In situ pulse respirometric methods for the estimation of kinetic and stoichiometric parameters in aerobic microbial communities

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ABSTRACT

In situ pulse respirometry was applied in an activated sludge bubble column treating synthetic wastewater for the estimation of the (i) maximum specific oxygen consumption rate, (ii) substrate affinity constant, (iii) biomass growth yield, (iv) maintenance coefficient, and (v) specific endogenous respiration rate. Parameters obtained from respirometry were compared to those obtained by the chemostat method, based on substrate and biomass measurements, under several dilution rates. The low sensitivity of substrate measurement methods and the difficulties of sampling heterogeneous biomass suspension are critical issues limiting the applicability of the chemostat method. Additionally, the extensive time consuming nature of this method allows concluding that chemostat method presents several disadvantages in comparison with *in situ* pulse respirometric techniques. Parameters were obtained from respirograms by fitting ASM1 and ASM3 models, and from experiments performed by injecting pulses of increasing substrate concentration. The injection of pulses of increasing concentration was the most adequate method, with several advantages such as a simpler experimental data interpretation, and results with better confidence.

Considering the assessment and comparison of the experimental and calculation methods presented, it is recommended that the estimation of kinetic and stoichiometric parameters in mixed aerobic cultures should preferentially be performed by using *in situ* respirometric techniques.

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1. Introduction

Respirometry is the measurement of the biological oxygen consumption rate under well defined conditions [1]. The interest of respirometry for parameter estimation, compared to techniques based on substrate concentration measurement, is that dissolved oxygen (DO) concentration can be measured easily and continuously with relative small input of experimental effort and obtaining high-quality data [2]. DO concentration changes in the order of ten parts-per-billion can be monitored online at high frequency.

Notwithstanding the potential advantages of respirometric methods, kinetic and stoichiometric parameters are still largely determined by substrate mass balances in batch or chemostat culture. From these, chemostat is still widely accepted as a suitable method for determining the substrate affinity constant (K_S) [3–5]. It consists in measuring the residual limiting substrate concentration for different dilution rates. The growth limiting substrate is directly measured at concentrations close to K_S [6], being

precision and accuracy of substrate measurements pointed as the main limitation of the method [7]. Additionally, reaching steady state takes considerable time, thus the method is especially time consuming as it requires the operation of the reactor under several steady states. Drawbacks of traditional methods may be overcome by respirometry [8,9].

Within many respirometric techniques, pulse respirometry, developed in the late 80s and mid 90s is probably one of the most used [10–13]. It consists in measuring DO concentration after the injection into the system of a defined substrate concentration pulse. The exogenous oxygen uptake rate (OUR_{ex}) curves reflect the kinetic of the aerobic biodegradation process and allow the estimation of kinetic and stoichiometric parameters. After the injection of substrate pulses, the kinetic parameters are usually estimated by direct model fitting to a respirometric curve [8,11,14]. Alternatively, Cech et al. [10] proposed a method in which kinetic parameters are obtained from the observed respirometric response of the endogenous system to the injection of substrate pulses of increasing concentration.

The determination of the oxidation yield ($Y_{O_2/S}$) and the growth yield ($Y_{X/S}$) by pulse respirometry is also commonly done by model fitting to a respirometric curve [2]. However, parameter identifiability analysis showed that $Y_{X/S}$ cannot be estimated accurately and

Nomenclature

ASM1, 3   activated sludge model 1, 3
ATU   allylthiourea
b_H specific endogenous respiration rate (h^{-1})
b_{Sto} specific respiration rate for X_{Sto} (ASM3) (h^{-1})
c maintenance constant of Neijssel and Tempest model [32] (–)
C DO concentration (mg O_2 L^{-1})
C_b baseline DO concentration (mg O_2 L^{-1})
C^* saturation DO concentration (mg O_2 L^{-1})
COD   chemical oxygen demand
COD-S   soluble COD fraction
COD-X   insoluble COD fraction
D dilution rate (d^{-1})
DO   dissolved oxygen (mg O_2 L^{-1})
$D_{(K-S)}$ Kolmogorov–Smirnov goodness of fit statistic
$F_{(i,j)}$ F ratio (ANOVA)
HRT   hydraulic retention time (d)
k maintenance constant of Pirt (1982) model [33] (–)
k_La oxygen volumetric mass transfer coefficient (h^{-1})
K_S substrate affinity constant (mg COD-S L^{-1})
k_{Sto} storage rate constant (ASM3) (g COD-S g COD-X^{-1} h^{-1})
K_{Sto} saturation constant for X_{Sto} (ASM3) (mg COD-X_{Sto} L^{-1})
m maintenance coefficient of Pirt (1965) [31] (g COD-S g COD-X^{-1} h^{-1})
m' maintenance coefficient of Neijssel and Tempest [32] (g COD-S g COD-X^{-1} h^{-1})
m'' maintenance coefficient of Pirt (1982) [33] (g COD-S g COD-X^{-1} h^{-1})
OUR_{ex} exogenous oxygen uptake rate (mg O_2 L^{-1} h^{-1})
OUR_{ex}max   maximum exogenous oxygen uptake rate (mg O_2 L^{-1} h^{-1})
p probability (p-value)
$q_{O_2 max}$ maximum specific oxygen consumption rate (g O_2 g COD-X^{-1} h^{-1})
RMSE   root mean squared error (–)
r^2 square correlation coefficient (–)
S substrate concentration (mg COD-S L^{-1})
$SOUR_{ex}$max   specific OUR_{ex}max (g O_2 g COD-X^{-1} h^{-1})
S_P pulse substrate concentration (mg COD-S L^{-1})
SRT   sludge retention time (d)
t time (h)
t_r response time of the process (h)
t_{mix} mixing time of the reactor (s)
X biomass concentration (mg COD-X L^{-1})
X_{Sto} internal storage material (ASM3) (mg COD-X_{Sto} L^{-1})
$Y_{O_2/S}$ substrate oxidation yield (g O_2 g COD-S^{-1})
Y_{Sto} storage yield (ASM3) (g COD-X_{Sto} g COD-S^{-1})
$Y_{X/S}$ biomass growth yield (g COD-X g COD-S^{-1})
$Y'_{X/S}$ true $Y_{X/S}$ (g COD-X g COD-S^{-1})
$Y_{X/Sto}$ biomass growth yield on X_{Sto} (ASM3) (g COD-X g COD-X_{Sto}^{-1})
μ specific growth rate (d^{-1})

Table 1
List of parameters estimated and method used.

