INDISIM-Denitrification, an individual-based model for study the denitrification process

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Abstract

Denitrification is one of the key processes of the global nitrogen (N) cycle driven by bacteria. It has been widely known for more than one hundred years as a process by which the biogeochemical N-cycle is balanced. To study this process, we develop an individual-based model called INDISIM-Denitrification. The model embeds a thermodynamic model for bacterial yield prediction inside the individual-based model INDISIM and is designed to simulate in aerobic and anaerobic conditions the cell growth kinetics of denitrifying bacteria. INDISIM-Denitrification simulates a bioreactor that contains a culture medium with succinate as a carbon source, ammonium as nitrogen source and various electron acceptors. To implement INDISIM-Denitrification, the individual-based model INDISIM was used to give sub-models for nutrient uptake, stirring and reproduction cycle. Using a thermodynamic approach, the denitrification pathway, cellular maintenance and individual mass degradation were modelled using microbial metabolic reactions. These equations are the basis of the sub-models for metabolic maintenance, individual mass synthesis and reducing internal cytotoxic products. The model was implemented in the open-access platform NetLogo. INDISIM-Denitrification is validated using a set of experimental data of two denitrifying bacteria in two different experimental conditions. This provides an interactive tool to study the denitrification process carried out by any denitrifying bacterium since INDISIM-Denitrification allows changes in the microbial empirical formula and in the energy-transfer-efficiency used to represent the metabolic pathways involved in the denitrification process. The simulator can be obtained from the authors on request.

Keywords: denitrification, bacterial yield prediction, individual-based model, Thermodynamic Electron Equivalents Model, NetLogo, INDISIM.

1. Introduction

For the past decades, scientists have experienced huge interest in crossing the frontiers between different disciplines such as mathematics, biology, chemistry, thermodynamics and modelling, among others. Thus, this is a study of microbial metabolism in the framework of non-equilibrium thermodynamics and individual-based modelling, both concepts being applied to bacterial denitrification systems evolving in a bioreactor.

Denitrification is the dissimilatory reduction of nitrate (NO_3^-) to (mainly) dinitrogen gas (N_2) by bacteria. Hence, one or both of the ionic nitrogen oxides, NO_3^- and nitrite (NO_2^-), can be reduced to the gaseous oxides, nitric oxide (NO) and nitrous oxide (N_2O), which consequently may also be reduced to N_2 [1, 2]. A complete denitrification pathway is defined as the assemblage of four subsequent reactions: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ [1, 3].

Denitrification has been described, studied and investigated over the last one hundred years [4] at many levels, ranging through gene expression of the enzymes involved in the process [5–10], describing microbial metabolic pathways [11–14], measuring global N-oxides flux [15–17], evaluating the impact of metal concentrations in the soil on the expression of enzymes in different species of denitrifying bacteria [18–20], contributing to wastewater treatments as well as other biological systems [21, 22], within mathematical modeling [23–28] and, in individual-based models [29–32].

Interest in denitrification is motivated by several key factors. First, it is a fundamental process in wastewater treatment to reduce NO_3^- excess and stimulate carbon removal in anoxic conditions [33]. Second, it contributes to nitrous N_2O and/or NO emissions when denitrifying bacteria do not complete the metabolic pathway implicated, which is involved in atmospheric phenomena like global warming and ozone damage [34, 35]. Third, it is the mechanism by which the global nitrogen cycle is balanced [36].

Denitrification is a process driven by bacteria species with a genetic capacity for denitrification; they are classified as facultative aerobes. The denitrification pathway is common among several microbial species: *Pseudomonas, Achomobacter (Alcaligenes)* [37], *Paracoccus, Thiobacillus, Bacillus*, *Halobacterium*, *Chromobacterium, Hyphomicrobium*, in addition to some species of *Moraxella* that are also able to denitrify [22, 36]. Most of them are commonly found in soils, sediments, surface and ground waters, and wastewater treatment plants [33]. Denitrifying bacteria are able to use N-oxides as electron acceptors (e-acceptors) instead of oxygen (O_2), by electron transport chains similar to the ones used in aerobic respiration [6]. This means that they shift to NO_3^- or NO_2^- or NO or N_2O respiration when O_2 becomes limiting [22].

There are a wide range of environmental factors that control the complex regulatory network involved in bacterial denitrification. These include low O_2 concentration and the availability of e-acceptors (NO_3^-, NO_2^-, NO, N_2O) and C-sources as electron donors (e-donors) in the local environment where the bacteria develop [1]. Further, there is some

evidence that the denitrifying bacteria have the ability to reduce their own biomass to avoid accumulation of cytotoxic intermediate products (N_2O and/or NO) and complete the denitrification pathway and maximize energy conservation [11, 22]. In addition, if any of the key factors that control the denitrification pathway provoke an interruption of the process, then cytotoxic gases (N_2O and/or NO) are released to the medium. This can be viewed as a negative environmental consequence of denitrification since NO participates in photochemical reactions to produce tropospheric ozone, a greenhouse gas. The soil emissions of NO to the atmosphere have been measured and modeled in order to control its production [15, 23]. N_2O is a potent greenhouse gas and dominant ozone-depleting substance [9, 18, 19, 36]. Further, these gases have bacterial cytotoxic properties [5, 20] such as essential cellular cofactor inactivation of B12-dependent enzymes [7, 38], loss of cell division and viability [1, 4].

To study and analyze a microbial system, it is crucial to recognize the *structured* nature of each cell and the *segregation* of the culture into individual units that may differ from each other [39]. Therefore, it is crucial to carefully select the modeling approach.

The modelling approach traditionally used in biological fields, is an approach to understanding population level, where the population parameters are time-dependent and modified directly using the model's equations [40]. Models built at a population level of description are a particular type of System-based Model (SBM) [41]. They consider variables that characterize the population and the set of laws governing it. These rules are usually formalized with differential equations, which are ultimately based on assumptions regarding the behavior of the individuals. SBMs consist in defining the relevant variables of the system and proposing a set of rules governing them, applying these rules, i.e. solving the equations, and assessing the validity of the model through the comparison of its results with experimental observations [42]. Some of the applications of these models are predictive microbiology in food and control of fermentation processes [43], optimization of microbial cultures and antibiotics production in the pharmaceutical industry [44], waste control and water treatment [21], or the study of microbial ecology and evolution of population diversity in wild and artificial ecosystems [33]. Population models are based on assumptions about the individual behavior of microbes, and they therefore also raise new questions regarding microbial physiology and cellular models [45, 46].

Individual-based modeling is implemented and used in many scientific contexts, such as biological, chemical, biotechnological, ecological, among others [40, 47]. In this type of modeling, the interactions of the agents (individuals and/or collective entities) with their environment are simulated and the population-level behavior is an emergent property [48]. The IBM of microbes is called the "microbial individual-based model" (μIBM) [49]. Such models provide some advantages over the population-level approaches commonly used to model microbes' processes since: (a) they describe the system evolution as a whole by establishing behavior-rules for the microbes and their relations; (b) they can reproduce system variability because they admit the introduction of randomness and specific characteristics for the microbes; (c)

they take into account the individual adaptive behavior to the local environmental conditions; (d) they have the capability to resolve population heterogeneity (intra-population variability) to deal with complete life cycles, and (e) they represent the individual adaptive behavior to deal with internal and external conditions that changing over the time [48, 50, 51]. Also IBMs have the ability to link mechanisms at the individual level to population level behavior (emergence), and they are very convenient to tackle the inapplicability of the continuum hypothesis [46, 52].Therefore, the individuals and their internal differences and actions are better represented with a μIBM, in which the population behavior is the consequence of a set of microbes growing and interacting with the local environment [42, 47, 49, 52–54]. However, the potential of IBMs has a cost. They are more complicated structurally than analytical models, they must be implemented and executed in computers with determinate computing capabilities (modelling large-scale systems), the lack of individual-based data is sometimes crucial for their progress, besides they present some difficulties at the time of analysis, understanding and communicating [55]. To mitigate some of these problems there has been established the ODD protocol which stands for Overview, Design and Details as the universal way used by the scientific community for presenting and describing their IBMs [40, 47, 48, 56]. The use of specific programming environments to implement these computational models facilitates their use [55, 57], which along with computer processing tools and statistical analysis of data provides parameter estimation and the corresponding sensitivity analysis. These facilities make the methodology of discrete modelling based on the individual a valid and attractive option for study of microbial systems, increasing its presence in academic [58–61] and scientific fields [56, 62–65].

INDISIM is an individual-based discrete simulation model developed to study bacterial cultures [66]. This model has been used as the core for other models such as INDISIM-SOM [67, 68] and INDISIM-SOM-NL [69], INDISIM-YEAST [70], INDISIM-COMP [71], and INDISIM-*Saccha* [53] to model: the soil organic matter dynamics, yeast fermentation, multi-species composting, and the dynamics of *Saccharomyces cerevisiae* in anaerobic cultures, respectively.

Commonly, the biomass volumetric productivity and the macro-molecular composition of the cells are studied with regards to the potential production of the cells in response to their environment within the cultivation system [72]. According to the principles of the thermodynamics of non-equilibrium systems, a microorganism keeps alive by taking energy from its environment to maintain its structures and functions [73].

Taking into account this perspective, in the past few decades, several approaches in bio-thermodynamics, non-equilibrium thermodynamics, and network thermodynamics have been developed and reported to study and describe a macroscopic growth model for biomass yield prediction and cellular bioenergetics [22, 72, 74–88]. These approaches can be useful in the calculation of: (a) the complete growth stoichiometry, (b) the maintenance coefficients and maximal growth yields, (c) the limit to growth yield posed by the second thermodynamic law, (d) the chemical-oxygen-demand-based growth yields, and (e) the maximal product yields in aerobic and anaerobic metabolism. Therefore, these thermodynamic

approaches aim to represent all reactions that occur in the microorganisms using a set of microbial metabolic reactions (MMRs) [30, 31]

Using INDISIM [66] as a core model, we developed a model called INDISIM-Paracoccus [31] which is the first μIBM to use thermodynamics concepts to write the MMRs for cellular maintenance and individual mass production. It was designed to investigate the order of preference in the use of various e-acceptors in the denitrification process driven by *Paracoccus denitrificans*. With that model, we were able to fix the sequence order of the reduction of NO_3^- semi reactions along the denitrification and obtained a set of model parameter values to get a reasonably good fit of the simulation outputs to experimental data. INDISIM-Paracoccus had two main limitations, one was that it is only useful for one species of bacteria, the *P. denitrificans*, while there are many other bacteria that are able to denitrify. The second limitation was that some of the simulation outputs related to the cytotoxic gas nitrous oxide in the electron-donor limited and electron-acceptor limited experiments were not predicted accurately enough when compared with experimental data [32]. This current work aims to improve INDISIM-Paracoccus in order to overcome these limitations and provide it with a greater use and predictive capacity.

We develop an μIBM that is called INDISIM-Denitrification (Fig. 1) to deal with the dynamics of any denitrifying bacterium in aerobic and anaerobic conditions, including a thermodynamic model based on bioenergetics efficiency to describe the microbial metabolism. In particular, (a) we select the common pathways expressed in any denitrifying bacterium and represent them using a thermodynamic approach as a set of MMRs (which are central to the formulation of the metabolic sub-models inside of the μIBM developed), (b) we include into these MMRs the elemental composition of the microbial cells using a generic empirical formula that considers the molar relationship between the four main elements (C, H, O and N), (c) we design and parameterize behavior-rules plausible for any denitrifying bacterium with three main metabolic purposes: cellular maintenance, mass synthesis and individual mass degradation to reduce internal cytotoxic products; (d) we simulate a bioreactor that contains a culture medium where denitrifying bacteria develop and grow; (e) we implement the model on the open-access platform NetLogo presenting an μIBM simulator; and (f) we test the adequacy of the model using a set of experimental data for the denitrifying bacteria *P. denitrificans* and *Achromobacter xylosoxidans* published by Felgate et al. (2012). The use of a broader set of experimental data of two different denitrifying bacteria *P. denitrificans* and *A. xylosoxidans* leads to a better agreement to *P. denitrificans* data than previously obtained and open the possibility to deal with a new bacterium (*A. xylosoxidans*).

2. Materials and methods

2.1 Thermodynamic approach

The Thermodynamic Electron Equivalents Model (TEEM) is designed for bacterial yield prediction [22, 74, 81, 88–90]. TEEM is based on terms of the Gibbs free energy involved in the overall metabolic process and in how the energy between catabolism and anabolism is coupled using a term of energy-transfer-efficiency (ε). TEEM has two versions, the first one, TEEM1 [22] considers a realistic formulation of the anabolic reaction taking into account different N-sources such as NH_4^+, NO_3^-, NO_2^- and N_2, and a complete explanation of ε between catabolism and anabolism. The second version, TEEM2 [74] complements TEEM1 because it considers oxygenase reactions involved and the aerobic heterotrophic oxidation of C1 organic compounds.

