Observation and mathematical description of the acceleration phenomenon in batch respirograms associated with ammonium oxidation

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Abstract Two-step nitrification models are generally calibrated using short-term respirometric batch experiments. Important discrepancies appear between model predictions and experimental observations just after the pulse addition since a fast transient in the OUR profile is experimentally observed. Acceleration of the OUR appears ongoing between the substrate addition and attainment of the maximum OUR value. Among the several phenomena that could contribute to this observation, the most probable cause is the limitation of reducing equivalents required for maximal ammonia monooxygenase activity at the time of substrate addition. Ignoring acceleration would result in large parameter estimation errors from respirometric batch experiments. This work proposes a simple methodology to successfully describe (not to explain) the acceleration phenomenon estimating only two parameters. This methodology consists of introducing a Gaussian-like expression in the model.

Keywords Acceleration; ammonia monooxygenase; modelling; nitrification; respirometry

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Introduction

Nitrification refers to the biological oxidation of ammonium to nitrate. There are two physiologically distinct groups of bacteria that sequentially and collectively perform nitrification. Thus, this process can be considered a two-step process. Ammonia oxidising biomass (AOB) performs nitritation (i.e. biological oxidation of ammonia to nitrite) and nitrite oxidising biomass (NOB) performs nitratation (i.e. biological oxidation of nitrite to nitrate). For a long period, nitrification has been modelled as a single step considering direct ammonia to nitrate oxidation, based on the frequent observation that the limiting step in the two-step process was the first step: oxidation of ammonia to nitrite. However, this simplification has recently been abandoned and, nowadays, two-step nitrification models are commonly applied in the literature (Gee *et al.*, 1990; Ossenbruggen *et al.*, 1996; Chandran and Smets, 2000a or Petersen *et al.*, 2003 among many others). These models are generally calibrated using respirometric batch tests and some of them include substrate measurements. The model identifiability, especially when using Oxygen Uptake Rate (OUR) as the sole measurement variable, is an important issue, and was deeply examined by Petersen *et al.* (2003) and Chandran and Smets (2000b).

As described in Vanrolleghem *et al.* (2004), important discrepancies appear in respirometric short-term batch experiments with COD as substrate between model predictions and experimental observations, particularly at the start of the experiment (i.e. just after substrate pulse addition). These discrepancies have been termed wake-up or start-up processes (Vanrolleghem *et al.*, 2004). Their work demonstrated that the dynamics of the experimental set-up (e.g. non-ideal mixing, DO probe response time and substrate

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diffusion) could only partially explain the experimentally observed phenomenon. They concluded that the short transient response probably had some physiological reason. They succeeded in modelling this transient phenomenon using a first-order model to describe the OUR profile, but the first-order rate constant had to be empirically estimated. However, the case of an ammonium pulse is different than the case of COD pulse because neither the wake-ups or the start-up modelled per Vanrolleghem *et al.* (2004) was able to capture the observed early transients in the OUR profile with ammonium as substrate. A different process, which we call acceleration, appears ongoing. This process requires a special focus because erroneous values of the stoichiometric and kinetic values would be estimated if this process were not explicitly accounted for in the mathematical model.

Methods

The respirometer used in this study is a 1 L LFS respirometer equipped with DO and pH monitoring. A LFS respirometer consists of a continuously aerated bioreactor without liquid flow-through, with the DO concentration measured in the liquid phase and the substrate introduced by small volume pulse additions. The procedure for OUR calculation with LFS respirometry can be found elsewhere (Spanjers *et al*., 1997). The airflow to the respirometer was controlled with a mass flow meter (Bronckhorst HiTec 825). The respirometer was thermally controlled by submersion in an isothermal bath and was mixed by magnetic stirring. The pH probe was a WTW Sentix 81 and the DO probe was WTW CellOx 325. These pH and DO probes were connected to a multiparametric reception device (INOLAB 3 of WTW). This in turn was connected via RS232 to a PC equipped with novel Visual Basic 6.0 software, allowing on-line data acquisition and storage in a Microsoft Excel worksheet. Moreover, the software was also capable of manipulating a microdispenser in order to keep the pH constant via acid/base dosage. The experiments were performed at pH $= 7.50 \pm 0.02$ and T $= 25 \pm 0.1$°C. The biomass used in this work was enriched in nitrifying biomass. It was withdrawn from a pilot plant fed with a low COD/N ratio influent and under conditions that favour nitrifying biomass growth. Plant operations were detailed in Jubany *et al.* (2005). Ammonium and nitrite were measured using Dr Lange kits (models LCK301 and LCK304).

