ARTICLE

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Kinetic and Stoichiometric Parameters Estimation in a Nitrifying Bubble Column Through "In-Situ" Pulse Respirometry

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ABSTRACT: This article proposes a simple "in-situ" pulse respirometric method for the estimation of four important kinetic and stoichiometric parameters. The method is validated in a suspended biomass nitrifying reactor for the determination of (i) maximum oxygen uptake rate ($OUR_{ex}max$), (ii) oxidation yield (f_E), (iii) biomass growth yield (f_S), and (iv) affinity constant (K_S). $OUR_{ex}max$ and f_E were directly obtained from respirograms. In the presented case study, a minimum substrate pulse of 10 $mgNH_4^+$-N L^{-1} was necessary to determine $OUR_{ex}max$ which was 61.15 ± 4.09 $mgO_2\,L^{-1}\,h^{-1}$ (5 repetitions). A linear correlation ($r^2 = 0.93$) obtained between $OUR_{ex}max$ and the biomass concentration in the reactor suggests that biomass concentration can be estimated from respirometric experiments. The substrate oxidation yield, f_E, was determined along 60 days of continuous operation with an average error of 5.6%. The biomass growth yield was indirectly estimated from the substrate oxidation yield f_E. The average obtained value (0.10 ± 0.04 $mgCOD\,mg^{-1}COD$) was in accordance with the f_S estimation by the traditional COD mass balance method under steady-state conditions (0.09 ± 0.01). The affinity constant K_S was indirectly estimated after fitting the ascending part of the respirogram to a theoretical model. An average value of 0.48 ± 0.08 $mgNH_4^+$-N L^{-1} was obtained, which is in the range of affinity constants reported in the literature for the nitrification process (0.16–2 $mgNH_4^+$-N L^{-1}).

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KEYWORDS: respirometry; growth yield; in-situ pulse technique; kinetic parameters

Introduction

Respirometry consists in the measurement of the biological oxygen consumption rate under well-defined conditions (Spanjers et al., 1999). Respirometry has been largely used for the characterization of numerous biological systems at least since the beginning of the last century (Meiklejohn, 1937; Thompson, 1932). More recently, respirometry combined with the injection of a substrate pulse has been applied in biological systems for the determination of kinetic parameters (Checchi and Marsili-Libelli, 2005; Ciudad et al., 2006; Jubany et al., 2005). This technique consists in measuring the dissolved oxygen concentration profile, after the injection of a defined concentration of substrate into the system. This can be done maintaining aeration (Ficara et al., 2000; Guisasola et al., 2003; Kong et al., 1994) or without aeration (Chandran and Smets, 2000; Guisasola et al., 2005; Jubany et al., 2005).

Pulse respirometry has been mainly used to estimate the substrate affinity constant (K_S), the substrate oxidation yield (f_E), the maximum substrate degradation rate (R_{max}) and the maximum growth rate (μ_{max}) (Chandran and Smets, 2005; Vanrolleghem et al., 2004). Other authors also refer the pulse respirometry technique to estimate the oxygen affinity constant—K_{O_2} (Guisasola et al., 2005), the biomass growth yield—f_S (Chandran and Smets, 2000, 2001; Dircks et al., 1999) and the aerobic endogenous decay constant—k_m (Avcioglu et al., 1998). The literature also reports the use of respirometry

for the evaluation of inhibitory effects of several compounds (Carrera et al., 2004; Kong et al., 1996; Villaverde et al., 2000).

The number of publications in the field of pulse respirometry underlines the large interest of this method for the kinetic and stoichiometric parameters characterization. So far, pulse respirometry has been applied in closed or open respirometers. The use of respirometers allows a better control of the test conditions and improves the results precision. However, this practice implies sampling that could be not fully representative of the reactor conditions, especially in heterogeneous, sequential or fixed biomass reactors. To avoid the potential drawbacks of sampling, Riefler et al. (1998) suggested a theoretical ''in-situ'' model and Yoong et al. (2000) presented results obtained with ''in-situ'' respirometry but without pulse addition and in a reactor without aeration. ''In-situ'' pulse respirometry, defined as pulses of substrate directly applied in biological reactors, has not been reported yet.

This articles aims to apply an ''in-situ'' pulse respirometric technique in order to estimate (i) the parameters that can be retrieved from ''in-situ'' respirograms, (ii) the error of the technique through an error propagation method, and (iii) the effect of the pulse respirometry on the bioreactor process. A nitrifying reactor with suspended biomass was used as a model system. A special emphasis was also given to the accuracy of the growth yield estimated by respirometry in comparison with a more traditional method based on chemical oxygen demand (COD) measurements.

Materials and Methods

Nitrifying Reactor

A glass bubble column was used (0.12 m diameter, 0.64 m height, 5.3 L useful volume). A porous plate was placed at the bottom of the reactor for air supply. The reactor was fed continuously with a mineral solution containing ($mg\,L^{-1}$); $(NH_4)_2SO_4$, 5,600; $NaHCO_3$, 10,000; KH_2PO_4, 190; $MgSO_4 \cdot 7H_2O$, 114; $CaCl_2$, 76; $FeCl_3 \cdot 6H_2O$, 12. The medium was fed with a peristaltic pump (Masterflex L/S precision, Cole-Parmer) with a flow rate of 0.040 $L\,h^{-1}$ (hydraulic retention time (HRT) of 5.3 days). Air was supplied continuously at an air flow rate of 0.56 $L\,min^{-1}$ using a mass flow controller (GFC171S, Aalborg, Monterrey, Mexico). pH was maintained at 8.0 ($\pm$0.3) with NaOH 1 M using a pH controller (Black Stone BL 7916, Cole-Parmer). Oxygen was measured with a polarographic dissolved oxygen bench meter (HI2400, Hanna Instruments, Mexico DF, Mexico). This bench meter was connected to a PC for data acquisition. The oxygen probe was placed at the top of the reactor. The reactor was inoculated with 1 L of mixed liquor obtained from a fixed-bed nitrifying reactor operated continuously in the same laboratory and the initial ammonia concentration was 100 $mgNH_4^+\text{-}N\,L^{-1}$. The reactor was maintained at ambient temperature (21–25°C).

