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Water impact

The nitritation/anammox process is an emerging technology to remove nitrogen from wastewater with the final goal to prevent eutrophication. To ensure long-term process stability, the activity of the slow-growing anammox bacteria must be known. Mass balances are often used to determine the anammox activity, but heterotrophic activity (e.g. denitrification) is usually neglected in such calculations. This is critical for wastewaters containing considerable amounts of organic substrate (e.g. municipal wastewater in the mainstream or urine). We show that, in theory, mass balances allow determining anammox activity also under such conditions, but, in practice, the variance of the calculated rates is too high to be meaningful for plant operation. Alternative methods must be used for determining the anammox activity.

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Observability of anammox activity in single-stage nitritation/anammox reactors using mass balances

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ABSTRACT

In nitritation/anammox reactors, several bacterial groups contribute to the overall nitrogen conversion. Knowing the activity of the main bacterial groups, especially of anaerobic ammonium-oxidising bacteria (AMX), is extremely helpful to understand the process and optimise its operation. Mass balances of dissolved compounds such as ammonium, nitrite and nitrate commonly allow determining bacterial activities in a nitritation/anammox process, but the activity of heterotrophic bacteria (HET) is usually neglected. However, even in wastewater with low contents of organic substrate, heterotrophic denitrification can contribute substantially to nitrogen removal. The goal of this study was to critically evaluate the applicability of mass balances for the determination of the relevant bacterial activities in a nitritation/anammox process with high HET activity. We set up and solved mass balances of different degrees of complexity. Both, with catabolic reactions alone and with balances according to the activated sludge model stoichiometry, the resulting linear equation system does not allow estimation of any of the considered bacterial activities. When kinetic rate expressions are included, it is possible to compute the concentrations of all considered bacterial groups, but the estimation uncertainty is far too high for practical purposes: the relative standard deviation for AMX is 5280%. In a completely autotrophic system the relative standard deviation for AMX is only 5%, which proves that the high standard deviations are due to the complexity of the nitration-anammox process with HET activity. The high standard deviations of the calculated bacterial concentrations can be significantly reduced by adding an additional mass balance for the total biomass (standard deviation for AMX activity 1210%). The required number of measurements to achieve an acceptable precision, in our example about 600 conversion rate measurements to reach a 50% standard deviation for the AMX concentration, is still far too high though for practical purposes. To conclude, mass balances including kinetics theoretically allow the observation of the bacterial activities in nitritation/anammox reactors with high HET activity. However, the required precision of the calculated conversion rates, the uncertainty of

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stoichiometric and kinetic parameters and reactor dynamics (unsteady conditions) makes mass balances unsuitable for practical estimation of AMX activity. Thanks to high frequency and new online instruments, mass balances might become a suitable tool in the future.

1. INTRODUCTION

Nitrogen removal using the nitritation/anammox process is a cost efficient alternative to conventional nitrification/denitrification, thanks to reductions in the requirements for oxygen and organic substrates in comparison to conventional nitrification/denitrification processes. However, maintaining a high activity of anammox bacteria (AMX) can be challenging.[1] Especially in reactors with high ratios of biodegradable organic carbon to nitrogen (COD/N) decreasing AMX activity might not be noticed in time, because heterotrophic bacteria (HET) take over a considerable part of the nitrogen removal from AMX.[2] Several analytical and experimental methods exist for the reliable determination of AMX concentrations or activities as Podmirseg *et al.*[3] have shown recently. However, all of these methods require instruments which are not available at typical wastewater treatment plants. It would be desirable to be able to calculate the activities of the involved bacterial groups from regularly measured variables for performance monitoring such as the concentrations of for example ammonium and nitrite.

Mass balances for nitrogen compounds, *i.e.*, ammonium, nitrite and nitrate, have frequently been used to calculate the activities of aerobic ammonium-oxidising bacteria (AOB), nitrite-oxidising bacteria (NOB) and AMX in nitritation/anammox reactors.[4,5] However, as elaborated by Mutlu *et al.*,[6] the calculation of AOB, NOB and AMX activity with such mass balances is coupled to the assumption that the activity of HET is negligible. Quite frequently, this assumption is incorrect. On one hand, it has been shown experimentally that even in biofilm systems without organic carbon in the influent, up to 50% of the biomass can be heterotrophic, supported by microbial decay products.[7,8] On the other hand, wastewater almost always contains biodegradable organic matter. Digester supernatant, which is the most common

