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# Multispecies Granular Biofilm Modelling for Simultaneous Anammox and Denitrification Processes in Batch Systems

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**Abstract:** The nitrogen removal pathways in anaerobic ammonium oxidation (anammox) process where anaerobic ammonium-oxidizing bacteria and denitrifying heterotrophs convert ammonium and nitrite directly to dinitrogen gas under anoxic conditions were investigated using a mathematical model. The Activated Sludge Model No. 1 (ASM1) was modified with two step denitrification and one-step anaerobic ammonia oxidation. A one dimensional granular biofilm model was implemented in Aquasim 2.1v (EAWAG, Switzerland) in order to describe a series of batch processes operated in a bioreactor treating synthetic wastewater. A sophisticated statistical method was used for parameter estimation. The model was not sensitive with respect to the detachment velocity in the biofilm matrix as well as the porosity coefficients of dissolved state variables. The final results showed a satisfactory goodness of fitness representing Pearson correlation coefficients of 0.96, 0.98 and 0.81 for modeled and measured concentrations of NH4-N, NO2-N and NO3-N respectively. Although for the validation period some discrepancies between modeled and measured data points were observed, the model performance was satisfactory in general.

***Keywords**:* Environmental modelling and software; biofilm model; granular sludge; parameter estimation; activated sludge model

## 1 INTRODUCTION

Anammox related technologies are currently applied for nitrogen removal in wastewater treatment. With respect to the identification of growth and decay kinetics as well as the consideration of physiological characteristics different methodologies have been investigated so far using numerical modelling approaches and experimental setups (Lotti et al. 2012, Winkler et al. 2015). Nevertheless, many aspects of the anammox process such as the estimation of kinetic and stoichiometric coefficients and uncertainty analysis of the model's structure are still subject to debate. In such models mathematical complexity is often a drawback for the application and modelling of kinetic and stoichiometric characteristic of such detailed systems. Simplification of such mathematical models can be found in previous works (Pérez et al. 2005, Boltz et al. 2010). This work focus to develop a simplified mathematical biofilm model for anammox-denitrificiation process and to estimate and understand the impact of the various parameters dealing with growth, decay and hydrolysis kinetics as well as initial conditions on model prediction, behaviour and error quantification. The next aim is to enhance the description of spatial distribution of organisms present in spherical granular-based biofilms during simultaneous anammox and denitrification (SAD) process during anaerobic batch assays. Alongside a multi-parameter sensitivity analysis and parameter identification platform is comprised and discussed. The validity of the model efficiency is studied for different independent batch scenarios. To avoid mathematical complexity, the simulations are carried out in a simple as one-dimensional (1D) model. The key hypothesis was whether we can use the mathematical models to

describe the interactions and coexistence and kinetics of nitrogen converting bacteria in a granular sludge system.

## 2 METHODS

The Activated Sludge Model No. 1 (ASM1) (Henze, 2000) was modified and formulated with two step denitrification and one-step anammox processes (Ni et al. 2009). A granular one dimensional biofilm model was implemented in Software Aquasim 2.1v (EAWAG, Switzerland). A one-dimensional continuum model of biofilm growth incorporating fluid flow and shear stress based general description of detachment is included in the model. Model is able to predict the temporal and partial microbial distributions of anammox bacteria and heterotrophs in mixed-population biofilms. The biofilm matrix phase is an immobilized but dynamic microbial environment through pore water and bulk liquid phase.

### 2.1 Experiments and Analytical Methods

Granular sludge was collected from a full-scale landfill leachate treatment plant (LTP) and synthetic wastewater was used as feed for the batch experiments (Ke et al. 2015). The batch assays were performed in serum bottles with a total volume of 300 ml and a liquid volume of 275 ml. The sludge concentration was 3.7 g VSS /l. The assays were performed with a temperature of 37°C, dissolved oxygen concentration of below 0.2 mgO2 /l and a pH value of around 7.5. Fluorescence in situ hybridization (FISH) using the anammox-probe AMX368 was performed directly on the thin sections adherent to the slides.

For the visualization of all cells in the thin sections, DAPI (4',6-diamidino-2-phenylindole) was used after hybridization to estimate the granules diameter and initial volumetric fractions. Concentrations of ammonium and nitrite were measured according to the German standards DIN 38406/5 and DIN EN 26777-D10. Nitrate was measured using reagent “Nitrate-Test” from MERCK (Darmstadt, Germany). The experimental period used for parameter estimation was based on a batch test performed anaerobically with 63.5 mg/l NH4-N, 63.5 mg/l NO2-N and without nitrate in the synthetic wastewater feed. A second independent batch test was used for model validation, with an influent concentration of 62.5 mg/l NO3-N, 8 mg/l NH4-N and with 75 mg/l of glucose.