Results and discussion

Acceleration phenomenon description

The acceleration phenomenon is defined as the period between the substrate addition and attainment of the maximum OUR value. This phase should be absent if Monod kinetics were applicable (Figure 1a); however it is routinely experimentally observed (Figure 1b). Figure 1a depicts the simulation results with a two-step nitrification model (with default parameter values) without any correction for the acceleration phenomenon. Clearly, an instantaneous increase to the maximum OUR value is predicted after ammonium is added. On the other hand, Figure 1b depicts a representative experimental OUR profile obtained after substrate addition (36.7 mg N-NH_4^+/L). The discrepancy between the experimental and model predicted profiles is clear. Apparently, OUR was very low just after the pulse addition and accelerated until it reached a maximum rate after approximately 15 minutes. Later decreases in the OUR are due to substrate exhaustion. We call acceleration the phenomenon observed at the start of the pulse. This observation is not unique to one experiment, but has been repeatedly observed in respirometric batch tests with ammonium as substrate performed by the authors. Moreover, this phenomenon is not only observed in the OUR profile, but also in the concomitantly measured substrate profiles. Figure 2 shows an experiment (36 mg N-NH_4^+/L pulse) with ammonium and nitrite measurements. The ammonium was initially consumed at a very low rate and then its

removal accelerated like the OUR. The nitrite profile was, moreover, consistent with the ammonium and OUR profiles indicating acceleration in the process rate.

Hypotheses for the existence of an acceleration phenomenon

A review of the literature suggests several processes that could contribute to the observed acceleration phenomenon. They are briefly introduced below, and their applicability to the situation discussed leading to rejection of several of them. Ultimately, we will introduce a modelling based approach alone to describe (not explain) the acceleration phenomenon.

a. *Overcome co-substrate limitation to ammonia monooxygenase (AMO):* By definition as a monooxygenase, AMO requires molecular oxygen and reducing equivalents to catalyze NH_3 oxidation. The electron flow diagram (Figure 3) suggests that there is a link between AMO and hydroxylamine oxyreductase (HAO) activity. The reducing equivalents produced during NH_2OH oxidation are channelled back to AMO

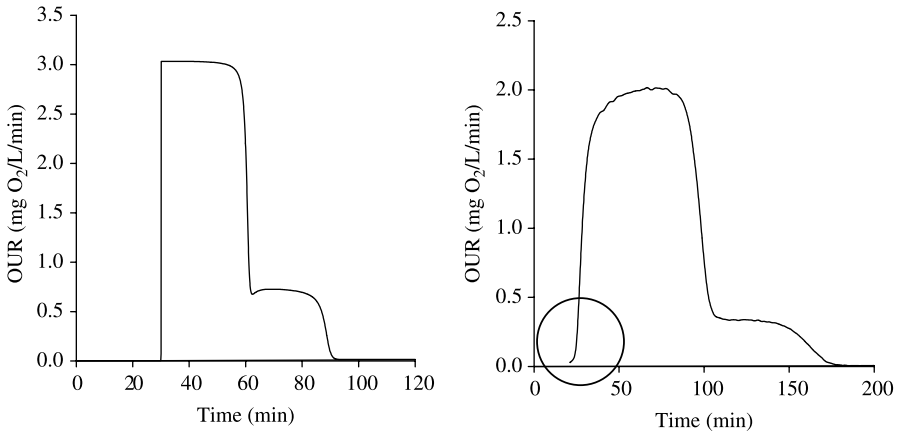

Figure 1 (Panel A, Left) Simulated OUR profile for a 35 mg $N\text{-}NH_4^+$/L pulse. (Panel B, Right) experimental OUR profile for a pulse of 36.7 mg $N\text{-}NH_4^+$/L