Methods

Ammonia, nitrate, and nitrite were measured in triplicate according to colorimetric standards methods (APHA, 1999) and confirmed with an SAN plus analyzer (Sampler SA 1000, Skalar, Boom, Belgium). Nitrogen mass balance was confirmed through total nitrogen measurement (Shimadzu Vcsn equipped with a TNM-1 module, Shimadzu, Mexico). Biomass concentration was estimated in triplicate from COD, determined by the closed reflux colorimetric method according to standard method (APHA, 1999). Mass transfer coefficient (k_La) was measured in triplicate before and after each respirometric experiment. The method used was the dynamic method as described by Badino et al. (2000). The oxygen probe was calibrated using nitrogen (0%) or air (100%) injected in a sample of sterilized mixed liquor obtained from the reactor. The concentration of the ammonia solution used to make the pulses was confirmed by analysis made in triplicate.

''In situ'' respirometric pulse experiments were done according to the following procedure; (i) the data acquisition system was switched on and the system was maintained until stable oxygen readings were obtained, (ii) the reactor feeding was then stopped, (iii) the oxygen concentration slowly increased until reaching a new stationary state, corresponding to the endogenous respiration state (Moussa et al., 2003), (iv) the k_La of the reactor was measured, (v) a pulse of 2.5, 5.0, or 10 $mgNH_4^+\,L^{-1}$ was injected and the oxygen concentration was acquired until the system returned to the previous steady-state, (vi) pulse was eventually repeated, and (vii) the k_La was measured again before the feeding of the reactor was switched back on.

Data Interpretation

In this study, nitrification was considered as a single step process (Eq. 1). The single step assumption allows simpler data interpretation and parameter retrieval from single ammonia pulse injection instead of ammonia plus nitrite pulse injection. The single step assumption is based on the fact that the ammonia oxidation is considered as the limiting step of the process (Khin et al., 2002; Langergraber et al., 2003; Sperandio et al., 2005), which is also considered valid by the Activated Sludge Model 1 under non-limiting oxygen concentration (Jubany et al., 2005). However, the literature reports that the validity of the single step assumption depends on the temperature. As the optimal temperature of the ammonia oxidation process is higher than the optimal temperature of nitrite oxidation, there is a boundary temperature above what the single step assumption is no more valid. Hellinga et al. (1998) suggests that this temperature is 20°C and Nowak et al. (1995) suggest 25°C. Kong et al. (1996) operated a respirometer at 25°C and assumed a single step process. In this work, the reactor was maintained at ambient temperature (21–25°C). As it will be presented in the result section, except during the first

few days of the experiment, no nitrite accumulation was observed in the reactor and the single step assumption was considered valid. The absence of nitrite during pulse experiments was also confirmed experimentally, from samples taken when the minimum oxygen concentration was reached.

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (1)$$

According to this equation, the total amount of oxygen needed to oxidize the ammonia injected is 4.57 $gO_2\,g^{-1}N$, which is often called the nitrogen oxygen demand (NOD). This amount of oxygen is theoretical since Equation (1) does not consider biomass growth. When biomass growth is considered Equation (1) becomes:

$$\alpha NH_4^+ + \beta O_2 + \gamma CO_2 \rightarrow \delta C_5H_7NO_2 + \varepsilon NO_3^- + \zeta H^+ + \eta H_2O \quad (2)$$

However, if reagents and products are expressed in oxygen demand, that is, the amount of oxygen needed for their complete oxidation, only ammonia, oxygen, and biomass have to be considered. An oxygen balance can be then written as follow:

$$63.98\alpha - 32.00\beta = 160.00\delta \quad (3)$$

In this equation 63.98 is the mass of oxygen (g) needed to oxidize 1 mole of ammonia (4.57 $gO_2\,g^{-1}N$) and 160.00 is the amount of oxygen (g) needed to oxidize 1 mole of biomass produced ($C_5H_7NO_2$, 1.42 $gO_2\,g^{-1}$Biomass). By rearranging Equation (3):

$$\frac{32\beta}{63.98\alpha} + \frac{160.00\delta}{63.98\alpha} = 1 \quad (4)$$

The first term of this equation represents the mass of oxygen consumed per unit of ammonia consumed, expressed as NOD and is defined as the substrate oxidation yield (f_E). The second term is the mass of biomass produced, expressed as COD per unit of nitrogen consumed, expressed as NOD and is defined as the biomass growth yield (f_S). According to Equation (4), f_S and f_E are complementary:

$$f_E + f_S = 1 \quad (5)$$

After a substrate pulse injection, the oxygen mass balance in the reactor can be described by a balance between the exogenous respiratory activity and the oxygen provided by continuous aeration. The oxygen mass balance can be expressed by Equation (6) (Kong et al., 1994) where OUR_{ex} is the exogenous respiration rate of the microorganism [$g\,L^{-1}\,h^{-1}$] and C_b is the oxygen concentration during the pseudo steady-state, defined as the baseline concentration.

$$\frac{dC}{dt} = k_L a(C_b - C) - OUR_{ex} \quad (6)$$

When a known amount of substrate is oxidized during the pulse injection, f_E is given by the amount of oxygen consumed per unit NOD of ammonia oxidized. The f_E can therefore be expressed as:

$$f_E = \frac{\int_0^t OUR_{ex}\,dt}{NOD[NH_4^+]_p} = \frac{k_L a \int_0^t (C_b - C)dt + (C_0 - C_f)}{4.57[NH_4^+]_p} \quad (7)$$