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influent for a nitritation/anammox system has biodegradable organic carbon to nitrogen (COD/N) ratios in the range of 0.2 to 0.5 g COD·g N^{-1}.[9,10,11] Some wastewaters even have elevated COD/N ratios in the range of 1 to 1.5 g COD·g N^{-1}, which is still not high enough for complete nitrogen removal via heterotrophic denitritation. Examples are stored urine with a theoretical COD/N ratio between 1 g COD·g N^{-1} and 1.5 g COD·g N^{-1}.[12,13] COD/N ratios of approximately 1 g COD·g N^{-1} are also expected in the recently discussed integration of anammox into mainstream wastewater treatment.[14,15]

To our knowledge, only three studies included COD consumption in their mass balances to assess the bacterial activities in a nitritation/anammox process.[16,17,18] These three studies used four equations representing the conversion of ammonium, nitrite, nitrate and COD. As only four unknowns can be determined with four independent equations, the authors considered only the activities of AOB, NOB, AMX and nitrate reduction by HET. However, in single-stage nitritation/anammox reactors, heterotrophic consumption of oxygen and nitrite is not negligible. For one thing, the yield of HET growth with oxygen is higher than with nitrite and nitrate and therefore, in the presence of all three electron acceptors, HET might prefer oxygen over nitrate and nitrite. Secondly, especially in the presence of high amounts of biodegradable organic matter, HET are able to take over a substantial part of the nitrite removal from AMX.[2]

The goal of this study is to critically evaluate whether mass balances with commonly measured compounds (for example ammonium and nitrite), can be used to observe the six main bacterial activities in a single-stage nitritation/anammox reactor: aerobic ammonium oxidation by AOB, nitrite oxidation by NOB, anaerobic ammonium oxidation by AMX, heterotrophic oxygen reduction, heterotrophic nitrite reduction and heterotrophic nitrate reduction. Mass balances with increasing complexity are analysed starting with catabolic reactions only and ending with a stoichiometric matrix which accounts for information on both catabolic and anabolic reactions, microbial kinetic rate functions and a balance for biomass. For all resulting

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mass balances, both structural and practical observability of the bacterial activities are evaluated.

2. MATERIAL AND METHODS

2.1 Definitions

In this paper, we use the following definitions:

Parameters: Parameters characterise the chemical, physical or biological processes and are assumed to be constant for a given system. Examples for biological processes are stoichiometric and kinetic constants such as the yield or the maximum growth rate. The parameters were taken from literature.

State variables: In this study, state variables are compounds, which are converted in the chemical, physical and biological processes. Examples are the ammonia concentration or the biomass concentration. In theory, state variables can be determined by analytical measurements.

Conversion rates (r_{Ci}) describe the conversion of a state variable per time unit. A net conversion rates describe the overall conversion of a state variable by all bacterial processes.

Bacterial reaction rates (r_{Ri}) quantify bacterial reactions (R_i) such as catabolic or anabolic reactions or, in terms of the activated sludge models (ASMs),[19] growth and decay processes.

Bacterial activities: The activity of a bacterial group is defined as the conversion of a characteristic substrate by this bacterial group. The characteristic substrate is ammonia in the case of AMX and AOB, nitrite in case of NOB and COD in case of heterotrophic COD degradation with oxygen, nitrite and nitrate.

Structural observability: In a linear equation system, all unknowns are structurally observable if the number of independent equations is equal to or higher than the number of unknowns. An equal number means that the equation system is determined; a larger number means that it is over-determined. Mathematically, the number of unknowns that can be

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estimated is evaluated by calculating the rank of the balancing matrix ***A*** (see equation 6). This rank will equal the number of unknowns, if they are all structurally observable.

Practical observability: To be practically observable, the unknowns have to fulfil two more conditions besides being structurally observable: first, the set of parameters must allow the calculation of meaningful values for the unknowns (*e.g.* positive concentrations of biomasses). In extreme cases, empirically determined parameters do not allow the estimation of all unknowns, although the unknowns are structurally identifiable. This can occur for example, if the particular choice for yield parameters causes one balance equation to become a linear combination of two or more of the remaining balancing equations. Second, the precision of the calculated values for the estimates must be sufficiently precise to be of practical use.

2.2 Choice of state variables

The considered mass balances involve seven compounds: ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), oxygen (O_2), dissolved organic substances (measured as chemical oxygen demand, COD), protons (H^+) and total inorganic carbon (TIC). These compounds and their net conversion rates can be determined on large wastewater treatment plants without highly sophisticated analytical methods. Most of these compounds and their conversion rates are directly accessible with measurements: NH_4^+, NO_2^-, NO_3^-, O_2, and organic substances. The H^+ conversion rate and the concentration and conversion rate of TIC can be calculated from alkalinity measurements, pH values and estimated of the CO_2 stripping (equation 2). It should be noted that in more highly concentrated wastewaters (for example digester supernatant or urine) additional bases such as phosphate species or free ammonia need to be measured and accounted for. To simplify the mass balance for dissolved biodegradable organic substances, we assume that all organic substances are degraded by bacteria. We choose acetate ($C_2H_3O_2^-$, abbreviated as Ac) to represent the organic compounds.

