

Kinetic Characterization of *Brocadia* spp.- Dominated Anammox Cultures.

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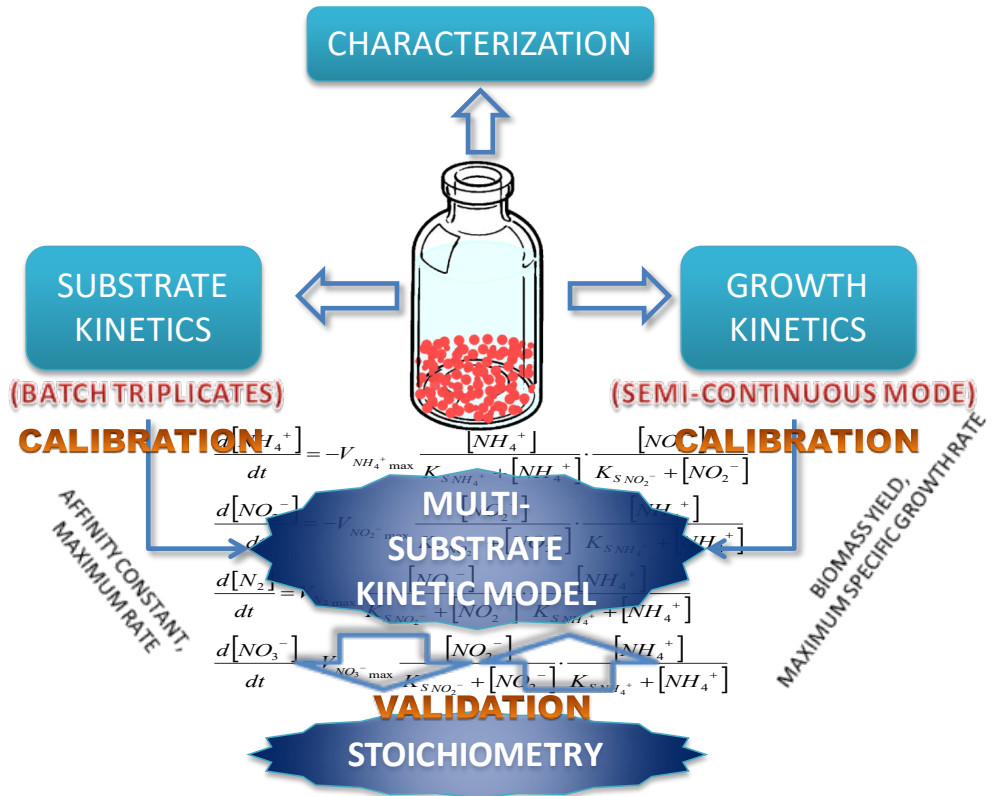
Abstract

The microbial anaerobic ammonium oxidation (anammox) process has interesting engineering applications for an efficient and economical removal of nutrient nitrogen from wastewater bearing high ammonium and relatively low organic matter composition. Currently, there is a need to have reliable estimates of kinetic parameters that can improve the understanding and the modeling of the anammox process for optimizing onsite applications. In this work, a multi-substrate kinetic model explaining the activity of the anammox process based on substrates consumption is proposed using well characterized granular (GS) and flocculent (FS) biomass sources mostly composed by *Candidatus Brocadia fungida* and *Candidatus Brocadia caroliniensis*, respectively. Kinetic parameters were very similar irrespective of the biomass source (GS: K_S of 0.64 (NH_4^+) and 0.35 (NO_2^-) mM, and specific V_{max} of 0.69 (NH_4^+), 0.88 (NO_2^-), 0.64 (N_2) and 0.23 (NO_3^-) mmol g^{-1} VSS h^{-1} . FS: K_S of 0.53 (NH_4^+) and 0.37 (NO_2^-) mM, and V_{Smax} of 0.67 (NH_4^+), 0.86 (NO_2^-), 0.64 (N_2) and 0.25 (NO_3^-) mmol g^{-1} VSS h^{-1}). The model respected the experimentally calculated stoichiometry of N-compounds, which serves as an independent validation. Also, growth kinetics of the FS was studied, calculating an Y_{XS} of 0.111 mol C-biomass mol^{-1} N_2 produced and a μ_{max} of 0.0041 h^{-1} , which corresponds to a doubling time of 6.9 d. The model data from FS was used for determining the equation of the anammox reaction. Slight differences with the previously established stoichiometries were found, overall with respect to the yield of NO_3^- and the biomass (reaching 0.353 mol NO_3^- and 0.105 mol C mol^{-1} NH_4^+ consumed), which were related with the r-metabolic strategy of FS. As a concluding remark, the kinetic understanding of the anammox biomass described in this work can be used for optimizing ulterior applications.

Key words: Anaerobic ammonium oxidation, *Brocadia* sp., kinetics, modeling, growth.

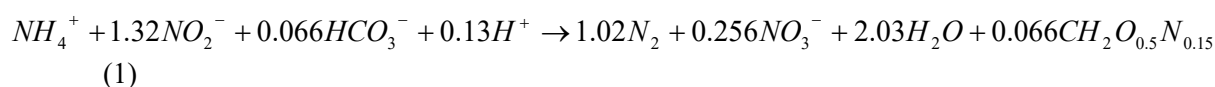
GRAPHICAL ABSTRACT

ANAMMOX BATCH TESTS



1. Introduction

Recent understanding of the microbiology of the nitrogen cycle have opened new possibilities for treating high-nitrogen bearing wastewater (Schmidt et al., 2003). Among them, the anaerobic ammonium oxidation (anammox) is the most promising to become a wide-spread technological option for high-ammonia and low-organic matter waste streams (Kuenen, 2008; Kartal et al., 2010). The anammox process is catalyzed by chemolithoautotrophic bacteria of the phylum planctomycetes. Several genera have been identified being able to perform this process: *Brocadia* sp., *Kuenenia* sp., *Scalindua* sp., *Anammoxoglobus* sp. and *Jetenia* sp. (Kuenen, 2008). This mesophilic process involves the anoxic oxidation of ammonia with nitrite as the main electron acceptor in a thermodynamically favorable fashion ($\Delta G = -357 \text{ kJ mol}^{-1} \text{ NH}_4^+$) (Jetten et al., 2001). The experimentally calculated stoichiometry from chemostat experiments has been established as follows (Strous et al., 1998):



Aside from functioning as electron acceptor, nitrite also functions as an electron donor for CO_2 fixation to support biomass growth (Jetten et al., 1998; Kuenen, 2008). Consequently there is some formation of NO_3^- and a molar relationship of NO_2^- to NH_4^+ consumption greater than 1.

Modeling biological processes is a critical step for the proper control of full-scale plants. A multi-variable control system is often used (such as supervisory control and data acquisition -SCADA-type), in which all the biological and abiotic processes are governed by differential-algebraic equations with dynamic state variables (Olsson et al., 2005; Ferrero et al., 2012; Olsson, 2012). In anaerobic conditions, biological processes are commonly rate-limiting (Gavala et al., 2003), so there is a great need to elucidate the kinetics of the process in order to optimize the subsequent step of modeling and control (Pavlostathis, 2011). The International Water Association (IWA) has developed

different simulation models for activated sludge (Activated Sludge Models, ASM). In these models, all the biological processes are derived from the Monod kinetics (Henze et al., 2000). These models include the nitrogen removal steps, so they have been recently extended to cover the anammox process (Dapena-Mora et al., 2004; Volcke et al., 2006).

