

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

Pablo Araujo-Granda^{1,2,*}, Anna Gras², Marta Ginovart³, Vincent Moulton⁴

¹ Chemical Engineering Faculty, Central University of Ecuador, Ciudad Universitaria – Ritter s/n y Bolivia, P.O.Box. 17-01-3972, Quito - Ecuador; e-mail: paraujo@iquce.edu.ec

² Department of Agri-Food Engineering and Biotechnology, Universitat Politècnica de Catalunya, Edifici D4, Esteve Terradas 8, 08860 Castelldefels, Barcelona - Spain; telephone: (34) 935521224; e-mail: anna.gras@upc.edu

³ Department of Mathematics, Universitat Politècnica de Catalunya, Edifici D4, Esteve Terradas 8, 08860 Castelldefels, Barcelona - Spain; telephone: (34) 935521133; e-mail: marta.ginovart@upc.edu

⁴ School of Computing Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ - United Kingdom; telephone: (44) 1603 592607; e-mail: v.moulton@uea.ac.uk

* Corresponding author: e-mail: paraujo@iquce.edu.ec or pablo@araujo.ec, telephone: (34) 636261560

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 behaviour 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 behaviour-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 behaviour-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.

Keywords: denitrification, *Paracoccus denitrificans*, bacterial yield prediction, individual-based model, Thermodynamic Electron Equivalent Model, NetLogo, INDISIM.

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 (N_2O) emissions when denitrifying bacteria do not complete the metabolic pathway implicated (Davidson et al., 1991; Snyder et al., 2009). The global warming potential of N_2O is 296 times greater than a unit of CO_2 (Richardson et al., 2009). In agricultural soils, N_2O 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 (O_2) availability, such as waterlogged soils, certain bacteria are able to use nitrate (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 (O_2) as the electron acceptor, and “*Nitrate reduction - Dissimilatory*” with nitrate (NO_3^-) as the electron acceptor (Baker et al., 1998; Beijerinck MW, 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 NO_3^- , nitrite (NO_2^-), nitric oxide (NO) or N_2O as electron acceptors (Baumann et al., 1996;

Bergaust et al., 2010; Bergaust et al., 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 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 behaviour 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 behaviour according to the changing individual internal and external conditions, linking mechanisms at the individual level to behaviour at the population level (Grimm, 1999; Mantzaris, 2007). IBMs consider individuals as discrete entities that follow behaviour-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 behaviours 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 modelling 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, and 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; Tijhuis et al., 1993; von Stockar and van der Wielen, 1997; Xiao and VanBriesen, 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 behaviour-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 behaviour 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 behaviour-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 (ε) 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 Rd , Ra and Rc , ii) other Gibbs energy potential terms, and iii) a term for energy-transfer efficiency (ϵ). 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 ϵ 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 to 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 microorganisms. Figure 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 24h. The aeration was then shut down and the system switched to continuous culture with a dilution rate of 0.05 h^{-1} . Under these conditions two experiments took place, one in which the reservoir medium feed contained 20 mM NO_3^- , 5 mM succinate and 10 mM NH_4^+ which was designed to achieve an electron donor-limited/electron acceptor-sufficient steady state (succinate-limited/ NO_3^- -sufficient). In the other experiment the reservoir medium feed contained 5 mM NO_3^- , 20 mM succinate and 10 mM NH_4^+ to achieve an electron donor-sufficient/electron acceptor-limited steady state (succinate-sufficient/ 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 hours) in a batch culture, and the second one during the anaerobic phase (from 24 to 120 hours) in a continuous culture. Also the bacteria will be handled in two different experiments: i) in conditions of succinate-sufficient/ NO_3^- -limited (experiment E1), and ii) in conditions succinate-limited/ 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 modelling 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 modelling 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 N₂ production, 1 point is assigned if the N₂ 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 modelling

3.1 Metabolic reactions

To develop the individual behaviour-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 I to write the energy reactions (Table II). 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 behaviour-rule for biomass synthesis it is necessary to write the metabolic pathways for aerobic and 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 O_2 and NO_3^- and in anaerobic conditions they are NO_3^- , NO_2^- , NO and N_2O (Table I). The stoichiometric coefficients for each metabolic pathway were obtained (Table III) from Gibbs free energy for each half-reaction with a different assigned ϵ 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 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 $\sim 0.5 \mu\text{m}$ and the largest one a bacterium with a diameter of $\sim 0.9 \mu\text{m}$ (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 $1.1 \text{ g}\cdot\text{cm}^{-3}$, 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 $\text{C}_3\text{H}_{5.4}\text{N}_{0.75}\text{O}_{1.45}$ (van Verseveld et al., 1979, 1983) so that each bacterium is assumed to have this elementary cell composition.

A two-dimensional lattice of 25 x 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 (μM) and dry mass in $\text{mg}\cdot\text{ml}^{-1}$.

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 μM), 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. Figure 2 shows the INDISIM-Paracoccus schematic diagram.

3.2.4 Design Concepts

3.2.4.1 Basic Principles: The model has two kinds of behaviour-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 II), iii) cellular growth when a micro-organism executes any of the metabolic reactions adjusted by TEEM2 (Table III), 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 O₂ 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 otherwise it uses the aerobic metabolism. The second individual rule is performing biomass synthesis (Table III) 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 II) and after updated amounts also allow execution of the corresponding synthesis reaction (Table III) 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 IV).

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 IV). 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 IV). 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 IV 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 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 $O_{2\text{-MIN}}$ threshold value are established from normal distributions with mean values determined by the experimental procedure (Table IV) 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 ($\text{mmol}\cdot\text{l}^{-1}$ or $\text{umol}\cdot\text{l}^{-1}$) of each culture medium component (succinate, NH_4^+ , O_2 , NO_3^- , CO_2 , HCO_3^- , NO_2^- , NO , N_2O and N_2), microbial biomass ($\text{mg}\cdot\text{ml}^{-1}$) and the population biomass distribution at each time step (the user can obtain all simulated data in the output file with the extension “.txt”).

3.2.5 Initialization

The user can adjust: i) the culture medium composition ($\text{mmol}\cdot\text{l}^{-1}$) of succinate, NH_4^+ , O_2 and NO_3^- , ii) $O_{2\text{-MIN}}$ value which is in the range of 0.01 to 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 shutdown O_2 input flow, and viii) the maintenance energy requirement

for aerobic and anaerobic phases ($\text{gC}_{\text{donor}} \cdot \text{gC}_{\text{mic}}^{-1} \cdot \text{h}^{-1}$).

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 ($U_i, \text{mol}_{\text{nutrient}} \cdot \text{h}^{-1}$) of the bacterium and the nutrient available in the culture medium ($A_i, \text{mol}_{\text{nutrient}} \cdot \text{h}^{-1}$), 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:

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

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 $\text{mol}_{\text{nutrient}} \cdot \text{mol}_{\text{mass}}^{-1} \cdot \text{h}^{-1}$, where mol_{mass} denotes the moles of microbial mass (the microbial mass equals $\text{C}_3\text{H}_{5.4}\text{N}_{0.75}\text{O}_{1.45}$).

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:

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

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

(μ_{\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 modelling process that the nutrient with maximum Dab has the highest availability; the other availability values are assigned proportionally (Table IV).

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 II). 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 $0.002 \text{ gC}_{\text{donor}} \cdot \text{gC}_{\text{mic}}^{-1} \cdot \text{h}^{-1}$, 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 $0.004 \text{ gC}_{\text{donor}} \cdot \text{gC}_{\text{mic}}^{-1} \cdot \text{h}^{-1}$ which was assumed for anaerobic phase. The energy reactions (Table II) 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 II), we establish the maintenance requirements for aerobic and anaerobic phases (see supplementary material for details). When the individual carries out its maintenance, the CO_2 and the reduced electron acceptors are expelled to the culture medium except for the 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 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, NH_4^+ and 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 modelling 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 $100 \times 100 = 10000$ patches. The number of individuals in these simulations was increased as well, from 3000 (in the smallest system) to around 50000 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 IV), 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 IV). 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/ NO_3^- -limited (Experiment E1) and succinate-limited/ NO_3^- -sufficient (Experiment E2) and for each of the hypotheses considered (the metabolic hypothesis and the Gibbs' hypothesis). The simulated cultures were initially grown under batch aerobic conditions (from 0 to 24 hours) following the switch to continuous culture where the populations shift to anaerobic metabolism (from 24 to 120 hours). 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, 4 and 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 hours) in batch culture, and the second one occurs in anaerobic phase (from 24 to 120 hours) 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 IV). In all cases, some simulation results shown in Figs. 3A, 3B and 3C 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 ($u_{\text{Nitrate-a}} = 0.27 \text{ mol}_{\text{nitrate-a}} \cdot \text{mol}_{\text{mass}}^{-1} \cdot \text{h}^{-1}$, the high value in Table IV) (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, NO_3^- , NO_2^- and N_2O . 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 ($u_{\text{Succinate}} = 0.52 \text{ mol}_{\text{succinate}} \cdot \text{mol}_{\text{mass}}^{-1} \cdot \text{h}^{-1}$) the model accuracy is good (Figs. 6A and 6B).

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 4D). 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 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, 6D and 6E, the value of nitrate-x that best fits the simulation results with the experimental points, for both experiments and hypotheses, is $0.119 \text{ mol}_{\text{nitrate-x}} \cdot \text{mol}_{\text{mass}}^{-1} \cdot \text{h}^{-1}$. Looking at the bubble charts of Figs. 6F, 6G and 6H, 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 IV. 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 modelling 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 V. 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, 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 V) 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 modelling the individual behaviour-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 modelling 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 behaviour 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 N₂O 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 N₂O emissions and other nitrous oxide productions.

Acknowledgments: The financial support of the Ecuador National Secretary of Science and Technology (SENESCYT) and the Plan Nacional I+D+i from the Spanish Ministerio de Educación y Ciencia (MICINN, CGL2010-20160). We would also like to thank David Richardson and Andrew Gates for helpful discussions at early stages in this project and for providing us with the full dataset presented (Felgate et al., 2012).