### 2.2 Model Geometry and Boundary Mass Transfer

Previously authors had shown images of granule for the biomass from the same origin. The results represented that the spatial distributions are in granular forms and mostly appear in spherical granular biofilms (Ke et al. 2015). Thus the model encompasses a geometrical simplified model to characterize the biofilm structure in which spherical granular particles were assumed. Biofilm matrix structure is assumed heterogeneous (layered with pore water separating solid biofilm matrix from the bulk liquid) and parameters concerning biofilm structure, mass transfer and the diffusion in the model are listed in Table S4 of the Supplementary Materials. Previously reported values in literature have been used to define and characterize the typical range of values. For four parameters a range of values and not a single value is found by previous researchers. Therefore, these four parameters were considered for further sensitivity analysis and uncertainty analysis described in next sections. Moreover, each of four parameters were identified independently for model verification scenarios within the defined range. These parameters are: initial biofilm thickness i.e. the average radius size of one spherical anammox granule in sludge, the estimated size of support one cell particle, external mass transfer boundary layer thickness (LL) and the total biomass density in the biofilm solid matrix which were kept constant during the simulation periods (rho). Boundary Layer Resistance is always equal to LL/D which D is diffusion constants for particulate or dissolved variables

### 2.3 Parameter Estimation and Statistical Analyses

The model’s main output variables were selected as NO3-N, NH4-N and NO2-N concentrations in the bulk volume of the biofilm system. The absolute-relative sensitivity analysis was initially carried out to identify the most important parameters influencing nitrogen conversion and the development of the biofilm (Reichert, 1998). Thereafter, the Simplex nonlinear parameter estimation algorithm was carried out followed by calculating the statistical confidence regions for each two jointly adjusted sensitive

parameters. The corresponding confidence regions indicate 95 percent confidence interval for calculated Chi-squared lower than an assumed threshold. All identified kinetic and stoichiometric parameters giving the best agreement between simulation and observation data points for calibration scenario were selected for the rest of model validation simulations. Statistical indices ($\chi 2$ and R2) were also calculated to evaluate the model performance for validation scenarios using the same kinetic and stoichiometric parameter sets. Further uncertainty analysis was performed for determining the sources of significant uncertainty in predicting nitrogen components in for three validation scenarios. For this mean, a simple linear regression equation was assumed to model the nitrogen loss using the observation data points. The confidence regions of parameters i.e. intercept and slope were assumed to be linear and a 95% confidence interval regions around the slope of a regression line was constructed by calculating the inverse of the cumulative Normal distribution function for an assumed value of x, and a supplied distribution mean and corresponding standard deviation of y values. In this case x values will be the time steps in hour and y values will be the relevant observed nitrogen components concentration. Statistical calculations were done in MATLAB matrix laboratory (Mathworks, Natick, USA) and Microsoft Excel spreadsheet.

## 3 RESULTS AND DISCUSSION

The model verification is based on the comparison between the experimental results and the model prediction using the same input kinetic and stoichiometric model parameters. Experimental data of two scenarios were considered and evaluated for calibration and validation. In these two scenarios respectively for calibration (Figure 3) simultaneous activity of denitrifiers and anammox in presence of ammonium and nitrite as substrates and secondly for validation single activity heterotrophic denitrifying activity in presence of nitrate and absence (Figure 4a and 4b) and presence (Figure 4c and 4d) of the soluble readily biodegradable organics in the bulk liquid phase was validated.

### 3.1 Model Calibration

The calibration period was implemented for a batch test performed anaerobically with 63.5 mg//l NH4-N and 63.5 mg/l NO2-N and no nitrate in feed synthetic wastewater. The validation period was selected for another batch test performed for 62.5 mg N/l of NO3-N input with and without addition if glucose as external carbon source and no nitrite in feed. The parameter estimation function was Chi-squared. The simplex nonlinear parameter estimation algorithm has been used followed by calculating the statistical confidence regions for each two jointly adjusted sensitive parameters. Pearson correlation coefficient has been used to check the linear regression between the modeled and measured data points.

