CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

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DEPARTAMENTO DE
BIOTECNOLOGÍA Y BIOINGENIERÍA

“Aplicaciones de la microrrespirometría para la caracterización de bioprocesos”

T E S I S

Que presenta:

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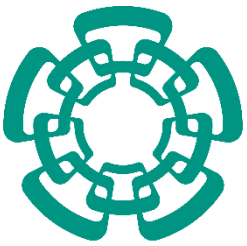

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"Applications of microrespirometry for the characterization of bioprocesses"

Doctoral thesis by:

Miguel Ángel Vital Jácome

To obtain the degree of:

DOCTOR OF SCIENCE
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Thesis co-directors:

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I. Vital-Jacome M, Dochain D, Thalasso F (2017). Microrespirometric model calibration applied to wastewater processes. *Biochemical Engineering Journal* (accepted for publication).

II. Vital-Jacome M, Dochain D, Thalasso F (2017). Microrespirometry for calibration and validation of the Activated Sludge Model No.3 (in preparation for publication).

III. Vital-Jacome M, Dochain D, Thalasso F (2017). Temperature effect on the kinetic and stoichiometric parameters of the ASM3 model by microrespirometry (ready for submission).

IV. Vital-Jacome M, Buitrón G, Moreno-Andrade I, Garcia-Rea V, Thalasso F. (2016). Microrespirometric determination of the effectiveness factor and biodegradation kinetics of aerobic granules degrading 4-chlorophenol as the sole carbon source. *Journal of Hazardous Materials* 313:112-121.

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RESUMEN

La estimación de parámetros en el modelado de bioprocesos es una tarea difícil, debido a varias dificultades que incluyen: la alta variabilidad de los sistemas biológicos, las pocas variables que se pueden medir con exactitud, los problemas de identificabilidad de los modelos y el correcto diseño de los experimentos para la estimación de parámetros. La microrrespirometría, es decir, la respirometría aplicada en sistemas de múltiples microrreactores, es un nuevo método recientemente desarrollado en nuestro grupo de investigación en Cinvestav-IPN, que puede ayudar a superar algunas de las dificultades antes mencionadas.

En esta tesis, quisimos explorar el potencial de la microrrespirometría, aplicando el método en varios casos de estudio en el área de tratamiento de aguas residuales. La caracterización de estos procesos se hizo a través de la calibración de modelos respirométricos específicos para cada caso. Prestamos especial atención a la estimación de los parámetros por el ajuste a modelos y a los problemas de identificabilidad que esto acarrea. Este enfoque nos permitió, entre otras cosas: establecer metodologías para la estimación de parámetros, evaluar las fuentes de incertidumbre del método microrrespirométrico y aplicar el método al estudio de otros temas de interés en el modelado bioprocesos.

Se demostró que la microrrespirometría fue útil para la calibración y validación de modelos, para la estimación del efecto de la temperatura en parámetros cinéticos y estequiométricos y para el estudio de la cinética microbiana y la transferencia de masa en gránulos aerobios. Encontramos que el estudio de casos prácticos fue la mejor manera de probar el potencial de microrrespirometría y evaluar sus limitaciones. Al final, el uso adecuado de este método nos ayudará a mejorar nuestra comprensión de los procesos biológicos, simplificando la tarea de calibración de modelos y mejorando la aplicación de los mismos.

ABSTRACT

Parameter estimation is a challenging task in bioprocess modelling, due to several difficulties that include: the high variability of biological systems, the few variables that can be accurately measured, the identifiability problems of models, and the correct design of experiments for parameter estimation. Microrespirometry, i.e., respirometry performed in microreactor systems, is a new method recently developed in our research group at Cinvestav-IPN, which can help to overcome some of the difficulties above mentioned.

In this thesis, we wanted to explore the potential of microrespirometry, by applying the method in various case studies in wastewater treatment. The characterization of these processes was done through the calibration of typical respirometric models. We paid a special attention to the estimation of parameters by model fitting and to the identifiability issues that this entails. This approach allowed us, among others: to establish methodologies for the estimation of parameters, to evaluate the sources of uncertainty of the microrespirometric method, and to apply the method to the study of other topics of interest in bioprocess modelling.

We showed that microrespirometry was useful for calibration and validation of models, for the estimation of the effect of temperature on kinetic and stoichiometric parameters, and for the study of microbial kinetics and mass transfer in aerobic granules. We found that the study of practical cases was the best way to test the potential of microrespirometry and to evaluate its limitations. In the end, the proper use of this method will help us to improve our understanding of biological processes by simplifying the model calibration task, and enhancing the application of models.

CHAPTER 1

INTRODUCTION

Bioprocesses are used in biotechnology to achieve the application of living organisms or their components to obtain desired products and services. These products are used in medicine, agriculture, food production and environmental applications.

Bioprocesses are complex systems, which require the knowledge and application of multiple disciplines and several fields of engineering. Bioprocess scientists and engineers use knowledge to design, to develop, to maintain, to research, to improve and to optimize, as well as to find suitable solutions to specific problems. Many of these tasks are achieved by creating mathematical models, which allow to analyze and to test potential solutions.

Mathematical models are the simplified representation of complex processes, and in biotechnology, they are specially used to describe the phenomena of substrate bioconversion and its relation to microbial growth, which is often called "microbial kinetics". It was Jacques Monod (1949) and his famous model (Eq. (1)), who initiated the mathematical modelling of microbial kinetics. Monod's model relates the microbial growth rate to the concentration of a single growth-controlling substrate via two parameters, the maximum specific growth rate (μ_m), and the half saturation constant (K_S). Monod also made the link between growth and substrate consumption (q_s), by using another parameter, the cell growth yield ($Y_{X/S}$), which is a measure of the conversion efficiency of substrate into cell material (Eq. (2)).

$$\mu = \frac{\mu_m \cdot S}{K_S + S} \tag{1}$$

$$q_s = \frac{\mu}{Y_{X/S}} \tag{2}$$

The study of microbial kinetics has gradually evolved since the Monod's model, other models emerged, some of them as a modification of the original model by introducing

new terms and parameters (Kovárová-Kovar and Egli 1998). Today, kinetic models are the base of advanced dynamic models, which are used for the design, study, characterization, control, and optimization of bioprocesses.

Models in general are made of three components, variables, constants, and parameters. Variables are the inputs and outputs of the model, which are related to constants and parameters through the structure of the model. Constants and parameters are different from each other; constants have always a fixed value, and parameters can change their value, depending on the circumstances of application of the model (Dochain and Vanrolleghem 2001).