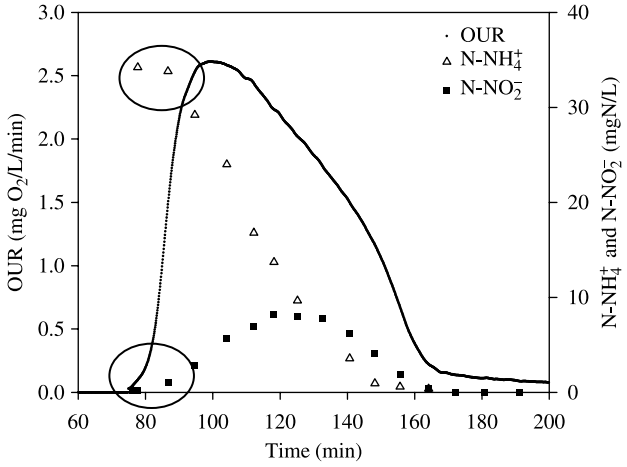

Figure 2 Experimental OUR, ammonium and nitrite profiles showing the acceleration effect for a pulse of 36 mg $N\text{-}NH_4^+$/L

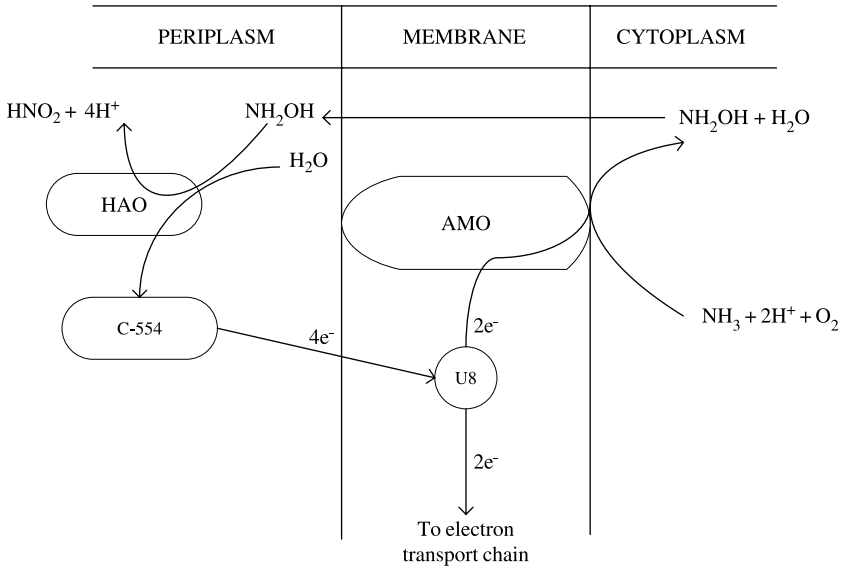

Figure 3 Schematic metabolic representation of the nitritation step (adapted from Schlegel and Bowien, 1989).C-554 – Cytochrome 554 and U8 – Ubiquinone 8

via ubiquinone U8. Therefore, the acceleration phase could be related to the oxidation of sufficient NH_2OH and in turn NH_3 before maximal AMO activity is attained. Indeed, during concurrent NH_3 and NH_2OH oxidation, the acceleration phase is nearly eliminated underlining a possible link between the two oxidation steps (Chandran, 1999). Recently, Zart *et al.* (2000) demonstrated that NO, a potential intermediate in NH_2OH to HNO_2 oxidation, can stimulate ammonium oxidation by *Nitrosomonas eutropha.* They hypothesized that NO provides the cells with NO_2 (or N_2O_4), which could act as the cosubstrate for AMO. Again, this could imply that sufficient down-stream product needs to be accumulated before AMO can attain maximal activity. There is however, little experimental evidence supporting this hypothesis since the biochemical details on the oxidation from NH_2OH to HNO_2 have not been fully resolved.

b. *Signal response of DO electrodes and mixing phenomena:* These can be rejected since the acceleration phase was not experimentally observed in NH_2OH or $N\text{-}NO_2^-$ oxidation respirograms (Chandran, 1999; Guisasola *et al.*, 2005a).