For the use of any version of TEEM, first, we need to identify the e-donor(s) (usually the C-source) and the e-acceptor(s) and write reduction-half-reactions for each one of them. Second, it is necessary to establish the N-source for biomass synthesis and the empirical chemical formula that will represent the cells.

According to TEEM, to write the energy equation (*Re*) which represents the microbial catabolism, we need to combine the reduction-half-reaction of an e-donor (*Rd*) with the reduction-half-reaction of an e-acceptor (*Ra*). Once the catabolic process is represented by the *Re* equation, it is necessary to write the reaction for the cellular synthesis (*Rs*) that will represent the microbial anabolism. To do this, we need to combine the reduction-half-reaction of *Rd* with the cell synthesis half-reaction (*Rc*). For *Rc* we have to write and balance a hypothetical half-reaction that consider as reactants: The N-source (NH_4^+, NO_3^-, NO_2^- or N_2), CO_2 and HCO_3^-, and as products: water and the microbial mass represented by an empirical chemical formula of cells ($C_nH_aO_bN_c$). This empirical chemical formula considers only the four basic elements: Carbon (*n*), Hydrogen (*a*), Oxygen (*b*) and Nitrogen (*c*). To estimate the Gibbs free energy of this half-reaction (*Rc*), TEEM uses a value of 3.33 KJ per gram cells [88] which is related to one generic microbial cell composition ($C_5H_7O_2N$). To couple the energy from catabolism to anabolism, TEEM establishes a relation with the electrons involved. The electrons that come from the e-donor are divided in two parts, a fraction (fe^o) is transferred to the e-acceptor to generate energy (catabolism) and another fraction (fs^o) is transferred to the N-source for cell synthesis (anabolism). TEEM calculates the relationship between fe^o and fs^o using: (a) Gibbs standard free energy of *Rd*, *Ra* and *Rc*, (b) other Gibbs standard free energy terms related to C1 carbon source and oxygenase's enzymes, and (c) a term for energy-transfer efficiency (ε). This term is included because TEEM assumes that a fraction of thermodynamic free energy is lost at each transfer energy between catabolism and anabolism. TEEM's developer [22, 74] used an extensive amount of information provided by several authors, Heijnen and Van Dijken, 1992; VanBriesen and Rittmann, 2000; VanBriesen, 2002; Xiao and VanBriesen, 2008, to calibrate and determine the best-fit energy-transfer-efficiency (ε) for the TEEM model.

Therefore, using TEEM we can get the complete chemical and energetic stoichiometry of microbial growth represented by a MMR. In this study, we will represent metabolic pathways as a set of MMRs using TEEM2 and use them as the basis of the behavior-rules (such as individual cellular maintenance or individual mass synthesis or individual mass degradation to reduce cytotoxic products) for each bacteria of the virtual system, and we assume the ε value as an individual value [30].

2.2 Experimental data

To study into a bioreactor the denitrification process, the experimental assays were designed to breed and develop bacteria in a bioreactor under two different conditions: one first stage in a batch culture (from 0 to 24 h) during the aerobic phase, and the second one in a continuous culture (from 24 to 120 h) during the anaerobic phase. Under these bioreactors procedures two experiments were performed with two different bacterial species by Felgate et al. (2012): (a) the reservoir medium feed contained 20 mM NO_3^-, 5 mM succinate and 10 mM NH_4^+ which was designed to achieve an e-donor limited with e-acceptor sufficient during the steady state and is designed as *succinate-limited/NO_3^--sufficient* (Experiment E1), and (b) the reservoir medium feed contained 5 mM NO_3^-, 20 mM succinate and 10 mM NH_4^+ to achieve an e-donor sufficient with e-acceptor limited during the steady state and is designed as *succinate-sufficient/NO_3^--limited* (Experiment E2). The data for the time evolutions of dry mass (biomass), NO_3^-, NO_2^- and N_2O were collected from 0 to 120 h, according to the experimental procedure presented in Felgate et al. (2012) that utilizes two different denitrifying bacteria, the *P. denitrificans* and *A. xylosoxidans*. With INDISIM-Denitrification we will carry on virtual experiments to reproduce the behavior of both bacteria growing in both media.

2.3 Programming environment

INDISIM-Denitrification is implemented in the widely used, free and open source platform NetLogo (Fig. 2), a multi-agent programming language to modeling environment for simulating natural phenomena [57]. This provides full access to the simulation model, including a graphical user interface and the model's source code. Given NetLogo's rather flat learning curve and comprehensive documentation [40], users without extensive modeling experience can also modify the code and, thus, investigate alternative mechanisms or adapt certain processes according to other approaches (e.g. introducing variations in the biomass empirical chemical formula of bacteria).

2.4 Model analysis

To assess the validity of the model, after the first visual techniques with subjective assessment, we carried out numerical validation techniques that provide a quantification of the difference (or similarity) between observed and simulated values. The goal is to find ranges of values for the model parameters' that make it possible to roughly reproduce the evolution of

214 a set of focus variables observed in the two trials using the experimental data for the two bacteria, *P. denitrificans* and *A.*
215 *xylosoxidans* [18].
216 In order to compare the simulation results with the experimental data we used the geometric reliability index (GRI) values,
217 a statistical method to determine the reliability of a model [93]. This index can deal with precise notions of model
218 accuracy; therefore, its value indicates how closely the simulation results match the experimental ones. For models with
219 simulation results reasonably close to experimental observations this GRI shows a resulting factor of 1 to 3, with 1
220 corresponding to 100% accuracy. The interpretation of GRI is that the simulation is accurate within a multiplicative factor,
221 e.g. with a GRI value equal to 1.32, this means that the simulated values fall between 1/1.32 and 1.32 times the
222 corresponding experimental values [94].
223 The combination of the use of multiple deviance measures with visual inspection in the exploratory data analysis can help
224 to identify deficiencies and capabilities of the model developed. To assess whether a certain combination of model
225 parameter values leads to acceptable model output, we include the GRI calculation within the main code of the simulator
226 for the evolution of four variables: microbial biomass (dry mass), NO_3^-, NO_2^- and N_2O, controlled for each one of the two
227 scenarios (Experiments E1 and E2) and for the two denitrifying bacteria tested (*P. denitrificans* and *A. xylosoxidans*).
228 The software tool "BehaviorSpace" incorporated in NetLogo was used for running simulation experiments varying
229 parameters values and writing model outputs data to files to be statistically analyzed. Each simulation is replicated three
230 times.

231 # 3. Results and discussion

232 INDISIM-Denitrification model was developed to reproduce a bioreactor experimental protocol for the denitrification
233 process carried out by denitrifying bacteria growing in batch and continuous culture, in aerobic and anaerobic growing
234 conditions. To describe our model we use the ODD protocol ("Overview, Design concepts, and Details"), which helps to
235 ensure that the model description is complete [40, 47, 95]. The complete and detailed description of this model can be
236 found in the Supplementary material 'ODD of the model INDISIM-Denitrification'. In this section only, the new features
237 of the model in relation to INDISIM-Paracoccus are highlighted.

238 ## 3.1. Microbial metabolic reactions

239 The reduction of cytotoxic products as a result of anaerobic metabolism through the individual-mass degradation has been
240 added to individual metabolism, joint with the cellular maintenance and the individual-mass synthesis (Supplementary
241 material). It seems feasible that in natural conditions when the level of cytotoxic compounds in the media is high then the
242 microorganisms follow different biological strategies to survive. We have assumed and modelled that the individual can

use its own mass as an e-donor and the NO and/or N_2O as e-acceptor to keep the levels of those products below toxic concentrations. We consider a degradation coefficient (h^{-1}) to stablish the amount of individual mass that is used to reduce cytotoxic products, its value is depending on the bacterial species. The individual mass decreases according to this quantity.

To raise the model to a wider number of bacterial species capable to denitrify, we considered the microbial biomass composition represented by the elemental formula of $C_nH_aO_bN_c$, being the sub index the elemental molar relation. The molar relation can be modified in the computational model by the user according to the microorganism to simulate and in consequence the thermodynamic calculations using TEEM2 have been generalized.

To derive the MMRs required for the individual behavior-rule for cellular maintenance, it is necessary to model the energy reactions for aerobic and anaerobic metabolism. We considered the reaction between succinate (which is always the e-donor) and O_2 (as e-acceptor) for the aerobic phase, while for the anaerobic phase; the e-acceptor is an N-oxide. We used the inorganic half-reactions for *Rd* and various *Ra* shown in Rittmann and McCarty, (2001) to write the energy reactions (*Re*) shown in Table 1. With these energy reactions and an appropriate maintenance requirement (gCdonor $gCmic^{-1}$ h^{-1}), we designed the individual rule for cellular maintenance.

For individual-mass synthesis, it is necessary to model the metabolic pathways for aerobic and anaerobic conditions for a general denitrifying bacterium and they are translated into a set of MMRs. To incorporate this in the model we took a rough approximation to the microbial biomass represented by an empirical chemical formula of cells ($C_nH_aO_bN_c$), which is written only with the molar relationship of the four main elements, *n* for carbon, *a* for hydrogen, *b* for oxygen and *c* for nitrogen. We consider that the microorganism increases its individual-mass when it executes any of the reactions described as a set of MMRs (Table 2), in aerobic phase executes aerobic respiration (Reaction I) and dissimilatory nitrate reduction IV (Reaction II), and in anaerobic phase executes denitrification (Reactions III to VI) [12]. To formulate these reactions and calculate the corresponding stoichiometric coefficients we used the TEEM methodology [74]. In all reactions succinate is the universal *Rd* and C-source, NH_4^+ is the universal N-source (*Rc*) for cell synthesis and the nutrients used as *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 2).

For the individual mass degradation, to reduce internal cytotoxic products, we write the half-reaction where the biomass is an e-donor which can be coupled with e-acceptor half-reaction to write the stoichiometry reaction (Table 3). With TEEM2 methodology all reactions, for cellular maintenance (Table 1), for individual-mass synthesis (Table 2) and for individual mass degradation (Table 3) are balanced for mass and energy.

3.2 Parametrization and sensitivity analysis

INDISIM-Denitrification has the capability to work with any denitrifying bacterium. To test the performance of INDISIM-Denitrification we used experimental data published for two different denitrifying bacteria, *P. denitrificans* and *A. xylosoxidans,* and compared them with the simulation results obtained with the NetLogo implementation of our model. To set up the thermodynamic model, we first used the empirical chemical formula ($C_3H_{5.4}O_{1.45}N_{0.75}$) for the denitrifying bacterium *P. denitrificans* published by van Verseveld et al., (1983, 1979, 1977). Taking into account the coefficients *n*, *a*, *b* and *c*, the molar relationship between carbon, hydrogen, oxygen and nitrogen, are 3, 5.4, 1.45 and 0.75, respectively, and the information provided by Table 2 and Table 3. The stoichiometric coefficients for each MMR related to individual-mass synthesis (Table 4) and to individual-mass degradation to reduce cytotoxic products (Table 5) were obtained applying TEEM2 [30, 46] with an assigned ε value in the range proposed for McCarty (1971, 2007) and Rittmann and McCarty (2001) (see supplementary material to detailed calculations).

To represent the microbial biomass of *A. xylosoxidans* through an empirical chemical formula we adopted $C_5H_9O_{2.5}N$ [26, 80, 83] and used the information provided by Table 2 and Table 3, the stoichiometric coefficients for each MMRs related to individual-mass synthesis (Table 6) and to individual-mass degradation to reduce cytotoxic products (Table 7) were obtained operating with TEEM2 [46], using a different assigned ε value for each reaction in the range proposed by McCarty (2007) and Xiao and VanBriesen (2008).

The model implementation in NetLogo allows the user to quickly and easily change many parameter's values involved, and specifically, in this new simulator:

(a) The molar relationship between the elements of the biomass empirical formula (C, H, O and N), with which the NetLogo simulator immediately recalculates all of the stoichiometric coefficients for the set of MMRs.

(b) The bacteria size, allowing the spherical equivalent diameter (expressed in μm) for the smallest and largest bacteria, where in all cases the bacterium is considered to be spherical shape. In the case of *P. denitrificans* the smallest individual represents a bacterium with a diameter of ~ 0.5 μm and the largest one a bacterium with a diameter of ~ 0.9 μm. In the case of *A. xylosoxidans* the smallest individual represents a bacterium with a spherical equivalent diameter of ~ 0.63 μm and the largest one a bacterium with a spherical equivalent diameter of ~ 1.40 μm.

(c) The maximum population growth rate (μ_{max} which is expressed in h^{-1}), a parameter which is used to estimate the individual maximum uptake-rates (*ui*) which are calculated adding the maintenance and growth requirements according the stoichiometric coefficients of the MMRs. van Verseveld 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 NO_3^--limited conditions to aerobic succinate-limited conditions. In the case of *A. xylosoxidans* a value equal to 0.25 h^{-1} is reported, which was obtained when the bacterium grew over 6-carbon compounds in aerobic conditions [99]. With this information, the

simulator recalculates the maximum uptake reference value to all nutrients considered in the virtual system. Using these values and performing calculations with the stoichiometric coefficients of each MMRs adjusted by TEEM2 (Table 4 and Table 6), we obtained the maximum uptake-rate for each nutrient and bacteria. Taking into account the maximum uptake-rate for each nutrient and bacteria, we established the values for the sensitivity analysis performed for this parameter (Table 8 and Table 9).