There is a need to have reliable estimates of kinetic parameters that can improve the understanding and the modeling of the anammox process, which enable an increased predictability and better design of the biological process, facilitating its scale up. Currently there is no fully developed and consensual kinetic anammox model. Early approaches consisted of simple models used for estimating maximum growth rate and affinity constants. Substrate anammox kinetics has been often explained by Monod (Strous et al., 1999; van der Star et al., 2008; Ni et al., 2010; Chen et al., 2011b; Oshiki et al., 2011; Ni et al., 2012), Haldane-type (Chen et al., 2011c) or even pseudo-first order models (Strous et al., 1998; Scaglione et al., 2009). An enormous variability in the values of the saturation constants (0.003-13.7 mM) and maximum specific activities (0.09-3.74 mmol N g⁻¹ VSS h⁻¹), linking with the lack of generalization in anammox models, makes the modeling a major concern for scaling-up. Moreover, multi-substrate biological processes are complex, requiring a more comprehensive approach to enable more accurate estimates of the kinetic parameters. A previous study reflected the necessity of improving the estimation of parameters for anammox kinetics to optimize the simulation of substrate consumption and biomass growth (Dapena-Mora et al., 2004).

The objective of this study is to present a multi-substrate modeling approach towards calculating the kinetic parameters of the anammox process using data from *Brocadia* sp. dominated anammox enrichment cultures cultivated under batch conditions favoring either the measurement of activity or growth. The model is calibrated with experimental data and validated by comparing the experimentally calculated with the model-predicted stoichiometries.

2. Materials and methods

2.1 Anammox biomass

Biomass used in this work was collected from two sources: a lab-scale expanded granular sludge bed (EGSB) reactor operated in continuous mode for 250 d (granular sludge, GS), and a lab-scale membrane bioreactor (MBR) with a culture enriched in anammox biomass for 360 d (flocculent sludge, FS). The GS was retrieved from the reactor while it was treating a synthetic wastewater composed by a stoichiometric relationship between NH_4^+ and NO_2^- (1:1.32). The N-loading rate of the reactor was $3.36 \text{ g N L}^{-1} \text{ d}^{-1}$. The sludge, consisted of red and medium size ($2.4 \pm 0.6 \text{ mm}$) granular sludge, had a specific anammox activity (SAA) of $0.424 \pm 0.014 \text{ mmol N}_2 \text{ g}^{-1} \text{ VSS h}^{-1}$, a volatile to total solids (VS/TS) ratio of 0.89 ± 0.11 and a VS concentration of $0.043 \pm 0.009 \text{ g g}^{-1}$ wet sludge. The FS was retrieved from the reactor treating the same synthetic wastewater than the EGSB reactor, but the N-loading rate was $0.35 \text{ g N L}^{-1} \text{ d}^{-1}$. The flocculent sludge was visually well dispersed, and the sludge volumetric index (SVI) was around 0.22 mL g^{-1} , indicating a very good settling characteristics. The SAA was $0.464 \pm 0.008 \text{ mmol N}_2 \text{ g}^{-1} \text{ VSS h}^{-1}$, the total to volatile suspended solids (TSS/VSS) relationship was 0.83 ± 0.01 and the biomass concentration was $765 \pm 0.03 \text{ g VSS L}^{-1}$.

Anammox bacteria in the flocculent and granular sludge were microbiologically characterized by generating a clone library as described in the Supplementary Data. The two different inocula were characterized by different anammox species. One unique anammox phylotype was found in both flocculent and granular sludge, showing very high similarity with the 16s rRNA gene of the genus *Brocadia* (> 99.5%). The anammox strain in the flocculent sludge was most closely related to *Candidatus Brocadia caroliniensis* (Magr  et al., 2012), whereas the granular sludge was composed mainly by *Candidatus Brocadia fulgida* (Kartal et al., 2008) (See Supplementary Data, Figure S1).

2.2 Basal medium

The basal mineral medium was prepared using ultrapure water (Milli-Q system, Millipore) and contained the following compounds (mg L^{-1}): $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (57.5); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (100); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200); and 1.0 mL L^{-1} of two trace element solutions. Trace element solution 1 contains

(in mg L⁻¹) FeSO₄ (5000) and ethylenediamine-tetraacetic acid (EDTA) (5000). Trace element solution 2 contains (in mg L⁻¹) EDTA (1500); ZnSO₄·7H₂O (430); CoCl₂·6H₂O (240); MnCl₂ (629); CuSO₄·5H₂O (250); Na₂MoO₄·2H₂O (220); NiCl₂·6H₂O (190); Na₂SeO₄·10H₂O (210); H₃BO₃ (14); NaWO₄·2H₂O (50).

Nitrite and ammonium were added to the basal medium using NH₄HCO₃ and NaNO₂ at different concentrations. NaHCO₃ was added at 4 g L⁻¹ to provide alkalinity and carbon source.

2.3 Substrate kinetics experiments

Batch experiments were performed in 160 mL flask serum bottles hermetically closed and inoculated with 1.875 g VSS L⁻¹ of the GS or 50 mL of the FS. The total volume was set at 100 mL. NO₂⁻ and NH₄⁺ were supplied at two molar relationships (1.2 and 1.54 mol NO₂⁻ mol⁻¹ NH₄⁺), corresponding to values 10% higher than the 1.32 stoichiometric relationship between NH₄⁺ and NO₂⁻ (Eq. (1)). Total N-concentration was 6.5 mM. Anaerobic conditions were achieved by flushing the media and headspace with He/CO₂ 80/20 %, which also serves to adjust the pH to around 7.2. Temperature was fixed at 30 ± 0.1 °C. A mixing speed of 200 rpm was used in order to minimize the external diffusion contribution. Liquid sampling (1.5 mL) was performed periodically for measuring the concentration of NO₂⁻, NO₃⁻, NH₄⁺ and the pH. The experiments were assayed in triplicate. Another experiment were also conducted and used for refilling the triplicates in each liquid sampling in order to maintain the liquid and headspace volumes unchanged, as well as the biomass concentration (refill bottle).

2.4 Growth kinetics experiments

The growth kinetics were studied inoculating reaction bottles with 2 mL of the FS in the same conditions described in the substrate kinetics experiments but adding successive substrate spikes at 2.71 and 3.57 mM of NH₄⁺ and NO₂⁻, respectively (semi-continuous mode operation). Each spike was performed once the N₂ production had ended and all the NO₂⁻ and NH₄⁺ were consumed. Before each spike, the liquid and headspace were flushed with He/CO₂ 80/20 for adjusting the pH. Liquid samples (1.5 mL) were retrieved at the beginning and the end for measuring the concentration of NO₂⁻, NO₃⁻ and NH₄⁺ and closing N balances. These and one more liquid sample (1 mL) were taken out at the

middle time of each spike for monitoring the time evolution of the biomass concentration. The experiments were assayed in triplicate and a refill bottle was also used.

2.5 Abiotic, control and activity tests

Abiotic controls were similar to biotic experiments but in absence of biomass. Controls were performed using the same biomass concentration as in the anammox treatments adding only NH_4^+ , NO_2^- or NO_3^- as the sole N source, to monitor other nitrogen-consuming biological processes such as endogenous denitrification. Activity tests were carried out in the same conditions described in the substrate kinetics experiments but using 2.71 and 3.57 mM of NH_4^+ and NO_2^- , respectively. The SAA was calculated from the data of N_2 production, using 4 data points in the linear range to calculate the slope by linear regression.