References

- Baker, S.C., Ferguson, S.J., Ludwig, B., Page, M.D., Richter, O.-M.H., van Spanning, R.J.M., 1998. Molecular Genetics of the Genus *Paracoccus*: Metabolically Versatile Bacteria with Bioenergetic Flexibility. *Microbiol. Mol. Biol. Rev.* 62, 1046–1078.
- Baumann, B., Snozzi, M., Zehnder, A., Van Der Meer, J., 1996. Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. *J. Bacteriol.* 178, 4367–4374.
- Beijerinck MW, M.D., 1910. Bildung und Verbrauch von Stickoxydul durch Bakterien. *Int. J. Med. Microbiol.* 2, 30–63.
- Bergaust, L., Mao, Y., Bakken, L.R., Frostegård, A., 2010. Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrous [corrected] oxide reductase in *Paracoccus denitrificans*. *Appl. Environ. Microbiol.* 76, 6387–96. doi:10.1128/AEM.00608-10
- Bergaust, L., van Spanning, R.J.M., Frostegård, A., Bakken, L.R., 2012. Expression of nitrous oxide reductase in *Paracoccus denitrificans* is regulated by oxygen and nitric oxide through FnrP and NNR. *Microbiology* 158, 826–34. doi:10.1099/mic.0.054148-0
- Blackburn, T.H., 1990. Denitrification in Soil and Sediment. Springer US, Boston, MA. doi:10.1007/978-1-4757-9969-9
- Bley, T., 2011. From single cells to microbial population dynamics: modelling in biotechnology based on measurements of individual cells. *Adv. Biochem. Eng. Biotechnol.* 124, 211–27. doi:10.1007/10_2010_79
- Button, D.K., 1998. Nutrient Uptake by Microorganisms according to Kinetic Parameters from Theory as Related to Cytoarchitecture. *Microbiol. Mol. Biol. Rev.* 62, 636–645.
- Caspi, R., Altman, T., Dreher, K., Fulcher, C.A., Subhraveti, P., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L.A., Ong, Q., Paley, S., Pujar, A., Shearer, A.G., Travers, M., Weerasinghe, D., Zhang, P., Karp, P.D., 2012. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res.* 40, D742–53. doi:10.1093/nar/gkr1014
- Christensen, D., McCarty, P., 1975. Multi-process biological treatment model. *J. (Water Pollut. Control Fed.* 47, 2652–2664.
- Davidson, E.A., Rogers, J., Whitman, W.B., 1991. Fluxes of nitrous oxide and nitric oxide from terrestrial ecosystems. 219–235.
- Felgate, H., Giannopoulos, G., Sullivan, M.J., Gates, A.J., Clarke, T.A., Baggs, E., Rowley, G., Richardson, D.J., 2012. The impact of copper, nitrate and carbon status on the emission of nitrous oxide by two species of bacteria with biochemically distinct denitrification pathways. *Environ. Microbiol.* 14, 1788–800. doi:10.1111/j.1462-2920.2012.02789.x
- Ferrer, J., Prats, C., López, D., 2008. Individual-based modelling: an essential tool for microbiology. *J. Biol. Phys.* 34, 19–37. doi:10.1007/s10867-008-9082-3
- Ginovart, M., Cañadas, J.C., 2008. INDISIM-YEAST: an individual-based simulator on a website for experimenting and investigating diverse dynamics of yeast populations in liquid media. *J. Ind. Microbiol. Biotechnol.* 35, 1359–66. doi:10.1007/s10295-008-0436-4
- Ginovart, M., López, D., Gras, A., 2005. Individual-based modelling of microbial activity to study mineralization of C and N and nitrification process in soil. *Nonlinear Anal. Real World Appl.* 6, 773–795. doi:10.1016/j.nonrwa.2004.12.005

- Ginovart, M., López, D., Valls, J., 2002. INDISIM, an individual-based discrete simulation model to study bacterial cultures. *J. Theor. Biol.* 214, 305–19. doi:10.1006/jtbi.2001.2466
- Gras, A., Ginovart, M., Valls, J., Baveye, P.C., 2011. Individual-based modelling of carbon and nitrogen dynamics in soils: Parameterization and sensitivity analysis of microbial components. *Ecol. Modell.* 222, 1998–2010. doi:10.1016/j.ecolmodel.2011.03.009
- Grimm, V., 1999. Ten years of individual-based modelling in ecology: what have we learned and what could we learn in the future? *Ecol. Modell.* 115, 129–148. doi:10.1016/S0304-3800(98)00188-4
- Grimm, V., Berger, U., DeAngelis, D.L., Polhill, J.G., Giske, J., Railsback, S.F., 2010. The ODD protocol: A review and first update. *Ecol. Modell.* 221, 2760–2768. doi:10.1016/j.ecolmodel.2010.08.019
- Grimm, V., Revilla, E., Berger, U., Jeltsch, F., Mooij, W.M., Railsback, S.F., Thulke, H.-H., Weiner, J., Wiegand, T., DeAngelis, D.L., 2005. Pattern-oriented modeling of agent-based complex systems: lessons from ecology. *Science* 310, 987–91. doi:10.1126/science.1116681
- Heijnen, J.J., 1999. Bioenergetics of microbial growth. *Encycl. Bioprocess Technol. Ferment. Biocatal. Biosep.* doi:10.1002/0471250589
- Heijnen, J.J., Van Dijken, J.P., 1992. In search of a thermodynamic description of biomass yields for the chemotrophic growth of microorganisms. *Biotechnol. Bioeng.* 39, 833–58. doi:10.1002/bit.260390806
- Heinen, M., 2006. Simplified denitrification models: Overview and properties. *Geoderma* 133, 444–463. doi:10.1016/j.geoderma.2005.06.010
- Hellweger, F.L., Bucci, V., 2009. A bunch of tiny individuals—Individual-based modeling for microbes. *Ecol. Modell.* 220, 8–22. doi:10.1016/j.ecolmodel.2008.09.004
- Holt, J., Krieg, N., Sneath, P., Staley, J., Williams, S., 1994. International edition: Bergey's manual of determinative bacteriology.
- Jachner, S., van den Boogaart, K.G., Petzoldt, T., 2007. Statistical Methods for the Qualitative Assessment of Dynamic Models with Time Delay (R Package qualV). *J. Stat. Softw.* 22, 1–30.
- Kampschreur, M.J., Kleerebezem, R., Picioreanu, C., Bakken, L., Bergaust, L., de Vries, S., Jetten, M.S.M., van Loosdrecht, M.C.M., 2012. Metabolic modeling of denitrification in *Agrobacterium tumefaciens*: a tool to study inhibiting and activating compounds for the denitrification pathway. *Front. Microbiol.* 3, 370. doi:10.3389/fmicb.2012.00370
- Kreft, J.-U., Plugge, C.M., Grimm, V., Prats, C., Leveau, J.H.J., Banitz, T., Baines, S., Clark, J., Ros, A., Klapper, I., Topping, C.J., Field, A.J., Schuler, A., Litchman, E., Hellweger, F.L., 2013. Mighty small: Observing and modeling individual microbes becomes big science. *Proc. Natl. Acad. Sci. U. S. A.* 110, 18027–8. doi:10.1073/pnas.1317472110
- Lee, M.W., Vassiliadis, V.S., Park, J.M., 2009. Individual-based and stochastic modeling of cell population dynamics considering substrate dependency. *Biotechnol. Bioeng.* 103, 891–9. doi:10.1002/bit.22327
- Leggett, R.W., Williams, L.R., 1981. A reliability index for models. *Ecol. Modell.* 13, 303–312. doi:10.1016/0304-3800(81)90034-X
- Liu, J.-S., Vojinović, V., Patiño, R., Maskow, T., von Stockar, U., 2007. A comparison of various Gibbs energy dissipation correlations for predicting microbial growth yields. *Thermochim. Acta* 458, 38–46. doi:10.1016/j.tca.2007.01.016

- Mantzaris, N. V., 2007. From single-cell genetic architecture to cell population dynamics: quantitatively decomposing the effects of different population heterogeneity sources for a genetic network with positive feedback architecture. *Biophys. J.* 92, 4271–88. doi:10.1529/biophysj.106.100271
- Maskow, T., von Stockar, U., 2005. How reliable are thermodynamic feasibility statements of biochemical pathways? *Biotechnol. Bioeng.* 92, 223–30. doi:10.1002/bit.20572
- McCarty, P., 1971. Energetics and bacterial growth. *Org. Compd. Aquat. Environ.* 1, 157–172.
- McCarty, P.L., 2007. Thermodynamic electron equivalents model for bacterial yield prediction: modifications and comparative evaluations. *Biotechnol. Bioeng.* 97, 377–388. doi:10.1002/bit
- Portell, X., Gras, A., Ginovart, M., 2014. INDISIM-Saccha, an individual-based model to tackle *Saccharomyces cerevisiae* fermentations. *Ecol. Modell.* 279, 12–23. doi:10.1016/j.ecolmodel.2014.02.007
- Prats, C., Ferrer, J., Gras, A., Ginovart, M., 2010. Individual-based modelling and simulation of microbial processes: yeast fermentation and multi-species composting. *Math. Comput. Model. Dyn. Syst.* 16, 489–510. doi:10.1080/13873954.2010.481809
- Prats, C., Giró, A., Ferrer, J., López, D., Vives-Rego, J., 2008. Analysis and IBM simulation of the stages in bacterial lag phase: basis for an updated definition. *J. Theor. Biol.* 252, 56–68. doi:10.1016/j.jtbi.2008.01.019
- Railsback, S.F., Grimm, V., 2012. *Agent-Based and Individual-Based Modeling: A Practical Introduction*. Princeton University Press.
- Resat, H., Bailey, V., McCue, L.A., Konopka, A., 2012. Modeling microbial dynamics in heterogeneous environments: growth on soil carbon sources. *Microb. Ecol.* 63, 883–97. doi:10.1007/s00248-011-9965-x
- Richardson, D., Felgate, H., Watmough, N., Thomson, A., Baggs, E., 2009. Mitigating release of the potent greenhouse gas N₂O from the nitrogen cycle - could enzymic regulation hold the key? *Trends Biotechnol.* 27, 388–97. doi:10.1016/j.tibtech.2009.03.009
- Rittmann, B.E., McCarty, P.L., 2001. *Environmental Biotechnology: Principles and Applications*, Biotechnology.
- Scheller, R.M., Sturtevant, B.R., Gustafson, E.J., Ward, B.C., Mladenoff, D.J., 2010. Increasing the reliability of ecological models using modern software engineering techniques. *Front. Ecol. Environ.* 8, 253–260. doi:10.1890/080141
- Snyder, C.S., Bruulsema, T.W., Jensen, T.L., Fixen, P.E., 2009. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. *Agric. Ecosyst. Environ.* 133, 247–266. doi:10.1016/j.agee.2009.04.021
- Thiele, J.C., Kurth, W., Grimm, V., 2014. Facilitating Parameter Estimation and Sensitivity Analysis of Agent-Based Models: A Cookbook Using NetLogo and “R.” *J. Artif. Soc. Soc. Simul.* 17, 11.
- Tijhuis, L., Van Loosdrecht, M.C., Heijnen, J.J., 1993. A thermodynamically based correlation for maintenance gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnol. Bioeng.* 42, 509–519. doi:10.1002/bit.260420415
- van Verseveld, H.W., Boon, J.P., Stouthamer, A.H., 1979. Growth yields and the efficiency of oxidative phosphorylation of *Paracoccus denitrificans* during two- (carbon) substrate-limited growth. *Arch. Microbiol.* 121, 213–223. doi:10.1007/BF00425058