(d) The maintenance coefficient (gCdonor $gCmic^{-1}$ h^{-1}), a parameter which is used in the aerobic and anaerobic growth phases of the denitrifying bacteria. In the case of cellular maintenance, Gras et al. (2011) consider an appropriate maintenance requirement for soil heterotrophic microorganisms of 0.002 (gCdonor $gCmic^{-1}$ h^{-1}), which was assumed in INDISIM-Paracoccus [31] for the aerobic phase. This is different in the implementation of INDISIM-Denitrification, due to that the model considering a unique parameter for the cellular maintenance coefficient (gCdonor $gCmic^{-1}$ h^{-1}) in both aerobic and anaerobic phase.

(e) The mass degradation coefficient (h^{-1}), another individual parameter related to the individual mass, which was introduced for the anaerobic phase only. In Table 10 we show the tested values for cellular maintenance and individual mass degradation parameters: these ranges of values will be the same for both bacteria.

To start the calibration and sensitivity analysis process, we combined the values from Table 8 and Table 9 using a full factorial design for each species of bacteria and each virtual experiment (E1, E2), and after that, we combined the values from Table 10 in a full factorial design for the cellular maintenance and mass degradation coefficients.

To assess whether a certain combination of model parameter values leads to acceptable model output, we calculated GRI value for four variables: microbial biomass (drymass), NO_3, NO_2^- and N_2O, controlled for both scenarios (Experiments E1 and E2) and for both denitrifying bacteria *P. denitrificans* and *A. Xylosoxidans*.

The simulation and experimental mean values of the three replications performed are presented with their corresponding standard errors in the following graphical representations. We established multiple fitting criteria using the model's parameters: uptake-rate for all nutrients involved, cellular maintenance rate and, mass degradation coefficient, with the experimental data of Felgate et al. (2012). The essential idea is to find a value or a range of values for these parameters that make it possible to roughly reproduce the evolution of a set of patterns observed in the two experiments and for both bacteria species. All full factorial designs were executed using the tool "BehaviorSpace" included in NetLogo, a task that was facilitated due to the simulator including in its code the complete experimental data set to calculate GRI. Each simulation result was compared to the experimental values and the GRI for each one was calculated. We selected the combination of the parameters with minimum GRI value to declare the best fit of INDISM-Denitrification.

3.3 Simulation outputs

In order to verify our model implementation, we checked several features to ensure its accurate quantification of the conceptual model. For instance, one of the main checks was to verify that the simulator accomplished mass-balances for C and N, which ensures that the chemical reactions and the bioreactor inputs/outputs are accurately implemented, and the simulator works as is expected. We also tested that the individuals were able to carry out all of the reactions in different 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. 2).

The outputs of the model (Fig. 2) are: (a) the concentration ($mmol \cdot l^{-1}$ or $umol \cdot l^{-1}$) of each culture medium component (succinate, NH_4^+, O_2, NO_3^- CO_2, HCO_3^-, NO_2^-, NO, N_2O and N_2), (b) microbial biomass ($mg \cdot ml^{-1}$), (c) the population mass distribution, (d) a graphical view to show the frequency of use of each metabolic reaction, (e) all MMRs written using TEEM for any denitrifying bacteria, and (f) GRI´s values for the time evolution of system variables, microbial biomass (dry mass), NO_3^-, NO_2^- and N_2O. The outputs of the model that are compared with experimental data are shown in the figures 3 to 6, in these figures the experimental data are drawn with means of the replicates and their standard errors, and the simulation results are drawn with a sequence of dots, each dot represents the mean of the replicates of the model in each step time.

3.3.1 Simulations for *P. denitrificans*

INDISIM-Denitrification was used to simulate the growth and behavior of the *P. denitrificans* in a bioreactor which works in aerobic conditions (batch culture) and in anaerobic conditions (continuous culture) in accordance with the experiments E1 and E2 and experimental protocols published by Felgate et al. (2012). The set of individual and environmental parameter values that generate model outputs with acceptable GRI coefficient are shown in Table 11. In figure 3 and figure 4, we present the outputs assessed for the bacterium *P. denitrificans*, namely the drymass, NO_3^-, NO_2^- and N_2O time evolutions for the two experiments succinate-limited/NO_3^--sufficient – E1 (Fig. 3) and succinate-sufficient/NO_3^--limited – E2 (Fig. 4) with the GRI score obtained in the statistical analysis.

According to the magnitude of the GRI coefficient, the results of the simulated experiment E1, succinate-limited/NO_3^--sufficient, are accurate to the experimental results for drymass, NO_3^-, NO_2^- and N_2O evolutions. The experimental data suggest that the culture achieved the steady state 40 hours from the start; the effect of moving from aerobic to anaerobic phase, is appreciable on the GRI values for NO_2^- (Fig. 3-C) and N_2O time evolution (Fig. 3-D), the highest values obtained are for E1, but still adequate. Furthermore, we consider that the results on figures 3-C and 3-D are due to the stochastic nature of the parameter related with the behavior-rule of the individual-mass degradation to reduce cytotoxic products are executed, observing these results we confirm that our model has the necessary stochasticity that an IBM must have.

The lowest value obtained for GRI corresponds to the drymass temporal evolution (Fig. 3-A), which confirms that the thermodynamic approach used to represent the metabolic pathways was properly selected.

The results obtained for the simulated experiment E2, succinate-sufficient/NO_3^--limited, are close to the experimental result for drymass evolution (Fig. 4-A). On the other hand, the GRI scores for NO_3^-, NO_2^- and N_2O evolutions in E2 (Fig. 4-B, Fig. 4-C and Fig. 4-D) are outside of the required GRI range, which suggests a rough adequacy of the model in the experiment e-donor-sufficient/e-acceptor-limited.

An explanation for this fact is that the amount of e-donor is able to reduce whole amount of e-acceptor of the system. This explains the fact that the temporal evolutions of NO_2^- and N_2O (Fig. 4-C and Fig. 4-D) in the anaerobic conditions hit zero, increasing the value of the GRI.

The simulated data for the drymass evolution in experiment E1 and E2 has the lowest GRI values. These results reinforce the idea that the contribution of TEEM2 to write the metabolic reactions is crucial in a model based on individuals and moreover that the metabolism is a central part of it. Also, it suggests that the formula $C_3H_{5.4}O_{1.45}N_{0.75}$ used to represent the biomass of *P. denitrificans* provides an acceptable agreement between the simulated and experimental system variables.

The system variables outputs for *P. denitrificans* with INDISIM-Denitrification simulator improve the GRI value, from 12.94 (INDISIM-Paracoccus) to 2.02 (INDISIM-Denitrification), for the N_2O time evolution for the experiment with e-donor limited (Fig. 3-D) in relation to the results presented in our previous work [31]. In light of this results it seems plausible that the individual-mass degradation could be an interesting individual strategy to reduce the accumulation of cytotoxic products in the surrounding media as has been pointed out by some authors [22].

In addition to those temporal evolutions which are compared to the experimental values through GRI values, INDISM-Denitrification gives the outputs (graphical and numerical) for other nutrients and metabolic products involved in the denitrification process, such as succinate, NH_4^+, O_2, NO, N_2, CO_2 and HCO_3^- (Fig. 2). These chemical compounds do not have the corresponding experimental temporal evolutions in the data set presented by Felgate et al. (2012), therefore it is not possible to calculate the GRI values for them. However, INDISIM-Denitrification provides the user with these data and thus, it makes possible a comparison when new experimental data become available.

3.3.2 Simulations for *A. xylosoxidans*

We took new experimental data published by Felgate et al. (2012), and not previously used, into account for comparing the adequacy of the simulations with INDISIM-Denitrification for *A. xylosidans*, to evaluate the goodness of the model, and to assess the improvements introduced to achieve the objectives of this work. Simulations were run with the empirical chemical formula $C_5H_9O_{2.5}N$, which is commonly used to represent the biomass composition of *A. xylosoxidans*. The

individual and environmental parameter values that caused model outputs with acceptable GRI coefficient are shown in Table 12.

In Figure 5 and Figure 6, the outputs assessed for the bacterium *A. xylosoxidans* are shown, namely the drymass, NO_3^-, NO_2^- and N_2O evolutions for the two experiments, experiment E1 with succinate-limited/NO_3^--sufficient (Fig. 5) and experiment E2 with succinate-sufficient/NO_3^--limited (Fig. 6), where the GRI scores obtained in the statistical analysis performed are included. According to the GRI values for the experiment e-donor limited (Fig. 5), the simulation results obtained with INDISIM-Denitrification for the bacterium *A. xylosoxidans* showed an acceptable behavior, because all of the values were in the acceptable range of GRI (from 1 to 3). The highest GRI value was obtained in the temporal evolution of NO_2^- (Fig. 5-C).

The acceptable range for GRI was only achieved in the drymass and NO_3^- evolution (Fig. 6-A and Fig. 6-B) for the experiment e-donor sufficient (E2). This model's behavior is a key point for future upgrades of this INDISIM branch because it could be necessary to include a new behavior-rule at the individual level to regulate the model's response when the e-acceptor is limited (e-donor sufficient).

4. Conclusions and final remarks

Considering the GRI values obtained for the temporal evolutions variables tested, INDISIM-Denitrification provides acceptable results for the experiments where the e-donor is limited, specifically for denitrifying bacterium *P. denitrificans*: (a) biomass, from 1.22 (INDISIM-Paracoccus) to 1.08 (INDISIM-Denitrification), (b) nitrate, from 1.26 (INDISIM-Paracoccus) to 1.23 (INDISIM-Denitrification), (c) nitrite, from 2.05 (INDISIM-Paracoccus) to 1.97 (INDISIM-Denitrification), and (d) nitrous oxide, from 12.94 (INDISIM-Paracoccus) to 2.02 (INDISIM-Denitrification) (Fig. 3 and Fig. 5). We consider that one of the reasons is due to TEEM being designed for bacterial yield prediction in microbial systems when the C-source is a limiting factor, e.g. the wastewater treatments [22].

One of the novelties of INDISIM-Denitrification simulator is that it offers a greater versatility in relation to the previous version (INDISIM-Paracoccus), because it can be used to work with any other bacteria in a pure culture. It is also possible to simulate a functional denitrifying group when the user works with mixed cultures and use mean molar coefficient for microbial biomass (*n,a,b,c*) defining a representative empirical formula for bacterial population. In consequence, all the stoichiometric coefficients for the set of MMRs for each metabolic pathway are automatically recalculated. Following the principle that all individuals could achieve the maximum growth rate, μ_{max} if the user changes this value, the individual maximum uptake-rate values are recalculated for all nutrients involved in metabolism, according to the stoichiometric coefficients of the MMRs related with individual mass synthesis. Since these improvements in the parameter calculations are incorporated in the code, the calibration for other denitrifying populations is easier. Therefore, the INDISIM-

Denitrification simulator allows the user to interact in a much more extensive way with significant biological parameters of the metabolic part of the bacteria, giving different values to parameters that can condition the growth dynamics, and which are notable for the denitrification results.

The model has been improved since we have assumed that individuals cannot live and develop in the same way in a favorable environment as in a hostile environment. From the moment in which an accumulation of cytotoxic products occurs in the medium, the individual develops a strategy to survive and it has an energy or mass cost. We have assumed that the individual consumes its own biomass to reduce some of the N-oxides which are toxic and we have implemented this sub model as a part of the metabolism. This assumption has given much better results in the calibration of the model in relation to the INDISIM-Paracoccus, specifically for the evolution of NO and N_2O for both bacteria in the experiment succinate-limited/NO_3^--sufficient, since accumulation did not occur in the simulated system just as in the experimental tests. So, we can conclude that our assumption or hypothesis is consistent and reflects how individuals maintain their viability in the presence of cytotoxic products.

The implementation of INDISIM-Denitrification in NetLogo offers easy access to the computer code for future and specific adaptations to the user interested in diverse academic and research applications. In particular, it facilitates the exploration of the effects of bacterial metabolic behavior on denitrification dynamics and allows users to test their own (virtual or measured) parameter values or to compare the model output to their own observations.

Based on results, it appears that INDISIM-Denitrification is a useful tool to model any denitrifying bacterium in batch and continuous cultures under different oxygen concentration to simulate aerobic or anaerobic metabolism. In this study, homogeneous, laboratory chemostat data, typically showing low spatial heterogeneity, have been used. Nevertheless, the developed model allows us to include the heterogeneous dynamics into the system. This heterogeneity is not only related with aerobic and anaerobic conditions, it is also reflected at the individual level with the behavior rules and alternatives in the use of metabolic pathways. For instance, the heterogeneity at individual level can be revealed using biomass distributions of the bacteria (or other distributions of cellular contents) and controlling which reactions are more often used than others by the microbes during the temporal evolution of the system (Figure 2). Nowadays, this perspective on the biological heterogeneity in individual behavior has been assumed and treated in other applications [100–102] in order to advance our understanding of microbial systems. Using highly controlled experimental conditions has offered the possibility to focus on the individual behavior rules (exception made of the bacterial movement) that are now validated and ready, in the near future, to deal with other medium conditions.