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2.3 Determining the net conversion rates

We assumed that measurements are taken from an ideally stirred continuous flow reactor with biomass retention in which all state variables are at their steady state values. For dissolved compounds, which have no gas phase exchange the net conversion rate can be calculated as

$$r_{S_i} = \frac{Q}{V} \cdot \left(S_i - S_{i,in}\right) \quad (1)$$

where Q is the flow rate ($m^3 \cdot d^{-1}$), V is the reactor volume (m^3), S_i is the concentration of the dissolved compound i (g $i \cdot m^{-3}$) in the reactor, and $S_{i,in}$ is the influent concentration of the dissolved compound i (g $i \cdot m^{-3}$).

Volatile compounds, such as O_2 and CO_2, are influenced by gas exchange processes. In this case, the net conversion rate r_{Si} becomes

$$r_{S_i} = \frac{Q}{V} \cdot \left(S_i - S_{i,in}\right) - r_{i,gas} \quad (2)$$

with

$$r_{i,gas} = \left(S_{i,G} - H_i \cdot S_i\right) \cdot \frac{Q_{air}}{V} \cdot \left(1 - exp\left(-\frac{K_L a_i \cdot V}{H_i \cdot Q_{air}}\right)\right) \quad (3)$$

where H_i is the Henry coefficient of compound i (g $i \cdot m^{-3}_{gas}$/g $i \cdot m^{-3}_{liquid}$), $S_{i,G}$ is the concentration of compound i in the gas used for aeration (g $i \cdot m^{-3}$), Q_{air} is the aeration rate ($m^3 \cdot d^{-1}$) and $K_L a_i$ is the mass transfer rate constant for compound i (d^{-1}).

Net conversion rates can also be given for particulate compounds such as bacteria and inert biomass:

$$r_{X_j} = \frac{Q}{V} \cdot \left(X_{j,eff} - X_{j,in}\right) \quad (4)$$

where $X_{j,in}$ is the influent concentration of bacteria type j (g COD $\cdot m^{-3}$), and $X_{j,eff}$ is the concentration of bacteria type j in the reactor (g COD $\cdot m^{-3}$). In this study, we assumed that no particulate material enters the reactor with the influent, so that $r_{X_j} = \frac{Q}{V} \cdot X_{j,eff}$ for all bacteria and inert biomass.

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2.4 Setting up the mass balances

In general, mass balance equations for a system with *n* compounds and *m* relevant bacterial reactions (*R*) result in an equation system with the following structure:

$$\begin{pmatrix} \upsilon_{i,j} & \cdots & \upsilon_{i,m} \\ \vdots & \ddots & \vdots \\ \upsilon_{n,j} & \cdots & \upsilon_{n,m} \end{pmatrix} \cdot \begin{pmatrix} r_{Rj} \\ \vdots \\ r_{Rm} \end{pmatrix} = \begin{pmatrix} r_{Ci} \\ \vdots \\ r_{Cn} \end{pmatrix} \quad (5)$$

where $\upsilon_{i,j}$ is the stoichiometric coefficient of compound *i* in the bacterial reaction *Rj*, r_{Rj} is the unknown reaction rate of reaction *Rj* and r_{Ci} is the measured net conversion rate of compound *i*. In most cases, C_i describes a dissolved compound but it can also be used for biomass.

Equation 5 can also be written in matrix notation as

$$\boldsymbol{A} \cdot \boldsymbol{r}_R = \boldsymbol{r}_C \quad (6)$$

where $\boldsymbol{A}$ is the matrix of the stoichiometric coefficients as shown in equation 5, $\boldsymbol{r}_R$ is the vector of the biomass reaction rates and $\boldsymbol{r}_C$ is the vector of the net conversion rates. Generally, $\boldsymbol{A}$ is also called the balancing matrix.

2.5 Mass balances with catabolic reactions only (equation system 1)

In the first equation system, we assumed that biomass growth and biomass decay are in equilibrium and no inert biomass is produced; this means the net biomass production is zero. For such a system, the net conversion rates are mainly determined by catabolic bacterial reactions. With this approach the stoichiometric coefficients are given by the chemical sum formulae of the catabolic reactions and do not require empirically determined parameters such as yield or nitrogen content of biomass.