2.6 Analytical methods

NO_3^- and NO_2^- were analyzed by suppressed conductivity ion chromatography (IC) using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS₁₈ analytical column (4×250 mm) and an AG₁₈ guard column (4×50 mm). During each run, the eluent (10 mM KOH) was used for 20 min. NH_4^+ was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH_4^+ electrode (Mettler Toledo, Columbus, OH, UAS). N_2 was analyzed using a Hewlett Packard 5890 Series II gas chromatograph (GC) (Agilent Technologies, Palo Alto, CA) fitted with a Carboxen 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector (TCD). The temperatures of the column, the injector port and the detector were 220, 110 and 100 °C, respectively. Helium was used as the carrier gas and the injection volume was 100 μL .

Biomass concentration in the growth kinetics experiments was measured by the Pierce BCA standard protein content method (Thermo Scientific Inc, Rockford, IL, USA). Calibration curves between volatile suspended solids (VSS) and protein concentration were performed, obtaining values of g VSS g^{-1} protein. Calibrations were performed on each spike, and calibration values were 0.42 ± 0.05 g protein g^{-1} VSS ($R^2 = 0.992 \pm 0.004$). Values of g VSS were transformed into mol-C L^{-1} by using the

generic equation for anammox biomass, $\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$ (Strous et al., 1998). The parameters pH, TS, VS, TSS, VSS and SVI were measured according to Standard Methods (APHA, 2005).

2.7 Modeling

The data collected from the kinetic batch experiments described above were used to model the anammox substrate and growth kinetics, and thereby determining the kinetic parameters. A multi-substrate model based on double Monod kinetics and using substrates concentration (NH_4^+ and NO_2^-) as calculation basis was used for modeling substrate kinetics. Simplification for the double Monod equations were done by assuming that anammox growth is negligible compared with substrate consumption at these operating conditions. So, biomass concentration can be considered as constant, and then the specific maximum activity (μ_{max}), the biomass concentration (X) and the biomass yield (Y_{XS}) can be lumped in a new parameter known as maximum rate (V_{max}). Then, the process can be described according to the following system of ordinary differential equations:

$$\frac{d[\text{NH}_4^+]}{dt} = -V_{\text{NH}_4^+ \max} \frac{[\text{NH}_4^+]}{K_{S \text{NH}_4^+} + [\text{NH}_4^+]} \cdot \frac{[\text{NO}_2^-]}{K_{S \text{NO}_2^-} + [\text{NO}_2^-]} \quad (2)$$

$$\frac{d[\text{NO}_2^-]}{dt} = -V_{\text{NO}_2^- \max} \frac{[\text{NO}_2^-]}{K_{S \text{NO}_2^-} + [\text{NO}_2^-]} \cdot \frac{[\text{NH}_4^+]}{K_{S \text{NH}_4^+} + [\text{NH}_4^+]} \quad (3)$$

$$\frac{d[\text{N}_2]}{dt} = V_{\text{N}_2 \max} \frac{[\text{NO}_2^-]}{K_{S \text{NO}_2^-} + [\text{NO}_2^-]} \cdot \frac{[\text{NH}_4^+]}{K_{S \text{NH}_4^+} + [\text{NH}_4^+]} \quad (4)$$

$$\frac{d[\text{NO}_3^-]}{dt} = V_{\text{NO}_3^- \max} \frac{[\text{NO}_2^-]}{K_{S \text{NO}_2^-} + [\text{NO}_2^-]} \cdot \frac{[\text{NH}_4^+]}{K_{S \text{NH}_4^+} + [\text{NH}_4^+]} \quad (5)$$

Where V_{max} represents the maximum rate of each component (mM h^{-1}) and K_S is the saturation or Monod constant (mM). In order to compare our results with literature, we expressed V_{max} in specific terms (maximum specific rate, V_{Smax} , $\text{mmol g}^{-1} \text{VSS h}^{-1}$).

Also, an empirical simple pseudo-Monod model, based on N_2 production in order to simplify the parameters estimation, was used to calculate the anammox growth kinetics by using the following system of ordinary equations:

$$\frac{dN_2}{dt} = \frac{\mu_{\max}}{Y_{X/S}} \cdot X \cdot \frac{N_{2\max} - N_2}{K_s + N_{2\max} - N_2} \quad (6)$$

$$\frac{dX}{dt} = Y_{X/S} \cdot \frac{dN_2}{dt} \quad (7)$$

$$\frac{dN_2}{dt} = \frac{\mu_{\max}}{Y_{X/S} \cdot K_s^2} \cdot X \cdot N_2 \cdot (N_{2\max} - N_2) \quad (8)$$

Where μ_{\max} represents the maximum growth rate (h^{-1}), $Y_{X/S}$ is the biomass yield (mmol C-biomass $mmol^{-1} N_2$), X represents the biomass concentration (mM C-biomass), K_s is the apparent saturation constant for N_2 production and $N_{2\max}$ is the maximum N_2 production for an infinite reaction time (mM N_2). Eq (8) is an empirical equation based on the Logistical Model (Kovács et al., 2004), a modification of pseudo-second order model, used for trying to reproduce a *Lag-phase* in the first spike that commonly appears in semi-continuous mode experiments.

The modeling was conducted with the following assumptions:

- There are negligible contributions of inhibition or activation processes derived from NO_2^- or NH_4^+ at the concentrations assayed.
- No other abiotic or biological processes occurred during the experimental time, so the fate of each substrate and product is only explained by the anammox reaction.

Integration of the systems of differential equations described in Eqs. (2-5) and (6-8) was accomplished by using the Episode numerical method for Stiff systems. In order to optimize the parameters estimation, the three sets of data from the triplicates at the two concentrations tested in the substrate kinetics experiments were used for the integration, resulting in 24 differential equations.

Initial conditions for the substrate kinetics were $t = 0$; $[NH_4^+]$, $[NO_2^-]$ = initial concentrations; $[NO_3^-] = 0$ (granular sludge) or = initial concentration (flocculent sludge); $[N_2] = 0$. Also, the three sets of

data from triplicates for each spike (7 spikes) in the growth experiments were used for the integration, resulting in 42 differential equations. Initial conditions were $t = 0$; $N_2 = 0$, $X =$ initial biomass concentration for each spike. Experimental data were fitted to the proposed models by means of a non-linear least squares minimization of the error using a simplex algorithm followed by a Powell minimization algorithm. Goodness of fitting was quantified by the coefficient of correlation. The software Micromath© Scientist 3.0 (Micromath, Saint Louis, MO, USA) was used for this purpose.

3. Results and discussion

3.1 Substrate kinetics

Results and modeling

Figure 1 shows the time evolution of the most relevant species involved in the anammox process using GS, NH_4^+ , NO_2^- , NO_3^- and N_2 . A similar profile was obtained with the experiments using FS, as depicted in Figure 2. These results demonstrate that the anammox process was the exclusive process during the experimental time. As can be seen, both NH_4^+ and NO_2^- were consumed during the experiment and N_2 and NO_3^- were concomitantly produced. Control experiments using only NO_2^- and NO_3^- revealed that no other biological processes (such as endogenous denitrification) were taking place during the batch experiments. Also, abiotic tests confirmed there was no removal of substrates without inoculum (data not shown). Consequently, data from Figures 1 and 2 were selected for modeling the anammox process.

Experimental data was fitted to the model described in Eqs. (2-5). Table 1 shows the estimated parameters values from the fitting with standard deviations and the coefficient of correlation for the entire fittings. The fitting curves for GS and FS are included in Figures 1 and 2, respectively. A very good fit was achieved, as can be deduced by (a): a high correlation obtained between experimental and predicted data and (b): low standard errors calculated for the parameters values, providing a strong statistical support for the good fit of the model. A more in depth statistical analysis of the fittings can be found in Table 1S (GS) and Table 2S (FS) in the Supplementary Data.