- van Verseveld, H.W., Braster, M., Boogerd, F.C., Chance, B., Stouthamer, A.H., 1983. Energetic aspects of growth of *Paracoccus denitrificans*: oxygen-limitation and shift from anaerobic nitrate-limitation to aerobic succinate-limitation. *Arch. Microbiol.* 135, 229–236. doi:10.1007/BF00414485
- van Verseveld, H.W., Meijer, E.M., Stouthamer, A.H., 1977. Energy conservation during nitrate respiration in *Paracoccus denitrificans*. *Arch. Microbiol.* 112, 17–23. doi:10.1007/BF00446649
- VanBriesen, J.M., 2002. Evaluation of methods to predict bacterial yield using thermodynamics. *Biodegradation* 13, 171–190. doi:10.1023/A:1020887214879
- von Stockar, U., van der Wielen, L.A.M., 1997. Thermodynamics in biochemical engineering. *J. Biotechnol.* 59, 25–37. doi:10.1016/S0168-1656(97)00167-3
- Wilensky, U., 1999. NetLogo [WWW Document]. Cent. Connect. Learn. Comput. Model. Northwest. Univ. Evanston, IL. URL <http://ccl.northwestern.edu/netlogo/> (accessed 3.5.14).
- Woolfenden, H.C., Gates, A.J., Bocking, C., Blyth, M.G., Richardson, D.J., Moulton, V., 2013. Modeling the effect of copper availability on bacterial denitrification. *Microbiologyopen* 2, 756–65. doi:10.1002/mbo3.111
- Xiao, J., VanBriesen, J.M., 2008. Expanded thermodynamic true yield prediction model: adjustments and limitations. *Biodegradation* 19, 99–127. doi:10.1007/s10532-007-9119-5
- Xiao, J., VanBriesen, J.M., 2006. Expanded thermodynamic model for microbial true yield prediction. *Biotechnol. Bioeng.* 93, 110–21. doi:10.1002/bit.20700
- Zumft, W., 1997. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616.

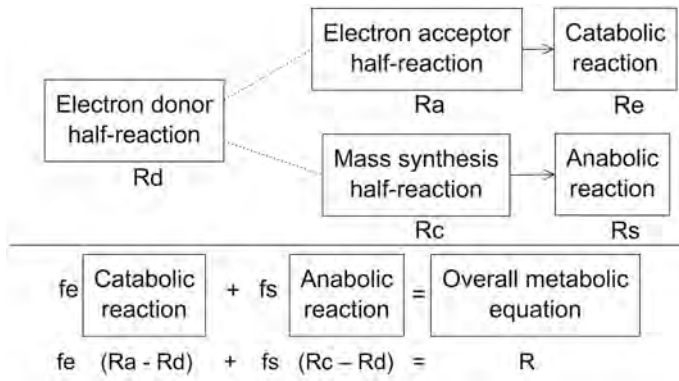


Figure 1. Schematic representation of TEEM, adapted from (Rittmann & McCarty, 2001).

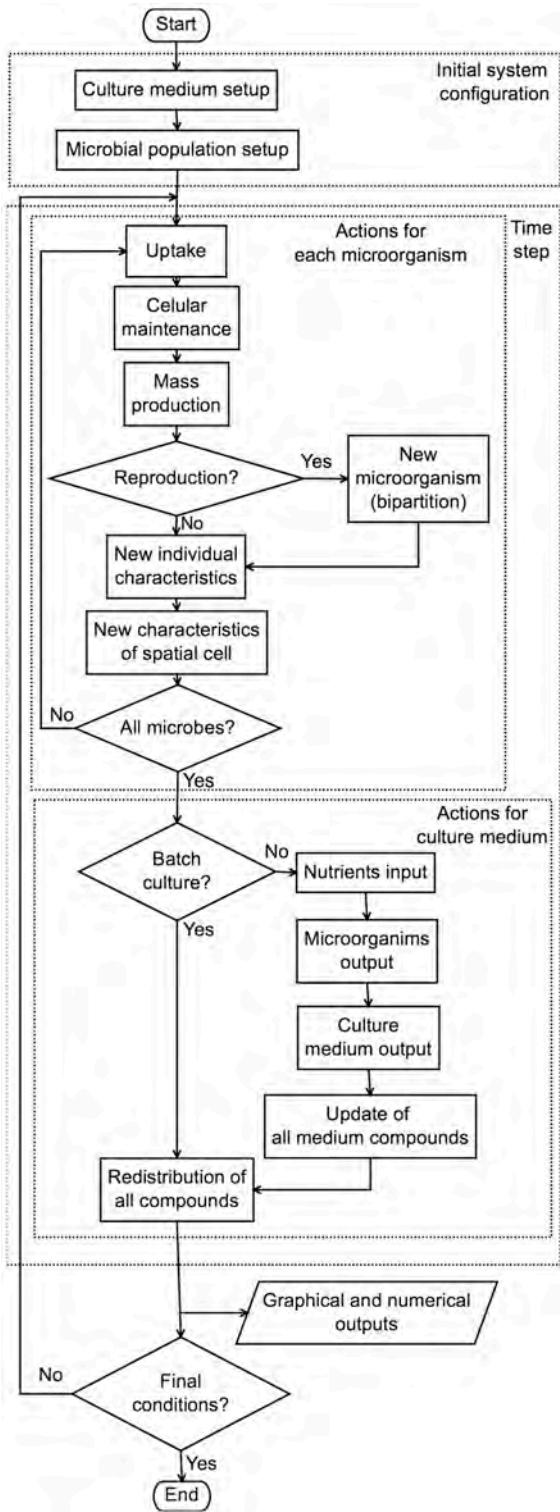


Figure 2. Flow chart of the INDISIM-Paracoccus model.

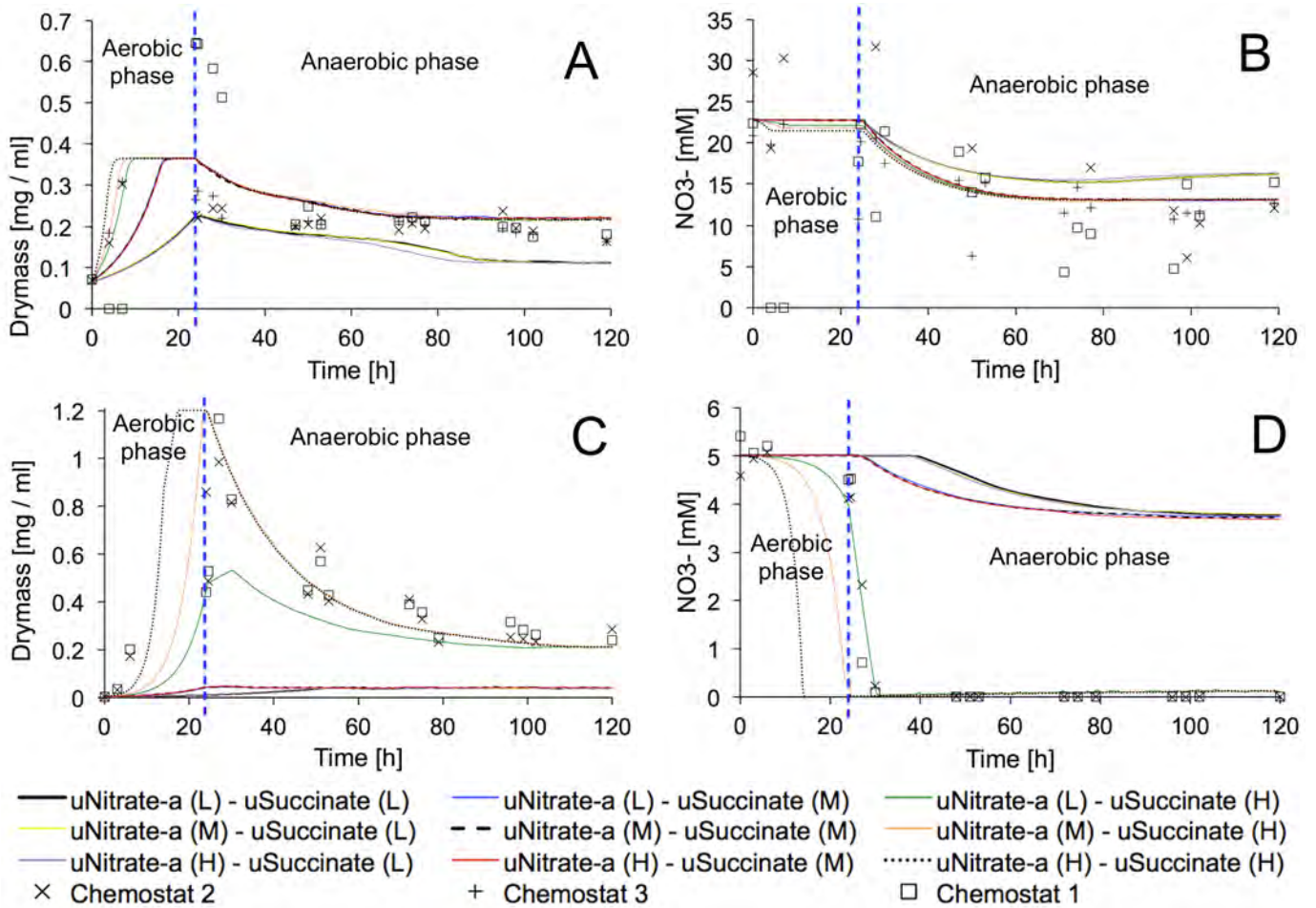


Figure 3. INDISIM-Paracoccus model calibration outputs for the aerobic phase. Each color line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and nitrate in aerobic phase. The u_i values are reported in Table IV (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/ NO_3^- -sufficient (A and B) and for the experiment E2, succinate-sufficient/ NO_3^- -limited (C and D). The aerobic phase (from 0 to 24 hours) in batch culture, and the anaerobic phase (from 24 to 120 hours) in continuous culture. The metabolic hypothesis has been assumed in the simulator to generate these results.