c. *Substrate inhibition by NH_3:* The effect of substrate inhibition and an initial acceleration phase would also result in lower initial OUR until the inhibition disappeared. However, the initial concentrations at which our experiments were performed (around 40 mg $N\text{-}NH_4^+$/L) are far below the self-inhibitory concentrations for NH_3 oxidation. For example, Anthonisen *et al.* (1976) stated that inhibition appears from 450 mg $N\text{-}NH_4^+$/L ($T = 25°C$ and $pH = 7.5$).

d. *Accumulation of NH_3 and NH_2OH before oxidation:* Recently, Schmidt *et al.* (2004) showed that uptake of nitrogen substrates may precede their oxidation. However, although in our experiments daily initial respirograms were collected with endogenous biomass, repeated injection of NH_3 consistently returned the same acceleration phenomenon (Guisasola, 2005). Furthermore, the acceleration phenomenon was absent with NH_2OH as substrate pulse (Chandran, 1999). Hence, our results are not compatible with this hypothesis.

e. *Regulation of amoA:* It has been shown that *amoA* expression decreases under starvation conditions, and thus responds to bulk $N\text{-}NH_4^+$ concentrations (e.g. Stein and

Arp, 1998). However, we have seen that prior N-NH_4^+ and NH_2OH pulses to the test biomass did not impact the extent of the acceleration phase (Chandran, 1999; Guisasola, 2005a).

Based on the above arguments, the co-substrate hypothesis deserves further evaluation. Restated, we contend that the cause of the acceleration phase is then, probably, the limitation of reducing equivalents required for maximal AMO activity at the time of substrate addition. This limitation might disappear once the process starts and some hydroxylamine is oxidised. Hence, the nitritation process acts as an autocatalytic reaction, accelerating as electrons are produced in the second nitritation step. Once the reducing equivalents are no longer limiting AMO activity, maximal specific removal rates might be attained. The metabolic details of electron transport and substrate oxidation have, to a large extent, been unravelled for *Nitrosomonas* (Figure 3) (Schlegel and Bowien, 1989; Hagopian and Riley, 1998; Poughon *et al.*, 2001). According to this figure, NH_2OH can serve as a direct energy substrate, although it can exert toxic responses at relatively low concentrations (e.g. Bock *et al.*, 1991; Hagopian and Riley, 1998). Molecular oxygen is only required in the first step, whereas the second step is a simple four-electron oxidation that uses oxygen derived from water dissociation. Clearly, the AMO catalyzed step requires reducing equivalents which are regenerated in excess in the second step.

Impact of acceleration phase on parameter estimation from nitrification respirograms

Ignoring the acceleration phase would result in large errors during parameter estimation from respirometric batch experiments. This error would be particularly significant when assessing the biomass growth yield. The area under the OUR profile is proportional to the total oxygen consumption; Figure 4 illustrates the importance of the acceleration and start-up processes in view of modelling. This figure shows the experimental results of two sequential pulses (30.5 and 17.6 mg N-NH_4^+/L in Panel A and B, respectively). The first sharp-peak in both OUR profiles is due to oxygen transfer limitations as detailed in Guisasola *et al.* (2005b). The acceleration phase (filled in the left figure) can be very significant. Therefore, if the acceleration phase was neglected alleging experimental error, the biomass growth yield would be notably overestimated. Clearly, the region in the acceleration phase must be included when calculating the total cumulative oxygen uptake to conduct the yield estimates.

On the other hand, some authors use yield values previously estimated (or assumed from the literature). Figure 4b compares an experimental OUR with a model prediction of the pulse assuming the yield is known and without any consideration of the acceleration or the start-up phases. Clearly, although unbiased estimates of the yield can be inferred, the best-fit biokinetic parameter estimates (μ_{max} and K_S) would be biased because the acceleration phenomenon (first curvature in the OUR) cannot be correctly described.

Although a detailed description of internal metabolic processes underlying the acceleration phenomenon may not be available to date, it may not be necessary for a successful description of its biokinetic manifestation. Indeed, a biochemically structured description of the process would require additional reactions and additional compounds (e.g. reaction intermediates such as hydroxylamine). More processes inclusion would increase demands on parameters to be estimated and, with OUR as the only output measurement, parameter identifiabilitiy would be a severe limitation.