The estimation of parameter values is part of the task known as "model calibration", and is a crucial step within the construction of models. Without these parameter values, it is impossible to apply models.

In bioprocesses modelling, the most important parameters are those related to microbial kinetics, because they describe the inherent properties of the biological processes. However, the estimation of biokinetic parameters is a challenging area, mainly for the following reasons:

(i) Working with living organisms, which change their behavior depending on the time and the environmental conditions; this causes a high variability of parameters reported in literature, even for the same microorganisms and substrates (Kovárová-Kovar and Egli 1998).

(ii) Kinetics are poorly known, because the metabolism is a network of complex reactions; this causes the development of complex nonlinear models, which are subject to many identifiability problems.

(iii) Only few variables can be measured on-line and off-line in bioprocesses, due to the lack or high cost of measurement methods; this makes very difficult to obtain reliable and reproducible experimental data for parameter estimation.

(iv) The experimental conditions used to estimate parameters have an important influence on the results; this causes that sometimes the estimated parameters do not reflect the true parameters of the process under actual conditions. For example, the initial substrate to biomass ratio (So/Xo) defines the type of parameters that we can estimate (intrinsic, defined, or extant; Grady et al. 1996).

In the research group on Environmental Bioprocesses at Cinvestav-IPN, we are dedicated to the development, study, implementation, and improvement of methods for the estimation of kinetic and stoichiometric parameters of biokinetic models, using for this purpose, respirometry based techniques.

Recently, our research group developed a new method that combines respirometry with the use of novel miniature bioreactors, which we have called "microrespirometry". This method has the potential to perform simultaneously multiple replicates of respirometric experiments, and can be used to improve and facilitate the parameter estimation task in many areas of biotechnology.

This thesis focuses on testing the advantages and limitations of microrespirometry for parameter estimation, and offers guidelines to exploit its maximum potential. In addition, we tested its application on two topics of interest for the modeling of bioprocesses: the effect of temperature on kinetic parameters and the study of microbial kinetics in aerobic granules.

CHAPTER 2

LITERATURE REVIEW

2.1 Respirometry

Respirometry was first defined as the measurement of the oxygen consumption rate of activated sludge under well-defined experimental conditions (Spanjers et al. 1999). As can be deduced from the definition, this method originated in wastewater treatment processes. Respirometry evolved from previous methods for determination of the biochemical oxygen demand (BOD) in wastewater. Respirometry uses the relations that exist in aerobic processes between the oxygen consumption, the substrate removal, and the microbial growth. Figure 1 shows a schematic representation of these relationships. When the substrate (electron donor) enters to the cell, it is oxidized through numerous metabolic reactions, its electrons flow through the electron transport chain, and eventually end to the oxygen (electron acceptor). Microorganisms convert the energy of the chemical bonds in the substrate to the high-energy phosphate bonds of adenosine triphosphate (ATP). This energy is used to synthesize new biomolecules required for cell growth. In this way, the oxygen consumption or respiration is an indirect indicator of the substrate uptake and the microbial growth. In respirometry, respiration is measured by changes in oxygen concentration in the liquid or the gas phase.

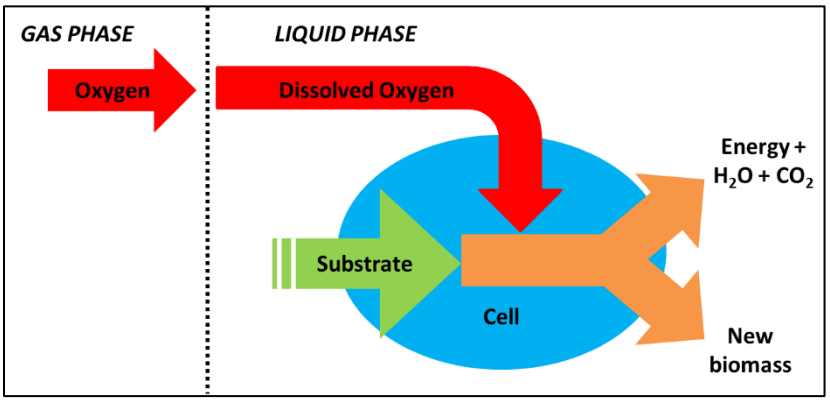

Figure 1. The relationship between respiration, substrate uptake and microbial growth (modified from Spanjers et al. 1998).

Respirometry is performed in devices called respirometers, which are basically small bioreactors equipped to measure the oxygen uptake rate (OUR). The classification of respirometers according to the Respirometry Group Task of the International Water Association (IWA) can be found in Spanjers et al. (1998). Respirometers are classified according to: the phase where oxygen is measured (liquid/gas), the gas operating conditions (flowing/static), and the liquid operating conditions (flowing/static).

The most widely used measurement method in respirometry, is the measurement of the dissolved oxygen concentration (DO) in the liquid phase, which is convenient for the following reasons: (i) DO can be measured "in situ" and in real time with the use of a specific, and available and low-cost probe; (ii) DO measures can be performed with high sensitivity and precision (on the order of 0.01 mg L^{-1}); (iii) DO changes are related to the respiration rate in any process where oxygen acts as the final electron acceptor; (iv) DO measured in the liquid corresponds to the total oxygen in the system, since intracellular oxygen is negligible, in contrast to the measurement of other substrates.

Respirometry has several applications, and most them, are in wastewater treatment processes. Among these applications, the most important are:

- Determination of different COD fractions in wastewater (Brouwer et al. 1998; Mathieu and Etienne 2000).
- Model calibration of the activated sludge models (ASM) for wastewater treatment (Henze et al. 2000; Petersen et al. 2003).
- Estimation of biological activity and inhibitory effects on activated sludge (Guisasola et al. 2004; Kong et al. 1996).
- Control of activated sludge processes (Copp et al. 2002; Spanjers et al. 1998).

In respirometry, the most commonly used methods for obtaining OUR data are the static and the dynamic methods. Static respirometry is based on the measurement of the DO concentration in a non-aerated respirometer, where the OUR is measured from the decreasing slope of DO during the experiment. Dynamic respirometry is based on the measurement of DO in an aerated respirometer. When it is combined with the injection of substrate pulses, the method is called “dynamic pulse respirometry” and in

that case, the OUR is measured from the DO curve obtained during the experiment, called respirogram, and for the correct interpretation of the data it is necessary to estimate the oxygen transfer rate of the system (Ramirez-Vargas et al. 2013).