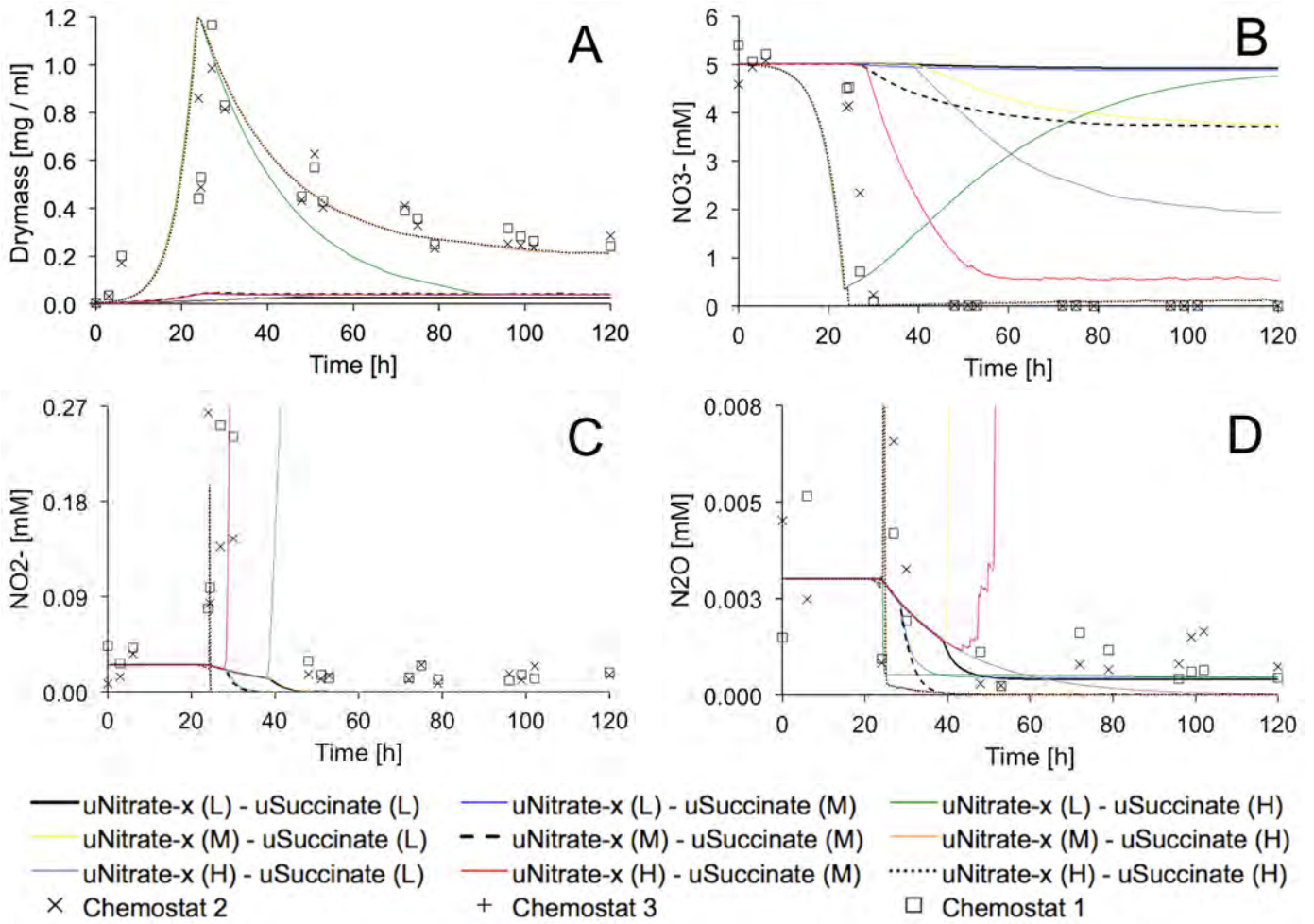


Figure 4. INDISIM-Paracoccus model calibration output for the experiment succinate-sufficient/ NO_3^- limited (E1). Time evolutions of: (A) Biomass, (B) Nitrate, (C) Nitrite and (D) Nitrous oxide. Each color line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and nitrate in anaerobic phase. The u_i values are reported in Table IV (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.

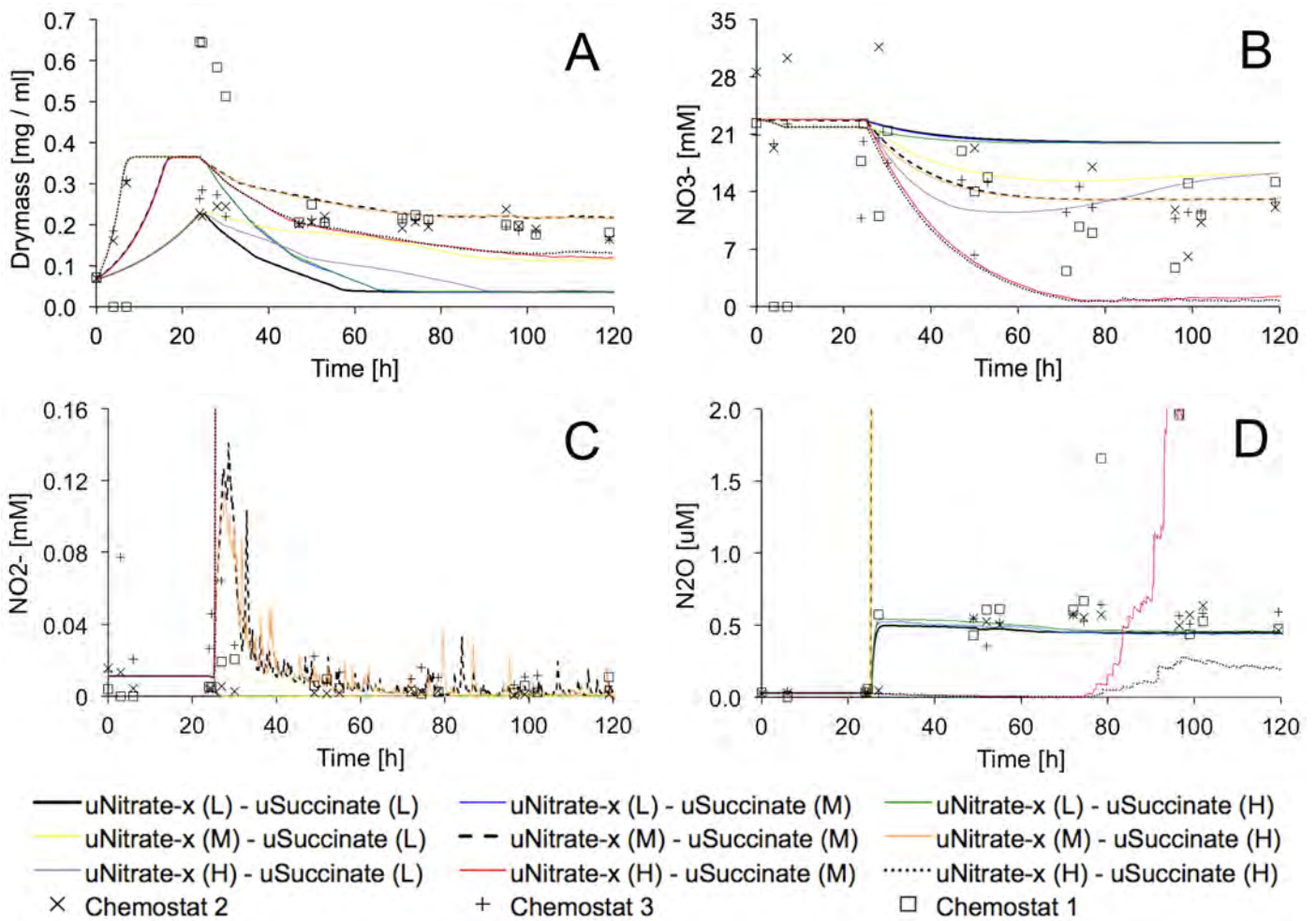


Figure 5. INDISIM-Paracoccus model calibration output for the experiment succinate-limited/ NO_3^- sufficient (E2). Time evolutions of: (A) Biomass, (B) Nitrate, (C) Nitrite and (D) Nitrous oxide. Each color line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and nitrate in anaerobic phase. The u_i values are reported in Table IV (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.

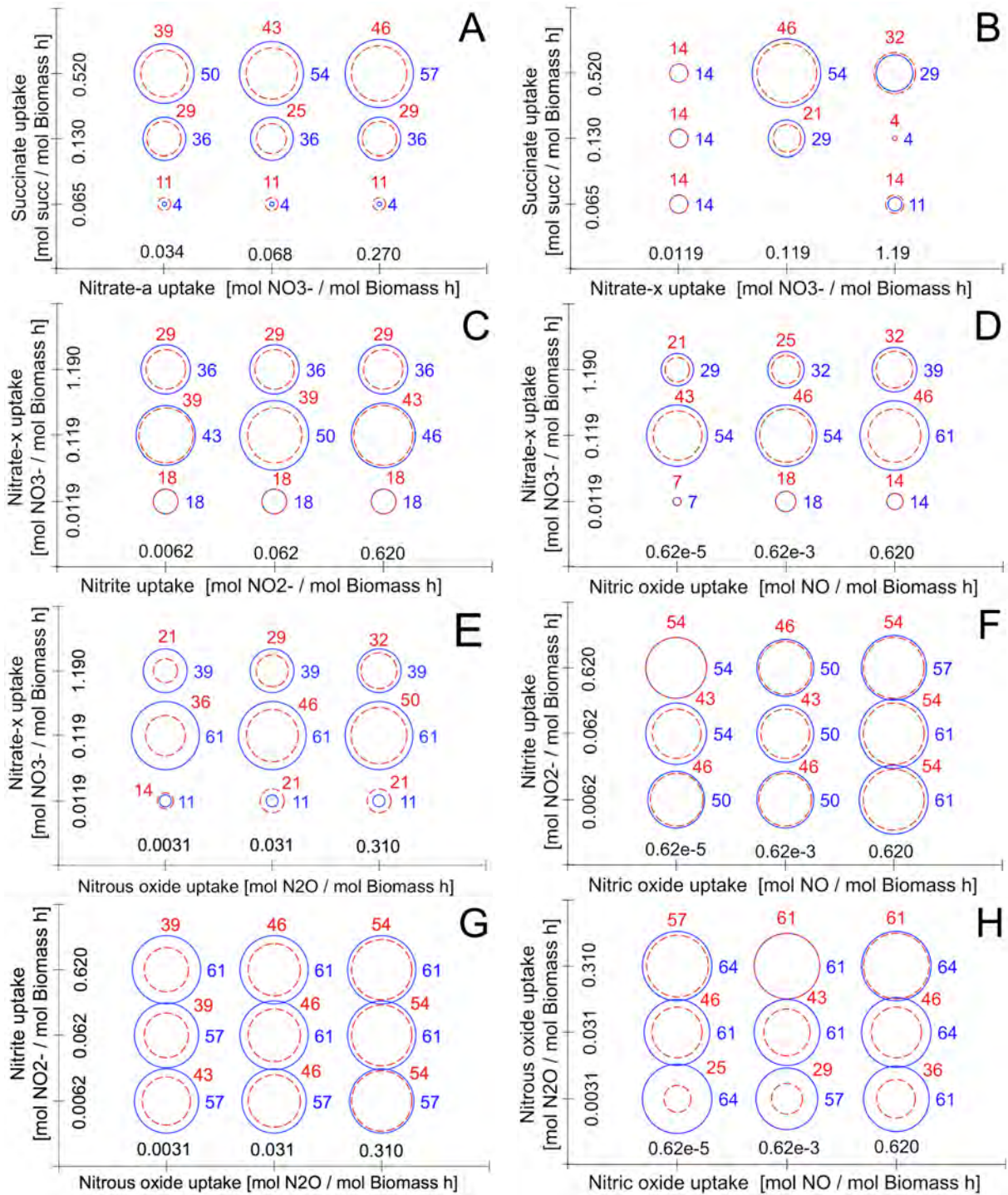


Figure 6. Bubble charts to illustrate the model adequacy for different uptake-rate values (u_i). The u_i values come from Table IV. Each circle radius represents the global adequacy of the model versus seven experimental time evolutions for two experiments (E1 and E2) presented by Felgate et al. (2012). Continuous blue line assuming the metabolic hypothesis, and dashed red line assuming Gibbs hypothesis.