The model prediction is highly sensitive to the yield coefficient of denitrifying heterotrophs and anammox autotrophs, the bacterial biomass density, the initial volumetric fractions and the initial particulate concentration of various biomass types present in granules. The detachment velocity has shown to be not sensitive in this biofilm model due to short time of simulation compared to other parameters such as initial values. Calculated Pearson correlation coefficients between the modeled and measured data points for the calibration scenario show a satisfactory goodness of fitness equal to 0.96, 0.98 and 0.81 for NH4-N, NO2-N and NO3-N respectively (Figure 1). In Figure 1 for nitrite simulation, a significant decline in ammonium concentration within the first 7 hours due to the activity of anammox bacteria has been simulated. The release of ammonia due to decay process of anammox bacteria can be observed from hour 7 to hour 10. The model can reliably simulate both anammox growth and decay process. The decline in nitrite concentration displayed by simulation and experimental results is explained by the growth and activity of anammox bacteria and denitrifies. In Figure 1 for nitrate the measured values indicate that there is an increase in nitrate production due to the activity of anammox bacteria outcompeting the denitrifiers within the first four time steps. This observation is similar to the simulated results by the model indicating the increase in nitrate due to the defined stoichiometric coefficients. Furthermore, the model behaviour considers a decline in nitrate concentration after 6 hours. The corresponding prediction is in compliance with the results of Figure 1 for nitrite outlining the release of ammonium concentration due to the decay of anammox autotrophs.

### 3.2 Model Validation

For the validation scenario, despite some discrepancies between modeled and observed data points were observed, the model performance was satisfactory. The grey shade area (Figure 2) represents the 95% confidence interval of, a range of values that we can be 95% certain contains the true actual value occurs. Therefore, it is expected that predicted values fall within the interval 95% interval, the model is able to simulate the variation trends well for both scenarios within the confidence region. The very good agreement between measured and predicted values suggest the validity of the model established in this work for nitrate prediction. A few discrepancies in the beginning time steps in the form of underestimating the observed concentration of nitrate was seen. Figure 2 corresponds to more intense underestimation in nitrate concentrations when glucose was added to the reactor hence more dispersion was seen during regression analysis (Figure 2d). The Figure 2c, d stated that by availability of glucoses as readily biodegradable carbon source for heterotrophic organisms, the nitrate depletion rate is higher. Within the first two hours a sharp reduction in nitrate consumption has been predicted. NO3-N reduction rate within the first two hours during availability of glucose is 10 mg N.l-1h-1 while this value for endogenous activity is 5 mg N.l-1h 1. This result is validated by observed data points. During the first 6 hours the both modeled and measured data marked 5 mg N.l-1h-1 nitrate reduction in absence of glucose. The nitrate reduction in presence of glucose for model and measurements is estimated around 7 mg N.l-1h-1. In general nitrate reduction prediction results for both scenarios produced a strong correlation between 0.93-9.96 using the simple least squares regression method and the simulation line lays inside the calculated 95% confidence region. It must be noted that due to the limited number of data points used, the confidence regions and correlation coefficient may not be reliable despite the approach is correct.
For the validation of ammonium reduction simulation, despite some more discrepancies between modelled and observed data points for nitrate were observed, the model performance was satisfactory.

Underrating the nitrate amount for calibration and validation might be due to excess growth rate of denitrifies more than actual amount and due to underestimating the specific anammox activity. The latter is because the model under severe constant nitrite limiting conditions estimated the anammox activity rate to zero while in actual conditions nitrite concentration is not physically zero. Nitrite can be produced during the partial denitrification by nitrate reductase (NaR). That is the reason during batch measurement NO2-N increased from 0.1 mg N/l to 2.2 mg N/l after two hours and then dropped to zero (Measured data and simulated data of nitrite is not shown). The introduction of nitrite into the system led to immediate consumption by anammox activity. Despite the model considers two pathways for denitrification over nitrite and denitrification over nitrate but it completely skips this possibility of partial denitrification additionally production and survival of NO2-, NO and N2O from partial reductase, meaning that denitrification would proceed until NO2- or NO or N2O only. Therefore, anammox activity is underestimated which will lead to underestimating the nitrate values during the time steps two to five hours.