The six main catabolic bacterial reactions in a nitritation/anammox reactor with heterotrophic activity are:

Heterotrophic COD degradation with O_2:

$$C_2H_3O_2^- + H^+ + 2O_2 \rightarrow 2CO_2 + 2H_2O \quad (7)$$

Heterotrophic COD degradation with NO_2^-:

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$$C_2H_3O_2^- + \frac{8}{3}NO_2^- + \frac{11}{3}H^+ \rightarrow 2CO_2 + \frac{4}{3}N_2 + \frac{10}{3}H_2O \quad (8)$$

Heterotrophic COD degradation with NO_3^-:

$$C_2H_3O_2^- + \frac{8}{5}NO_3^- + \frac{13}{5}H^+ \rightarrow 2CO_2 + \frac{4}{5}N_2 + \frac{14}{5}H_2O \quad (9)$$

Aerobic ammonium oxidation:

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (10)$$

Aerobic nitrite oxidation:

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \quad (11)$$

Anaerobic ammonium oxidation:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (12)$$

Nine compounds are produced or consumed in these six reactions, but two of the compounds, H_2O and N_2, are not considered in the mass balances, because the produced amounts are too low compared to the background concentration in water and air.

An overview of the equation system is given in Table 1, the complete equation system is given in the Supporting information S1. The unknowns to be calculated are the bacterial reaction rates r_{Rj} (d^{-1}), which could be later used to calculate bacterial activities by multiplying the bacterial reaction rates with the respective stoichiometric coefficients for the substrates.

2.6 Mass balances based on the activated sludge models (equation systems 2 to 4)

According to ASMs, biological metabolism can be represented by growth and decay of biomass. Both processes are modelled with empirical stoichiometric coefficients. In ASMs, growth inherently includes catabolic and anabolic reactions, whereas decay can be due to anabolic reactions, predation and chemical decomposition (*e.g.* hydrolysis). In this study, biomass decay was simulated as endogenous respiration. Median values of a literature review were used for the stoichiometric coefficients for growth and decay (Table 2). The biomass

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composition was assumed to be $C_5H_7O_2N$ for HET, AOB and NOB, while for AMX, the biomass composition given by Strous *et al*.[20] was used ($CH_2O_{0.5}N_{0.15}$).

Three different equation systems were tested. In equation system 2 (Table 1) only mass balances for dissolved compounds were considered and the mass balances were set up according to equation 6: instead of the bacterial reaction rates r_{Rj}, bacterial activities (α_{Rj}) were used as unknowns

$$\boldsymbol{A} \cdot \boldsymbol{a}_R = \boldsymbol{r}_C \quad (13)$$

with

$$\alpha_{R,j} = r_{R,j} \cdot X_j \quad (14)$$

This approach was used because the stoichiometry of ASMs is given per biomass unit. It should be noted that this approach does not require that X_j are calculated.

In equation systems 3 and 4 (Table 1), balances for the bacterial groups and inert biomass were also included (see equation 4). Most of the previously published models (for example Kaelin *et al*.[31]) assumed that one type of HET can use all three electron acceptors (O_2, NO_2^-, NO_3^-). In reality, HET biomass will consist of a mixture of heterotrophic bacteria that can use one, two or all three electron acceptors.[32] In our study, we compared mass balances with one type of HET that can use all three electron acceptors (equation system 3) and three types of HET which specifically use only one of them (equation system 4).

An overview of the equations systems is given in Table 1. The complete equation systems are given in the Supporting information S2.

2.7 Mass balances based on the activated sludge model including kinetics (equation system 5 and 6)

In equation system 5 and 6 (Table 1) the bacterial reaction rates are described with more detailed kinetic expressions

$$r_{Ri} = \rho_j \cdot X_j \quad (15)$$

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where ρ_j is the process rate, which is the product of saturation and inhibition terms, and the maximum growth rate $\mu_{max,j}$ (d^{-1}) or the maximum endogenous respiration rate $b_{max,i}$ (d^{-1}). When kinetic constants are included, growth and endogenous respiration of each type of bacteria can be combined in one equation and merged with the stoichiometric coefficients. In this equation system, biomass concentrations instead of bacterial rates are the unknowns:

$$\boldsymbol{A} \cdot \boldsymbol{X} = \boldsymbol{r}_C \tag{16}$$

with the balancing matrix $\boldsymbol{A}$

$$\boldsymbol{A} = \begin{pmatrix} \upsilon_{i,j} \cdot \rho_j & \cdots & \upsilon_{i,m} \cdot \rho_m \\ \vdots & \ddots & \vdots \\ \upsilon_{n,j} \cdot \rho_j & \cdots & \upsilon_{n,m} \cdot \rho_m \end{pmatrix} \tag{17}$$

