Kinetics and thermodynamics of anaerobic ammonium oxidation process using *Brocadia* spp. dominated mixed cultures

D. Puyol, J. M. Carvajal-Arroyo, B. Garcia, R. Sierra-Alvarez and J. A. Field

ABSTRACT

Anaerobic ammonium oxidation (anammox) is a recently discovered microbial process commonly applied to treat ammonium pollution in effluents with low organic carbon content. Modeling anammox processes is important for simulating and controlling full-scale plants. In this study, the anammox process was simulated using three models, and substrate and growth parameters obtained by different research groups. Two *Brocadia* spp.-dominated mixed cultures, one granular and the other flocculent, were used for this purpose. A very good correlation between experimental data using both sludges and model predictions was achieved by one of the models, obtaining correlation coefficients higher than 0.997. Other models and stoichiometric equations tested were unable to predict the anammox kinetics and stoichiometry. Furthermore, the thermodynamic behavior of the two mixed cultures was compared through the determination of the energy of activation of the anammox conversion at temperatures ranging from 9 to 40 °C. Optimum temperature for anammox activity was established at 30–35 °C in both cases. The energy of activation values calculated for granular sludge and flocculent sludge were 64 and 124 kJ mol^{-1}, respectively.

Key words | anammox, *Brocadia* spp., kinetics, modeling, simulation, thermodynamics

D. Puyol (corresponding author)
J. M. Carvajal-Arroyo
B. Garcia
R. Sierra-Alvarez
J. A. Field
Department of Chemical and Environmental Engineering,
The University of Arizona,
Tucson, AZ 85721,
USA
E-mail: *danielpuyol@email.arizona.edu*; *daniel.puyol@uam.es*

INTRODUCTION

Anammox (anaerobic ammonium oxidation) is a recently discovered microbial process involved in the nitrogen biogeocycle (Schmidt *et al.* 2003). Anammox has interesting engineering applications and allows efficient and economic removal of nutrient nitrogen from wastewaters with high NH_4^+ concentration and relatively low chemical oxygen demand (COD)/ammonium ratio (e.g. landfill leachate and sewage sludge centrate) (Kuenen 2008). The anammox process involves the anaerobic oxidation of ammonium (NH_4^+) using nitrite (NO_2^-) as a terminal electron acceptor. This reaction results in the formation of dinitrogen gas. Some nitrate (NO_3^-) is also produced from partial oxidation of nitrite for carbon fixation from inorganic carbon dioxide. The stoichiometry has been calculated as (Strous *et al.* 1998)

$$NH_4^+ + 1.32 \cdot NO_2^- + 0.066 \cdot HCO_3^- + 0.13 \cdot H^+ \rightarrow 1.02 \cdot N_2 + 0.26 \cdot NO_3^- + 2.03 \cdot H_2O + 0.066 \cdot CH_2O_{0.5}N_{0.15} \quad (1)$$

Anammox bacteria are slow-growing microorganisms with a complex cell biology comprising an intracellular organelle surrounded by ladderane lipids (anammoxosome) which have to deal with an inert substrate (ammonium) under highly competitive and anoxic conditions (Kartal *et al.* 2012). Anammox activity has been observed at temperatures ranging from 10 to 45 °C, with an optimum at 30–35 °C. The energy of activation (E_a) of the anammox reaction has been reported to be around 60 kJ mol^{-1} (Strous *et al.* 1999; Dosta *et al.* 2008).

To improve the understanding and the modeling of the anammox process there is a need to have reliable estimates of kinetic parameters, which enable an increased predictability and better design of the biological process, facilitating its scale-up (Pavlostathis 2011). Modeling of the anammox process has received increasing attention in the last decade. Early approaches mainly consisted of simple Monod models that were used to estimate the maximum specific

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growth rate (μ_{max}) and the half-saturation constants for the substrates of the anammox reaction (Ks_{NH4+} and Ks_{NO2-}) (Strous *et al.* 1998; van der Star *et al.* 2008; Ni *et al.* 2010). However, multi-substrate biological processes are complex, so simulation of the anammox reaction has been previously described by double Monod equations, which are adaptations of the general IWA activated sludge models (Dapena-Mora *et al.* 2004; Volcke *et al.* 2006; Cema *et al.* 2012). Recently, an improved model considering all the nitrogen species in the parameters' estimation has been proposed (Puyol *et al.* 2013). This model was calibrated and validated with very strong statistical and stoichiometric support.