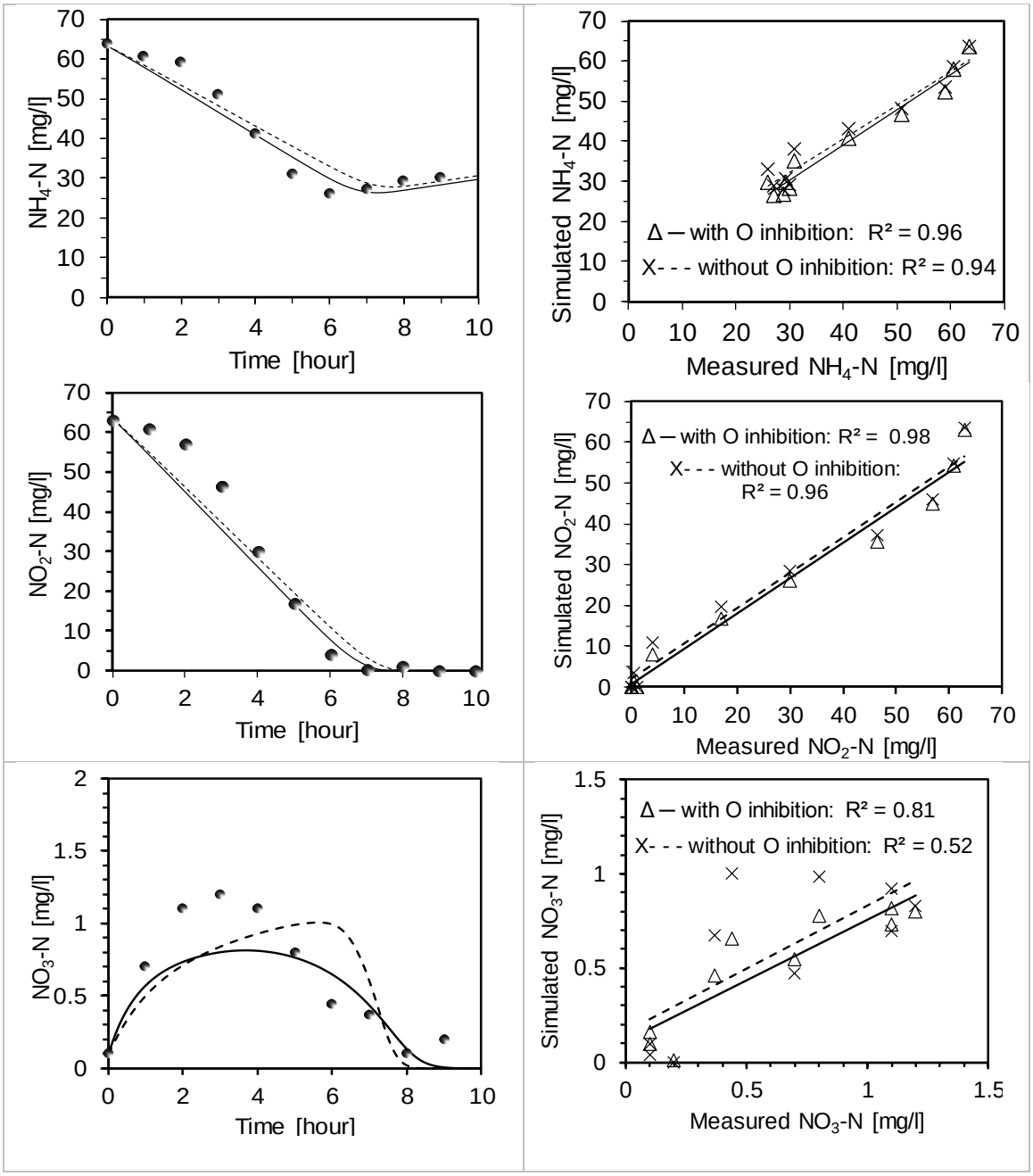


**Figure 1.** Model calibration results (left) and analysis of regressions (right) for three output variables. Dotted lines and X symbols indicate the simplified versio of the model without oxygen inhibition term and continous line and Δ symbols are the main model considered with oxygen inhibition term in anammox growth rate, Black spheres are the hourly measured values for each nitrogen component

