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Developing an individual-based model to study the bacterial denitrification process

By

Pablo Alejandro Araujo Granda

Supervisors:

Dra *Anna Gras Moreu*
Departament d'Enginyeria Agroalimentària i Biotecnologia

Dra *Marta Ginovart Gisbert*
Departament de Matemàtiques

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Pablo Alejandro Araujo Granda

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Tomad, Señor, y recibid toda mi libertad, mi memoria, mi entendimiento y toda mi voluntad, todo mi haber y mi poseer; Vos me lo distes, a Vos, Señor, lo torno; todo es vuestro, disponed a toda vuestra voluntad; dadme vuestro amor y gracia, que esta me basta.

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Cuenta la leyenda que aquel o aquella persona que beba agua de la Font de Canaletes volverá a Barcelona.

Catalunya m'ha robat el cor. La màgia dels castellers i l'emoció de veure a l'enxaneta coronar la torre són indescriptibles. No és possible viure un hivern sense gaudir d'una deliciosa calçotada en unió de la colla d'amics. I arriba la primavera, i amb ella el fastuós carnaval de Sitges i el festival de flors a Girona és un delit als sentits, tot allò s'enalteix amb l'alegria de veure l'ou ballant en les fonts de Barcelona en dies de Corpus, temps en el qual el Tabal ens convida a Patum. El ritme del ball de gitanes anuncia que a Castellar del Vallès els dies de paella estan pròxims. Màgics dies d'estiu es gaudeixen a la Costa Brava i en totes i cadascuna de les platges catalanes. Els colors de la tardor són màgics en caminar per la muntanya i descobrir antics assentaments ibèrics. És digne d'admiració gaudir del naixement d'un nou dia des del Cap de Creus i viatjant cap a l'interior descobrir pobles que conserven la màgia del medieval, Peratallada, Rupit i Pruit, Besalú, Monells, Castellfollit de la Roca, Pals, Beget, Tossa de Mar, Guimerà són només un exemple. No puc amagar que en cada lloc que he tingut l'honor de conèixer a quedat part de la meva ànima. Seguir la ruta dels mercats medievals per Manresa i Vic acompanyat de les caminades per la muntanya sagrada de Montserrat no té comparació. I el menjar, únic plaer que pots viure, amb una sopa de galets, fuet, llonganissa, bull blanc i bull negre, pa amb tomàquet, coques per Sant Joan, Mandonguilles amb sèpia, recuit de forteta i els panellets, no puc enumerar totes les delícies que he provat, però sense cap dubte el millor de tots i cada un d'ells va ser haver-los compartits amb grans amics. L'espai és curt i encara queda molt per dir, per tant acabo expressant que: !! moltes gràcies Barcelona, tornaria a beure l'aigua de canaletes les vegades que calguin !!.

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En el cuaderno de los Ejercicios Espirituales de san Ignacio de Loyola, en la anotación 234, está escrito: *El primer punto es traer a la memoria los beneficios recibidos de creación, redención y dones particulares, ponderando con mucho afecto cuanto ha hecho Dios nuestro Señor por mí y cuanto me ha dado de lo que tiene, y consequenter el mismo Señor desea dárseme en cuanto puede según su ordenación divina. Y con esto reflectir, en mí mismo, considerando con mucha razón y justicia lo que yo debo de mi parte ofrecer y dar a la su divina majestad, es a saber, todas mis cosas y a mí mismo con ellas, así como quien ofrece afectándose mucho.*

Pablo A.

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Abstract

It is crucial to study the denitrification process driven by bacteria as it is one of the most important environmental processes for several reasons: i) it has an application in the removal of nitrogen (N) from high-N waste materials ii), it is one of the mechanisms to N-fertilizer's loss, iii) it contributes to the emissions of gasses with large global warming potential, and iv) it is the mechanism by which the global nitrogen cycle is balanced.

Many models have been developed in the framework of continuous models to deal with the complexity of the denitrification process in order to become predictive models, but some of the assumptions contained in them are not realistic enough in those contexts, and also they have their own constraints and limitations. On the other hand, the researchers have paid more attention to the role of microbial activity with the advance of experimental techniques.

Discrete models, such as individual-based models (IBMs), can be developed and applied to microbial systems due to the fact that they allow representation of some intracellular characteristics regarding the complexity of the micro-organisms, which constitutes a key advantage of this modelling approach in the study of the different biotechnological processes. The IBM is able to incorporate and accommodate the behaviour of denitrifying bacteria, and investigate their metabolism from different and attractive perspectives.

A key factor in modelling the microbial activity is the methodology followed to represent metabolic pathways. A cellular metabolic model could be based on a non-equilibrium thermodynamic approach such as the Thermodynamic Electron Equivalents Model (TEEM), which is developed for biomass yield prediction using the associated standard Gibbs free energies and the bioenergetics growth efficiency between cell anabolism and catabolism.

The main objective of this doctoral thesis is to develop an IBM to study denitrification processes driven by denitrifying bacteria, using TEEM to write microbial metabolic reactions (MMRs) which represent the metabolic pathways as the center of the individual sub-model.

Two new computational models of the INDISIM family, INDISIM-Paracoccus and INDISIM-Denitrification have been designed, implemented on the NetLogo platform, parameterized and calibrated with experimental data to analyze the system dynamics in a bioreactor in batch and continuous culture with denitrifying bacteria growing in it. The bioreactor conditions can be aerobic and/or anaerobic, and the growing media is liquid medium with an electron donor, C-source, N-source, and oxygen and all N-oxides as electron acceptors.

An open access and open source tool has been developed to write MMRs based on TEEM. It is called MbT-tool which stands for Metabolism-based on Thermodynamics. Using MbT-Tool three sets of MMRs have been written, which are the centre of the individual sub-model for INDISIM-Paracoccus and INDISIM-denitrification, representing reactions involved in: i) cellular maintenance, ii) individual mass synthesis, and iii) individual mass degradation to reduce cytotoxic products.

The simulation results obtained with INDISIM-Paracoccus and INDISIM-Denitrification have been compared with experimental data published by Felgate et al. (2012) regarding the growth of *Paracoccus denitrificans* and *Achromobacter xylosoxidans* in a bioreactor. According to the statistical analysis of the simulations results, for both denitrifying bacteria tested, the IBMs developed show better adjustments in the assays with electron donor limited than in the assays with electron acceptor limited.

The IBM's development and application with some intracellular detail and complexity constitute a key advantage in the investigation and understanding of the different steps of denitrification carried out by denitrifying bacteria.

Resumen

Es relevante estudiar la desnitrificación ya que es un importante proceso medioambiental, debido a que: i) es uno de los mecanismos que explica la pérdida de fertilizantes de nitrógeno (N), ii) tiene aplicación en la remoción de N proveniente de residuos con alto contenido de N, iii) contribuye a las emisiones de gases que presentan gran potencial de calentamiento global, y iv) es el mecanismo por el cual se balancea el ciclo del N.

Varios modelos han sido desarrollados usando el enfoque de la modelización continua para hacer frente a la complejidad del proceso de la desnitrificación, con la finalidad de obtener modelos predictivos, pero algunas de sus suposiciones no son suficientemente reales en este contexto, además estos modelos tienen sus propias restricciones y limitaciones. Por otro lado, los investigadores están prestando más atención al rol de la actividad microbiana, desde que en los últimos años se han desarrollado y avanzado las técnicas experimentales de manera importante.

Modelos discretos, como los modelos-basados en el individuo (IBMs), pueden ser desarrollados y aplicados a sistemas microbianos ya que permiten representar algunas de las características intracelulares relacionadas con la complejidad de los microorganismos, lo cual constituye una ventaja clave de este enfoque de modelización en el estudio de diversos procesos biotecnológicos. El IBM es capaz de incorporar y adaptar el comportamiento de las bacterias desnitrificantes e investigar su metabolismo desde perspectivas distintas. Un factor clave para modelizar la actividad microbiana es la metodología utilizada para representar las rutas metabólicas. Un modelo metabólico celular puede estar basado en la termodinámica del no equilibrio como el Modelo Termodinámico de Electrones Equivalentes (TEEM), el cual está desarrollado para la predicción del rendimiento de la biomasa usando las energías estándar de Gibbs junto con la eficiencia bioenergética de crecimiento entre anabolismo y catabolismo celular.

El objetivo principal de esta tesis doctoral es desarrollar un IBM para estudiar la desnitrificación bacteriana, usando el TEEM para escribir reacciones metabólicas microbianas (MMRs) las cuales representan a las rutas metabólicas y son el centro del sub-modelo individual. Dos nuevos modelos computacionales de la familia INDISIM, el INDISIM-Paracoccus y el INDISIM-Denitrification, han sido diseñados, implementados en la plataforma NetLogo, parametrizados y calibrados con datos experimentales para estudiar la dinámica del crecimiento de las bacterias desnitrificantes dentro de un bioreactor en cultivos cerrados y continuos, en condiciones aerobias o anaerobias. El medio de cultivo es líquido y contiene un donador de electrones, oxígeno y los óxidos de N como aceptores de electrones, una fuente de carbono y una fuente de N. Una herramienta de acceso libre y código abierto ha sido desarrollada para escribir las MMRs basadas en TEEM y es llamada MbT-Tool (Metabolismo basado en la Termodinámica). Utilizando MbT-Tool se pueden escribir tres grupos de MMRs que han sido el centro del sub-modelo individual para INDISIM-Paracoccus e INDISIM-Denitification, y que representan las reacciones involucradas en: i) el mantenimiento celular, ii) la síntesis de masa individual, y iii) la degradación de la masa individual para reducir productos citotóxicos.

Los resultados de simulación obtenidos con INDISIM-Paracoccus e INDISIM-Denitrification han sido comparados los datos experimentales publicados por Felgate et al. (2012) relacionados con el crecimiento de *Paracoccus denitrificans* y *Achromobacter xylosoxidans* dentro de un bioreactor. De acuerdo con el análisis estadístico de los resultados de simulación, los IBMs desarrollados muestran mejores ajustes para los experimentos con donador de electrones limitado que para los ensayos con aceptor de electrones limitado. El desarrollo y aplicación de IBMs con algunos detalles y complejidad intracelular, constituyen una ventaja clave en la investigación y comprensión de los diferentes pasos de la desnitrificación bacteriana.

Extracte

L'estudi del procés de desnitrificació és rellevant, ja que és un important procés mediambiental, a causa que: i) és un dels mecanismes que ocasiona una pèrdua dels fertilitzants de nitrogen (N), ii) és d'utilitat en l'eliminació de N en residus amb un alt contingut en N, iii) contribueix en les emissions de gasos d'alt potencial sobre l'escalfament global, i iv) és el mecanisme pel qual es tanca el cicle del N.

S'han desenvolupat diversos models continus per tractar la complexitat del procés de desnitrificació en sistemes ambientals per tal de que puguin ser models amb capacitat predictiva, però alguns dels supòsits que es fan no són prou realistes i tenen les seves mancances i limitacions. D'altra banda, els investigadors estan posant més atenció en el rol que juga l'activitat microbiana, des de que en els darrers anys s'ha desenvolupat i avançat en tècniques experimentals de manera important.

Models discrets, com els models basats en l'individu (IBMs), poden ser desenvolupats i utilitzats en sistemes microbians ja que permeten la representació d'algunes característiques intracel·lulars atenent a la complexitat dels microorganismes, cosa que resulta clau a l'hora d'abordar aquest nou enfoc per l'estudi de diversos processos biotecnològics. Els IBMs són capaços d'incorporar el comportament de les bactèries desnitrificants i d'investigar el seu metabolisme des de perspectives diferents.

Un factor clau en la modelització de l'activitat microbiana és la metodologia seguida per a representar les rutes metabòliques. Un model de metabolisme cel·lular podria estar basat en l'enfoc de la termodinàmica de no-equilibri, com per exemple el denominat Model Termodinàmic d'Electrons Equivalents (TEEM). El TEEM va ser desenvolupat per predir el rendiment de la biomassa utilitzant les energies lliures estàndards de Gibbs associades i l'eficiència bioenergètica de creixement entre els processos anabòlics i catabòlics de la cèl·lula.

L'objectiu principal d'aquesta tesis doctoral és desenvolupar un IBM per l'estudi dels processos de desnitrificació duts a terme per bactèries desnitrificants, mitjançant l'ús del TEEM per descriure les reaccions metabòliques microbianes (MMRs) que representen les vies metabòliques i són el centre del sub-model individual.

Dos nous models computacionals de la família d'INDISIM, l'INDISIM-Paracoccus i l'INDISIM-Denitrification han estat dissenyats, implementats en la plataforma de NetLogo, i parametritzats i calibrats amb dades experimentals per analitzar la dinàmica d'un sistema format per bactèries desnitrificants en un bioreactor en un cultiu tancat i/o continu, en condicions aeròbies i/o anaeròbies. El medi de cultiu és líquid i conté un donador d'electrons, oxigen i òxids de N com acceptors d'electrons, una font de carboni i una de N.

S'ha creat una eina d'accés i codi obert per escriure les MMRs basades en el TEEM i és anomenada MbT-tool (Metabolisme basat en la Termodinàmica). Utilitzant MbT-tool es poden descriure tres grups de MMRs, que seran el centre del sub-model individual per l'INDISIM-Paracoccus i l'INDISIM-denitrification, i que són reaccions involucrades en: i) el manteniment cel·lular, ii) la síntesi individual de massa, i iii) la degradació individual de massa per reduir els productes citotòxics.

Els resultats de simulació obtinguts amb INDISIM-Paracoccus i INDISIM-Denitrification han estat comparats amb les dades experimentals publicades per Felgate et al. (2012), sobre el creixement de *Paracoccus denitrificans* i *Achromobacter xylosoxidans* en un bioreactor. Segons l'anàlisi estadístic dels resultats, per les dues bactèries desnitrificants amb les quals s'ha testat, els IBMs desenvolupats mostren millors ajustos en els assajos amb donadors d'electrons limitants que en els assajos amb acceptors d'electrons limitants.

El desenvolupament d'un IBM i la seva aplicació amb cert nivell de detall i complexitat intracel·lular constitueix una avantatge clau per la investigació i la comprensió dels diferents passos de la desnitrificació bacterià.

Contents

Chapter I – Introduction

For the past several decades, many scientists have experienced great interest in crossing the frontiers between different academic and technological disciplines such as mathematics, physics, biology, chemistry, thermodynamics and modelling, among others. At present these interdisciplinary domains still have many unexplored areas that could provide fruitful and exciting results and more so when innovative approaches are conjugated.

This thesis gathers together four years of research carried out by a chemical engineer in the framework of a bio-thermodynamic, microbiology and individual-based modelling approach. 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.

In this chapter, we present first an introduction to bacterial denitrification, followed by general ideas in regard to non-equilibrium thermodynamics applied to microbial systems and ending with the aims statement and outline of this thesis, along with a background of our research group.

1.1 Bacterial denitrification

Denitrification is one of the key processes of the global nitrogen (N) cycle driven by bacteria (Blackburn, 1990; Zumft, 1997). It has been widely known for more than one hundred years as a process by which the biogeochemical nitrogen cycle is balanced (Figure 1.1). Briefly, we could define it as the dissimilatory reduction of nitrate (NO_3^-) to (mainly) dinitrogen gas (N_2) driven by bacteria (Caspi et al., 2012; Knowles, 1982).

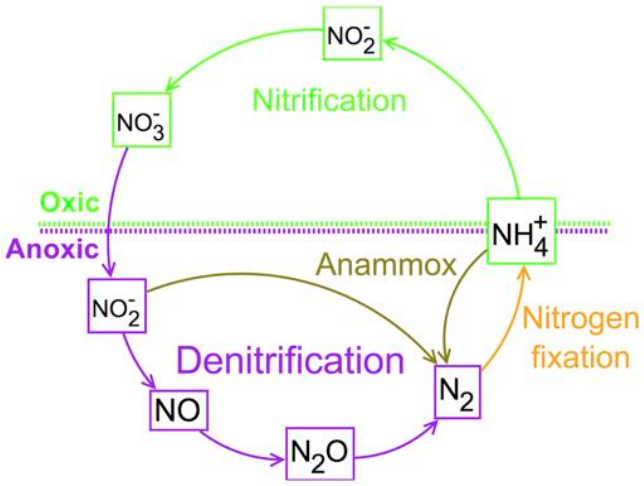

Figure 1.1 Representation of the global nitrogen cycle.

In conditions of low oxygen (O_2) availability, such as waterlogged soils, certain bacteria are able to use NO_3^- as a final electron acceptor (e-acceptor) and carry out respiratory metabolism in anaerobic conditions (denitrification). These bacteria are known as heterotrophic denitrifying bacteria and are widespread in agricultural soils (Felgate et al., 2012; Richardson et al., 2009).

They are facultative aerobes and the most common genera are *Pseudomonas, Achomobacter (Alcaligenes), Paracoccus, Thiobacillus, Bacillus*, *Halobacterium*, *Chromobacterium, Hyphomicrobium* and some species of *Moraxella*. They are commonly found in soils, sediments, surface and ground waters, and wastewater treatment plants (Holt et al., 1994; Knowles, 1982; Rittmann and McCarty, 2001).

Denitrifying bacteria are able to use N-oxides as a respiratory substrate (e-acceptors) in place of O_2 when it becomes limited. For instance, NO_3^-, nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) all become e-acceptors in anaerobic conditions. A complete denitrification pathway (Figure 1.1) is defined as the assemblage of four subsequent reactions: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$ (Berks et al., 1995; Rittmann and McCarty, 2001).

Denitrification has been investigated at many levels, ranging from gene expression to global nitrogen flux (Bergaust et al., 2012; Davidson et al., 1991). Some denitrification models have been reviewed by Heinen (2006). Most of them incorporate a large number of parameters including NO_3^- concentration, soil moisture, soil temperature and pH. The simplest models are obtained by adjusting empirical functions to the experimental results used for their studies. More recently, Kampschreur et al. (2012) and Woolfenden et al. (2013) published denitrification models describing the process carried out by microbes in terms of a set of differential equations according to Monod and Michaelis Menten kinetics.

One of the reasons for studying denitrification is that it contributes to NO and/or N_2O emissions when denitrifying bacteria do not complete the metabolic pathway implicated (Davidson et al., 1991; Snyder et al., 2009). A wide range of factors control this complex regulatory network carried out by denitrifying bacteria, one of which is the low concentration of electron donors (usually carbon (C) sources), which limits the supply of electrons to drive the reductive reactions.

Therefore, the electron flows over the successive denitrification steps are lopsided, and this can cause accumulation of the denitrification intermediates (Baumann et al., 1996). Another factor is the low concentration of metals as iron (Fe) or copper (Cu), which causes some denitrifying enzyme not to be expressed, e.g. nitrite reductase (Nir), which is solely dependent on Fe as a cofactor in the bacterium *Paracoccus denitrificans* or is solely dependent on Cu in the case of *Achromobacter xylosoxidans* (Felgate et al., 2012; Streminska et al., 2012).

In agricultural soils, N_2O emissions are of great importance due to the large amount of N-fertilizer in crops and soil organic matter mineralization, which depends on the conditions the microorganism encounters in its surrounding environment (Snyder et al., 2009; Woolfenden et al., 2013). Environmental conditions that have a dominant influence on N_2O emissions are: water filled pore space, temperature, soluble C availability, and when available soil N, especially in NO_3^- form, exceeds crop uptake, so that the risk of increased N_2O emissions rises (Richardson et al., 2009; Snyder et al., 2009; Streminska et al., 2012).

The global warming potential of N_2O is 296 times greater than a unit of carbon dioxide (CO_2) (Richardson et al., 2009). An excessive NO emission from soil to the atmosphere is undesirable because it promotes an increase of tropospheric ozone, a greenhouse gas, and the N_2O is a potent greenhouse gas and air pollutant due to its dominating the decrease of the ozone layer (Davidson et al., 2000).

Furthermore, these gases show microbial cytotoxic properties, NO as a free-radical and oxidant, while N_2O binds and inactivates vitamin B12, an essential cellular cofactor in B12-dependent enzymes involved in DNA synthesis, provoking loss of cell division and cell viability (Sullivan et al., 2013). Consequently, to identify the environmental factors that control N_2O and/or NO production and consumption by microbes is essential to progress in

the formulation of the strategies to reduce NO and/or N_2O emissions (Davidson et al., 2000; Farquharson and Baldock, 2007; Felgate et al., 2012; Richardson et al., 2009).

To understand and control how the environmental factors control the denitrification process it is convenient to investigate the bacterial denitrification dynamics in a controlled environment such as a bioreactor (Baker et al., 1998; Baumann et al., 1996; Felgate et al., 2012; Richardson et al., 2009).

1.2 Modelling approaches

Modelling is the process of building a theoretical scheme, usually in mathematical form, which is made in order to understand, study and simulate a process or a concept, commonly with the aid of a computer (Dictionary, 2016). In the case of mathematical modelling, this scheme is made using mathematical and statistical tools. Before starting a modelling project, and according to this definition, the first thing to be aware of is that a model is not a faithful reproduction of the reality: a model is an abstraction of it, a simplification of a system, which is an idealized representation of the real world.

Models are sometimes criticized for being unrealistic because they do not represent in detail the real environment so they are not able to accurately reproduce the natural or laboratory systems evolving in different physical, chemical or biological conditions.

One remarkable difference compared with experimental work is that scientists try to explain a fact under experimental conditions or from direct observation, while models do not investigate the nature of the phenomenon; they investigate the validity of our thoughts, in essence, if the logic behind an argument is correct. Somehow we can see the models as aids to human thinking, due to the fact that our brain suffers simple constraints and is not able to consider all the possibilities of a complicated argument in a balanced manner (Kokko, 2007).

The art of modelling lies in recognizing those aspects of the reality that can be sacrificed and those, which are crucial to maintain. Often we may be tempted to take into account all known aspects of the process or system to be modelled, instead of taking into account those that are really relevant, with the thought that having a model with more details of the reality will be more useful.

To avoid such temptation we have to follow a simple rule: the model will have sufficient complexity to prove or disprove the aspects of the system that will be modelled (Kokko, 2007; Railsback and Grimm, 2012). Once the model is developed and contrasted with the real system, it may be of interest to incorporate new aspects to test different mechanisms or to extend it (Reuter et al., 2011).

The modelling approach traditionally used in biological fields, such as in ecology, is an approach to understanding population level, where the population parameters are time-dependent and modified directly using the model's equations (Railsback and Grimm, 2012). Models built at a population level of description are a particular type of System-based Model (SBM) (Cardona et al., 2010).

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 behaviour 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 (Ferrer et al., 2008).

One of the best-known examples of this kind of approach is the modelling of systems of predator-prey (Lotka-Volterra model - LVM), which studies population dynamics of interacting species. These models have been considered as the basis for understanding

ecological processes, and a number of studies have been done on them. In a few words, we could say that LVM uses a system of first order non-linear differential equations, which are time dependent, to represent the changes in the population densities of predators and prey.

Therefore, LVM parameters specifying the dynamical system are given in terms of groups and not in terms of individuals (Matsuda et al., 1992). In addition to the system of differential equations that attempt to describe the change rate of population densities over time, it is also necessary to consider a set of parameters that determine: i) how fast prey species breed, ii) how many prey are eaten by the predators taking into account the density of predators and prey, iii) how efficiently the consumed prey become the offspring of predators, and iv) how quickly the predators die when food is limited. Predator-prey models can show qualitatively good behaviour, but may be considered only as conceptual models because the parameters used are unrealistic or not connected directly with the real system (Kokko, 2007; Matsuda et al., 1992; Railsback and Grimm, 2012).

In the case of microbial systems, the population level models deal with population variables and fix a set of governing laws (equations) which are based on, or at least consistent with, an assemblage of assumptions about the individual behaviour of microbes. Population models are often built for predictive purposes and by means of phenomenological laws for the behaviour of the whole system.

Some of the applications of these models are predictive microbiology in food and control of fermentation processes (Garcia-Ochoa et al., 2010), optimization of microbial cultures and antibiotics production in the pharmaceutical industry (Raaijmakers et al., 2002), waste control and water treatment (Henze et al., 2006), or the study of microbial ecology and evolution of population diversity in wild and artificial ecosystems (Lu et al., 2014). Population models are based on assumptions about the individual behaviour of microbes, and they therefore also raise new questions regarding microbial physiology and cellular models (Resat et al., 2012).

Alternatively, it is possible to simulate the interactions of autonomous agents (individual and collective entities) and their environment, using agent-based models (ABMs) or, more specifically, individual-based models (IBMs).

Grimm (1999) defines IBMs as simulation models that treat individuals as unique and discrete entities, which have at least one property in addition to age that changes during the life cycle. IBMs have been used in ecology since 1970s.

For instance, using an IBM for the study of a prey-predator system, we approach this problem from a different angle; we are concerned primarily with specifying the behaviour of individuals. The relevant question here is, what kinds of actions must an individual predator or individual prey follows so that populations of such individuals will exhibit the well-known characteristic oscillations? Which would be the rules that each organism might follow in order for the given population-level patterns to occur?

A plausible and very simple formulation could be to consider predators and prey as wandering randomly around the landscape, while the predators look for prey to prey on. Each time step costs the predators energy, and they must eat prey in order to replenish their energy - when they run out of energy they die. To allow the population to continue, each predator or prey has a fixed probability of reproducing at each time step depending on their own internal characteristics. The prey must eat grass from their surroundings in order to maintain their energy - when they run out of energy they die. Once grass is eaten it will only regrow after a fixed or random amount of time (Wilensky and Reisman, 2006).

During the last fifteen years IBMs have also come to be used in microbiology. Some of the reasons are: i) IBMs have the ability to incorporate both lower and higher levels of organization into the individual-based framework, ii) IBMs incorporate models of intracellular dynamics, which define the behaviour of the individual cell more

mechanistically, rather than being based on phenomenological descriptions of the cell's behaviour, iii) using IBMs is straight forward as a way of modelling diverse communities comprising potentially many different species, and iv) indirect interactions between individuals of different species (e.g., mediated by diffusible molecules, such as nutrients, toxins, or chemical signals) are an emergent property of the simulation (Hellweger et al., 2016; Hellweger and Bucci, 2009; Kreft et al., 2013).

1.3 Microbial thermodynamics

For building mathematical models of microorganisms, it is necessary to describe physical and biochemical constraints, a set of metabolic reactions (pathways) carried out by them, their interactions and their responses to environmental disturbances (Grimm, 1999; Railsback and Grimm, 2012).

A robust modelling of any microbial metabolism must include chemical conservation principles, through stoichiometric approaches, for quantifying substrate utilization, product formation, and biomass generation, and link this mass balance to energy conservation laws, i.e. to the first principle of thermodynamics and to the constraints imposed by the second law of thermodynamics (Toure and Dussap, 2016).

Most biochemical processes involve a near-equilibrium reaction, which can be assessed with a non-equilibrium thermodynamic approach. This approach aims at showing a global overview of microbial metabolism taking into account thermodynamic principles, i.e. the global thermodynamic representation of whole reactions that occur within cells (Heijnen and Van Dijken, 1992; VanBriesen, 2002).

With the word metabolism we could summarize all the biochemical and physiochemical processes that happen within a living cell. The microbial metabolism is comprised of two general sub-processes, catabolism and anabolism: catabolism incorporates all the processes involved in the substrates oxidation or in the use of sunlight in order to gain metabolic energy, and anabolism is the synthesis of cellular components. Therefore, the energy required by the anabolic processes could come from catabolic processes (Heijnen, 1999).

1.3.1 Microbial biomass yield ratios

Since the introduction of biotechnology processes in industry, engineers frequently require an estimation of the expected microbial biomass production in relation to the substrate consumed or the product obtained, above all the microbiological and biochemical properties of the microorganism(s) involved (VanBriesen, 2002).

An important parameter to describe any biotechnological process is the biomass yield production (Y) achieved for a population growing on a substrate. Many authors have suggested different ratios to define Y, in that sense Monod (1949) considered that it was a characteristic constant for microorganism species growing on a specific substrate. So he suggested using the term of yield as the relation between biomass production and substrate consumption.

A few years later, Bauchp and Elsden (1960) proposed the concept of expressing the biomass yield in terms of ATP consumed (Y_{ATP} in grams of biomass dry weight/mol ATP). Mayberry et al. (1967) proposed the concept of biomass yield (Y_{Ave} in grams of biomass per mole of available electrons). Minkevich and Eroshin (1973) proposed the oxygen efficiency (η_o), which is defined as the ratio of the amount of electrons conserved in the biomass over the amount of electrons available in the organic substrate by aerobic combustion to bicarbonate (HCO_3^-). Linton and Stephenson (1978) proposed the carbon yield of biomass on the C-source (Y_C in C-mol biomass/C-mol C-source). Heijnen and Van

Dijken (1992) defined the Y_{DX} as C-mol of biomass produced per mole of electron donor (e-donor) consumed (C-mol for organic or in moles for inorganic donors), therefore, Y_{DX} is in C-mol/(C)-mol.

Actually due to its prime importance, biomass yield for many different microbial systems has been studied extensively, and it is currently known for a wide variety of substrates that support microbial growth. They can be within the range of 0.01 to 1.0 C-mol biomass/(C)-mol, and depend strongly on the microorganism and the corresponding growth substrates (Heijnen, 1993; Heijnen et al., 1992; Heijnen and Van Dijken, 1992; McCarty, 2007; Smolders et al., 1994; Tijhuis et al., 1993; von Stockar, 2010)..

1.3.2 Models to yield prediction

In addition to defining the ratios for Y_{DX}, various authors have suggested some models. In that sense Borden et al. (1986) developed an equations system to predict the simultaneous growth, decay, and transport of microorganisms jointly with the transport and removal of hydrocarbons and O_2. This model is called BIOPLUME, and the O_2 transport will be rate limiting and the consumption of O_2 and hydrocarbon can be approximated as an instantaneous reaction.

Widdowson et al. (1988) propose a model to simulate organic C biodegradation by facultative bacteria using O_2-based and/or NO_3^--based respiration where the denitrification process was modelled with differential equations. It is relevant to notice that in this model the authors begin to consider the biomass formed by a group of individuals. Unfortunately, the authors consider that the effects of this group of individuals (microbial population) over the biodegradation, are likely to be secondary and the inclusion of such effects introduces unnecessary complexity.

MacQuarrie et al. (1990) propose a physically and biochemically based numerical solution for the transport of biodegradable organic solutes with emphasis on an efficient numerical approach. A dual-Monod relationship combined with an equation to model contaminant transport through porous media (advection-dispersion equation) is used to represent the biological and physical processes affecting the organic solute, e-acceptor, and microbial population. In general terms, calculating Y_{DX} using the previous models involves the selection and quantifying of at least one *black box* parameter, which must be applied to all microbial growth systems. This poses a difficult problem to solve due to the fact that is difficult to predict the value of this parameter without having information about the intracellular biochemical properties of the microorganism(s) involved in the process.

1.3.3 Yield prediction based on thermodynamics

The thermodynamic approaches to predict the biomass yield have been described, studied and investigated during the last twenty five years (Heijnen and Van Dijken, 1992; VanBriesen, 2002).

The most important principles, on which non-equilibrium thermodynamics are based, lie in the constraints for two extensive properties, energy and entropy, which are represented in the universal thermodynamics' laws (Roels, 1980a).

The first law of thermodynamics states that energy is a conserved quantity; in essence, the conversion term appearing in the balance equation is zero. The first law generalizes this constraint to apply irrespective of the nature of the system in which the process takes place.

The second law of thermodynamics states that the entropy production in any possible process must exceed zero. Therefore, considering the second law of thermodynamics, the maximum Y_{DX} in a microbiological system has a theoretical upper limit. Thus, the

maximum Y_{DX} could be determined by the electrons available in the e-donor and the electrons required to synthesize cells (VanBriesen, 2002).