In this work, a multi-substrate solution approach to an anammox kinetic model was utilized to simulate the anammox process. The approach was tested on substrate consumption data obtained with two mixed cultures dominated by *Brocadia* spp. A thermodynamic study was also conducted to investigate the effect of temperature from 9 to 40 °C on the anammox activity of these cultures and calculate the E_a of the anammox reaction according to the Arrhenius equation.

MATERIALS AND METHODS

The anammox granular sludge (GS) and the flocculent sludge (FS) were collected from an expanded granular sludge bed reactor (EGSB) and a membrane bioreactor (MBR), respectively. The EGSB reactor was operated in continuous mode for 250 d 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 g N L^{-1} d^{-1}, and the sludge retention time (SRT) was around 145 d. The original inoculum was granular biomass from a full-scale anammox plant treating sludge centrate in The Netherlands, which was provided by Paques BV. The MBR was operated for 360 d treating the same synthetic wastewater as the EGSB reactor, but the N-loading rate was 0.35 g N L^{-1} d^{-1} and the SRT was 78 d. The original inoculum was an anammox enrichment culture derived from return activated sludge (Sun *et al.* 2011) originally obtained from Ina Road treatment plant in Tucson, AZ (USA). Both reactors were located in a temperature-controlled room set at 30 ± 2 °C and the influents were always prepared and maintained in anaerobic conditions.

The average measured particulate size was 2.4 ± 0.6 and 0.22 ± 0.18 mm for GS and FS, respectively. The microbial diversity in both GS and FS was characterized by generating a clone library (Sun *et al.* 2011). Two unique anammox species were detected, the GS with *Candidatus B. fulgida* and the FS with *Candidatus B. caroliniensis*.

Activity kinetics were studied in triplicate batch assays using 1.62 g volatile solids (VS) L^{-1} and 0.36 g volatile suspended solids (VSS) L^{-1} of the GS and FS, respectively. NO_2^- and NH_4^+ were spiked at 3.93 and 2.71 mM, respectively. Experimental values used in the thermodynamic study were obtained in triplicate batch assays inoculated with GS (0.36 g VS L^{-1}) and FS (0.77 g VSS L^{-1}), and then spiked with NO_2^- (3.57 mM) and NH_4^+ (2.71 mM). Batch assays were performed as previously described (Sun *et al.* 2011).

VS and VSS content were determined according to *Standard Methods* (APHA 2005). Biomass particle size was measured by means of the software ImageJ using five representative pictures of GS and FS (Puyol *et al.* 2013). The N_2 content in gas samples was measured by gas chromatography with thermal conductivity detection, NO_2^- and NO_3^- using ionic chromatography with suppressed conductivity detection, and NH_4^+ using an ammonium selective electrode (Sun *et al.* 2011).

Double Monod kinetics was used to describe the dependency of the growth rate of the anammox bacteria on NH_4^+ and NO_2^- concentrations as follows:

$$r = \mu_{max} \frac{[NH_4^+]}{Ks_{NH_4^+} + [NH_4^+]} \frac{[NO_2^-]}{Ks_{NO_2^-} + [NO_2^-]} X \quad (2)$$

where μ_{max} is the anammox specific growth rate (h^{-1}), Ks_{NH4+} and Ks_{NO2-} are the saturation constants for NH_4^+ and NO_2^- (mM) and X is the biomass concentration (mM C-biomass). Simulations were conducted using the model parameters and stoichiometry determined by Puyol *et al.* (2013), and the model parameters of Ni *et al.* (2009) and Oshiki *et al.* (2011) using the stoichiometry proposed by Strous *et al.* (1998). The stoichiometric and kinetic matrixes, as well as model parameters, are presented in Table 1. The experimental data were compared with the simulated data and the statistics were provided according to Puyol *et al.* (2012). The biomass stoichiometry used was $CH_2O_{0.5}N_{0.15}$ (Strous *et al.* 1998). Both simulation and statistical calculations were performed using MicroMath Scientist 3.0 (http://www.micromath.com/).