and the vector of the biomass concentrations

$$\boldsymbol{X} = \begin{pmatrix} X_j \\ \vdots \\ X_m \end{pmatrix} \tag{18}$$

The bacterial reaction rates can subsequently be calculated according to equation 15. The relevant process rates for a nitritation/anammox reactor with heterotrophic activity are listed in Table 3. Whenever possible we used median values based on a literature review (Table 4). While the affinity and inhibition constants of AOB, NOB and HET have been documented in several studies, the number of values is sparse for the saturation coefficient of AMX for ammonium ($K_{NH4,AMX}$) and nitrite ($K_{NO2,AMX}$) and the inhibition coefficient of AMX for oxygen ($K_{I,O2,AMX}$). Strous *et al.*[42] reported that both, the affinity constants for the substrates ammonium and nitrite, lower than 0.1 mg $N \cdot L^{-1}$. We assumed that $K_{NH4,AMX}$ and $K_{NO2,AMX}$ were 0.1 mg $N \cdot L^{-1}$ each. Strous *et al.*[43] reported that AMX were completely inhibited at 0.5% air saturation, which equals 0.036 mg $O_2 \cdot L^{-1}$ at 30°C. In this study, we assumed that $K_{I,O2,AMX}$, which corresponds to the oxygen concentration at 50%, was 0.1 mg $O_2 \cdot L^{-1}$. For the concentrations of

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O_2, NO_2^-, NO_3^-, NH_4^+ and Ac we used simulated reference values (Section 2.5, Table 5). The complete equation systems are given in the Supporting information S3.

2.8 Mass balances based on the activated sludge model including kinetics and a biomass balance (equation system 7)

Equation system 5 can be extended with a biomass balance according to

$$\sum_j^m \left(\rho_{j,growth} + (1 - f_{XI}) \cdot \rho_{j,end\ resp}\right) \cdot X_j = r_{X_{tot}} \quad (19)$$

$\rho_{j,growth}$ (d^{-1}) is the process rate for bacterial growth and $\rho_{j,end\ resp}$ (d^{-1}) is the process rate for endogenous respiration. The kinetic expressions for $\rho_{j,growth}$ and $\rho_{j,end\ resp}$ are given in Table 4. f_{XI} (-) is the fraction of biomass converted into inert biomass X_I (Table 2). r_{Xtot} ($gCOD \cdot m^{-3} \cdot d^{-1}$) is the conversion rate for the total biomass. The term (1- f_{XI}) ensures that the production of inert biomass is included in the total production of biomass. An overview of equation system 7 is given in Table 1. The complete mass balances are given in the Supporting information S4.

2.9 Reference data for conversion rates

Computer simulations with the software Aquasim[44] were used to obtain reference data for solving the mass balances (equation systems 5 to 7). Measurements from a single-stage sequencing batch reactor with five-times diluted urine (influent COD/N ratio 1.27 g $COD \cdot g\ N^{-1}$)[45] were used as influent concentrations and as initial conditions (Supporting information S5). All compounds included in the model are listed in the Supporting information S6. The stoichiometric constants and the kinetic constants were the same as given in Table 2 and Table 4. Three groups of HET were introduced to represent the heterotrophic activity with oxygen, nitrite and nitrate as electron acceptors. In case of equation system 6, the initial biomass concentrations of the three groups of HET were set to zero and all heterotrophic processes were inactivated. The simulation of pH equilibria and aeration effects is described in the Supporting

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information S7. Long-term simulations with constant influent rates were used to approximate concentrations at steady state. The simulated net conversion rates and the biomass concentrations at steady state are listed in Table 5.

2.10 Evaluating structural observability

To solve a linear equation system we need at least as many independent equations (mass balances) as unknown variables (bacterial reaction rates, bacterial activities or biomass concentrations). Independent means that none of the equations can be linearly combined and transformed to another of the available equations. Practically, the classification of the considered equation systems and observability of the unknowns is based on the evaluation of the rank of the linear equation system: the rank must be equal to the number of unknowns otherwise the linear equation system is under-determined.

When empirically determined stoichiometric parameters are used, some equations might be dependent due to a particular choice of parameter values and not due to an under-determination of the equation system for all feasible parameter values. In this case, the lack of observability would not be structural but only practical. To test whether the lack of observability is not only practical but also structural, we performed Monte Carlo simulations: 10,000 simulations were done with uniformly and randomly distributed parameter values in a range of ± 50% of the default values (median values from literature according to Table 2 and Table 4). If this test does not provide any parameter sets, which make the equation system observable, it is very likely that the lack of observability is structural. A stringent test of the structural observability would be considerably more complex[46] and was considered to be unnecessary to obtain conclusive results. All computations were executed by means of MATLAB (R2013b, The MathWorks Inc., Natick MA, USA). The Matlab codes are given in the Supporting information S8 and S9.