Thereby, several approaches have been reported to develop a rigorous thermodynamic description for Y_{DX} prediction: i) *The degree of reductance* (Roels, 1983, 1980a, 1980b), ii) *The Gibbs energy dissipation* (Heijnen, 1999; Heijnen et al., 1992; Heijnen and van Dijken, 1993; Heijnen and Van Dijken, 1992; Tijhuis et al., 1993), and iii) *The energy-transfer-efficiency* (Christensen and McCarty, 1975; McCarty, 2007, 1971, 1965; Rittmann and McCarty, 2001; Stratton and McCarty, 1969).

1.3.3.1 The degree of reductance

Roels (1983, 1980a, 1980b) presented a Y_{DX} prediction approach based on the idea that the combined application of the first and second laws of thermodynamics allows the definition of a fundamental measure for the efficiency of the energy transformations inside a system. This is the called thermodynamic efficiency (n^{bb}) and it is defined as the ratio between the sum of the Gibbs energy associated with all consumed chemicals (reactants) and the sum of the Gibbs energy associated with all produced chemicals (products).

The attractive feature of this approach is that it can be applied to any microbial system and has a maximum limit due to the second law of thermodynamics. This limit of n^{bb} is equal to 1 and it is related to the theoretical maximum limit of Y_{DX}. Besides, Roels, (1980a, 1980b) taking into account the second law of thermodynamics and principles of heat production, correlated the degree of reductance (relation between the available electrons and the atoms of C of the substrate) with Y_{DX}.

It was concluded that, when the degree of reductance of C in the e-donor substrate is equal or greater than 4.55, the Y_{DX} appears to be nearly constant to approximately 0.55 C-mol cells/C-mol substrate (Roels, 1983, 1980a). However, when the degree of reductance is lower than 4.55, Y_{DX} decreases in the same sense as the degree of reductance. This approach is clearly an empirical approximation for a limited subgroup of microorganisms; but it provides a first yield estimate for aerobic heterotrophic growth (VanBriesen, 2002).

1.3.3.2 The Gibbs energy dissipation

Heijnen and Van Dijken (1992) presented a Y_{DX} prediction method, which is based on a statistical relationship among: i) C chain length, ii) e-donor substrate degree of reductance, iii) the Gibbs energy dissipation for the biological reaction that involves oxidation of the primary e-donor substrate, iv) reduction of the primary e-acceptor substrate, and v) production of biomass as the only product.

In a few words, the Gibbs energy dissipation coupled with the Gibbs energy values of the catabolic and anabolic reactions, is used to predict Y_{DX} (Heijnen, 1999; Heijnen and van Dijken, 1993; Heijnen and Van Dijken, 1992; Tijhuis et al., 1993). The Gibbs energy dissipation could be determined experimentally or it can be calculated using an empirical correlation between the number of C atoms in a mole of the substrate and the reductance degree of the C in the e-donor substrate (Heijnen and Van Dijken, 1992).

This correlation was calculated from multiple data sets collected from aerobic and anaerobic growth on a variety of substrates (Heijnen, 1999; Tijhuis et al., 1993; VanBriesen, 2002). Having once calculated or experimentally obtained the estimation of the Gibbs energy dissipation, Y_{DX} is computed taking into account: i) four elementary reactions and mass balances, ii) the experimental yield of bacterial cells on the e-donor substrate, iii) the degree of reductance of the e-donor substrate, iv) the degree of reductance of the carbon in the bacterial cells, v) the Gibbs free energy of the e-donor

oxidation half-reaction, vi) the Gibbs free energy of the e-acceptor half reaction, and vii) the Gibbs free energy of the formation of biomass (Liu et al., 2007; von Stockar, 2010).

Therefore, this approach which is based on the Gibbs energy dissipation per C-mol biomass produced, provides an estimated biomass yield with an error of about 13% for a wide range of microbial growth systems, in which Y_{DX} varies between 0.01 and 0.80 C-mol/(C)-mol (Heijnen et al., 1992; Heijnen and Van Dijken, 1992). The value of Y_{DX} and its Gibbs energy dissipation for different microorganisms, different e-donors, C-sources, and e-acceptors has been measured under carbon- and energy-limited growth conditions (Heijnen, 1999; Heijnen et al., 1992).

1.3.3.3 The energy-transfer efficiency

Christensen and McCarty (1975) presented BIOTREAT a computerized BIOlogical TREATment model, which is used to study the stoichiometry and kinetics of a wide variety of biological treatment of wastewaters. Using BIOTREAT Y_{DX} is determined from thermodynamic considerations. BIOTREAT writes a stoichiometric equation to describe the overall cellular reaction taking into account half-reactions for the e-donor, e-acceptor and cell synthesis.

One of the key features of BIOTREAT is that C-source will be a limiting nutrient. The key features of McCarty's approach lie in the idea that microorganisms capture energy released by redox reactions for maintenance and growth. In redox reactions the electrons are obtained from an e-donor and transferred to intracellular electron carriers, and carriers bring the electrons towards the e-acceptor; as a result the acceptor suffers a reduction that causes the regeneration of the initial carrier.

Taking into account these ideas BIOTREAT will be updated to the thermodynamic approach called Thermodynamic Electron Equivalents Model (TEEM) (McCarty, 2007; Rittmann and McCarty, 2001). TEEM considers that when microorganisms use an e-donor substrate for synthesis, a portion of their electrons (fe^o) is transferred to the e-acceptor to generate energy and metabolic products and the other portion of electrons (fs^o) is transferred to the N-source for cell synthesis (Rittmann and McCarty, 2001).

Therefore, McCarty's approach is a thermodynamic model based on bioenergetics growth efficiency that can make an adjustment between anabolic reaction with catabolic reaction to predict bacterial yield with the associated standard Gibbs free energies for these reactions (McCarty, 2007).

The catabolic reaction is the combination of the half-reaction for the e-donor and the half-reaction for the e-acceptor. The anabolic reaction is the combination of e-donor half-reaction with the half-reaction for the biomass synthesis that considers ammonium (NH_4^+) or other N-sources for new biomass generation (Rittmann and McCarty, 2001).

As stated, several approaches have been reported to develop a rigorous thermodynamic description for biomass yield prediction (Christensen and McCarty, 1975; Heijnen, 1999; Heijnen et al., 1992; Heijnen and Van Dijken, 1992; Liu et al., 2007; Maskow and von Stockar, 2005; McCarty, 2007; Rittmann and McCarty, 2001; Roels, 1983, 1980a; Tijhuis et al., 1993; von Stockar and van der Wielen, 1997; Xiao and VanBriesen, 2008, 2006).

These approaches consider the Gibbs energy for cell synthesis from C-sources and N-sources, the energy available from substrate transformation, the specific Gibbs energy consumption for cellular maintenance, and the energy efficiency transfer to the overall process to describe growth of micro-organisms in a standard mathematical and thermodynamic model.

All of the described thermodynamic approaches could be useful in the calculation of: i) the complete growth stoichiometry, ii) maintenance coefficients and maximal growth yields, iii) the limit to growth yield posed by the second thermodynamic law, iv) chemical-oxygen-demand-based growth yields, and v) maximal Y_{DX} in anaerobic metabolism. Therefore, to choose one of them it is necessary to take into account the existing knowledge about the microbial system, the growth conditions and the general application in which the information will be used.

1.4 Research framework

BIOCOM-SC – BIOlogía COMputacional y Sistemas Complejos (Computational Biology and Complex Systems) is an accepted and consolidated research group by the Generalitat of Catalunya under the number: 2014GR1093. The aim of the group is to use computational methods to address complex problems in biological systems, biomedicine and biophysics. BIOCOM-SC have three divisions: MOSIMBIO (MOdelización y SIMulación discreta de sistemas BIOlógicos), NOLIN (Non-linear Physics and out of Equilibrium Systems) and Microgravity Lab (BIOCOMSC, 2016).

The current thesis is born from the collaboration between:

- MOSIMBIO research group (Universitat Politècnica de Catalunya – Barcelona Tech - UPC) with project support from the Ministerio de Educación y Ciencia Español (MICINN, CGL2010- 20160).
- School of Biological Sciences and the School of Computing Sciences, University of East Anglia, Norwich Research Park – United Kingdom, with the full experimental data set of two denitrifying bacteria growing into a bioreactor (Felgate et al., 2012).
- Facultad de Ingeniería Química, Universidad Central del Ecuador with the financial support of the Secretaria Nacional de Educación Superior, Ciencia, Tecnología e Innovación del Ecuador (SENESCYT).

MOSIMBIO is a research group comprised of physicists, mathematicians, biologists and agricultural engineers, that investigates topics on microbiology from an interdisciplinary perspective (MOSIMBIO, 2016).

MOSIMBIO's focus is to study the general properties of microbial populations and ecosystems using IBMs. This approach allows the combination of the techniques and experience of different disciplines: the biological rules governing the individuals could be framed with the chemical and physical laws that also describe their local environment, and the collective outcome could be drawn from the statistical treatment of the population ensemble (Ferrer et al., 2008).

This research group developed a modelling methodology which was revised, tested, standardized and finally published with the acronym INDISIM which stands for INDividual DIScrete SIMulations (Ginovart et al., 2002a) and it was presented as a discrete simulation model to study bacterial cultures.

This model was used as a core model in other works (Ginovart et al., 2002b, 2002c), it was expanded to address other microbiological systems of interest and to raise other models such as INDISIM-SOM (Ginovart et al., 2005; Gras et al., 2011, 2010; Gras and Ginovart, 2006), INDISIM-YEAST (Ginovart et al., 2011a, 2011b, 2006; Ginovart and Cañadas, 2008), INDISIM-COMP (Prats et al., 2010) and INDISIM-Saccha (Portell et al., 2014) to deal with soil organic matter dynamics, yeast fermentations, multi-species composting, and dynamics of *Saccharomyces cerevisiae* anaerobic cultures, respectively.

Also the INDISIM philosophy and methodology was applied to develop models in other fields like biomedicine and epidemiology (Caceres et al., 2013; Cardona and Prats, 2016; Ferrer et al., 2008; Ferrer Savall, 2010; Prats et al., 2016, 2015; Vilaplana et al., 2014).

The motivation to deepen knowledge of denitrification modelling is twofold. First, agricultural soils are partially responsible for N_2O emissions to the atmosphere. Second, it opens the possibility of including this N cycle pathway into INDISIM-SOM (Ginovart et al., 2005; Gras et al., 2011, 2010; Gras and Ginovart, 2006). In that model the mineralization of C and N as well as the nitrification processes are driven by different microbial functional groups: e.g. heterotrophs, autotrophs and nitrifiers are already incorporated. This simulator was able to reproduce the kinetics of these processes for different soils in laboratory conditions.

The use of IBMs to study denitrifying bacteria is based on the use of INDISIM methodology. Although INDISIM and its different adaptations have been able to study processes of biotechnological interest, it would be very valuable to increase the level of detail in individual metabolic sub-models to progress in the study of microbial communities growing in different environmental conditions and using different substrates.

1.5 Objectives

The general objective of this doctoral thesis is to develop an IBM to study the denitrification process driven by denitrifying bacteria, using thermodynamic principles to write microbial metabolic reactions as the centre of the individual sub-model.

The specific goals are:

- To develop an open access and open source computational tool to systematize the writing of microbial metabolic reactions based on the thermodynamic principles to be used as the starting point of modelling projects dealing with biotechnological process carried out by microbes.
- To use microbial metabolic reactions in the design, implementation and parameterization of the thermodynamic behaviour-rules embedded in the metabolic sub-model of an IBM for denitrifying bacteria in the framework of INDISIM, implementing the model in an open-access programming platform to achieve a simulator that facilitates exploring the effects of denitrifying bacterial metabolic sub-model.
- To use the simulator obtained to test hypotheses and diverse metabolic strategies for the individual behaviour of *Paracoccus denitrificans* in relation to the use of substrates growing in aerobic and anaerobic conditions in a bioreactor, testing the adequacy of the simulation outputs with experimental published data.
- According to the results previously obtained, to improve the model design, modifying the individual rules required in the individual-based modelling context and to generalize the model to tackle other denitrifying bacteria using a wider set of published experimental data, performing the sensitivity analysis for some models' parameters in order to learn how the system works, and complete the modelling cycle.

1.6 Chapters description

In order to present the theoretical framework and the IBM developed in the current thesis, this manuscript contains the following chapters:

Chapter 2, named *Modelling And Simulation Framework* describes the theoretical fundamentals of the individual-based model methodology and the core IBM called INDISIM, followed by the explanation of the Thermodynamic Electron Equivalents Model (TEEM), which will be used to represent metabolic pathways through microbial metabolic reactions. Further, we present the basic outlines of how the microbial cells could be represented using an empirical formula that considers the molar relationship between the

elements that constitute it. Finally, a specific section describing NetLogo, the open source software used to implement our IBMs concludes this chapter.

Chapter 3, named *A Computational Tool To Write Microbial Metabolic Reactions Based On Thermodynamics.* In this chapter we present the thermodynamic principles on which the microbial metabolism is based, which is useful to describe the basic concepts on how to represent metabolic pathways using microbial metabolic reactions (MMRs). Further, taking into account TEEM we present an open-source software to write MMRs, and finally, we present some examples related to writing some MMRs for diverse metabolic functional groups of microorganisms. This chapter is based on the published paper entitled: *MbT-Tool: An Open-access Tool Based On Thermodynamic Electron Equivalents Model To Obtain Microbial-metabolic Reactions To Be Used In Biotechnological Processes* (Araujo Granda et al., 2016a).

Chapter 4, named *An Individual-based And Thermodynamic Model To Deal With Paracoccus Denitrificans In A Bioreactor* describes step by step how we have developed the IBM called INDISIM-Paracoccus to deal with the denitrification process in a bioreactor. Also, using the accepted ODD protocol, we present INDISIM-Paracoccus.

Chapter 5, named *Study Of Two Metabolic Hypotheses To Denitrification Pathway Using INDISIM-Paracoccus* describes the performance, application and some simulation results achieved with INDISIM-Paracoccus. The simulator will allow us to test hypotheses about actions in the individual metabolic behaviour and facilitate the exploration of new individual strategies. Both chapters are based on the published paper entitled: *INDISIM-Paracoccus, An Individual-based And Thermodynamic Model For A Denitrifying Bacterium* (Araujo Granda et al., 2016c).

Chapter 6, named *INDISIM-Denitrification: The First Update Of INDISIM-Paracoccus.* describes the IBM called INDISIM-denitrification, which is the first update of INDISIM-Paracoccus, extending its design and performance to other denitrifying bacteria. New elements have been included in this IBM that improve the adequacy of the model taking into account a wider set of experimental data. The simulation results are compared with experimental observations for two distinct denitrifying bacteria (*Paracoccus denitrificans* and *Achromobacter xylosoxidans*). This chapter is the content of a manuscript already prepared to be summited to the special issue of *Frontiers in Microbiology*, section Systems Microbiology, research Topic titled: "*The Individual Microbe: Single-Cell Analysis and Agent-Based Modelling*".

Chapter 7, named *Conclusions And Future Perspectives*, presents some final remarks and perspectives for this research work related to the use of the thermodynamic-based model developed, in exploring some individual characteristics of the denitrifying bacteria, e.g. the possibility to use different values for the individual energy-transfer-efficiency when the environmental conditions change, or the possibility to complete the global nitrogen cycle in the IBM named INDISIM-SOM, or using INDISIM-Denitrification as the basis to develop a new IBM that takes into account the expression of the denitrifying enzymes.

Chapter II – Modelling and simulation framework

For addressing the specific research questions of this thesis, centred on developing a bacterial IBM to investigate the denitrification process, a general introduction of this type of modelling approach is offered in this chapter. Also, the core model INDISIM used to develop the simulation models for denitrifying bacteria is presented here. The microbial IBMs describe the bacteria and media actions in a simple way focusing the attention on the individual bacterial maintenance and growth; thus, this chapter gives some insights into the thermodynamic principles linked with the microbial metabolism. The IBMs and computational tools developed in this doctoral thesis will be implemented in the widely used, free and open source software platform NetLogo that will also be introduced in this chapter.

2.1 The individual-based models in microbiology

The use of IBMs in microbiology allows deeper knowledge of living entities (individuals, cells and microorganisms) as part of a complex system, so we could study, for example, competition phenomena, synergies or antagonisms between biotic (microorganisms) and abiotic factors (substrates, end products, growth factors or boundary conditions).

IBMs consider individuals as discrete entities that follow behaviour-rules that drive how the individuals interact with their surrounding environment and other individuals, so that the individual and the environment can change and adapt their characteristics over time. This makes it possible to explore connections between micro-level behaviours of individuals to macro-level patterns that emerge from their interactions (Prats et al., 2008; Wilensky, 1999).

Additionally, IBMs take into account that all individuals inside a population are not equals and cellular properties are distributed unevenly between the cells and the growth curve of the population emerges from the interaction of individuals with their environment (Fredrickson et al., 1967; Lee et al., 2009; Mantzaris, 2007). IBMs have the ability to simulate variability among individuals, local interactions, complete life cycles and individual behaviour according to the changing individual internal and external conditions, linking mechanisms at the individual level to behaviour at the population level (Grimm, 1999; Mantzaris, 2007).

Arguments for using IBMs in microbiology are: the ability to resolve population heterogeneity (intra-population variability), to deal with complete life cycles and the possibility of representing the behaviour adapted to internal and external conditions changing thorough time and space (Grimm et al., 2006; Railsback and Johnson, 2011; Reuter et al., 2011). Also IBMs have the ability to link mechanisms at the individual level to population level behaviour (emergence), and they are very convenient to tackle the inapplicability of the continuum hypothesis (Hellweger and Bucci, 2009).

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, and presents some difficulties at the time of analysis, understanding and communicating (Hellweger et al., 2016). 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 (Grimm, 1999; Grimm et al., 2006; Railsback and Grimm, 2012; Thiele and Grimm, 2010). The use of specific programming environments to implement

these computational models facilitates their use (Hellweger et al., 2016; Wilensky, 1999), 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 (Font-Marques and Ginovart, 2016; Ginovart, 2014; Ginovart et al., 2012; Ginovart and Prats, 2012) and scientific fields (Thiele, 2014; Thiele et al., 2014, 2012a, 2012b; Thiele and Grimm, 2010).

The importance of intra-population variability is related to increasing availability of observations based on individuals (Individual-based Observations IBOs), which come from the use of various experimental techniques: fluorescence, flow cytometry, microscopy and scanning micro-spectroscopy, among others. These techniques can be used to measure and identify cells, measuring respiratory activity, protein expression, substrate uptake, intracellular pH, nutrient content and toxins at individual cell level (Brehm-stecher and Johnson, 2004; Hellweger et al., 2016; Kreft et al., 2013).

Classic models based on continuous functions, were intended to predict the growth of a population as a whole, since the individual inner workings of microorganisms were not known or cared about. However, in recent years there have been advances in the fields of molecular biology and biochemistry that have increased our knowledge of the inner workings of microorganisms (Jun and Taheri-Araghi, 2015; Taheri-Araghi et al., 2015). With the addition of this new type of information and data, and the degree of nonlinearity of many of these processes, the error associated with the average population models is getting bigger (Hellweger and Bucci, 2009). Also, as the complexity increases, it becomes more difficult to develop equations based on a population level that are able to incorporate various processes and different behaviours of individuals currently becoming known and documented (Kreft et al., 2013). Anyway, it seems more immediate to encode these processes through the behaviour-rules of an individual and then allow the behaviour of the population to emerge (Reuter et al., 2011).

The population level approach approximates a population of discrete individuals using a continuous density (continuum hypothesis). For populations of microorganisms that can reach very high densities, the continuum hypothesis typically is a good assumption. However, due to the high growth rates that they can reach, there are also cases where the population-level response is affected by the fate of a few individuals. As incredible as it may seem in populations with a very high population density, a single individual can be important (Hellweger and Bucci, 2009; Partensky et al., 1999; Prats et al., 2008).

The two approaches, the continuous-macroscopic and the discrete-microscopic approaches, are not incompatible or exclusive, but are complementary. Population-level approaches are mostly used for predictive purposes, due to their simplicity and computational efficiency. Moreover, they have been widely tested and, nowadays, many modelling frameworks exist. IBMs have had their own place in microbial research and have also been used for some predictive purposes, but their strength lies in the means they offer to disentangle and understand the dynamics of bio-systems (Hellweger and Bucci, 2009; Kreft et al., 2013). In summary, in addition to the characteristics just described, IBMs are useful to study the relations between experimental data and theoretical proposals, allowing testing of the consistency of different microbial models, and supplying holistic knowledge of the systems under study (Ferrer et al., 2008).

The choice of a modelling approach to study a bacterial system, either population-level (top-down, usually continuous with differential equations) or individual-based (bottom-up, discrete and computational model) is an important decision depending on the project's specific aspects, the characteristics of the system and the questions to be answered. For a review of some microbial system evolutions using the IBM methodology see, for instance, Bley, 2011; Ferrer et al., 2008; Hellweger and Bucci, 2009; Kreft et al., 2013; Lee et al., 2009; Resat et al., 2012, and Tack et al., 2015, among others.

2.2 The core model INDISIM (INDividual DIScrete SIMulation)

INDISIM is an IBM approach to studying bacterial cultures and it was developed by Ginovart et al. (2002) in order to simulate the growth and behaviour of bacterial populations. INDISIM simulates bacterial growth in a certain culture medium. The growth takes place in a two-dimensional space, but it can be extended to three dimensions when it is required by the system under study (Portell et al., 2014). The basic unit of INDISIM is the bacterial cell, each one being defined by a group of time-dependent variables regarding its spatial position and its individual characteristics (mass, mass to start the reproduction cycle, or reproduction cycle status, among others). Some of these properties may change throughout the bacterial life (Ginovart et al., 2002a).

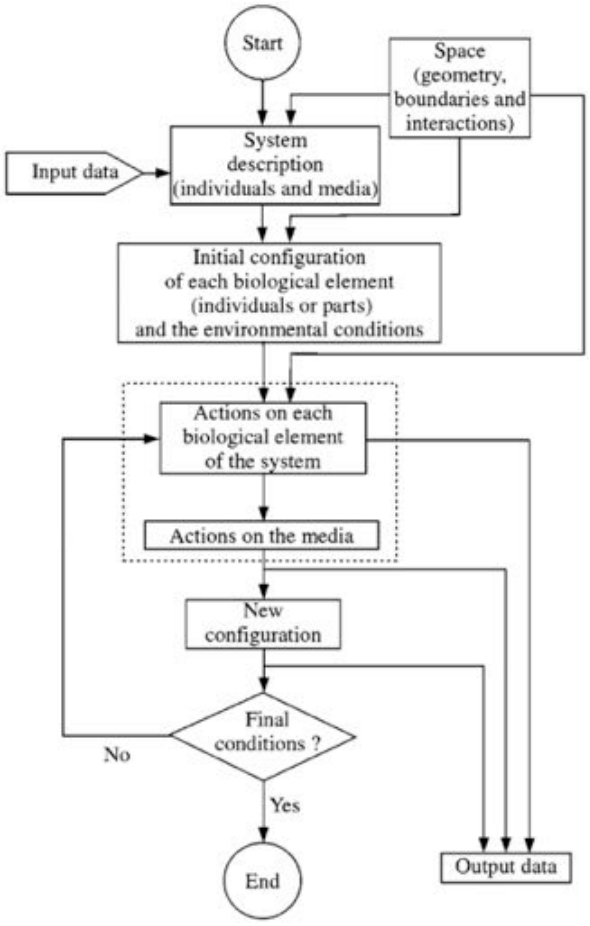

Figure 2.1 General flow chart of INDISIM. From: Ginovart et al. (2002).

The environment is divided into spatial cells that contain the bacteria and the nutrient particles. Each spatial cell is labelled with its coordinates (x,y) and its properties are also gathered by time-dependent variables (nutrient content, end product, pH, or temperature, among others). The bacterial activity (movement in the space, uptake of nutrients or substrates, metabolism of these products taken up with the excretion of some end products, reproduction, viability and lysis, the external manipulations such as agitation or medium renewals, and the diffusion processes are the major activities that may modify the environment properties and their spatial distribution. The processes affecting the bacterial and the environment are constituted by a set of rules. INDISIM is discrete in space and time. The time is split into discrete time steps. At each time step, every bacterium of the system acts sequentially and, after that, actions on the environment are carried out. In each application, this model can be adapted to the system under study by taking into account the kind of microorganism, the environmental conditions and the specific features of the system.

In Figure 2.1 we present the general flow chart of INDISIM, note that there are three main parts: i) the initialization, where the input parameters are read, the spatial structure is defined and the environmental and initial population is configured, ii) the main loop, where at each time step the actions for individuals and medium are described, and iii) the final part, where the necessary calculations are made to obtain the model's results.

2.3 The Thermodynamic Electron Equivalents Model

The Thermodynamic Electron Equivalents Model (TEEM) is the evolution of BIOTREAT (Christensen and McCarty, 1975). TEEM is designed to study the

stoichiometry and kinetics of a wide variety of biological treatments of wastewaters. (McCarty, 2007, 1971, 1965; Rittmann and McCarty, 2001; Stratton and McCarty, 1969). TEEM writes a stoichiometric reaction to describe the overall cellular metabolism from reduction-half-reactions for: the e-donor (Rd), e-acceptor (Ra) and cell synthesis (Rc). TEEM is based on terms of the standard 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 (Rittmann and McCarty, 2001) 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 (McCarty, 2007) complements TEEM1 because it considers oxygenase reactions involved and the aerobic heterotrophic oxidation of C1 organic compounds.

For the use of TEEM, no specific and detailed knowledge of microbial metabolism is required. First, we have to identify the e-donor(s) 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 third, it is essential to determine the empirical chemical formula that will represent the microbial cells.

According to TEEM, the metabolic energy is obtained from the redox reaction between an e-donor with an e-acceptor. The electrons are obtained from an e-donor and transferred to intracellular intermediates compounds, in TEEM1 the intermediate is the pyruvate with its half-reaction standard Gibbs free energy equal to 35.09 kJ/eeq, and in TEEM2 the intermediate is acetyl-CoA with its half-reaction standard Gibbs free energy equal to 30.9 kJ/eeq. The intermediate compounds bring the electrons towards the e-acceptor, which is reduced and causes the initial carrier regeneration. TEEM calculates Y_{DX} using a relation between the standard Gibbs free energy of the catabolic and anabolic reactions and an appropriate ε value.

The microbial catabolism is represented by the energy reaction (Re). To write it we have to combine Rd with Ra. Once Re is known, it is necessary to represent the microbial anabolism by writing the reaction for microbial biomass synthesis (Rs), and to do this we have to combine Rd with Rc. (Rittmann and McCarty, 2001) (Figure 2.2).

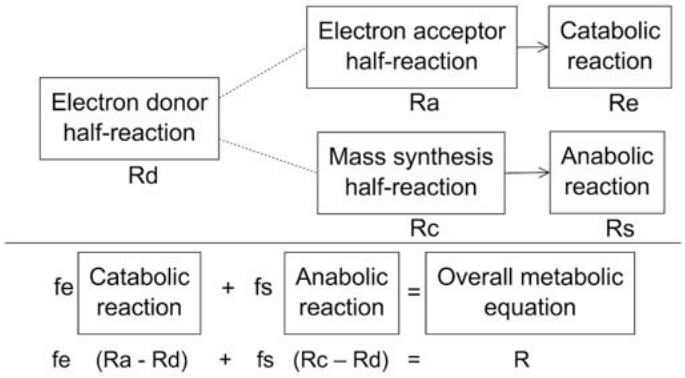

Figure 2.2. Schematic representation of TEEM, adapted from (Rittmann and McCarty, 2001).

Rc is a hypothetical half-reaction, which considers as reactants the N-source (NH_4^+, NO_3^-, NO_2^- or N_2), carbon dioxide (CO_2) and bicarbonate (HCO_3^-), and as products water and the microbial biomass represented by an empirical chemical formula of cells ($C_nH_aO_bN_c$).

This empirical chemical formula considers the molar relationships only for four basic elements: Carbon (n), Hydrogen (a), Oxygen (b) and Nitrogen (c). To establish the adequacy of this formula, researchers compared theoretical thermodynamic calculations using the cell's empirical chemical formula with the thermodynamics of growth of the same micro-organism on several substrates using batch cultures growing in the exponential phase at μ_{max} (Battley, 2007, 1995, 1993, 1987).

If the formula only considers four elements, the fitness is close to 95%, but if we include two more elements, e.g phosphorous and sulphur, the fitness increases its value to around 98-99% (Battley, 2013; LaRowe and Amend, 2016).

To estimate the standard Gibbs free energy of Rc (ΔG_{pc}), TEEM proposes a value of 3.33 KJ per gram cells (McCarty, 2007, 1971; Rittmann and McCarty, 2001), which is related to one generic microbial cell composition, $C_5H_7O_2N$, when NH_4^+ serves as the N-source for cell synthesis. The ΔG_{pc} value is valid in the context in which TEEM was developed.

This does not make a great deal of difference in calculated microbial yields, but is felt to be an acceptable theoretical choice (McCarty, 2007). In other research fields, e.g. bio-geochemical processes in marine environments, and taking into account different pressure, temperature, pH and N-sources for cell synthesis the reported ΔG_{pc} value is 302 J per gram cells (Amend et al., 2013; LaRowe and Amend, 2016, 2015).

Moreover, TEEM considers in its internal structure the possibility of using four different N-sources to write Rc (Rittmann and McCarty, 2001). The theoretical explanation is that microorganisms prefer to use NH_4^+ as an inorganic nitrogen source for cell synthesis, because it is already in the (-III) oxidation state, the status of organic nitrogen within the cell.

However, when NH_4^+ is not available for cell synthesis, many prokaryotic cells could use oxidized forms of nitrogen as alternatives. Therefore, NO_3^-, NO_2^- and N_2 are included as nitrogen sources (Table 2.1). When an oxidized form of nitrogen is used, microorganisms must reduce it to the (-III) oxidation state of NH_4^+, a process that requires electrons and energy, thus reducing their availability for synthesis. Therefore, different N-sources will obtain different results in the microbial yield (McCarty, 2007; Rittmann and McCarty, 2001).

Table 2.1. Cell synthesis half-reaction (*Rc*) with different N-sources. Adapted from Rittmann and McCarty (2001).

N-Source	**Half-reaction (Rc)**
NH_4^+	$\frac{(n-c)}{d}\ CO_2 + \frac{c}{d}\ NH_4^+ + \frac{c}{d}\ HCO_3^- + H^+ + e^- = \frac{1}{d} C_n H_a O_b N_c + \frac{(2n-b+c)}{d}\ H_2O$ $where, d = (4n + a - 2b - 3c)$
NO_3^-	$\frac{n}{d}\ CO_2 + \frac{c}{d}\ NO_3^- + \frac{(4n+a-2b+6c)}{d} H^+ + e^- = \frac{1}{d} C_n H_a O_b N_c + \frac{(3c+2n-b)}{d}\ H_2O$ $where, d = (4n + a - 2b + 5c)$
NO_2^-	$\frac{n}{d}\ CO_2 + \frac{c}{d}\ NO_2^- + \frac{(4n+a-2b+4c)}{d} H^+ + e^- = \frac{1}{d} C_n H_a O_b N_c + \frac{(2c+2n-b)}{d}\ H_2O$ $where, d = (4n + a - 2\mathrm{b} + 3c)$
N_2	$\frac{n}{d}\ CO_2 + \frac{c}{2d}\ N_2 + H^+ + e^- = \frac{1}{d} C_n H_a O_b N_c + \frac{(2n-b)}{d}\ H_2O$ $where, d = (4n + a - 2b)$

To couple the energy from catabolism to anabolism, TEEM considers a relation between the electrons involved. The electrons that come from the e-donor will be divided into two portions. The first portion (fe^o) is transferred to the e-acceptor to generate energy (catabolism) and the other portion of electrons (fs^o) is transferred to the N-source for cell synthesis (anabolism).