Early kinetic approaches for the anammox process showed low statistical support for the parameters estimation. In some cases, the lack of residual analysis avoided to have an overview of the good of fitting (Strous et al., 1998; Strous et al., 1999; Chen et al., 2011a). In other cases, high standard errors in the parameters estimation were showed, conferring low credibility to their values (van der Star et al., 2008; Oshiki et al., 2011). A statistical analysis of any kinetic model from a critical point of view is absolutely necessary for corroborating the assumptions made in the model proposal.

Another strong support for the model proposed is found in its reproducibility. As can be seen, up to 6 sets of data were used for calculating the parameters values and the good of fitting were always higher than 95% in all cases (see Supplementary Data). A high number of sets of data can reduce the relative error in the determination of repetitive parameters. In addition, the parameter values using the two different sludges were very close with each other, which is coherent since the dominant anammox species of both sludges belong to the same genus (*Brocadia* spp.). These evidences support the reproducibility of the model and, consequently, its suitability for using in field applications. Once the model is calibrated, it is necessary to give physical meaning to the parameters values.

Figures 1 & 2

Table 1

Kinetic parameters

The specific maximum rates for GS and FS were similar, resulting in values between 0.64-0.88 mmol g⁻¹ VSS h⁻¹ for N₂, NH₄⁺ and NO₂⁻, and around 0.24 mmol g⁻¹ VSS h⁻¹ for NO₃⁻. There is a wide range of V_{Smax} values reported in the literature, from less than 0.3 (Scaglione et al., 2009; Chen et al., 2010) up to higher than 1.5 mmol g⁻¹ VSS h⁻¹ (Strous et al., 1998; Strous et al., 1999; Chen et al., 2011b; Ni et al., 2012). Maximum anammox rates depends on degree to which biomass is enriched with anammox bacteria, and the type of this bacteria. Most active biomass seems to be associated with continuous reactors working at very high N-loading rates, where the enrichment in anammox strains is elevated (Chen et al., 2011b; Ni et al., 2012). Although our cultures were highly enriched in anammox strains, the reactors where they were retrieved dealt with N-loading rates lower than those reported previously, presumably affecting the specific maximum rates.

With respect to the saturation constants, our results suggest that saturation for nitrite is reached at lower concentrations than for ammonium, resulting in K_s values slightly higher for ammonium (see Table 1), which are in accordance with other works (Chen et al., 2011a; Chen et al., 2011b; Ni et al., 2009). However, the values of saturation constants were lower than previously estimated using continuous reactors (Ni et al., 2010; Chen et al., 2011a; Ni et al., 2012), but higher than those obtained

by batch experiments (Strous et al., 1999; van der Star et al., 2008; Oshiki et al., 2011). An explanation for this divergence is developed in the following section.

As commented before, multiple substrates processes are hard to model. Using simple Monod models are prone to make erroneous estimation of the kinetic parameters. The values predicted by simple Monod would most likely be very much dependent on whether second substrate is limiting or not-limiting. The saturation constants for NO_2^- and NH_4^+ also depend on the concentration of each other. Then, saturation constant values are potentially different using simple Monod equations rather than multiple-substrate equations. This could be the case with the estimation of the saturation constants by some authors (Strous et al., 1999; Chen et al., 2011b; Ni et al., 2012).

Other authors simplified the modeling by using zero order approximations due to the use of very high substrate concentration. In these cases, as the high substrate concentration leads to saturated scenarios, the NH_4^+ and NO_2^- consumption rates, as well as the N_2 and NO_3^- production rates are arbitrarily considered as constants (Trimmer et al., 2005; Magrı et al., 2012).

The empirical approximations can also cause common mistakes in the parameters estimation. A particular case can be found in the work of Chen et al. (Chen et al., 2011a). They considered the process following a Monod model, but they included saturation parameters not only for the substrates (NH_4^+ , NO_2^-), but also for the products (N_2). Curiously, they could properly estimate the saturation constant for ammonium, whereas the estimation of that for nitrite was accompanied by high standard error, so the resulting modeling was poor. Using different calculation basis in modeling (in this case, both substrates and products concentrations for parameters estimation) leads unavoidably to empirical approximations.

Also, modeling multi-substrate processes should have a very good numerical support in order to avoid the effect of the degrees of freedom on the fitting quality, which can be achieved by collecting a high number of experimental data. However, this is unable in most of the cases because the change of working volumes and pressures. This problem can be solved by using data from lots of replicates, as

in our case, in which up to 6 different sets of data for each sludge were used to estimate the kinetic parameters.

Some authors, however, claimed that diffusion of substrate through the biofilms and the bacterial membranes can affect the estimation of the saturation constants, thus resulting in an over-estimation of their values (Strous et al., 1998; Strous et al., 1999; Oshiki et al., 2011). We suggest that the contribution of diffusivity problems in the parameter estimation of our experiments can be considered as negligible. It is supporting by two considerations. First, we have applied a very high mixing rate (200 rpm), which can neglect the external diffusion (Zaiat et al., 1996; Chou et al., 2008). Also, it is known that the aggregation state leads to different internal diffusion rates (Gonzalez-Gil et al., 2001), but very close values for saturation constants using biomass in different state of aggregation (GS and FS) were obtained.

Balance of Nitrogen as a validation of the model

The stoichiometric parameters of the anammox process were determined by using the experimental data from the substrate kinetics experiments. A mass balance of N (Table 2) half-way through and at the end of the experimentation was performed. The N balance predicted by the model described in Eqs. (2-5) is also shown. These data can be used as an external validation of the model. As can be seen, the measured and model predicted N balances were within 95% agreement of each other for N species. Also, in almost all the cases the model respected the stoichiometry during all the running time, with the only exception of the middle time using GS, where small deviations in the comparison between modeled and real stoichiometries of N-compounds between 11-18% were observed, which is presumably due to low experimental errors. The validation is also strongly supported with the high good of fitting, giving the model a high grade of robustness.

Table 2

3.2 Growth kinetics

Results and modeling

Anammox growth kinetics using FS was studied. Figure 3 shows the time course of biomass and N₂ production of a typical experiment. The first spike in the three replicates has a different kinetic behavior than all the additional spikes in the experiment, which is attributed to a *lag-phase* of the anammox process. As can be extracted from Figure 3, the slope of N₂ production increased with time in parallel with biomass production. This increment was explained only by the biomass growth, so data from Figure 3 were used for modeling the anammox growth.

The experimental data were fitted to the model described in Eqs. (6-8) using the same procedure described for the substrates kinetics. Table 3 shows the growth kinetic parameter values and the goodness of fitting. The simulated curves are plotted in Figure 3 alongside the measured data. As in the case of the substrate kinetics, the parameters determination had a very good statistical support. This affirmation is strengthened by a really high value of the correlation coefficient (0.991) and very low standard deviations of the model parameters. Also, a more in depth evaluation of the statistical analysis concerning the goodness of fit can be found in Table 3S (Supplementary Data). From this approach, a doubling time of 6.9 ± 1.4 d was estimated.