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2.11 Evaluating practical observability

If the test for structural observability was successful, we tested the practical observability by estimating the theoretical standard deviations of the estimates. To this end, we assumed that the net conversion rates r_{Ci} have a standard deviation of 5% of their steady-state value. In practice these standard deviations are due to measurement errors and can be higher than 5% so that the computed standard deviations are rather optimistic (*i.e.*, low). In real systems, the variation is probably even larger due to imprecise parameter values. Furthermore, parameter values and analytical measurements of variables can be systematically wrong, leading to systematic estimation errors (bias). Assuming that measurement errors for the rate measurements, r_{Ci}, are drawn independently from a normal distribution with zero mean and a given standard deviation ($\sigma_{r_{Ci}}$), one can compute the expected value for the bacterial concentrations X_j in equation systems 5 to 7 (Table 1) as follows:[47]

$$\boldsymbol{A} \cdot \boldsymbol{X} = \boldsymbol{r}_C \tag{20}$$

$$\boldsymbol{X} = (\boldsymbol{A}^{\mathrm{T}} \cdot {\Sigma_{r_C}}^{-1} \cdot \boldsymbol{A})^{-1} \cdot \boldsymbol{A}^{\mathrm{T}} \cdot {\Sigma_{r_C}}^{-1} \cdot r_C = \boldsymbol{P} \cdot r_C \tag{21}$$

$$\forall k, l : k = l \Rightarrow \Sigma_{r_C}(k,l) = \sigma_{r_C}^2 \tag{22}$$

$$\forall k, l : k \neq l \Rightarrow \Sigma_{r_S}(k,l) = 0 \tag{23}$$

The variance-covariance matrix for the rate estimates is then:

$$\boldsymbol{\Sigma}_{\boldsymbol{X_j}} = \boldsymbol{P} \cdot \boldsymbol{\Sigma}_{\boldsymbol{r_C}} \cdot \boldsymbol{P}^T \tag{24}$$

With the individual standard deviations for the rate estimates computed from the variances on its diagonal, the standard deviation of the biomass estimate becomes

$$\sigma_{X_j} = \left(\boldsymbol{\Sigma}_{\boldsymbol{X_j}}(j,j)\right)^{1/2} \tag{25}$$

In practice, the standard deviation of the net conversion rates can be reduced by means of independent repetitions of the measurements under the same experimental conditions. The

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standard deviation of the average of *r* independent measurements can be estimated by dividing the standard deviation of a single measurement by the square root of the total number of measurements *r*:

$$\sigma_{X_j,r} = \sigma_{X_j,r}/r^{1/2} \quad (26)$$

3 RESULTS

The setup of the mass balances and the main results are summarised in Table 1. The results will be explained in more detail in the following paragraphs.

3.1 Mass balances based on catabolic reactions only

Catabolic reactions alone do not allow determining the activities of the different bacterial groups. The linear system consists of seven equations but the rank of the matrix is only four (Table 1, system 1), which means that three out of the seven equations can be expressed as linear combinations of four independent equations. The lack of observability is thus structural in the sense that it is impossible to conceive of any experiment, even idealised, that permits simultaneous estimation of all reaction rates. In contrast to ASMs reactions (see section 3.2), the stoichiometric coefficients are known constants that follow directly from the definition of the considered chemical reactions. As such, this result is universal in the sense that it does not depend on any adjustable model parameter.

Even if the linear equation system for the catabolic reactions were solvable, the resulting bacterial reaction rates would most probably not be accurate due to some coarse simplifications. The basic assumption that biomass growth and decay are in equilibrium is practically never the case in a wastewater treatment plant. Biomass losses via the effluent or biomass withdrawal cannot be avoided. Furthermore, the catabolic reaction for AMX does not consider an important contribution of AMX to nitrate production: in order to generate the required energy for carbon

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fixation, AMX oxidise nitrite to nitrate, accounting for 11% of the total N conversion by AMX.[20]

3.2 Mass balances based on the activated sludge model

When considering microbial metabolism according to ASMs the number of unknowns increases to twelve: for each of the six bacterial groups, two bacterial rates - growth and endogenous respiration - are included. In the equation system there are now more unknowns (twelve) than mass balances (seven) which means no unique solution exists under any scenario (Table 1, system 2). Concentrations for NOB, AOB, AMX, HET and for inert biomass can be included to provide additional measurements and associated balancing equations. Despite the inclusion of such measurements which are hard to obtain in practice, it remains impossible to determine the microbial activities (Table 1, system 3). This is also the case when the HET are divided in three subgroups, which use oxygen, nitrite or nitrate as electron acceptors (Table 1, system 4). The same classification was obtained for 10'000 random sets for the parameter values. This suggests that the lack of observability is most probably structural and not due to a particular combination of estimated parameters.

Even if it were possible to compute values for the considered respiration rates, it is worth considering that empirical stoichiometric coefficients have to be included in the mass balances (Table 2). These are considered to be known exactly although such parameters are estimated in practice. This means that the balancing equations as described here are subject to additional unaccounted uncertainty in practice.