TEEM calculates the relationship between fe^o and fs^o using: (i) standard Gibbs free energy of Rd, Ra and Rc, (ii) standard Gibbs free energy of the intracellular intermediates compounds, and (iii) an appropriate ε value.

This term is included because TEEM considers that a fraction of the thermodynamic free energy involved is lost at each energy transfer between catabolism and anabolism (Xiao and VanBriesen, 2008, 2006). With this information, a global reaction (R= fe^oRa + fs^oRc – Rd) is written. This reaction represents the full stoichiometric relationships for a biological process without the experimental yield values commonly required to obtain it (VanBriesen, 2002).

TEEM's author (McCarty, 2007) used a large set of yield 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.

The ε value is considered by McCarty (1971) to be in the range between 0.2 and 0.8. Christensen and McCarty (1975) and VanBriesen (2002) suggested a value of 0.2 to 0.3 for aerobic heterotroph micro-organisms, and McCarty (2007) and Xiao and VanBriesen (2006, 2008) proposed a value between 0.4 and 0.7 for anaerobic heterotroph microorganisms.

But in general terms, to start a modelling project McCarty (2007) recommends us to use a value for energy-transfer-efficiency equal to 0.37. This value permits accurate predictions of bacterial growth with a precision around 85% (McCarty, 2007). For a detailed description of this thermodynamic approach the reader can refer to McCarty (2007) and Rittmann and McCarty (2001).

Therefore, using TEEM we can get the complete chemical and energetic stoichiometry of microbial growth represented by a microbial metabolic reaction (MMR). In this study we will represent metabolic pathways as a set of MMR and use them as the basis of the behaviour-rules (such as individual cellular maintenance or individual mass synthesis or individual mass degradation to reduce cytotoxic products among other processes) for each bacteria of the virtual system.

To do this we will consider the energy-transfer-efficiency (ε) value as an individual value. All of the mathematical expressions of TEEM1 and TEEM2 are presented in the Table 2.2.

2.4 Programing framework: NetLogo

In the last few years the software technology to develop, execute and analyze IBMs has progressed significantly, making it easier for researchers to use IBMs, and to assist in the interpretation and understanding of patterns within experimental data.

Hellweger et al. (2016) presented a list of open-source platforms (OSP). An OSP is a software tool that allows the user to create models within a range of systems and, also gives the option to read and rewrite parts of the source-code of the software itself in order to adapt or generate a simulator.

Before choosing the OSP option to start a modelling project, it is convenient that the user defines: (i) the physical processes involved (e.g., diffusion, convection and

mechanical interactions), (ii) the type of environment that interacts with the individuals to be modelled (e.g., liquid and stirring culture, agar plate, biofilm flow cell or gut), (iii) the set of microbial species and their biological processes (e.g., growth, cell-cell communication or motility), and (iv) the purposes of the simulator developed. These characteristics can help in the decision of which OSP to use in the modeling project.

One of the key advantages of some OSPs is the community-based development that they bring to the user, which is one of the most effective ways to implement an ever-wider range of processes. However, specific applications may be better served by software that is more specialized (Hellweger et al., 2016).

The computational tools and IBMs developed in this work will be implemented in NetLogo, a multi-agent programming language and modelling environment for simulating natural phenomena (Tisue and Wilensky, 2004; Wilensky, 1999). NetLogo can coordinate all the instructions given to a set of individuals so that they all operate independently among themselves and with the environment.

Key features of Netlogo are: (i) free and open source, (ii) cross-platform: runs on Mac, Windows and Linux, i.e., any operating system that supports Java, (iii) present an international character set support, (iv) mobile agents (turtles) move over a grid of stationary agents (patches), (v) link agents connect turtles to make networks, graphs, and aggregates, (vi) user can rapidly develop a model by programming agent behaviour and using a graphical user interface to control the simulation and view model's outputs, (vii) simulation state or time series data can be saved/loaded as CSV files (Hellweger et al., 2016; Tisue and Wilensky, 2004), and (viii) there are extensions to link NetLogo with: Matlab (Matthew and Jason, 2013), R (Thiele, 2014; Thiele et al., 2014, 2012a, 2012b; Thiele and Grimm, 2010) and GIS (Wilensky, 1999).

Besides these features, the NetLogo user could have the possibility of being part of its community-based development called "Modelling Commons", which is a web-based collaboration system for NetLogo modellers.

Users of the "Modelling Commons" can share, download, modify, create versions or comment on, and run NetLogo models, both those that are a part of the NetLogo models library and those that have been uploaded by other NetLogo users (http://modelingcommons.org).

Use of the "Modelling Commons" is free of charge. You may use it for your own personal work, for your research group or company, or for a class in which you are a student or teacher. The "Modelling Commons" is sponsored by the CCL, the same group that develops and distributes NetLogo (Center for Connected Learning, 2016).

NetLogo is continually being updated by the Center for Connected Learning (CCL) in the Northwestern University (http://ccl.northwestern.edu/netlogo/). The models library included into NetLogo software, programming and transition guide, interface and info tab guide, and NetLogo dictionary are distributed free of charge.

NetLogo provides a friendly interface that can be managed by non-expert users, and at the same time any advanced user could modify the NetLogo's source-code to develop extensions and update it.

Therefore, NetLogo can be freely used in order to explore existing models, as well as to implement and use new models (Wilensky, 1999). This is a strong reason to choose it because one of the purposes of this modelling project is that we would like to distribute the source-code among the scientific community to promote its use and to encourage them to extend and adapt it, improving the current work carried out, and advance and progress in this research field.

Table 2.2. Core equations of non equilibrium thermodynamic models: TEEM1 (Rittmann and McCarty, 2001) and TEEM2 (McCarty, 2007).

<table>
<tr><th>TEEM1</th><th>TEEM2</th></tr>
<tr><td>$A = -\frac{\Delta G_s}{\varepsilon \Delta G_e} = -\frac{\frac{(\Delta G_{in} - \Delta G_d)}{\varepsilon^n} + \frac{\Delta G_{pc}}{\varepsilon}}{\varepsilon(\Delta G_a - \Delta G_d)} = \frac{f_e^o}{f_s^o}$</td><td>$A = -\frac{\Delta G_s}{\varepsilon \Delta G_e} = -\frac{\frac{(\Delta G_{fa} - \Delta G_d)}{\varepsilon^m} + \frac{(\Delta G_{in} - \Delta G_{fa})}{\varepsilon^n} + \frac{\Delta G_{pc}}{\varepsilon}}{\varepsilon\left(\Delta G_a - \Delta G_d - \frac{q}{p}\Delta G_{xy}\right)} = \frac{f_e^o}{f_s^o}$</td></tr>
<tr><td>$f_s^o = \frac{1}{1+A}$; $f_e^o = \frac{A}{1+A}$; $f_s^o + f_e^o = 1$</td><td>$Y_{c/c} = \frac{\gamma_d}{\gamma_x} f_s^o$</td></tr>
<tr><td rowspan="6">ΔG_{in} = Reduction potential for Pyruvate half-reaction (35.09 kJ/eeq).</td><td>ΔG_{in} = Reduction potential for Acetyl-CoA half-reaction (30.9 kJ/eeq).</td></tr>
<tr><td>ΔG_{fa} = Reduction potential for formaldehyde half-reaction (46.53 kJ/eeq for C1 compounds, 0 for others).</td></tr>
<tr><td>ΔG_{xy} = Reduction potential for NADH oxidation (219.2 kJ/mol).</td></tr>
<tr><td>m = +1 if $\Delta G_{fa} > 0$, otherwise = n.</td></tr>
<tr><td>p = Number of electron equivalents per mole of substrate from half-reaction reduction equation.</td></tr>
<tr><td>q = Number of oxygenase reactions per mole substrate.</td></tr>
<tr><td colspan="2">fs^o = Fraction of e-donor electrons converted for synthesis (eeq cells/eeq donor).</td></tr>
<tr><td colspan="2">fe^o = Fraction of e-donor electrons used for energy and converted to reaction products (eeq products/eeq donor)</td></tr>
<tr><td colspan="2">ΔG_{pc} = Gibbs free energy for intermediate conversion to cells (kJ/eeq) = 3.33 kJ/gcells (Molecular weight Cells/pcells) = 3.33(113/20) = 18.8 kJ/eeq with NH_4^+ as nitrogen source and cell formulation of $C_5H_7O_2N$. With NO_3^-, NO_2^-, or N_2 as nitrogen source, pcells equals 28, 26 and 23 kJ/eeq, respectively (Rittmann and McCarty, 2001).</td></tr>
<tr><td colspan="2">ε = Energy transfer efficiency.</td></tr>
<tr><td colspan="2">ΔG_e = Gibbs free energy for energy reaction (kJ/eeq).</td></tr>
<tr><td colspan="2">ΔG_s = Gibbs free energy for cell synthesis reaction (kJ/eeq).</td></tr>
<tr><td colspan="2">ΔG_d = Reduction potential for e-donor half-reaction (kJ/eeq).</td></tr>
<tr><td colspan="2">ΔG_a = Reduction potential for e-acceptor half-reaction (kJ/eeq).</td></tr>
<tr><td colspan="2">n = +1 if m = n and (ΔG_{in} - ΔG_d) > 0, otherwise n = -1.</td></tr>
<tr><td colspan="2">γ_d = Degree of reduction of e-donor.</td></tr>
<tr><td colspan="2">γ_x = Degree of reduction of cells.</td></tr>
<tr><td colspan="2">Yc/c = Maximum bacterial yield ($molC_{mic}/molC_{substrate}$).</td></tr>
</table>

Chapter III – A computational tool to write Microbial Metabolic Reactions based on Thermodynamics

3.1 Introduction

Modelling cellular metabolism is a strategic issue to investigate microbial behaviour and interactions, especially for biotechnological processes driven by microorganisms. A key factor in modelling the cellular metabolism is the technique used for representing metabolic pathways.

Commonly the pathway information shows us the main chemical species involved and its corresponding product with the information about the enzyme responsible for the reaction and in some cases the genes involved (Caspi et al., 2012). This information is only descriptive and lacks important terms, such as mass balance; therefore, it is not possible to use this information directly for modelling the cellular metabolism. A robust metabolic model of any living system must include chemicals and conservation principles, and relate stoichiometric mass balance with the laws of energy conservation (Toure and Dussap, 2016).

One of the most fundamental properties of living cell systems is their ability to utilize and transform energy. This energy could come from different forms: mechanical, electrical, electromagnetic, chemical and thermal energy. The energy transformations are also fundamental for the transport of substrates along pH and electrical gradients across the cellular membrane. Therefore, we could define the microbial growth as the organized increase of all chemical (microbial) components (Maskow and Paufler, 2014). Besides, living cell systems have the capability to utilize and transform energy involving thousands of individual enzyme-catalyzed chemical reactions. Since every reaction involves a loss or gain of electrons, the amount of energy released or used depends on the oxidation-reduction potential difference. In order to maintain its integrity, gain and loss of energy must be balanced via a controlled electron transport and energy transformation, which follows thermodynamic laws. The first thermodynamic law says that the total amount of energy in nature is constant, and the second thermodynamic law establishes that the total amount of entropy in nature is increasing (Doelle, 1969).

The chemical reactions in living organisms occur in characteristically organized sequences, called metabolic pathways. There are two main types of metabolic pathways: (i) pathways which lead from large (low oxidative state) to smaller molecules (high oxidative state), which are called catabolic pathways or catabolism, and (ii) pathways which lead from small (high oxidative state) to large molecules (low oxidative state) essential for the formation of cellular material, which are referred to as anabolic or biosynthetic pathways, or anabolism (von Stockar et al., 2006).

Consequently, all catabolic pathways are oxidative and thus energy producing: the anabolic pathways are reductive and energy consuming. Therefore, studying cell metabolism consists, basically, in describing how to couple energy between catabolism and anabolism and the transfer mechanisms involved, which are all based on thermodynamic laws (Figure 3.1).

For the construction and development of models for living organisms, it is necessary to describe physical constants, metabolic pathways, and interactions and responses between living organisms and their environment. Intuitively, a complete model has a number of sub-models that take into account the compartmentalized structure of living organisms and, specifically, the metabolic sub-model should include a quantitative representation of the catabolic and/or anabolic pathway involved according to the thermodynamic laws.

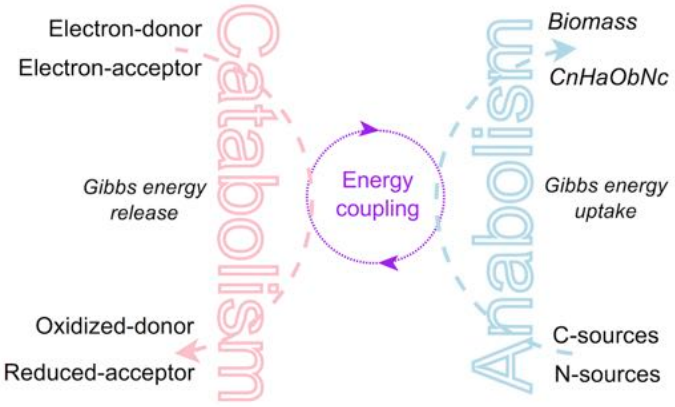

Figure 3.1. Scheme of energy coupling between catabolism and anabolism. The all-cellular material is represented using the term microbial biomass, which is denoted by the empirical formula CnHaObNc.

3.2 Basis to write Microbial Metabolic Reactions

Modellers have met a problem that needs to be solved and that is how to represent quantitatively the pathway(s) that will be used by the individuals in their models. The common modelling way is to take some population values, e.g. the experimental biomass yield, and use it as a system value. From our point of view this assumption causes the modellers to explore the individual differences in other values, e.g. C/N relationship of cell composition or cell size, and not the possibility that the individuals have to execute different metabolic pathways according to the different media composition and nutrient availability.

Consequently, we could say that a microbial metabolic reaction (MMR) is the quantitative written version of a metabolic pathway or part of it, and therefore, it must include the main chemical species involved properly balanced with stoichiometric principles according to the laws of energy and entropy conservation.

The stoichiometry is a part of the chemistry that permits the writing of balanced reactions because it studies the molar relation between the reactants and products involved. There are several reasons to suggest that the biochemical reactions inside a metabolic pathway complicate the stoichiometry. First, biochemical reactions often involve oxidation and reduction of more than one chemical species. Second, the microorganisms, solely responsible for the reaction, have two different roles: they are performing the reaction and also they are the final products of the reaction. Third, the microorganisms carry out a lot of chemical reactions in order to capture a part of the energy liberated for cell synthesis and for conserving cellular activity (Rittmann and McCarty, 2001; VanBriesen and Rittmann, 2000).

Therefore, the use of MMR to represent the metabolic pathway, with all-cellular material characterized through an empirical formula to microbial mass ($C_nH_aO_bN_c$ wherein n, a, b and c are the molar ratios of carbon, hydrogen, oxygen and nitrogen, respectively) as a product or as a reactant, is a significant duty for modelling microbial activity and sometimes a complex task for biologists, ecologists, modelers, and engineers, among others.

To develop the computational tool to write MMRs following thermodynamic principles, we have chosen the TEEM approach (Section 2.3) because it includes, inter alia: i) the standard Gibbs energy for cell synthesis from different carbon-sources and nitrogen-

sources, ii) the energy available from substrate transformation, and iii) the energy-transfer-efficiency (ε) to the overall metabolic process.

3.3 The computational tool to write Microbial Metabolic Reactions: MbT-tool

The computational tool is implemented on NetLogo, which was chosen mainly for the way that this platform is organized; it is very well documented and gives the option to share this developed tool with other researchers. This straightforward interaction will facilitate the upgrading and updating of the computational tool by the scientific community interested in writing and using biochemical reactions for the microbial processes.

We have named the computational tool MbT-tool, standing for Metabolism based on Thermodynamics. With this tool it is possible to write MMRs conducted by microbes. MbT-tool is based on the two versions of the TEEM, TEEM1 (Rittmann and McCarty, 2001) and TEEM2 (McCarty, 2007). The MbT-tool user interface displays all variables and parameters regarding the empirical cells formula and substrates that can be selected to describe the metabolic processes. The complexity of the implemented thermodynamic models as well as the programming code can be avoided by the user, however if it is required and the user has knowledge of the matter, the user could incorporate redox reactions that are not covered already.

MbT-tool considers Battley's arguments (Battley, 2013) about the empirical composition of the cells and allows writing of MMRs using the same formula of cells over different substrates, and also gives the option of using different cell compositions and using them over the substrates which have been used to determine the empirical chemical formula. Figure 3.2 shows the schematic description of how MbT-tool is based on TEEM, Figure 3.3 shows the schematic programming structure of MbT-tool and Figure 3.4 shows the user interface of the implemented tool. Besides the "Interface" window, MbT-tool includes the "Information" and the "Code" windows.

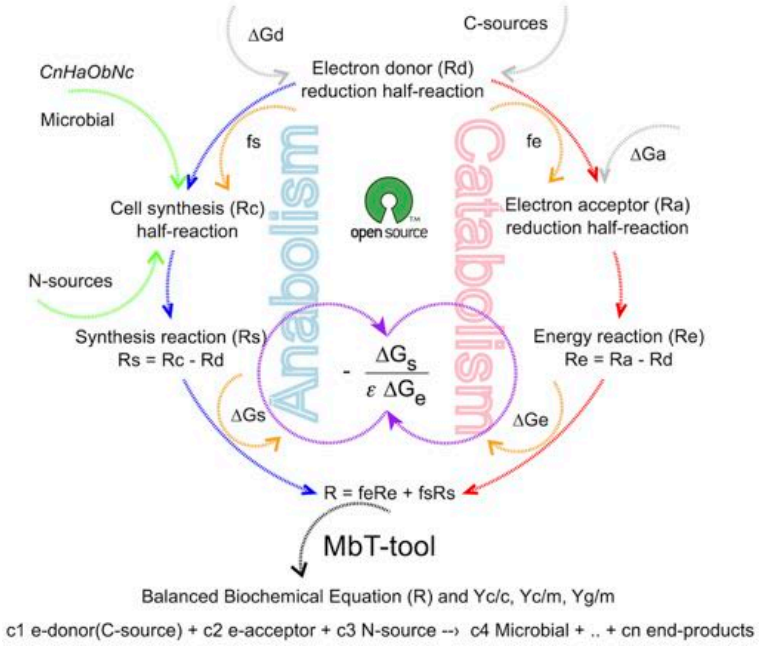

Figure 3.2. MbT-tool is the computational tool to write the microbial metabolism through a microbial metabolic reaction using a thermodynamic approach based on the energy-transfer-efficiency between catabolic and anabolic processes.

3.3.1 How to use MbT-Tool?

To use the computational tool, it is necessary to install NetLogo on the computer (https://ccl.northwestern.edu/netlogo/download.shtml), and download MbT-tool (http://mosimbio.upc.edu/en/publications/publications-by-year/years-2010-now). It runs on almost any operating system (OS) that supports JAVA.

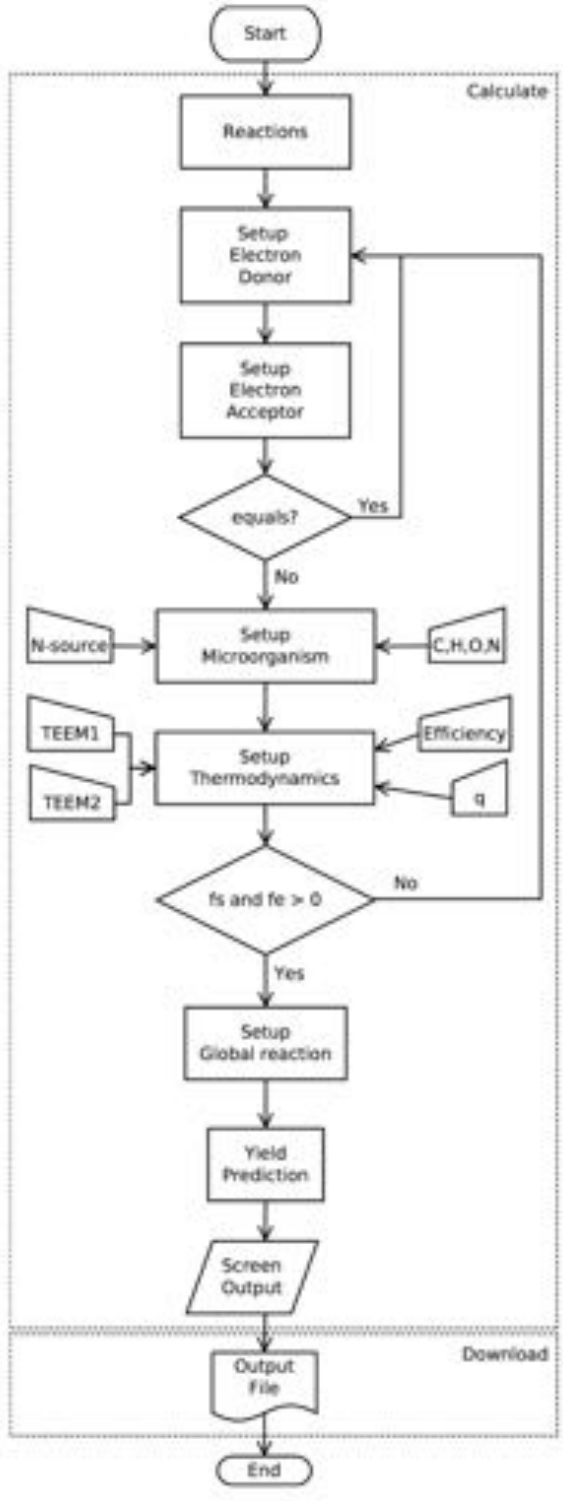

Figure 3.3. Flow chart of MbT-tool.

On opening MbT-tool the interface window appears (Figure 3.4). First the user has to select one or two e-donors and one e-acceptor from the organic (Table 3.1) or inorganic (Table 3.2) reduction-half-reactions. After that, the empirical chemical formula of the cells (Table 3.3) will be selected or the molar relationship between the four main elements (C, H, O and N) could be inputted. Then the user has to define the N-source between four sources: NH_4^+, NO_3^-, NO_2^- or N_2 to the cell synthesis half-reaction (Table 2.1).

With this setup information the user has to select the thermodynamic approach TEEM1 or TEEM2 to write the MMRs (Table 2.2). If the user chooses TEEM2 it is necessary to define the number of oxygenase reactions per mole of substrate, introducing an integer number in the "q" parameter.

Finally, the user has to fix the ε value for the process. With all this data, the MbT-tool displays the following outputs: the Rd, Ra and Rc half-reactions, the energy reaction (Re), the synthesis reaction (Rs), fe^o, fs^o, ε, the MMR (R) and the calculated microbial yield (Figure 3.4). This MMR is the reaction that McCarty defines as global reaction, which arises from the combination between the catabolic reaction with the anabolic reaction and the value of ε (McCarty, 2007).

The user could also download these outputs in an archive with a ".txt" extension. The file name is written with the information of the Rd, Ra, Rc, N-source and the thermodynamic approach used (TEEM1 or TEEM2).

Table 3.1. Organic half-reactions and their Gibb´s standard free energy according to Rittmann and McCarty (2001).

#	Name	Half-reaction	ΔG° (kJ/e$^-$eq)
O-1	Acetate	$^1/_8\ CO_2 + {}^1/_8\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_8\ CH_3COO^- + {}^3/_8\ H_2O$	27.40
O-2	Alanine	$^1/_6\ CO_2 + {}^1/_{12}\ HCO_3^- + {}^1/_{12}\ NH_4^+ + {}^{11}/_{12}\ H^+ + e^- \rightarrow {}^1/_{12}\ CH_3CHNH_2COO^- + {}^5/_{12}\ H_2O$	31.37
O-3	Benzoate	$^1/_5\ CO_2 + {}^1/_{30}\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{30}\ C_6H_5COO^- + {}^{13}/_{30}\ H_2O$	27.34
O-4	Citrate	$^1/_6\ CO_2 + {}^1/_6\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{18}\ (COO^-)CH_2COH(COO^-)\ CH_2COO^- + {}^4/_9\ H_2O$	33.08
O-5	Ethanol	$^1/_6\ CO_2 + H^+ + e^- \rightarrow {}^1/_{12}\ CH_3CH_2OH + {}^1/_4\ H_2O$	31.18
O-6	Formate	$^1/_2\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_2\ HCOO^- + {}^1/_2\ H_2O$	39.19
O-7	Glucose	$^1/_4\ CO_2 + H^+ + e^- \rightarrow {}^1/_{24}\ C_6H_{12}O_6 + {}^1/_4\ H_2O$	41.35
O-8	Glutamate	$^1/_6\ CO_2 + {}^1/_9\ HCO_3^- + {}^1/_{18}\ NH_4^+ + H^+ + e^- \rightarrow {}^1/_{18}\ COOHCH_2CH_2CHNH_2COO^- + {}^4/_9\ H_2O$	30.93
O-9	Glycerol	$^3/_{14}\ CO_2 + H^+ + e^- \rightarrow {}^1/_{14}\ CH_2OHCHOHCH_2OH + {}^4/_9\ H_2O$	38.88
O-10	Glycine	$^1/_6\ CO_2 + {}^1/_6\ HCO_3^- + {}^1/_6\ NH_4^+ + H^+ + e^- \rightarrow {}^1/_6\ CH_2NH_2COOH + {}^1/_2\ H_2O$	39.80
O-11	Lactate	$^1/_6\ CO_2 + {}^1/_{12}\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{12}\ CH_3CHOHCOO^- + {}^1/_3\ H_2O$	32.29
O-12	Methane	$^1/_8\ CO_2 + H^+ + e^- \rightarrow {}^1/_8\ CH_4 + {}^1/_4\ H_2O$	23.53
O-13	Methanol	$^1/_6\ CO_2 + H^+ + e^- \rightarrow {}^1/_6\ CH_3OH + {}^1/_6\ H_2O$	36.84
O-14	Palmitate	$^{15}/_{19}\ CO_2 + {}^1/_{92}\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{92}\ CH_3(CH_2)_{14}COO^- + {}^{31}/_{92}\ H_2O$	27.26
O-15	Propionate	$^1/_7\ CO_2 + {}^1/_{14}\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{14}\ CH_3\ CH_2COO^- + {}^5/_{14}\ H_2O$	27.63
O-16	Pyruvate	$^1/_5\ CO_2 + {}^1/_{10}\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{10}\ CH_3\ COCOO^- + {}^2/_5\ H_2O$	35.09
O-17	Succinate	$^1/_7\ CO_2 + {}^1/_7\ HCO_3^- + H^+ + e^- \rightarrow {}^1/_{14}\ (CH_2)_2(COO^-)_2 + {}^3/_7\ H_2O$	29.09

To avoid potential errors when using the MbT-Tool, we recommend not selecting the same chemical species for the e-donor and for the e-acceptor; they must be different.

If e-donor and e-acceptor are the same, the result of the calculations using their standard Gibbs free energy could provoke an inconsistent value on the fraction of electrons destined for cellular synthesis or electrons destined for energy. An inconsistent value will be obtained when these fractions of electrons are greater than one or below zero. If this numerical inconsistency occurs, the MbT-Tool stops and shows an alert message to the user.

Regarding the decision of using TEEM1 or TEEM2, it only depends on: (i) the growth substrate, (ii) the microorganism(s) involved, and (iii) the metabolic pathway that will be represented using the MbT-Tool. For instance, if the growth substrate is a C1 compound, it is better to use TEEM2 over TEEM1.

If one of the intermediates metabolic compounds in the pathway is pyruvate, it is better to use TEEM1 over TEEM2, If Acetyl-CoA is an intermediate compound in the pathway, it is better to use TEEM2 over TEEM1. Finally, if oxygenase reactions are involved, it is better to use TEEM2 over TEEM1.

Basically, micro-organisms utilize the oxygenase reactions to create more biodegradable forms of substrates. Some examples are: when alkanes are hydroxylated (Schirmer et al., 2010), alkenes are converted into the corresponding epoxides (Peters and Witholt, 1994), carbon monoxide is oxidized to carbon dioxide (Timkovich and Thrasher, 1988), NH_4^+ is oxidized to hydroxylamine (Arp et al., 2002), and some aromatic compounds and cyclic alkanes are hydroxylated (Kauppi et al., 1998).

To determine the "q" value, the user must establish if the reaction is mono-oxygenase-catalyzed or di-oxygenase-catalyzed. But in the most common cases, the "q" value ranges from 0 to 4. For a detailed description of this procedure the reader can refer to Xiao and VanBriesen (2008, 2006).

Table 3.2. Inorganic half-reactions and their Gibb's standard free energy according to Rittmann and McCarty (2001).

#	Half-reaction	ΔG^o (kJ/e$^-$eq)
I-1	$^1/_8\ NO_3^- + {}^5/_4\ H^+ + e^- \rightarrow {}^1/_8\ NH_4^+ + {}^3/_8\ H_2O$	- 35.11
I-2	$^1/_6\ NO_2^- + {}^4/_3\ H^+ + e^- \rightarrow {}^1/_6\ NH_4^+ + {}^1/_3\ H_2O$	- 32.93
I-3	$^1/_6\ N_2 + {}^4/_3\ H^+ + e^- \rightarrow {}^1/_3\ NH_4^+$	26.70
I-4	$Fe^{3+} + e^- \rightarrow Fe^{2+}$	- 74.27
I-5	$H^+ + e^- \rightarrow ½\ H_2$	39.87
I-6	$½\ NO_3^- + H^+ + e^- \rightarrow ½\ NO_2^- + ½\ H_2O$	- 41.65
I-7	$^1/_3\ NO_3^- + {}^4/_3\ H^+ + e^- \rightarrow {}^1/_3\ NO + {}^2/_3\ H_2O$	- 39.00
I-8	$^1/_4\ NO_3^- + {}^5/_4\ H^+ + e^- \rightarrow {}^1/_8\ N_2O + {}^5/_8\ H_2O$	- 57.54
I-9	$^7/_{24}\ NO_3^- + {}^{31}/_{24}\ H^+ + e^- \rightarrow {}^1/_6\ NO + {}^1/_{16}\ N_2O + {}^{31}/_{48}\ H_2O$	- 48.27
I-10	$^1/_5\ NO_3^- + {}^6/_5\ H^+ + e^- \rightarrow {}^1/_{10}\ N_2 + {}^3/_5\ H_2O$	- 72.20
I-11	$2H^+ + NO_2^- + e^- \rightarrow NO + H_2O$	- 33.72
I-12	$^1/_5\ NO_2^- + {}^4/_3\ H^+ + e^- \rightarrow {}^1/_6\ N_2 + {}^2/_3\ H_2O$	- 92.56
I-13	$H^+ + NO + e^- \rightarrow ½\ N_2O + ½\ H_2O$	- 115.83
I-14	$H^+ + ½\ N_2O + e^- \rightarrow ½\ N_2 + ½\ H_2O$	- 133.47
I-15	$^1/_8\ SO_4^{2-} + {}^{19}/_{16}\ H^+ + e^- \rightarrow {}^1/_{16}\ H_2S + {}^1/_{16}\ HS^- + {}^1/_2\ H_2O$	20.85
I-16	$^1/_6\ SO_3^{2-} + {}^5/_4\ H^+ + e^- \rightarrow {}^1/_{12}\ H_2S + {}^1/_{12}\ HS^- + {}^1/_2\ H_2O$	11.03
I-17	$^1/_2\ SO_4^{2-} + H^+ + e^- \rightarrow {}^1/_2\ SO_3^{2-} + {}^1/_2\ H_2O$	50.30
I-18	$^1/_6\ SO_4^{2-} + {}^4/_3\ H^+ + e^- \rightarrow {}^1/_6\ S + {}^2/_3\ H_2O$	19.15
I-19	$^1/_4\ SO_4^{2-} + {}^5/_4\ H^+ + e^- \rightarrow {}^1/_8\ S_2O_3^{2-} + {}^5/_8\ H_2O$	23.58
I-20	$¼\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$	- 78.72

Table 3.3. Empirical chemical formulas of some microbes. Adapted from Rittmann and McCarty (2001).

