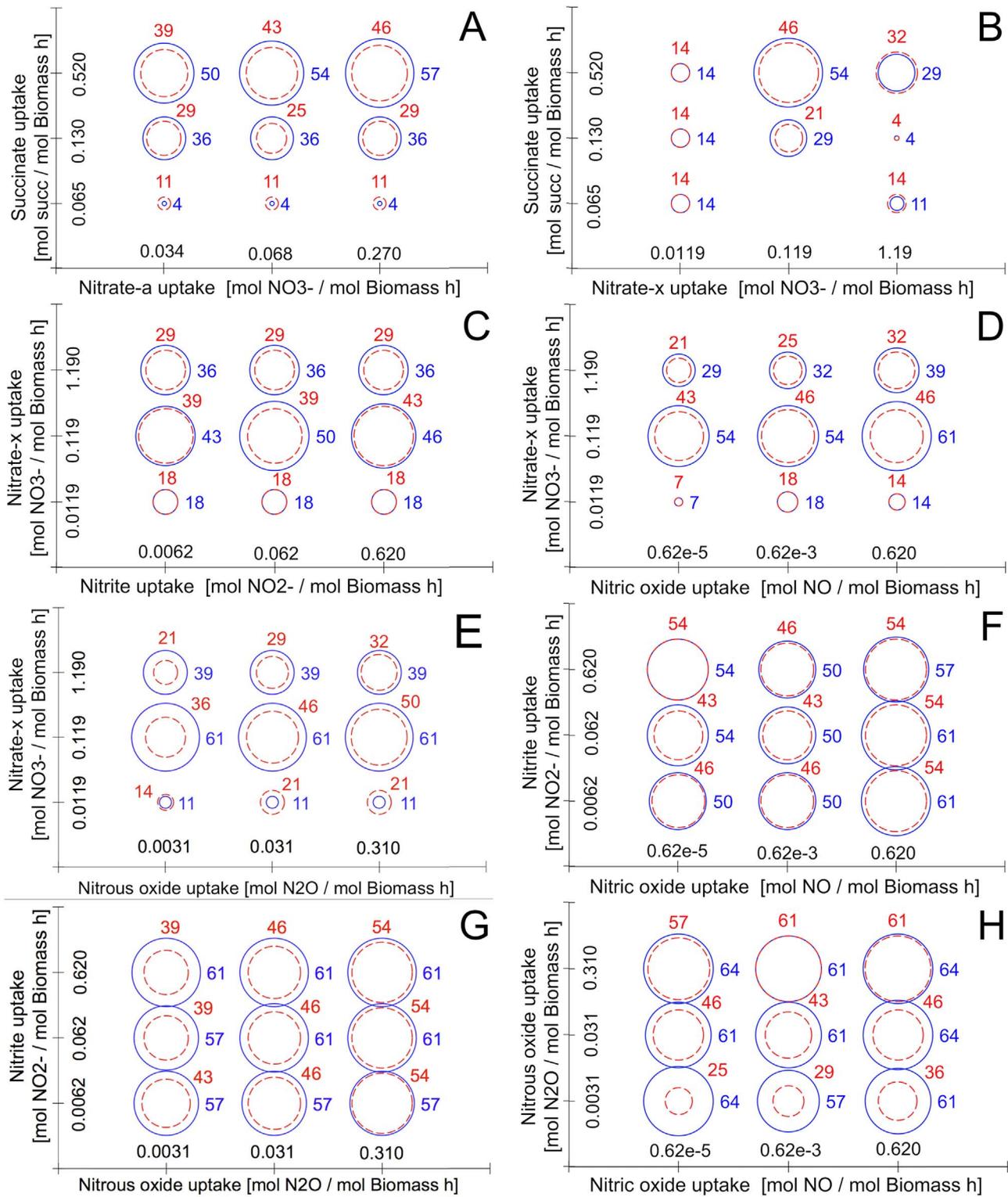
If the bacterial cell prioritizes the use of those nitrogen oxides with a higher degree of oxidation over others during the denitrification process (metabolic hypothesis), the simulation results are in better agreement with the experimental data presented by Felgate et al. (2012), than when the bacterial cell goes first for the more spontaneous reaction inside the denitrification pathway (Gibbs hypothesis). The idea that the metabolic hypothesis works better at the individual level than Gibbs hypothesis is supported using the values of the statistic GRI (Table 5) as a reference and from the radius of the circles in bubble charts obtained in the calibration process (Fig. 6).

Based on our results, it appears that TEEM2, one of the thermodynamic models based on bioenergetics growth efficiency, seems to be a useful tool for modeling the individual behavior-rules for maintenance and mass generation in the INDISIM-Paracoccus model.

INDISIM-Paracoccus is a promising tool to model *P. denitrificans* in batch and continuous cultures under both aerobic and anaerobic conditions. In contrast to previous modeling approaches, our model is the first attempt to study denitrification process using the IBM approach jointly with thermodynamic reactions for the cellular activity. The capacity to embed thermodynamic properties into individual cells, which can simulate the behavior of the bacterial population more realistically and mechanistically than other

modeling approaches, makes this model very attractive for future investigations. Besides, the NetLogo implementation of the model allows the user to have control of the input parameters and initial conditions for the simulations from a very friendly interface, as well as giving easy access to the computer code for future adaptations.

The development and application of IBMs with some intracellular detail and complexity constitutes the key advantage of this model to study and understand the different steps of denitrification carried out by a denitrifying bacterium. Exploring model behavior regarding its input parameters and assessing alternative submodels provides a way to advance the construction of a complete simulator to control factors that help to understand how major or minor  $\text{N}_2\text{O}$  generation is a consequence of this denitrifier metabolic individual activity. In particular, it is hoped that this NetLogo simulator will facilitate new fruitful interactions between modelers and experts in the field of denitrification. For example, this denitrification model could be incorporated into INDISIM-SOM to complement the mineralization and nitrification processes already incorporated to deal with a mixed microbial community to understand *in silico* what the consequences are of different media conditions and different microbial functional groups (heterotrophs, autotrophs and denitrifiers) on the  $\text{N}_2\text{O}$  emissions and other nitrous oxide productions.



**Fig. 6.** Bubble charts to illustrate the model adequacy for different uptake-rate values ( $u_i$ ). The  $u_i$  values come from Table 4. Each circle radius represents the global adequacy of the model versus seven experimental time evolutions for two experiments (E1 and E2) presented by Felgate et al. (2012). Continuous blue line assuming the metabolic hypothesis, and dashed red line assuming Gibbs hypothesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 5**

Values of the geometric relatively index (GRI) for the temporal evolutions of biomass, nitrate, nitrite and nitrous oxide, taking into account the INDISIM-Paracoccus outputs versus experimental data presented by Felgate et al. (2012).

Hypothesis	Experiment	Biomass	Nitrate	Nitrite	Nitrous oxide
Metabolic	Succinate-limited/NO <sub>3</sub> <sup>-</sup> -sufficient	1.22	1.26	2.05	12.94
	Succinate-sufficient/NO <sub>3</sub> <sup>-</sup> -limited	1.66	9.39	17.79	7.10
Gibbs	Succinate-limited/NO <sub>3</sub> <sup>-</sup> -sufficient	1.22	1.26	11.79	11.77
	Succinate-sufficient/NO <sub>3</sub> <sup>-</sup> -limited	1.64	9.40	17.87	10.37

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jtbi.2016.05.017>.

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