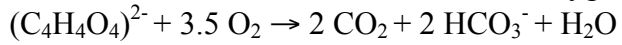
Table I. Inorganic and organic half-reactions and their Gibbs standard free energy according to Rittmann and McCarty (2001).

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

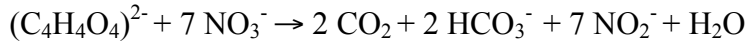
(a) This value was estimated from reported value of 18.8 kJ/eq for an assumed cell relative composition of $\text{C}_5\text{H}_7\text{O}_2\text{N}$ (Rittmann and McCarty, 2001) and considering NH_4^+ as source for cell synthesis of $\text{C}_3\text{H}_{5.4}\text{O}_{1.45}\text{N}_{0.75}$ (van Verseveld et al., 1983).

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

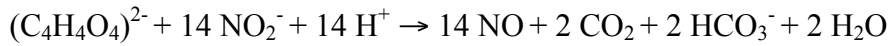
Aerobic maintenance with succinate and oxygen:



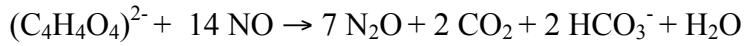
Anaerobic maintenance with succinate and nitrate:



Anaerobic maintenance with succinate and nitrite:



Anaerobic maintenance with succinate and nitric oxide:



Anaerobic maintenance with succinate and nitrous oxide:

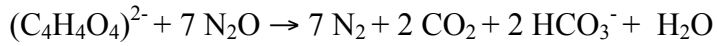


Table III. Balanced chemical equations (R) for biomass synthesis in aerobic and anaerobic phase. ($R = fe^oRa + fs^oRc - Rd$) according to TEEM2 (McCarty, 2007).	
R1	Aerobic conversion of succinate: $(C_4H_4O_4)^{2-} + 0.60 NH_4^+ + 1.04 O_2 \rightarrow 0.81 C_3H_{5.4}O_{1.45}N_{0.75} + 0.19 CO_2 + 1.40 HCO_3^- + 0.34 H_2O$
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): $(C_4H_4O_4)^{2-} + 0.58 NH_4^+ + 2.28 N_2O \rightarrow 0.77 C_3H_{5.4}O_{1.45}N_{0.75} + 2.28 N_2 + 0.26 CO_2 + 1.42 HCO_3^- + 0.36 H_2O$

Nutrient	Culture medium initial concentration [mM] according to Felgate et al. (2012)	Availability coefficient – a_i – (h^{-1}) fixed according to <i>Dab</i>	Uptake-rate – u_i – ($\text{mol}_{\text{nutrient}} \cdot \text{mol}_{\text{mass}}^{-1} \cdot \text{h}^{-1}$)			
			Testing values			Calibrated Values
			Low (L)	Medium (M)	High (H)	
Succinate	$5^c - 20^d$	$0.28^{a,b,e,f}$	0.065	0.13	0.52^g	$0.52^{a,b,e,f}$
Ammonium	$10^{c,d}$	$0.84^{a,b,e,f}$	-----	-----	0.31^g	$0.31^{a,b,e,f}$
Oxygen	$0.236^{c,d}$	$0.79^{a,b,e,f}$	-----	-----	0.54^g	$0.54^{a,e,f}$
Nitrate-a (aerobic)	$4.9983^d - 21.6095^c$	$0.63^{a,b,e,f}$	0.034	0.068	0.27^g	$0.27^{a,e,f}$
Nitrate-x (anaerobic)			0.019	0.119	$1.19^{g,h}$	$0.119^{b,e,f}$
Nitrite	$0.0255^c - 0.0112^d$	$0.79^{a,b,e,f}$	0.0062	0.062	$0.62^{g,h}$	$0.062^{b,e} - 0.62^{b,f}$
Nitric Oxide	-----	$1.00^{a,b,e,f}$	0.0000062	0.00062	$0.62^{g,h}$	$0.62^{b,e,f}$
Nitrous Oxide	$0.003^c - 0.000028^d$	$0.50^{a,b,e,f}$	0.0031	0.031	$0.31^{g,h}$	$0.31^{b,e,f}$
Other bacterial parameters						
Parameter	Testing range	Calibrated value			Reference	
Cellular maintenance ($\text{gC}_{\text{donor}} \cdot \text{gC}_{\text{mic}}^{-1} \cdot \text{h}^{-1}$)	-----	$0.0020^a - 0.0040^b$			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 (μm)	$0.4 - 0.6^{a,b}$	$0.5^{a,b}$			Holt et al. (1994)	
Big bacterium size (μm)	$0.8 - 1.0^{a,b}$	$0.9^{a,b}$				
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 ($\mu_{\text{max}} = 0.418 \text{ h}^{-1}$, van Verseveld et al., 1983) and the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table III). 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.						

Table V. 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 ₃ ⁻ -sufficient	1.22	1.26	2.05	12.94
	Succinate-sufficient/NO ₃ ⁻ -limited	1.66	9.39	17.79	7.10
Gibbs	Succinate-limited/NO ₃ ⁻ -sufficient	1.22	1.26	11.79	11.77
	Succinate-sufficient/NO ₃ ⁻ -limited	1.64	9.40	17.87	10.37

INDISIM-Paracoccus - Supplementary Material

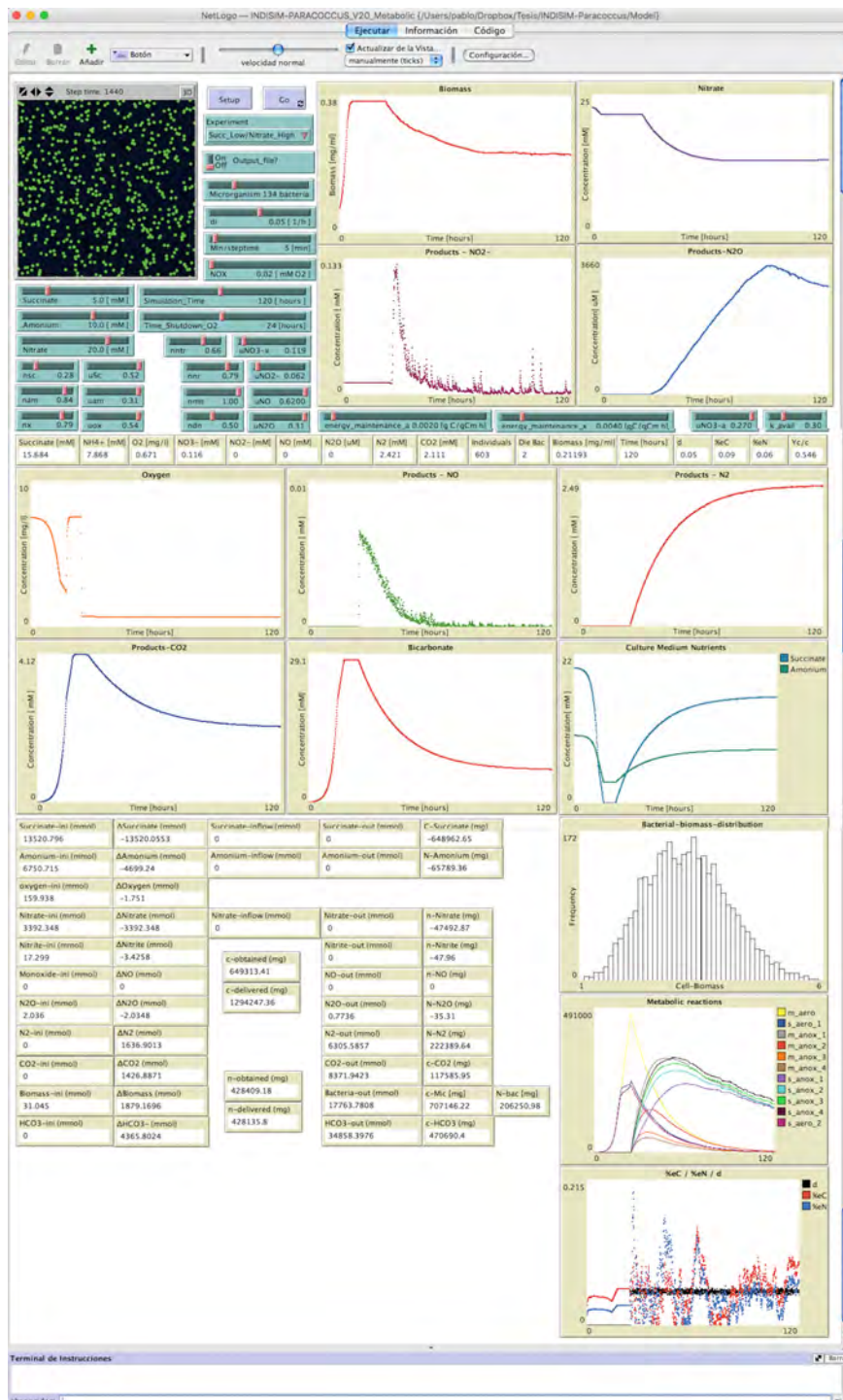


Figure S1. A screenshot of the user interface of the INDISIM-Paracoccus simulator in NetLogo. The sliders allow changing initial values, simulated time and a set of parameters of the model. Observations are provided with numerical monitors and plots of temporal evolutions of the modelled compounds over time. Mass distributions of the bacteria and the number of times that each metabolic reaction have been used by bacteria are also presented in the simulator interface.