TEEM2, one of the thermodynamic models based on bioenergetics growth efficiency, also appears to be a useful tool for modeling the individual metabolism in the INDISIM-Denitrification model. In contrast to other modeling approaches, it allows the user to embed thermodynamic properties into individual cells, which can simulate the behavior of the bacterial

population more realistically than the continuous and traditional population-based approaches.

With µIBM as the INDISIM-Denitrification it should be possible to investigate the theory for the coupling energy between catabolism and the anabolism, which is the principal assumption in the TEEM2 because it considers that thermodynamic free energy is lost at each transfer by including a term for this efficiency (ε). TEEM2 considers ε value constant, but there is no clear reason why it should do this. Therefore, experiments could be developed with some specific environmental conditions where the same metabolic pathway would be adjusted with different values of ε, The use of IBMs allow to model individuals that can change their (ε) value according to the local environmental conditions. This will be an interesting contribution because some authors consider that ε value is not constant in the metabolic process [76, 77].

The development and application of µIBM with some intracellular detail and complexity is the key advantage of our new model for studying the different steps of denitrification carried out by a denitrifying bacterium. Exploring model behavior via its input parameters and assessing alternative sub-models provides a way to progress with the development of a simulator able to control factors that contribute to our understanding of how major or minor N_2O generation is a consequence of this denitrified metabolic individual activity.

In a broader context, and in connection with other models where the process of nitrification can be significant, this model can give insights into the representation of microbial activity existing in diverse environments, as for instance, in organic matter transformations. For instance, the mineralization and nitrification processes involved in those transformations are mainly driven by bacteria (and other microbes), and consequently, the standpoint used in this denitrifying model can be adapted or incorporated to represent these processes [68, 69]. The cycles of carbon and nitrogen require the integration of these interacting processes. The challenges associated with the distribution and activity of microorganisms at a microscale, for instance, in soils, is being investigated both from experimental data with advanced and innovative techniques and with the use of models and simulations [103]. Insights of the microscale heterogeneity of the spatial distribution of organic matter connected with microbial activity need spatially explicit modelling approaches. In the recent past computer simulations focusing on the microscale are resulting in some additions to our understanding of such complex environments [104–106]. The denitrifying model achieved in this study would highly benefit those spatially explicit models, because it can be treated as a module in order to build the backbone of a more ambitious biophysical model for transformations of organic matter.

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Authors Contributions

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. Microbial metabolic reactions (Energy Reactions - Re) for cellular maintenance in aerobic (I) and anaerobic phase (from II to V). Re = Ra – Rd according to TEEM2 [22, 74].

I	$(C_4H_4O_4)^{2-} + 3.5\ O_2 = 2\ CO_2 + 2\ HCO_3^- + H_2O$
II	$(C_4H_4O_4)^{2-} + 7\ NO_3^- = 7\ NO_2^- + 2\ CO_2 + 2\ HCO_3^- + H_2O$
III	$(C_4H_4O_4)^{2-} + 14\ NO_2^- + 14\ H^+ = 14\ NO + 2\ CO_2 + 2\ HCO_3^- + 8\ H_2O$
IV	$(C_4H_4O_4)^{2-} + 14\ NO = 7\ N_2O + 2\ CO_2 + 2\ HCO_3^- + H_2O$
V	$(C_4H_4O_4)^{2-} + 7\ N_2O = 7\ N_2 + 2\ CO_2 + 2\ HCO_3^- + H_2O$

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Table 2. Microbial metabolic reactions for individual-mass synthesis in aerobic[(a)] and anaerobic[(b)] conditions for any denitrifying bacteria when succinate is C-source, NH_4^+ is N-source and different e-acceptors involved in common denitrification pathway. (R = fe^o Ra + fs^o Rc – Rd) according to TEEM2 [22, 74].

I[(a)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d}\right) NH_4^+ + \left(\frac{1}{4} f_e^o\right) O_2 + (f_e^o + f_s^o - 1)\, H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d} - \frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - \frac{1}{2} f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$
II[(a)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d} - \frac{1}{8} f_e^o\right) NH_4^+ + \left(\frac{1}{8} f_e^o\right) NO_3^- + \left(\frac{5}{4} f_e^o + f_s^o - 1\right) H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d} - \frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - \frac{3}{8} f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$
III[(b)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d}\right) NH_4^+ + \left(\frac{1}{2} f_e^o\right) NO_3^- - \left(\frac{1}{2} f_e^o\right) NO_2^- + (f_e^o + f_s^o - 1) H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d} - \frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - \frac{1}{2} f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$
IV[(b)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d}\right) NH_4^+ + (f_e^o) NO_2^- - (f_e^o) NO + (2f_e^o + f_s^o - 1) H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d} - \frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$
V[(b)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d}\right) NH_4^+ + (f_e^o) NO - \left(\frac{1}{2} f_e^o\right) N_2O + (f_e^o + f_s^o - 1) H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d}\frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - \frac{1}{2} f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$
VI[(b)]	$\left(\frac{1}{14}\right) C_4H_4O_4^{2-} + \left(f_s^o \frac{c}{d}\right) NH_4^+ + \left(\frac{1}{2} f_e^o\right) N_2O - \left(\frac{1}{2} f_e^o\right) N_2 + (f_e^o + f_s^o - 1) H^+$ $-\left(f_s^o \frac{1}{d}\right) C_nH_aO_bN_c + \left(f_s^o \frac{n-c}{d} - \frac{1}{7}\right) CO_2 + \left(f_s^o \frac{c}{d} - \frac{1}{7}\right) HCO_3^- + \left(\frac{3}{7} - \frac{1}{2} f_e^o - f_s^o \frac{2n-b+c}{d}\right) H_2O$

$C_nH_aO_bN_c$ is the general empirical chemical formula of cells, where the coefficients n, a, b and c are the molar relationship between the elements: carbon, hydrogen, oxygen and nitrogen, respectively. Also, $d = (4n + a - 2b - 3c)$. fe^o and fs^o are the portion of electrons for coupling energy between catabolic and anabolic process according to TEEM2 [22, 74]. NH_4^+ is the N-source for biomass synthesis. When the coefficient is evaluated if the result is positive indicates "reaction reactant" and if is negative indicates "reaction product".

Table 3. Microbial Metabolic Reactions for individual mass degradation to reduce cytotoxic products NO or N_2O in anaerobic phase according to TEEM2 [22, 74].

NO	$\left(\frac{1}{d}\right) C_n H_a O_b N_c + (1)NO - \left(\frac{1}{2}\right) N_2O - \left(\frac{c}{d}\right) NH_4^+ - \left(\frac{n-c}{d}\right) CO_2 - \left(\frac{c}{d}\right) HCO_3^- + \left(\frac{2n-b+c}{d} - \frac{1}{2}\right) H_2O$
N_2O	$\left(\frac{1}{d}\right) C_n H_a O_b N_c + \left(\frac{1}{2}\right) N_2O - \left(\frac{1}{2}\right) N_2 - \left(\frac{c}{d}\right) NH_4^+ - \left(\frac{n-c}{d}\right) CO_2 - \left(\frac{c}{d}\right) HCO_3^- + \left(\frac{2n-b+c}{d} - \frac{1}{2}\right) H_2O$

$C_nH_aO_bN_c$ is the general empirical chemical formula of cells, where the coefficients n, a, b and c are the molar relationship between the elements: carbon, hydrogen, oxygen and nitrogen, respectively. Also, $d = (4n + a - 2b - 3c)$. When the coefficient is evaluated if the result is positive indicates "reaction reactant" and if is negative indicates "reaction product".

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Table 4. Microbial metabolic reactions that represent aerobic[(a)] and anaerobic[(b)] pathways for the denitrifying bacterium *Paracoccus denitrificans* for individual-mass synthesis using different values of energy-transfer-efficiency (ε) according to TEEM2 [74] used for test INDISIM-Denitrification model [46].

I[(a)]	$(C_4H_4O_4)^{2-} + 0.66\ NH_4^+ + 0.79\ O_2 =$ $0.89\ C_3H_{5,4}O_{1,45}N_{0,75} + 0.01\ CO_2 + 1.34\ HCO_3^- + 0.27\ H_2O$	ε=0.84
II[(a)]	$(C_4H_4O_4)^{2-} + 0.08\ NH_4^+ + 0.52\ NO_3^- + 1.05\ H^+ + 0.18\ H_2O =$ $0.80\ C_3H_{5,4}O_{1,45}N_{0,75} + 0.20\ CO_2 + 1.4\ HCO_3^-$	ε=0.90
III[(b)]	$(C_4H_4O_4)^{2-} + 0.30\ NH_4^+ + 4.56\ NO_3^- =$ $4.56\ NO_2^- + 0.4\ C_3H_{5,4}O_{1,45}N_{0,75} + 1.10\ CO_2 + 1.70\ HCO_3^- + 0.67\ H_2O$	ε=0.41
IV[(b)]	$(C_4H_4O_4)^{2-} + 0.57\ NH_4^+ + 4.67\ NO_2^- + 4.67\ H^+ =$ $4.67\ NO + 0.76\ C_3H_{5,4}O_{1,45}N_{0,75} + 0.30\ CO_2 + 1.43\ HCO_3^- + 2.71\ H_2O$	ε=0.84
V[(b)]	$(C_4H_4O_4)^{2-} + 0.58\ NH_4^+ + 4.60\ NO =$ $2.30\ N_2O + 0.77\ C_3H_{5,4}O_{1,45}N_{0,75} + 0.27\ CO_2 + 1.42\ HCO_3^- + 0.37\ H_2O$	ε=0.56
VI[(b)]	$(C_4H_4O_4)^{2-} + 0.58\ NH_4^+ + 2.29\ N_2O =$ $2.29\ N_2 + 0.77\ C_3H_{5,4}O_{1,45}N_{0,75} + 0.27\ CO_2 + 1.42\ HCO_3^- + 0.37\ H_2O$	ε=0.53

I[(a)] represents the pathway: Aerobic respiration, II[(a)] represents the pathway: Nitrate Reduction - Dissimilatory in aerobic phase, and gathering the reactions III[(b)], IV[(b)], V[(b)] and VI[(b)] the pathway: Nitrate Reduction – Denitrification, all of them are represented according to Caspi et al., (2012); Knowles, (1982) and Zumft, (1997).

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Table 5. Microbial metabolic reactions for individual mass degradation to reduce cytotoxic products NO and/or N_2O in anaerobic phase. For the denitrifying bacterium *Paracoccus denitrificans*, used for test INDISIM-Denitrification model [46].

NO	$C_3H_{5,4}O_{1,45}N_{0,75} + 12.25\ NO =$ $6.125\ N_2O + 0.75\ NH_4^+ + 2.25\ CO_2 + 0.75\ HCO_3^- + 0.825\ H_2O$
N_2O	$C_3H_{5,4}O_{1,45}N_{0,75} + 6.125\ N_2O =$ $6.125\ N_2 + 0.75\ NH_4^+ + 2.25\ CO_2 + 0.75\ HCO_3^- + 0.825\ H_2O$

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Table 6. Microbial metabolic reactions that represent aerobic[(a)] and anaerobic[(b)] pathways for the denitrifying bacterium *Achromobacter xyloxidans*, for individual-mass synthesis using different values of energy-transfer-efficiency (ε) according to TEEM2 [74] used for test INDISIM-Denitrification model [46].

I[(a)]	$(C_4H_4O_4)^{2-} + 0.50\ NH_4^+ + 0.89\ O_2 =$ $0.50\ C_5H_9O_{2.5}N + 0.01\ CO_2 + 1.50\ HCO_3^- + 0.01\ H_2O$	ε = 0.76
II[(a)]	$(C_4H_4O_4)^{2-} + 0.52\ H_2O + 0.77\ NO_3^- + 1.54\ H^+ =$ $0.37\ C_5H_9O_{2.5}N + 0.51\ CO_2 + 1.63\ HCO_3^- + 0.40\ NH_4^+$	ε = 0.65
III[(b)]	$(C_4H_4O_4)^{2-} + 0.24\ NH_4^+ + 4.49\ NO_3^- =$ $4.49\ NO_2^- + 0.24\ C_5H_9O_{2.5}N + 1.05\ CO_2 + 1.76\ HCO_3^- + 0.52\ H_2O$	ε = 0.41
IV[(b)]	$(C_4H_4O_4)^{2-} + 0.45\ NH_4^+ + 4.54\ NO_2^- + 4.54\ H^+ =$ $4.54\ NO + 0.45\ C_5H_9O_{2.5}N + 0.20\ CO_2 + 1.55\ HCO_3^- + 2.37\ H_2O$	ε = 0.84
V[(b)]	$(C_4H_4O_4)^{2-} + 0.50\ NH_4^+ + 3.53\ NO =$ $1.77\ N_2O + 0.50\ C_5H_9O_{2.5}N + 0.01\ CO_2 + 1.50\ HCO_3^- + 0.003\ H_2O$	ε = 0.66
VI[(b)]	$(C_4H_4O_4)^{2-} + 0.24\ NH_4^+ + 4.50\ N_2O =$ $4.50\ N_2 + 0.24\ C_5H_9O_{2.5}N + 1.05\ CO_2 + 1.76\ HCO_3^- + 0.52\ H_2O$	ε = 0.27

I[(a)] represents the pathway: Aerobic respiration, II[(a)] represents the pathway: Nitrate Reduction - Dissimilatory in aerobic phase, and gathering the reactions III[(b)], IV[(b)], V[(b)] and VI[(b)] the pathway: Nitrate Reduction – Denitrification, all of them are represented according to Caspi et al., (2012); Knowles, (1982) and Zumft, (1997).