3.3 Mass balances based on the activated sludge model including kinetics

When the kinetics are known, one can combine the growth and endogenous respiration for each type of bacteria into a single net growth rate. This reduces the number of unknowns from twelve to six and allows determining the biomass concentrations and thereby the bacterial

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activities (Table 1, system 5). In this case, and for the first time in this study, the balancing matrix A is full-rank. This means that the concentrations of AOB, NOB, AMX and the hypothetical concentrations of the three groups of HET can be estimated (structural observability). The activities for growth and endogenous respiration can later be calculated with the assumed kinetic expressions.

Although the biomass concentrations can be determined, the uncertainty of the resulting values is immense when using a single set of rate measurements (Table 6). Assuming that all necessary stoichiometric and kinetic parameters (Table 2 and Table 4) are known exactly, the relative standard deviations of the bacterial activities equal the relative standard deviations of the calculated biomass concentrations and only depend on the measurement accuracy of the net conversion rates. Even if a low standard deviation of 5% is assumed for the conversion rate measurements, the resulting standard deviations for the biomass concentrations are extremely high: the relative standard deviation for AMX is 5280% (Table 6).

The most likely explanation for the high uncertainties of the calculated biomass concentrations is that the mass balances are close to linearly dependent. Removing the heterotrophic activities from the equation system in particular is expected to improve the estimation precision. Nitrogen can then only be removed via nitritation/anammox and not also by a second parallel reactions scheme (nitrification/denitrification), which strongly reduces the number of estimated unknowns. We demonstrate that this is indeed the case by applying the same mass balances for a completely autotrophic system (Table 1, system 6). With a relative standard deviation of 5% for the net conversion rates, the resulting relative standard deviations for the calculated concentrations of AOB, NOB and AMX are now below 10% (Table 6).

The high relative standard deviations of the calculated biomass concentrations can also be reduced by including a mass balance for the biomass (Table 1, equation system 7) to the original equations system (including HET activity). In our example, the relative standard deviation becomes 1210%. However, this standard deviation is still unrealistically high for

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practical purposes. The standard deviations of the net conversion rates can be reduced though with multiple measurements. As an example, the required number of measurements and the corresponding standard deviation of the net conversion rate to reach a certain standard deviation for the calculated biomass concentration of AMX are listed in Table 7. To achieve a relative standard deviation of 50% for the AMX concentration, about 600 measurements of the net conversion rates would be necessary under the same experimental conditions. This number is however still too high for measurements based on conventional grab sampling. Therefore, we conclude that neither AMX activity nor any other of the considered bacterial activities is practically observable with mass balances and conventional grab sampling.

4 DISCUSSION

4.1 Constraints are necessary for the structural observability of the linear equation system

By including constraints, *i.e.*, kinetic expressions, we achieved complete structural observability of all unknown parameters (Table 1, systems 5 and 6). This approach is similar to the flux balance analysis, which is a common method to analyse the metabolic networks of single microorganisms.[48] To overcome the lack of detailed information about the metabolism of a bacterial cell, the metabolic network is represented by a stoichiometric matrix describing the relation of conversion rates and metabolic reactions at steady state. The resulting equation system[49] has essentially the same form as our equation systems and it is usually not observable because the number of unknown reactions is larger than the number of compounds.[50] Constraints are introduced, *e.g.*, measured fluxes or boundaries for certain rates, to allow quantitative predictions. Concentrations, *e.g.*, of metabolites, cannot be predicted, because accurate kinetic parameters are usually not available.[50] Here is the difference to our approach: our systems do not consist of single cells but of bacterial populations, for which macroscopic

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kinetic data are available. By including the kinetic data (systems 5 and 6 in Table 1), all unknown variables, *i.e.* bacterial concentrations, are observable.

Including kinetics makes the linear equation system structurally observable but also affects the accuracy of the final result. Systematic errors can be included in the mass balance. As the data compilation in Table 4 shows, literature values for kinetic parameters vary widely and the chosen kinetic expressions might not include critical influences such as inhibition of AMX or AOB. However, unexpected inhibition effects by unknown compounds are a frequent problem in wastewater treatment plants.[51]

It would be desirable to achieve observability of all reaction rates by increasing the number of independent mass balance equations and not using kinetics. In theory, at least two additional mass balances could be included in our systems: one for H_2O and one for N_2. Unfortunately, no conversion rates for the two compounds can be measured, because their background concentrations are far too high. Furthermore, the two additional equations are not independent from the others (data not shown), so that the rank of the previous mass balances does not increase. Another option would be to include side-products such as nitrous oxide (N_2O) or nitric oxide (NO). Both compounds can be measured in the off-gas[52] or with sensors directly in the water.[53] The isotopic signatures of N_2O even allow the differentiation of the production pathway.[54] However, both compounds are side-products, which only occur under certain circumstances. Additional reaction rates would have to be included to balance those compounds, so that structural observability of the bacterial activities would still not be achieved without introducing kinetics. Based on these considerations, including constraints (*e.g.* as kinetic expressions) seems to be the only way to achieve structural observability of the bacterial activities with the linear equation systems in our study.