Parameter	COD balance/chemostat	Pulse respirometry
$Y_{X/S}$	✓	✓
$Y_{O_2/S}$		✓
$q_{O_2 max}$	✓	✓
K_S	✓	✓
m	✓	✓
b_H		✓

(m) and endogenous respiration rates (b_H), which are both important for a proper description of microbial kinetics [22,23].

So far, literature on respirometry has given a special emphasis to parameters retrievability, identifiability, precision, and sensitivity, but, it has comparatively been given less focus to the accuracy of the retrieved parameters through the comparison between parameters obtained by respirometry and other methods [24]. The aim of the present research paper is to find a suitable method for the estimation of kinetic and stoichiometric parameters in mixed cultures, using suspended activated sludge as model system. With this purpose two categories of estimation methods are assessed: (i) chemostat method, and (ii) *in situ* pulse respirometry. Additionally, two respirometric methods are compared: respirograms model fitting in the context of ASM1 and ASM3; and a method based on the injection of pulses of increasing substrate concentration.

2. Materials and methods

2.1. Experimental strategy

A lab-scale reactor was operated during 270 days under several dilution rates (D). This allowed the estimation of the main stoichiometric and kinetic parameters, namely $Y_{X/S}$ (biomass growth yield), $Y_{O_2/S}$ (substrate oxidation yield), $q_{O_2 max}$ (maximum specific oxygen consumption rate), K_S, and maintenance coefficient (m) by respirometry and by traditional chemostat method. Additionally, the endogenous respiration rate (b_H) was only estimated by respirometry (Table 1).

2.2. Experimental setup

A transparent acrylic reactor was used (0.14 m internal diameter, 0.56 m height, 8.6 L working volume). Air was continuously supplied through a porous plate (0.09 m diameter) located at the bottom of the reactor, with a constant air flow rate of 0.60 L min^{-1}. The air flow rate was controlled by a mass flow-controller (Aalborg, Model GFC 17, Denmark). The reactor was continuously fed with synthetic wastewater, containing (mg L^{-1}): gelatine peptone, 640; meat extract, 440; urea, 120; NaCl, 28; CaCl$_2$·2H$_2$O, 16; MgSO$_4$·6H$_2$O, 8; K$_2$HPO$_4$, 34; Na$_2$HPO$_4$, 134; NH$_4$Cl, 6.8; total chemical oxygen demand (COD), 1198 ± 11; soluble COD, 1087 ± 17; C/N ratio, 2.6 (g/g). Allylthiourea (ATU) was added to the synthetic wastewater solution (10 mg ATU L^{-1}) in order to inhibit nitrification. The synthetic wastewater was fed continuously with peristaltic pumps (Watson Marlow 101 U/R, 405 U/R1 or 401 U/D1, depending on the flow rate). Effluent was collected by overflow in a refrigerated tank (4 °C) for posterior analysis. pH was maintained at 7.0 ± 0.5, by addition of 0.5 M NaOH or 0.2 M H$_2$SO$_4$ (Control System/Pump BL7917, Hanna Instruments, USA). The reactor was inoculated with 1 L of mixed liquor obtained from a conventional wastewater treatment plant (Cambados-Maia, Portugal). The reactor was maintained at ambient room temperature (19–23 °C).

The reactor was characterised in terms of mixing time (t_{mix}) by lithium chloride pulse experiments [9]. t_{mix} was defined as the time required for the lithium concentration in the reactor, after the injection of a pulse, to reach 90% of the final lithium concentration. The

simultaneously with kinetic parameters by model fitting [15]. An alternative strategy for $Y_{X/S}$ determination is to consider the total exogenous oxygen consumed during the pulse injection [16].

Pulse respirometry is also of interest to determine inhibition constants [17,18], oxygen affinity constants [19], wastewater biodegradability [20,21], and to estimate maintenance coefficients

t_{mix} was determined as follows: (i) the reactor feeding was stopped, (ii) a known concentration of lithium chloride was injected at the bottom of the reactor, (iii) samples were taken from the top of the reactor, and (iv) after stable lithium concentration was observed, the reactor feeding was switched back on. The lithium concentration was measured by atomic absorption (Varian SPECTRAA 250 Plus, USA).

2.3. Reactor operation

After inoculation, the reactor was operated under fed-batch mode for 5 days. During this period, about 20% of the mixed liquor was substituted every day. After this adaptation period, the reactor was operated continuously during 270 days under five D (from 0.2 to 2.0 d^{-1}) and, consequently, five organic loading rates (from 0.2 to 2.3 g COD-S L^{-1} d^{-1}). Each D tested was maintained at least until steady-state was reached, the steady state is defined as constant degradation rate and biomass concentration (variations within normal standard deviations).