#	Empirical formula	Growth substrate and environmental conditions	Reference
	Pure cultures		
M-1	$C_5H_8O_2N$	Bacteria, acetate, aerobic	(Bailey and Ollis, 1986)
M-2	$C_5H_{8.33}O_{0.81}N$	Bacteria, undefined	
M-3	$C_4H_8O_2N$	Bacteria, undefined	
M-4	$C_{4.17}H_{7.42}O_{1.38}N$	*Aerobacter aerogenes*, undefined	
M-5	$C_{4.54}H_{7.91}O_{1.95}N$	*Klebsiella aerogenes*, glycerol, μ=0.1 h^{-1}	
M-6	$C_{4.17}H_{7.21}O_{1.79}N$	*Klebsiella aerogenes*, glycerol, μ=0.85 h^{-1}	
M-7	$C_{4.16}H_8O_{1.25}N$	*Escherichia coli*, undefined	(Battley, 1993)
M-8	$C_{3.85}H_{6.69}O_{1.78}N$	*Escherichia coli*, glucose	(Battley, 1987)
M-9	$C_{6.33}H_{10.21}O_{3.53}N$	*Saccharomyces cerevisiae*, glucose	(Battley, 2013)
M-10	$C_4H_{7.2}O_{1.93}N$	*Paracoccus denitrificans*, succinate	(van Verseveld et al., 1983)
M-11	$C_5H_9O_{2.5}N$	*Agrobacterium tumefaciens*, succinate	(Kampschreur et al., 2012)
M-12	$C_{4.17}H_8O_{1.75}N$	Bacteria, undefined	(Holt et al., 1994)
	Mixed cultures		
M-13	$C_5H_7O_2N$	Casein, aerobic	(Porges et al., 1956)
M-14	$C_7H_{12}O_4N$	Acetate, NH_4^+ N-source, aerobic	(Symons and McKinney, 1958)
M-15	$C_9H_{15}O_5N$	Acetate, NO_3^- as N-source, aerobic	
M-16	$C_9H_{16}O_5N$	Acetate, NO_2^- as N-source, aerobic	
M-17	$C_{4.9}H_{9.4}O_{2.9}N$	Acetate, methanogenic	(Speece and McCarty, 1964)
M-18	$C_{4.7}H_{7.7}O_{2.1}N$	Octanoate, methanogenic	
M-19	$C_{4.9}H_9O_3N$	Glycine, methanogenic	
M-20	$C_5H_{8.8}O_{3.2}N$	Leucine, methanogenic	
M-21	$C_{4.1}H_{6.8}O_{2.2}N$	Nutrient broth, methanogenic	
M-22	$C_{5.1}H_{8.5}O_{2.5}N$	Glucose, methanogenic	
M-23	$C_{5.3}H_{9.1}O_{2.5}N$	Starch, methanogenic	
M-24	$C_nH_aO_bN_c$	Bacteria, generic	------

3.3.2 Program and computer code

MbT-tool is programmed using built-in language primitives and the extension "matrix" of NetLogo. The code contains all the instructions and mathematical formulas to write MMRs. When the user clicks on the button *calculate*, the first procedure that the MbT-tool executes is the creation of a square matrix of i-rows by i-columns called "Reactions". The number (i) of rows and columns is the same and represents the number of chemical species programmed in the source code of the tool. Each row stores all numerical coefficients of each reduction-half-reaction with its Gibb's standard free energy. Therefore each chemical species uses a different column (Table 3.4) and each half-reaction uses a different row. In all cases (except Gibb's standard free energy), if the numerical coefficient is positive it means *reaction reactant* and if it is negative it means *reaction product*.

Table 3.4. Column positions of chemical species on the matrix *reactions* in the computer code of MbT-Tool.

Column	Chemical Specie	Column	Chemical Specie
1	O_2	21	HS^-
2	H^+	22	$S_2O_3^{2-}$
3	e^-	23	S
4	CO_2	24	Alanine
5	NH_4^+	25	Benzoate
6	HCO_3^-	26	ΔG
7	Biomass	27	Citrate
8	H_2O	28	Ethanol
9	Acetate	29	Formate
10	NO_3^-	30	Glucose
11	NO_2^-	31	Glutamate
12	NO	32	Glycerol
13	N_2O	33	Glycine
14	N_2	34	Lactate
15	H_2	35	Methane
16	Fe^{3+}	36	Methanol
17	Fe^{2+}	37	Palmitate
18	SO_4^{2-}	38	Propionate
19	SO_3^{2-}	39	Pyruvate
20	H_2S	40	Succinate

When the matrix *reactions* is ready, MbT-tool selects from its rows the e-donor (*Rd)* and the e-acceptor (*Ra)* taking into account the user selection of these half-reactions, and places the corresponding information in different matrices (the matrix called "Rd" for *Rd* and the matrix called "Ra" for *Ra*). In the case of *Rd* it is also established what chemical compound is the electron source, and this last information will be used in the yield

calculations. With the matrices Rd and Ra ready, MbT-tool sets up *Rc* taking into account the user selection of the microorganism empirical chemical formula and the N-source. This information is stored in a matrix called “b-biomass”. The next step is to make the thermodynamic calculations following the mathematical expressions of TEEM (taking into account the user selection between TEEM1 or TEEM2). If the TEEM2 model is selected, MbT-tool uses a value of the parameter “q” that represents the number of oxygenase reactions per mole of substrate (the default value for “q” is zero).

In the thermodynamic calculations, the Gibb’s standard free energy for *Rc* (ΔG_{pc}) is evaluated depending on the N-source and the estimated value of 3.33 kJ per gram cell. This value is referred to through the empirical cell formula of $C_5H_7O_2N$ (McCarty, 2007, 1971) and MbT-tool adjusts it depending on the user selection of the micro-organism empirical chemical formula.

The label to mark each empirical chemical formula presented in Table 3.3 is made with two words, the first one indicates the type or name of the micro-organism and the second one indicates the substrate where the experiments for the microbial growth took place. But in any case this nomenclature conditions the normal operation of MbT-tool, because the information valid to make the calculations is only the molar relationship between the four main elements (C, H, O, N).

To complete the information MbT-tool uses the value of ε selected by the user. With all this information, MbT-tool displays the half-reactions of Rd and Ra with their Gibb’s free energy, the Rc, Re, Rs, the portion of electrons (fe^o) to generate energy, the portion of electrons (fs^o) for cell synthesis, the energy-transfer-efficiency, the balanced reaction (R) in which the micro-organisms responsible for the process are included, and also the values of microbial yield using the units $g_{cells}\ mol_{donor}^{-1}$, $molC_{cells}\ mol_{donor}^{-1}$ and $molC_{cells}\ molC_{donor}^{-1}$. MbT-tool allows the user to download the displayed results or outputs generated when the button called download is used.

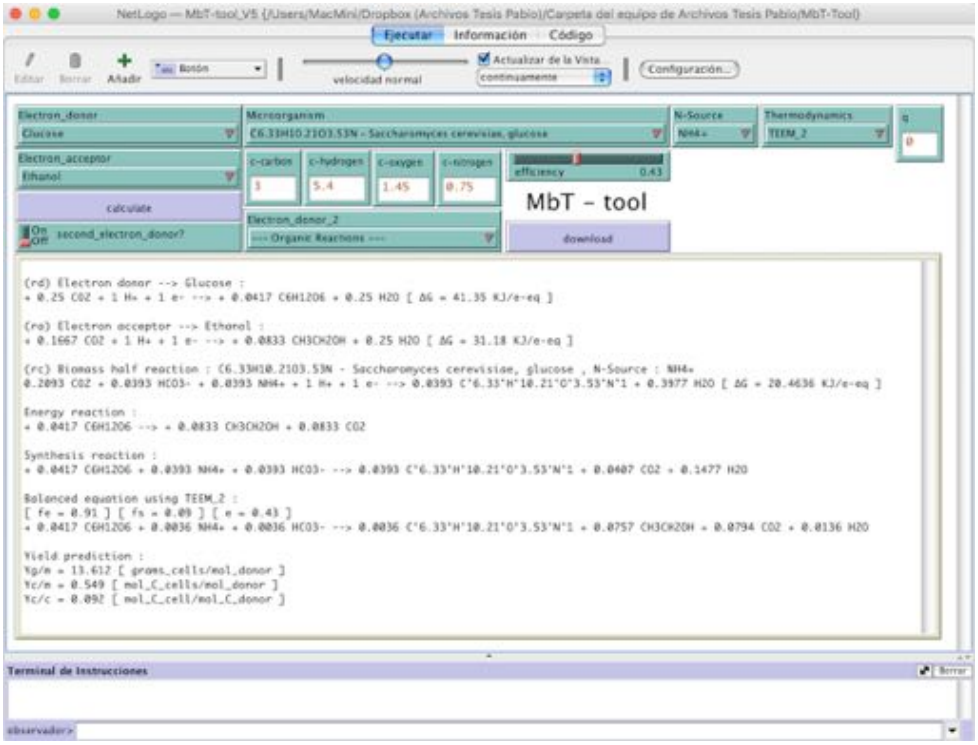

Figure 3.4. Screenshot of the MbT-tool’s user interface and the outputs generated in one execution.

3.3.3 How to add other half-reactions for e-donor and e-acceptor

The user could add another half-reaction using the chemical species programmed just by adding another row in the matrix *reactions*. It is also possible to add another chemical species just by adding another column to the matrix *reactions*. In the case that a new chemical species is added, it's necessary to add another reduction-half-reaction.

As an example of this procedure we will present the steps to add a new chemical species (NTA – Acid nitrilotriacetic) and it's half-reaction into the source code of MbT-Tool. The user must follow the steps below, to do it:

Step 1: Modify (increase) the number called "column_matrix": this indicates the number of chemical species programmed in the current version of MbT-tool. With the changes to this number the user modifies the number of rows and columns of the matrix *reactions*.

According to Table 3.4 the number of chemical species programmed is forty: since we want to add a new one this number will be increased by one unit. In the procedure *setup-reactions*, the NetLogo code will be:

set column_matrix 41

Step 2: Write the new half-reaction according to MbT-Tool's format (if the stoichiometric coefficient is positive it means *reaction reactant* and if it is negative it means *reaction product*) and the positions of the matrix *reactions* showed in Table 3.4

The half-reaction for NTA is:

$^{1}/_{18}\ NH_4^+ + {}^{6}/_{18}\ HCO_3^- + {}^{20}/_{18}\ H^+ + 1\ e^- \rightarrow {}^{1}/_{18}\ (C_6H_6O_6N)^{3-} + {}^{12}/_{18}\ H2O$ with ΔG^o = 68.889 KJ/e-eq

In the procedure *setup-reactions*, the NetLogo code will be:

matrix:set reactions 37 1 (20 / 18) ; H+

matrix:set reactions 37 2 (1) ; e-

matrix:set reactions 37 4 (1 / 18) ; NH4+

matrix:set reactions 37 5 (6 / 18) ; HCO3-

matrix:set reactions 37 7 (-12 / 18) ; H2O

matrix:set reactions 37 40 (-1 / 18) ; NTA - Acid nitrilotriacetic

matrix:set reactions 37 25 (68.889) ; ∆G

Each line of this code has a special structure: first, the NetLogo primitive "matrix:set", followed by the name of the matrix, in our case *reactions*, followed by a number which indicates the row of the matrix where the half-reaction will be added, followed by a number which indicates the column position for the stoichiometric coefficient according to Table 3.4, followed by a number between parentheses, this number is the stoichiometric coefficient of the chemical species in the reduction-half reaction (positive = reactant and negative = product, except in the case of the standard Gibbs energy this rule does not apply, see column 25 in matrix *reactions*), and finally a comment with the name of the chemical species.

Step 3: Add the new reaction name on the sliders to select "Electron_donor" and "Electron_acceptor".

Due to the fact that the new chemical species to be added is an organic one, the label for both sliders will be: "NTA – Acid nitrilotriacetic" into the label "organic reactions".

Step 4: Add new lines of code in the section-code to define the e-donor and the e-acceptor.

In the case of the e-donor, in the procedure *setup-electron-donor*, the NetLogo code will be:

if Electron_donor = "NTA - Acid nitrilotriacetic" [set rd matrix:submatrix reactions 37 0 38 column_matrix set c 40]

The structure of this code shows us: first, the condition to choose the e-donor labelled, and second the commands to execute if the condition is accomplished.

Using the NetLogo's primitive "matrix:submatrix" we define the matrix "rd" to storage the half-reaction to the e-donor, the numbers after the name of the source matrix (*reactions)* points out the row that will be taken from the matrix *reactions* according to the selection in the slider and finally the "c" value points out the position of this organic compound in the matrix *reaction* (procedure *setup-reaction*). This value is used in the procedure called *yield-prediction*.

In the case of the e-acceptor, in the procedure *setup-electron-acceptor*, the NetLogo code will be:

if Electron_acceptor = "NTA - Acid nitrilotriacetic" [set ra matrix:submatrix reactions 37 0 38 column_matrix]

The structure of this code is exactly the same as in the e-donor, but in the case of the e-acceptor we define the matrix "ra" to storage of the half-reaction for it.

Step 5: Add new lines of code in the procedure called "output_all".

The procedure *output_all* allows MbT-Tool to display its results. Therefore, the user has to add two new lines of code, one line to reactants and one line to products. The code for reactant will be:

ifelse (matrix:get to-print 0 40) <= 0 [ ][output-type "+ " output-type precision (matrix:get to-print 0 40) 4 output-type " (C6H6O6N)3- "]

And the code for products will be:

ifelse (matrix:get to-print 0 40) >= 0 [ ][output-type "+ " output-type precision abs (matrix:get to-print 0 40) 4 output-type " (C6H6O6N)3- "]

The structure of this code follow the syntaxes for the NetLogo's primitives: matrix:get, output-type and precision. The main difference is in the condition (<= 0 or >= 0) with this structure MbT-Tool establishes if the stoichiometric coefficient is a reactant or product.

3.4 Testing MbT-Tool

With MbT-tool the user could write MMRs for diverse metabolic functional groups of microorganisms such as aerobic heterotrophs, nitrifiers, denitrifiers, methanogens, sulphate reducers, sulphide oxidizers and alcoholic and lactic fermenters (Table 3.5).

We have chosen some different microbial processes to demonstrate the potential and versatility of MbT-tool showing the output of the simulator, which is a set of MMRs with the corresponding yield value.

In the experiments carried out by Battley (2013, 2007, 1995) related to the *Saccharomyces cerevisiae*, he has established $C_{6.33}H_{10.21}O_{3.53}N$ as the empirical chemical formula for this yeast. Considering this information, using the MbT-Tool it is possible to write two metabolic pathways: the biomass synthesis from glucose, and the biomass synthesis from pyruvate to ethanol. Before executing the MbT-Tool, it is necessary to determine some parameters. We used TEEM1 because we consider that there is no oxygenase reaction involved and the c-source is not a C1 compound. We used NH_4^+ because this chemical species is the universal N-source to biomass synthesis, and for ε

we used a value that allows us to obtain the reported cell yield growing on glucose of the 0.098 mol Ccells/mol Cdonor (Battley, 2013). Using the MbT-tool with the established inputs, theoretical considerations and TEEM1 with ε value of 0.57 for the first pathway and ε value of 0.84 for the second one, the MMRs and the yields are:

Biomass synthesis from glucose:

$C_6H_{12}O_6$ + 0.094 NH_4^+ + 2.25 HCO_3^- → 0.094 $C_{6.33}H_{10.21}O_{3.53}N$ + 2.16 CH_3COCOO^- + 1.17 CO_2 + 3.59 H_2O with an Yc/c = 0.099 mol Ccells/mol Cdonor.

Biomass synthesis from pyruvate to ethanol:

CH_3COCOO^- + 0.047 NH_4^+ + 1.325 H_2O → 0.047 $C_{6.33}H_{10.21}O_{3.53}N$ + 0.734 CH_3CH_2OH + 0.283 CO_2 + 0.953 HCO_3^- with an Yc/c = 0.098 mol Ccells/mol Cdonor.

In the MbT-tool, we selected a gram-negative bacterium with the empirical formula of $C_5H_7O_2N$ (VanBriesen and Rittmann, 2000). This bacterium degrades nitrilotriacetic acid (NTA) in the absence of molecular oxygen. To write the metabolic equation using NO_3^- as e-acceptor, NTA is used as the e-donor, and TEEM1 is used as the thermodynamic approach with an ε value of 0.33 (McCarty, 2007, 1971; Rittmann and McCarty, 2001; Stratton and McCarty, 1969; Xiao and VanBriesen, 2008, 2006). Wanner et al. (1990), reported a cell yield growing on NTA equal to 50.760 g cells/mol NTA for this microbial process, while the bacterial yield prediction obtained with the MbT-tool is 51.311 g cells /mol NTA. All outputs are presented in Figure 3.5.

```
(rd) Electron donor --> NTA - Acid nitrilotriacetic :
+ 0.0556 NH4+ + 0.3333 HCO3- + 1.1111 H+ + 1 e- --> + 0.0556 (C6H6O6N)3- + 0.6667 H2O
[ ΔG = 68.889 KJ/e-eq ]

(ra) Electron acceptor --> NO3- -> N2 :
+ 0.2 NO3- + 1.2 H+ + 1 e- --> + 0.1 N2 + 0.6 H2O [ ΔG = -72.2 KJ/e-eq ]

(rc) Biomass half reaction : C5H7O2N - Casein, aerobic , N-Source : NH4+
0.2 CO2 + 0.05 HCO3- + 0.05 NH4+ + 1 H+ + 1 e- --> 0.05 C'5'H'7'O'2'N'1 + 0.45 H2O
[ ΔG = 18.799 KJ/e-eq ]

Energy reaction :
+ 0.0556 (C6H6O6N)3- + 0.0667 H2O + 0.2 NO3- + 0.0889 H+ --> + 0.0556 NH4+ + 0.3333
HCO3- + 0.1 N2

Synthesis reaction :
+ 0.0556 (C6H6O6N)3- + 0.2 CO2 + 0.2167 H2O --> 0.05 C'5'H'7'O'2'N'1 + 0.1111 H+ +
0.0056 NH4+ + 0.2833 HCO3-

Balanced equation using TEEM_1 :
[ fe = 0.5 ] [ fs = 0.5 ] [ e = 0.33 ]
+ 0.0556 (C6H6O6N)3- + 0.1008 CO2 + 0.1423 H2O + 0.0992 NO3- --> 0.0252
C'5'H'7'O'2'N'1 + 0.0119 H+ + 0.0304 NH4+ + 0.3081 HCO3- + 0.0496 N2

Yield prediction :
Yg/m = 51.311 [ grams_cells/mol_donor ]
Yc/m = 2.268 [ mol_C_cells/mol_donor ]
Yc/c = 0.378 [ mol_C_cell/mol_C_donor ]
```

Figure 3.5. General outputs of MbT-tool for the calculations related to the degradation of nitrilotriacetic acid in the absence of molecular oxygen (using NO_3^- as e-acceptor) by a gram-negative bacterium.

In the published research carried out by H.W. van Verseveld (van Verseveld et al., 1979, 1977; van Verseveld and Stouthamer, 1978) related to the growth of *Paracoccus denitrificans*, and considering succinate as e-donor and various final e-acceptors, the

reported formula for this denitrifying bacterium was established as $C_3H_{5.4}O_{1.45}N_{0.75}$. To write MMRs, we considered succinate as C-source, NH_4^+ as N-source for biomass synthesis and the main e-acceptors involved in the de-nitrification pathway. Taking into account this information we used the MbT-tool with TEEM2 and different ε values to represent a sequence of four reduction reactions ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) using MMRs for this denitrifying bacterium.

First reaction ($NO_3^- \rightarrow NO_2^-$): $(C_4H_4O_4)^{2-}$ + 0.30 NH_4^+ + 4.55 **NO_3^-** → 0.40 $C_3H_{5.4}O_{1.45}N_{0.75}$ + 4.55 **NO_2^-** + 1.10 CO_2 + 1.70 HCO_3^- + 0.67 H_2O. (ε = 0.41).

Second reaction ($NO_2^- \rightarrow NO$): $(C_4H_4O_4)^{2-}$ + 0.58 NH_4^+ + 4.55 **NO_2^-** + 4.55 H^+ → 0.77 $C_3H_{5.4}O_{1.45}N_{0.75}$ + 4.55 **NO** + 0.26 CO_2 + 1.42 HCO_3^- + 2.64 H_2O. (ε = 0.84).

Third reaction ($NO \rightarrow N_2O$): $(C_4H_4O_4)^{2-}$ + 0.58 NH_4^+ + 4.55 **NO** → 0.77 $C_3H_{5.4}O_{1.45}N_{0.75}$ + 2.28 **N_2O** + 0.26 CO_2 + 1.42 HCO_3^- + 0.36 H_2O (ε = 0.56).

Final reaction ($N_2O \rightarrow N_2$): $(C_4H_4O_4)^{2-}$ + 0.58 NH_4^+ + 2.28 **N_2O** → 0.77 $C_3H_{5.4}O_{1.45}N_{0.75}$ + 2.28 **N_2** + 0.26 CO_2 + 1.42 HCO_3^- + 0.36 H_2O. (ε = 0.53).

Battley (1987) established $C_{3.85}H_{6.69}O_{1.78}N$ as the empirical chemical formula for *Escherichia coli*. Considering this information, using the MbT-Tool it is possible to represent its diauxic growth on glucose and lactose. With the obtained MMR, it is possible to begin a modelling project related to the quantitative determination of metabolic fluxes during co-utilization of two C-sources (Wendisch et al., 2000) or a modelling project related to the organization of metabolic reaction networks (Kremling et al., 2001). We used TEEM2 because we consider that at least one mono-oxygenase-catalyzed reaction is involved (q = 1) (Xiao and VanBriesen, 2008, 2006). We used NH_4^+ as N-source to biomass synthesis. We used two e-donors (glucose and lactate) and one e-acceptor (oxygen), and for ε we used a 0.37 value (McCarty, 2007). Using the MbT-tool with the established inputs, the MMRs and the yields are:

$C_6H_{12}O_6$ + $CH_3CHOHCOO^-$ + 5.05 O_2 + 1.018 NH_4^+ + 0.018 HCO_3^- → 1.018 $C_{3.85}H_{6.69}O_{1.78}N$ + 5.097 CO_2 + 7.133 H_2O

Bacterial yield: Yg/m = 97.139 (grams cells/mol donor); Yc/m = 3.917 (molC cells/mol donor) and Yc/c = 0.435 (molC cell/molC donor).

Table 3.5. Microbial types taking into account the e-donor and the e-acceptor. Adapted from Rittmann and McCarty (2001).

Microbial Group	e-donor	e-acceptor
Aerobic Heterotrophs	Organic half-reaction	O_2
Nitrifiers	NH_4^+ NO_2^-	O_2 O_2
Denitrifiers	Organic half-reaction H_2 S	NO_3^-, NO_2^- NO_3^-, NO_2^- NO_3^-, NO_2^-
Methanogens	H_2	CO_2
Sulfate reducers	Acetate H_2	SO_4^{2-} SO_4^{2-}
Sulfide oxidizers	H_2S	O_2
Fermenters	Organic half-reaction	Organic half-reaction

3.5. Final remarks

The main purpose of this chapter is to present the development of the MbT-Tool and make it available to a wide spectrum of readers, showing how this tool could be used in different research frameworks. This tool is a tangible way of achieving the compression of a thermodynamic application connected with microbial metabolism, therefore, it transfers a theoretical knowledge to a diverse range of applications of interest.

The MbT-tool is, as far as we know, the only open-access and open-source software, that allows the writing of MMRs based on thermodynamic concepts. To use the MbT-Tool, non-expert knowledge about microbial metabolism is necessary, and only the most basic organic and inorganic chemistry is enough. The MbT-Tool by itself does not simulate any microbiological, ecological or biotechnological process, but the results obtained from the MbT-Tool allow the user to start with the calculations or the simulations for the study of any of these research fields.

However, this tool has a limitation: it is not possible to compare the MbT-Tool's outputs directly with experimental results. We consider that only after the construction of a model with the MbT-Tool's results would the non-expert user be able to interpret its results. A non-expert user is considered here as a person whose expertise is not in the field of non-equilibrium thermodynamics, and therefore, to develop and apply all the conceptual elements involved in this thermodynamic approach for the construction of living models could be a time-consuming task. In contrast, a person with experience in these MMRs could have enough criteria to use and interpret the output reactions from MbT-tool in his academic or research context.

In the research field on IBMs, using the INDISIM methodology as a core model, for instance (Ginovart et al., 2005, 2002a; Ginovart and Cañadas, 2008; Gras et al., 2011; Gras and Ginovart, 2004; Portell et al., 2014), we realized that is essential to know the metabolic reactions carried out by the micro-organisms to increase or decrease their own biomass. These metabolic reactions could be used to design the individual metabolism model as well as to parameterize the IBM models. In this sense, we think that the MbT-Tool's outputs are a convenient means to advance with this type of model (Araujo Granda et al., 2016b, 2016c, 2015).

Finally, we consider that the user could take advantage of the MMRs provided by the MbT-tool in a wide range of academic or research fields, such as, for example: i) as a source to design processes that take advantage of the microbial system [4], and (ii) as the bedrock to make a connection between the microbial biomass and the substrates used by the micro-organism for pollution control for instance (Christensen and McCarty, 1975; Henze et al., 2006). Additionally, the NetLogo's rather flat learning curve and comprehensive documentation (Railsback and Grimm, 2012) make this a user-friendly tool, easily accessible to chemists, biologists, ecologists, engineers and modellers, among others.

In conclusion, the description of the cellular metabolism by means of thermodynamic concepts is strategic for investigating microbial activity and modelling bio-technological processes. We are convinced that the MbT-tool will facilitate users to think about the biochemistry of metabolism due to its simplicity of use, and its results could be an interesting starting point for a microbial modelling approach.

Chapter IV – An individual-based and thermodynamic model to deal with *Paracoccus denitrificans* in a bioreactor

4.1 Introduction

The individual-based model developed to deal with *Paracoccus denitrificans* is called INDISIM-Paracoccus (Araujo Granda et al., 2016c). It embeds a thermodynamic model for bacterial yield prediction in the individual-based model INDISIM, and is designed to simulate the bacterial cell population behaviour and the product dynamics within the culture.

The INDISIM-Paracoccus model assumes a culture medium containing succinate as a carbon source, NH_4^+ as a nitrogen source and various e-acceptors such as O_2, NO_3^-, NO_2^-, NO and N_2O to simulate in continuous or batch culture the different nutrient-dependent cell growth kinetics of the bacterium *Paracoccus denitrificans*.

The individuals in the model represent microbes and the IBM INDISIM gives the behaviour-rules that they use for their nutrient uptake and reproduction cycle. Three previously described metabolic pathways for *P. denitrificans* were selected and translated into MMR using MbT-Tool (Araujo Granda et al., 2016a). These stoichiometric reactions are an intracellular model for the individual behaviour-rules for metabolic maintenance and biomass synthesis, and result in the release of different N-oxides to the medium. The model was implemented using the NetLogo platform and it provides an interactive tool to investigate the different steps of denitrification carried out by a denitrifying bacterium.

In this chapter we show a detailed description on how to use TEEM to represent the metabolic pathways of the denitrifying bacterium *P. denitrificans*, and we explain the IBM named INDISIM-Paracoccus using ODD protocol (Grimm et al., 2010) .

4.2. *Paracoccus denitrificans* metabolic pathways

Intuitively, a complete model has a number of sub models that take into account the compartmentalized structure of living organisms. Therefore, the intracellular sub model for the individual behaviour-rules for cellular maintenance and biomass synthesis comes from representing the common metabolic pathways expressed in *P. denitrificans* through an MMR (Araujo Granda et al., 2016a, 2015) using TEEM (McCarty, 2007; Rittmann and McCarty, 2001).

P. denitrificans can survive in ecosystems with fluctuating aerobic and anaerobic conditions, because it can obtain the chemical energy from respiratory metabolism using molecular oxygen or nitrogen oxides dissolved in the medium; thus in aerobic phase conditions it can execute "*Aerobic respiration*" with O_2 as the e-acceptor and "*Nitrate reduction - Dissimilatory*" with NO_3^- as e-acceptor (Baker et al., 1998; Beijerinck, 1910; Caspi et al., 2012). Further, in anoxic conditions it has a respirative metabolism as well and it executes "*Nitrate reduction - Denitrification*" because it is capable of anaerobic growth in the presence of NO_3^-, NO_2^-, NO or N_2O as e-acceptor (Baumann et al., 1996; Bergaust et al., 2010; Bergaust et al., 2012; Caspi et al., 2012; van Verseveld et al., 1983).

Respiration is a process by which electrons are passed from an e-donor to a terminal e-acceptor. However, in respiration the electrons do not pass directly from the donor to the acceptor. Instead, they pass a number of membrane-bound electron carriers that function as a transport chain, passing the electrons from one to another in steps that follow the electrochemical gradients between the e-donor and the e-acceptor (Caspi et al., 2012). Each oxidized member of the electron transfer chain can be reduced by the reduced form

of the previous member, and the electrons flow through the chain all the way to the terminal acceptor, which could be oxygen in the case of aerobic respiration, or another type of molecule in anaerobic respiration (*dissimilatory reduction*). *Denitrification* is part of the global nitrogen cycle, returning biological nitrogen to the atmosphere and maintaining the balance of the nitrogen budget. In four reactions, catalyzed by the enzymes nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos), NO_3^- is successively reduced to N_2, with the nitrogen being transformed to the next lower oxidation state in each successive reaction (Caspi et al., 2012; Knowles, 1982) (Figure 4.1).

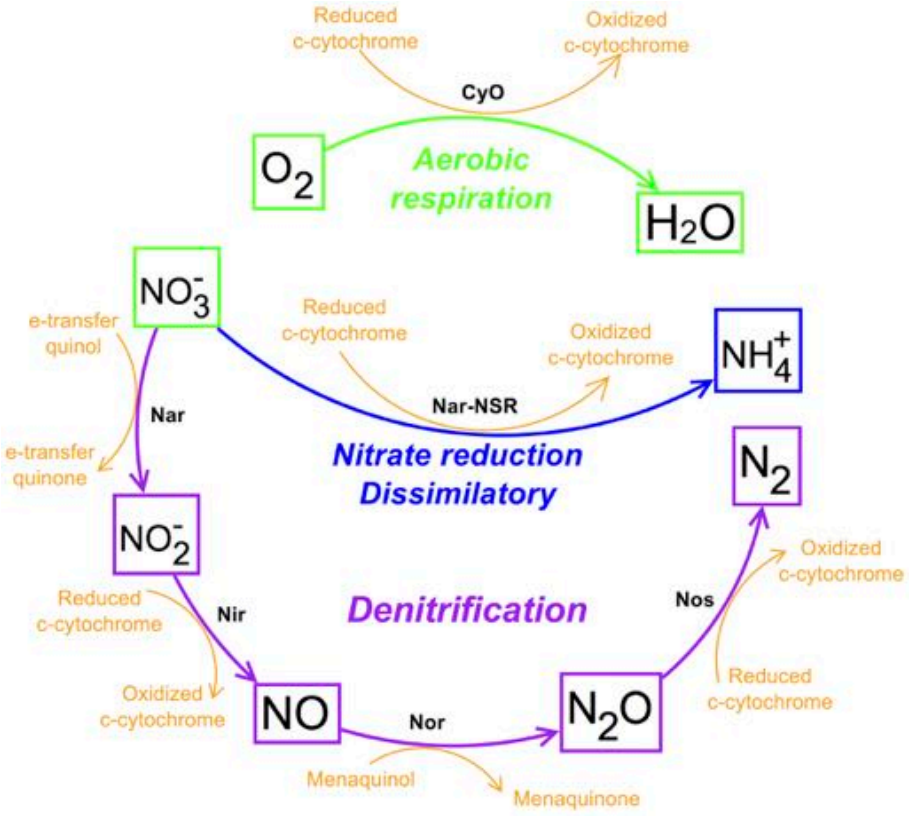

Figure 4.1 Metabolic pathways for *Paracoccus denitrificans*. Adapted from Caspi et al. (2012).