The E_a was calculated by estimating the maximum specific growth rate (μ_{max}) of the anammox bacteria by using the empirical growth parameters determined by Puyol *et al.* (2013). The natural logarithm of μ_{max} was compared with the inverse of the temperature (1,000/K), and the linear regression parameters were used for calculating

Table 1 | Stoichiometric and kinetic matrix of the models used for the simulation, parameter values and goodness of fitting of every simulation model to the experimental data. Kinetic equation for the simulation is described in Equation (2)

	Components					Parameters					
Process[a]	S_{NH4+}	S_{NO2-}	S_{NO3-}	S_{N2}	X	Ks_{NH4+}	Ks_{NO2-}	$Y_{X/S}$	μ_{max}	R^2	Reference
X-GS	$-1/Y_{X/S}$	−1.278/	0.353/	0.944/	1	0.64	0.35	0.105	0.0017	0.997	Puyol *et al.* (2013)[b]
X-FS	$-i_{NBM}$	$Y_{X/S}$	$Y_{X/S}$	$Y_{X/S}$		0.53	0.37		0.0034	0.999	
X-GS	$-1/Y_{X/S}$	$-1/Y_{X/S}$	1/	1.02/	1	0.05	0.04	0.06	0.0006	0.987	Ni *et al.* (2009)[c]
X-FS	$-i_{NBM}$	−1/0.258	0.258	$Y_{X/S}$					0.0013	0.992	
X-GS	$-1/Y_{X/S}$	−1.32/	0.256/	1.02/	1	0.03	0.09	0.034	0.0003	0.943	Oshiki *et al.* (2011)[c]
X-FS	$-i_{NBM}$	$Y_{X/S}$	$Y_{X/S}$	$Y_{X/S}$					0.0007	0.974	

i_{NBM} (N content of biomass) = 0.15 mmol N mmol^{-1} C. S and Ks are in mM.
$Y_{X/S}$ is in mmol C mmol^{-1} NH_4^+, μ_{max} is in h^{-1}.
[a]X-GS = growth of X using GS. X-FS = growth of X using FS.
[b]According to the stoichiometry by Puyol *et al.* (2013).
[c]According to the stoichiometry by Strous *et al.* (1998).

the E_a values for both GS and FS as follows:

$$\frac{d[N_2]}{dt} = \frac{\mu_{max}}{Y_{X/S}} X \frac{([N_{2\,max}] - [N_2])}{Ks_{N_2} + ([N_{2\,max}] - [N_2])} \quad (3)$$

$$\mu_{max} = A \cdot e^{-E_a/R \cdot T} \Rightarrow \ln(\mu_{max}) = \ln A - \frac{E_a}{R \cdot T} \quad (4)$$

where $Y_{X/S}$ is the biomass yield (mmol C-biomass mmol^{-1} NH_4^+ produced), Ks_{N2}, $[N_2]$ and N_{2max} are the pseudo-Monod constant, the nitrogen produced and the maximum nitrogen production for an infinite time of reaction, respectively (mM N_2), E_a is the energy of activation (kJ mmol^{-1} N_2 produced), A is the pre-exponential factor (h^{-1}), T is the temperature (K), and R is the universal gas constant (8.314×10^{-6} kJ mmol^{-1} N_2 K^{-1}).

RESULTS AND DISCUSSION

Kinetics of the anammox reaction

Figure 1 shows the time evolution of the nitrogen species involved in the anammox process using both GS and FS. Ammonium and nitrite were consumed in both cases, with concomitant production of nitrogen and nitrate. Specific anammox activities (SAA) of 0.414 ± 0.004 and 0.848 ± 0.021 mmol N_2 g^{-1} VSS h^{-1} were calculated for GS and FS, respectively. The higher SAA of the FS can be related to the growth conditions of both sludges, since a higher specific loading rate was maintained in the MBR than in the EGSB (1.33 versus 0.60 mmol N_2 g^{-1} VSS h^{-1}, respectively). The SAA values determined are comparable to those reported by other authors for sludge from high-rate