2.4. Methods

2.4.1. Analytical procedures

Influent and effluent were characterised through triplicate measurements of the total and soluble COD. COD was determined using the closed reflux colorimetric method, according to standard methods [25]. Substrate concentration (S) was considered to be the soluble COD fraction (COD-S), and biomass concentration (X) the insoluble COD fraction (COD-X), estimated as the difference between total COD and soluble COD. Soluble and insoluble COD fractions were separated by filtration (0.45 μm).

2.4.2. Pulse respirometry and data interpretation

The DO concentration was measured online with a polarographic oxygen probe, located at the top of the reactor and connected to a DO-meter (Hannah Instrument HI2400, USA) and a computer for data acquisition. DO readings were corrected for temperature, salinity, and altitude through DO-meter automatic compensation adjustments. The oxygen probe was calibrated before each respirometric experiment. Saturation DO concentration (C^*) was experimentally measured under the experimental conditions of the reactor, using sterilised effluent coming out from the reactor. Oxygen mass transfer coefficient ($k_L a$) was determined from the dynamic method, as it was described by Badino et al. [26]. The response time of the electrode was taken into account during respirometric and $k_L a$ measurements.

In situ respirometric pulse experiments were done according to the following procedure: (i) the reactor was maintained until stable DO readings were obtained; (ii) the substrate feeding was stopped and the aeration maintained; (iii) the DO concentration slowly increased until reaching a new pseudo-stationary state, called baseline oxygen concentration (C_b) corresponding to endogenous respiration [27]; (iv) a pulse of synthetic wastewater, containing ATU (reactor concentration of 10 mg ATU L^{-1}), was injected in order to obtain a substrate concentration in the reactor (S_P) of approximately 20 mg COD-S L^{-1}; (v) the DO concentration was acquired until the system returned to C_b; (vi) additional pulses were eventually injected (10–40 mg COD-S L^{-1}); and (vii) the $k_L a$ was measured in triplicate before the feeding of the reactor was restored. The initial substrate to biomass ratio (S_0/X_0) applied with the pulses was between 0.03 and 0.2 g COD-S g COD-X^{-1}.

The respirometric data interpretation method was as previously reported [28–30]. Briefly, after the injection of a known substrate pulse concentration (S_P), the $Y_{O_2/S}$ was given by the amount of oxygen consumed per unit COD of substrate oxidised

(Eq. (1)). $Y_{X/S}$ expressed in COD units was estimated from $Y_{O_2/S}$ (Eq. (2)).

$$Y_{O_2/S} = \frac{\int_0^t OUR_{ex}\, dt}{S_P} = \frac{k_L a \int_0^t (C_b - C)\, dt}{S_P} \qquad (1)$$

$$Y_{X/S} = 1 - Y_{O_2/S} = 1 - \frac{k_L a \int_0^t (C_b - C)\, dt}{S_P} \qquad (2)$$

The average $Y_{X/S}$ estimated at each D by respirometry were used to estimate the maintenance coefficient (m) according to the Pirt method [31] (Eq. (3)). Models including a variable maintenance were also considered such as the Neijssel and Tempest model [32] (Eq. (4)), and the 1982 Pirt model [33] (Eq. (5)). In these models, since the system was considered at steady state and completely mixed, the specific growth rate (μ) was considered equal to D.

$$\frac{1}{Y_{X/S}} = \frac{1}{Y'_{X/S}} + m\frac{1}{\mu} \qquad (3)$$

$$\frac{1}{Y_{X/S}} = \frac{1}{Y'_{X/S}} + m'\frac{1}{\mu} + cm' \qquad (4)$$

$$\frac{1}{Y_{X/S}} = \frac{1}{Y'_{X/S}} + m\frac{1}{\mu} + m''\left(\frac{1 - k\mu}{\mu}\right) \qquad (5)$$

During pulse respirometry, C_b was typically inferior to C^*. This difference was accounted for endogenous respiration [27,34]. In this work, m estimated from Eqs. (3)–(5), was compared to the specific endogenous respiration rate (b_H) estimated from Eq. (6).

$$b_H = \frac{k_L a(C^* - C_b)}{X} \qquad (6)$$

$q_{O_2\,max}$ and K_S were estimated by model fitting to respirometric data, as it was previously described [28]. Briefly, after the injection of a substrate pulse, the DO concentration in the reactor was described by a balance between the exogenous respiratory activity and the oxygen provided by continuous aeration [35], being C the DO concentration in the liquid phase. Additionally, the response time of the process (t_r) was taken into account in a similar manner as described previously by Vanrolleghem et al. [36].

$$\frac{dC}{dt} = [k_L a\,(C_b - C) - OUR_{ex}](1 - e^{-t/t_r}) \qquad (7)$$

In Eq. (7), two models were used to describe OUR_{ex} and fitted to experimental DO data: the ASM1 model [37], and the ASM3 model [38]. Table 2 shows the matrix representation of the models used. The main difference between both models is that ASM3 model considers substrate storage.

Eq. (7) was adjusted to the experimental data obtained from the pulse experiments with a fitting procedure based on Runge–Kutta method and a Marquardt optimisation method with 20 convergence steps (Model Maker, Cherwell Scientific Publishing, UK). $q_{O_2\,max}$ was obtained by dividing the estimated $OUR_{ex}max$ by the biomass concentration in the reactor (X).

Additionally to model fitting, $q_{O_2\,max}$ and K_S were also estimated after the injection of pulses of increasing substrate concentration, as it was reported by Orupold et al. [39]. The observed $SOUR_{ex}max$ (specific $OUR_{ex}max$, obtained by dividing $OUR_{ex}max$ by X) was plotted against the substrate concentration pulse. The graph obtained showed a clear Monod-type shape (Eq. (8)), used to estimate $q_{O_2\,max}$ and K_S.

$$SOUR_{ex}\,max = q_{O_2\,max}\left(\frac{S_P}{K_S + S_P}\right) \qquad (8)$$

2.4.3. Chemostat method

$Y_{X/S}$ was estimated from the biomass produced (difference between total and soluble COD of the effluent) and the substrate

Table 2
Simplified matrix of ASM1-like and ASM3 models for organic carbon removal, considering soluble biodegradable COD.