Biomass Growth Yield Estimation

During a pulse injection, the initial (C_0) and final (C_f) dissolved oxygen concentrations are usually equal to the baseline concentration (C_b). The biomass growth yield expressed in COD units can be easily estimated from pulse respirometry (Eq. 8). If biomass composition is considered as $C_5H_7NO_2$, the biomass growth yield expressed in weight units can be expressed by Equation (9).

$$f_s = 1 - \frac{k_L a \int_0^t (C_b - C)dt + (C_0 - C_f)}{4.57[NH_4^+]_p} \quad (8)$$

$$Y_{X/S} = \frac{4.57}{1.42} f_s \quad (9)$$

K_S Estimation

The nitrification rate (R) can be related to the OUR_{ex} using the substrate oxidation yield ($Y_{O_2/S}$).

$$R = \frac{OUR_{ex}}{Y_{O_2/S}} = \frac{OUR_{ex}}{4.57 f_E} \quad (10)$$

The nitrification process can be expressed by the Monod Equation (Ficara et al., 2000). The Monod Equation, expressed in oxygen demand is given by Equation (11).

$$OUR_{ex} = \frac{S}{S + K_S} OUR_{ex}max \quad (11)$$

By substitution of OUR_{ex} in Equation (6), Equation (12) is obtained.

$$\frac{dc}{dt} = k_L a(C_b - C) - \frac{S}{S + K_S} OUR_{ex}max \quad (12)$$

Equation (12) was adjusted to the experimental data obtained from pulse experiments. For that purpose, k_La was measured as described above. The $OUR_{ex}max$ was experimentally determined from the injection of pulses of increasing concentration (2.5, 5, and 10 $mgNH_4^+$-N L^{-1}) as it will be described in the Results and Discussion Section. Equation (12) was adjusted with a fitting procedure based on Runge-Kutta method with a Marquardt optimization with 20 convergence steps (Model Maker, Cherwell Scientific Publishing, Oxford, UK). The adjustment was made taking into account the response time as described previously by Vanrolleghem et al. (2004).

Error Estimation

The absolute error (Δf) was estimated trough error propagation according to Equation (13) (Elden and Wittmeyer-Koch, 1990).

$$\Delta f^2 \approx \sum_{k=1}^{n} \left| \frac{\partial f}{\partial x_k} \Delta x_k \right|^2 \tag{13}$$

The errors on the dissolved oxygen measurement (1.5%) and on the volume of ammonia injected (transferpette, 0.6%) were given by the equipment suppliers. The error made on the k_La ($3.33 \pm 1.03\%$) and the error on the actual ammonia concentration injected ($0.93 \pm 0.58\%$) were estimated after nine repetitions. The standard error (S_E) was estimated from Equation (14), using the standard deviation (σ) of n measures (Freund and Wilson, 1996).

$$S_E = \frac{\sigma}{\sqrt{n}} \tag{14}$$

The dissolved oxygen data obtained from respirometric experiments were softened by a standard 7-point smooth (UN-SCAN-IT software, Silk Scientific Corporation, Orem, UT, 1996).

Results and Discussion

The nitrifying reactor was inoculated and operated during 60 days under a constant loading rate of 210 ($\pm$24) $mgNH_4^+$-N L^{-1} d^{-1}. Since the onset, a clear nitrification was observed (Fig. 1). The ammonia concentration rapidly decreased from 1,000 mg L^{-1} to less than 0.1 mg L^{-1}. As a consequence of the nitrification activity, a clear increase of the nitrate concentration was observed as well as a biomass concentration increase. A temporary accumulation of nitrite was also observed from days 0 to 15. From the data presented in Figure 1, it can be concluded that the system reached a steady-state approximately after 40 days of continuous operation. By the end of this operation, it was observed that the nitrate concentration was inferior to the ammonia

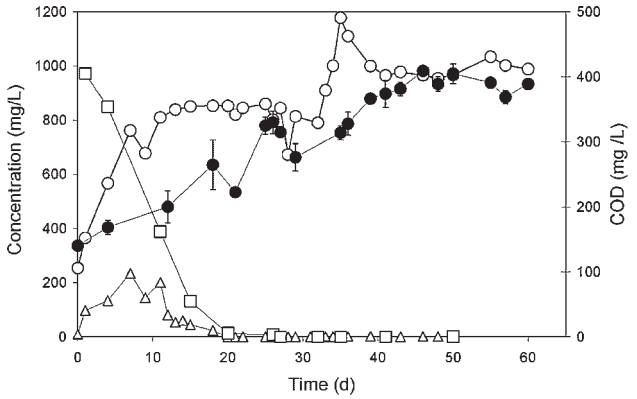

Figure 1. Ammonia (-□-, $mgNH_4^+$-N L^{-1}), nitrite (-Δ-, $mgNO_2^-$-N L^{-1}), nitrate (-○-, $mgNO_3^-$N), biomass (-●-, mgCOD L^{-1}) concentrations observed in the reactor.

concentration fed to the reactor. Indeed, the ammonia concentration in the feeding solution was 1,140 ($\pm$41) $mgNH_4^+$-N L^{-1} while the effluent nitrate concentration appeared steadily around 983 ($\pm$26) $mgNO_3^-$-N L^{-1}. In order to understand such a difference, a nitrogen balance over the reactor was estimated taking into account ammonia oxidation, biomass growth (Eq. 2), and stripping processes. Ammonia oxidation accounted for 86.2%, biomass growth for 3.0%, and stripping for 10.8% of the total ammonia loading rate. Ammonia stripping from 10% to 30% of the ammonia loading rate has been also reported by Jokela et al. (2002) in a fixed-bed nitrifying reactor operated under similar working conditions (pH 7.5–8.5, temperature 25°C).

To discard stripping during short-term pulse experiments that could reduce the amount of ammonia actually oxidized, an abiotic experiment was done. This experiment consisted of injecting a pulse of ammonia in a bubble column operated as the biological reactor but without biomass. The actual ammonia concentration was followed during 2 h. No detectable reduction of ammonia concentration was detected. This concludes that ammonia was not subject to significant stripping during short-term experiments although about 10% was probably lost by stripping in the reactor over the HRT of 5.6 days.