4.3 Cellular maintenance

To develop the individual behaviour-rule for cell maintenance it is necessary to write the energy reactions (Re) for aerobic and anaerobic conditions. For the aerobic phase we consider the reaction between succinate (which is always the e-donor) and O_2 as e-acceptor, while for the anaerobic phase the e-acceptors are N-oxides. To formulate MMRs that represent the cellular maintenance and calculate the corresponding stoichiometric coefficients, we used the reduction-half-reactions for Rd and different Ra shown in Table 3.1 and Table 3.2.

As an example, we explain step by step how to obtain the MMR for the cellular maintenance in aerobic phase.

Step 1. Choose from Table 3.1 the half-reaction for the e-donor (succinate) and from Table 3.2 the half-reaction for the e-acceptor (in this example, O_2). According to Rittmann and McCarty, (2001) following the equation (Re = Ra – Rd) a MMR can be written for the

energy reaction as follows.

Ra	$0.25\ O_2 + H^+ + e^- \rightarrow 0.50\ H_2O$
– Rd	$0.0714\ (C_4H_4O_4)^{2-} + 0.4285\ H_2O \rightarrow 0.1428\ CO_2 + 0.1428\ HCO_3^- + H^+ + e^-$
Re	$0.0714\ (C_4H_4O_4)^{2-} + 0.25\ O_2 \rightarrow 0.1428\ CO_2 + 0.1428\ HCO_3^- + 0.0714\ H_2O$

Step 2. To write the energy reaction in a standard way, we divide all of the stoichiometric coefficients by the e-donor coefficient (succinate).

Therefore, the aerobic maintenance with succinate and oxygen is represented by the equation: $\mathbf{(C_4H_4O_4)^{2-} + 3.5\ O_2 \rightarrow 2\ CO_2 + 2\ HCO_3^- + H_2O,}$ which is the MMR for the energy reaction to fit the individual behaviour-rule for aerobic maintenance.

Taking into account this methodology and using different e-acceptors for the anaerobic phase we can write all of the energy reactions for cellular maintenance in aerobic and anaerobic phase (Table 4.1).

Table 4.1. Balanced energy reactions (Re) for cellular maintenance in aerobic and anaerobic phase. Re = Ra – Rd according to (Rittmann and McCarty, 2001).

Rd	Re	Ra
Succinate	$(C_4H_4O_4)^{2-} + 3.5\ O_2 \rightarrow 2\ CO_2 + 2\ HCO_3^- + H_2O$	O_2
	$(C_4H_4O_4)^{2-} + 7\ NO_3^- \rightarrow 2\ CO_2 + 2\ HCO_3^- + 7\ NO_2^- + H_2O$	NO_3^-
	$(C_4H_4O_4)^{2-} + 14\ NO_2^- + 14\ H^+ \rightarrow 14\ NO + 2\ CO_2 + 2\ HCO_3^- + 2\ H_2O$	NO_2^-
	$(C_4H_4O_4)^{2-} + 14\ NO \rightarrow 7\ N_2O + 2\ CO_2 + 2\ HCO_3^- + H_2O$	NO
	$(C_4H_4O_4)^{2-} + 7\ N_2O \rightarrow 7\ N_2 + 2\ CO_2 + 2\ HCO_3^- + H_2O$	N_2O

With these energy reactions and appropriate maintenance requirements, the individual rule for cell maintenance is then designed, and will be executed before biomass synthesis.

4.4 Biomass synthesis

To develop the individual behaviour-rule for biomass synthesis it is necessary to write MMR for aerobic and anaerobic conditions. To formulate these reactions and calculate the corresponding stoichiometric coefficients we used the TEEM2 methodology. In all reactions succinate is the universal e-donor (Rd) and C-source, and NH_4^+ is the universal N-source to the cell synthesis (Rc). The nutrients used as e-acceptors (Ra) are different; in aerobic conditions they are O_2 and NO_3^- and in anaerobic conditions they are NO_3^-, NO_2^-, NO and N_2O.

As an example, we present step by step how to represent the first reaction in the denitrification pathway ($NO_3^- \rightarrow NO_2^-$) through an MMR using succinate as e-donor and C-source, NH_4^+ as N-source and NO_3^- as e- acceptor.

Step 1. To choose inorganic (Table 3.2) and organic (Table 3.1) half-reactions and their Gibb's standard free energy according to Rittmann and McCarty (2001) for Rd (succinate) and Ra (NO_3^-).

Step 2. To write the cell synthesis half-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) with NH_4^+ as N-source (Table 2.1).

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

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

Step 3. To adjust the standard Gibbs free energy of Rc with NH_4^+ as N-source. To do this Rittmann and McCarty (2001) proposed a general Rc with its standard Gibbs free energy:

$$^1/_5\,CO_2 + {}^1/_{20}\,NH_4^+ + {}^1/_{20}\,HCO_3^- + H^+ + e^- \rightarrow {}^1/_{20}\,C_5H_7O_2N + {}^9/_{20}\,H_2O$$

ΔG_{pc}^o = 18.80 kJ/eeq.

Taking into account this value and the reaction written in step 2.

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

Therefore, for *P. denitrificans* elementary cell composition the cell half-reaction (Rc) with NH4+ as N-source with Gibb's standard free energy is:

$$^9/_{49}\,CO_2 + {}^3/_{49}\,NH_4^+ + {}^3/_{49}\,HCO_3^- + H^+ + e^- \rightarrow {}^4/_{49}\,C_3H_{5,4}O_{1,45}N_{0,75} + {}^{106}/_{245}\,H_2O$$

ΔG_{pc}^o = 20.398 kJ/eeq

Step 4. To calculate the degree of reduction 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 5. To calculate the f_s^o, f_e^o and $Y_{c/c}$ according to McCarty (2007) (Table 2.2).

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

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 ε = 0.41, and assuming that standard conditions apply:

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

$$f_s^0 = \frac{1}{1+A} = \frac{1}{1+1.857} = 0.35$$

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

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

Step 6. To write the microbial metabolic reaction R = fe°Ra + fs°Rc – Rd according to Rittmann and McCarty (2001).

fe°Ra	0.3250 NO_3^- + 0.65 H^+ + 0.65 e^- → 0.3250 NO_2^- + 0.3250 H_2O
fs°Rc	0.064 CO_2 + 0.0214 NH_4^+ + 0.021 HCO_3^- + 0.35 H^+ + 0.35 e^- → 0.0286 $C_3H_{5,4}O_{1,45}N_{0,75}$ + 0.1514 H_2O
– Rd	0.0714 $(C_4H_4O_4)^{2-}$ + 0.4285 H_2O → 0.1428 CO_2 + 0.1428 HCO_3^- + 1.00 H^+ + 1.00 e^-
R	0.0714 $(C_4H_4O_4)^{2-}$ + 0.0214 NH_4^+ + 0.3250 NO_3^- → 0.0286 $C_3H_{5,4}O_{1,45}N_{0,75}$ + 0.3250 NO_2^- + 0.0786 CO_2 + 0.1214 HCO_3^- + 0.0479 H_2O

R is the MMR using TEEM2 to represent the first reaction of the denitrification pathway ($NO_3^- \rightarrow NO_2^-$).

Step 7. To write this reaction in a standard way, we divide all of the stoichiometric coefficients by the e-donor coefficient (succinate). Using TEEM2 methodology each MMR that represents the reaction inside the metabolic pathway accomplishes balances for carbon, nitrogen, electron and energy. In the same way we proceed with the calculations for the other reactions inside the denitrification pathway. The stoichiometric coefficients for each MMR were obtained following TEEM2 methodology with a different assigned ε value (Table 4.2).

The different ε value selected for each MMR is in agreement with those proposed by McCarty (1971, 2007) and Rittmann and McCarty (2001). Besides, the selected ε value provokes that the bacterial yield of each MMR is according to the experimental values reported. For *P. denitrificans* in aerobic phase growth, considering succinate as e-donor, Heijnen and Van Dijken (1992) proposed a maximum population growth yield (Yc/c) of 0.48 C_{mic}/C_{succ} and van Verseveld et al. (1983) of 0.51 C_{mic}/C_{succ}, and for *P. denitrificans* in anaerobic phase growth considering succinate as e-donor and NO_3^- as e-acceptor, Heijnen and Van Dijken (1992) published a Yc/c value of 0.387 C_{mic}/C_{succ} and van Verseveld et al. (1977) of 0.352 C_{mic}/C_{succ}. These population growth yields are the reference used to adjust each reaction using TEEM2 (Table 4.2).

Table 4.2. Microbial metabolic reactions (R) that represent aerobic and anaerobic pathways for *P. denitrificans* biomass synthesis, using different values of energy-transfer-efficiency (ε) according to TEEM2 (R = fe^oRa + fs^oRc – Rd) (McCarty, 2007).

#	Microbial metabolic reactions (R)	ε
R1	$(C_4H_4O_4)^{2-} + 0.60\ NH_4^+ + 1.04\ \mathbf{O_2}$ $\rightarrow 0.81\ C_3H_{5.4}O_{1.45}N_{0.75} + 0.19\ CO_2 + 1.40\ HCO_3^- + 0.34\ \mathbf{H_2O}$	0.84
R2	$(C_4H_4O_4)^{2-} + 0.08\ NH_4^+ + 0.52\ \mathbf{NO_3^-} + 1.05\ H^+ + 0.18\ H_2O$ $\rightarrow 0.80\ C_3H_{5.4}O_{1.45}N_{0.75} + 0.20\ CO_2 + 1.40\ HCO_3^-$	0.90
R3	$(C_4H_4O_4)^{2-} + 0.30\ NH_4^+ + 4.55\ \mathbf{NO_3^-}$ $\rightarrow 0.40\ C_3H_{5.4}O_{1.45}N_{0.75} + 4.55\ \mathbf{NO_2^-} + 1.10\ CO_2 + 1.70\ HCO_3^- + 0.67\ H_2O$	0.41
R4	$(C_4H_4O_4)^{2-} + 0.58\ NH_4^+ + 4.55\ \mathbf{NO_2^-} + 4.55\ H^+$ $\rightarrow 0.77\ C_3H_{5.4}O_{1.45}N_{0.75} + 4.55\ \mathbf{NO} + 0.26\ CO_2 + 1.42\ HCO_3^- + 2.64\ H_2O$	0.84
R5	$(C_4H_4O_4)^{2-} + 0.58\ NH_4^+ + 4.55\ \mathbf{NO}$ $\rightarrow 0.77\ C_3H_{5.4}O_{1.45}N_{0.75} + 2.28\ \mathbf{N_2O} + 0.26\ CO_2 + 1.42\ HCO_3^- + 0.36\ H_2O$	0.56
R6	$(C_4H_4O_4)^{2-} + 0.58\ NH_4^+ + 2.28\ \mathbf{N_2O}$ $\rightarrow 0.77\ C_3H_{5.4}O_{1.45}N_{0.75} + 2.28\ \mathbf{N_2} + 0.26\ CO_2 + 1.42\ HCO_3^- + 0.36\ H_2O$	0.53

R1 represents the pathway: *Aerobic respiration*, R2 represents the pathway: *Nitrate Reduction - Dissimilatory* in aerobic phase, and gathering the reactions R3, R4, R5 and R6 the pathway: *Nitrate Reduction – Denitrification* is represented (Caspi et al., 2012; Knowles, 1982; Zumft, 1997).

4.5 Model description

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

4.5.1 Purpose

To develop a computational model for the denitrification process carried out by the bacteria *P. denitrificans* growing in batch and continuous culture, in aerobic and anaerobic growing conditions, in order to reproduce a bioreactor experimental protocol and explore the consequence of different priorities in the individual use of nutrients on the system dynamics.

The first hypothesis (the Gibbs hypothesis) is that the denitrification reactions succeed sequentially according to their standard Gibbs energy, assuming that the bacterium first goes for the more spontaneous reactions. Reactions with lower Gibbs energy are expected to occur first. In this case the order is: R3, R6, R5 and R4 (Table 4.2). The second hypothesis (the metabolic hypothesis) is that the bacterial cell prioritizes the use of those nitrogen oxides with a higher degree of oxidation over others, which is the common order established in the denitrification pathway (Caspi et al., 2012). In this case the order is: R3, R4, R5 and R6 (Table 4.2).

4.5.2 Entities, State Variables, and Scales

The INDISIM-Paracoccus model has two types of entities: individuals and square patches of culture medium. Each individual represents a unique bacterium of *P. denitrificans* and has the variables: unique identification number, location (XY grid cell coordinates of where it is), mass, reproduction mass, and counters for each metabolic pathway and reproduction cycle.

Therefore, the model assumes that the smallest individual represents a bacterium with a diameter of ~ 0.5 μm and the largest one a bacterium with a diameter of ~ 0.9 μm (Holt et al., 1994). All bacteria have spherical shape and their individual mass is deduced from cell volume by assuming the microbial mass density equal to 1.1 $g \cdot cm^{-3}$, which has been used in previous INDISIM models (Gras et al., 2011). In order to characterize the composition of the microbial cells, the model uses the empirical formula $C_3H_{5.4}N_{0.75}O_{1.45}$ (van Verseveld et al., 1979, 1983) so that each bacterium is assumed to have this elementary cell composition.

A two-dimensional lattice of 25 x 25 grid cells represents the bioreactor that contains the culture medium; each cell represents 1 pl, so that the total bioreactor volume is 625 pl. The spatial cell variables are: unique position identifier in XY coordinates, total amount of each nutrient: succinate, NH_4^+, O_2, NO_3^-, and metabolic products, NO_2^-, NO, N_2O, N_2 and CO_2. All microbial and culture medium processes are discretized in time steps. One time step represents 5 min; for the current work the simulations were run for 1440 time steps (120 h).

With these units, graphical and numerical model outputs are the molar concentration of nutrients and metabolic products expressed in millimolar (mM) or micromolar (μM) and dry mass in $mg \cdot ml^{-1}$.

4.5.3 Process Overview and Scheduling

The initial configuration of our model has two parts: the first one for the system and the second one for the entities (culture medium and bacteria). The initial system setup sets the world size and topology, and the time scaled factor (time step).

The topology of the world is programmed using the torus mode; therefore, rectangular periodic boundary conditions were used. The initial culture medium concentrations and the initial bacterial population are established using random variables, normal probability distributions with mean values that are determined by the experimental procedure (Felgate et al., 2012).

At each time step a group of individuals are controlled using a set of time-dependent variables for each bacterium. All individuals perform the following processes: nutrient uptake, cellular maintenance, biomass synthesis, metabolic products generation and bipartition.

Culture medium processes are different depending on the management bioreactor protocol, but in any case the culture medium is randomly homogenized to simulate chemostat agitation. At the beginning of the simulation the bioreactor works as a batch culture with oxygen saturated conditions (236 μM), and the user can choose at what time to end this phase, and switch to continuous culture in anoxic conditions, with input-output culture media (with nutrients, metabolic products and micro-organisms) according to the dilution rate fixed.

For each time step the time-dependent variables of microorganisms and culture medium are calculated, updating the graphics and digital outputs according to the time scale proposed. The model also controls the whole carbon and nitrogen mass inside and outside of the system to ensure the carbon and nitrogen are balanced. During the

simulation processes the bacteria are called in a different random order in each time step and the state variables changes are immediately assigned generating an asynchronous update (Figure 4.2).

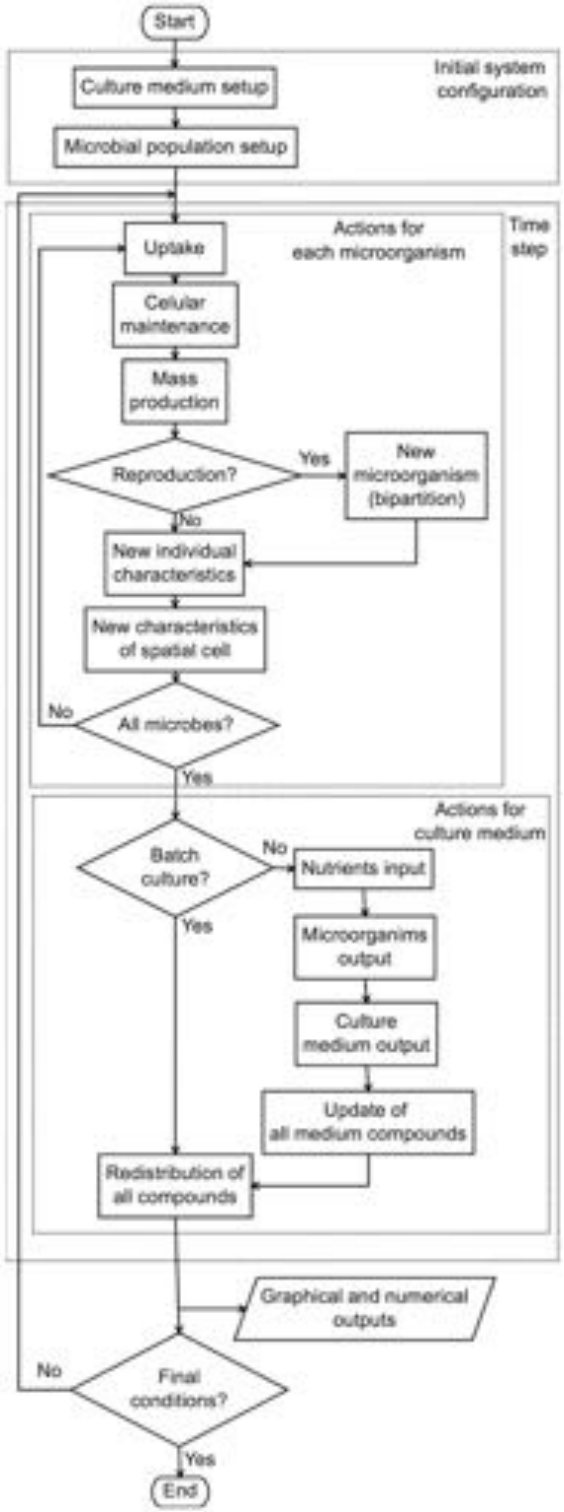

Figure 4.2. Flow chart of the INDISIM-Paracoccus model.

4.5.4 Design Concepts

4.5.4.1 Basic Principles

The model has two kinds of behaviour-rules, rules for the individuals (bacteria) and different rules for the environment (culture medium). The set of individuals and the environment is called the system (bioreactor). All of the rules are used at the level of the sub-models and they are explained in the corresponding section.

The individual rules are: i) nutrient uptake, ii) cellular maintenance (Table 4.1), iii) cellular growth when a micro-organism executes any of the metabolic reactions adjusted by TEEM2 (Table 4.2), and iv) cellular division following binary fission.

The system rules are those that mimic the general bioreactor procedures when it works as: i) a batch culture with constant oxygenation, with O_2 input flow, ii) a continuous culture with a dilution rate, with the entrance of fresh medium, and output of medium and bacteria, and iii) the stirring culture, with redistribution of compounds, which permits the exclusion of local diffusion limitations.

4.5.4.2 Emergence

The system dynamics emerge as the result of the interaction between bacteria and the culture medium that they find inside of the bioreactor.

The model outputs are: the biomass evolution, nutrient consumption, metabolic and/or denitrification products generation, or other parameters that appear at the system level as a consequence, and from, the individual bacterial activity.

4.5.4.3 Adaptation

All the individuals (bacteria) are programmed with the same rules; some of these rules will be executed and others not, depending on the internal changes of the individuals and/or the characteristics of their local environments. Individuals act one after another, not in parallel.

Hence, after one individual carries out all of its actions the composition of the spatial cell where it lives changes and the next individual meets a different medium composition in relation to the previous acting or post-acting individuals. In particular, the metabolic pathway that it might employ could be different.

The first individual rule is how to respond to the O_2 dissolved level in the culture medium: if the O_2 dissolved level in the spatial cell is lower than a threshold value ($O_{2\text{-}MIN}$) the bacterium uses the anaerobic metabolism and otherwise it uses the aerobic metabolism.

The second individual rule is performing biomass synthesis (Table 4.2) for growth and metabolic product generation. This rule is executed only when the amounts of nutrients taken in the uptake are enough to accomplish the maintenance requirement (Table 4.1) and after updated amounts also allow execution of the corresponding synthesis reaction (Table 4.2) in the aerobic or anaerobic phase.

The third individual rule is whether to divide or not, depending on whether or not it has reached the minimum reproduction mass. The reproduction mass (m_R) is the mass the bacterium must reach to start the bipartition (m_R is obtained from a normal random distribution with a mean value of 75% of the larger bacterium size) (Table 4.3).

4.5.4.4 Interaction

P. denitrificans 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.

4.5.4.5 Collective

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

4.5.4.6 Stochasticity

Several processes are modelled on criteria of randomness: i) the reproduction sub-model, ii) a portion of the uptake sub-model, iii) some parameters involved in the bioreactor management or operating protocol, and iv) a part of the initial system configuration. Inside the reproduction sub-model we consider that the reproduction threshold biomass for each bacterium is determined using a value from a normal random distribution (Table 4.3).

For the physical separation of the two bacteria the original mass is separated into two new bacteria with masses according to a value from the normal random distribution with mean value 0.5 and standard deviation 0.075 (Table 4.3). Thus, the mass of the original bacterium does not divide exactly in the proportion 50-50.

Inside the uptake sub-model we consider that at each time step, each individual nutrient uptake-rate *(u_i)* for each nutrient is established from a normal random distribution

with the mean value shown in Table 4.3 and a standard deviation of 5% of this value. Regarding the bioreactor management: i) the dilution rate parameter, for each input-output, is obtained by using the normal random distribution with mean value 0.05 h^{-1} and standard deviation 0.0025, in order to represent experimental error, and ii) each bacterium position randomly changes at some time steps in order to represent the mixing effects from the stirred tank.

For the initial system configuration we consider that the initial culture medium composition, the initial population biomasses and $O_{2\text{-MIN}}$ threshold value are established from normal distributions with mean values determined by the experimental procedure (Table 4.3) and standard deviations of 5% of these values.

To represent the small reactor with constant agitation, we introduce a redistribution of nutrients and metabolic products in random time steps. When the simulation starts each bacterium has a position randomly assigned in the culture medium.

4.5.4.7 Observation

The graphical and numerical outputs of the model are 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), microbial biomass ($mg \cdot ml^{-1}$) and the population biomass distribution at each time step (the user can obtain all simulated data in the output file with the extension ".txt").

4.5.5 Initialization

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

4.5.6 Sub models

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

4.5.6.1 Uptake

Each nutrient uptake depends on the individual's capacity to capture nutrients through the cell membrane-associated proteins (Button, 1998) and on the nutrient availability in the medium (Gras et al., 2011).

In our model, to determine the amount of each nutrient captured (absorbed) by each bacterium at each time step, two values are compared, the maximum uptake capacities (Ui, $mol_{nutrient} \cdot h^{-1}$) of the bacterium and the nutrient available in the culture medium (Ai, $mol_{nutrient} \cdot h^{-1}$), and the lowest value is chosen.

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

Ui = u_i * individual-mass

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

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

Ai = a_i * nutrient-amount

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

Following the INDISIM framework (Gras et al., 2011) the maximum population growth rate (μ_{max}) has been used to estimate the individual maximum uptake-rates (u_i). vanVerseveld et al. (1983) reported for *P. denitrificans* a growth rate value equal to 0.418 h^{-1} which was obtained in the change from a culture growing in anaerobic NO_3^--limited conditions to aerobic succinate-limited conditions.

Using this value and performing calculations with the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2, we obtained the maximum uptake-rate for each nutrient (Table 4.3).

In order to give values to the availability coefficient (a_i), and only as a macro reference to sort and represent numerically the availabilities of the nutrients in the culture medium, the Fick's law binary diffusion coefficients (Dab) in water was used. Therefore we assumed in the modelling process that the nutrient with maximum Dab has the highest availability; the other availability values are assigned proportionally (Table 4.3).

4.5.6.2 Maintenance

Before biomass synthesis, it is necessary that each bacterium achieve some energetic requirements to ensure its viability. The cellular maintenance sub-model has two main components, the maintenance requirement and the energy reactions (Re) written with TEEM2 (Table 4.1).

The maintenance requirements are proportional to the individual's mass. The coefficients determine an amount of nutrients per time step for cellular maintenance. Gras et al. (2011) consider an appropriate maintenance requirement for soil heterotrophic microorganisms of 0.002 $gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$, which was assumed in the model for aerobic phase. For *P. denitrificans* in anaerobic phase growth and taking succinate as e-donor and NO_3^- as e-acceptor, van Verseveld et al. (1977) give a maintenance coefficient of 0.004 $gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$ which was assumed for anaerobic phase.

The energy reactions (Table 4.1) indicate the stoichiometry that the nutrients follow when each bacterium executes this action or rule. Each bacterium achieves its maintenance when the amount of nutrients taken in is enough to accomplish the maintenance requirement and these amounts also allow it to execute the corresponding energy reaction.

Performing calculations with the energy reactions (Table 4.1), we establish the maintenance requirements for aerobic and anaerobic phases. When the individual carries out its maintenance, the CO_2 and the reduced e-acceptors are expelled to the culture medium except for the NO_2^-, which is added to its corresponding intake.

In anaerobic phase the first individual option is to accomplish the maintenance requirement, carrying out the energy reaction with succinate and NO_3^-. If the bacterium cannot reach its maintenance requirements, it can try with succinate and another e-acceptor following other reactions according to the hypothesis test.

After the maintenance, if the remaining succinate taken up and the quantity of e-acceptors are higher than zero, the individual can perform biomass synthesis.

4.5.6.3 Biomass synthesis and metabolic products

With the nutrient intakes updated and using the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table 4.2), each bacterium divides the amount of each nutrient taken up by its respective stoichiometric coefficient and selects the smallest value (the limiting nutrient).

This information provides the demands of each one of the nutrients and drives the creation of new mass and metabolic products generation. After executing any metabolic reaction the CO_2 produced is released to the culture medium.

When the bacterium executes the reactions of denitrification, nitrogen oxides are produced, and they are not expelled into the culture medium; only the nitrogen gas is expelled, and the amounts of nitrogen oxides generated are added to its corresponding intakes.

The execution of each metabolic reaction is limited to the existence of sufficient quantities of e-donors and acceptors. After this, if there are any intakes, the microbe can perform the next metabolic reaction. When this condition is not fulfilled the syntheses finish and the remaining intakes are returned to the culture medium.

4.5.6.4 Bioreactor operation

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

- *Agitation.* To represent the agitation inside the small experimental reactor, which causes homogeneity in the culture medium, nutrients and metabolic products are redistributed in various time steps. In the culture medium the microorganism positions change randomly,
- *Input flow.* The bioreactor is refilled with fresh culture medium (succinate, NH_4^+ and NO_3^-) with a composition equal to the initial one, and
- *Output flow.* A fraction of the individuals and culture medium are randomly removed. The input and output flows are performed according to the dilution rate parameter.

Table 4.3. INDISIM-Paracoccus model parameters values.

Nutrient	Culture medium initial concentration [mM] Felgate et al. (2012)	Availability coefficient – a_i (h^{-1}) fixed according to *Dab*	Uptake-rate – u_i ($mol_{nutrient} \cdot mol_{mass}^{-1} \cdot h^{-1}$)			
			Testing values			Calibrated Values
			Low (L)	Medium (M)	High (H)	
Succinate	5 [c] – 20 [d]	0.28 [a,b,e,f]	0.065	0.13	0.52 [g]	0.52 [a,b,e,f]
Ammonium	10 [c,d]	0.84 [a,b,e,f]	------	------	0.31 [g]	0.31 [a,b,e,f]
Oxygen	0.236 [c,d]	0.79 [a,b,e,f]	------	------	0.54 [g]	0.54 [a,e,f]
Nitrate-a (aerobic)	4.9983 [d] – 21.6095 [c]	0.63 [a,b,e,f]	0.034	0.068	0.27 [g]	0.27 [a,e,f]
Nitrate-x (anaerobic)			0.019	0.119	1.19 [g,h]	0.119 [b,e,f]
Nitrite	0.0255 [c] – 0.0112 [d]	0.79 [a,b,e,f]	0.0062	0.062	0.62 [g,h]	0.062 [b,e] – 0.62 [b,f]
Nitric Oxide	------	1.00 [a,b,e,f]	0.0000062	0.00062	0.62 [g,h]	0.62 [b,e,f]
Nitrous Oxide	0.003 [c] – 0.000028 [d]	0.50 [a,b,e,f]	0.0031	0.031	0.31 [g,h]	0.31 [b,e,f]

Other bacterial parameters

Parameter	Testing range	Calibrated value	Reference
Cellular maintenance ($gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$)	-----	0.0020 [a] – 0.0040 [b]	Gras et al. (2011) and van Verseveld et al. (1983)
Mass split	-----	0.50 (15% coefficient of variation)	Derived from (Ginovart et al., 2002a)
Small bacterium size (μm)	0.4 – 0.6 [a,b]	0.5 [a,b]	Holt et al. (1994)
Big bacterium size (μm)	0.8 – 1.0 [a,b]	0.9 [a,b]	
Minimum bacterium size at reproduction	-----	75% of big bacterium size (15% coefficient of variation)	Derived from (Gras et al., 2011) and (Ginovart et al., 2002a)

Phase: (a) Aerobic, (b) Anaerobic. Experiment: (c) Succinate-limited/NO_3^--sufficient, (d) Succinate-sufficient/NO_3^--limited. Hypothesis: (e) Metabolic, (f) Gibbs. The values (g) are the result of 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 3.2-3). The values (h) are the result of dividing each high uptake-rate by 4 due to the maximum growth rate being achieved when the four reactions are carried out by the bacterium.

Chapter V – Study of two metabolic hypotheses to denitrification pathway using INDISIM-Paracoccus

5.1 Introduction

In previous chapters it was explained how metabolic pathways are represented through a set of microbial metabolic reactions formulated by a non-equilibrium thermodynamic approach. With this stoichiometric information the metabolic dependent sub-models are designed and included in the IBM INDISIM-Paracoccus.

The objective of this chapter is to investigate the effects of the priority in the use of different e-acceptors at the microbial level, formulating two hypotheses about the order in which the reactions are followed by the bacteria *P. denitrificans* while the denitrification process occurs.

These two hypotheses are tested with the simulator developed, INDISIM-Paracoccus, and comparing the simulation outputs with a set of experimental data reported in Felgate et al. (2012).

The first hypothesis (the "Gibbs hypothesis") assumes that the denitrification reactions succeed sequentially according to their standard Gibbs energy; the bacterium goes first for the more spontaneous reactions. Reactions with lower Gibbs energy are expected to occur first, in this case the order is: R3, R6, R5 and R4 (Table 4.2).

The second hypothesis (the "Metabolic hypothesis") assumes that the bacterial cell prioritizes the use of those N-oxides with a higher degree of oxidation over others, which is the common order established in the denitrification pathway (Caspi et al., 2012). In this case the order is: R3, R4, R5 and R6 (Table 4.2).

5.2. Experimental data

To examine the denitrification process, Felgate et al. (2012) cultured *P. denitrificans* in a bioreactor growing under batch conditions in an O_2-saturated medium for 24 hours. The aeration was then shut down and the system switched to continuous culture with a dilution rate of 0.05 h^{-1}. Under these conditions two experiments took place, one in which the reservoir medium feed contained 20mM NO_3^-, 5mM succinate and 10mM NH_4^+, which was designed to achieve an e-donor-limited/e-acceptor-sufficient steady state (succinate-limited/NO_3^--sufficient). In the other experiment the reservoir medium feed contained 5mM NO_3^-, 20mM succinate and 10mM NH_4^+ to achieve an e-donor-sufficient/e-acceptor-limited steady state (succinate-sufficient/NO_3^--limited).