Component process	S	X_{Sto}	X	C	Rate
Substrate consumption (ASM1)	$-\frac{1}{Y_{O_2/S}}$			-1	OUR$_{ex}$ max $\frac{S}{K_S+S}$
Storage of S (ASM3)	-1	Y_{Sto}		$-(1-Y_{Sto})$	$k_{Sto}X\frac{S}{K_S+S}$
Growth on X_{Sto} (ASM3)		$-\frac{1}{Y_{X/Sto}}$	1	$-\frac{(1-Y_{X/Sto})}{Y_{X/Sto}}$	$\mu_{max}X\frac{(X_{Sto}/X)}{K_{Sto}+(X_{Sto}/X)}$
Respiration of X_{sto} (ASM3)		-1		-1	$b_{Sto}X_{Sto}$

consumed (difference between influent and effluent soluble COD). The average of $Y_{X/S}$ estimated at each D, were used to determine the maintenance coefficient (m) according to the Pirt method [31] (Eq. (3)), as it was done with data obtained by respirometry. $q_{O_2\,max}$ and K_S were estimated by fitting the Monod equation to the average experimental data (SOUR$_{ex}$ and S) determined at each dilution rate, under steady-state. With that purpose, SOUR$_{ex}$ was estimated from the total COD removed.

2.4.4. Statistical analysis of the results

Models goodness of fit was estimated through the comparison of three parameters: (i) the correlation factor (r^2), (ii) the root mean squared error (RMSE), and (iii) the Kolmogorov–Smirnov test [40]. Significance of difference between parameters was estimated through One-way ANOVA and Tukey's HSD post hoc test, using PASW Statistics 18 (SPSS Inc. Package).

Average values are presented with the corresponding standard error.

3. Results and discussion

3.1. Reactor operation

The reactor was inoculated with activated sludge from a local wastewater plant, and operated under fed-batch mode. After 5 days, a clear biomass growth was observed (approximately from 400 to 500 mg COD-X L^{-1}), corroborated by an increase in substrate degradation activity. Time was reset and the reactor was then operated under continuous mode for 270 days. The reactor worked with a constant influent concentration of 1.06 ± 0.09 g COD-S L^{-1}. Five dilution rates were tested, from 0.19 to 1.95 d^{-1}. The first tested D (0.45 d^{-1}) was maintained for 170 days. The additional D (i.e., 0.19, 0.28, 0.91, and 1.95 d^{-1}) were maintained for a time corresponding to six hydraulic retention times (HRT) at least. Fig. 1 shows the reactor's behaviour during the continuous operation. The system adapted adequately to the variations of the organic loading rate, imposed by the variations of D, presenting a stable COD removal efficiency during the whole operation (90–100% soluble
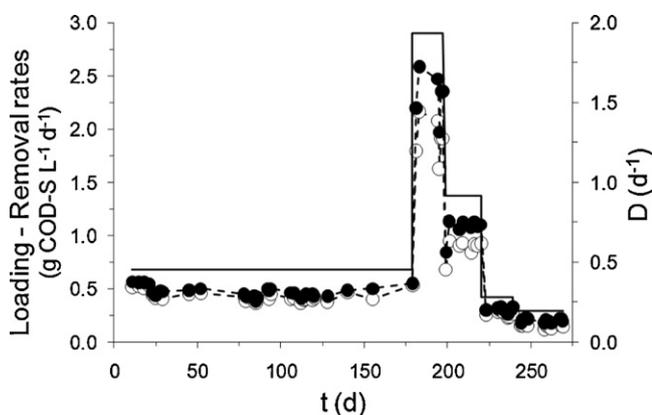
Table 3
Sludge retention time (SRT) and biomass concentration (X) achieved at each of the tested dilution rates (D).

D (d^{-1})	SRT (d)	X (mg COD-X L^{-1})
2.0	0.5	674.6 ± 203.0
0.9	1.1	595.4 ± 156.4
0.5	2.2	454.4 ± 191.2
0.3	3.6	377.7 ± 148.4
0.2	5.2	193.2 ± 57.2

COD removal). Table 3 presents information on sludge retention time (SRT) and biomass concentration (X) achieved at each tested D. A quasi-linear ($r^2 = 0.98$) decrease of X was observed with the SRT increase. This can be explained by the increase of substrate limitations to biomass growth as dilution rate decreased, and SRT increased.

The mixing time of the reactor (t_{mix}) was determined in two periods: at the beginning of operation, on day 10, when a t_{mix} of 19 ± 2 s was obtained; and at the end of operation, on day 250, when a t_{mix} of 22 ± 2 s was obtained. These low t_{mix} values, comparatively to HRT, show that the bubble-column reactor was a well-mixed system.

The first pulse experiments were done on day 20. Fig. 2 shows a superposition of two respirograms obtained after feeding suppression and injection of two successive pulses of 22 mg COD-S L^{-1}. Fig. 2 shows that DO concentration decreased sharply immediately after pulse injection (time 0) and then, after 2 h, returned to the baseline value (C_b) of 8.2 mg L^{-1}. Both respirograms showed a similar shape with a square correlation coefficient (r^2) between them of 0.99. At the end of the second pulse, after 6 h of respirometric experiments, the substrate feeding was turned back on, and the system returned to normal operation. No changes were observed in DO, biomass, nor substrate concentrations compared to the situation observed before the respirometric experiments (results not shown). These results confirm that in situ pulses are reproducible and that feeding suspension during approximately 6 h did not affect significantly the behaviour of the system.