2

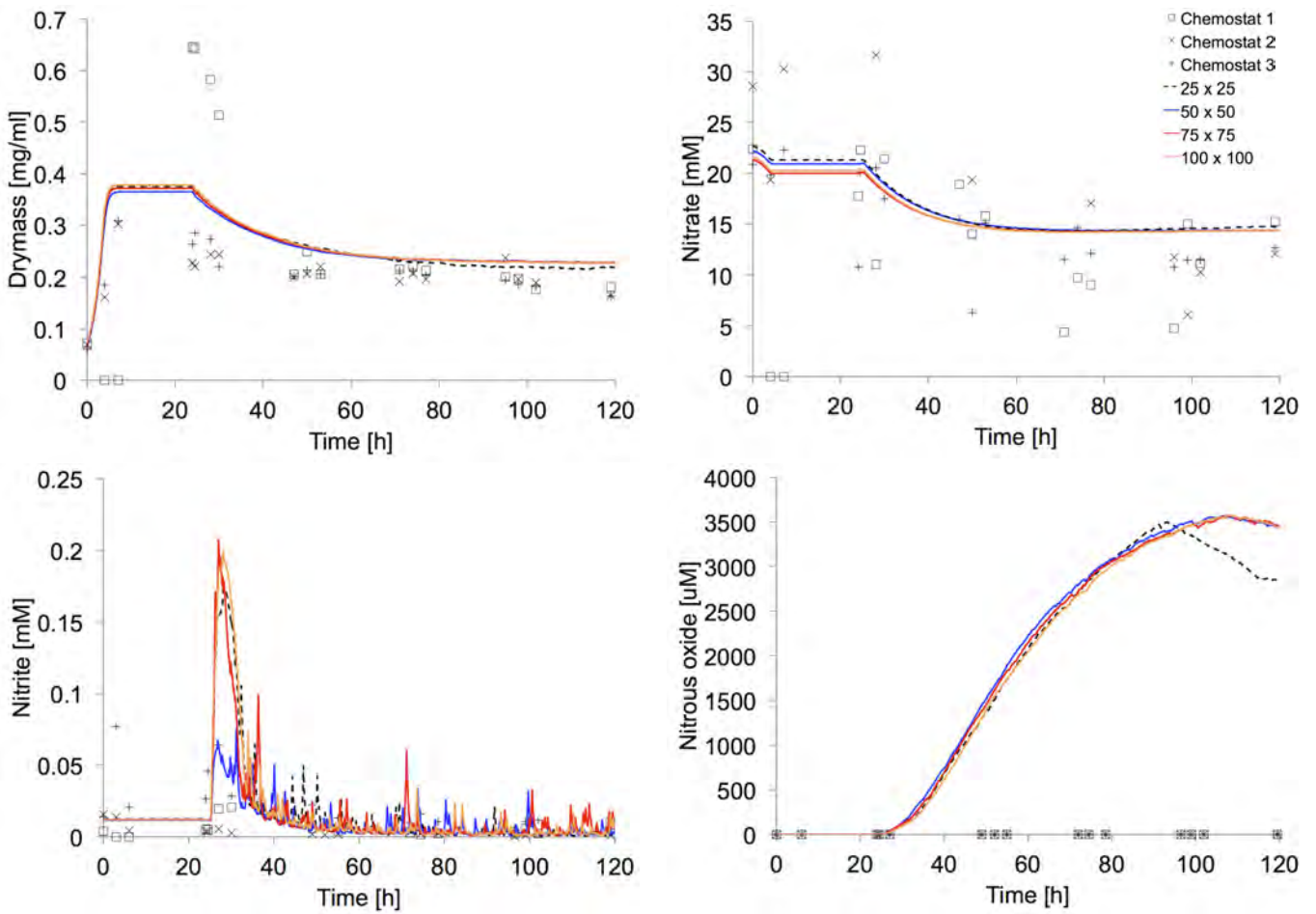


Figure S2. INDISIM-Paracoccus model calibration tests: experiment succinate-limited/ NO_3^- -sufficient in aerobic and anaerobic phase. Each color line represents a simulation result with different values of the system size. Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. The time step assumed in the simulator to generate these results was 5 minutes.

3

4

5

2

6

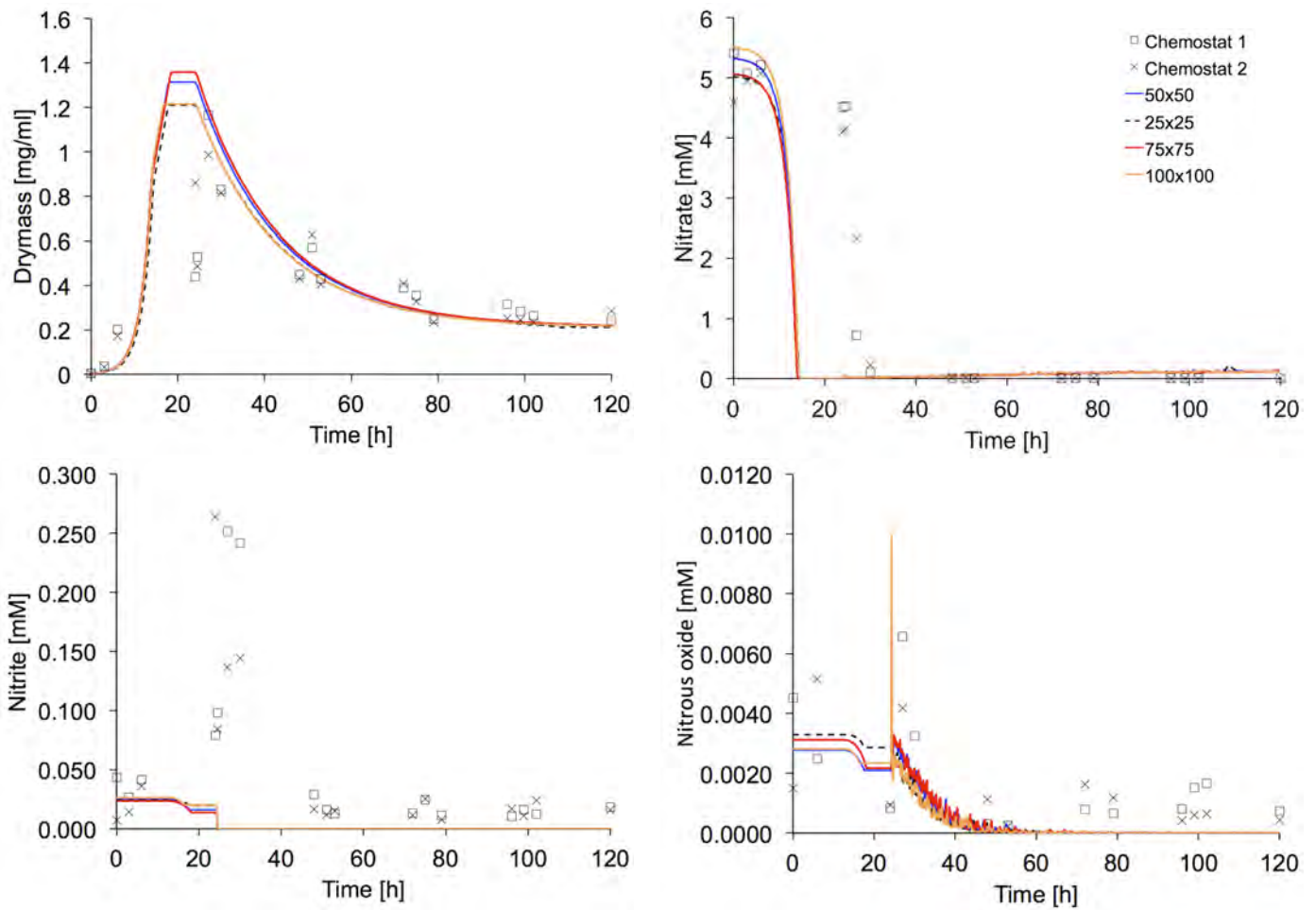


Figure S3. INDISIM-Paracoccus model calibration tests: experiment succinate-sufficient/ NO_3^- -limited in aerobic and anaerobic phase. Each color line represents a simulation result with different values of the system size. Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. The time step assumed in the simulator to generate these results was 5 minutes.

7

8

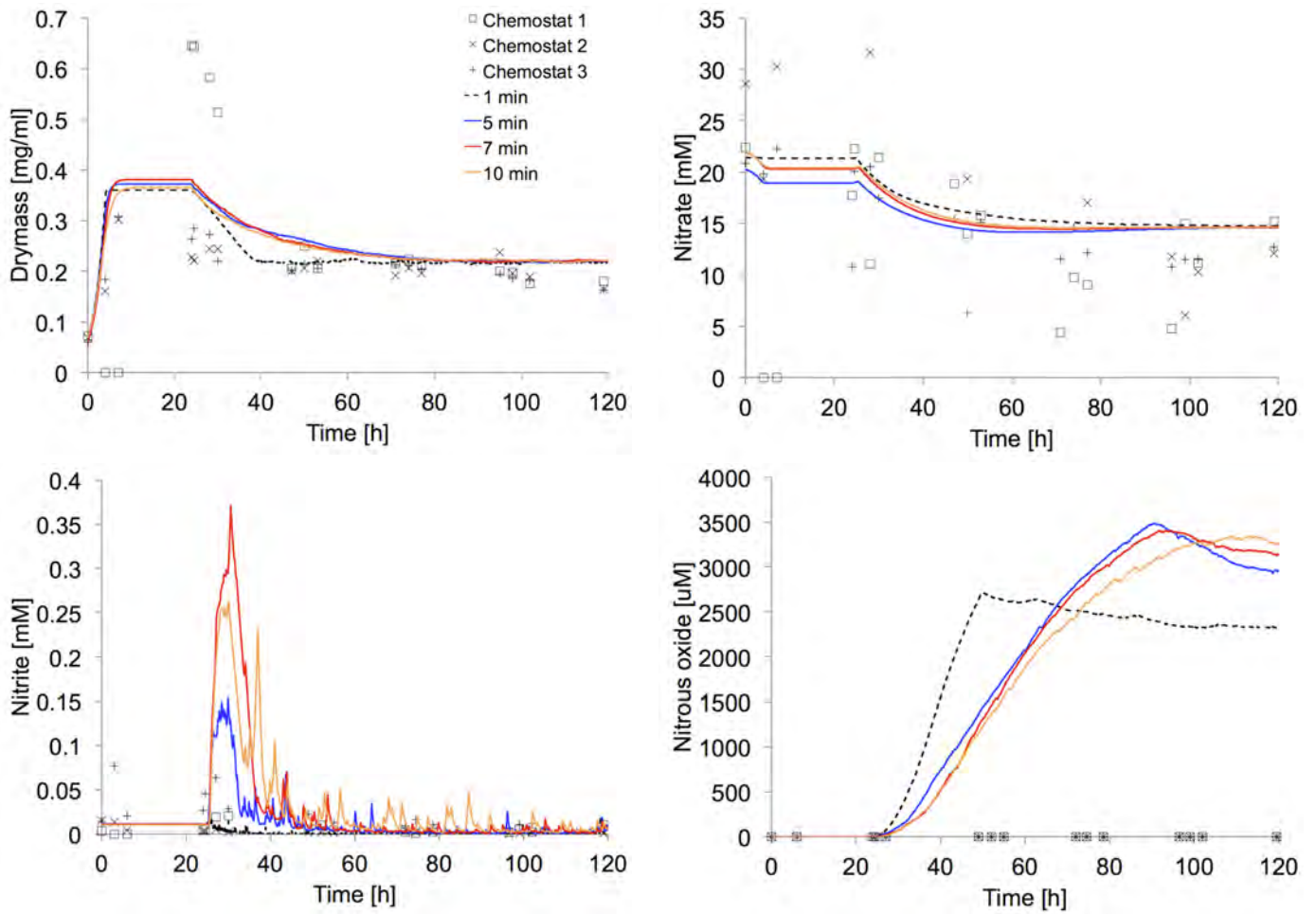


Figure S4. INDISIM-Paracoccus model calibration tests: experiment succinate-limited/ NO_3^- -sufficient in aerobic and anaerobic phase. Each color line represents a simulation result with different values of the step-time. Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. The system size assumed in the simulator to generate these results was 25×25 patches.