Table 7. Microbial metabolic reactions for individual mass degradation to reduce cytotoxic products NO and/or N_2O in anaerobic phase. For the denitrifying bacterium *Achromobacter xyloxidans*, used for test INDISIM-Denitrification model [46].

NO	$C_5H_9O_{2.5}N + 21\ NO = 10.5\ N_2O + NH_4^+ + 4\ CO_2 + HCO_3^- + 2\ H_2O$
N_2O	$C_5H_9O_{2.5}N + 10.5\ N_2O = 10.5\ N_2 + NH_4^+ + 4\ CO_2 + HCO_3^- + 2\ H_2O$

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Table 8. Uptake-rate (u_i) parameter´s values with units ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1}) for the sensitivity analysis of the uptake-rate (u_i) parameter for denitrifying bacterium *P. denitrificans* used for test INDISIM-Denitrification model [46].

Nutrient	Uptake-rate ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1})		
	Testing values		
	Low (L)	Medium (M)	High (H)
Succinate	0.051	0.102	0.204 [a]
Ammonium	------	------	0.105 [a]
Oxygen	------	------	0.125 [a]
Nitrate-a (aerobic)	0.000911	0.00911	0.0911 [a]
Nitrate-x (anaerobic)	0.00398	0.0398	0.398 [a,b]
Nitrite	0.00214	0.0214	0.214 [a,b]
Nitric Oxide	0.00209	0.0209	0.209 [a,b]
Nitrous Oxide	0.00104	0.0104	0.104 [a,b]

The values (a) are the result of performing calculations between the maximum growth rate (μ_{max} = 0.418 h^{-1}, van Verseveld et al., 1983) and the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table 4). The values (b) are the result of dividing each high uptake-rate by 4 due to the maximum growth rate being achieved when the four reactions (III[(b)], IV[(b)], V[(b)] and VI[(b)]) are carried out by the bacterium.

Table 9. Uptake-rate (u_i) parameter´s values with units ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1}) used in the sensitivity analysis of this parameter for denitrifying bacterium *A. xylosoxidans* used for test INDISIM-Denitrification model [46].

Nutrient	Uptake-rate ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1})		
	Testing values		
	Low (L)	Medium (M)	High (H)
Succinate	0.036	0.072	0.144 [a]
Ammonium	------	------	0.050 [a]
Oxygen	------	------	0.089 [a]
Nitrate-a (aerobic)	0.001031	0.01031	0.1031 [a]
Nitrate-x (anaerobic)	0.00235	0.0235	0.235 [a,b]
Nitrite	0.00126	0.0126	0.126 [a,b]
Nitric Oxide	0.00089	0.0089	0.089 [a,b]
Nitrous Oxide	0.00236	0.0236	0.236 [a,b]

The values (a) are the result of performing calculations between the maximum growth rate (μ_{max} = 0.250 h^{-1}, Nielsen et al., 2006) and the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table 6). The values (b) are the result of dividing each high uptake-rate by 4 due to the maximum growth rate being achieved when the four reactions (III[(b)], IV[(b)], V[(b)] and VI[(b)]) are carried out by the bacterium.

Table 10. Values used in the sensitivity analysis performed with the parameter for cellular maintenance and individual mass degradation coefficient, for two denitrifying bacteria (*P. denitrificans* and *A. xylosoxidans*) used for test INDISIM-Denitrification model [46].

Cellular maintenance ($gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$)	$2.0x10^{-3}$ [(a)]	$4.0x10^{-3}$	$2.0x10^{-2}$	$4.0x10^{-2}$
Mass degradation (h^{-1})	$2.2x10^{-2}$ [(a)]	$4.0x10^{-2}$	$6.0x10^{-2}$	$8.5x10^{-2}$

[(a)] Reference value obtained from initial model calibration.

Table 11. INDISIM-Denitrification model parameters values for *P. denitrificans* [46].

Nutrient	Culture medium initial concentration [mM] Felgate et al. (2012)	Availability coefficient – a_i (h^{-1}) fixed according to Dab	Uptake-rate – u_i – ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1})
Succinate	5 [c] – 20 [d]	0.28 [a,b]	0.204 [a,b]
Ammonium	10 [c,d]	0.84 [a,b]	0.105 [a,b]
Oxygen	0.236 [c,d]	0.79 [a,b]	0.125 [a]
Nitrate-a (aerobic)	4.9983 [d] – 21.6095 [c]	0.63 [a,b]	0.00911 [a]
Nitrate-x (anaerobic)			0.039 [b]
Nitrite	0.0255 [c] – 0.0112 [d]	0.78 [a,b]	0.214 [b]
Nitric Oxide	------	1.00 [a,b]	0.209 [b]
Nitrous Oxide	0.003 [c] – 0.000028 [d]	0.50 [a,b]	0.104 [b]

Other bacterial parameters		
Parameter	**Calibrated value**	**Reference**
Cellular maintenance coefficient (gC_{donor}·gC_{mic}^{-1}·h^{-1})	0.0020 [a,b]	Gras et al. (2011)
Mass degradation coefficient (h^{-1})	0.022	Calibrated
Mass split	0.50 (15% coefficient of variation)	Derived from [66]
Small bacterium size (μm)	0.5 [a,b]	Holt et al. (1994)
Big bacterium size (μm)	0.9 [a,b]	
Minimum bacterium size at reproduction	75% of big bacterium size (15% coefficient of variation)	Derived from [68] and [66]

Phase: (a) Aerobic, (b) Anaerobic. Experiment: (c) Succinate-limited/NO_3^--sufficient, (d) Succinate-sufficient/NO_3^--limited.

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Table 12. INDISIM-Denitrification model parameters values for A xylosoxidans [46].

Nutrient	Culture medium initial concentration [mM] Felgate et al. (2012)	Availability coefficient – a_i (h^{-1}) fixed according to Dab	Uptake-rate – u_i – ($mol_{nutrient}$·C-mol_{mic}^{-1}·h^{-1})
Succinate	5 [c] – 20 [d]	0.28 [a,b]	0.144 [a,b]
Ammonium	10 [c,d]	0.84 [a,b]	0.050 [a,b]
Oxygen	0.236 [c,d]	0.79 [a,b]	0.089 [a]
Nitrate-a (aerobic)	5.1538 [d] – 21.7469 [c]	0.63 [a,b]	0.01031 [a]
Nitrate-x (anaerobic)			0.235 [b]
Nitrite	0.00765 [c] – 0.36863 [d]	0.77 [a,b]	0.00126 [b]
Nitric Oxide	------	1.00 [a,b]	0.0089 [b]
Nitrous Oxide	0.00001818 [c] – 0.00006263 [d]	0.50 [a,b]	0.236 [b]

Other bacterial parameters

Parameter	Calibrated value	Reference
Cellular maintenance coefficient (gC_{donor}·gC_{mic}^{-1}·h^{-1})	0.0020 [a,b]	Gras et al. (2011)
Mass degradation coefficient (h^{-1})	0.085	Calibrated
Mass split	0.50 (15% coefficient of variation)	Derived from [66]
Smallest bacterium size (μm)*	0.63 [a,b]	Holt et al. (1994)
Big Biggest bacterium size (μm)*	1.40 [a,b]	
Minimum bacterium size at reproduction	75% of big bacterium size (15% coefficient of variation)	Derived from [68] and [66]

Phase: (a) Aerobic, (b) Anaerobic. Experiment: (c) Succinate-limited/NO_3^--sufficient, (d) Succinate-sufficient/NO_3^--limited. (*) This size refers to a spherical equivalent diameter.

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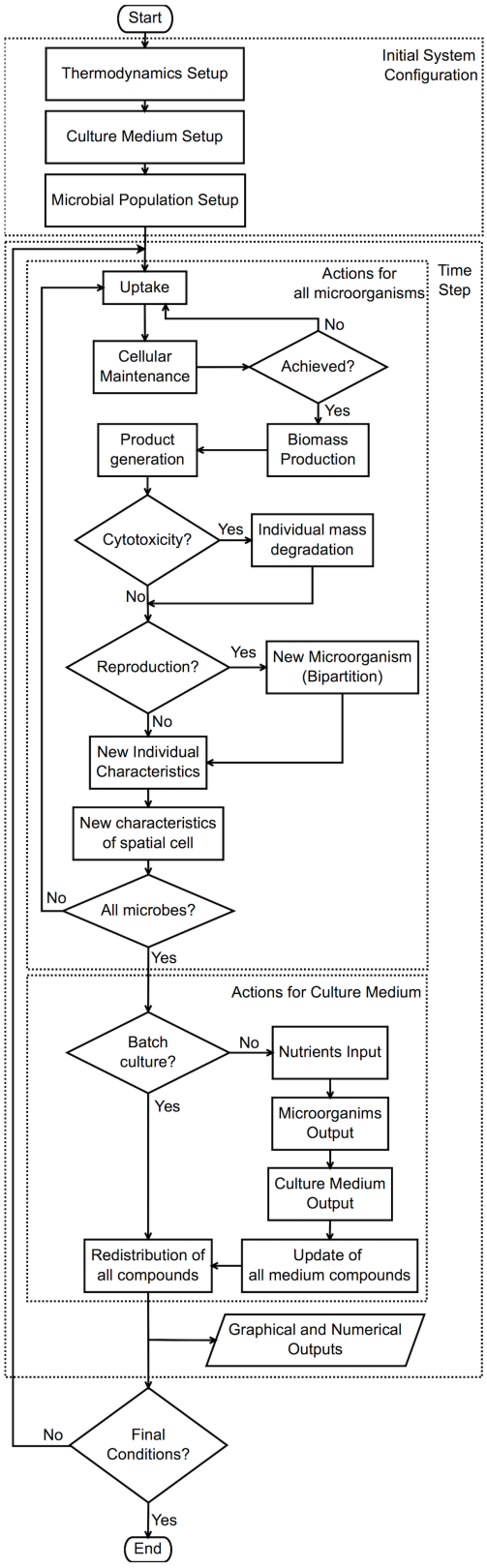

Figure 1. Schematic diagram of the INDISIM-Denitrification model [46].

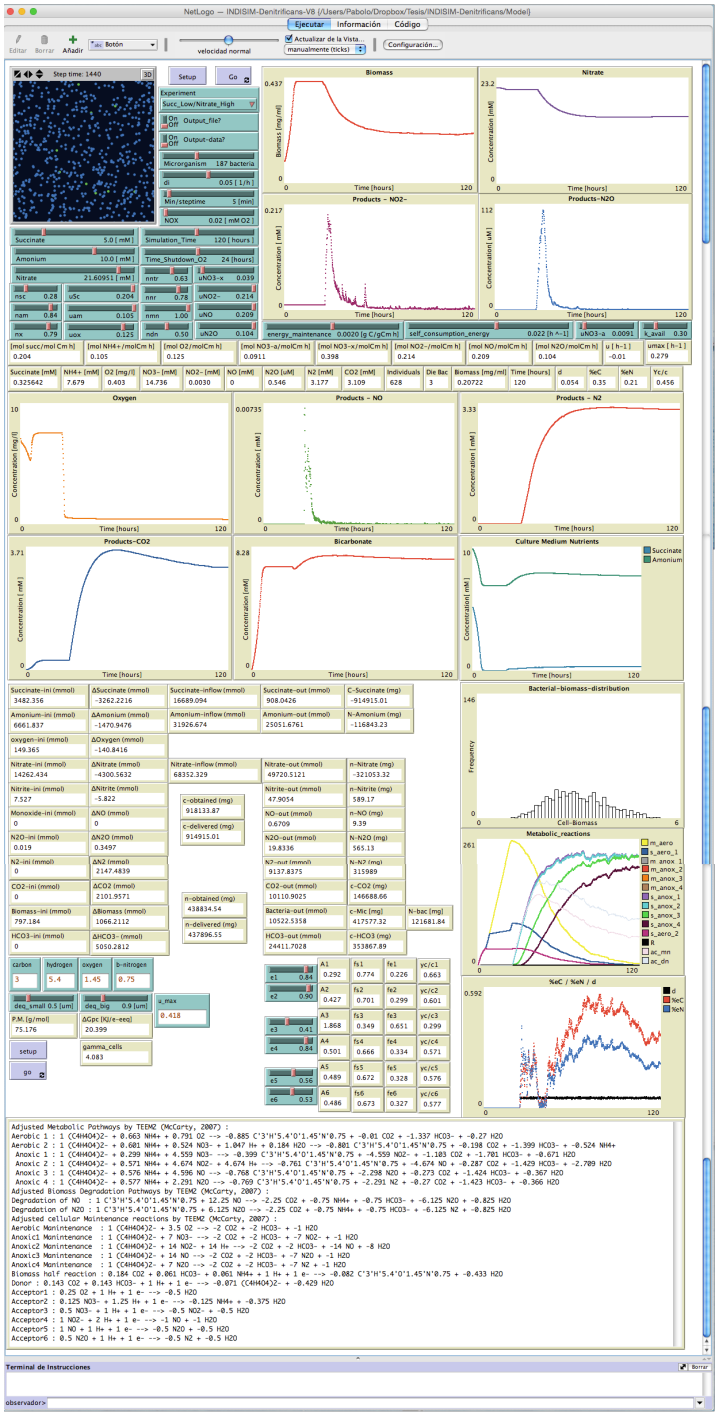

Figure 2. A screenshot of the user interface of INDISIM-Denitrification simulator in NetLogo. The sliders allow initial values, simulated time and the models´ parameters to be varied. Observations are provided with monitors and plots of the modelled compounds over time. Bacteria Mass distributions and the number of times that each metabolic reaction has been used by bacteria are also presented in the simulator interface with TEEM2 results when the C-source is succinate, NH_4^+ is the N-source and the e-acceptors are O_2 and N-oxides using an empirical chemical formula of any denitrifying bacteria.