Fig. 1. Behaviour of the reactor: dilution rate (D, –), loading rate (●), and removal rate (○).
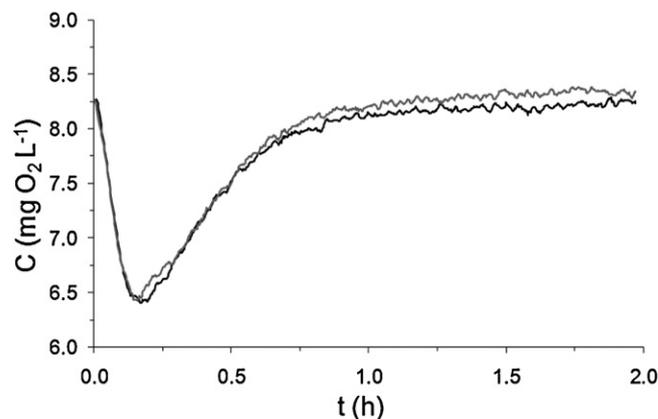
Fig. 2. Superposition of two respirograms observed after the injection of two consecutive pulses of 22 mg COD-S L^{-1}: first pulse (darker line), and second pulse (lighter line).
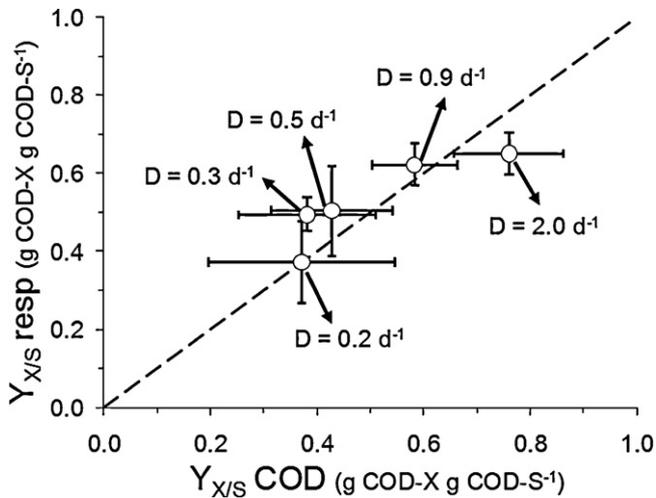
Fig. 3. Correlation between the biomass growth yields ($Y_{X/S}$) estimated by respirometry and by COD mass balance. The dotted line represents the linear regression forced to cross the origin.

3.2. $Y_{X/S}$ and m

$Y_{X/S}$ was estimated by respirometry (Eq. (2)) from the area of the 51 respirograms obtained during the whole reactor operation, and also by COD mass balance. Fig. 3 presents a comparison of the average $Y_{X/S}$ values for each dilution rate. The slope of the linear regression forced to cross the origin was 1.01, with a r^2 of 0.98, meaning that the values obtained with both methods were comparable. Very recently, Di Trapani et al. [41] found, in a membrane bioreactor, a significant difference between the $Y_{X/S}$ values obtained by pulse respirometry and by COD mass balance. This discrepancy of values was suggested to be related with two terms, (i) the fact that respirometric pulses were done with a readily biodegradable substrate for characterisation of biomass acclimated to real wastewater, and (ii) the influence of biomass decay phenomenon, which is taken into account by the COD mass balance method, but not by the respirometric method due to the short time of experiments Biomass decay was probably an important factor in Di Trapani et al. [41] work, as they used a membrane bioreactors with high sludge retention. In the present work no discrepancy was observed between $Y_{X/S}$ values obtained by pulse respirometry and by COD mass balance, probably because the same complex substrate was used to feed the reactor and for respirometric pulses, and because the reactor used was completely mixed with no biomass retention in excess to hydraulic retention, thus where decay phenomenon was negligible. Regardless of the proximity of the $Y_{X/S}$ values obtained by pulse respirometry and by COD mass balance, the average standard deviation of the respirometric method (15%) was lower than the average standard deviation of the COD balance method (27%). The higher standard deviation of the COD method is likely due to the required biomass sampling procedures, which are not necessary with *in situ* respirometric methods. This constitutes one advantage of respirometry.

From the $Y_{X/S}$ determined by respirometry, and by COD mass balance, m was determined graphically (Fig. 4) according to the Pirt's method [31] (Eq. (3)). Maintenance coefficients, m, estimated by the COD mass balance and by respirometry, were, respectively, 0.012 ± 0.012 and $0.010 \pm 0.006\,\mathrm{h}^{-1}$. Considering that the results were obtained over 270 days with a mixed culture that was certainly changing in composition over time [42], it is not surprising that with neither one of the methods a perfect linear correlation was obtained. Nevertheless, the r^2 of the respirometric method was significantly higher (0.90) than the correlation of the COD method
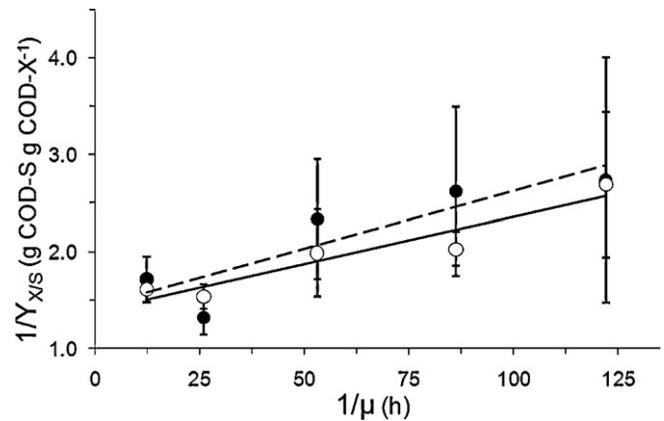
(0.78), confirming that methods based on biomass sampling are affected by intrinsic errors due to the difficulty of sampling heterogeneous suspensions. The 1982 Pirt [33], and the Neijssel and Tempest [32] models were unsuccessfully applied ($r^2 < 0.70$) to the same experimental data (results not shown).