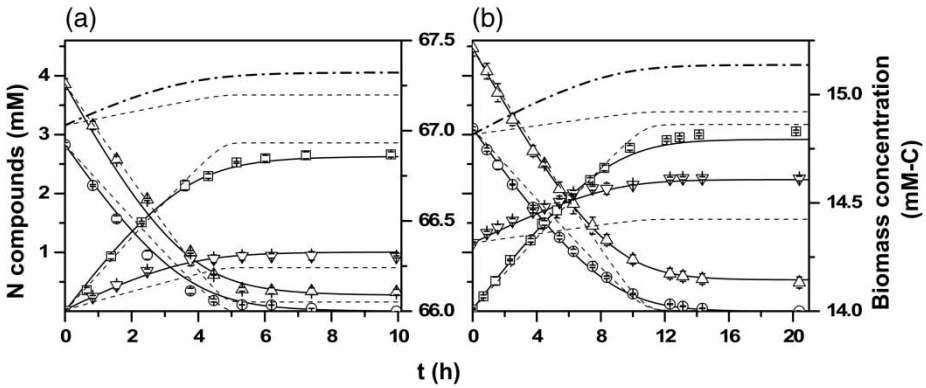

Figure 1 | Time course of N_2 (□), NH_4^+ (○), NO_2^- (△) and NO_3^- (▽) in the kinetic experiments using granular (a) and flocculent (b) sludge. Continuous lines and dash-dot lines are simulations of the N-compounds and biomass growth, respectively, obtained using the model described by Puyol *et al.* (2013). Dot lines are simulations obtained using the Oshiki *et al.* (2011) (a) and Ni *et al.* (2009) (b) models. Error bars represent standard deviations from triplicate experiments.

anammox reactors (from 0.42 to 0.94 mmol N_2 g^{-1} VSS h^{-1}) (Chen *et al.* 2011; Ni *et al.* 2012).

The molar ratio between the NO_2^- and NH_4^+ uptakes were very similar in the assays with GS and FS (1.25 ± 0.04 versus 1.28 ± 0.00), suggesting the same stoichiometry for the anammox reaction.

Simulation of the anammox reaction

Simulations of the anammox reaction and biomass growth were performed according to models described by Ni *et al.* (2009), Oshiki *et al.* (2011) and Puyol *et al.* (2013). Table 1 lists the substrate and growth parameters as well as the goodness of fitting through the correlation coefficient (R^2). Simulation curves for the consumption of nitrate and ammonium and for the production of N_2, nitrate and biomass by the GS and FS cultures are depicted in Figure 1. As can be seen, the model described by Puyol *et al.* (2013) accurately predicted the conversion of N species by both mixed cultures, GS and FS, and simulated the corresponding biomass growth. The two literature models were unable to describe properly the anammox reaction under the experimental conditions used in his study, as can be seen in Figure 1. Main differences are related to the stoichiometry of the nitrate production, since the Strous equation (Strous *et al.* 1998) predicts a nitrate stoichiometry significantly lower than that predicted by Puyol *et al.* (2013) (0.256 versus 0.353 mol NO_3^- mol^{-1} NH_4^+). Statistical analysis was used to further evaluate the accuracy of every model.

A high correlation between the simulation data from the Puyol *et al.* (2013) model and the experimental values was achieved with both biomass sources ($R^2 = 0.997$ and 0.999 for GS and FS simulation, respectively), whereas simulations of data using the models of Ni *et al.* (2009) and Oshiki *et al.* (2011) were somewhat less reliable, which can be deduced by lower values of the correlation coefficients (Table 1).

The main difference between the three simulation models was the values estimated for the substrate saturation constants (Table 1). Generally, model parameters used for calibrating the anammox reaction have been extracted from those calculated by Strous *et al.* (1999). In all the cases analyzed, calibration of the model parameters led to significantly increased values of the saturation constants of both nitrite and ammonium, compared with the initial parameters. The authors tried to explain those findings as kinetic limitations due to substrate diffusion through biogranules (Dapena-Mora *et al.* 2004; Volcke *et al.* 2006; Cema *et al.* 2012). However, in this work two anammox cultures with very different aggregation state (granular and flocculent) were used, and the saturation constants determined in both cases are very similar (Table 1). This finding suggests that kinetic limitation by diffusion of substrates seems to be negligible in comparison with the anammox reaction under the present working conditions, as has been previously claimed (Puyol *et al.* 2013).