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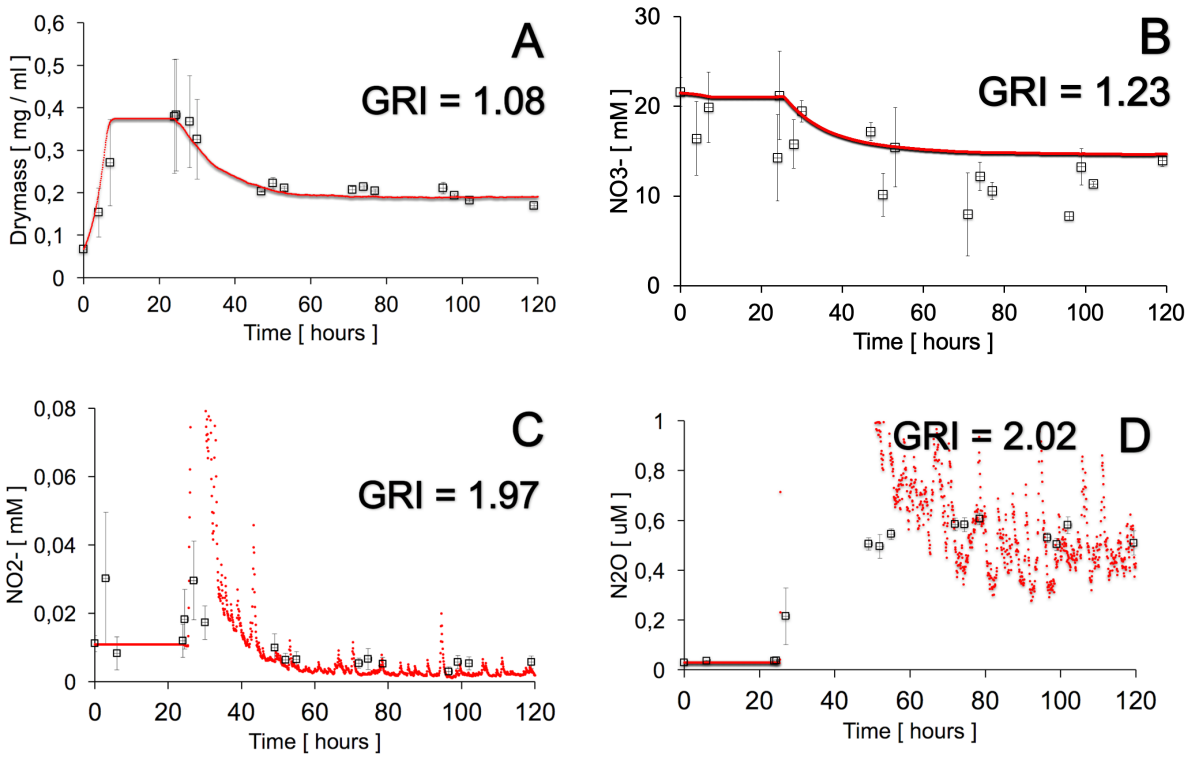

Figure 3. INDISIM-Denitrification simulation results using the empirical chemical formula of *Paracoccus denitrificans* (dots and continuous line) and experimental values (squares) are presented with their standard error [18] for the experiment E1: succinate-limited/NO_3^--sufficient. Temporal evolution of biomass (A), nitrate (B), nitrite (C) and nitrous oxide (D) in aerobic and anaerobic phases. The simulation results are compared with the experimental values through GRI (Geometric Reliability Index).

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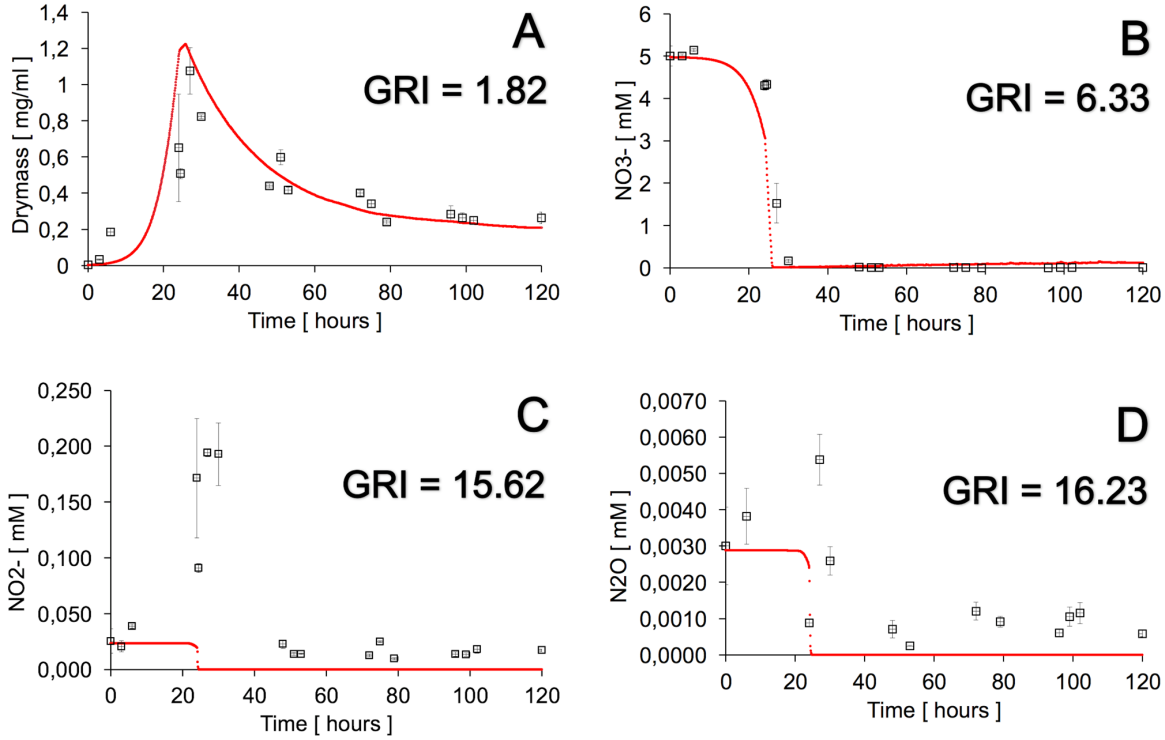

Figure 4. INDISIM-Denitrification simulation results using the empirical chemical formula of *Paracoccus denitrificans* (dots and continuous line) and experimental values (squares) are presented with their standard error [18] for the experiment E2: succinate-sufficient/NO_3^--limited. Temporal evolution of biomass (A), nitrate (B), nitrite (C) and nitrous oxide (D) in aerobic and anaerobic phases. The simulation results are compared with the experimental values through GRI (Geometric Reliability Index).

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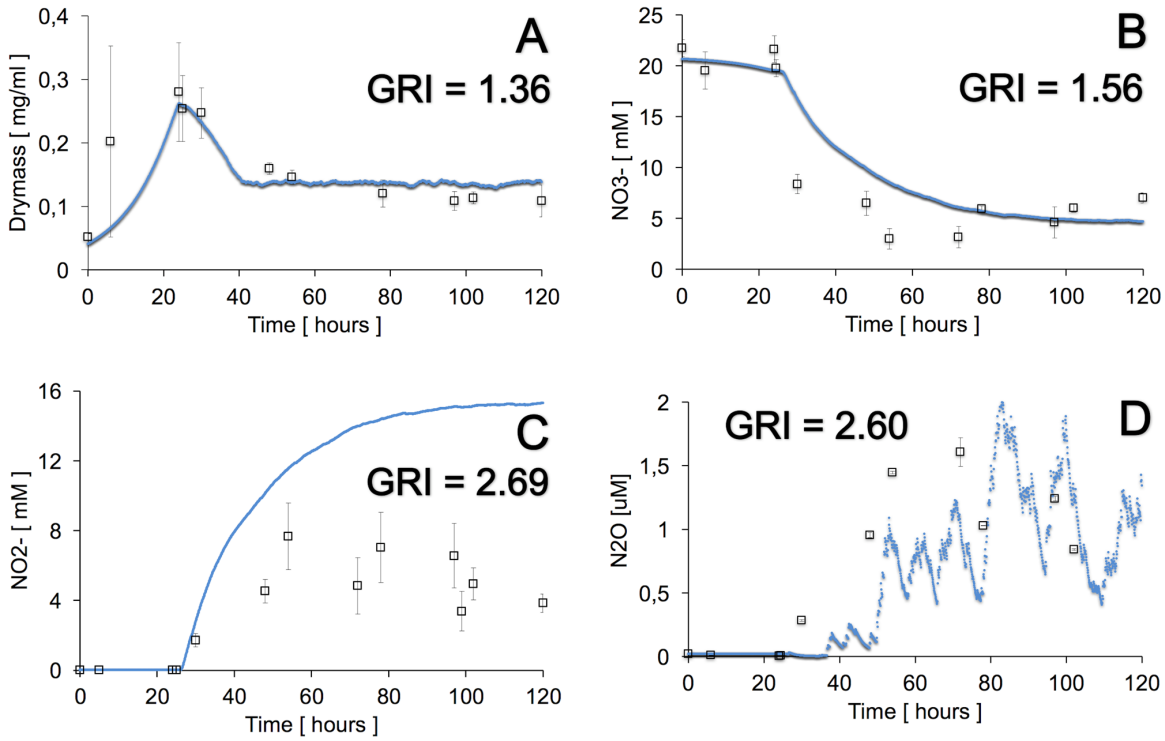

Figure 5. INDISIM-Denitrification simulation results using the empirical chemical formula of *Achromobacter xylosoxidans* (dots and continuous line) and experimental values (squares) are presented with their standard error [18] for the experiment E1: succinate-limited/NO_3^--sufficient. Temporal evolution of biomass (A), nitrate (B), nitrite (C) and nitrous oxide (D) in aerobic and anaerobic phases. The simulation results are compared with the experimental values through GRI (Geometric Reliability Index).