10

11

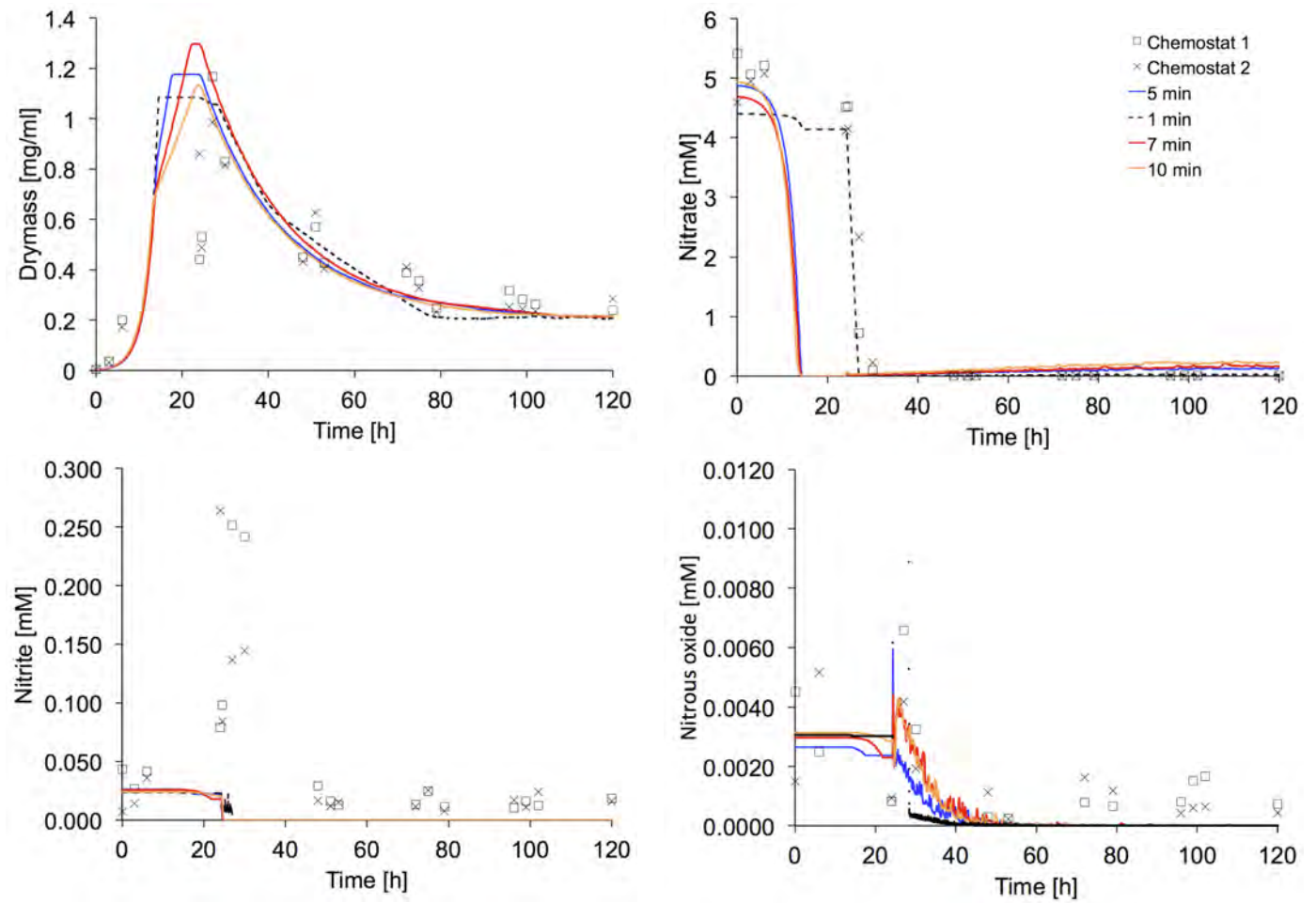


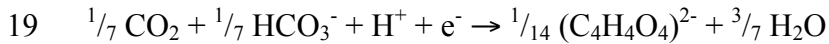
Figure S5. INDISIM-Paracoccus model calibration tests: experiment succinate-sufficient/ NO_3^- -limited in aerobic and anaerobic phase. Each color line represents a simulation result with different values for the time step. Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The metabolic hypothesis has been assumed in the simulator to generate these results. The system size assumed in the simulator to generate these results was 25×25 patches.

15 **Cellular maintenance**

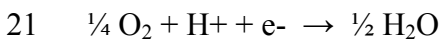
16 *How to write the energy reaction?*

17 **Step 1.** Write inorganic and organic half-reactions for electron donor and electron acceptor.

18 Electron donor (succinate) half-reaction (Rd):

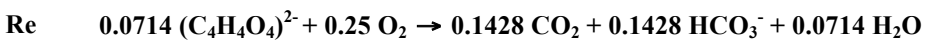
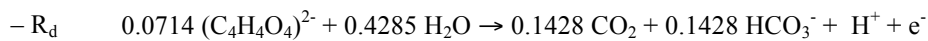
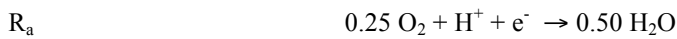


20 Electron acceptor (oxygen) half-reaction (Ra):



22 **Step 2.** According to (Rittmann & McCarty, 2001) following the equation ($\text{Re} = \text{Ra} - \text{Rd}$) a

23 balanced stoichiometric equation can be written for the energy reaction as follows.



24 Re is the balanced chemical equation for the energy reaction to fit the individual behavior-

25 rule for aerobic maintenance in INDISIM-Paracoccus model.

26 *How to use the energy reaction inside the rule for cellular maintenance?*

27 Before biomass synthesis, each individual in INDISIM-Paracoccus model executes a

28 behavior-rule for cellular maintenance. The maintenance requirements are different for

29 aerobic and anaerobic phases. For example, for aerobic phase we employ an appropriate

30 maintenance requirement for heterotrophic microorganisms of 0.002 gCdonor·gCmicrobial

31 h^{-1} proposed by Gras et al., (2011) and the energy reaction (Re) between succinate and

32 oxygen:

33 The first action that each individual does is to calculate the specific maintenance

34 requirements for aerobic phase using the elementary cell composition for *P. denitrificans*

35 ($\text{C}_3\text{H}_{5.4}\text{N}_{0.75}\text{O}_{1.45}$) proposed by (van Verseveld et al., 1979, 1983).

$$\begin{aligned}
36 \quad & 0.002 \frac{\text{gC}_{\text{succinate}}}{\text{gC}_{\text{microbial}} \cdot \text{h}} \times \frac{1 \text{ mol Succinate}}{48 \text{ gC}_{\text{Succinate}}} \times \frac{36 \text{ gC}_{\text{microbial}}}{1 \text{ mol } C_3H_{5.4}O_{1.45}N_{0.75}} = 0.0015 \frac{\text{mol Succinate}}{\text{mol Biomass} \cdot \text{h}} \\
& 0.0015 \frac{\text{mol Succinate}}{\text{mol Biomass} \cdot \text{h}} \times \frac{0.25 \text{ mol Oxygen}}{0.0714 \text{ mol Succinate}} = 0.0052 \frac{\text{mol Oxygen}}{\text{mol Biomass} \cdot \text{h}}
\end{aligned}$$

37 After that, taking into account the individual mass, the amount of each nutrient (nutrient-
38 maintenance) is calculated. Then the individual compares the nutrient-maintenance with the
39 amount of each nutrient after the uptake sub-model (nutrient-useful). If nutrient-useful is
40 higher than the nutrient-maintenance the individual executes the corresponding energy
41 reaction. If this condition is not reached the individual stops, and waits for the next time step.
42 After the execution of any energy reaction, the individual updates its nutrient-useful values.

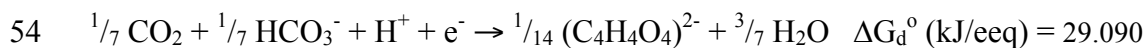
43 ***Biomass generation***

44 *How to write the balanced biochemical equation (BBE) that represents a metabolic pathway*
45 *to biomass synthesis?*

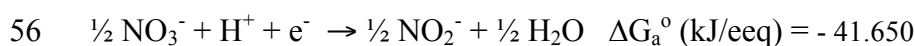
46 Example of calculations for anaerobic nitrate reduction with succinate as electron donor and
47 C-source, ammonium as N-source and nitrate as electron acceptor with $e = 0.41$, to fit the
48 individual behavior-rule for biomass generation in INDISIM-Paracoccus model for the
49 reaction 1 ($\text{NO}_3^- \rightarrow \text{NO}_2^-$) in metabolic pathway 3.

50 **Step 1.** Write inorganic and organic half-reactions and their Gibb's standard free energy at
51 pH = 7.0 according to Rittmann and McCarty (2001) for electron donor, electron acceptor and
52 cell synthesis reaction with ammonium as N-source.