The data was collected from 0 to 120 hours (Felgate et al., 2012). Therefore, the bacteria will grow and develop in two different conditions: the first during the aerobic phase (from 0 to 24 h) in a batch culture, and the second one during the anaerobic phase (from 24 to 120 h) in a continuous culture.

Therefore, the bacteria will be handled in two different experiments: (i) in conditions of succinate-sufficient/NO_3^--limited (experiment E1), and (ii) in conditions succinate-limited/NO_3^--sufficient (experiment E2). The two hypotheses proposed (the "Metabolic hypothesis" and the "Gibbs hypothesis") will be tested using the INDISIM-Paracoccus and taking into account all these laboratory conditions.

5.3. Utilization of the model and analysis of the results

The model is implemented in the widely used, free and open source IBM software platform NetLogo (Wilensky, 1999) (Figure 5.1). Given that the main purposes of the virtual experiments performed with the computational model obtained are principally exploratory as to the nature and dynamics of the bio-system, the model is not designed for predictive purposes.

A best-fit calibration (i.e., a calibration aiming for one unique set of parameter values giving model results best matched to some exact criteria) was avoided. Instead a categorical calibration, which searches for parameter values producing results within a category or range defined as acceptable, was performed (Railsback and Grimm, 2012).

The multiple outputs used to test models is one of the main features of the pattern-oriented modelling strategy and very valuable for IBMs. Once a system representation is built, a depth exploration of how well the model really explains observed phenomena can be carried out with a quantitative analysis (Thiele, 2014; Thiele et al., 2014).

To assess the two hypotheses (the "Metabolic hypothesis" and the "Gibbs hypothesis") and to facilitate parameter estimation, we established multiple fitting criteria using the parameter uptake-rate for all nutrients involved with the experimental data of Felgate et al. (2012). The basic idea is to find ranges of these uptake-rate values that make it possible to roughly reproduce the evolution of a set of focus variables or patterns observed in the two experiments.

Taking into account that the bacteria grow in aerobic and anaerobic conditions, to calibrate the model in aerobic conditions we combined the uptake-rates for succinate (uSuccinate) and NO_3^- in aerobic phase (uNitrate-a). To calibrate the model in anaerobic conditions we first combined the uptake-rates for succinate and NO_3^- in anaerobic phase (uNitrate-x). After that we combined the uptake-rate of nitrate-x with the uptake-rates of NO_2^-, NO and N_2O. Then we combined the up-take-rate of NO_2^- with the uptake-rates of NO and N_2O, and finally we combined the uptake-rate of NO with the uptake-rate of N_2O. Therefore, the parameters are combined in pairs, and in all cases two parameters change and the others remain constant.

To assess whether a certain combination of parameter values leads to acceptable model output, we calculate a score based on the evaluation of the seven patterns controlled for each of the experiments E1 and E2. Therefore, to appraise: (i) in each of the temporal evolutions of microbial biomass, NO_3^-, NO_2^- and N_2O, if the simulation result agrees well with the experimental data 1 point is assigned, if agreement is fair 0.5 points are assigned and if agreement is poor 0 points are assigned, (ii) regarding the NO concentration in the culture medium, 1 point is assigned if the maximum NO value is under 10^{-3} mM, 0.5 points if the value is in the range [10^{-3}, 0.5] mM and 0 points in all other cases, iii) regarding the N_2 production, 1 point is assigned if the N_2 production is in the range reported by Felgate et al. (2012) and 0 points in other cases, and (iv) regarding the role of succinate or NO_3^- as a limited-nutrient during the steady state, if the simulation result agrees well with the experimental data 1 point is assigned, if agreement is fair 0.5 points are assigned and if agreement is poor 0 points are assigned.

Taking into account the sum of points achieved for each combination and each experiment (7 patterns x 2 experiments = 14 items assessed), we use a percentage to represent a global model adequacy for the experimental data. This percentage is represented using bubble graphs, where the bubble radius is proportional to the percentage.

The tool "Behaviour-Space" incorporated in NetLogo was used for running simulation experiments with varying parameters and writing model outputs to files that were used for the fitting criteria.

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

5.4 Results and discussion

INDISIM-Paracoccus was implemented in the NetLogo platform (Figure 5.1). It is straightforward to change parameter values, to modify the source code of the model and to investigate alternative mechanisms or add additional processes relevant to a particular study. It is hoped that this NetLogo simulator will facilitate new fruitful interactions between modellers and experts in the field of denitrification.

We used a variety of measures and basic techniques in order to verify that our implementation was in accordance with the conceptual model and its quantification. For instance, to ensure that the stoichiometry and the bioreactor inputs/outputs are accurately implemented, one of the main tasks was to control the differences between C and N levels, to ensure that the simulator accomplished balances for C and N. For each time step the following is controlled: i) the entrance and exit of all chemical species involved, ii) the product generations and nutrient consumptions from each balanced chemical equation executed, and iii) the bacteria inside and outside of the system.

At the end of every time step, from each one of the patches, the simulator obtains the amount of each nutrient and metabolic product, and for each one of the bacteria the value of its mass.

To ensure that the C and N are balanced, the model implementation summarizes all of the C and N inside and outside of the system and compares this value with the same calculation obtained in the previous time step. We expected that these two values should be the same, but there exist small differences (not higher than 0.05%); the simulator registers these and presents them as graphical and numerical outputs (Figure 5.1).

We also tested that the individuals were able to carry out all of the reactions in a variety of culture media compositions. In addition, we systematically investigated internal model logic and behaviours by collecting global and individual data through the simulation, which were numerically and visually tested (Figure 5.1).

The control of the different metabolic pathways used in each time step and for each bacterium is programmed in the computer code, and the simulator facilitates which pathways are in use and which not.

The main metabolic differences are present when the model runs with different conditions from experiments (E1 or E2), corresponding to the experimental condition of succinate limited or NO_3^- limited. Also, in the anaerobic conditions, it is possible to follow (control) the number of bacteria, which do not complete the denitrification pathway or follow (control) the metabolic pathway (synthesis or maintenance) that is the most used by the bacteria. This control is a graphical output in the model's implementation on NetLogo that appears in the user interface of the simulator (Figure 5.1).

Such tests are essential for increasing the reliability of the computational model, and for contributing to the understanding of the virtual system and the consequences of the modeling assumptions (Scheller et al., 2010).

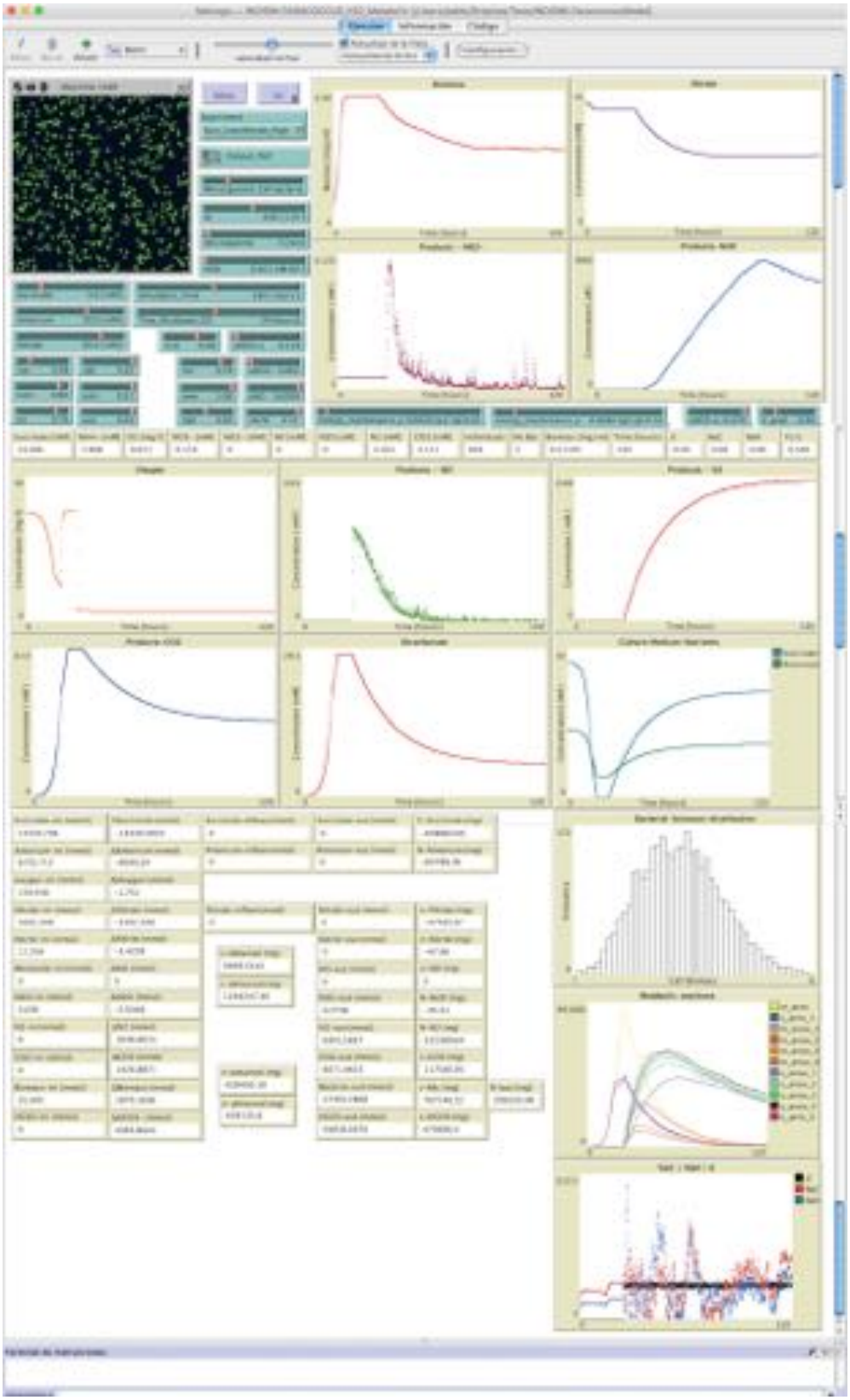

Figure 5.1. User interface of the INDISIM-Paracoccus simulator in NetLogo. The sliders allow the user to set all model parameters. Simulation results are provided with numerical monitors and graphical outputs of temporal evolutions for some system variables, individual mass distributions and frequency that bacteria use each metabolic reaction.

5.4.1 Sensitivity analysis of the grid size and time step

We have carried out simulations to study the influence of the grid size and the number of individuals on the evolution of the focus variables. Then four different grid sizes were planned and the number of individuals in these simulations was increased proportionally. We changed the size of the system, from 25 x 25 = 625 patches of culture medium to 100 x 100 = 10000 patches. The number of individuals in these simulations was increased as well, from 3000 (in the smallest system) to around 50000 individuals (in the biggest system). The model's outputs for these sets of simulations were very similar (Figs. Figure 5.2 and Figure 5.3).

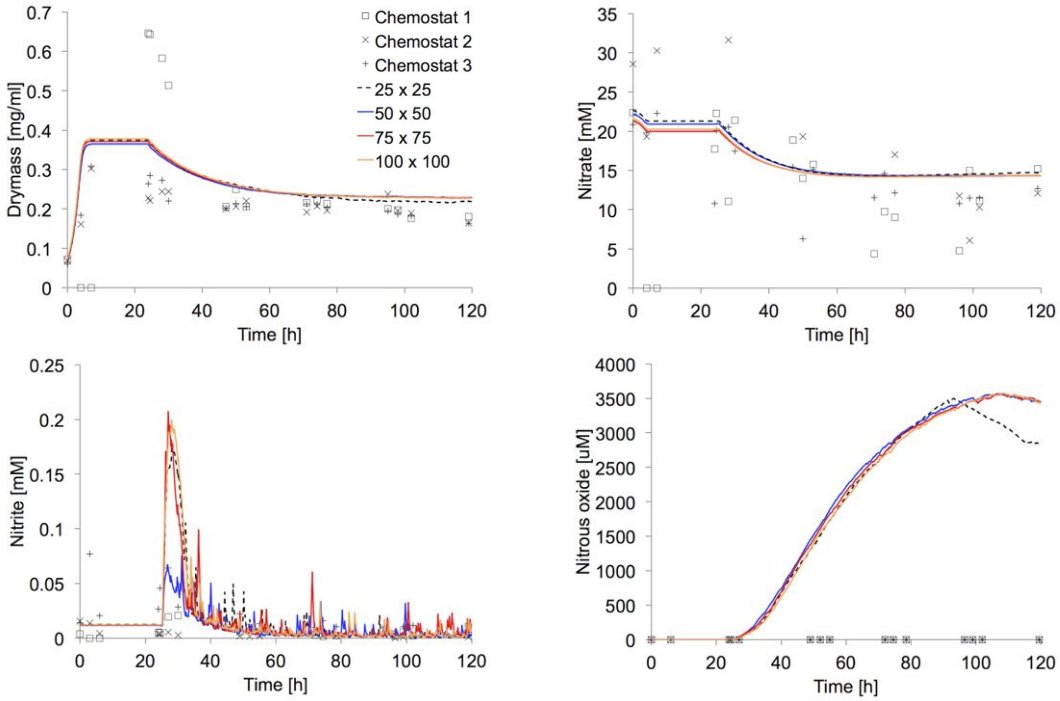

Figure 5.2. INDISIM-Paracoccus model calibration tests: experiment succinate-limited/NO_3^--sufficient in aerobic and anaerobic phase. Each coloured line represents a simulation result with different values of the system size. Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results. The time step assumed in the simulator to generate these results was 5 minutes.

With the increase of the system sizes the time spent in the simulations increased along with computational requirements. Taking into account these results we decided to establish a system size in 25 x 25 patches that was not too large, but was sufficient to allow us to obtain simulation results that we could compare with the experimental data available.

Also, we investigated the time step before fixing it in our simulations, and finally, this was established at 5 min. We tested the model using values from 1 to 10 min (Figure 5.4 and Figure 5.5). This is one of the parameters that can be changed in the initialization of the system before starting the simulation.

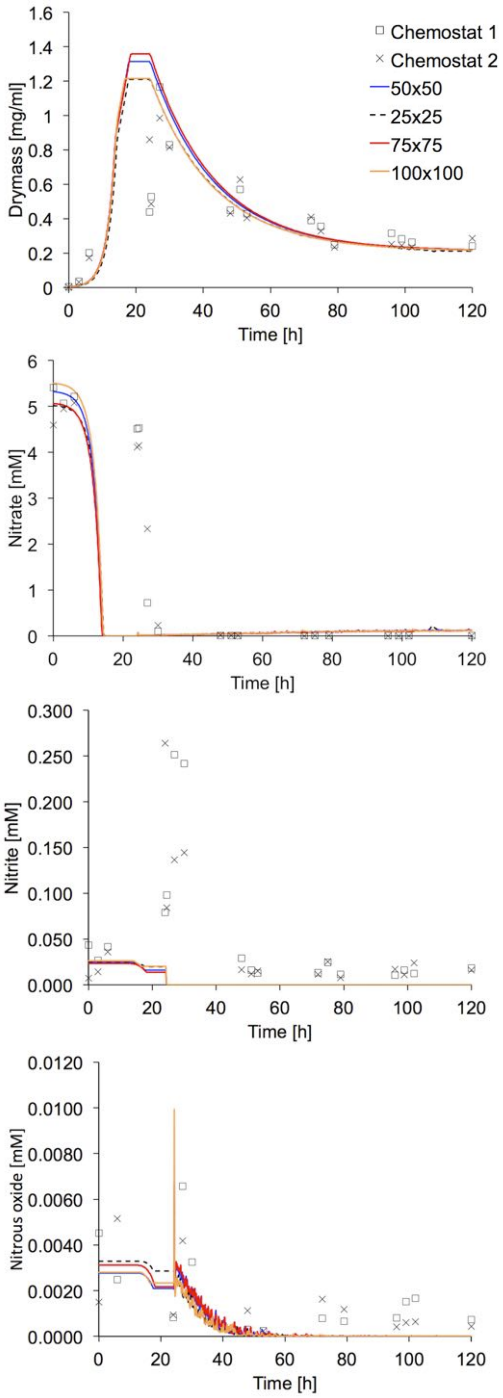

Figure 5.3. INDISIM-Paracoccus model results: experiment succinate-sufficient/NO_3^--limited in aerobic and anaerobic phase. Each coloured line represents a simulation result with different values of the system size. Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results. The time step assumed in the simulator was 5 minutes.

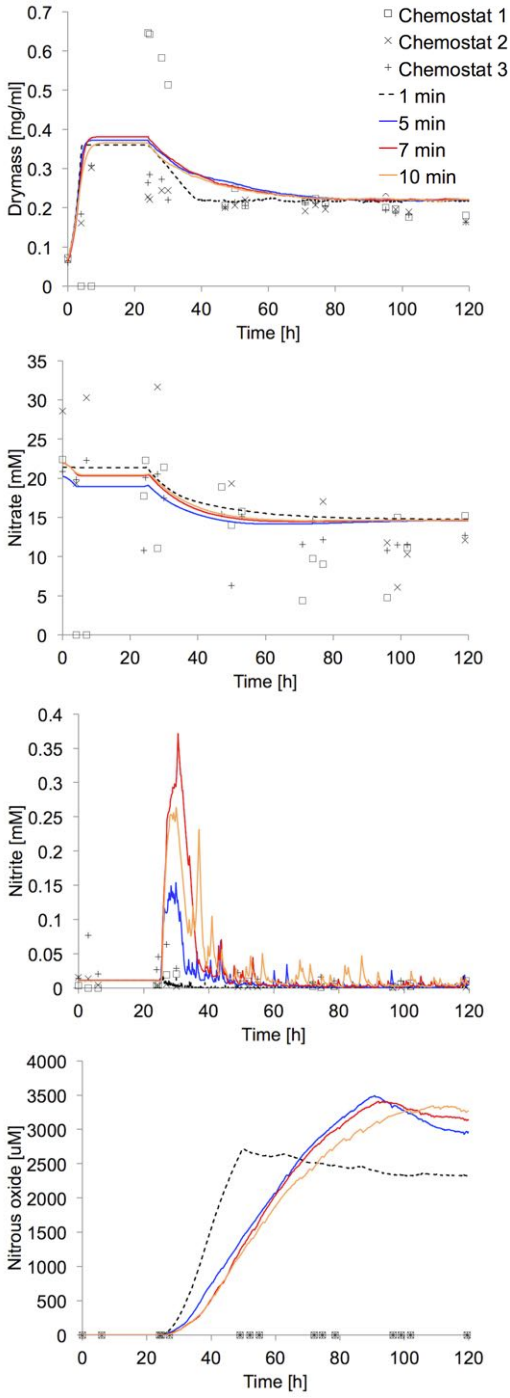

Figure 5.4. INDISIM-Paracoccus model results: experiment succinate-limited/NO_3^--sufficient in aerobic and anaerobic phase. Each coloured line represents a simulation result with different values of the step-time. Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results. The system size assumed in the simulator to generate these results was 25x25 patches.

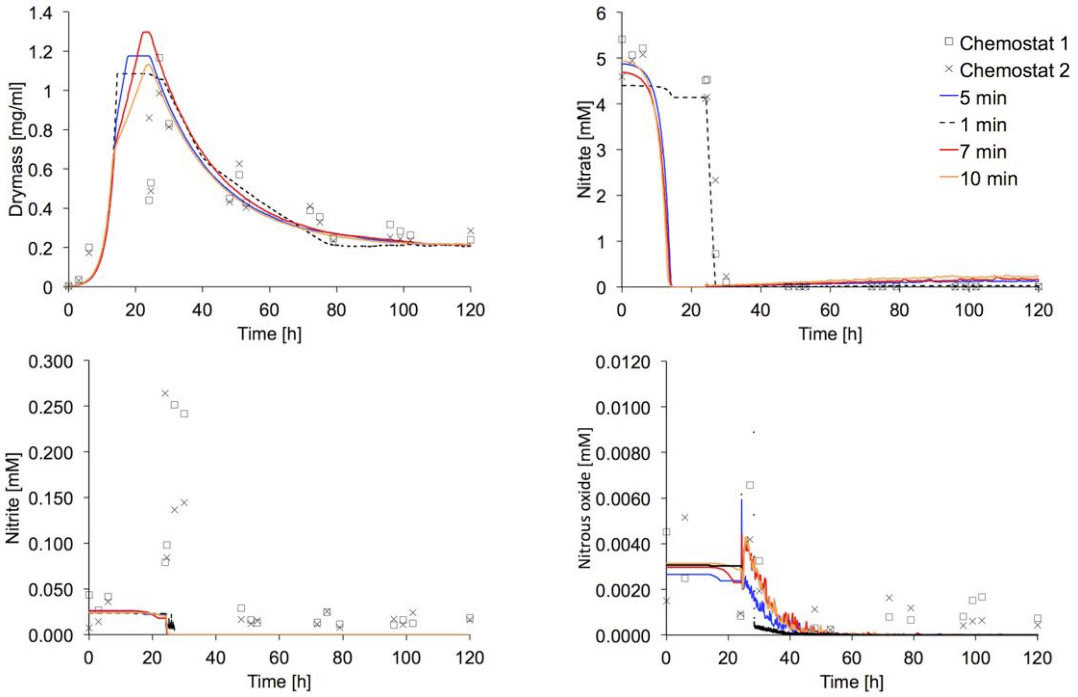

Figure 5.5. INDISIM-Paracoccus model calibration tests: experiment succinate-sufficient/NO_3^--limited in aerobic and anaerobic phase. Each color line represents a simulation result with different values for the time step. Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results. The system size assumed in the simulator to generate these results was 25x25 patches.

5.4.2 Results from the categorical calibration process with both experiments and the two hypotheses

During the development of the model some parameter values were obtained from biological constraints or references and were used in all simulations performed (Table 4.3), but others were not fixed due to the uncertainty in, or complete lack of, observational data.

We used the uptake-rate parameters with a set of simulation series during the categorical calibration process (Table 4.3). The simulation outputs were compared with the experimental data under the two different scenarios corresponding to the two experimental conditions of Felgate et al. (2012): succinate-sufficient/NO_3^--limited (Experiment E1) and succinate-limited/NO_3^--sufficient (Experiment E2) and for each of the hypotheses considered (the "Metabolic hypothesis" and the "Gibbs hypothesis").

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

Each curve in Figure 5.6 , Figure 5.7 and Figure 5.8 represents the simulation result for one combination of values of the parameters "uptake-rate" for the different nutrients. The bacteria in our model grow and develop in two very different conditions: the first condition occurs in aerobic phase (from 0 to 24 hours) in batch culture, and the second one occurs in anaerobic phase (from 24 to 120 hours) in continuous culture.

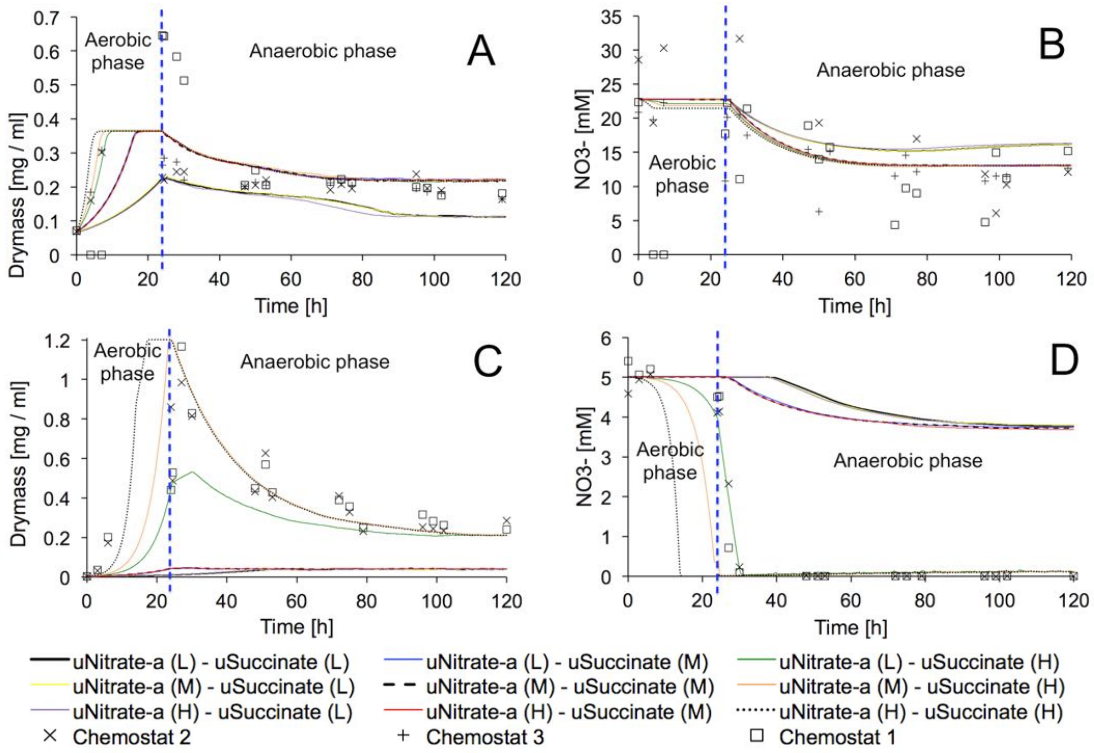

Figure 5.6 INDISIM-Paracoccus model calibration outputs for the aerobic phase. Each coloured line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and NO_3^- in aerobic phase. The u_i values are reported in Table 4.3 (being L = low, M = medium and H = high). Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). For the experiment E1, succinate-limited/NO_3^--sufficient (A and B) and for the experiment E2, succinate-sufficient/NO_3^--limited (C and D). The aerobic phase (from 0 to 24 hours) in batch culture, and the anaerobic phase (from 24 to 120 hours) in continuous culture. The "Metabolic hypothesis" has been assumed in the simulator to generate these results.

The first series of simulations (Figure 5.6) were carried out to explore the succinate and NO_3^- uptake-rate values during the aerobic phase (Table 4.3). In all cases, some simulation results shown in Figure 5.6 -A, Figure 5.6 -B and Figure 5.6 -C reproduce the experimental trend (Felgate et al., 2012) in a better way than other simulation results shown in Figure 5.6 -D, but in any case the state achieved in the aerobic phase determined the subsequent denitrification.

As the two hypotheses we tested (metabolic and Gibbs) do not play any role in the aerobic phase, the results obtained with a different combination of uptake-rate values for succinate and NO_3^- in the aerobic phase are a consequence of nutrient usage. When the uptake-rate for NO_3^- in aerobic phase takes the value of the maximum nutrient uptake-rate (uNitrate-a = 0.27 $mol_{nitrate\text{-}a} \cdot mol_{mass}^{-1} \cdot h^{-1}$, the high value in Table 4.3) the fit of the model is acceptable (Figure 5.9-A).

With the uptake-rate value of NO_3^- (aerobic phase) fixed, our second series of simulations explored combinations of uptake-rates for succinate (uSuccinate) and NO_3^- in the anaerobic phase (uNitrate-x). In Figure 5.7 we present the simulation results with the experimental data corresponding to experiment E1 and in Figure 5.8 to experiment E2.

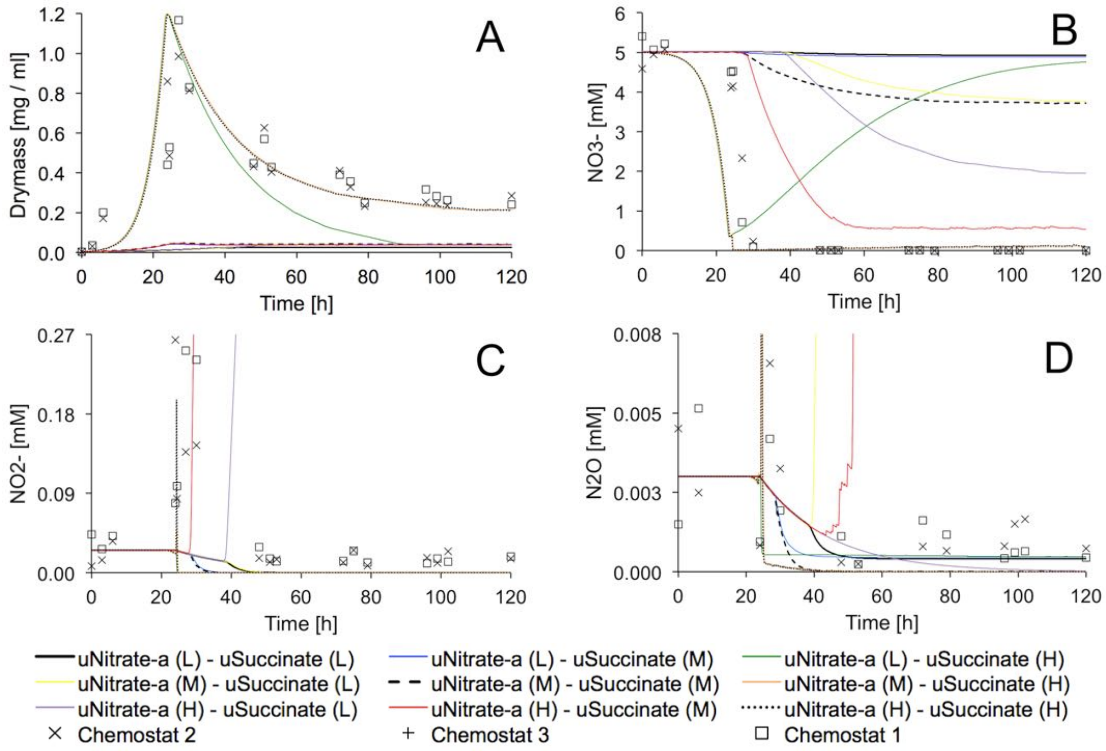

Figure 5.7 INDISIM-Paracoccus model calibration output for the experiment succinate-sufficient/NO_3^--limited (E1). Time evolutions of: (A) Biomass, (B) NO_3^-, (C) NO_2^- and (D) N_2O. Each coloured line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and NO_3^- in anaerobic phase. The u_i values are reported in Table 4.3 (being L = low, M = medium and H = high). Points (squares and crosses) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results.

These results are generated using the "Metabolic hypothesis" and compared with the experimental temporal evolutions of biomass, NO_3^-, NO_2^- and N_2O. Therefore, we can see the model behaviour during the denitrification process.

Taking into account these series of simulations when the uptake-rate for succinate takes the value of the maximum nutrient uptake-rate (uSuccinate = 0.52 $mol_{succinate} \cdot mol_{mass}^{-1} \cdot h^{-1}$) the model accuracy is good (Figure 5.9-A and Figure 5.9-B).

When the e-donor (succinate) is not a limiting nutrient (experiment E1), the model results regarding the time evolutions of NO_2^- and N_2O show values equal or close to zero (Figure 5.7 -C and Figure 5.7 -D). This is due to the fact that there exists an e-donor, and the e-acceptors can be reduced.

When the e-donor (succinate) is limited (experiment E2) the model shows low sensitivity in the time evolution of NO_2^- (Fig. Figure 5.8-C) and in the N_2O (Fig. Figure 5.8-D). For both experiments (E1 and E2) and for the two hypotheses ("Gibbs hypothesis" and "Metabolic hypothesis") the model shows a good response and sensitivity to the time evolutions of dry mass (Figure 5.7 -A and Figure 5.8-A) and NO_3^- (Figure 5.7 -B and Figure 5.8-B).

This is because NO_3^- is the nutrient with which the denitrification process begins when bacteria meet in its environmental anoxic conditions.