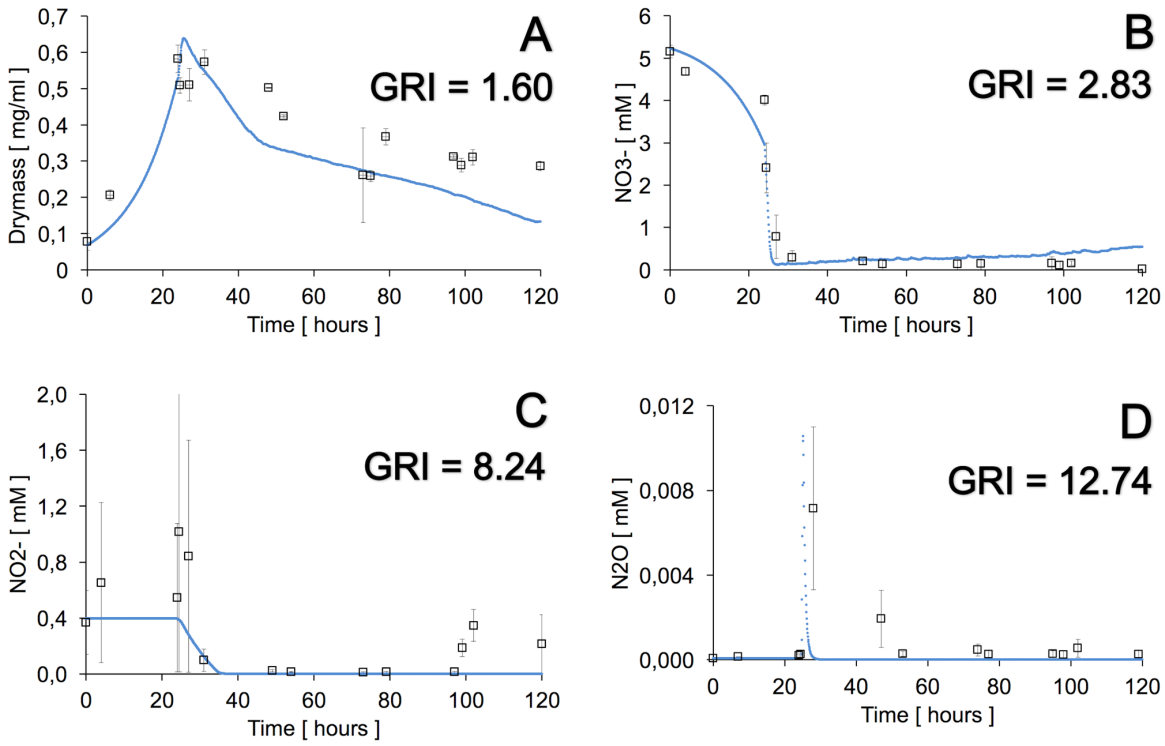

Figure 6. INDISIM-Denitrification simulation results using the empirical chemical formula of *Achromobacter xylosoxidans* (dots and continuous line) and experimental values (squares) are presented with their standard error [18] for the experiment E2: succinate-sufficient/NO_3^--limited. Temporal evolution of biomass (A), nitrate (B), nitrite (C) and nitrous oxide (D) in aerobic and anaerobic phases. The simulation results are compared with the experimental values through GRI (Geometric Reliability Index).

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Supplementary Material

Section I. Cellular maintenance

Before biomass synthesis, each individual in INDISIM-Denitrification model executes a behavior-rule for cellular maintenance. For example to fit the individual behavior-rule for the first reaction in the anaerobic phase we employ an appropriate maintenance requirement for heterotrophic microorganisms of 0.002 gCdonor·gCmicrobial^{-1}·h^{-1} proposed by Gras et al., (2011), and the energy reaction (Re) between succinate and nitrate:

Step 1. Write inorganic and organic half-reactions for e-donor and e-acceptor.

E-donor (succinate) half-reaction (Rd):

$^1/_7\ CO_2 + {}^1/_7\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{14}\ (C_4H_4O_4)^{2-} + {}^3/_7\ H_2O$

E-acceptor (nitrate) half-reaction (Ra):

$½\ NO_3^- + H^+ + e^- \rightarrow ½\ NO_2^- + ½\ H_2O$

Step 2. According to [2] following the equation (Re = Ra – Rd) a balanced stoichiometric equation can be written for this energy reaction as follows.

R_a	$0.50\ NO_3^- + H^+ + e^- \rightarrow 0.50\ NO_2^- + 0.50\ H_2O$
$-R_d$	$0.0714\ (C_4H_4O_4)^{2-} + 0.4285\ H_2O \rightarrow 0.1428\ CO_2 + 0.1428\ HCO_3^- + H^+ + e^-$
Re	$\mathbf{0.0714\ (C_4H_4O_4)^{2-} + 0.50\ NO_3^- \rightarrow 0.50\ NO_2^- + 0.1428\ CO_2 + 0.1428\ HCO_3^- + 0.0715\ H_2O}$

Re is the balanced chemical equation for the energy reaction to fit the individual behavior-rule for aerobic maintenance in INDISIM-Denitrification model.

Step 3. Computation of specific maintenance requirements for the first reaction in anaerobic phase each step time using the elementary cell composition for *P. denitrificans* ($C_3H_{5.4}N_{0.75}O_{1.45}$) proposed by [3, 4].

$$0.002\,\frac{gC_{succinate}}{gC_{microbial}\cdot h} \times \frac{1\ mol\ Succinate}{48gC_{succinate}} \times \frac{36gC_{microbial}}{1\ mol\ Biomass} \times \frac{0.08333\ h}{step\ time} = 0.000125\ \frac{mol\ Succinate}{mol\ Biomass\cdot step\ time}$$

$$0.000125\ \frac{mol\ Succinate}{mol\ Biomass\cdot step\ time} \times \frac{0.50\ mol\ NO_3^-}{0.0714\ mol\ succinate} = 0.000875\ \frac{mol\ NO_3^-}{mol\ Biomass\cdot step\ time}$$

Step 4. Maintenance requirements computation, for the first reaction in anaerobic phase each step, according to the individual mass. Consider an individual who has a diameter of 0.9 µm (individual mass of 6 pmol approximately).

$$0.000125\ \frac{mol\ Succinate}{mol\ Biomass\cdot step\ time} \times\ 6\ pmol\ biomass = 0.00075\ pmol\ Succinate$$

$$0.000875\ \frac{pmol\ NO_3^-}{pmol\ Biomass\cdot step\ time} \times\ 6\ pmol\ biomass = 0.00525\ pmol\ NO_3^-$$

Then the individual compares these quantities to the corresponding uptakes and picks the lowest values to execute the energy reaction. First establish which is the reactant limiting, with this information run the reaction and finally update the

773 corresponding uptakes.

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Section II. Biomass generation

Example of calculations for anaerobic nitric oxide reduction with succinate as e-donor and C-source, ammonium as N-source and nitric oxide as e-acceptor with $e = 0.56$, to fit the individual behavior-rule for biomass generation in INDISIM-Denitrification model for the reaction ($NO \rightarrow N_2O$) in metabolic pathway 3.

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

E-donor (succinate) ½ reaction (R_d):

$^1/_7\, CO_2 + {}^1/_7\, HCO_3^- + H^+ + e^- \rightarrow {}^1/_{14}\, (C_4H_4O_4)^{2-} + {}^3/_7\, H_2O \quad \Delta G_d^o$ (kJ/eeq) = 29.090

E-acceptor (nitric oxide) ½ reaction (R_a):

$H^+ + NO + e^- \rightarrow ½\, N_2O + ½\, H_2O \quad \Delta G_a^o$ (kJ/eeq) = - 115.829

Cell ½ reaction (Rc) with ammonium as N-source:

$^1/_5\, CO_2 + {}^1/_{20}\, NH_4^+ + {}^1/_{20}\, HCO_3^- + H^+ + e^- \rightarrow {}^1/_{20}\, C_5H_7O_2N + {}^9/_{20}\, H_2O \quad \Delta G_{pc}^o$ (kJ/eeq) = 18.80

Step 2. Adjust the cell ½ reaction (Rc) to *P. denitrificans* elementary cell composition $C_3H_{5.4}N_{0.75}O_{1.45}$ (van Verseveld et al., 1979, 1983) following the methodology proposed by Rittmann and McCarty (2001). Where, n = 3, a = 5.4, b = 0.75 and c = 1.45.

$$\left(\frac{n-c}{4n+a-2b-3c}\right) CO_2 + \left(\frac{c}{4n+a-2b-3c}\right) NH_4^+ + \left(\frac{c}{4n+a-2b-3c}\right) HCO_3^- + H^+ + e^-$$
$$\rightarrow \left(\frac{1}{4n+a-2b-3c}\right) C_nH_aO_bN_c + \left(\frac{2n-b+c}{4n+a-2b-3c}\right) H_2O$$

$$^9/_{49}\, CO_2 + {}^3/_{49}\, NH_4^+ + {}^3/_{49}\, HCO_3^- + H^+ + e^- \rightarrow {}^4/_{49}\, C_3H_{5.4}O_{1.45}N_{0.75} + {}^{106}/_{245}\, H_2O$$

$$18.80\ \frac{kJ}{eqq} \times \frac{20\ eqq}{1\ mol\ C_5H_7O_2N} \times \frac{1\ mol\ C_5H_7O_2N}{113.11\ g_{microbial}} \times \frac{75.17\ g_{microbial}}{1\ mol\ C_3H_{5.4}O_{1.45}N_{0.75}} \times \frac{1\ mol\ C_3H_{5.4}O_{1.45}N_{0.75}}{^{49}/_4\ eqq} = 20.398\ \frac{kJ}{eqq}$$

For *P. denitrificans* elementary cell composition the cell ½ reaction (Rc) with ammonium as N-source with Gibb's standard free energy at pH = 7.0 is

$$^9/_{49}\, CO_2 + {}^3/_{49}\, NH_4^+ + {}^3/_{49}\, HCO_3^- + H^+ + e^- \rightarrow {}^4/_{49}\, C_3H_{5,4}O_{1,45}N_{0,75} + {}^{106}/_{245}\, H_2O \qquad \Delta G_{pc}^o \text{ (kJ/eeq)} = 20.398$$

Step 3. Degree of reduction computation for e-donor and cells:

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

$$\gamma_x = \frac{electron\ cells}{Carbon\ cells} = \frac{^{49}/_4}{3} = 4.083$$

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

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

ΔG_{in} = 30.90 kJ/eqq. Since no oxygenase is involved, q = 0. Since succinate is not a C1 compound, ΔG_{fa} = 0 and m = n. Since (ΔG_{in} - ΔG_d) > 0 → (30.9 – 29.09) > 0, n = 1, m = 1. Using e = 0.41, and if standard conditions apply.

$$A = -\frac{\frac{(0 - 29.09)}{0.56^1} + \frac{(30.90 - 0)}{0.56^1} + \frac{20.398}{0.56}}{0.56(-115.829 - 29.09 - 0)} = 0.489$$

$$f_s^0 = \frac{1}{1 + A} = \frac{1}{1 + 0.489} = 0.672$$

$$f_e^o = A \cdot f_s^o = 0.489 \times 0.672 = 0.328$$

$$Y_{C/C} = \frac{\gamma_d}{\gamma_x} f_s^o = \frac{3.5}{4.083} \times 0.672 = 0.576 \left[\frac{mol\ C_{cells}}{mol\ C_{succinate}}\right]$$

Step 5. A balanced stoichiometric equation can then be written. The overall reaction R is equal to R = $fe^oR_a + fs^oR_c - R_d$ according to Rittmann and McCarty (2001) and the coefficients present on Table III for one mole of succinate we can write.

$$\mathbf{R} \quad 1\ (C_4H_4O_4)^{2-} + 0.576\ NH_4^+ + 4.596\ NO \rightarrow 0.768\ C_3H_{5,4}O_{1,45}N_{0,75} + 2.298\ N_2O + 0.273\ CO_2 + 1.424\ HCO_3^- + 0.367\ H_2O$$

R is the balanced chemical equation using the Thermodynamic Electron Equivalents Model second version to fit the individual behavior-rule for biomass generation in INDISIM-Denitrification model for metabolic pathway (NO → N_2O).

Section III. Individual mass degradation to reduce cytotoxic products

To develop the new individual behaviour-rule to reduce the concentration of cytotoxic products (NO and/or N_2O), the individual mass will be used by the bacterium as e-donor when the C-source is a limiting substrate in the media. To obtain this new metabolic process in the context of IBM, the bacterial biomass of each individual diminishes; the biomass half-reaction acts as e-donor and is combined with the e-acceptor half-reaction, and the MMR that represents the individual mass degradation reaction can be written.

For instance, we take the bacterium *P. denitrificans* to show how to write this reaction.

Step 1. Considering the elementary cell composition for *P. denitrificans* ($C_3H_{5.4}N_{0.75}O_{1.45}$) [3, 4], the general biomass half-reaction equation [2] may be written as:

$$^{4}/_{49}\, C_3H_{5.4}O_{1.45}N_{0.75} + {}^{106}/_{245}\, H_2O \rightarrow {}^{9}/_{49}\, CO_2 + {}^{3}/_{49}\, HCO_3^- + {}^{3}/_{49}\, NH_4^+ + H^+ + e$$

This reaction is the e-donor half-reaction (Rd) which considers the individual mass as electron source, breaking it down into CO_2, HCO_3^- and NH_4^+. These electrons will be transferred to the e-acceptor.

Step 2. It is necessary to write the half-reactions for the e-acceptors considered, as follows:

$$NO + H^+ + e^- \rightarrow ½\, N_2O + ½\, H_2O$$

$$½\, N_2O + H^+ + e^- \rightarrow ½\, N_2 + ½\, H_2O$$

Step 3. Following TEEM's methodology a balanced stoichiometric reaction can be written to represent the individual mass degradation to reduce cytotoxic products.

Ra	$NO + H^+ + e^- \rightarrow ½\, N_2O + ½\, H_2O$
– Rd	$^{4}/_{49}\, C_3H_{5.4}O_{1.45}N_{0.75} + {}^{106}/_{245}\, H_2O \rightarrow {}^{9}/_{49}\, CO_2 + {}^{3}/_{49}\, HCO_3^- + {}^{3}/_{49}\, NH_4^+ + H^+ + e^-$
Rg	$^{4}/_{49}\, C_3H_{5.4}O_{1.45}N_{0.75} + NO \rightarrow {}^{9}/_{49}\, CO_2 + {}^{3}/_{49}\, HCO_3^- + ½\, N_2O + {}^{3}/_{49}\, NH_4^+ + {}^{33}/_{490}\, H_2O$

Therefore, Rg is the microbial metabolic reaction using TEEM to represent the individual mass degradation to reduce NO which will be a cytotoxic product.