In Figure 2, we show an example of a respirogram obtained with the dynamic pulse respirometric method, which was the main method used in this thesis. The general procedure to obtain the respirogram described in Figure 2 is as follows: (i) the biomass is aerated until reaching a "pseudo-stationary" state, corresponding to the endogenous respiration (α) (Spanjers and Vanrolleghem 1995); (ii) a pulse of known concentration of substrate is injected (β), and the evolution of the DO is recorded until the endogenous respiration state is reached again (γ).

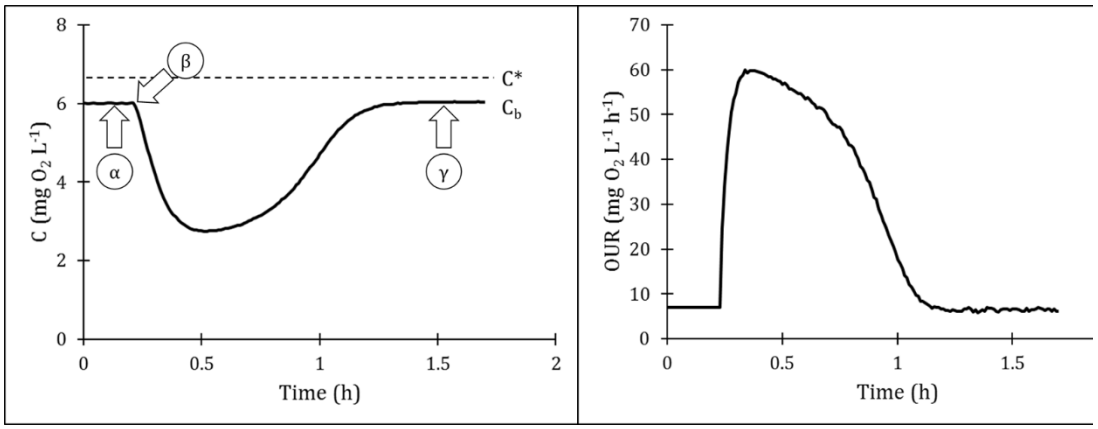

Figure 2. Example of respirogram obtained using the dynamic method shown as DO concentration (left) and OUR (right).

The dynamical changes observed during the respirometric experiments showed in Figure 2, are representative of the biodegradation kinetics of the substrate injected. Once the experimental data are obtained, the next objective of respirometry is to identify a kinetic model and the parameters that describe the dynamics observed in the respirometer.

2.2 Respirometric based-models

Respirometric models are those that describe the dynamical changes inside a respirometer. These models consider the respirometer as a batch reactor, where the DO mass balance equation in the bulk liquid of the respirometer has two components (Eq. (3)): the oxygen transfer rate (OTR) and the oxygen uptake rate (OUR). The OTR is described by Eq. (4), where $K_L a$ is the volumetric oxygen mass transfer coefficient, C^* is the DO saturation concentration, and C is the DO concentration at each instant of time. The OUR is described by Eq. (5) and has two components: the endogenous oxygen uptake rate (OUR_{end}), which is the oxygen consumption in absence of readily biodegradable substrate (Spanjers and Vanrolleghem 1995); and the exogenous oxygen uptake rate (OUR_{ex}), which is the oxygen consumption needed to degrade the amount of substrate injected.

$$\frac{dC}{dt} = OTR - OUR \tag{3}$$

$$OTR = K_L a \cdot (C^* - C) \tag{4}$$

$$OUR = OUR_{end} + OUR_{ex} \tag{5}$$

The term OUR_{ex} depends on the biodegradation kinetics of the k substrates (S_i) present in the pulse injected (Eq. (6); Dochain and Vanrolleghem 2001).

$$OUR_{ex} = \sum_{i=1}^{k} (1 - Y_i) r_{S_i} \tag{6}$$

In Eq. (6), Y_i is the yield coefficient or the fraction of substrate S_i that is not oxidized but converted into new biomass (X), and r_{S_i} is the consumption rate of S_i. Depending on the case study and the objective of the model, the OUR_{ex} (Eq. (6)) can be related to the consumption of one or more substrates ($k = 1, \dots, N$). In the same batch conditions of the respirometric experiments, the mass balance of S_i is described by Eq. (7).

$$\frac{dS_i}{dt} = -r_{S_i} \tag{7}$$

Typically, the change of the biomass concentration in respirometric experiments is considered negligible, i.e., $\frac{dX}{dt} = 0$, because the initial ratio So/Xo is usually low, and the experiments are of short duration. Another useful assumption is to consider that oxygen consumption is only due to exogenous respiration (OUR_{ex}), i.e., the endogenous respiration (OUR_{end}) is considered constant during the experiment, and therefore can be removed from the data or analyzed separately.

The simplest respirometric models, also used during the development of this thesis, are those that consider the consumption of a single growth limiting substrate ($k = 1$). These models are useful when determining the kinetics of readily biodegradable substrates, and may involve different types of kinetic models, for example:

1) Monod kinetics:

$$\frac{dS}{dt} = -r_S = -\frac{\mu_m \cdot X}{Y_{X/S}} \cdot \frac{S}{K_S + S} = -\frac{OUR_{max}}{\left(1 - Y_{X/S}\right)} \cdot \frac{S}{K_S + S} \tag{8}$$

$$OUR_{ex} = \frac{\mu_m \cdot X \cdot \left(1 - Y_{X/S}\right)}{Y_{X/S}} \cdot \frac{S}{K_S + S} = OUR_{max} \cdot \frac{S}{K_S + S} \tag{9}$$

2) Haldane kinetics:

$$\frac{dS}{dt} = -r_S = -\frac{\mu_m \cdot X}{Y_{X/S}} \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}} = -\frac{OUR_{max}}{\left(1 - Y_{X/S}\right)} \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}} \tag{10}$$

$$OUR_{ex} = \frac{\mu_m \cdot X \cdot \left(1 - Y_{X/S}\right)}{Y_{X/S}} \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}} = OUR_{max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}} \tag{11}$$

In these models, the parameters that we want to estimate with respirometric data are: μ_m, K_S and $Y_{X/S}$ (plus K_I in the Haldane case); but the success in the estimation depends on the method of data analysis and the identifiability problems associated to the model (section 2.4). Sometimes, it is useful to combine some parameters into new ones, for example, the parameter OUR_{max} (Eq. (8-11)) that indicates the maximum oxygen uptake rate; this combination results in parameters that provide useful and additional information, but can also help us to solve certain identification problems (Dochain and Vanrolleghem 2001).