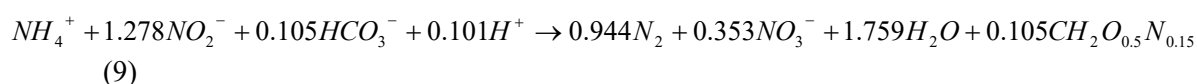
In contrast to the substrate kinetics, the parameters of the anammox growth kinetics were in the same magnitude order to those reported in the literature. The relatively high growth rate determined in this experiment is compared with some other cultures, in which the derived doubling times are reported to be at around 10 d (Strous et al., 1998; van der Star et al., 2007). However, doubling times of similar or slightly lower values were also reported (van der Star et al., 2007; van der Star et al., 2008; Chen et al., 2010; Oshiki et al., 2011). The biomass yield was slightly higher than most values reported in the literature (0.111 versus around 0.06 mmol C-biomass mmol⁻¹ N₂) (Strous et al., 1998; Strous et al., 1999; Oshiki et al., 2011). However, some other works reported lower (0.034 mmol C-biomass mmol⁻¹ N₂, (Ni et al., 2009)) or much higher (0.279 mmol C-biomass mmol⁻¹ N₂, (Chen et al., 2010)) values for Y_{XS} .

Figure 3

Table 3

Stoichiometry and equation of the anammox process using FS

The kinetic parameters from the substrate and growing models using FS (*Candidatus Brocadia caroliniensis*) were used for determining the global anammox equation. For this purpose, mass balances of N, C, H and O were carried out. N balance was accounted for 99.1% using the specific maximum rates from substrate kinetics (Table 2). The C balance was accounted for 100% by using the growth parameters in Table 3. The H balance was accounted for by 88.6% by simulating the H⁺ consumption in the substrate kinetics experiments. Simulation of the pH evolution was performed considering all the components of the medium that can impact the pH, such as EDTA, NH₄HCO₃, CO₂, NaHCO₃ and Na₂HPO₄. Simulation was accomplished using Microsoft Excel© (Microsoft, Redmont, WA, USA) and a group of macros called CurTiPot 3.6.1, developed by I. G. R. Gutz (Gutz, 2010). The O balance was closed by difference (100%). The mass balances resulted in the following chemical equation:



This equation is slightly different to that presented by Strous et al. (1998) (Eq. (1)), which is the most cited equation for the anammox process. The main differences are the parameters affecting biomass production (0.105 versus 0.066, this study compared to Strous et al (1998), respectively) and the nitrate production (0.353 versus 0.256). Interestingly, the variation of these parameters is almost proportional (around 1.5 times higher in both cases).

There are some evidences indicating that the anammox stoichiometry can be strongly related with the physiological state of the biomass, which can be affected by the N-load (Ahn, 2006; Dosta et al., 2008; Yang et al., 2009), the temperature (Dosta et al., 2008) or the pH (Carvajal-Arroyo et al., 2013). These aspects lead to a modification of the stoichiometric parameters, overall in the growth parameters that can directly affect to nitrate and biomass production. This variation reflects the different growth rate of the FS compared with the work by Strous et al. (1998) (doubling times of 6.9 d versus 10 d), and also affects the nitrate stoichiometry since this compound is produced by partial oxidation of nitrite only when biomass is growing (Jetten et al., 1998).

3.3 Implications: strategy of growth.

In this section, the metabolic strategy of the GS and FS used in this study is discussed. Taking into account all the experimental considerations and the kinetic parameters values, it can be deduced that both type of biomass are clear types of r-strategists. This kind of microorganisms usually shows relatively low substrate affinity and high growth rates, in contrast to K-strategists, which behave the contrary (Fontaine et al., 2003; Jeschke et al., 2008). As have been previously commented, both GS and FS have relatively high saturation constants and specific substrate-utilization rates. FS have been showed to have also a relatively high growth rate, resulting in doubling times lower than 7 d. Also, the r-strategy has been previously related with the type of anammox genus. As hypothesized by Oshiki et al. (2011), the genus *Brocadia* sp. seems to be an r-strategist in contrast to *Kuenenia* sp., which usually chooses to optimize the substrate affinity. This is accordance with our experiments, in which the two enrichment cultures have been described as dominant in *Brocadia* sp. Another fact than can promote the development of r-strategists is the absence of competitor for the substrate (Fontaine et al., 2003; Kim and Kim, 2006). The contribution of some other biological processes to N balances have been described here as negligible, so it can be deduced that anammox biomass from GS and FS does not need to compete with other N-utilizers bacteria, as aerobic autotrophic nitrifiers or anaerobic denitrifiers. In addition, the environmental conditions (temperature, pH and non-inhibitory substrate concentrations) seems to be ideal for growing, which gives the anammox bacteria the chance of using more energy in growing rather than in multiplying their enzymatic content, which indeed would diminish the saturation constants values. Finally, the N-loading rate in which the GS and FS have been enriched is comparatively higher than other environments in which anammox K-strategists have been developed (Strous et al., 1999; Ni et al., 2009). In practice, this kind of biomass will be able to compete and develop in those environments with high ammonium and nitrite concentrations and in optimum conditions, as high-rate anaerobic reactors, and it is possible that the natural environments would be not very feasible for them. Understanding the kinetic behavior of anammox cultures seems to be absolutely necessary for optimizing the use of this anaerobic technology for treating wastewaters or for onsite applications.

4. Conclusions

The substrate and growth kinetics of the anammox process has been studied using two different anammox enriched biomass from bioreactors, one granular and the other flocculent, dominated by anammox bacteria closely related to *Candidatus Brocadia fulgida* and *Candidatus Brocadia caroliniensis*, respectively. Both species shows slight differences in specific activities. Substrate kinetics for both sludge are described by a physical multi-substrate kinetic model based on substrate consumption. A high similarity in the substrate kinetic parameters (specific maximum rates and affinity constants) is presented, suggesting the model has universal applicability for different bioreactor systems. Growth kinetics is described by an empirical global model based on Monod kinetics using N₂ production as the basis for calculation and *C. B. caroliniensis* as inoculum. The statistical analysis confirms both models have a high goodness of fit to the experimental data. Also, the experimentally measured N balances and stoichiometry were very close to those predicted by the multi-substrate model, providing an independent validation of the model. Using the kinetics parameters of *C. B. caroliniensis*, the equation of the anammox reaction has been studied by closing the N, C, and H, while O was close by difference. A slight variation in the stoichiometric parameters with respect to the previously reported stoichiometry is found, and related with the different growth and substrate consumption strategy (metabolism) of *C. B. caroliniensis*, which uses more energy in growing, decreasing the catabolism in favor of the anabolism, a typical r-strategy.

5. Acknowledgements

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6. References

Ahn, Y.-H., 2006. Sustainable nitrogen elimination biotechnologies: A review. *Process Biochemistry* 41, 1709-1721.