At day 5, a first pulse respirometry experience was done with the injection of two pulses of 10 $mgNH_4^+$-N L^{-1}. Figure 2A presents the first respirogram observed. Figure 2B presents both respirograms after being softened to allow comparison. These respirograms show a similar behavior. The dissolved oxygen concentration descended sharply from 5.9 to 5.6 mg L^{-1}, followed by a stable period and then by a slow increase up to the initial steady-state. In Figure 2B, the thin line represents the second pulse, made immediately after the first one. The sole difference between both pulses is a slight delay of the second pulse. This delay generated a small difference in the determination of the oxidation yield (f_E), which was 0.69 for the first pulse and 0.71 (+2.9%) for the second one.

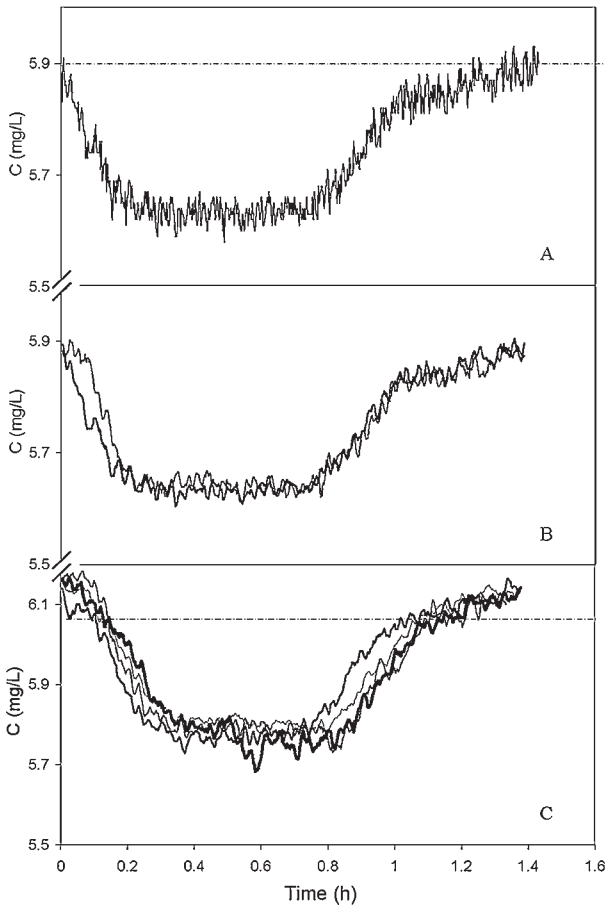

Figure 2. Dissolved oxygen concentration profile observed in the reactor after the injection of ammonia pulses; (**A**) non-softened oxygen profiles, (**B**) and (**C**) softened oxygen profiles.

"In-situ" respirometry involves feeding suspension that could have a significant impact on the process itself and on the repeatability of the pulses. To evaluate the effect of the feeding suspension, the feeding pump was switched off and four pulses of 10 mgNH_4^+-N L^{-1} were injected in a row over a total time of 5.5 h. No significant difference was observed between the pulses (Fig. 2C). A standard deviation of 4.23% on the substrate oxidation yield (f_E) was observed between the four pulses. At the end of the fourth pulse, the system was returned to normal operation and no change was observed in the dissolved oxygen concentration or the biomass and ammonia concentration. These results show

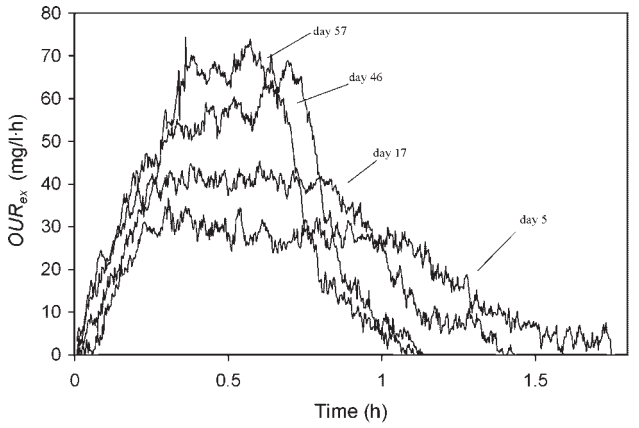

Figure 3. Oxygen uptake rates observed after the injection of 10 mgNH_4^+-N L^{-1} pulses at days 5, 17, 46, and 57.

that the application of repeated pulse during 5.5 h did not affect significantly the behavior of the system.

Figure 3 shows different pulses (10 mgNH_4^+-N L^{-1}) made at days 5, 17, 46, and 57. This figure shows OUR_{ex} respirograms instead of dissolved oxygen profile, for a clearer representation. The OUR_{ex} observed at four different times show the same sharp increase followed by a pseudo steady-state. This pseudo steady-state corresponds to a short period of time during which the process is independent of the substrate concentration. These OUR_{ex} were considered as the OUR_{ex}max as it was confirmed by the injection of higher substrate concentration without any change in the maximum OUR_{ex} observed (Table I).

From days 5 to 57, a progressive increase of the OUR_{ex}max and the biomass concentration was observed. A similar trend of both variable along the time was observed with a ratio of 0.15 ($\pm$0.01) $mgO_2\,mg^{-1}COD\,h^{-1}$ ($r^2 = 0.93$). These results indicate that the biomass concentration can be estimated "in-situ" from direct respirometric experiment, after proper calibration. However, this assumption, commonly used in respirometry, would be only valid if most of the biomass present in the reactor is nitrifying and, additionally, if substrate degradation is the only limiting step of the process in absence of, for instance, mass transfer limitation.