2.2.1 Respirometry for calibration of ASM models

To describe the removal of pollutants in wastewater by activated sludge, in some instances, it is convenient to have an exhaustive description of the process. The Activated Sludge Models (ASM) are complex models, developed by the IWA and the Task Group of Mathematical Modelling for Design and Operation of Biological Wastewater Treatment (Henze et al. 2000). The ASM models are partially structured models based on Monod kinetics that comprise the knowledge about the different components and reactions that occur in activated sludge processes. Respirometry is the basis of the calibration of ASM models (Petersen et al. 2003), because the respiration of activated sludge is affected by the concentration of aerobically biodegradable components, which are most of pollutants in wastewater. Parameter estimation by respirometry is performed using simplified versions of ASM models, considering only the processes and state variables that are important to the experiment (Guisasola et al. 2005; Ordaz et al. 2012). The original ASM models include: ASM1, ASM2, ASM2d and ASM3, but since their publication, other ASM based models including many model extensions have been published. As an example, the models ASM1 and ASM3 are briefly described below, together with their relationship with respirometry. In this description, only the growth of heterotrophs based on degradation of the chemical oxygen demand (COD) is considered, however, both models may include the growth and oxygen consumption by autotrophs.

In Activated Sludge Model No.1 (ASM1), heterotrophic biomass uses COD in a cyclic reaction scheme (Henze et al. 2000). This scheme is shown in Figure 3, where readily biodegradable COD (S_S) is consumed together with oxygen for growth of heterotrophic biomass (X_H); decay processes produce inert suspended organic matter (X_I) and feeds hydrolysis with slowly biodegradable COD (X_S).

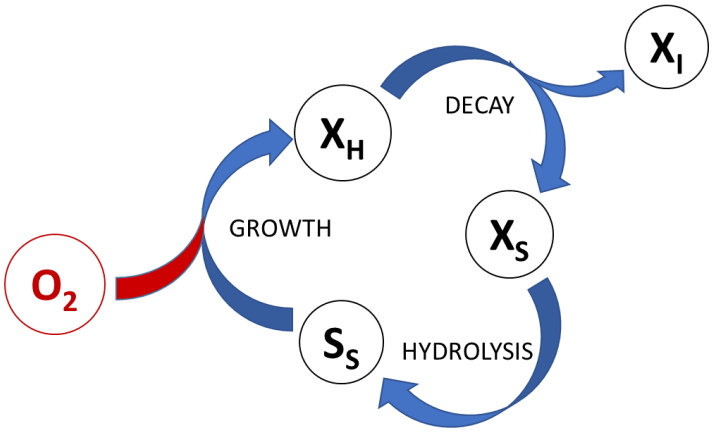

Figure 3. Flow of COD in ASM1

A detailed description of the calibration of the ASM1 model can be found in Petersen et al. (2003). Most of the wastewater component concentrations of the model, including S_S, X_H and X_S, can be estimated using respirometric experiments. In ASM1, there is only one process consuming oxygen for degradation of COD, and their kinetic and stoichiometric parameters are obtained by respirometry. The ASM1 model is considered in many cases the state-of-the-art for modelling activated sludge systems, and is the most used model in practice. However, it is known that this model is difficult to calibrate, because as shown in Figure 3, one needs to calibrate other processes that indirectly influence the only process that consume oxygen (Van Loosdrecht et al. 2015).

In contrast, the reaction scheme of Activated Sludge Model No.3 (ASM3) is shown in Figure 4. In ASM3 as in ASM1, readily biodegradable COD (S_S) is present in the wastewater and is produced by hydrolysis of slowly biodegradable COD (X_S). In ASM3, S_S is first stored in the form of cell internal products (X_{STO}), such products

include poly-hydroxy-alkanoates (PHA), glycogen, etc. Once S_S is depleted under feast conditions, X_{STO} is used under famine conditions for the growth of heterotrophic biomass (X_H). An important difference with respect to ASM1, is that in ASM3 the idea of decay processes is replaced with endogenous respiration processes (Henze et al. 2000).

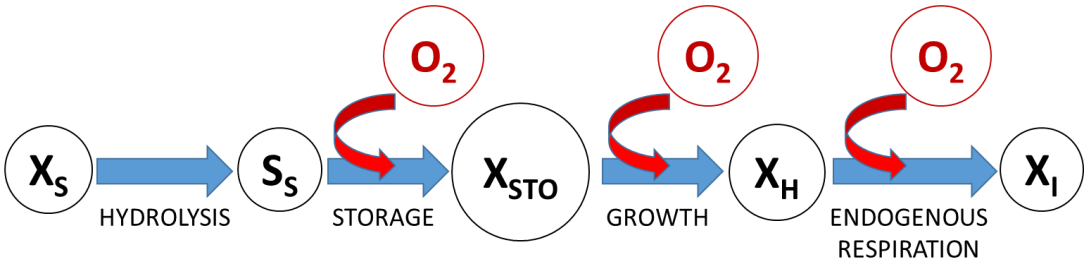

Figure 4. Flow of COD in ASM3

The ASM3 model is not cyclic like ASM1 and has more oxygen entry points. This means that the parameters of the three processes: the rate of oxygen consumption for degradation or storage of S_S, the rate of growth associated with degradation of X_{STO}, and the endogenous respiration, can be estimated by respirometric methods. Besides, due to the greater number oxygen entry points, the ASM3 model is considered easier to calibrate compared to the ASM1 model. In the cyclic scheme of ASM1, any change in the parameter values influences more the state variables compared to ASM3. Thus, the ASM3 model has better identification properties than the ASM1 model (Gernaey et al. 2004). In this thesis, for the characterization of activated sludge processes, the use of the ASM3 model over ASM1 was preferred because of the advantages above mentioned.

2.3 Estimation of parameters using respirometry

There are several methods and approaches to process the data obtained from respirometric experiments to estimate parameters, using either DO or OUR measures. The most common methods for parameter estimation in respirometry are:

1. Direct analysis or interpretation of respirograms. These methods involve the identification of baselines, points of inflection and area analysis of respirograms (Spanjers et al. 1999). This approach, for example, allows to estimate different waste water component concentrations, as well as stoichiometric parameters like substrate or storage yields (Karahan-Gül et al. 2002; Petersen et al. 2003). Direct methods are easy to implement, and their great advantage is that they do not cause parameter identifiability problems (Spanjers et al. 1999). However, these methods can only be applied when individual substrate components determine the shape of the respirograms, because these kind of respirograms have an easier and less ambiguous interpretation (Decubber 2014).