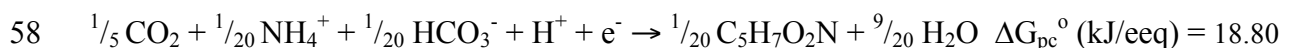
53 Electron donor (succinate) $\frac{1}{2}$ reaction (R_d):



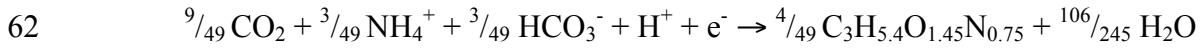
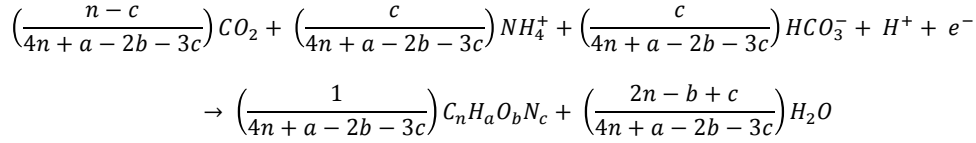
55 Electron acceptor (nitrate) $\frac{1}{2}$ reaction (R_a):



57 Cell $\frac{1}{2}$ reaction (R_c) with ammonium as N-source:



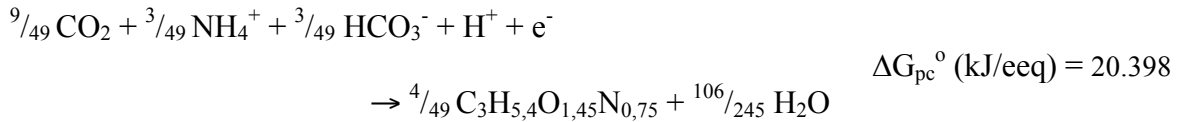
59 **Step 2.** Adjust the cell $\frac{1}{2}$ reaction (Rc) to *P. denitrificans* elementary cell composition
60 $C_3H_{5.4}N_{0.75}O_{1.45}$ (van Verseveld et al., 1979, 1983) following the methodology proposed by
61 Rittmann and McCarty (2001).



$$18.80 \frac{kJ}{eqq} \times \frac{20 eqq}{1 mol C_5H_7O_2N} \times \frac{1 mol C_5H_7O_2N}{113.11 g_{microbial}} \times \frac{75.17 g_{microbial}}{1 mol C_3H_{5.4}O_{1.45}N_{0.75}} \times \frac{1 mol C_3H_{5.4}O_{1.45}N_{0.75}}{49/4 eqq} = 20.398 \frac{kJ}{eqq}$$

$$18.80 \frac{kJ}{eqq} \times \frac{20 eqq}{1 mol C_5H_7O_2N} \times \frac{1 mol C_5H_7O_2N}{113.11 g_{microbial}} \times \frac{75.17 g_{microbial}}{1 mol C_3H_{5.4}O_{1.45}N_{0.75}} = 249.8755 \frac{kJ}{mol}$$

63 For *P. denitrificans* elementary cell composition the cell $\frac{1}{2}$ reaction (Rc) with ammonium as
64 N-source with Gibb's standard free energy at pH = 7.0 is



65 **Step 3.** Degree of reduction computation for electron donor and cells:

$$\gamma_d = \frac{\text{electrons donor}}{\text{Carbon donor}} = \frac{14}{4} = 3.5$$

$$\gamma_x = \frac{\text{electron cells}}{\text{Carbon cells}} = \frac{49/4}{3} = 4.083$$

66 **Step 4.** Computation of f_s° , f_e° and $Y_{c/c}$ according to McCarty (2007).

$$A = -\frac{\Delta G_s}{\varepsilon \Delta G_e} = \frac{\frac{(\Delta G_{fa} - \Delta G_d)}{\varepsilon^m} + \frac{(\Delta G_{in} - \Delta G_{fa})}{\varepsilon^n} + \frac{\Delta G_{pc}}{\varepsilon}}{\varepsilon \left(\Delta G_a - \Delta G_d - \frac{q}{p} \Delta G_{xy} \right)} = \frac{f_e^{\circ}}{f_s^{\circ}}$$

67 $\Delta G_{in} = 30.90$ kJ/eqq. Since no oxygenase is involved, $q = 0$. Since succinate is not a C1
68 compound, $\Delta G_{fa} = 0$ and $m = n$. Since $(\Delta G_{in} - \Delta G_d) > 0 \rightarrow (30.9 - 29.09) > 0$, $n = 1$, $m = 1$.
69 Using $e = 0.41$, and assuming that standard conditions apply.

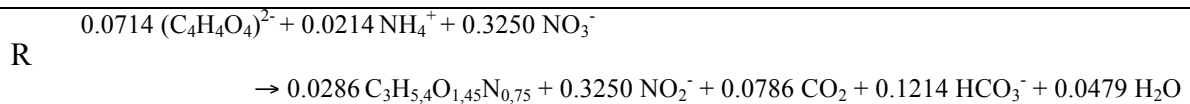
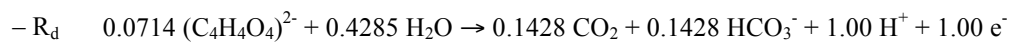
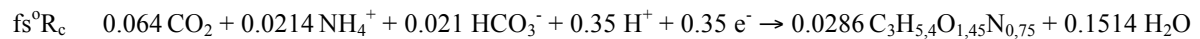
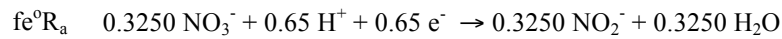
$$A = \frac{\frac{(0 - 29.09)}{0.41^1} + \frac{(30.90 - 0)}{0.41^1} + \frac{20.398}{0.41}}{0.41(-41.65 - 29.09 - 0)} = 1.857$$

$$f_s^o = \frac{1}{1+A} = \frac{1}{1+1.857} = 0.35$$

$$f_e^o = A \cdot f_s^o = 1.857 \times 0.35 = 0.65$$

$$Y_{C/c} = \frac{\gamma_d}{\gamma_x} f_s^o = \frac{3.5}{4.083} \times 0.35 = 0.30 \left[\frac{\text{mol } C_{\text{cells}}}{\text{mol } C_{\text{succinate}}} \right]$$

70 **Step 5.** A balanced stoichiometric equation can then be written. The overall reaction R is
 71 equal to $R = f_e^o R_a + f_s^o R_c - R_d$ according to Rittmann and McCarty (2001).



72 R is the balanced chemical equation using the Thermodynamic Electron Equivalent Model
 73 second version to fit the individual behavior-rule for biomass generation in INDISIM-
 74 Paracoccus model for metabolic pathway ($\text{NO}_3^- \rightarrow \text{NO}_2^-$).

75 In the same way we proceed in the calculations to the other reactions in the other pathways.

76 For *P. denitrificans* in aerobic phase growth, considering succinate as electron donor, Heijnen
 77 and Van Dijken (1992) proposed a maximum population growth yield ($Y_{C/c}$) of 0.48
 78 $C_{\text{mic}}/C_{\text{succ}}$ and van Verseveld et al. (1983) of 0.51 $C_{\text{mic}}/C_{\text{succ}}$, and for *P. denitrificans* in
 79 anaerobic phase growth considering succinate as electron donor and NO_3^- as electron
 80 acceptor, Heijnen and Van Dijken (1992) published a $Y_{C/c}$ value of 0.387 $C_{\text{mic}}/C_{\text{succ}}$ and van
 81 Verseveld et al. (1977) of 0.352 $C_{\text{mic}}/C_{\text{succ}}$. These population growth yields are the reference
 82 used for each reaction adjusted by TEEM2 (Table III).

83 *How to use the BBE inside the rule for biomass synthesis?*

84 This rule begins with the nutrient-useful values obtained after executing the cellular
 85 maintenance. If all the amounts of the nutrient-useful are higher than zero, following the

86 stoichiometry of each BBE, the individual increases its biomass (individual mass) and
 87 produces metabolic products. Once executed each reaction the individual updates its nutrient-
 88 useful values. If these values are higher than zero the individual can execute the next reaction.
 89 The sub model ends when the individual has executed all the chemical reactions that make
 90 the metabolic pathway and then the individual can execute the next sub model (reproduction).
 91 Nevertheless, the sub model can end when any of the nutrient-useful is lower or equal to zero,
 92 in this case the individual stops until the next time step.

93 *How we established the order of the reactions inside the denitrification pathway for the Gibbs*
 94 *hypothesis?*

95 Using the free energies of formation for various chemical species, with the balanced
 96 biochemical equations presented in Table III, we performed several calculations. For
 97 example:

Chemical specie	ΔG° (KJ/mol), 25°C	Stoichiometric Coefficient
$(C_4H_4O_4)^{2-}$	-690.23	0.0714
NH_4^+	-79.37	0.0214
NO_3^-	-111.34	0.3250
$C_3H_{5.4}O_{1.45}N_{0.75}$	249.87	0.0286
NO_2^-	-37.20	0.3250
CO_2	-394.36	0.0786
HCO_3^-	-586.85	0.1214
H_2O	-237.18	0.0479

$$\Delta G = \sum_{i=1}^{i=n} (v_i \Delta G_i^0)_P - \sum_{i=1}^{i=n} (v_i \Delta G_i^0)_R$$

98 Where v_i is the stoichiometric coefficient of i in the reaction and ΔG_i is the free energy of
 99 formation. With this data and the equation we calculated the ΔG for each equation.

$$\begin{aligned} \Delta G = & [(0.0286 * 249.87) + (0.3250 * -37.20) + (0.0786 * -394.36) \\ & + (0.1214 * -586.85) + (0.0479 * -237.18)] \\ & - [(0.0714 * -690.23) + (0.0214 * -79.37) + (0.3250 * -111.34)] \end{aligned}$$

$$\Delta G = - 31.48 \text{ KJ/mol}$$

100 The final values for all the equations in the denitrification pathway are as follows:

Reaction	ΔG (KJ/mol)	Efficiency (ϵ)
R3	- 31.48	0.41
R4	7.99	0.84
R5	-18.58	0.56
R6	-24.17	0.53

101

102 If we consider that with lower Gibbs energy the reaction will occur first, the order of the
103 reaction inside the denitrification pathway is: R3, R6, R5, R4.

104 Also the reader could see the values for the energy-transfer-efficiency (ϵ) used to write each
105 equation using TEEM2.

106

107 ***Maximum nutrient uptake-rate***

108 To estimate these values we use $\mu_{\max} = 0.418 \text{ h}^{-1}$, reported for *P. denitrificans* by van
109 Verseveld et al. (1983). With this value we calculate the maximum uptake for each nutrient
110 (the high value) according to the stoichiometric coefficients adjusted by TEEM2 for the
111 reaction 1 in metabolic pathway 1 ($\text{O}_2 \rightarrow \text{H}_2\text{O}$).

$$0.418 \frac{1}{h} \times \frac{0.0714 \text{ mol Succinate}}{0.0575 \text{ mol Biomass}} = 0.52 \frac{\text{mol Succinate}}{\text{mol biomass} \cdot h}$$

$$0.418 \frac{1}{h} \times \frac{0.0431 \text{ mol Ammonium}}{0.0575 \text{ mol Biomass}} = 0.31 \frac{\text{mol Ammonium}}{\text{mol biomass} \cdot h}$$

$$0.418 \frac{1}{h} \times \frac{0.0740 \text{ mol Oxygen}}{0.0575 \text{ mol Biomass}} = 0.54 \frac{\text{mol Oxygen}}{\text{mol biomass} \cdot h}$$

112 These values are the maximum values that the nutrient-uptake (u_i) parameter could take. We
113 use these values as a reference to start the calibration process of our model. The values
114 obtained after the calibration process are presented in Table IV.

115