Hence, we chose for a mathematically simple, but sufficient, approach to capture the acceleration phenomenon. The first-order approach, used to describe the somewhat similar start-up phase observed with COD oxidation associated respirograms, failed

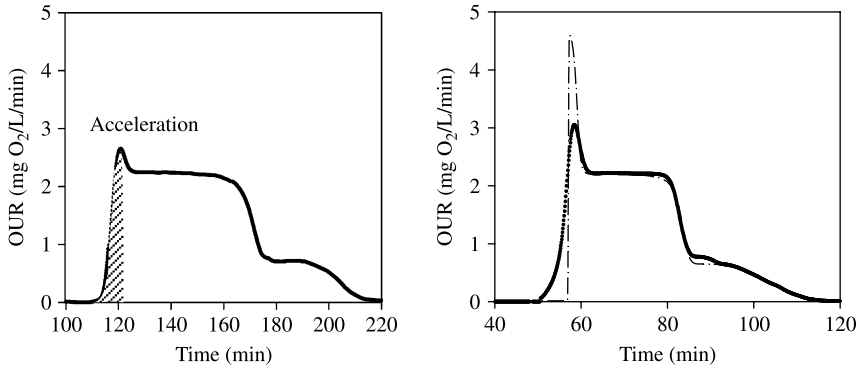

Figure 4 (Panel A, left) Experimental OUR profile for a 30.5 mg N-NH_4^+/L pulse with the acceleration phenomenon filled in black. (Panel B, right) Experimental OUR profile for a 17.6 mg N-NH_4^+/L pulse and simulation (dash-dotted) ignoring the acceleration phenomenon

(Vanrolleghem *et al.*, 2004): the first-order model fits convex OUR patterns only, whereas the curvature changes from concave to convex in the experimental OUR profiles with ammonium. This change in the curvature requires a two parameter description. The first trial considered an overdamped second-order system (which is the next logical step to the first-order model). The response of these systems in front of a step input change is a slow initial change followed by an increase in the speed. It results in an S-shape response which may fit the acceleration phenomenon. However, this equation did not provide a good fit to the observed acceleration phenomenon.

Several numerical expressions were proposed and examined. Table 1 describes and shows the parameter estimation and fit goodness for each of them. The term f_i stands for biomass active fraction. This value cannot be reliably estimated in a separate way from the maximum specific growth rate in these short-term experiments.

As can be observed in Table 1, the best fit was obtained with the Gaussian-like curve. The parameter estimates obtained when acceleration was ignored have a significant error, particularly the AOB maximum growth rate. The fit goodness obtained with the Gompertz expression is comparable with the Gaussian-like curve. A graphical analysis of the fits (results not shown) confirms that the Gaussian-like curve is the best expression among the other examined. Equation 1 shows a simplification of the differential equation describing substrate. The first term corresponds to the substrate consumption due to the nitritation process and the second term corresponds to the Gaussian-like expression.

$$\frac{dS_{NH4}}{dt} = -\mu_{MAX} \cdot \frac{S_{NH4}}{K_{NH4} + S_{NH4}} \cdot \frac{S_O}{K_O + S_O} \cdot X_A \cdot e^{\frac{-(t-\beta)^2}{\sigma}} \quad (1)$$

Table 1 Description, parameter estimation values and cost function (CF) calculated as the squared sum errors between the predicted and the experimental OUR profiles

Expressions tested\Parameters	$\mu_{MAXA} f_A$ (1/d)	$\mu_{MAXN} \cdot f_N$ (1/d)	K_{NH} (mg N/L)	K_{NO} (mg N^-/L)	β	σ	CF
Ignoring acceleration	0.61	0.79	1.01	2.78	–	–	7.3
First order $(1 - e^{-t/\beta})$	4.83	0.99	2.47	4.47	71		5.9
Second order $\left[1 + \frac{1}{\sigma-\beta} \cdot (\beta \cdot e^{-t/\beta} - \sigma \cdot e^{-t/\sigma})\right]$	3.32	1.56	3.95	9	3.64	22.6	4.4
Gaussian-like curve $e^{-\frac{(t-\beta)^2}{\sigma}}$ for t < β	0.91	0.83	0.92	2.84	60.1	16.5	1.7
Chapman $(1 - e^{-t/\beta})^{\sigma}$	0.925	1.17	0.47	6.81	0.28	4.16	3.6
Gompertz $e^{-e^{-(t-\beta)/\sigma}}$	1.04	0.96	0.89	4.02	6.22	2.21	1.9