2. Optimization by model fitting to respirograms. In this approach, numerical optimization methods are used to estimate the parameter values that best describe the experimental data of respirograms (Spanjers and Vanrolleghem 1995). This approach allows us to estimate wastewater component concentrations, as well as kinetic and stoichiometric parameters, including maximum rates, saturation constants and stoichiometric yields (Brouwer et al. 1998; Lagarde et al. 2005; Petersen et al. 2003). Optimization methods are the most used in the literature; these methods are useful for parameter estimation of complex models, because they allow to estimate numerous parameters and to make a better description of the experimental data. However, the main disadvantage of these methods are the identifiability problems associated with the large number of parameters to be estimated, of a single measured state variable (Checchi and Marsili-Libelli 2005).

3. Estimation of parameters by linearization methods. These methods take advantage of the similarity between model structures of the Michaelis-Menten model for enzyme kinetics, and the Monod model for microbial kinetics. In this approach, linear graphs are constructed using linearization methods commonly applied in enzymatic kinetics, for example, Hanes-Woolf or Lineweaver-Burk. The points of these graphs come from respirometric experiments at increasing concentrations of substrate pulses. The parameters of the Monod model, the maximum specific growth rate (μ_m) and the half saturation constant (K_S) are estimated from the slope and the intersection of the axes of the linear graphs (Ramirez-Vargas et al. 2013). Linearization methods are easy to implement and to interpret. Another advantage of these methods is that they can be used for studying the effects or certain compounds, which have an unknown effect on the process, and that cannot be therefore added to more complex models. However, the main disadvantages of these methods, are the errors associated with the linearization methods and the limited number of parameters that can be estimated (Decubber 2014). In our research group at Cinvestav, these methods are known by the name of "pulses of increasing substrate concentration", and have been successfully applied for "in situ" characterization of different types of bioprocesses and reactor configurations (Oliveira et al. 2011; Ordaz et al. 2008; Ordaz et al. 2012; Ordaz et al. 2013).

In this thesis, we performed the estimation of parameters combining elements of the previously described approaches. For example, the estimation of stoichiometric yields was performed by direct interpretation of the respirograms area, the estimation of kinetic parameters was performed by model fitting to the experimental data, and pulses of increasing concentrations were used to obtain data to solve identifiability problems and perform model validation.

2.4 Parameter identifiability

Parameter identifiability is one of the major concerns of parameter estimation by respirometric methods. This topic has been extensively studied in literature (Checchi and Marsili-Libelli 2005; Dochain and Vanrolleghem 2001; Petersen 2000; Sin 2004).

The problem of identifiability is fundamental in bioprocess modelling, because of the nonlinear nature of microbial kinetics. The main objective of an identifiability analysis is to answer the following question (Dochain and Vanrolleghem 2001):

If only certain state variables are available for measurement, given the structure of the model (*structural identifiability*) and given the quality of the experimental data (*practical identifiability*), can we estimate unique values of the model parameters?

When structural or practical identifiability problems are present, the parameters values cannot be estimated or only with a large uncertainty, i.e., the estimated values are one among several possible solutions.

2.4.1 Structural identifiability

The structural identifiability, refers to the capacity to find unique values of the parameters of a model, having perfect measurements, i.e., noiseless data of the available state variables. A simple example borrowed from MacLean (2012) can help to illustrate this concept: in a linear model defined by $y = (a + 2b)x + c$, the parameters a and b can not be distinguished from each other; the parameters $(a + 2b)$ and c are structurally identifiable, but a and b are not identifiable because they can take any possible combination of values.

A structural identifiability analysis has two objectives: first, to show if the parameters of a model are identifiable; and second, in the case of finding identifiability problems, to show if there are some subsets or combinations of the model parameters that may be identifiable. Several methods to test structural identifiability in linear and nonlinear

models can be found in literature; these methods are complex and require an advanced degree of math expertise, and sometimes the use of symbolic software (e.g. Mathematica, Maple). In this thesis, structural identifiability analyzes were beyond the scope of the project, but a detailed review of concepts and methods applied to respirometric models can be found in Dochain and Vanrolleghem (2001).

2.4.2 Practical identifiability

The practical identifiability, evaluates the information content of the available data, i.e., if it is possible to find unique values of the model parameters having noise corrupted data of the available state variables. The classical example in bioprocess is the Monod model (Eq. (1)), where the parameters μ_m and K_S are usually highly correlated. Parameter correlation means that a change in one parameter can be compensated almost completely by a proportional shift in another parameter (Dochain and Vanrolleghem 2001); if after this shift, the fit between the model and the experimental data is the same, the parameters have practical identifiability problems. In the case of the Monod model, if only a few measurements are taken at low substrate concentrations, many combinations of μ_m and K_S can fit the data, and under these conditions only the ratio μ_m/K_S is identifiable. The objective of a practical identifiability analysis is to find the uncertainty of the parameter estimates, usually in terms of the confidence intervals. If the uncertainty of a certain parameter is large, it has practical identifiability problems, and therefore, its estimation cannot be relied with the available experimental data.

Several methods can be found in the literature for practical identifiability and uncertainty analysis. These methods include Bootstrapping, the Fisher Information Matrix, and the Profile Likelihood (Fröhlich et al. 2014; Joshi et al. 2006; Weber et al. 2011). Among them, the practical identifiability analysis in respirometric models is often performed by means of the Fisher Information Matrix (FIM), combined with information of the parameter sensitivity functions. In fact, most of the information generated in wastewater treatment, respirometric methods, and ASM models, was

achieved by FIM based methods. For instance, Vanrolleghem et al. (1995) performed a practical identifiability analysis of the single Monod respirometric model using the FIM; Weijers and Vanrolleghem (1997) proposed a method for selecting the best identifiable parameter subsets for calibration of ASM1 model based on the FIM; later, Brun et al. (2002) developed a methodology for selecting the best identifiable parameter subsets for calibration of ASM2d, based on sensitivity functions and the collinearity index. In the meantime, Petersen (2000) studied how to improve identifiability of ASM based models combining respirometry with titrimetric methods, using experiments based on Optimal Experimental Design (Dochain and Vanrolleghem 2001).

2.5 Microreactor systems

Microreactor systems, also described in the literature as microbioreactors, miniature bioreactors or microtiter plates, are small volume devices specially designed for clonal selection, strain screening, and optimization of process and culture media (Isett et al. 2007). These systems have been developed for working volumes of microliters to milliliters, in commercial versions ranging from 12 and 24, to 96 wells or plates in parallel (Duetz 2007).

Microreactor systems were made possible thanks to the development of optical fluorescence quenching sensors of DO and pH (Arain et al. 2006). These sensors can be miniaturized and have several advantages compared to their traditional electrochemical counterparts. In the case of DO, for example: DO fluorescence-based sensors have significantly lower response times (<5s), are more stable after calibration, are less susceptible to interference by chemical compounds, and are more sensitive to low DO concentrations (Wolfbeis 2015).