In order to further validate the model developed by Puyol *et al.* (2013), the stoichiometry of the anammox process was evaluated by comparing the measured with the literature values (Table 2). A nitrogen mass balance at the end of the experimentation was performed. N balances predicted by the Strous stoichiometry are also shown. The Strous stoichiometry was not able to predict the behavior of the anammox biomass under the experimental conditions used in this work. Differences in the strategy of growth (K-strategy versus r-strategy) of the anammox cultures used in the different works could explain these divergences (Puyol *et al.* 2013). In fact, values of μ_{max} calibrated by using the Ni and Oshiki models, where the Strous stoichiometry was used, seem to be not reliable, since they are much lower than the experimentally calculated by Puyol *et al.* (2013) ($\mu_{max} = 0.0041\ h^{-1}$), whereas values of μ_{max} calibrated by using the Puyol *et al.* model were very close to those experimentally calculated, overall for the FS (see Table 1). The low values of K_s and $Y_{X/S}$ of Ni and Oshiki models had a relevant effect on the low μ_{max} determined.

Effect of temperature on the anammox reaction: thermodynamics

Analysis of the effect of temperature on anammox kinetics was performed in bioassays with GS and FS, thus mirroring the simulation experiments. The time course of N_2 production at temperatures ranging from 9 to 40 °C is depicted in Figure 2. The highest activities were achieved at 30 °C for both cultures, and a rapid activity decrease was observed at lower and higher temperatures. At the lowest temperature tested (9 °C), the GS was more active than the FS. This observation may be related to the different

Table 2 | Experimental and literature molar stoichiometries of the anammox reaction based on ammonium consumption

	Experimental data		Literature stoichiometry
Element	GS	FS	Strous *et al.* (1998)
NO_2^-	1.249 ± 0.038	1.283 ± 0.002	1.32
N_2	0.942 ± 0.027	0.985 ± 0.006	1.02
NO_3^-	0.319 ± 0.012	0.354 ± 0.007	0.256

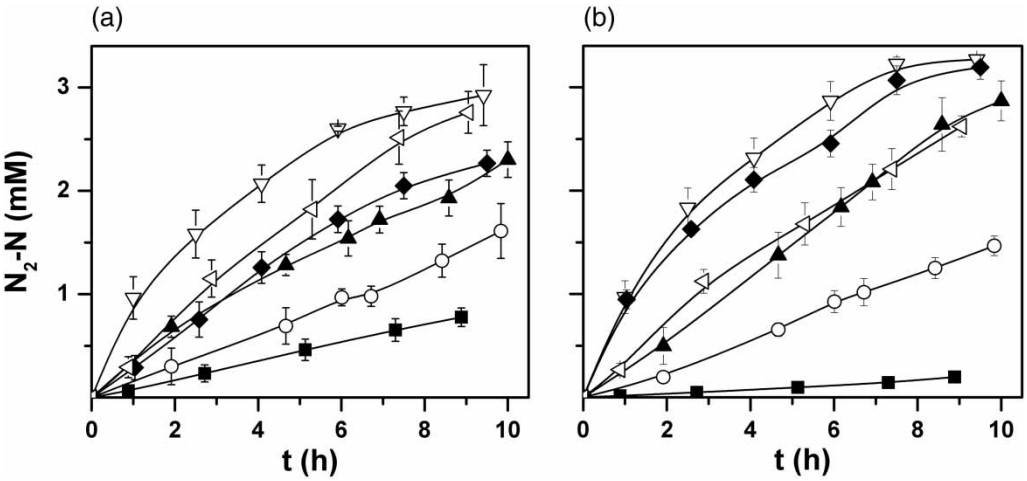

Figure 2 | Time course of the N_2 production in thermodynamics assays conducted at 9 (■), 18 (○), 25 (▲), 30 (▽), 35 (◆), and 40 °C (◁) using GS (a) and FS (b). Error bars are standard deviations from triplicates.

temperature sensitivity of the dominant anammox bacteria in the GS (*Candidatus B. fulgida*) and FS (*Candidatus B. caroliniensis*).

The experimental N_2 production data were fitted to Equation (3). Growth parameters were extracted from Puyol *et al.* (2013), the $Y_{X/S}$, [N_{2max}] and saturation constant values were fixed in the model calibration, and maximum specific growth rates (μ_{max}, h^{-1}) were calculated. Figure 3(a) shows the effect of the temperature in the μ_{max}. As can be seen, μ_{max} values increased exponentially up to a temperature around 30–35 °C. At higher temperatures, the μ_{max} decreased, which can be explained by deactivation caused by temperature. Other authors have reported similar values for the optimum temperature of the anammox reaction (Strous *et al.* 1999; Dosta *et al.* 2008).