Step 4. To write Rg in a standard way, we divide all of the stoichiometric coefficients by the e-donor coefficient (biomass). Taking into account this methodology and using different e-acceptors and the empirical cell composition of each bacterium, we can write the individual mass degradation reactions for both.

Section IV. INDISIM-Denitrification model description

To describe our model we use the ODD protocol ("Overview, Design concepts, and Details"), which helps to ensure that the model description is complete [6–8].

1 Purpose

To develop a computational model for the denitrification process carried out by denitrifying bacteria growing in batch and continuous culture, in aerobic and anaerobic growing conditions, and to reproduce a bioreactor experimental protocol. To carry out the sensitivity analyses for the individual uptake parameters, the cellular maintenance, the individual mass degradation coefficient for different culture media compositions.

2 Entities, State Variables, and Scales

The INDISIM-Denitrification model has two types of entities: individuals and square patches of culture medium. Each individual represents a denitrifying bacterium and is identified by a number, its individual variables are: location (XY grid cell coordinates of where it is), mass (molar units), reproduction mass (molar units), internal metabolic product amounts (molar units) and counters for each metabolic reaction and reproduction cycle. To assign the initial mass, the model assumes that each bacterium has spherical shape with a minimum and maximum diameter (μm) which is defined by the user. The individual mass is then deduced from cell volume by assuming the microbial mass density equal to 1.1 $g \cdot cm^{-3}$, which has been used in previous INDISIM models [1].

A two-dimensional lattice of 25x25 grid cells represents the bioreactor that contains the culture medium; each cell represents 1 nl, so that the total bioreactor volume is 625 nl.

Each spatial cell has a position identifier in XY coordinates, and the variables are: total amount (molar units) 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) and normally with all of the fixed parameters one simulation takes 6 or 7 minutes (using a desktop PC) and when you change all of the parameters of the model, for example, in the sensitivity analysis, one run could takes 6-7 hours (using a desktop PC). The model variable outputs are: (a) the concentration of nutrients and metabolic products (mM or μM) and dry biomass ($mg \cdot ml^{-1}$) in a text file, (b) a histogram to show the biomass distribution, (c) a plot to show the frequency of use of each metabolic reaction, (d) all MMRs written using TEEM for any denitrifying bacteria, and (e) numerical values of calculated GRI for four time evolutions: microbial biomass (dry mass), NO_3^-, NO_2^- and N_2O.

3 Process Overview and Scheduling

The initial system configuration has three main aspects to consider: (a) thermodynamics setup, in which the empirical chemical formula of the denitrifying bacterium is required and established by the user. All the MMRs for cellular maintenance, individual-mass synthesis and individual-mass degradation to reduce cytotoxic products following the TEEM methodology are written, (b) culture medium setup; the grid cells setup its values according to the experimental protocols published by Felgate et al. (2012) and considering the experiment (E1 or E2) under study, and (c) microbial population setup; the initial population setup its values considering the Felgate et al. (2012) population values, the bacteria size, which is defined by the user and the empirical chemical formula for the denitrifying bacterium under study.

At each time step all of the individuals are controlled using a set of time-dependent variables for each bacterium. All individuals have the opportunity to perform the following processes: nutrient uptake, cellular maintenance, individual mass synthesis, individual mass degradation to reduce internal cytotoxic products, and bipartition.

Culture medium processes are different depending on the management bioreactor protocol, but in any case, the culture medium is homogenized after some time steps 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 anaerobic conditions, with certain dilution rate that force the input and output of culture media (with nutrients in the input and metabolic products and microorganisms in the output) according to the dilution rate fixed by the user. For each time step the time-dependent variables of individuals and culture medium are recalculated and the state variables changes are immediately assigned generating an asynchronous update, and then the graphics and digital outputs are updated. Figure 1 shows the INDISIM-Denitrification schematic diagram.

4 Design Concepts

4.1 Basic Principles

The individual behavior-rules are: (a) nutrient uptake, (b) cellular maintenance, (c) growth when a microorganism executes any of the metabolic reactions adjusted by TEEM (Table II), (d) individual mass degradation to reduce internal cytotoxic products, and (e) cell division following binary fission. The system actions are those conducted by the general chemostat procedures when it works as a batch culture with constant oxygenation or a continuous culture with a dilution rate.

4.2 Emergence

Model outputs are the result of the interaction between individuals and the culture medium. The model is not forced to reproduce the biomass evolution, nutrient depletion, metabolic and/or denitrification products generation, or other patterns that appear at the system level.

4.3 Adaptation

All the individuals follow the same behavior-rules. 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 is run within a different medium composition. In consequence, the metabolic pathway that it might follow could be different. Before starting the individual actions, it is required to check the O_2 dissolved concentration in the culture medium: if the O_2 dissolved in the spatial cell is lower than a threshold value ($O_{2\text{-MIN}}$) the bacterium uses the anaerobic metabolism and otherwise it uses the aerobic metabolism. After that, the individual can perform cellular maintenance and mass synthesis to growth in aerobic or anaerobic phase. The last metabolic action is to respond to the internal concentrations of cytotoxic gases (NO and/or N_2O). This individual rule is executed only in the anaerobic phase and when the internal amount of the e-donor (C-source); is not enough to execute the next reaction in the denitrification pathway and the internal amount of cytotoxic products (NO and/or N_2O) are accessible in the bacterial cell. Then, the individual can degrade its own mass and reduce it according to the MMRs presented in Table III. At the end of time-step the individual check whether to divide or not, depending on whether or not it has reached the minimum reproduction mass.

NetLogo platform uses an explicit time scheme and a runtime approach that is not naturally parallelizable. Each time step, NetLogo agents act sequentially following a randomly chosen order. Under these circumstances, the most immediate updating scheme is the asynchronous implementation of the model, although a synchronous scheme is also achievable. The asynchronous scheme has been considered as a good approximation of real continuous time [10–12]. The reader should notice that the implementation of the model using a synchronous or asynchronous scheme could lead different model outputs and diverse types of discrepancies with the reality being represented. This should be specifically taken into account if the model is transferred or extended to other modeling frameworks.

4.4 Interaction

The denitrifying bacterium is the only bacteria species in the virtual bioreactor. The microorganisms 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.

4.5 Collective

Simulated microorganisms do not form aggregates; each individual acts uniquely.

4.6 Stochasticity

The reproduction threshold biomass for each bacterium is determined using a normal distribution, which has also been used to generate the initial population biomasses. For the physical separation of the two bacteria the original mass is split into two new bacteria with masses according to a value from normal random distribution. At each time step, each individual nutrient uptake capacity for each nutrient is establish from a normal random distribution with the mean value and a standard deviation of 5% of this value. The dilution rate for each input-output is obtained by using the normal random distribution with mean value $d = 0.05\ h^{-1}$ and standard deviation 0.0025, in order to represent the experimental error. When the individual used the behavior-rule to reduce internal cytotoxic products, we consider that the bacterium could determine the portion of its own biomass that will be degraded to reduce cytotoxic products according to a value from the normal random distribution with mean value given by the mass degradation coefficient, with units (h^{-1}), and standard deviation of 5% of this value. The initial culture medium composition and $O_{2\text{-MIN}}$ threshold value are established from normal distributions with mean values determined by the experimental procedure [9] and standard deviations of 5% of these values. When the simulation starts, each bacterium has a position randomly assigned in culture medium and this position randomly changes at some time steps.

4.7 Observation

The graphical and numerical outputs of the model are (a) the concentration ($mmol \cdot l^{-1}$ or $umol \cdot l^{-1}$) of each culture medium component (succinate, NH_4^+, O_2, NO_3^- CO_2, HCO_3^-, NO_2^-, NO, N_2O and N_2), (b) microbial biomass ($mg \cdot ml^{-1}$), the population mass distribution, (c) a graphical view to show the frequency of use of each metabolic reaction, (d) all MMRs written using TEEM for any denitrifying bacteria, and (e) GRI´s values for four time evolutions: microbial biomass (dry mass), NO_3^-, NO_2^- and N_2O.

At each time step (the user can obtain all simulated data in output file with extension ".txt").

5 Initialization

The user can adjust: (a) the culture medium composition ($mmol \cdot l^{-1}$) of succinate, NH_4^+, O_2 and NO_3^-, (b) $O_{2\text{-}MIN}$ value which is in the range of 0.01 to 0.31 mM O_2, (c) dilution rate (h^{-1}), (d) initial amount of viable microorganisms (bacteria), (e) total simulation time (h), (f) the equivalent step time (min), (g) time (h) for shutdown O_2 input flow, (h) the general maintenance energy requirement ($gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$), (i) the mass degradation coefficient (h^{-1}), (j) sizes for the smallest and biggest microorganism (µm), (k) the coefficients for the molar relationship between the elements carbon, hydrogen, oxygen and nitrogen to establish the empirical chemical formula of the denitrifying bacteria, and (l) the µmax value (h^{-1}) as reference to establish the maximum individual uptake.

6 Individual 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 INDISIM-Denitrification model.

Uptake:

Each nutrient uptake depends on the maximum uptake capacity of the individual to capture nutrients through the cell membrane-associated proteins [13] and on the nutrient availability in the medium. Individual uptake is assumed to be proportional to the individual mass and to the uptake rate (u_i being i the nutrient), which represents the amount of nutrient that could be captured per unit of time, and mass ($mol_{nutrient} \cdot molC_{mic}^{-1} \cdot h^{-1}$).

Following the INDISIM framework (Gras et al., 2011) the maximum population growth rate (μ_{max}) has been used to estimate the maximum individual uptakes. Using this value and performing calculations with the stoichiometric coefficients of each metabolic reaction adjusted by TEEM, we obtained the maximum uptake rate for each nutrient. The nutrient availability (a_i) is the fraction of each nutrient (i) in a spatial cell that is accessible per unit of time (h^{-1}) and for the individual. This parameter is directly related to the nutrient characteristics. In order to give values to this parameter, a_i, we use Fick's law binary diffusion coefficients (Dab) in water as a reference [14]. Therefore, we assumed that the nutrient with maximum Dab has the highest availability; the other availability values are assigned proportionally. To determine the individual nutrient uptake at each time step, each bacterium compares its maximum uptake capacities with the nutrient available and takes the lowest value.

Maintenance:

it is necessary that each bacterium achieve some energetic requirements to ensure its viability. The maintenance requirements are proportional to individual's mass. Gras et al., (2011) consider an appropriate maintenance requirement for soil heterotrophic microorganisms of 0.002 $gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$, which was assumed for aerobic and anaerobic phases, the stoichiometric coefficients are calculated according with the energy reactions (Table I).

When the individual carries out its maintenance, the CO_2 and the reduced e-acceptors are expelled to the culture medium except the NO_2^- which is added to its corresponding intake and being able to be used in the same time step. In anaerobic phase the first individual option is to accomplish the maintenance requirement carrying out the energy reaction with succinate and NO_3^-, if the bacterium cannot reach its maintenance requirements, it can try it with succinate and another e-acceptor, following the reaction sequence shown in Table I.

After the maintenance, if the remaining succinate uptaken and the quantity of some e-acceptors are higher than zero, the individual can perform individual mass synthesis.

Mass synthesis and metabolic products:

With the nutrient intakes updated and after the maintenance, the individual can generate its own mass following the sequence reactions presented in Table II. Using the stoichiometric coefficients of each metabolic reaction (Table II). Each bacterium divides the amount of each nutrient up-taken by its respective stoichiometric coefficient and selects the smallest value (the limiting nutrient). This information provides the demands of each one of the nutrients, the creation of new mass and metabolic products generation. The CO_2 produced is released to the culture medium and the amounts of N-oxides generated are added to its corresponding intakes. After this, if there are remaining amounts of e-donor and some e-acceptor intakes, the microbe can perform the next metabolic reaction.

Mass degradation:

if there are internal quantities of the cytotoxic gases NO and/or N_2O and the C-source quantity is not enough to execute a metabolic reaction, the microbe executes the mass degradation behavior-rule. It first establishes the amount of its mass that will be used to reduce internal cytotoxic products based on the mass degradation coefficient (h^{-1}), and with this quantity established, the cytotoxic product is reduced following the reactions coefficients (Table III), the individual mass is reduced, and the remaining unused intakes are expelled to the medium.

The sub models related to the bioreactor's procedure are:

Agitation*:*

Nutrients and metabolic products are redistributed in the culture medium and microorganism positions change randomly.

Input flow:

The bioreactor is filled with fresh medium (succinate, NH_4^+ and NO_3^-) with a composition equal to the initial one. A dilution rate is defined as a fraction (volume) of the culture media removed and filled by unit of time.

Output flow:

According to the dilution rate a fraction of the media in the bioreactor is removed and the same fraction of individuals are randomly removed.

Supplementary Material - References

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