Figure 4 presents the biomass growth yield (f_S) measured by pulse respirometry along the experiment. This figure presents also the apparent biomass growth yield determined

Table I. Maximum OUR_{ex} observed after the injection of pulses from 2.5 to 15.0 mgN-NH_4^+ L^{-1}.

Pulse concentration (mg N-NH_4^+ L^{-1})	Maximum OUR_{ex} observed ($mgO_2\,L^{-1}\,h^{-1}$)			
	Day 32	Day 43	Day 46	Day 57
2.5	47.77 $\pm$ 2.4	40.80 $\pm$ 2.9	39.96 $\pm$ 2.9	36.93 $\pm$ 1.8
5.0	60.11 $\pm$ 3.0	61.57 $\pm$ 4.3	63.30 $\pm$ 4.6	50.94 $\pm$ 2.5
10.0	60.19 $\pm$ 3.0	64.15 $\pm$ 4.5	66.50 $\pm$ 4.9	67.45 $\pm$ 3.2
15.0	61.24 $\pm$ 3.1		61.65 $\pm$ 4.5	68.13 $\pm$ 3.3

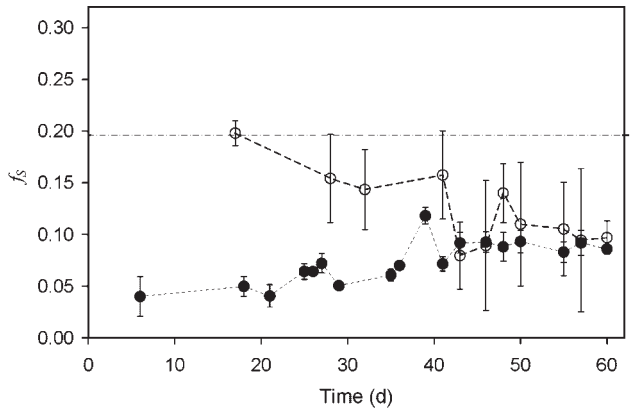

Figure 4. Biomass growth yield (f_S) measured by respirometry (○) and by COD mass balance (●) along the experiment. Dotted line represents the maximum theoretical growth yield that ensures exergonic process.

from COD mass balance. From day 43 onwards, the reactor had reached a steady-state and the values of growth yield obtained through respirometry and COD were similar, 0.10 (±0.04) and 0.09 (±0.01), respectively. However, before steady-state was reached, a significant difference between the biomass growth yield measured through COD and respirometry was observed. The initial respirometry values (f_S) are probably largely overestimated, as the literature on nitrifying process reports biomass growth yield from 0.03 (Gapes et al., 2003) to 0.13 (Gee et al., 1990). However, to our knowledge, the literature does not mention growth yields in nitrifying reactor during start-up. Thermodynamically, according to Equation (2), the maximum theoretical growth yield that ensures exergonic reaction is 0.20. This confirms, that at least the first growth yield (day 6, $f_S = 0.30$) was largely overestimated. One of the reasons could be that on day 6 the reactor showed a small accumulation of nitrite (Fig. 1). The single step nitrification assumption could be no longer valid under these conditions and a partial ammonia oxidation to nitrite would suggest a higher oxidation yield (f_E) and a lower growth yield (f_S) than those actually observed. Sampling during the pulses made on day 6 showed a low nitrite concentration when the lowest DO concentration was reached but no nitrite by the end of the pulse. No nitrite was observed during the pulses made afterwards which confirm that the single step assumption was valid for the pulses made after day 6. Another reason of the large difference between f_S estimated by COD and respirometry could be simply accounted to large experimental errors as it will be discussed latter. The results presented in Figure 4 suggest that "in-situ" respirometry allows the determination of the biomass growth yield when the process is under steady-state. The applicability of respirometry on processes under transient state requires further research to understand why the oxygen consumption after pulse injection is relatively low compared to those observed under steady-state.

The global ammonia affinity constant (K_S) was also estimated. In a first step, K_S constant was estimated from complete respirograms. Figure 5A shows an example of how the theoretical model (Eq. 12) fitted to the experimental data. Even taking into account a biological response time (Vanrolleghem et al., 2004), the correlation was only reasonable ($r^2 = 0.81 \pm 0.03$). In a second step, K_S was estimated from the second half of the respirograms (Fig. 5B), in which the ammonia concentration becomes limiting and the oxygen concentration starts to rise. Under these conditions, the respiration rate observed is probably less sensitive to external factors such as the biological and electrode response time or the external mass transfer limitations. This is probably why the correlations were better than those made from the complete respirograms ($r^2 = 0.89 \pm 0.04$). From days 32 to 57, the affinity constant of the process was estimated from 15 pulses of 2.5 to 10 mgNH$_4^+$-N L^{-1}. The affinity constant was 0.48 (±0.08) mgNH$_4^+$-N L^{-1}. This figure is close to the affinity constants reported by Sperandio et al. (2005) (0.24–0.32 mgNH$_4^+$-N L^{-1}) in a membrane nitrifying reactor. The global affinity constant estimated here by respirometry is also close to the affinity constant reported for the ammonia oxidation step by Ciudad et al. (2006), 0.30 mgN-NH$_4$ L^{-1}, by Carrera et al. (2004), 0.16 mgN-NH$_4$ L^{-1}, and by Ficara et al. (2000), 0.30–2 mgN-NH$_4$ L^{-1}.