As mentioned before, the difference between C_b and the C^* during respirometric experiments is typically accounted for endogenous respiration [27,34]. In order to compare maintenance (m) and endogenous respiration (b_H), the baseline DO concentration was measured before each of the 51 pulses made along the reactor operation. From these data, the estimation of b_H from Eq. (6) was $0.025 \pm 0.015\,\mathrm{h}^{-1}$. This result shows that b_H was superior to m ($0.010 \pm 0.006\,\mathrm{h}^{-1}$) estimated with respirometric data from Pirt's model [31]. The difference between b_H and m deserves a close attention. The difference between m and b_H can be explained by the fact that different non-growth processes are considered in each case [22,43]. The endogenous respiration includes osmoregulation, cell mobility, defence mechanisms, proofreading, and internal turnover of macromolecular compounds. According to these authors, m neglects some of these processes and is therefore expected to be lower than b_H, as it was observed in this work.

3.3. K_S and $q_{O_2\,max}$

ASM1 and ASM3 models were adjusted to the respirograms obtained along the reactor operation. Fig. 5 shows an example of data fitting using both models. Table 4 shows average values of the correlation factor, the RMSE and the Kolmogorov–Smirnov goodness of fit ($D_{(K-S)}$) after the analysis of 51 pulses made

Fig. 4. Pirt [27] linearisation of the experimental growth yield estimated from COD mass balance (●, *dotted line*), and by respirometry (○, *solid line*).
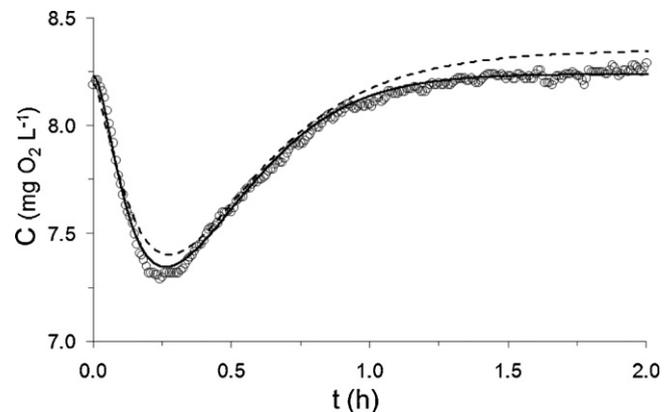
Fig. 5. Example of a respirogram (●), model fitting using ASM1 ($r^2 = 0.99$, *solid line*) and ASM3 ($r^2 = 0.95$, *dotted line*).

Table 4
Average values of the correlation factor, the RMSE and the $D_{(K-S)}$ value for ASM1 and ASM3 fitting to respirograms.

	ASM1	ASM3
r^2	0.97	0.91
RMSE	0.07	0.13
$D_{(K-S)}$	0.19	0.39

during the reactor operation. The correlation factor of ASM1 model was superior to that of ASM3. Both RMSE and $D_{(K-S)}$ were lower for the ASM1 than for the ASM3 model. These results confirm that ASM1 model adjusted better the experimental data, which is an indication that storage was not significant in this process. This is in accordance with literature, as storage mechanism is generally observed in sludge subjected to dynamic conditions. Krishna and van Loosdrecht [44] stated, for instance, that, under continuous feeding, activated sludge systems have a low storage capacity. More recently, Ciggin et al. [45] indicated that biomass acclimated to continuous feeding could not store the excess substrate even when a sudden change in the feeding was done. In the present work, the reactor was maintained under steady-state, and the biomass never experienced dynamic conditions of substrate concentration, except when substrate pulses were done. However, a maximum of 2 pulses per day were done and each sequence of pulses was parted by 15 to 70 h. According to Vanrolleghem et al. [46] it is expected that the biomass had no time to adapt its enzymatic system to storage. Prendl and Kroiss [47] did not observe storage when the sludge age was below 5 days. In the present work, except in the last D tested (0.2 d^{-1}), the sludge age was below 5 days. At last, it has been reported that storage occurs when sludge is subjected to nutrient or oxygen limitation with excess of substrate. In this work, the pulses were made of complete medium, including nutrients, and DO was always above 2 mg L^{-1}. According to the results obtained and the literature reports, storage was discarded and ASM1 model was used further on.

K_S and $q_{O_2 \, max}$ were estimated by ASM1 model fitting to the respirograms obtained during the reactor operation. Table 5 presents average results obtained from 51 pulses made over 270 days of experiment. K_S values estimated from model fitting were within the range generally observed in wastewater treatment plants [48], and they were also similar to the K_S estimated by respirometry by Carucci et al. [49] in real filtered wastewater ($K_S = 12.3 \pm 0.3$ mg COD-S L^{-1}). The response time of the process was approximately 0.06 h (3.4 min), similar to those previously reported by Vanrolleghem et al. [36]. The One-way ANOVA test revealed that K_S was not significantly affected by D ($F_{(4,46)} = 0.253$, $p = 0.91$). Regarding $q_{O_2 \, max}$, there was a significant difference between different D ($F_{(4,46)} = 4.617$, $p < 0.05$). Tukey's HSD post hoc test showed that this difference was between the extremes, i.e., between $q_{O_2 \, max}$ at $D = 0.2$ and 2.0 d^{-1} (Table 5).