Several microreactor designs have been developed. The simplest systems require an external incubator to control temperature and agitation, while other more advanced systems have their own agitation system as well as DO, temperature, pH, and aeration controls (Betts and Baganz 2006). A detailed review about classification and special features of microreactor systems can be found in Kim et al. (2012). The current trend of this technology is toward more automation and even smaller reaction vessel sizes (Lattermann and Büchs 2015).

Microreactors have been successfully applied for high-throughput selection of microbial and animal cell cultures (Chen et al. 2009; Isett et al. 2007). Moreover, a special attention has been paid to assessing the oxygen transfer capacity of these systems, which is compared to traditional lab-scale bioreactors (Funke et al. 2009; Kirk and Szita 2013). These features, in combination with the DO fluorescence-based sensors, make microreactors a very attractive system for applications in the measurement of the oxygen consumption in biological processes, and therefore, with high potential to develop respirometric techniques.

2.6 Microrespirometry

Nowadays, traditional respirometric methods are well-established and have been extensively studied; however, these methods are still subject to some drawbacks and methodological limitations, which affect the accuracy and precision of parameter estimation. These drawbacks include: mass transfer limitations and microbial aggregation changes due to mixing conditions (Chu et al. 2003); high variability between replicates of experiments (Magbanua Jr et al. 1998); high response times in measurements due to the use of electrochemical DO sensors (Betancur et al. 2008); transient response phenomena in the experiments (Vanrolleghem et al. 2004); among others. Fortunately, some of these drawbacks can be overcome with the application of new technologies and the evolution of the respirometric methods.

In this respect, "microrespirometry" is a relatively new method developed in our research group at Cinvestav-IPN. Basically, microrespirometry is a respirometry based method performed in microreactor systems, transforming traditional respirometry into a high-throughput method. The idea behind microrespirometry is to perform multiple simultaneous respirometric experiments, not only by multiplying the number of replicates but also the quantity and quality of the data available for parameter estimation. The study of microrespirometry in our research group started with the characterization of two different microreactor systems:

On one hand, Esquivel-Rios et al. (2014b) performed the characterization of an unbaffled 24-well (2.5 mL) microreactor system (PreSens, Mexico). This system requires an external incubator to achieve the agitation that causes the transfer of oxygen in the medium, and to maintain the temperature condition. These authors determined that the oxygen mass transfer capacity of the system, in terms of K_La, ranged from 2.2 to 48.0 h^{-1} under different operating conditions (liquid volume, agitation speed and number of glass beads used as stirrers).

On the other hand, Ramirez-Vargas et al. (2014) performed the characterization of a 24-well (10 mL) microreactor system (Micro-24 System, Pall Corporation, USA). This system is more specialized, and includes control and monitoring of pH, temperature,

DO, agitation and aeration. These authors determined that the oxygen mass transfer was characterized by $K_L a$ values ranging from 9 to 80 h^{-1}. This range was obtained by testing several features and operating conditions: 2 types of cassette and 3 types of cap designs, liquid volume, temperature, aeration rate and agitation speed.

Microrespirometry has many advantages over traditional respirometry, for example: the volume of sample required to perform the experiments is smaller; the number of simultaneous experimental replicates increases drastically; the quality of experimental data increases due to the use of better DO sensors; the oxygen mass transfer can be controlled in the same range as in traditional respirometers without the use of mechanical stirrers; and the number of experimental conditions that can be tested in one single experiment reduces the experimental effort.

However, together with these attractive advantages, there are also certain challenges that must be overcome before the use of microrespirometry can be extended. For example:

(i) New approaches for the treatment of the large amount of data that must be developed.

(ii) The positive or negative impact of the large amount of data available for parameter estimation must be assessed.

(iii) The experimental methodologies for the collection of data with this method must be standardized.

(iv) The impact of typical parameter estimation problems, like identifiably, must be evaluated.

(v) The impact of new sources of uncertainty in the parameter estimates must be evaluated.

2.7. Applications of microrespirometry

Some authors have measured the OUR in microreactor systems to monitor the respiratory activity of microbial cultures (Arain et al. 2006; Puskeiler et al. 2005). However, to the best of our knowledge, only in our research group the DO data produced by microreactors have been used for the estimation of kinetic and stoichiometric parameters. For instance: Esquivel-Rios et al. (2014b) developed a microrespirometric method based on linearization of the Monod model to estimate the parameters μ_m, K_S and $Y_{X/S}$ in heterotrophic and autotrophic cultures; Ramirez-Vargas et al. (2014) showed that a microreactor system was suitable for the measurement of OUR and the acquisition of data with potential use for parameter estimation by model fitting; and Esquivel-Rios et al. (2014a) characterized the inhibition by heavy metals (copper and zinc) on activated sludge, by evaluating the effect of different concentrations of these metals and other parameters on the kinetic parameters of the Monod model.

Potential applications of microrespirometry include many possibilities in various areas of biotechnology, as there is still many cases where model calibration is required. In this thesis, we selected two applications: the effect of temperature on kinetic parameters and the study of microbial kinetics of aerobic granules; the relevance of these topics is detailed in the following sections.

2.7.1 Effect of temperature on kinetic parameters

Temperature is the environmental factor that most affects the rate of biochemical reactions, and therefore, it also affects the metabolism and the growth rate of microorganisms on a macroscale. In biotechnology, temperature is of practical importance for the control of bioprocesses and the safe handling of products in the food industry (Rosso et al. 1993).

In terms of models, the effect of temperature is reflected by changes in the values of the model parameters. These changes have been extensively described for the parameter μ_m of the Monod model, used to reflect changes in the growth rate, and for which several models have been developed (see Table I of **paper IV** for details). However, there is a lack of information and reliable data on the effect of temperature on other kinetic parameters, including the parameter K_S of the Monod model, the stoichiometric parameter $Y_{X/S}$, and especially in parameters of other kinetic models. This lack of information and data is mainly due to the difficulties of parameter estimation, which could be partially solved with the use of better experimental methods.

Due to the characteristics of microrespirometry, which includes the possibility of performing simultaneous experiments at different experimental conditions, the evaluation of the effect of temperature on kinetic parameters is one of the most obvious applications, considering the significant saving of time and resources.

Our research on this subject began in the work of the master's thesis (Vital-Jacome 2013). In that work, we estimated the effect of temperature on the kinetic parameters of the Monod model ($\mu_m, K_S, Y_{X/S}$) for activated sludge and a nitrifying consortium; however, no attention was paid to the possible identifiability problems of the model, and how these problems could affect the trends of the parameters that we observed. Therefore, a more rigorous analysis considering all these omissions and testing more complex models would be useful.