Figure 6 shows a sensitivity analysis for the correlation (Δr^2) of the model (Eq. 12) to the experimental data. This sensitivity analysis was based on a typical respirogram observed after the injection of a 10 mgNH$_4^+$-N L^{-1} pulse

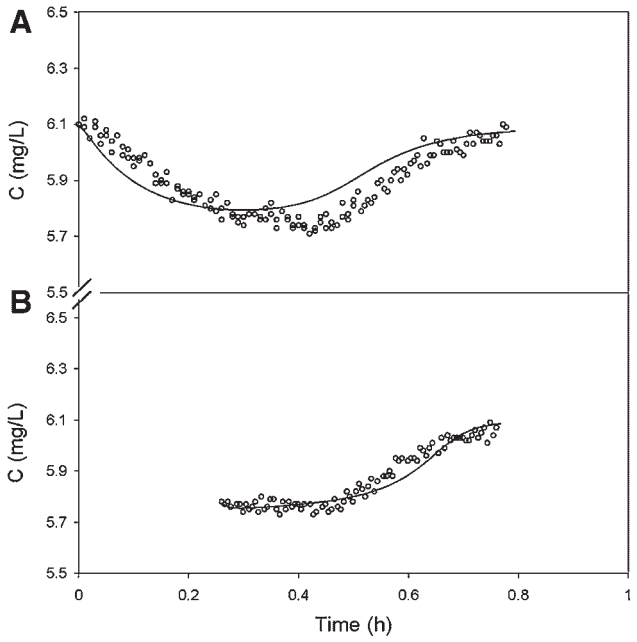

Figure 5. Example of best model fitting to the experimental data, considering complete respirogram (**A**) and the second half of the respirogram (**B**).

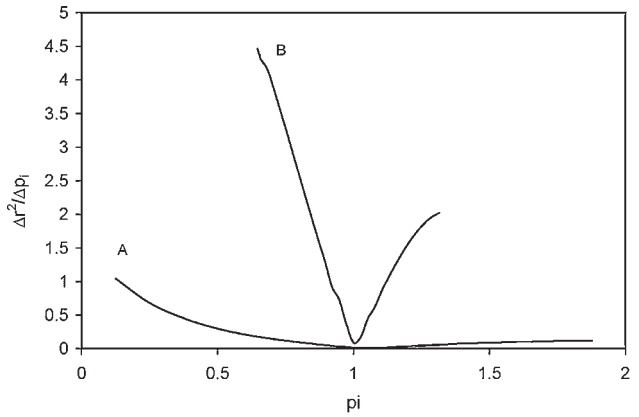

Figure 6. Sensitivity analysis of K_S (**A**) and f_E (**B**) estimation.

($K_S = 0.40$ mgNH$_4^+$-N L^{-1}, $f_E = 0.88$ and $OUR_{ex}max = 53.55$ mgO$_2$ L^{-1} h^{-1}). Figure 6 shows the impact of varying K_S and f_E on the sensitivity coefficient ($\Delta r^2/\Delta p_i$) where Δp_i is the variation of the parameter analyzed. According to this figure, $\Delta r^2/\Delta p_i$ shows a high sensitivity to K_S and f_E below their optimal value, which means that small errors on the parameter estimation would generate large error on the correlation factor. On the contrary, $\Delta r^2/\Delta p_i$ did not show a high sensitivity to $OUR_{ex}max$ (data not shown).

Parameter estimation from the second part of respirograms was previously used by Pratt et al. (2004) to estimate the k_La. The method is based on the assumption that, when oxygen concentration increases after a pulse injection, no more substrate is available and therefore the shape of the oxygen increase depends on the mass transfer coefficient. To confirm this point, k_La estimated according to the Pratt method were compared to k_La estimated by the Badino et al. (2000) method (Table II). The k_La estimated from the Pratt method was systematically about 10 times lower than the k_La estimated from the dynamic method. The k_La estimated from the Pratt method were clearly erroneous, as the biomass growth yield (f_S) estimated from Equation (7) with these k_La values would have been systematically around 0.90, which is almost five times higher than the maximum theoretical exergonic growth (0.20).

Ammonia and nitrite concentrations were measured in the reactor when the dissolved oxygen started to increase after the pulse injection, and both concentrations were below the detection limit of the technique used (0.04 mgNO$_2^-$-N L^{-1} and 0.10 mgNH$_4^+$-N L^{-1}). This was made to corroborate the initial hypothesis of the absence of significant substrate concentration when dissolved oxygen is increasing after pulse injection, as suggested by Pratt et al. (2004). However the absence of measurable nitrite and ammonia in the bulk phase does not mean they are totally absent from the system. According to Costa et al. (2006), ammonia oxidation and nitrite oxidation occur in the cytoplasm of the cells. Both ammonia and nitrite could be undetectable by the method used in this work but still available for biomass. This would explain why, at least under the conditions of this work, the method suggested by Pratt et al. (2004) gave erroneous results. A finer analyze based on more precise nitrite and ammonia concentrations measurement associated to a two step (ammonia and nitrite oxidation) model would allow to understand the difference observed.

Table II. Comparison of the k_La measured by respirometry according to Pratt et al. (2004) and measured by a dynamic method (Badino et al., 2000).

	k_La (h^{-1})	
Day	Respirometric method	Dynamic method
32	10 ± 2	99 ± 5
39	9 ± 3	76 ± 7
43	10 ± 2	116 ± 8
46	12 ± 4	123 ± 9
57	12 ± 5	125 ± 6

According to the results obtained along the 60 days experiment, typical error on the substrate oxidation yield (f_E) was 5.56%. According to Equation (5) f_S is linearly proportional to f_E. Therefore the estimated error on f_E is proportionally propagated to the estimated error on f_S, as it can be observed in Figure 7.