K_S and $q_{O_2 \, max}$ were also estimated by respirometry from the injection of pulses of increasing concentration. Pulses of 10.5, 21.0, and 42.3 mg COD-S L^{-1} were injected and between pulses the feeding was restored until stable DO readings were obtained to

Table 5
Average K_S and $q_{O_2 \, max}$ obtained by ASM1 model fitting of 51 pulses injected at five dilution rates (D).

D (d^{-1})	Number of pulses	K_S (mg COD-S L^{-1})	$q_{O_2 \, max}$ (h^{-1})
0.2	12	19.6 ± 2.7	0.11 ± 0.02
0.3	9	18.7 ± 2.5	0.09 ± 0.01
0.5	18	17.0 ± 2.6	0.07 ± 0.01
0.9	6	15.8 ± 2.1	0.04 ± 0.00
2.0	6	18.4 ± 2.7	0.08 ± 0.01
Average		17.9 ± 1.1	0.08 ± 0.01
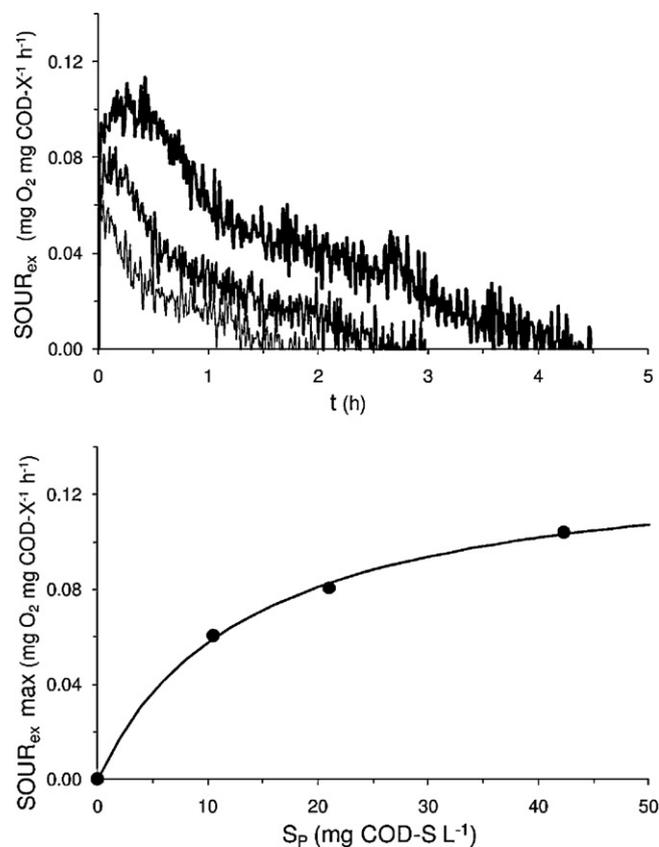
Fig. 6. Respirograms observed at three pulse concentrations (mg L^{-1}): 10.5 (*darker line*), 21.0 (*medium line*), and 42.3 (*lighter line*) (a); and relation between the maximum specific exogenous OUR ($q_{O_2 \, max}$) and pulse concentration (S_P) with corresponding Monod adjustment (*solid line*, $r^2 = 1.00$) (b).

ensure repeatability of the experimental conditions. Fig. 6a shows an example of the OUR$_{ex}$ response after the injection of three increasing concentration pulses. In each case, OUR$_{ex}$max depended on S_p. Fig. 6b shows that SOUR$_{ex}$max followed a Monod-type kinetic in relation to the substrate concentration ($r^2 = 1.00$). This allowed determining K_S and $q_{O_2 \, max}$ (Eq. (8)), 15.5 ± 2.4 mg COD-S L^{-1} and 0.12 ± 0.01 h^{-1}, respectively. By fitting ASM1 model simultaneously to the four respirograms used in the method of pulses of increasing substrate concentration (pulses of 10.5, 21.0 and 42.3 mg COD-S L^{-1}), the values obtained for K_S and $q_{O_2 \, max}$ were 19.9 ± 0.06 mg L^{-1} and 0.10 ± 0.02 h^{-1}, respectively, with a correlation factor of 0.92. By individual model fitting to the respirograms, model fitting to the 10.5 mg COD-S L^{-1} pulse failed; with the 21.0 mg COD-S L^{-1} pulse, the K_S and $q_{O_2 \, max}$ values estimated were 22.3 ± 0.13 mg COD-S L^{-1} and 0.12 ± 0.03 h^{-1}, respectively ($r^2 = 0.96$); and with the 42.3 mg COD-S L^{-1} pulse the values 33.7 ± 0.8 mg COD-S L^{-1} and 0.12 ± 0.02 h^{-1} were obtained for K_S and $q_{O_2 \, max}$, respectively ($r^2 = 0.93$). The discrepancy of values obtained by model fitting a single pulse confirms previous observations which state that a number of pulses higher than one further improve parameters practical identifiability [13,50]. However, this compromises an increment in the computational effort, which has to be taken into account. The obtained values with the two respirometric data treatment methods were similar. Though the K_S associated error for model fitting was lower, the correlation factor of the model was worse than the one corresponding to the increasing substrate concentration pulses method.