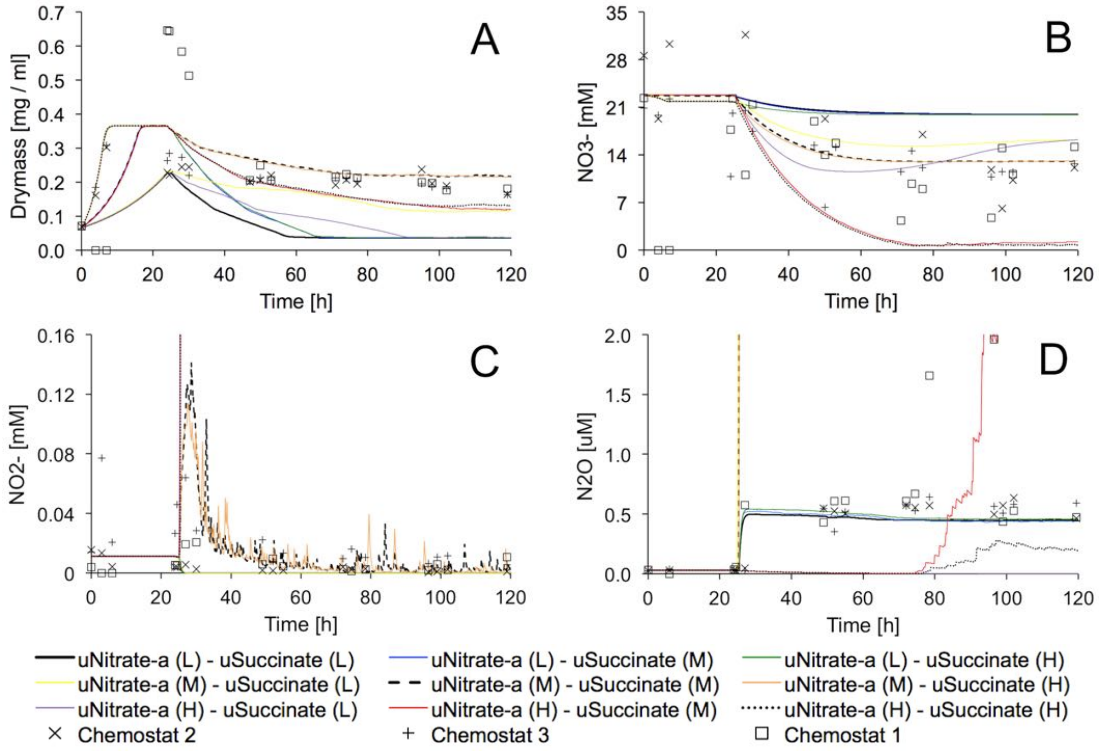

Figure 5.8 INDISIM-Paracoccus model calibration output for the experiment succinate-limited/NO_3^--sufficient (E2). Time evolutions of: (A) Biomass, (B) NO_3^-, (C) NO_2^- and (D) N_2O. Each coloured line represents the mean of three simulations obtained with different combinations of the values for the parameters uptake-rate (u_i) for succinate and NO_3^- in anaerobic phase. The u_i values are reported in Table 4.3 (being L = low, M = medium and H = high). Points (squares, crosses and sums) are the experimental data presented by Felgate et al. (2012). The "Metabolic hypothesis" has been assumed in the simulator to generate these results.

In the bubble charts of Figure 5.9, each circle radius represents the percentage of the global adequacy of the model compared with the experimental data presented by Felgate et al. (2012). From examination of the bubble charts corresponding to Figs. Figure 5.9-C, Figure 5.9-D and Figure 5.9-E, the value of nitrate-x that best fits the simulation results with the experimental points, for both experiments and hypotheses, is 0.119 $mol_{nitrate\text{-}x} \cdot mol_{mass}^{-1} \cdot h^{-1}$.

Looking at the bubble charts of Figs. Figure 5.9-F, Figure 5.9-G and Figure 5.9-H, it is possible to say that the model's sensitivity is low with the changes of the values corresponding to the uptake-rates of NO_2^-, NO and N_2O, since the radius of the circles are similar to each other.

Taking into account this calibration process, the calibrated values for all the uptake-rate nutrients, and for both hypotheses, are presented in Table 4.3. None of the tested parameter combinations met 100% of the calibration criteria defined with the 14 assessment criteria, but some are considerably better than others.

The multiple fitting criteria results used to explore these values and to contrast the two hypotheses are presented in bubble charts, which offer some hints as to the delimited range of values and help to discern between the two hypotheses. In all of the cases the "Metabolic hypothesis" shows a bigger radius of the circle than the "Gibbs hypothesis" (Figure 5.9).

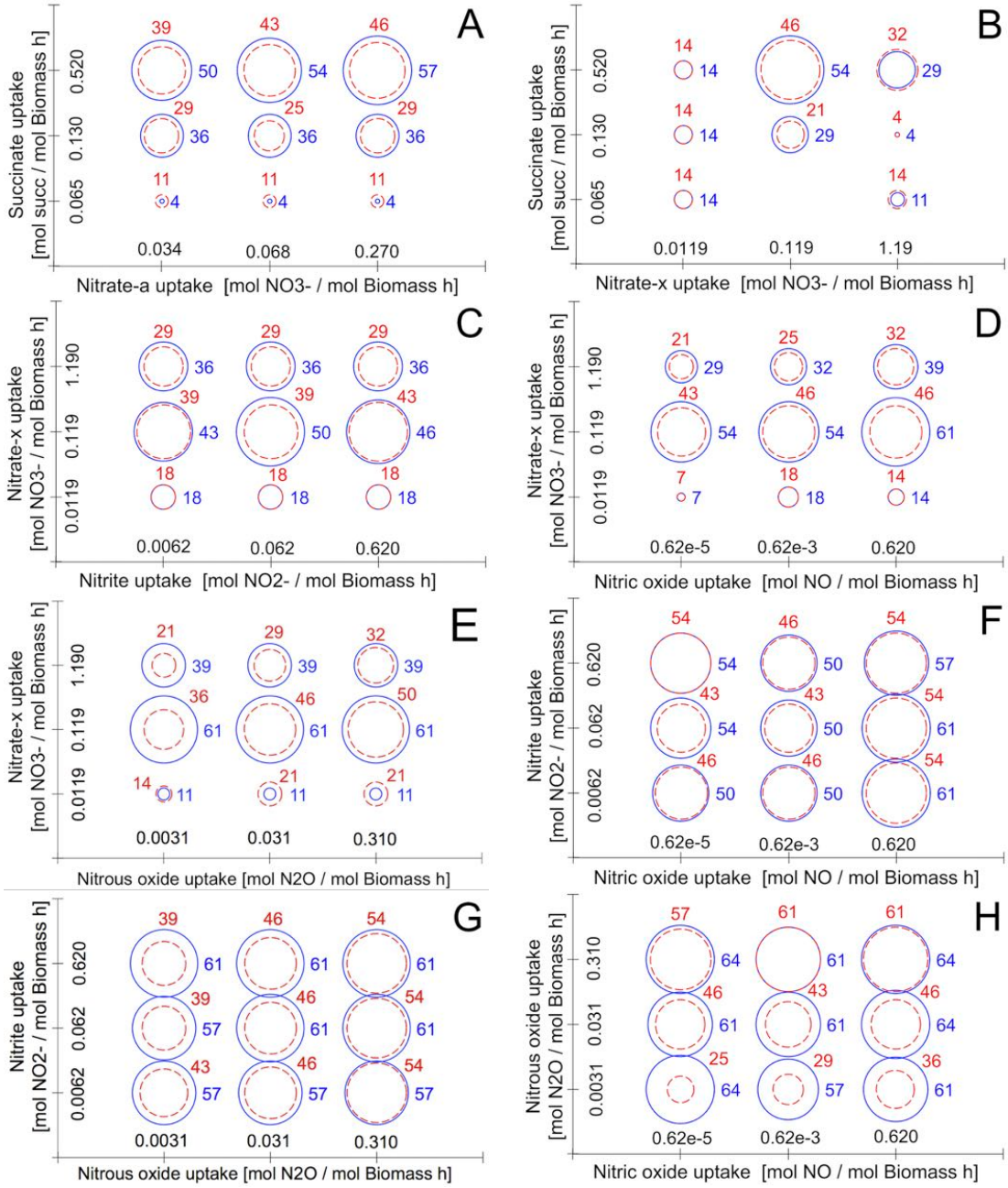

Figure 5.9 Bubble charts to illustrate the model adequacy for different uptake-rate values (u_i). The u_i values come from Table 4.3. Each circle radius represents the global adequacy of the model versus seven experimental time evolutions for two experiments (E1 and E2) presented by Felgate et al. (2012). The continuous blue line assumes the "Metabolic hypothesis", and the dashed red line assumes "Gibbs hypothesis".

In general, fitting a single response variable is straight-forward, but a global fitting for the whole system is much more demanding and challenging to achieve, and even more so if there are different experimental medium conditions jointly with aerobic and anaerobic metabolisms (Woolfenden et al., 2013).

We prioritized the diversity of the results because we are convinced that the use of multiple outputs (patterns) to test models is one of the main and most relevant features of the pattern-oriented modelling strategy used in the framework of IBMs (Grimm et al., 2005).

The GRI values for both hypotheses, for the four-temporal evolutions studied (biomass, NO_3^-, NO_2^- and N_2O) and for the two experiments (E1 and E2), are shown in Table 5.1.

For the "Gibbs hypothesis" the GRI values are higher than the GRI values of the "Metabolic hypothesis". It is noteworthy that the temporal evolutions of NO_2^- and N_2O are outside of the adequate GRI range for both hypotheses and for the two experiments, which suggests it is necessary to include new elements in the individual rules for this denitrifying bacteria which must be relevant in the dynamic of this oxide. Further developments of this model will need to take into account the role of the N_2O in the metabolic reactions and specific experimentation could help to identify the key factors, which control the amount of this product.

Table 5.1. Values of the geometric reliability index (GRI) for the temporal evolutions of biomass, NO_3^-, NO_2^- and N_2O, taking into account the INDISIM-Paracoccus outputs versus experimental data presented by Felgate et al. (2012).

Hypothesis	**Experiment**	**Biomass**	**NO_3^-**	**NO_2^-**	**N_2O**
Metabolic	E2	1.22	1.26	2.05	12.94
	E1	1.66	9.39	17.79	7.10
Gibbs	E2	1.22	1.26	11.79	11.77
	E1	1.64	9.40	17.87	10.37

Chapter VI – INDISIM-Denitrification: The first update of INDISIM-Paracoccus.

6.1 Introduction

INDISIM-Paracoccus was designed mainly to investigate the order in the use of various e-acceptors in the denitrification process driven by the denitrifying bacterium *Paracoccus denitrificans*. After evaluating the simulation results of INDISIM-Paracoccus with the set of the experimental data published by Felgate et al. (2012) (Chapter V), we realized that it was necessary to increase the model's complexity to improve the fitting of some system variables.

At the same time, we believed that to increase its scope and expand it to other denitrifying bacterial species would be worthwhile. So, the goal is to advance the development of an IBM that can manage different denitrifying bacterial species.

To achieve this objective the work is focused on updating INDISIM-Paracoccus by adding a new individual behaviour-rule in the metabolic sub-model and expanding the model to any denitrifying bacterium, including in the simulator's code the possibility of defining the empirical cell composition of the bacterium. With this input of information TEEM will be executed and MMRs obtained that represent all the pathways implicated in the denitrification process modelled.

The new behaviour-rule is introduced to represent the individual mass degradation in order to reduce internal cytotoxic products. These cytotoxic products appear inside each bacterium when they do not complete the denitrification pathway, due to low concentration of e-donor. The updated INDISIM-Paracoccus is named INDISIM-Denitrification.

INDISIM-Denitrification considers that each simulated individual represents a single denitrifying bacterium, each following the individual behaviour-rules concerning their motion, nutrient uptake and reproduction (cellular bipartition) according to the INDISM framework (Ginovart et al., 2002a) and the behaviour-rules concerned with the cellular maintenance and individual mass synthesis according to INDISIM-Paracoccus (Araujo Granda et al., 2016c).

The NetLogo's implementation of INDISIM-Denitrification embeds all the thermodynamics calculations using TEEM2, which allows the user to simulate different denitrifying bacteria because it is possible to change the molar relationships of the four main elements (C, H, O, N) that describe the microbial cell composition.

To test the performance of INDISIM-Denitrification we planned to use the empirical chemical formula of two different denitrifying bacteria: *Paracoccus denitrificans* and *Achromobacter xylosoxidans*. We compared the simulation outputs with the sets of experimental data of two different experiments for the two bacteria that have been published by Felgate et al. (2012).

Further, to establish the model fit we used a deviance measure for relative scale (the geometric reliability index – GRI) to measure, in a quantitative way, the consistency of patterns between experimental observations and model results (Jachner et al., 2007; Leggett and Williams, 1981).

6.1.1 Achromobacter xylosoxidans

The species named *Achromobacter xylosoxidans* was first proposed and published by Yabuuchi and Yano (1974) for the seven strains isolated from human ear discharge. *Achromobacter xylosoxidans* (also named *Alcaligenes xylosoxidans*) is a gram-negative,

aerobic, non-fermentative, non-spore-forming, oxidase- and catalase- positive, non-halophilic and non-hemolytic bacterium; a straight rod of 0.5 to 1.0 by 0.5 to 2.6 μm with rounded ends (Figure 6.1), motile with sheathed flagella arranged peritrichously. The number of flagella is 1 to 20 per cell (Holt et al., 1994). It is able to grow anaerobically with NO_3^- as e-acceptor, performing NO_3^- respiration combined with NO_2^- and N_2O respiration (Yabuuchi et al., 1998).

Therefore, NO_3^- is reduced either to N_2 or to NO_2^-. Nitrite reductase (Nir) expressed in *A. xylosoxidans* is solely dependent on copper (Cu) as a co-factor, therefore this bacterium is considered as a denitrifying bacterium (Knowles, 1982; Zumft and Körner, 1997). Soil and water are regarded as its natural habitats, but sometimes *A. xylosoxidans* is isolated from a hospital environment and from human clinical specimens with pathological significance or just as a contaminant (Abraham et al., 1993).

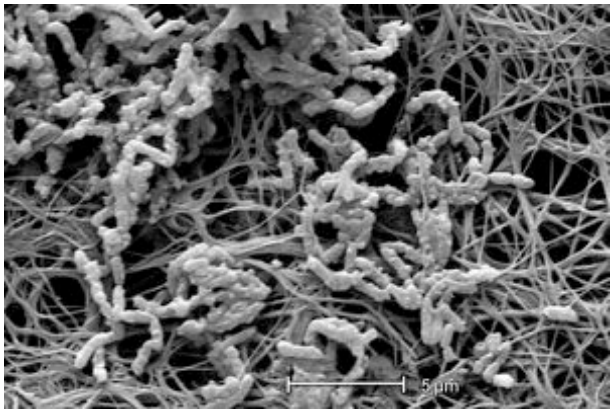

Figure 6.1 Rod-shaped bacteria (*A. xylosoxidans)* in association with fibrin like material (Kim et al., 2008). Provider: Dr. Rodney M. Donlan.

6.1.2 Metabolic reactions for *A. xylosoxidans*

There are three sets of metabolic reactions that are required to write for *A. xylosoxidans*: i) reactions for cellular maintenance, ii) reactions for individual mass synthesis, and iii) reactions for individual mass degradation to reduce cytotoxic products.

Using MbT-Tool it is possible to write MMRs to represent cellular maintenance for *A. xylosoxidans*; therefore, it is necessary to write the energy reactions for aerobic and anaerobic conditions.

For the aerobic phase the reaction between succinate, which is always the e-donor, and O_2 as e-acceptor, is considered, while for the anaerobic phase the e-acceptors are N-oxides involved in the denitrification process. Therefore, these reactions will be the same as the energy reactions written for *P. denitrificans* (Table 4.1).

Also using MbT-Tool we could write the set of MMRs to represent the pathways by which *A. xylosoxidans* could synthetize individual mass and generate metabolic products. In all reactions succinate is the universal e-donor (Rd) and C-source, NH_4^+ is the universal N-source to the cell synthesis (Rc), and the nutrients used as e-acceptors (Ra) are different; in aerobic conditions they are O_2 and NO_3^- and in anaerobic conditions they are NO_3^-, NO_2^-, NO and N_2O.

To represent the microbial biomass of *A. xylosoxidans* through an empirical chemical formula we adopted $C_5H_9O_{2.5}N$ (Heijnen, 1999; Kampschreur et al., 2012; Roels, 1983). Therefore, the stoichiometric coefficients for each microbial metabolic reaction were

obtained (Table 6.1) with a different assigned ε value for each reaction in the range proposed by McCarty (2007) and Xiao and VanBriesen (2008).

Table 6.1. Microbial metabolic reactions (R) that represent aerobic and anaerobic pathways for *A. xylosoxidans*, using different values of energy-transfer-efficiency (ε) according to TEEM2 (R = fe^{o}Ra + fs^{o}Rc – Rd) (McCarty, 2007).

#	Microbial metabolic reactions (R)	ε
R1	$(C_4H_4O_4)^{2-}$ + 0.50 NH_4^+ + 0.89 **O_2** → 0.50 $C_5H_9O_{2.5}N$ + 0.013 CO_2 + 1.50 HCO_3^- + 0.006 **H_2O**	0.76
R2	$(C_4H_4O_4)^{2-}$ + 0.77 **NO_3^-** + 1.54 H^+ + 0.52 H_2O → 0.37 $C_5H_9O_{2.5}N$ + 0.51 CO_2 + 1.63 HCO_3^- + 0.40 **NH_4^+**	0.65
R3	$(C_4H_4O_4)^{2-}$ + 0.24 NH_4^+ + 4.49 **NO_3^-** → 0.24 $C_5H_9O_{2.5}N$ + 4.49 **NO_2^-** + 1.05 CO_2 + 1.76 HCO_3^- + 0.52 H_2O	0.41
R4	$(C_4H_4O_4)^{2-}$ + 0.45 NH_4^+ + 4.54 **NO_2^-** + 4.54 H^+ → 0.45 $C_5H_9O_{2.5}N$ + 4.54 **NO** + 0.20 CO_2 + 1.55 HCO_3^- + 2.37 H_2O	0.84
R5	$(C_4H_4O_4)^{2-}$ + 0.50 NH_4^+ + 3.53 **NO** → 0.50 $C_5H_9O_{2.5}N$ + 1.77 **N_2O** + 0.006 CO_2 + 1.50 HCO_3^- + 0.006 H_2O	0.66
R6	$(C_4H_4O_4)^{2-}$ + 0.24 NH_4^+ + 4.50 **N_2O** → 0.24 $C_5H_9O_{2.5}N$ + 4.50 **N_2** + 1.05 CO_2 + 1.76 HCO_3^- + 0.52 H_2O	0.27

R1 represents the pathway *Aerobic respiration*; R2 represents the pathway *Nitrate Reduction - Dissimilatory* in aerobic phase; and gathering the reactions R3, R4, R5 and R6 represents the pathway *Nitrate Reduction – Denitrification* (Caspi et al., 2012; Knowles, 1982; Zumft, 1997).

6.1.3 Experimental data

To study the denitrification process in a bioreactor, the bacteria were bred and developed in two different conditions: the first in a batch culture (from 0 to 24 hours) during the aerobic phase, and the second one in a continuous culture (from 24 to 120 hours) during the anaerobic phase.

Under these conditions two experiments were performed: (i) 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 (ii) 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 hours, for each one of the bacteria involved (*Paracoccus denitrificans* and *Achromobacter xylosoxidans*) according to the experimental procedure presented and reported by Felgate et al. (2012).

6.2 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* ($C_3H_{5.4}N_{0.75}O_{1.45}$) (van Verseveld et al., 1983, 1979), the general biomass half-reaction equation (Rittmann and McCarty, 2001) 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:

$$NO + H^+ + e^- \rightarrow \tfrac{1}{2}\, N_2O + \tfrac{1}{2}\, H_2O$$

$$\tfrac{1}{2}\, N_2O + H^+ + e^- \rightarrow \tfrac{1}{2}\, N_2 + \tfrac{1}{2}\, H_2O$$

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

Ra	$NO + H^+ + e^- \rightarrow \tfrac{1}{2}\, N_2O + \tfrac{1}{2}\, H_2O$
– 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	$^{4}/_{49}\, C_3H_{5.4}O_{1.45}N_{0.75} + NO \rightarrow {}^{9}/_{49}\, CO_2 + {}^{3}/_{49}\, HCO_3^- + \tfrac{1}{2}\, N_2O + {}^{3}/_{49}\, NH_4^+ + {}^{33}/_{490}\, H_2O$

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 bacteria (Table 6.2).

Table 6.2. Microbial metabolic reactions (Rg) for *P. denitrificans* and *A. xylosoxidans* to represent the individual mass degradation to reduce cytotoxic products (NO and/or N_2O), according to TEEM (Rittmann and McCarty, 2001).

Bacteria	Cytotoxic gas	Microbial metabolic reaction (Rg)
P. denitrificans	NO	$C_3H_{5.4}O_{1.45}N_{0.75} + {}^{49}/_4\,NO \rightarrow {}^{9}/_4\,CO_2 + {}^{3}/_4\,HCO_3^- + {}^{49}/_8\,N_2O + {}^{3}/_4\,NH_4^+ + {}^{33}/_{40}\,H_2O$
	N_2O	$C_3H_{5.4}O_{1.45}N_{0.75} + {}^{49}/_8\,N_2O \rightarrow {}^{9}/_4\,CO_2 + {}^{3}/_4\,HCO_3^- + {}^{49}/_8\,N_2 + {}^{3}/_4\,NH_4^+ + {}^{33}/_{40}\,H_2O$
A. xylosoxidans	NO	$C_5H_9O_{2.5}N + 21\,NO \rightarrow 4\,CO_2 + HCO_3^- + {}^{21}/_2\,N_2O + NH_4^+ + 2\,H_2O$
	N_2O	$C_5H_9O_{2.5}N + {}^{21}/_2\,N_2O \rightarrow 4\,CO_2 + HCO_3^- + {}^{21}/_2\,N_2 + NH_4^+ + 2\,H_2O$

6.3 INDISIM-Denitrification description

The description of INDISIM-Denitrification model follows the ODD protocol, it stands for “Overview, Design concepts, and Details” (Grimm, 1999; Grimm et al., 2010; Railsback and Grimm, 2012).

6.3.1 Purpose

To develop a computational model for the denitrification process carried out by a denitrifying bacteria growing in batch and continuous culture, in aerobic and anaerobic conditions, in order to reproduce a bioreactor experimental protocol and explore the consequences of the individual uptake parameters, the cellular maintenance, the individual mass degradation coefficient and the sufficient and/or limiting of e-donor and e-acceptors on the system dynamics.

6.3.2 Entities, State variables and Scales

The INDISIM-Denitrification model has the same two entities as INDISIM-Paracoccus: individuals and square patches of culture medium. The individual variables are the same in both models (see chapter IV – section 4.5.2).

Also, INDISIM-Denitrificans assumes that each bacterium has a spherical shape and the user could define the spherical equivalent diameter (expressed in µm) for the smallest and largest individuals.

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.

The individual mass is then deduced from cell volume and assuming the microbial mass density equal to 1.1 $g \cdot cm^{-3}$, which has been used in previous INDISIM models (Gras et al., 2011). In order to characterize the elementary cell composition of the individuals, INDISIM-Denitrification allows the user to input the molar relationship between the four main elements (C, H, O, N) to define the biomass empirical formula.

Therefore, in the case of *P. denitrificans* the model uses $C_3H_{5.4}O_{1.45}N_{0.75}$ (van Verseveld et al., 1979, 1983) and for *A. xylosoxidans* $C_5H_9O_{2.5}N$ (Kampschreur et al., 2012; Roels, 1983, 1980a). The variables and the units designed for the square patches of culture medium, the time scaled factor (time step) and the units for the graphical and model outputs are the same as in INDISIM-Paracoccus (Chapter IV - section 4.5.2).

6.3.3 Process overview and scheduling

The initial configuration of INDISIM-Denitrification has three parts: the first one for thermodynamics calculations, the second one for the system (bioreactor) and the third one for the entities (culture medium and bacteria).

The thermodynamics setup considers the reduction-half-reaction of succinate as universal e-donor and the reduction-half-reactions of O_2, NO_3^-, NO_2^-, NO and N_2O as e-acceptors with their corresponding standard Gibbs free energy, and the standard Gibbs free energy for the intermediate compounds required by TEEM2 methodology.

Then the user could define the value for the energy-transfer-efficiency to obtain the microbial metabolic reactions for cellular maintenance, individual mass synthesis and individual mass degradation to reduce cytotoxic products.

The initial system setup sets the world size and topology, and the time scaled factor (time step). The topology of the world is programmed using the torus mode; therefore, rectangular periodic boundary conditions were used. The initial culture medium concentrations and the initial bacterial population are established using random variables, normal probability distributions with mean values that are determined by the experimental procedure (Felgate et al., 2012).

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

The culture medium processes, the beginning of the simulation and the protocols for managing the bioreactor (batch and chemostat conditions) are well described in chapter 4 - section 4.5.2.

Figure 6.2 shows the INDISIM-Denitrification flow diagram, which is essentially the same as INDISIM-Paracoccus but with the incorporation of the thermodynamic calculations as a first procedure to execute in the initial system configuration and the decision to execute the behaviour-rule related to reducing cytotoxic products using the individual mass.

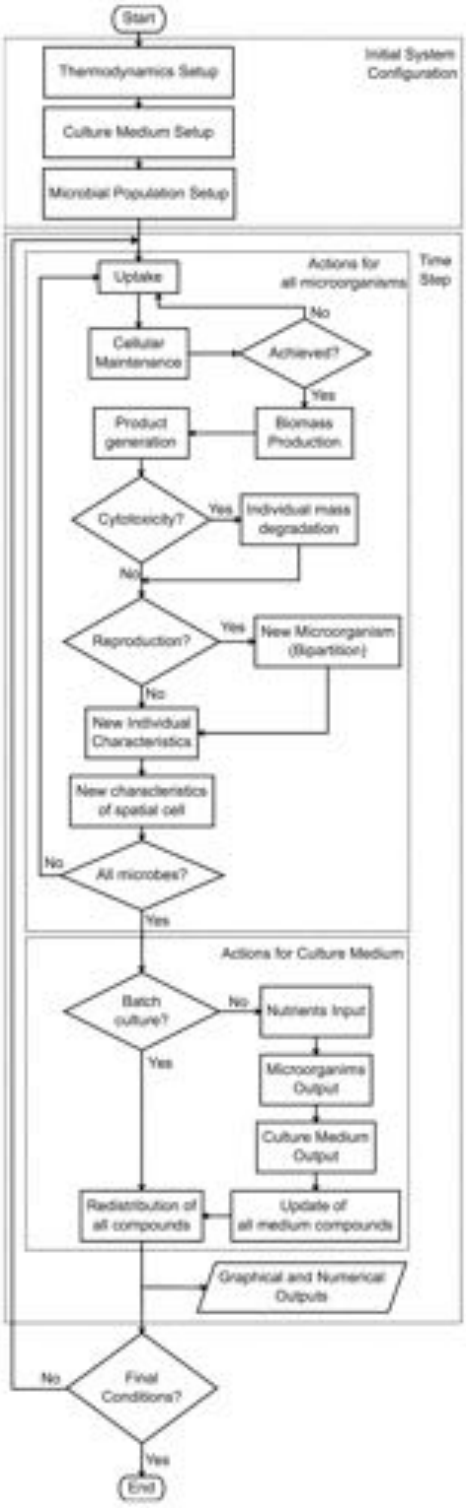

Figure 6.2 Flow chart of the INDISIM-Denitrification model.

6.3.4 Design concepts

The basic principles, emergence, adaptation, interaction, collective and stochasticity are explained in chapter IV - section 4.5.4.1 and the sub-sections initialization and sub models are explained in chapter IV - sections 4.5.6.1 and 4.5.6.2. In this part we only describe the novelty that INDISIM-Denitrification shows over INDISIM-Paracoccus.

For the adaptation sub-section to improve the individual model we include the behaviour rule to perform individual mass degradation to reduce cytotoxic products.

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 biomass and reduce it according to the MMRs presented in Table 6.2.

With regard to the stochasticity included in the individual mass degradation sub-model, we consider that the bacterium could determine the portion of its own biomass that will be degraded for reducing 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.

Inside the sub-models we programmed a new one, which is called *Individual mass degradation*. It takes place when there are internal quantities of the cytotoxic gases NO and/or N_2O and the C-source quantity is not enough to execute the next metabolic reaction inside the denitrification pathway; then the microbe executes the mass degradation behaviour-rule.

First it establishes the maximum amount of mass that will be used to reduce internal cytotoxic products based on the mass degradation coefficient (h^{-1}).

Having established this quantity, the cytotoxic product, NO or N_2O, is reduced following the reactions coefficients that appear in Table 6.2. The individual mass is reduced and the non-metabolized intakes are expelled to the medium.

6.4 Implementation of the model and analysis of the outputs of the simulator

INDISIM-Denitrification is implemented in NetLogo. The tool "Behaviour-Space" incorporated in NetLogo was used for running simulation experiments, varying parameters and writing model outputs to files that were used for the statistical analysis.

To assess the general validity of the model, and after the first visual techniques with subjective assessment, numerical validation techniques that provide measures of difference (or similarity) between experimental and simulated data are carried out.

The basic idea is to find ranges for the model parameters' values that make it possible to roughly reproduce the evolution of a set of focus variables observed in the two trials (Experiments E1 and E2) using the sets of experimental data for *P. denitrificans* and *A. Xylosoxidans* published by Felgate et al. (2012).

According to the statistical classification provided by Jachner et al., (2007) for the comparison of simulated results with experimental time evolutions, it is possible to determine the geometry reliability index (GRI).

The GRI values determine the reliability of the model; in the case where the simulated results are close to the experimental values GRI shows a value from 1 to 3, with 1 corresponding to 100% accuracy.

The interpretation of GRI is that the simulation is accurate within a multiplicative factor, 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 corresponding experimental values (Leggett and Williams, 1981). A combination of the use of this deviance measure with visual inspection in an exploratory data analysis can help to identify limitations and capabilities of the new model developed.

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

Each simulation result that we presented is the average of three simulated replicates and the sets of experimental values are presented with their corresponding standard error. Therefore, we calculate the GRI value, which will be the value to declare the fit or non-fit of the simulation results with the experimental ones (Jachner et al., 2007).

We established multiple fitting criteria using the model's parameters: i) uptake-rate for all nutrients involved, ii) cellular maintenance and, iii) mass degradation coefficient, with the experimental data of Felgate et al. (2012). The basic 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.

6.5 Parameterization and calibration of INDISIM-Denitrification

Following the INDISIM framework (Gras et al., 2011) the maximum population growth rate (μ_{max}) has been used to estimate the individual maximum uptake-rates (u_i). 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 (Nielsen et al., 2006).

Using these values and performing calculations with the stoichiometric coefficients of each metabolic reaction adjusted by TEEM2 (Table 4.2 and Table 6.1), 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 6.3 and Table 6.4).

In the case of cellular maintenance, Gras et al. (2011) consider an appropriate maintenance requirement for soil heterotrophic microorganisms of 0.002 with the units (gC_{donor} gC_{mic}^{-1} h^{-1}), which was assumed in INDISIM-Paracoccus 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 (gC_{donor} gC_{mic}^{-1} h^{-1}) in both aerobic and anaerobic phase.

Also, another individual parameter related to the individual mass, the mass degradation coefficient (h^{-1}), has been introduced for the anaerobic phase only. In Table 6.5 we show the tested values for these parameters: these ranges of values will be the same for both bacteria.