In our case, we observed through respirometry a growth yield of about 0.09 and therefore the standard error on the growth yield can be estimated to 56%. Of course, this error can be substantially reduced by increasing the number of repetitions. From Equation (14), the effect of the replicates number on the standard deviation was calculated for one to five repetitions. Standard deviation can be represented by a second order polynomial function in terms of the number of replicates (Eq. 15). From this equation, it was observed that the standard error (S_E) on f_S decreases from 54% to 12% as n increases from 1 to 5 (Table III). This behavior is in accordance with the central limit theorem.

$$\sigma = 0.264n^2 - 8.528n + 62.988 \qquad (15)$$

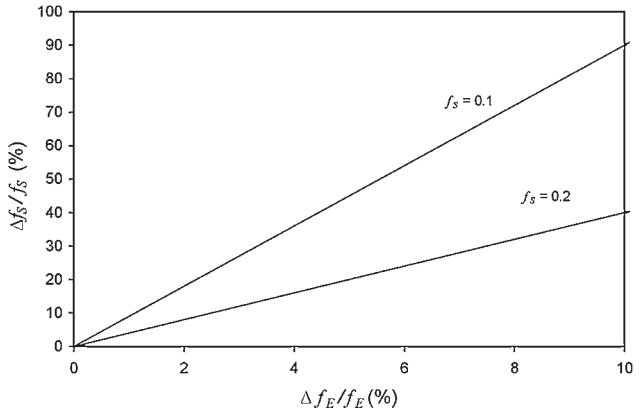

Figure 7. Sensitivity of the biomass growth yield (f_S) to the oxidation yield (f_E).

Table III. Standard error inherent to each parameter estimation.

	Standard error (%)	
Parameter	n = 3	n = 5
f_E	4.36	3.37
f_S	19.56	12.34
$OUR_{ex}max$	7.36	6.69
K_S	30.12	27.08

The same error estimation procedure was repeated for all the parameters that can be retrieved from the respirometric experiments (Table III). As it can be observed, the main error is made on the affinity constant and on the biomass growth yield.

Concluding Remark

The injection of substrate pulses in a nitrifying reactor under endogenous respiration state, allowed the retrieval of four important kinetic and stoichiometric parameters, namely (i) the maximum oxygen uptake rate, (ii) the oxidation yield, (iii) the growth yield, and (iv) the affinity constant.

The injection of pulses of increasing concentration was a suitable method to determine at which substrate concentration the $OUR_{ex}max$ is reached. Once this concentration is identified, the $OUR_{ex}max$ can be directly measured in subsequent experiments. A clear correlation ($r^2 = 0.93$) was observed between biomass concentration and $OUR_{ex}max$. Respirometry could be therefore considered also as a potential method for biomass estimation, after proper calibration, in reactor where most of the biomass is nitrifying and in absence of other limiting phenomena.

The substrate oxidation yield was also measured directly from the respirograms, with a standard error around 5.6%. As the oxidation yield and the growth yield are complementary, the same respirograms brought both parameters. Due to the low biomass growth yield observed, this parameter was estimated with a large error (about 56% for single measurement and 20% for triplicate measurement). Additionally, it was observed that when the reactor was under transient-state, the oxidation yield was probably underestimated and the growth yield overestimated. Complementary research is needed to understand this.

The affinity constant was also determined from ''in-situ'' pulse respirometry but with significant standard error (about 30% when measurements made in triplicate). The interpretation of the second part of the respirograms brings better correlated results than the interpretation of complete respirograms. This is probably due to the inability of the chosen model to interpret fast changing conditions. A more complex model taking into account biological and electrode response time as well as a two step biological model should give better correlation.

It is concluded that the pulse respirometry method can be usefully applied ''in-situ.'' Further research is needed to understand the response of the method under transient state and to verify the applicability of the method to other processes and other reactor designs especially in larger scale reactor where mixing and hydrodynamics play a key role.

Nomenclature

C	dissolved oxygen concentration [$mg\,L^{-1}$]
C_o	initial dissolved oxygen concentration [$mg\,L^{-1}$]
C_f	final dissolved oxygen concentration [$mg\,L^{-1}$]
C_b	baseline dissolved oxygen concentration ($mg\,L^{-1}$).
COD	chemical oxygen demand ($mgO_2\,L^{-1}$)
S_E	standard error (%)
f_E	substrate oxidation yield ($mgO_2\,mg^{-1}NOD$)
f_S	biomass growth yield ($mgCOD\,mg^{-1}NOD$)
k_La	volumetric mass transfer coefficient (h^{-1})
K_S	affinity constant ($mgNH_4^+\text{-}N\,L^{-1}$)
K_{O_2}	oxygen affinity constant
k_m	aerobic endogenous decay constant
n	number of experimental replicates
NOD	nitrogenous oxygen demand ($mgO_2\,L^{-1}$)
OUR_{ex}	oxygen uptake rate ($mgO_2\,L^{-1}\,h^{-1}$)
$OUR_{ex}max$	maximum oxygen uptake rate ($mgO_2\,L^{-1}\,h^{-1}$)
R	nitrification rate ($mgNH_4\text{-}N\,L^{-1}\,h^{-1}$)
R_{Smax}	maximum nitrification rate ($mgNH_4\text{-}N\,L^{-1}\,h^{-1}$)
$Y_{O_2/S}$	substrate oxidation yield ($mgO_2\,mg^{-1}NH_4\text{-}N$)
$Y_{X/S}$	biomass growth yield ($mgC_5H_7NO_2\,mg^{-1}NH_4\text{-}N$)
σ	standard deviation (%)
Δf	absolute error of a given function (−)
$\Delta r^2/\Delta p_i$	sensitivity coefficient

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References

APHA. 1999. Standard methods for the examination of water and wastewater. 20th edn. Washington, DC: American Publishers Health Association.

Avcioglu E, Orhon D, Sözen S. 1998. A new method for the assessment of heterotrophic endogenous respiration rate under aerobic and anoxic conditions. Water Sci Technol 38(8–9):95–103.

Badino AC, Facciotti MCR, Schmidel W. 2000. Improving k_La determination in fungal fermentation, taking into account electrode response time. J Chem Technol Biotechnol 75:469–474.

Carrera J, Jubany I, Carvallo L, Chamy R, Lafuente J. 2004. Kinetic models for nitrification inhibition by ammonia and nitrite in a suspended and an immobilized biomass systems. Process Biochem 39:1159–1165.

Chandran K, Smets B. 2000. Single-step nitrification models erroneously describe batch ammonia oxidation profiles when nitrite oxidation becomes rate limiting. Biotechnol Bioeng 68(1):396–406.