Table 3 shows the optimum temperature and the temperature range of the main biological N-removal processes (BNPs). As can be seen, optimal temperature levels range from 20 to 30 °C in all cases, with the exception of the nitratation and anammox processes. The temperature of operation is key for the operation of BNPs and, in particular for hybrid processes in which more than one BNP occurs simultaneously (Van Hulle *et al.* 2010). In this sense, selection of a suitable working temperature to favor growth of the desired microbial population can be critical to achieve complete N removal in systems such as the CANON process, where nitritation and anammox processes succeed in the same reactor, and the efficiency of the anammox process is strongly dependent on the stability of the nitritation process.

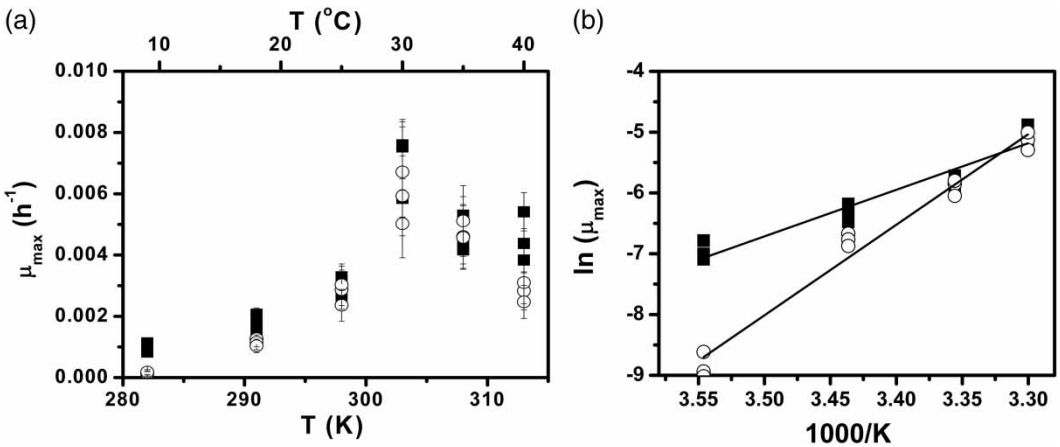

Figure 3 | Effect of temperature on the maximum SAA (a) and Arrhenius plots (b) for GS (■) and FS (○). Error bars represent standard deviations of the μ_{max} values resulted from fitting of every set of data to Equation (2).

Table 3 | Optimum and working ranges of temperatures of the main nitrogen-consuming biological processes

Process	Sub-process	*T* range[a] (°C)	Optimum *T* (°C)	Reference
Nitrification	Nitritation	4–50	22–30	Grunditz & Dalhammar (2001)
	Nitratation	4–52	25–35	
Denitrification	Autotrophic	< 15–50	20–25	Koenig & Liu (2001)
	Heterotrophic	(0)–10–35–(75)	20–25	Saleh-Lakha *et al.* (2009)
Anammox		10–45	30–35	This work; Strous *et al.* (1999); Dosta *et al.* (2008)

[a]Extreme (psychrophylic and thermophylic) values in brackets.

Values of μ_{max} in the exponential range (up to 30 °C) were used for estimating E_a values according to Equation (4). Plots of $\ln(\mu_{max})$ versus 1,000/K are presented in Figure 3(b). Estimated values for the E_a by linear regression were different for GS (0.064 ± 0.006 kJ mmol^{-1}, $R^2 = 0.91$) and FS (0.124 ± 0.006 kJ mmol^{-1}, $R^2 = 0.97$). The E_a value determined for GS, unlike that for FS, is comparable with those previously reported by other authors for anammox cultures (Strous *et al.* 1999; Dosta *et al.* 2008). The observed differences between GS and FS thermodynamics may be related to the presence of different dominant anammox species in the various cultures.

CONCLUSIONS

Activity and growth kinetics of *Brocadia* spp. anammox cultures were successfully predicted by using a multi-substrate anammox model. Statistical analysis confirmed that the model has a high goodness of fit to the experimental data. Also, the experimentally measured N balances were very close to those predicted by the model, providing an independent validation. The optimum temperature of the two anammox cultures tested was very similar (30–35 °C); however, thermodynamic studies revealed a marked difference in their E_a values, which may be related to the dominance of different *Brocadia* spp. in these anammox cultures.

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