- APHA, 2005. Standard methods for the examination of water and wastewater. Eaton, A. D., Clesceri, L. S., Rice, E. W., Greenberg, A. E., Eds., 21st Ed. Washington D.C. American Public Health Association.
- Carvajal-Arroyo, J.M., Sun, W., Sierra-Alvarez, R., Field, J.A., 2013. Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents. *Chemosphere*.
- Chen, J., Zheng, P., Yu, Y., Tang, C., Mahmood, Q., 2010. Promoting sludge quantity and activity results in high loading rates in Anammox UBF. *Bioresource technology* 101, 2700-2705.
- Chen, T., Zheng, P., Shen, L., Ding, S., Mahmood, Q., 2011a. Kinetic characteristics and microbial community of Anammox-EGSB reactor. *Journal of Hazardous Materials* 190, 28-35.
- Chen, T., Zheng, P., Tang, C., Wang, S., Ding, S., 2011b. Performance of ANAMMOX-EGSB reactor. *Desalination* 278, 281-287.
- Chen, T.T., Zheng, P., Shen, L.D., Ding, S., Mahmood, Q., 2011c. Kinetic characteristics and microbial community of Anammox-EGSB reactor. *Journal of Hazardous Materials* 190, 28-35.
- Chou, H.-H., Huang, J.-S., Jheng, J.-H., Ohara, R., 2008. Influencing effect of intra-granule mass transfer in expanded granular sludge-bed reactors treating an inhibitory substrate. *Bioresource technology* 99, 3403-3410.
- Dapena-Mora, A., Van Hulle, S.W.H., Luis Campos, J., Méndez, R., Vanrolleghem, P.A., Jetten, M., 2004. Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results. *Journal of Chemical Technology & Biotechnology* 79, 1421-1428.
- Dosta, J., Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Álvarez, J., Méndez, R., 2008. Short- and long-term effects of temperature on the Anammox process. *Journal of Hazardous Materials* 154, 688-693.
- Ferrero, G., Rodríguez-Roda, I., Comas, J., 2012. Automatic control systems for submerged membrane bioreactors: A state-of-the-art review. *Water research* 46, 3421-3433.
- Fontaine, S., Mariotti, A., Abbadie, L., 2003. The priming effect of organic matter: a question of microbial competition? *Soil Biology and Biochemistry* 35, 837-843.
- Gavala, H., Angelidaki, I., Ahring, B., 2003. Kinetics and Modeling of Anaerobic Digestion Process. Biomethanation I. in: Ahring, B., Angelidaki, I., de Macario, E., Gavala, H., Hofman-Bang, J., Macario, A., Elferink, S., Raskin, L., Stams, A., Westermann, P., Zheng, D. (Eds.). Springer Berlin / Heidelberg, pp. 57-93.
- Gonzalez-Gil, G., Seghezzo, L., Lettinga, G., Kleerebezem, R., 2001. Kinetics and mass-transfer phenomena in anaerobic granular sludge. *Biotechnol Bioeng* 73, 125-134.
- Gutz, I.G.R., 2010. Curtipot program, Version 3.5.4, pH and Acid-Base titration curves: Analysis and Simulation. [Online]. Available: http://www2.iq.usp.br/docente/gutz/Curtipot_.html.
- Henze, M., Gujer, W., Mino, T., Van Loosdrecht, M.C.M., 2000. Activated Sludge Models ASM1, ASM2, ASM2D and ASM3. Scientific and Technical Report No. 9. IWA Publishing, London.
- Jeschke, J.M., Gabriel, W., Kokko, H., 2008. r-Strategist/K-Strategists. in: Editors-in-Chief: Sven Erik, J., Brian, F. (Eds.). *Encyclopedia of Ecology*. Academic Press, Oxford, pp. 3113-3122.
- Jetten, M.S.M., Strous, M., van de Pas-Schoonen, K.T., Schalk, J., van Dongen, U., van de Graaf, A.A., Logemann, S., Muyzer, G., van Loosdrecht, M.C.M., Kuenen, J.G., 1998. The anaerobic oxidation of ammonium. *Fems Microbiology Reviews* 22, 421-437.
- Jetten, M.S.M., Wagner, M., Fuerst, J., van Loosdrecht, M., Kuenen, G., Strous, M., 2001. Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Current Opinion in Biotechnology* 12, 283-288.
- Kartal, B., Kuenen, J.G., van Loosdrecht, M.C.M., 2010. Sewage Treatment with Anammox. *Science* 328, 702-703.
- Kartal, B., Van Niftrik, L., Rattray, J., Van De Vossenberg, J.L.C.M., Schmid, M.C., Sinnighe Damsté, J., Jetten, M.S.M., Strous, M., 2008. Candidatus 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *Fems Microbiology Ecology* 63, 46-55.
- Kim, D.-J., Kim, S.-H., 2006. Effect of nitrite concentration on the distribution and competition of nitrite-oxidizing bacteria in nitrification reactor systems and their kinetic characteristics. *Water research* 40, 887-894.

- Kovács, K.A., Gróf, P., Burai, L., Riedel, M., 2004. Revising the Mechanism of the Permanganate/Oxalate Reaction. *The Journal of Physical Chemistry A* 108, 11026-11031.
- Kuenen, J.G., 2008. Anammox bacteria: from discovery to application. *Nature Reviews Microbiology* 6, 320-326.
- Magrí, A., Vanotti, M.B., Szögi, A.A., 2012. Anammox sludge immobilized in polyvinyl alcohol (PVA) cryogel carriers. *Bioresource technology* 114, 231-240.
- Ni, B.J., Chen, Y.P., Liu, S.Y., Fang, F., Xie, W.M., Yu, H.Q., 2009. Modeling a Granule-Based Anaerobic Ammonium Oxidizing (ANAMMOX) Process. *Biotechnology and bioengineering* 103, 490-499.
- Ni, S.-Q., Lee, P.-H., Sung, S., 2010. The kinetics of nitrogen removal and biogas production in an anammox non-woven membrane reactor. *Bioresource technology* 101, 5767-5773.
- Ni, S.-Q., Sung, S., Yue, Q.-Y., Gao, B.-Y., 2012. Substrate removal evaluation of granular anammox process in a pilot-scale upflow anaerobic sludge blanket reactor. *Ecological Engineering* 38, 30-36.
- Olsson, G., 2012. ICA and me – A subjective review. *Water research* 46, 1585-1624.
- Olsson, G., Nielsen, M., Yuan, Z., Lynggaard-Jensen, A., 2005. *Instrumentation, Control and Automation in Wastewater Systems*. International Water Association.
- Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H., Okabe, S., 2011. Physiological characteristics of the anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sinica'. *Microbiology-Sgm* 157, 1706-1713.
- Pavlostathis, S.G., 2011. 6.31 - Kinetics and Modeling of Anaerobic Treatment and Biotransformation Processes. in: Editor-in-Chief: Murray, M.-Y. (Ed.). *Comprehensive Biotechnology (Second Edition)*. Academic Press, Burlington, pp. 385-397.
- Scaglione, D., Caffaz, S., Bettazzi, E., Lubello, C., 2009. Experimental determination of Anammox decay coefficient. *Journal of Chemical Technology and Biotechnology* 84, 1250-1254.
- Schmidt, I., Slikers, O., Schmid, M., Bock, E., Fuerst, J., Kuenen, J.G., Jetten, M.S.M., Strous, M., 2003. New concepts of microbial treatment processes for the nitrogen removal in wastewater. *Fems Microbiology Reviews* 27, 481-492.
- Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M., 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Applied Microbiology and Biotechnology* 50, 589-596.
- Strous, M., Kuenen, J.G., Jetten, M.S.M., 1999. Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology* 65, 3248-3250.
- Trimmer, M., Nicholls, J.C., Morley, N., Davies, C.A., Aldridge, J., 2005. Biphase Behavior of Anammox Regulated by Nitrite and Nitrate in an Estuarine Sediment. *Applied and Environmental Microbiology* 71, 1923-1930.
- van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picoreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water research* 41, 4149-4163.
- van der Star, W.R.L., Miclea, A.I., van Dongen, U., Muyzer, G., Picoreanu, C., van Loosdrecht, M.C.M., 2008. The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. *Biotechnology and Bioengineering* 101, 286-294.
- Volcke, E.I.P., van Loosdrecht, M.C.M., Vanrolleghem, P.A., 2006. Continuity-based model interfacing for plant-wide simulation: A general approach. *Water research* 40, 2817-2828.
- Yang, Z., Zhou, S., Sun, Y., 2009. Start-up of simultaneous removal of ammonium and sulfate from an anaerobic ammonium oxidation (anammox) process in an anaerobic up-flow bioreactor. *Journal of Hazardous Materials* 169, 113-118.
- Zaiat, M., Cabral, A.K.A., Foresti, E., 1996. Cell wash-out and external mass transfer resistance in horizontal-flow anaerobic immobilized sludge reactor. *Water research* 30, 2435-2439.