Chandran K, Smets B. 2001. Estimating biomass yield coefficients for autotrophic ammonia and nitrite oxidation from batch respirograms. Water Res 35(13):3153–3156.

Chandran K, Smets B. 2005. Applicability of an extant batch respirometric assay in describing dynamics of ammonia and nitrite oxidation in a nitrifying bioreactor. Water Sci Technol 52(10):503–508.

Checchi N, Marsili-Libelli S. 2005. Reliability of parameter estimation in respirometric models. Water Res 39:3686–3696.

Ciudad G, Werner A, Bornhardt C, Muñoz C, Antileo C. 2006. Differential kinetics of ammonia and nitrite oxidizing bacteria: A simple kinetic study based on oxygen affinity and proton release during nitrification. Process Biochem 41(8):1764–1772.

Costa E, Perez J, Kreft JU. 2006. Why is metabolic labour divided in nitrification? Trends Microbiol 14(5):213–219.

Dircks K, Pind PF, Mosbaek H, Henze M. 1999. Yield determination by respirometry—The possible influence of storage under aerobic conditions in activated sludge. Water SA 25(1):69–74.

Elden L, Wittmeyer-Koch L. 1990. Numerical analysis. An introduction. New York: Academic Press.

Ficara E, Musumeci A, Rozzi A. 2000. Comparison and combination of titrimetric and respirometric techniques to estimate nitrification kinetic parameters. Water SA 26(2):217–224.

Freund R, Wilson W. 1996. Statistical methods. Boston: Academic Press.

Gapes D, Pratt S, Yuan ZG, Keller J. 2003. Online titrimetric and off-gas analysis for examining nitrification processes in wastewater treatment. Water Res 37:2678–2690.

Gee CS, Pfeffer JT, Suidan MT. 1990. Nitrosomonas and Nitrobacter interactions in biological nitrification. J Environ Eng—ASCE 116(1):4–17.

Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J. 2003. An off-line respirometric procedure to determine inhibition and toxicity of biodegradable compounds in biomass from an industrial WWTP. Water Sci Technol 48(11–12):267–275.

Guisasola A, Jubany I, Baeza JA, Carrera J, Lafuente J. 2005. Respirometric estimation of the oxygen affinity constants for biological ammonia and nitrite oxidation. J Chem Tech Biotech 80(4):388–396.

Hellinga C, Schellen AAJC, Mulder JW, van Loosdrecht MCM, Heijnen JJ. 1998. The SHARON process: An innovative method for nitrogen removal from ammonium-rich wastewater. Water Sci Technol 37(9):135–142.

Jokela JPY, Kettunen RH, Sormunen KM, Rintala JA. 2002. Biological nitrogen removal from municipal landfill leachate: Low-cost nitrification in biofilters and laboratory scale in-situ denitrification. Water Res 36:4079–4087.

Jubany I, Baeza JA, Carrera J, Lafuente J. 2005. Respirometric calibration and validation of a biological nitrite oxidation model including biomass growth and substrate inhibition. Water Res 39(18):4574–4584.

Khin T, Gheewala SH, Annachhatre AP. 2002. Modeling of nitrification inhibition with aniline in suspended-growth processes. Water Environ Res 74(6):531–540.

Kong Z, Vanrolleghem P, Verstraete W. 1994. Automated respiration inhibition-kinetics analysis (ARIKA) with a respirographic biosensor. Water Sci Technol 30(4):275–284.

Kong Z, Vanrolleghem P, Willems P, Verstraete W. 1996. Simultaneous determination of inhibition kinetics of carbon oxidation and nitrification with a respirometer. Water Res 30(4):825–836.

Langergraber G, Wuchty M, Fleischmann N, Lechner M. 2003. Rapid automated detection of nitrification kinetics using respirometry. Water Sci Technol 47(2):149–155.

Meiklejohn J. 1937. The oxygen uptake of suspensions and cultures of a free-living bacterium. J Exp Biol 14:158–170.

Moussa MS, Lubberding HJ, Hooijmans CM, Van Loosdrecht MCM, Gijsen HJ. 2003. Improved method for the determination of ammonia and nitrite oxidation activities in mixed bacterial cultures. Appl Microbiol Biotechnol 63:217–221.

Nowak O, Svardal K, Shweighofer P. 1995. The dynamic behaviour of nitrifiying activated sludge systems influenced by inhibiting wastewater compounds. Water Sci Technol 31(2):115–124.

Pratt S, Yuan Z, Keller J. 2004. Modeling aerobic carbon oxidation and storage by integrating respirometric, tritrimetric and off-gas CO_2 measurements. Biotechnol Bioeng 88:135–147.

Riefler RG, Ahlfeld DP, Smets BF. 1998. Respirometric assay for biofilm kinetics estimation: Parameter identifiability and retrievability. Biotechnol Bioeng 57(1):35–45.

Spanjers H, Takacs I, Brouwer H. 1999. Direct parameter extraction from respirograms for wastewater and biomass characterization. Water Sci Technol 39(4):137–145.

Sperandio M, Masse A, Espinoza-Bouchot MC, Cabassud C. 2005. Characterization of sludge structure and activity in submerged membrane bioreactor. Water Sci Technol 52(10–11):137–145.

Thompson W. 1932. Studies in respirometry I. A combined gas burette-interferometer respirometer. J Gen Physiol 16(1):5–22.

Vanrolleghem PA, Sin G, Gernaey KV. 2004. Transient response of aerobic and anoxic activated sludge activities to sudden substrate concentration changes. Biotechnol Bioeng 86(4):277–290.

Villaverde S, Fdz-Polanco F, Lacalle ML, Garcia PA. 2000. Influence of the suspended and attached biomass on the nitrification in a two submerged biofilters in series system. Water Sci Technol 41:169–176.

Yoong ET, Lant PA, Greenfield PF. 2000. In situ respirometry in an SBR treating wastewater with high phenol concentrations. Water Res 34(1):239–245.