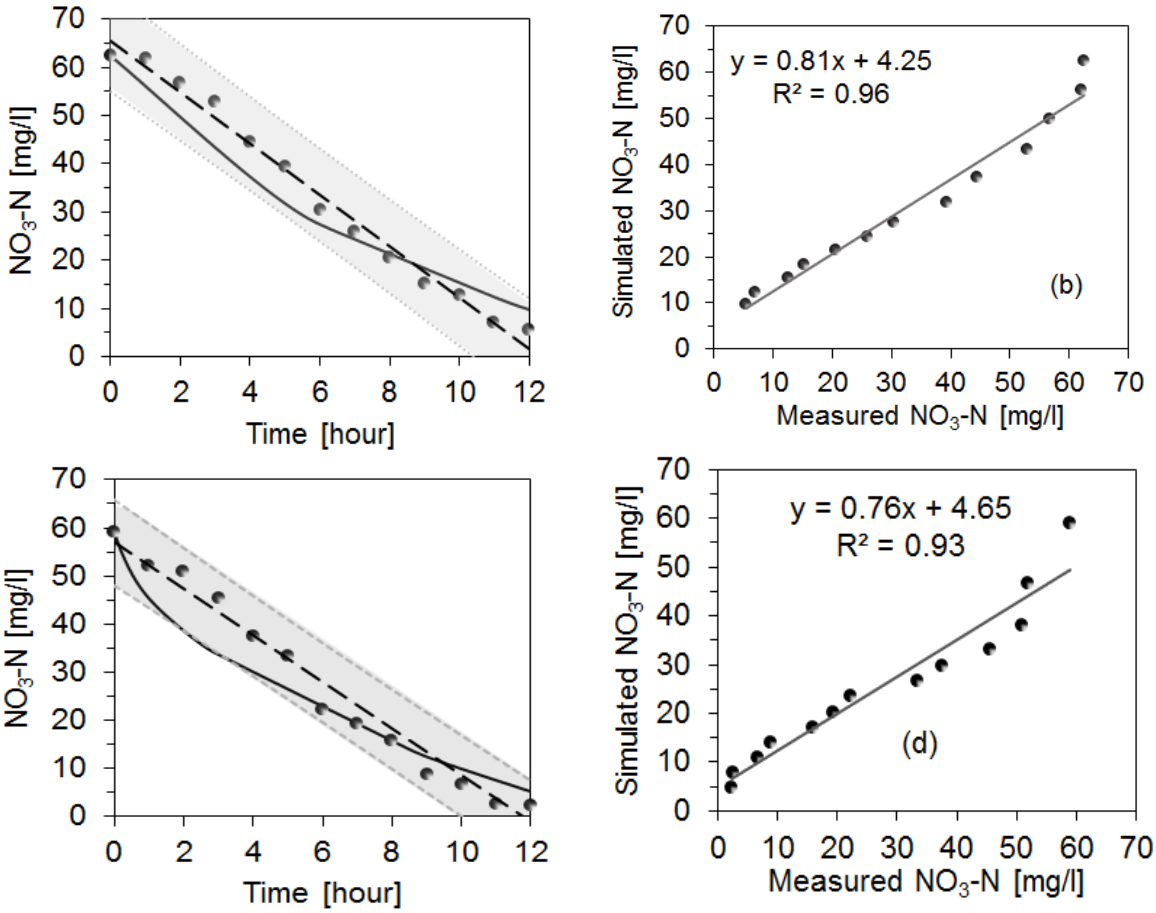


**Figure 2.** Model validation results (left) and analysis of regressions (right) for nitrate nitrogen. Dotted lines indicate the lower and upper limit of 95% confidence regions to check whether simulated points could fall in this confidence range and continuous black line is the main simulation line. The grey shade area represents the 95% confidence interval. Black spheres are the hourly measured values for each nitrogen component. The model validation for top graphs for Figure 4a and 4b is without any glucose while Figure 4c and 4d are the results of simulation during addition of 75 mg/l glucose.

## 4 CONCLUSIONS AND RECOMMENDATIONS

The model was successfully calibrated using statistical method for parameter estimation. The calibrated model has been validated and achieved satisfactory results with the same parameter set to simulate different experimental scenarios. In detail, the model was used to explain the physiological processes and kinetics of nitrogen converting bacteria in a granular sludge system.

Moreover, since in such biofilm models many constant and formula parameters and initial assumptions are significantly different or not clearly discussed and analysed in the current state of the art, this research is also aimed to use analytical and in-situ detection methods for an efficient parameter estimation procedure. It was investigated whether the calibrated model can be validated or not using one sets of kinetic and stoichiometric coefficients. The results generally indicate the higher sensitivity of anoxic yield and decay coefficients for heterotrophs and autotrophs. Besides parameters representing the initial volume fractions and biomass density were relatively sensitive. The calibration results show a satisfactory goodness of fitness representing 0.96, 0.98 and 0.81 for NH4-N, NO2-N and NO3-N respectively. For the validation period although few discrepancies between modeled and observed data points were observed but the model performance was enhanced and satisfactory.

However, the results suggested that the model can be further improved to more reliably simulate various experimental and operational data. As next steps for model evaluation and uncertainty analysis more data points will be investigated and more batch-operated experiments for validation

scenarios will be conducted. Measurements of the abundances of bacteria by multiplex real-time PCR can be useful for more accurate parametrization of the corresponding biofilm model.

## 5 CONFLICT OF INTEREST AND COPYRIGHT

The authors declare the work is a product of the intellectual and original contribution of the whole team of authors and that all members have been active in various degrees to the methods used for collecting data and information, literature review, running experiments and model setup, and to the discussion and conclusion as well as editing and final preparation of paper for submission. The authors hereby also declare that there is no conflict of interests in this research.

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