Table 1. Values of kinetic parameters, standard deviation at 95% and fitting goodness (expressed as correlation coefficient) of experimental data from Figures 2 and 3 to model described in Eqs. (2-5).

Parameter	Granular sludge	Flocculent sludge*
$V_{NH_4^+max}$ (mmol g ⁻¹ VSS h ⁻¹)	0.692 ± 0.001	0.668 ± 0.008
V_{NO_2-max} (mmol g ⁻¹ VSS h ⁻¹)	0.879 ± 0.001	0.857 ± 0.010
V_{N_2max} (mmol g ⁻¹ VSS h ⁻¹)	0.642 ± 0.000	0.641 ± 0.008
V_{NO_3-max} (mmol g ⁻¹ VSS h ⁻¹)	0.232 ± 0.000	0.247 ± 0.003
$K_{SNH_4^+}$ (mM)	0.64 ± 0.13	0.53 ± 0.05
K_{SNO_2-} (mM)	0.35 ± 0.09	0.37 ± 0.04
R^2	0.996	1.00

*The standard deviation of V_{max} values of FS is conditioned by a higher error in VSS measurements.

Table 2. Values of kinetic parameters, standard deviation at 95% and fitting goodness (expressed as correlation coefficient) of experimental data from Figure 4 to model described in Eqs. (6-8).

Parameter	Value
μ_{max} (h ⁻¹)	0.0041 ± 0.0008
$Y_{X/S}$ (mmol C-biomass mmol ⁻¹ N ₂ produced)	0.111 ± 0.013
K_S (mM N ₂)	1.098 ± 0.017
R^2	0.991
<i>Doubling time</i> (d)	6.919 ± 1.354

Table 3. Measured and predicted stoichiometry of the N compounds of the anammox process using granular and flocculent sludge.

	NH_4^+	N_2	NO_2^-	NO_3^-	N closing balance (% final-initial)
<i>Granular (final)</i>	1	0.96 ± 0.03	1.31 ± 0.06	0.32 ± 0.01	102.5
<i>Flocculent (final)</i>	1	0.97 ± 0.01	1.28 ± 0.00	0.35 ± 0.01	99.6
<i>Granular (middle)*</i>	1	0.82 ± 0.05	1.08 ± 0.12	0.32 ± 0.05	106.3
<i>Flocculent (middle)*</i>	1	0.92 ± 0.06	1.25 ± 0.08	0.38 ± 0.05	101.5
<i>Calculated granular (proposed)</i>	1	0.93	1.27	0.34	103.6
<i>Calculated flocculent (proposed)</i>	1	0.96	1.28	0.37	99.7
<i>Precision (granular, final) (%)</i>	100	96.7	98.0	104.7	
<i>Precision (flocculent, final) (%)</i>	100	99.9	99.9	104.2	
<i>Precision (granular, middle) (%)</i>	100	113.2	117.8	106.0	
<i>Precision (flocculent, middle) (%)</i>	100	104.3	102.6	98.1	

*Middle time granular= 1.55 h; middle time flocculent= 4.48 h.

Figure captions

Figure 1. Time evolution of NH_4^+ (●), NO_2^- (▲), NO_3^- (▼) and N_2 (■) using granular sludge in 10% stoichiometric excess of initial NO_2^- (a) and NH_4^+ (b) concentrations. Continuous lines are model fittings to Eqs. (2-5).

Figure 2. Time evolution of NH_4^+ (●), NO_2^- (▲), NO_3^- (▼) and N_2 (■) using flocculent sludge in 10% stoichiometric excess of initial NO_2^- (a) and NH_4^+ (b) concentrations. Continuous lines are model fittings to Eqs. (2-5).

Figure 3. Time evolution of nitrogen production (■) and biomass concentration (○) in the growth kinetics experiments. (a), (b) and (c) are replicates. Continuous lines are model fittings to Eqs. (6-8).