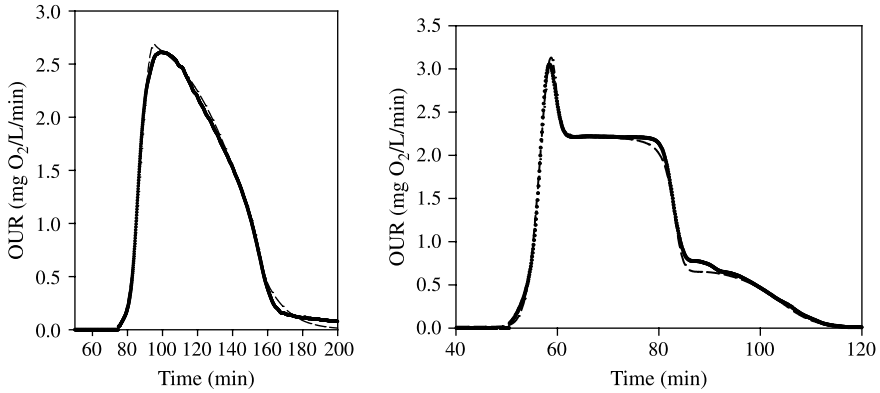

Figure 5 Examples of experimental OUR profiles with model fittings considering the acceleration phenomenon as described in equation 1 for a pulse of 36 mg N-NH_4^+/L (Panel A, Left) and a pulse of 17.6 mg N-NH_4^+/L (Panel B, Right)

where S_{NH4}: ammonium concentration, μ_{MAX}: maximum growth rate, K_{NH4}: ammonium affinity constant, S_O: oxygen concentration, K_O: oxygen affinity constant, X_A: biomass concentration, β: time of substrate addition and σ: width of the pulse.

The Gaussian-like expression predicts a symmetric profile, which also slows down after the value of β. Clearly, this expression is not totally perfect since slow substrate consumption is only needed at the start of the pulse. Hence, equation 1 should be only applied until time equals β (first half of the Gaussian profile). Figure 5 shows examples of this approach for the modelling of the experimental OUR profile of the experiments shown in Figure 2 and Figure 4-right.

The Gaussian-like expression successfully describes the acceleration phenomenon observed in ammonium batch respirograms. This expression, however, has no metabolic basis and it is descriptive and not explanatory of the observed phenomenon. For a proper modelling, a deeper insight in the metabolic basis of the acceleration phenomenon is required. Then, some of the hypotheses proposed in this work might be rejected and the real cause of the acceleration phenomenon, probably the AMO limitation by reducing equivalents, could be thoroughly and mechanistically modelled.

Conclusions

The acceleration phenomenon is a transient phase observed just after the pulse addition in respirometric batch experiments with ammonium as substrate. This phase can be very significant and non-reliable parameter estimates will be obtained if it is neglected when fitting the OUR profile. There are some processes that could contribute individually to this acceleration phenomenon. Among them, the most probable cause is the reducing equivalents limitation of AMO. The nitritation process acts as an autocatalytic reaction, accelerating as reducing equivalents are generated in the second nitritation step. Once the reducing equivalents are no longer limiting AMO activity, maximal specific removal rates might be attained**.** A detailed metabolic description of the acceleration phenomenon may not be necessary for a successful description of the process. Several expressions were examined and the best fit was obtained with a Gaussian-like expression. This simple mathematical approach was sufficient for a plausible description (not explanation) of the acceleration phenomenon as shown in this work. However, despite this successful description, the metabolic causes of the acceleration phenomenon deserve further study.

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