For further comparison between both respirometric data treatment methods, results obtained by model fitting after the injection of a pulse of 21.8 mg COD-S L^{-1} were compared to those obtained

Fig. 8. Average specific exogenous OUR ($SOUR_{ex}$) *versus* average S for each dilution rate ($\bigcirc$), and best fitting Monod model (*solid line, $r^2 = 0.61$*).
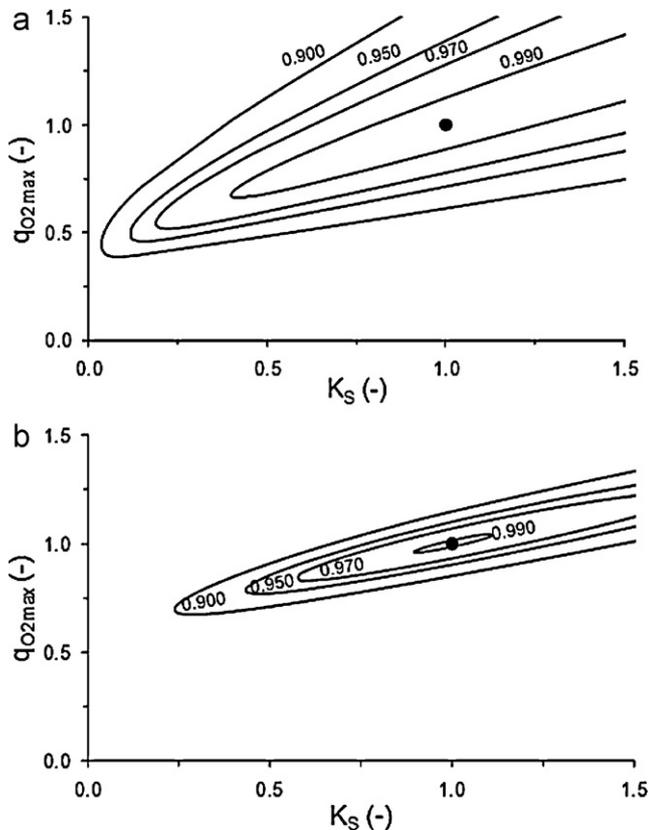
Fig. 7. Contour-plot of the correlation factor as a function of the substrate affinity constant (K_S) and the maximum specific exogenous OUR ($q_{O_2\,max}$), for the model fitting method (a), and for the increasing substrate concentration pulses method (b).

after the injection of pulses of increasing concentration. The results of these sensitivity analyses are presented in Fig. 7, where the lines represent the dimensionless K_S and $q_{O_2\,max}$ pairs for a specified r^2, as it is indicated. It is shown that the r^2 of the model fitting method is less sensitive to changes in K_S and $q_{O_2\,max}$ than the r^2 of the method based on pulses of increasing concentration. This indicates that the method based on the injection of pulses of increasing concentration is more precise than simple single pulse model fitting and it is confirmed as a suitable method to estimate $q_{O_2\,max}$ and K_S. In order to improve the identifiability of parameters through ASM model fitting, more than one pulse is recommended. In the increasing substrate concentration pulses method three pulses are needed, which means the experimental effort of both methods is equivalent. Nevertheless, the ASM model fitting requires a much higher computational effort in order to treat a large amount of data, while the increasing substrate concentration pulses method uses a basic Monod-type model fitting.

K_S and $q_{O_2\,max}$ were also determined from COD mass balance according to the chemostat method [3]. Fig. 8 shows the average specific substrate uptake rate expressed in oxygen demand *versus* the average substrate concentration at each dilution rate. The low r^2 obtained (0.61) did not allow the estimation of K_S nor $q_{O_2\,max}$. The low sensitivity of substrate measurement methods and the difficulties of sampling heterogeneous biomass suspension are critical issues limiting the applicability of the chemostat method. Furthermore, the chemostat method applied in mixed cultures may be questioned in the sense that it involves changing the HRT, which is a selection pressure for microorganisms in continuous culture. Consequently, the selection of slower- or faster-growing microorganisms occurs [42]. The poor results obtained are in accordance with these drawbacks. Additionally, the time consuming nature of
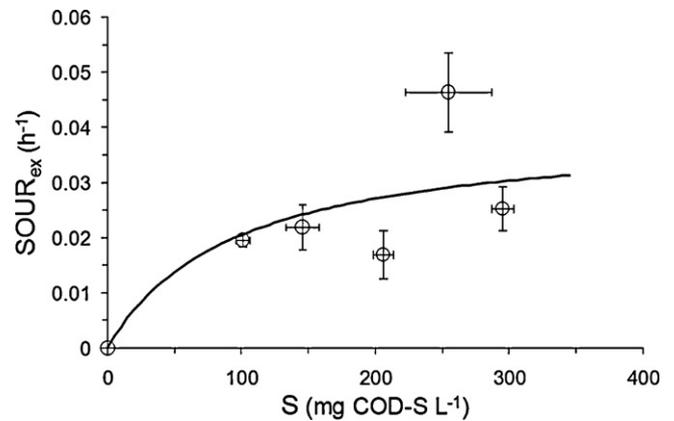
this method allows to conclude that no one single advantage can be pointed out to the chemostat method, in comparison with the simple *in situ* pulse respirometric techniques whatever the method used for mixed cultures parameters retrieval, although different methods of calculation have different precision and sensitivity. Therefore, the estimation of kinetic and stoichiometric parameters in mixed aerobic cultures should be always performed by using *in situ* respirometric techniques.

4. Conclusions

In situ pulse respirometry was applied to estimate stoichiometric and kinetic parameters in activated sludge systems, and a comparison with the chemostat method based on COD mass balances was done. $Y_{X/S}$, m, b_H, $q_{O_2\,max}$ and K_S were better estimated by respirometry than by chemostat mass balance. A further comparison of two different respirometric methods, namely the injection of pulses of increasing concentration and model fitting to respirograms gave similar results. It is concluded that respirometry is a more satisfactory method than mass balance and that within respirometric methods, the injection of pulses of increasing concentration presents several advantages compared to model fitting to respirograms, mainly simpler experimental data interpretation and better results confidence.

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