To start the calibration and sensitivity analysis process, we combine the values from Table 6.3 and Table 6.4 using a full factorial design for each species of bacteria and each virtual experiment, and after that, we combine the values from Table 6.5 in a full factorial design for the cellular maintenance and mass degradation coefficients.

Table 6.3 Uptake-rate (u_i) parameter 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.*

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 3.2-1). 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 (R3, R4, R5 and R6) are carried out by the bacterium.

Table 6.4 Uptake-rate (u_i) parameter values with units ($mol_{nutrient} \cdot C\text{-}mol_{mic}^{-1} \cdot h^{-1}$) used in the sensitivity analysis of this parameter for denitrifying bacterium *A. xylosoxidans.*

Nutrient	**Uptake-rate ($mol_{nutrient} \cdot C\text{-}mol_{mic}^{-1} \cdot h^{-1}$)**		
	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. 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 (R3, R4, R5 and R6) are carried out by the bacterium.

Table 6.5. Values used in the sensitivity analysis performed with the parameter for cellular maintenance and individual mass degradation coefficient.

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.

All full factorial designs were executed using the tool behaviour-space included in NetLogo, a task that is facilitated due to the simulator including in its code the complete experimental data set to calculate GRI. Each simulation result is compared to the experimental values and the GRI for each one is calculated. We selected the combination of the parameters with minimum GRI value to declare the best fit of INDISM-Denitrification.

6.6 Assessing the adequacy of INDISIM-Denitrification

6.6.1 Results and discussion for *P. denitrificans*

INDISIM-Denitrification was used to simulate the growth and behaviour of the denitrifying bacterium *P. denitrificans* in a bioreactor with regard to experiments E1 and E2 according to the 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 6.6.

Table 6.6. INDISIM-Denitrification model parameters values for *P. denitrificans*.

Nutrient	**Culture medium initial concentration [mM] Felgate et al. (2012)**	**Availability coefficient – a_i (h^{-1}) fixed according to *Dab***	**Uptake-rate – u_i – ($mol_{nutrient} \cdot C\text{-}mol_{mic}^{-1} \cdot h^{-1}$)**
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 ($gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$)	0.0020 [a]	Gras et al. (2011)
Mass degradation coefficient (h^{-1})	0.022	Calibrated
Mass split	0.50 (15% coefficient of variation)	Derived from (Ginovart et al., 2002a)
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 (Gras et al., 2011) and (Ginovart et al., 2002a)

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

In Figure 6.3 and Figure 6.4, we present the outputs assessed for the bacterium *P. denitrificans*, namely the drymass, NO_3^-, NO_2^- and N_2O evolutions for the two experiments succinate-limited/NO_3^--sufficient – E1 (Figure 6.3) and succinate-sufficient/NO_3^--limited – E2 (Figure 6.4) with the GRI score obtained in the statistical analysis. In these figures the experimental data are drawn with means of the replicates and their standard errors; and each simulated result is the mean of three simulated replicates.

According to the magnitude of the GRI coefficient, the results of the simulated experiment E1, succinate-limited/NO_3^--sufficient, (Figure 6.3) are accurate to the experimental results for drymass, NO_3^-, NO_2^- and N_2O evolutions (if the difference between the simulated and the experimental were reasonably narrow this index shows a value in a range from 1 to 3).

The experimental data suggest that the culture steady state is reached after 40 hours; simulated individuals reached the steady state later, the effect of phase change is appreciable on the GRI values for NO_2^- and N_2O time evolution (Figure 6.3-D), which is the highest obtained in E1, but still adequate.

Also, taking into account the lowest value of GRI for the drymass temporal evolution (Figure 6.3-A), this is clear support for the thermodynamic approach used to represent the metabolic pathways, due to TEEM being principally designed for biomass yield prediction.

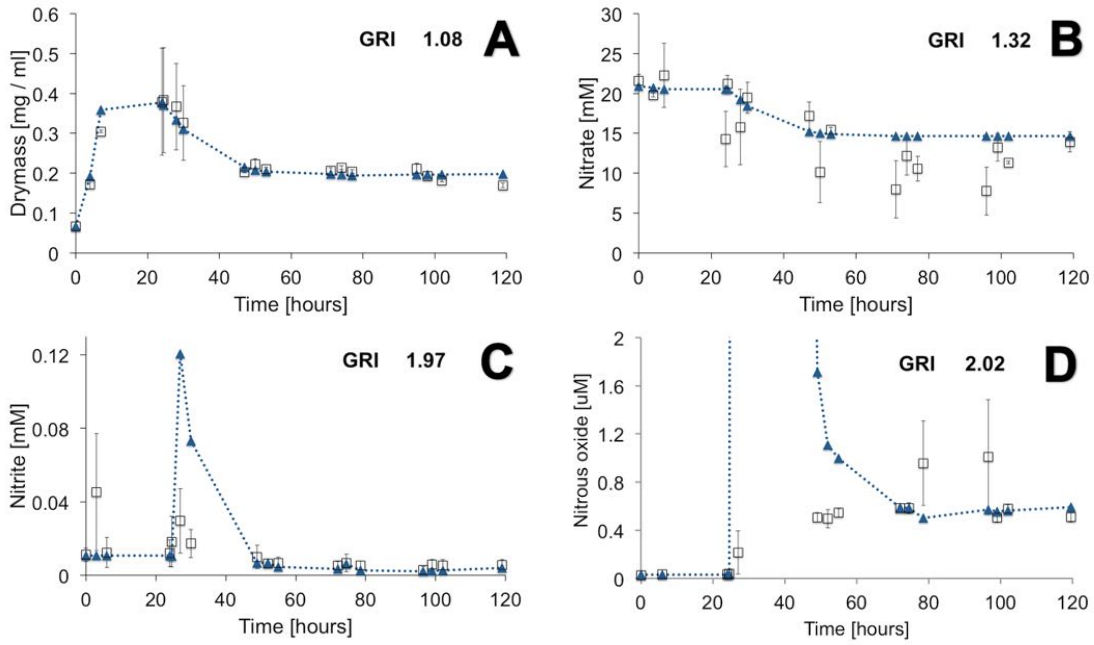

Figure 6.3. INDISIM-Denitrification simulation results (triangles) for *P. denitrificans* in experiment succinate-limited/NO_3^--sufficient (E1). Experimental mean values (squares) are presented with their standard error and each simulated value is the mean of three simulated replicates. Time evolutions of: (A) Drymass, (B) NO_3^-, (C) NO_2^- and (D) N_2O with GRI value.

Also, the results for the simulated experiment E2, succinate-sufficient/NO_3^--limited, are close to the experimental result for drymass evolution (Figure 6.4-A). On the other hand, the GRI scores for NO_3^-, NO_2^- and N_2O evolutions in E2 (Figure 6.4-B, Figure 6.4-C and Figure 6.4-D) are outside of the necessary GRI range, which suggests only rough adequacy of the model in the experiment e-donor-sufficient/e-acceptor-limited.

If the e-donor is sufficient and there is a small amount of e-acceptor, the reaction will occur reducing the entire acceptor; this justifies the fact that the temporal evolutions of NO_2^- (Figure 6.4-C) and N_2O (Figure 6.4-D) in the anaerobic phase hit zero, increasing the value of the GRI.

The simulated result for the drymass evolution in experiment E2 (Figure 6.4-A) has the lowest GRI value, which coincides with the experiment E1 (Figure 6.3-A). These results give us support in confirming the contribution of TEEM as the key part of the metabolic sub model.

They also suggest that the empirical chemical formula $C_3H_{5.4}O_{1.45}N_{0.75}$ used to represent the biomass of *P. denitrificans*, provokes an acceptable agreement of the model results compared to the experimental ones.

The behaviour-rule related to the individual mass degradation to reduce cytotoxic products, provokes an improvement in the N_2O time evolutions for the experiment with e-donor limited.

We based this discussion on comparison of INDISIM-Paracoccus's results (see chapter V, Table 5.1) with the results of INDISIM-Denitrification (Figure 6.3). Using INDISIM-Paracoccus the GRI value was 12.94 and using INDISIM-Denitrification the GRI value is 2.02, in view of which it seems plausible that the biomass degradation could be an

interesting individual strategy to reduce the accumulation of toxic products as has been pointed out by some authors (Rittmann and McCarty, 2001).

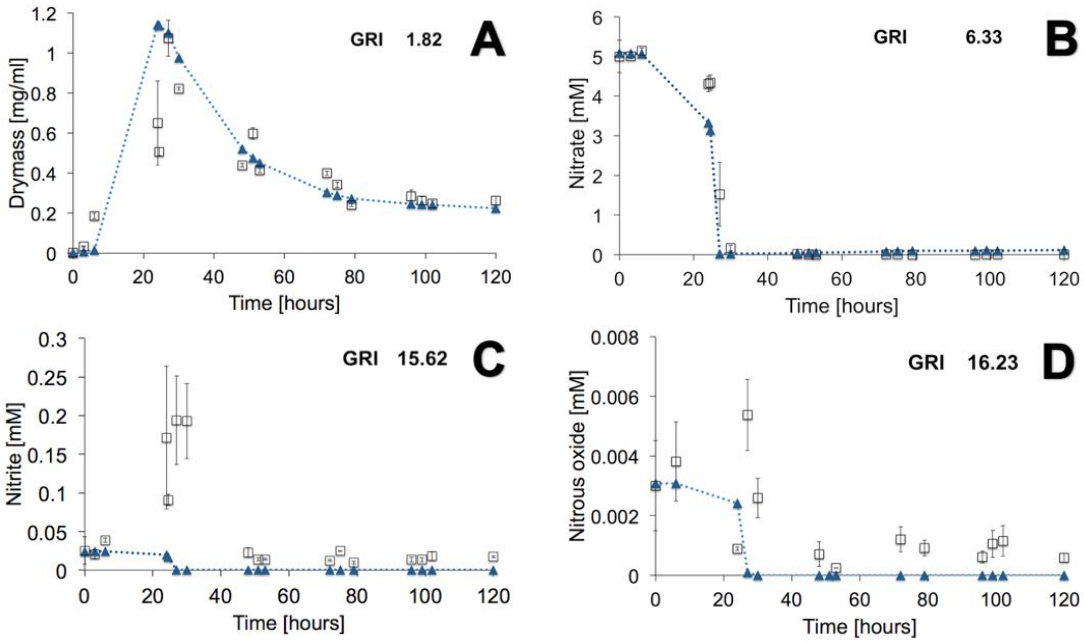

Figure 6.4. INDISIM-Denitrification simulation results (triangles) for *P. denitrificans* in experiment succinate-sufficient/NO_3^--limited (E2). Experimental mean values (squares) are presented with their standard error (Felgate et al., 2012) and each simulated value is the mean of three simulated replicates. Time evolutions of: (A) Biomass, (B) NO_3^-, (C) NO_2^- and (D) N_2O with GRI value.

In addition to the four temporal evolutions related to drymass, NO_3^-, NO_2^- and N_2O which are compared to the experimental values through GRI values, INDISM-Denitrification gives the user 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^-.

Some of 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 values and thus, it makes possible a comparison when a future and extra set of experimental data are available.

Fitting a single response variable is straight-forward, but a global fitting for the whole system variables is much more demanding and challenging to achieve, and even more so if there are different experimental medium conditions jointly with aerobic and anaerobic metabolisms. It is in this line that this research project is working.

6.6.2 Results and discussion for *A. xylosoxidans*

Now we use INDISIM-Denitrification to simulate the growth and behaviour of the denitrifying bacterium *A. xylosoxidans* in a bioreactor with regard to experiments E1 and E2 and according to the experimental protocols published by Felgate et al. (2012). The individual and environmental parameter values that cause model outputs with acceptable GRI coefficient are shown in Table 6.7.

In Figure 6.5 and Figure 6.6, 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 (Figure 6.5) and experiment E2 with succinate-sufficient/NO_3^--limited (Figure 6.6), where the GRI scores obtained in the statistical analysis performed are included.

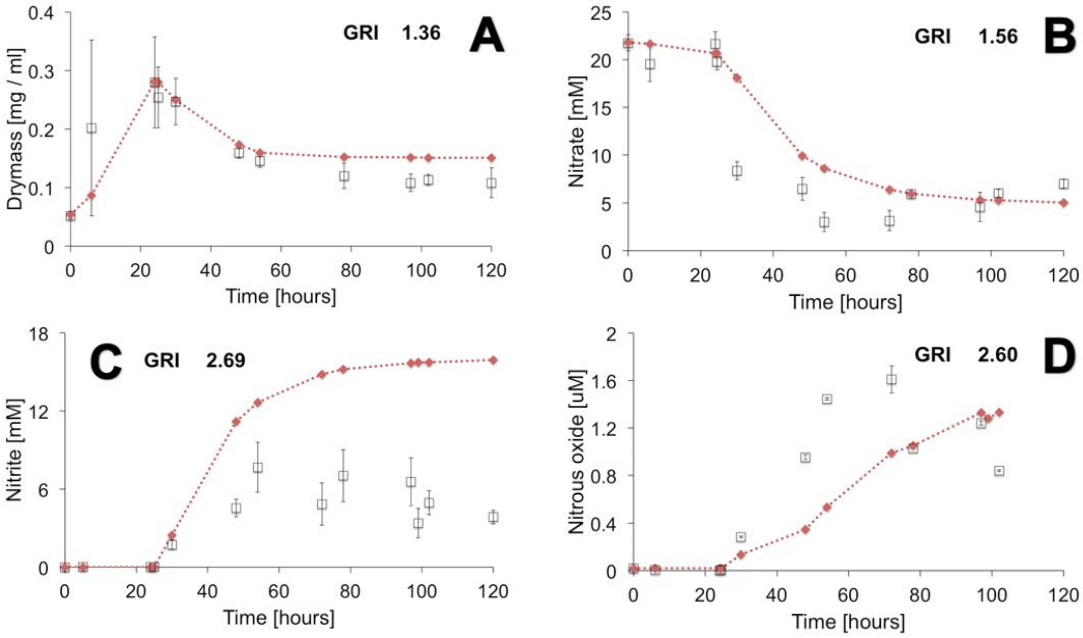

Figure 6.5. INDISIM-Denitrification simulation results (diamonds) for *A. xylosoxidans* in experiment succinate-limited/NO_3^--sufficient (E1). Experimental mean values (squares) are presented with their standard error and each simulated value is the mean of three simulated replicates. Temporal evolutions of: (A) Drymass, (B) NO_3^-, (C) NO_2^- and (D) N_2O with GRI value.

In these figures the experimental data are drawn with means and their corresponding standard errors, and each simulated result is the mean of three simulated replicates. It is necessary to pay attention to these results, due to their being obtained using the empirical chemical formula $C_5H_9O_{2.5}N$, which is a generic formula used to represent the biomass composition of *A. xylosoxidans*.

According to the GRI values for the experiment e-donor limited (Figure 6.5), the simulation results obtained with INDISIM-Denitrification for the bacterium *A. xylosoxidans* show an acceptable behaviour compared with the simulation model, because all of the values are in the acceptable range of GRI (from 1 to 3). The highest GRI value is obtained in the temporal evolution of NO_2^- (Figure 6.5-C).

In general terms, taking into account the GRI value obtained for all of the temporal evolutions tested, INDISIM-Denitrification provides acceptable results in the experiments where the e-donor is limited (Figure 6.3 and Figure 6.5). We consider that one of the reasons for obtaining these acceptable GRI values 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 (Rittmann and McCarty, 2001).

The acceptable range for GRI is only achieved in the drymass and NO_3^- evolution (Figure 6.6-A and Figure 6.6-B) for the experiment e-donor sufficient (E2). This model's behaviour is a key point for future upgrades of this INDISIM branch, because it could be

necessary to include a new behaviour-rule at the individual level to regulate the model's response when the e-acceptor is limited (e-donor sufficient).

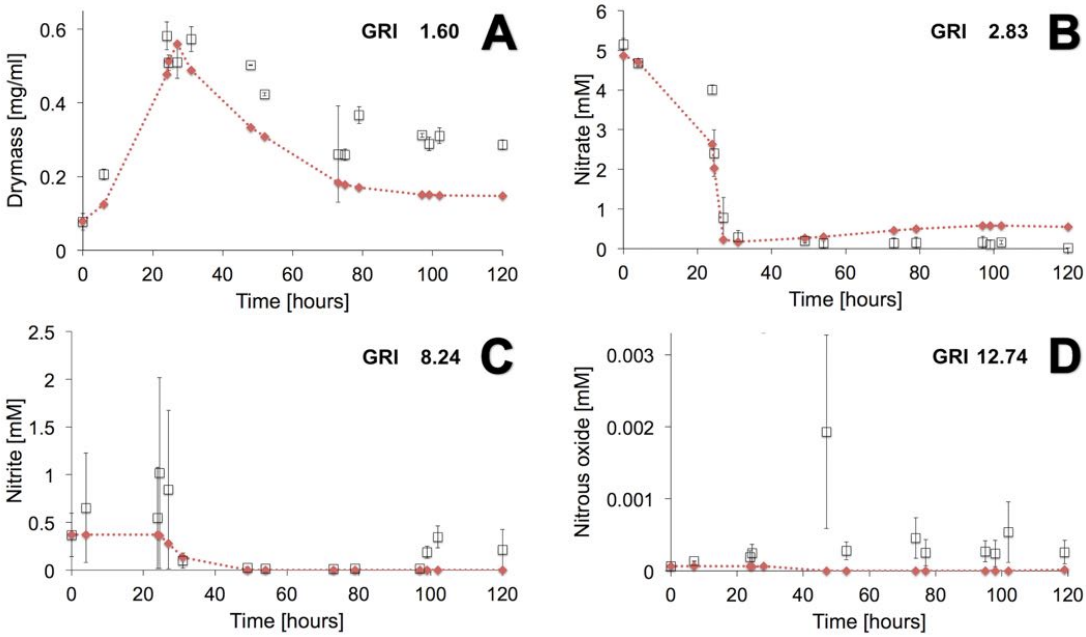

Figure 6.6. INDISIM-Denitrification simulation results (diamonds) for *A. xylosoxidans* in experiment succinate-sufficient/NO_3^--limited (E2). Experimental mean values (squares) are presented with their standard error (Felgate et al., 2012) and each simulated value is the mean of three simulated replicates. Temporal evolutions of: (A) Biomass, (B) NO_3^-, (C) NO_2^- and (D) N_2O with GRI value.

6.7 Final remarks

INDISIM-Denitrification has the capability to work with different empirical cellular formula for denitrifying bacteria. The model implementation in NetLogo allows the user to quickly and easily change the molar relationship between the atoms of the biomass empirical formula (C, H, O, N), which recalculate immediately all of the stoichiometric coefficients for the set of MMRs for each metabolic pathway.

It will also recalculate the individual maximum uptake-rate reference value of all nutrients considered in the virtual system, according to the INDISIM framework and μ_{max} value.

Besides, the user can modify the range of the cell size choosing the values for the smallest and for the biggest one, provided that the cell is always considered to be of spherical shape.

The simulators developed allow the user to work with batch or continuous cultures, being able to define the simulation time, formulate different culture medium, define different denitrifying bacterium, adjust the dilution ratio, setup the initial number of viable microorganisms, and setup the oxygen dissolved level in the culture medium which is the key factor to carry out the aerobic and anaerobic metabolic pathways, among others.

Besides, the NetLogo implementation of the model allows the user to have control of the input parameters and initial conditions for the simulations from a very friendly interface. In addition of all of this, the implementation of INDISIM-Denitrification gives easy access to

the computer code for future and specific adaptations to the user interested in diverse academic and research applications.

Table 6.7. INDISIM-Denitrification model parameters values for *A xylosoxidans*.

Nutrient	**Culture medium initial concentration [mM] Felgate et al. (2012)**	**Availability coefficient – a_i (h^{-1}) fixed according to *Dab***	**Uptake-rate – u_i – ($mol_{nutrient} \cdot C\text{-}mol_{mic}^{-1} \cdot h^{-1}$)**
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 ($gC_{donor} \cdot gC_{mic}^{-1} \cdot h^{-1}$)	0.0020 [a]	Gras et al. (2011)
Mass degradation coefficient (h^{-1})	0.085	Calibrated
Mass split	0.50 (15% coefficient of variation)	Derived from (Ginovart et al., 2002a)
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 (Gras et al., 2011) and (Ginovart et al., 2002a)

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.

Chapter VI – Conclusions and future perspectives.

7.1 Conclusions

An open access and open source tool has been developed to write microbial metabolic reactions based on Thermodynamic Electron Equivalents Model. It is called MbT-tool, which stands for Metabolism-based on Thermodynamics and, as far as we know, it is the first free computational tool available in this context for academics or researchers.

- MbT-tool writes the metabolic microbial reactions; in which one of the products is the biomass of the microorganism, as well as calculating the biomass yield. The users have the option of changing the molar relationship between the main four elements to define the empirical chemical formula that represents the microbial biomass.
- Using MbT-tool it is possible to write metabolic reactions for diverse microbial functional groups, such as aerobic heterotrophs, nitrifiers, denitrifiers, methanogens, sulphate reducers, sulphide oxidizers and fermenters.
- With MbT-tool outputs it is possible: i) to construct a model, ii) to incorporate the information into an existing model or iii) to start the calculations of the mass balance for a bioreactor.
- The users are able to modify the source-code to extend the scope of the MbT-Tool into their specific expertise and research field in order to deal with a modelling project in the framework of microbial ecology or bio-technological processes that require metabolic reactions. They can introduce other chemical species and their corresponding reduction-half-reactions to investigate diverse metabolic pathways.
- A set of metabolic reactions applying Thermodynamic Electron Equivalents Model has been written to represent three respiratory metabolic pathway followed by denitrifying bacteria, which are in aerobic conditions "*Aerobic respiration*" with oxygen as the electron acceptor and "*Nitrate reduction - Dissimilatory*" with nitrate as electron acceptor, and in anoxic conditions "*Nitrate reduction - Denitrification*" in the presence of N-oxides as electron acceptors.

The individual-based model named INDISIM-Paracoccus has been developed, including metabolic reactions as the basis of the individual behaviour-rules for the cellular maintenance and biomass synthesis sub-model, to deal with *Paracoccus denitrificans* growing in a bioreactor working as a batch and/or continuous culture. It has been verified that the corresponding simulator implemented in NetLogo platform works in accordance with its conceptual design.

- Taking into account the simulation results achieved with INDISIM-Paracoccus the Thermodynamic Electron Equivalents Model, one of the thermodynamic models based on bioenergetics growth efficiency, has been a suitable methodology to deal with metabolic actions in the framework of INDISIM, and it has been crucial to parameterize and to calibrate this individual-based model.
- The values of individual growth yields obtained for each metabolic reaction were always higher than the published values as population yield; however the population value obtained with our simulator was in accordance with published values for *P. denitrificans* and *A. xylosoxidans*.
- Two hypotheses have been successfully tested with INDISIM-Paracoccus to assess the sequence in the use of different electron acceptors in the denitrification pathway, which was a previous requirement to establish the order of the metabolic reactions and implement them in the individual behaviour model of the bacteria.

- The hypothesis named as ‘Gibbs’ assumes that the bacterium executes the reactions following the decreasing sequence of standard Gibbs energy, a plausible and attractive strategy at individual level because it represents the spontaneity of a reaction carried out by a microorganism. Nevertheless, the simulation results achieved show that nitrous oxide concentration in the system reached higher values than those reported by experimental data.
- The hypothesis named as ‘Metabolic’ assumes that the bacterium executes the reactions following the decreasing sequence in the degree of oxidation of nitrogen oxides during the denitrification process, linking with the idea of the sequential use of the production and consumption of electron acceptors. The corresponding simulations results are better than those corresponding to Gibbs hypothesis after the graphical and numerical analysis of the data.
- Assuming the ‘Metabolic’ hypothesis, the majority of the variables controlled by the simulator, biomass, nitrate, nitrite, dinitrogen production, succinate, are in good agreement with their corresponding experimental measures, but the temporal evolution of nitrous oxide and nitric oxide need to be enhanced, according to the statistical analysis of data performed.

To improve the first simulator INDISIM-Paracoccus, a new individual-based model named INDISIM-Denitrification has been produced, which includes the new individual rule to reduce cytotoxic products, nitric oxide and/or nitrous oxide, through the degradation of individual mass.

- The individual rule to reduce cytotoxic products seems to be plausible and correctly implemented in view of the successful results obtained. The simulation results are more suitable to the experimental data reported for *P. denitrificans.*
- With INDISIM-Denitrification the user has the option to change the molar relationship between the atoms of the biomass empirical formula (C, H, O, N), which represents a denitrifying bacterium, readjusting the stoichiometric coefficients for the attainment of the set of microbial metabolic reactions of each metabolic pathway. This improvement in the model provides it with a greater range of application.
- The adequacy of the model INDISIM-Denitrification has been tested successfully with another set of experimental data, which correspond to the denitrifying bacterium *A. xylosoxidans* growing in a bioreactor in both aerobic and anaerobic conditions.
- According to the statistical analysis of the simulations results, INDISIM-Denitrification, for both denitrifying bacteria tested, gives better adjustments in the experiments with electron donor limited than in the experiments with electron acceptor limited.
- INDISIM-Denitrification is a promising tool to study any denitrifying bacterium in batch and continuous cultures under both aerobic and anaerobic conditions, in that it has been implemented in the freely available programming platform NetLogo, making the simulation model accessible to users and allowing increased and better manipulation and comprehension of denitrification process.
- As far as we know INDISIM-Denitrification is the first attempt to study the denitrification process using the individual-based model approach jointly with thermodynamic reactions for the metabolic sub-model.

7.2 Perspectives and future work

We believe that the use of an approach in the field of non-equilibrium thermodynamics to describe the microbial metabolism has shown successful results and this methodology could be extended to other modelling frameworks. The use of MbT-tool outputs could be

assumed as a starting point to design the metabolic sub models in other INDISIM branches to improve the design and parametrization of the model.

Using the individual-based model developed it is possible to model bacteria that can adapt their behaviour according to the local environmental conditions and phase of the system evolution. Therefore, virtual experiments can be developed with some specific environmental characteristics where the bacteria execute a metabolic pathway using some value of energy-transfer-efficiency (ε) while in another environmental condition it executes the same pathway using a different ε value. This is an interesting approach, from our point of view, because it is well known that ε value is not a constant in a metabolic process, and it can change over time and according to the biological, physical or chemical environmental conditions.

The individual-based model has been developed and adjusted to reproduce the experimental protocols of denitrifying bacteria in a bioreactor under controlled conditions. Therefore, the model can be adapted to more complex systems, e.g. wastewater treatments, soil management, and composting processes, among others.

Part of this denitrification model (INDISIM-Denitrification) could be incorporated into INDISIM-SOM, extending this soil model to complement the soil nitrogen cycle to deal with a mixed microbial community. This type of model can contribute to understanding *in vitro* what the consequences are of different media conditions and different microbial functional groups (heterotrophs, autotrophs and denitrifies) that can execute different metabolic pathways depending on the presence of oxygen in soil atmosphere which will determinate the nitrous oxide emissions and other nitrogen oxide productions.

There are some experimental works, which make reference to the role played by some elements in the denitrification process such is copper and/or iron, because they are a co-factor in activating some denitrifying enzymes. The dependence or not is specific to some bacteria species. Study of this relation through the modeling process will be of great interest. Using a model such as INDISIM-Denitrification could be the next step to progress in knowledge of denitrification.

List of Figures

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List of Acronyms

Definition	*Acronym*	*Units*
Agent-based model	ABM	
Ammonium	NH_4^+	
Bacterial yield	Yc/c	$molC_{mic}/molC_{substrate}$
Bicarbonate	HCO_3^-	
BIOlogía COMputacional y Sistemas Complejos	BIOCOM-SC	
BIOlogical TREATment model	BIOTREAT	
Biomass yield production	Y	
Biomass yield per mole of ATP consumed	Y_{ATP}	
Biomass yield per mole of available electrons	Y_{AVE}	
Biomass yield per mole of electron donor consumed	Y_{DX}	
Carbon	C	
Carbon dioxide	CO_2	
Center for Connected Learning in Northwestern University	CCL	
Comma-separated values (in computing)	CSV	
Cooper	Cu	
Degree of reduction of cells	γ_x	
Degree of reduction of electron donor	γ_d	
Dinitrogen gas	N_2	
Electron acceptor	e-acceptor	
Electron donor	e-donor	
Energy reaction	Re	
Energy-transfer-efficiency	ε	
Fick's law binary diffusion coefficients in water	Dab	h^{-1}
Fraction of each nutrient in a spatial cell that is accessible per unit of time and for the individual	a_i	h^{-1}

Definition	*Acronym*	*Units*
Fraction of electron donor electrons converted for synthesis	fs^o	eeq cells/eeq donor
Fraction of electron donor electrons used for energy and converted to reaction products	fe^o	eeq products/eeq donor
Generic empirical chemical formula of cells	CnHaObNc	
Geometric reliability index	GRI	
Global reaction according with TEEM	R	
High	H	
INDividual DIScrete SIMulations	INDISIM	
Individual nutrient uptake-rate	ui	$mol_{nutrient} \cdot mol_{mass}^{-1} \cdot h^{-1}$ used in INDISIM-Paracoccus, and $mol_{nutrient} \cdot C\text{-}mol_{mic}^{-1} \cdot h^{-1}$ used in INDISIM-Denitrification
Individual-based model	IBM	
Individual-based Observation	IBO	
Iron	Fe	
Lotka-Volterra model	LVM	
Low	L	
Maximum population growth rate	μ_{max}	h^{-1}
Maximum uptake capacities	U_i	$mol_{nutrient} \cdot h^{-1}$
Medium	M	
Metabolism-based on Thermodynamics	MbT	
Microbial Metabolic Reaction	MMR	
MOdelización y SIMulación discreta de sistemas BIOlógicos	MOSIMBIO	
Nitrate	NO_3^-	
Nitrate reductase	Nar	
Nitric oxide	NO	
Nitric oxide reductase	Nor	
Nitrite	NO_2^-	
Nitrite reductase	Nir	

Definition	*Acronym*	*Units*
Nitrogen	N	
Nitrous oxide	N_2O	
Nitrous oxide reductase	Nos	
Non-linear Physics and out of Equilibrium Systems	NOLIN	
Number of electron equivalents per mole of substrate from half-reaction reduction equation	p	
Number of oxygenase reactions per mole substrate	q	
Nutrient available in the culture medium	A_i	$mol_{nutrient} \cdot h^{-1}$
Open-source platforms	OSP	
Operating system (in computing)	OS	
Overview, Design and Details	ODD	
Oxygen	O_2	
Reaction to represent biomass degradation to reduce cytotoxic products	Rg	
Reduction potential for Acetyl-CoA half-reaction (TEEM2) or for Pyruvate half-reaction (TEEM1)	ΔGin	kJ/eeq
Reduction potential for electron acceptor half-reaction	ΔGa	kJ/eeq
Reduction potential for electron donor half-reaction	ΔGd	kJ/eeq
Reduction potential for formaldehyde half-reaction	ΔGfa	kJ/eeq
Reduction potential for NADH oxidation	ΔGxy	kJ/mol
Reduction-half-reaction for cell synthesis	Rc	
Reduction-half-reaction for electron acceptor	Ra	
Reduction-half-reaction for electron donor	Rd	
Secretaria Nacional de Educación Superior, Ciencia, Tecnología e Innovación del Ecuador	SENESCYT	
Standard Gibbs free energy for cell synthesis reaction	ΔGs	kJ/eeq
Standard Gibbs free energy for energy reaction	ΔGe	kJ/eeq

Definition	*Acronym*	*Units*
Standard Gibbs free energy for intermediate conversion to cells	ΔGpc	kJ/eeq
Synthesis reaction	Rs	
System-based Model	SBM	
Thermodynamic efficiency	n^{BB}	
Thermodynamic Electron Equivalents Model	TEEM	

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