2.7.2 Microbial kinetics of aerobic granules

Aerobic granules are defined as aggregates or biofilms of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs (de Kreuk et al. 2007). Aerobic granules are one of the most promising technologies for the treatment of wastewater from municipal and industrial sources, as well as several organic toxic compounds and emerging contaminants (Adav

et al. 2008; Sarma et al. 2017). Because of their compact structure, these aggregates have many advantages compared to traditional processes of activated sludge flocs, for example: aerobic granules have better settle-ability, higher biomass retention, higher ability to resist high organic loading rates, and higher tolerance to toxicity (Adav et al. 2008).

Mathematical modelling of aerobic granular sludge has proven to be very useful to study these complex biofilm systems (Ni and Yu 2010). The bioconversion processes in the granules are determined by concentration gradients of oxygen and diverse substrates, which can be simulated using models. These models also require the estimation of kinetic parameters, and because of the aerobic nature of the granules, respirometric based methods can be useful for accomplishing this task.

Parameters estimated in biofilm systems can be divided into two categories: intrinsic parameters, if they are obtained by minimizing mass transfer resistance; and apparent parameters, when mass transfer resistance affects their estimation. The intrinsic kinetic parameters are the true parameter values, which should be used in biofilm models based on diffusion and reaction. The apparent kinetic parameters are the observed parameter values, which encompass both the biological reaction and the internal/external mass transfer in a biofilm.

Other useful parameters that can be estimated for biofilms are the effectiveness factor (η), defined by Eq. (12), and the Thiele (ϕ) modulus, defined by Eq. (13) (Liu et al. 2005). These parameters, which derived from models used in chemical engineering to describe diffusion and reaction in porous catalyst particles, provide valuable information in the design of biofilm processes. Typically, η is a measure of the effective usage of the catalyst particle space (Álvarez-Ramírez et al. 2005), and can be calculated as a function of ϕ.

$$\eta = \frac{Reaction\ rate\ with\ mass\ transfer\ resistance}{Reaction\ rate\ without\ mass\ transfer\ resistace} \tag{12}$$

$$\phi = \frac{Reaction\ rate\ at\ the\ surface}{Diffusion\ rate\ through\ the\ biofilm} \quad (13)$$

Unfortunately, in the modeling of aerobic granules, much attention has been paid to the development of models but little attention has been paid to the estimation of kinetic parameters, among other reasons, due to the difficulties of their estimation. Therefore, an approach to this problem based on microrespirometry may be useful.

CHAPTER 3

PROBLEM STATEMENT

Parameter estimation is a crucial task of model calibration, and is essential for the construction of models in bioprocesses. However, in biological systems, there are many difficulties that limit the reliable estimation of kinetic and stoichiometric parameters. At lab-scale, the development and further improvement of the existing experimental methods can help to overcome these difficulties, facilitating, and improving the accuracy of parameter estimation.

In this context, in our research group at Cinvestav-IPN, we developed a high quality and high throughput method, which combines the use of novel microreactor technologies and respirometry based methods for parameter estimation. This method, called "microrespirometry", allows to perform multiple simultaneous respirometric experiments, saving time, resources and improving the quality and quantity of the experimental data. We recently applied microrespirometry for the estimation of the kinetic parameters of the classical Monod model by linearization methods; however, the full potential of this method has not been exploited yet.

Reaching this potential will provide us with a powerful tool for the estimation of kinetic parameters in bioprocesses. However, we must solve some of the new challenges related to the application of microrespirometry. For that reason, in this thesis we developed a study applying microrespirometry in various case studies, with the aim of explore the potential of this method for the characterization of bioprocesses. For the first time, we assessed a methodology for parameter estimation by model fitting; we considered the problems of identifiability and evaluated the impact of other possible sources of parameter uncertainty. In addition, we applied the microrespirometric method in two relevant topics: the estimation of the effect of temperature on kinetic parameters and the estimation of kinetic parameters on aerobic granules. The results of this thesis will provide valuable information for the use of microrespirometry and the modelling of bioprocesses.

CHAPTER 4

OBJECTIVES AND HYPOTHESIS

3.1 General objective

To explore the potential of microrespirometry for the characterization of bioprocesses, by applying this method in various case studies for the estimation of kinetic and stoichiometric parameters of biokinetic models.

3.2 Specific objectives

To achieve the general objective, this thesis was divided into the following specific objectives:

1. To establish the methodology for data analysis, parameter estimation and minimization of parameter uncertainties.

2. To characterize the effect of temperature on kinetic parameters of the ASM3 model in activated sludge processes, by applying microrespirometry in combination with identifiability analysis.

3. To characterize the microbial kinetics and mass transfer phenomena in aerobic granules degrading 4-cholorophenol, by applying microrespirometry for the estimation of kinetic parameters.

3.3 Hypothesis

To address the objectives previously mentioned, we defined the following hypotheses (each one related to one of the corresponding papers):

1. In microrespirometry, parameter estimation is affected by several sources of uncertainty, which can be measured and minimized (**paper I**).

2. Microrespirometric data from pulses of increasing concentrations can be used for calibration and validation of activated sludge models (**paper II**).

3. The temperature effect on the parameters of the ASM3 model can be determined by a combination of identifiability analysis and microrespirometry experiments (**paper III)**.

4. The intrinsic parameters, the apparent parameters and the effectiveness factor of aerobic granules degrading 4-chlorophenol can be estimated using data from the microrespirometry method (**paper IV**).

5. The effectiveness factor in aerobic granules with substrate inhibition kinetics can be predicted by its relationship with the Thiele modulus (**paper V**).

6. The bacterial community structure in a SBR degrading 4-chlorophenol will specialize after the formation of aerobic granules by changes in the operating conditions (**paper VI**).

CHAPTER 5

RESULTS AND DISCUSSION

In this thesis, we applied microrespirometry for the characterization of bioprocesses related to wastewater treatment. The results of these studies are found in the following publications, which are the main products of this thesis:

In **paper I,** we used microrespirometry to characterize two wastewater processes: the aerobic degradation of 4-chlorophenol by acclimated sludge, and synthetic wastewater treatment by activated sludge. We estimated the parameters of a Haldane model and a modified ASM3 model by model fitting. For data analysis, we defined two different approaches for parameter estimation: mean parameters (MP) and multiple replicate parameters (MRP). In addition, we evaluated the impact of the main sources of parameter uncertainty: measurement errors, number of replicates, and parameter correlations. In the paper, we compared between MP and MRP, and discussed the best way to reduce the uncertainty of the parameter estimates.