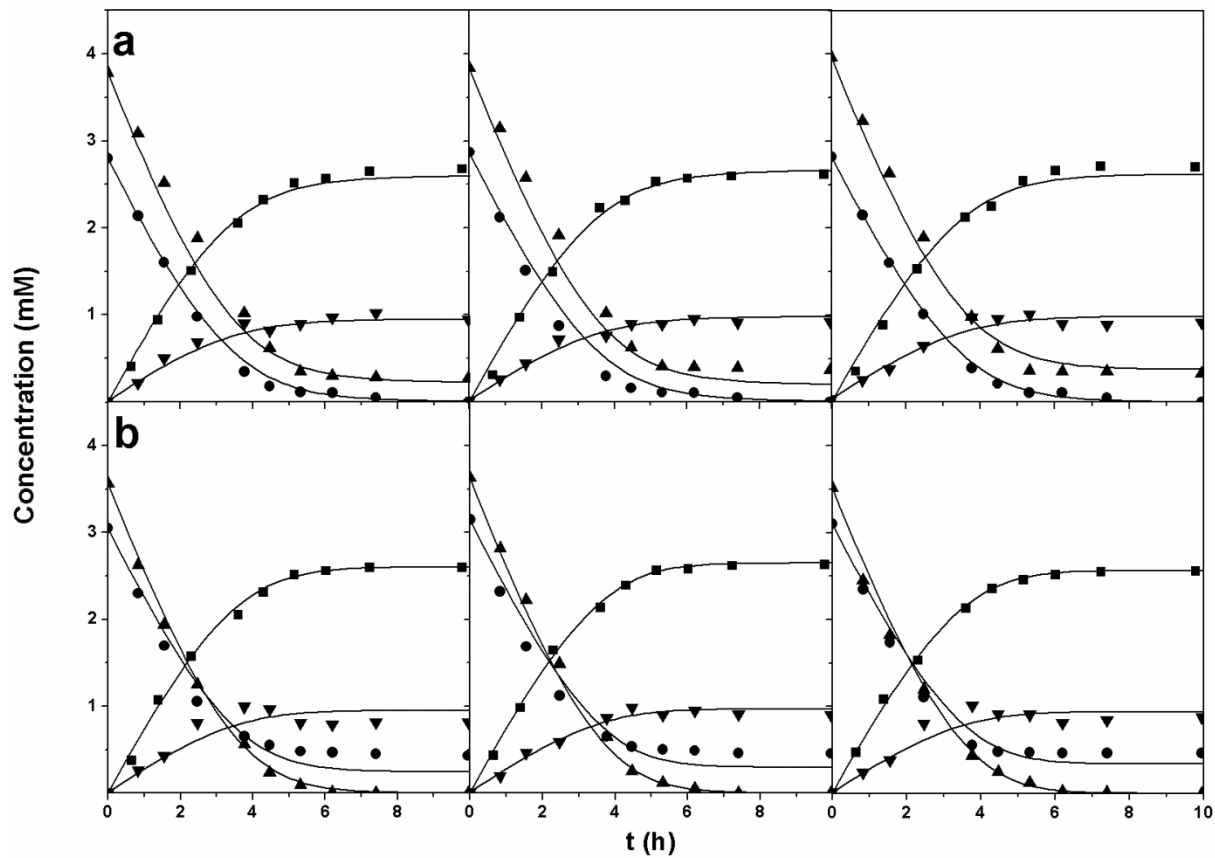


Figure 1

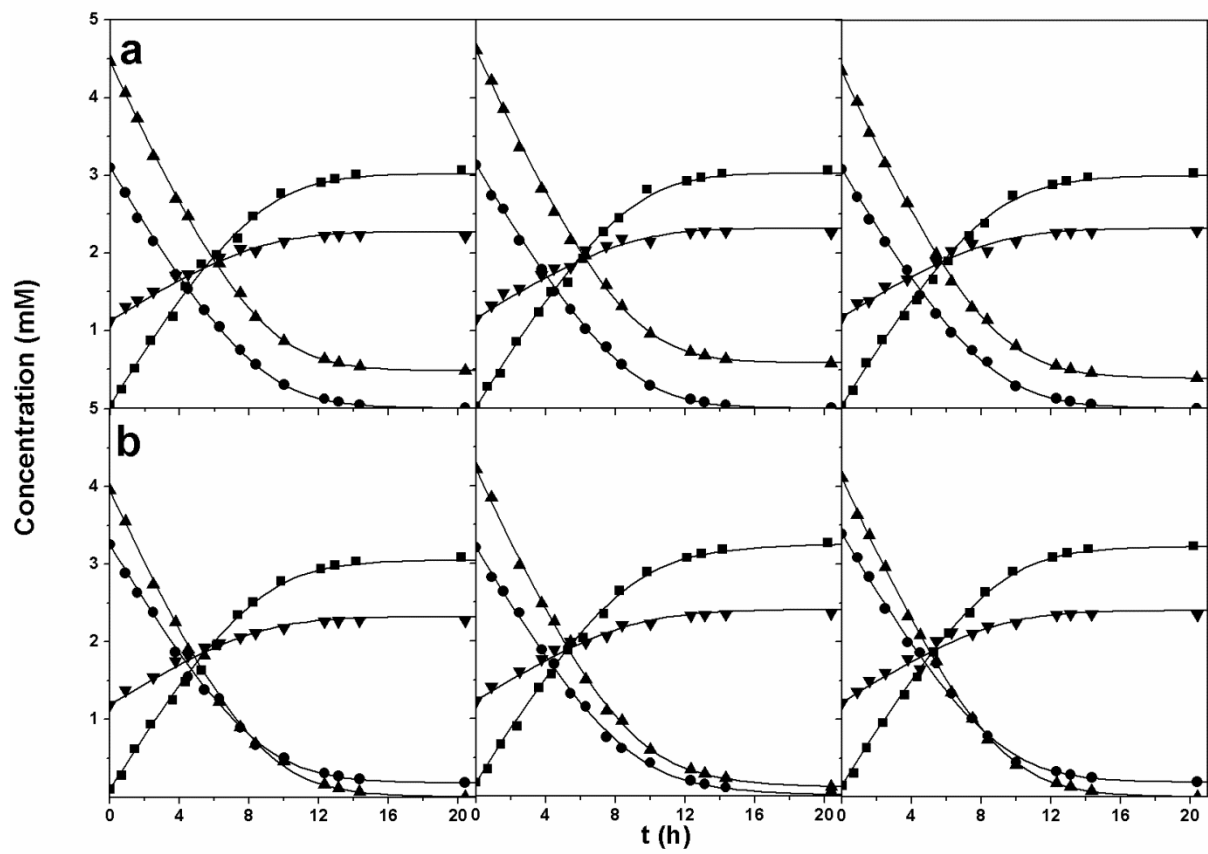
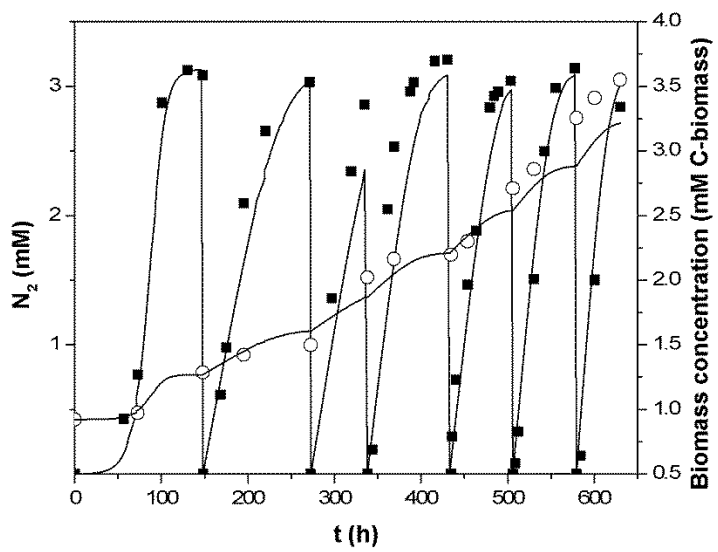
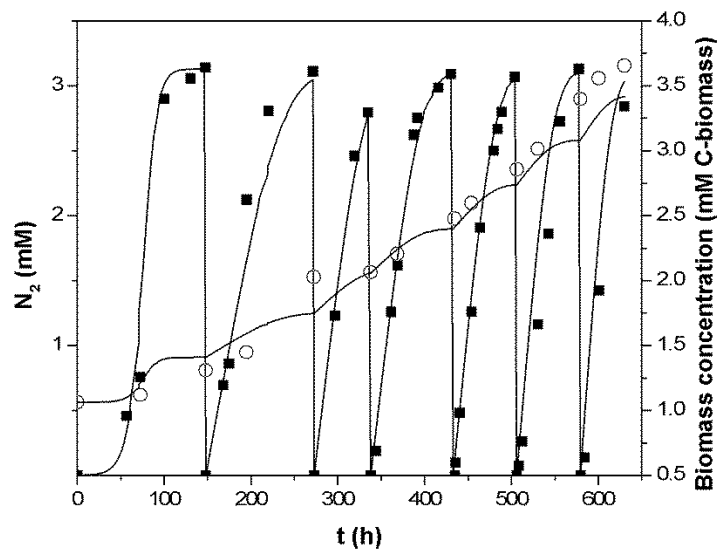
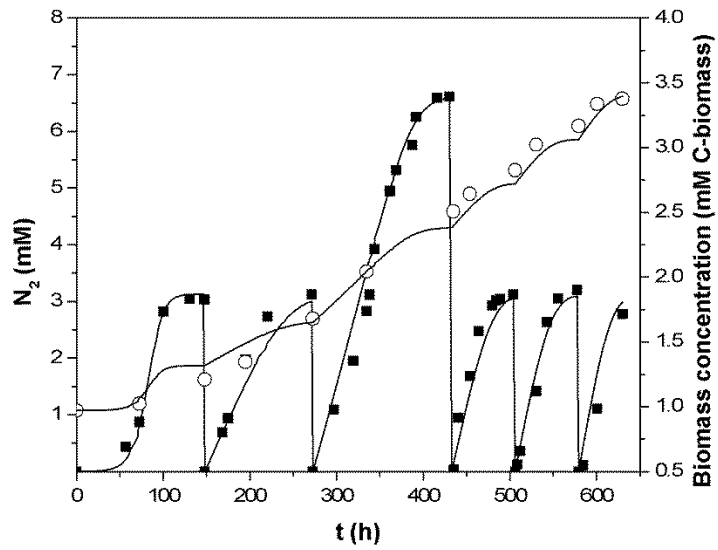


Figure 2



* In the third spike of the first replicate, double quantity of substrate was added by mistake.

Figure 3