In **paper II**, first, we selected an identifiable parameter subset of the ASM3 model based on previously published methods (Brun et al. 2002). Then, we used data generated by microrespirometry from pulses of different substrate concentrations, and performed the calibration and validation of the model. After analyzing the data, we found significant modelling limitations of the ASM3 model, and proposed a multi-response approach to overcome these issues. This paper is not ready for publication; it will be enriched with data from other activated sludge processes used in the research group, with the aim of having more experimental evidence to generalize the conclusions. However, the data analysis strategy that we developed in this paper was of great importance, because it was the basis of the strategy used in **paper III**.

In **paper III**, we addressed the effect of temperature with a rigorous analysis, considering the identifiability problems of the ASM3 model, and we carried out a model calibration and validation at each experimental condition. For this, we used the strategy developed in **paper II** (parameter subset selection plus model calibration and validation), to estimate the kinetic and stoichiometric parameters of the ASM3 model at different temperature conditions (20-38 °C). We discussed the effect of temperature on the selected parameters and their possible implications. In addition, we performed simulations of a continuous stirred thank reactor (CSRT) considering the ASM3 model calibrated by temperature, and discussed the possible implications for reactor operation. This paper is in the final stages of review, almost ready to be submitted.

In this thesis, we paid special attention to the reliability of the parameter estimates. For that reason, we performed practical identifiability analyses using previous methods described in literature, based on the FIM and parameter sensitivity functions. In **paper I** and **V**, the FIM was used to determine the confidence intervals of the estimated parameters, in **paper II** and **III**, the methodology proposed by Brun et al. (2002) was used to find identifiable parameter subsets, and the FIM to determine the confidence intervals.

In **paper IV**, we applied microrespirometry on an aerobic granular sludge degrading 4-chlorophenol (4-CP). We followed the granulation process on a Sequencing Batch Reactor feeding with 4-CP as sole carbon source. Besides some typical analyses as granule size, volatile suspended solids, and organic removal rate, we followed the changes of the intrinsic and apparent kinetic parameters of a Haldane model during the granulation process. Finally, we proposed an innovative approach to use data of the oxygen uptake rate of granules and disaggregated granules, to estimate the effectiveness factor as a function of the substrate concentration.

Paper V is a personal contribution to the understanding of kinetics and mass transfer on aerobic granules. This paper is in preparation for publication, and still needs some peer review. In this paper, I presented the calibration of a biofilm model using Haldane kinetics for aerobic granules degrading organic toxic pollutants. The model was calibrated using data from microrespirometric experiments. The calibrated model allowed the simulation of respirograms obtained with granular biomass, and it was used to evaluate the impact of particle size on the estimation of apparent parameters. Besides, I presented a generalization of the model that allows the prediction of the effectiveness factor based on the evaluation of the Thiele modulus and some dimensionless parameters. The results of this study have implications for the understanding and design of aerobic granular systems.

Paper VI resulted from collaboration with the research group of Dr. Luc Dendooven (Cinvestav-IPN). For this paper, we took several samples during the operation of the Sequencing Batch Reactor with aerobic granules degrading 4-chlorophenol, previously studied in **paper IV**. The new objective was to evaluate the changes of the microbial populations during the granulation process. Dr. Dendooven and his team performed the molecular biology experiments and the special data analysis needed for this paper.

CHAPTER 6

CONCLUSIONS

In **paper I**, we showed that the best way to analyze microrespirometric data, is to combine the information of all replicate experiments at the same time (MRP approach), in a single optimization step to estimate the model parameters; and to performed the FIM analysis for the same approach to determine the confidence intervals. We also showed that the replicates of the experiments in the microreactor system are completely reproducible and that only 5 replicates are enough for reliable parameter estimation.

We demonstrated in **paper II** that in one single experiment in microreactors, we can produce enough information to perform model calibration and still have enough information for a preliminary validation of the model. This is convenient for the saving of time and resources and can help us to identify modeling limitations, as we found for the ASM3 model.

The effect of temperature on kinetic parameters can be determined by microrespirometry using a very simple experimental design (**paper III**). A convenient strategy to calibrate the effect of temperature on models, is by preselecting an identifiable parameter subset and determining the effect of temperature only on the parameters of that subset. In the ASM3 model, some of the kinetic parameters followed a typical bell curve as a function of temperature, while other kinetic parameters did not follow a clear trend. Meanwhile, temperature did not significantly affect the stoichiometric parameters. Knowing the effect of temperature on the model parameters, can help us to have a better prediction and control of the operation of bioprocesses.

Microrespirometry can be used to estimate intrinsic and apparent kinetic parameters in aerobic granular processes. The parameters change significantly during the formation of aerobic granules. The strategy that we proposed in **paper IV,** which is based on the measurement of oxygen uptake rates, is useful for the estimation of the effectiveness factor of aerobic granules.

Apparent parameters describing aerobic granules with Haldane kinetics are strongly affected by mass transfer limitations. These effects can be modeled using the approach that we proposed in **paper V,** plus microrespirometry data for model calibration. The theoretical approach that we developed for the prediction of the effectiveness factor, has important implications for the understanding and design of aerobic granular systems.

Finally, the general conclusion of this thesis, is that microrespirometry is a powerful method for the estimation of parameters of biokinetic models, specially, when it is combined with the rest of the knowledge gained through many years of experience in parameter estimation. The proper use of microrespirometric methods will help us to improve our understanding of biological processes, and it will facilitate significantly the model calibration task in biotechnology.

CHAPTER 7

FUTURE RESEARCH

Future research on the topics addressed in this thesis should include:

1. Other case studies. Microrespirometry should not only be applied to wastewater treatment processes, but also to other areas of biotechnology where an important improvement in model calibration is needed.

2. Other models. When working with other case studies we must use other models. The strategies followed to estimate the parameters of other models will change the current ways to use microrespirometry.

3. Other methods. The knowledge gained from working with ASM models in literature is extensive, and there are other methods that can help to reduce practical identifiability problems in model calibration. Microrespirometry can benefit from the application of such methods; for example, the combination with titrimetric measures and the optimal experimental design methods (Petersen 2000), or other methods for selecting identifiable parameter subsets (Ruano et al. 2007).

4. Other sensors. The current trend of technology is the miniaturization of sensors, thus new sensors can be part of the future designs of microreactor systems. The estimation of biokinetic parameters will benefit greatly from having information from other variables measured online and accurately.

In the end, we should state that extending its application to other practical cases, is the only way we can assess the scope and limitations of microrespirometry.

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