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4.2 Additional mass balances could be used to achieve practical observability

Our study shows that not only increasing the number of measurements but also increasing the number of equations can improve the precision of the biomass concentration estimates. Adding one more equation (biomass balance) to equation 5 led to a substantial reduction of the variability of the estimated biomass concentrations in equation 6. This is due to the fact that some conversion rates are redundant and could also be calculated by combining other mass balances. In other words, some conversion rates are balanceable.[55] In practical systems, such redundancy can be used to detect and remove systematic balancing errors (gross error detection) and to reduce random measurement errors in measured data. The latter is also known as data reconciliation.[56]

Data reconciliation is a common procedure for industrial processes. It has been proposed for wastewater treatment as well,[57] but is not commonly applied. This is partly due the fact that municipal wastewater treatment plants usually do not comply with certain requirements of the most basic data reconciliation methods: most of the processes are not at steady state, the variances of process variables are not known and some measurements often have gross errors.[58] Recently, methods have been adapted for wastewater treatment and now allow the identification of periods with gross measurement errors, *e.g.* based on the CUSUM statistic for linear mass balancing errors[59] or based on bilinear mass balancing equations.[60] Furthermore, the ability to detect measurement errors via mass balancing can be ensured by optimising the location of sampling.[61] Long periods at quasi steady state conditions and without gross errors can provide a sufficiently high number of measurements to allow the calculation of precise biomass concentrations (Table 7). The result would be an average biomass concentration during an extended period of time.

Our study showed that not only increasing the number of measurements but also increasing the number of equations while retaining the same number of unknowns can improve the

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precision of the biomass concentrations. Adding one more equation (biomass balance) led to a substantial reduction of the variability of the final result.

4.3 Observing dynamics and separating bacterial processes can provide additional information

The approach used in this study is based on two basic requirements for reactor operation: first, the soluble and particulate compounds are in steady state, and second, all processes occur concomitantly in one single reactor. However, reactors with higher dynamics, such as sequencing batch reactors, and separating the aerobic and the anoxic process steps in two different reactors, are likely suited better to determine the bacterial activities on a regular basis. Short-term changes from single-stage operation to a cyclic multi-stage operation could also be used as an online experimental design method to obtain observability at regular time intervals.

Dynamic measurements allow for more information about the processes, but a prerequisite for practical applications is the use of sensors. If online measurements are available, not only the actual concentrations but also mathematical derivatives such as the oxygen consumption or the change of the oxygen consumption can be used to determine unknown activities.[62] Oxygen and ammonia sensors have been applied successfully for online observation of AMX activity in large scale wastewater treatment plants.[9] Nitrite sensors would further simplify the observation of AMX activity, but reliable nitrite sensors, especially for high nitrite concentrations, still have to be developed.[1,63]

By operating the nitritation/anammox process in two reactors[64] or during two phases in the same reactor[1], aerobic processes such as the activities of AOB, NOB and aerobic HET could be separated from anoxic activities such as AMX and heterotrophic denitrification. While such an approach would greatly simplify the quantification of AMX activity, operational problems such as N_2O production,[65] high NOB activity[66] or inhibition of AMX can occur due to nitrite overloading.[9] For such reasons, most full scale nitritation/anammox reactors are operated as

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single-stage systems,[51] although two-stage systems would easily render the AMX activity observable. However, in single-stage systems short phases with alternating aeration could be introduced to determine the AMX activity.

5 CONCLUSIONS

- In theory mass balances can be used to determine the AMX activity in a nitritation/anammox reactor with heterotrophic activity, but the requirements are challenging. Based on our study, three necessary conditions are:
  - Accurate values for the stoichiometric and the kinetic parameters are available for all considered reactions.
  - The process can be assumed to operate in steady state.
  - A large number of reliable measurements are available for flow rates, COD, nitrite, nitrate, ammonium, alkalinity, TIC, pH and oxygen.
- To achieve a satisfying precision for the estimated AMX activity, an immense number of independent measurements are required. In our example, the conversion rates would need to have standard deviation as low as 0.2% to achieve a precision of 50% for the AMX concentration. This high precision for the conversion rates is practically unachievable with grab samples and laboratory measurements. In the future, high-frequency measurements with sensors and data reconciliation methods could allow for such a high precision of conversion rates.

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650 **Table 1** Overview of the tested mass balances.

System number	System description	Number of mass balances	Number of unknowns	Rank	Rel. std. dev. of AMX conc.
1	Catabolic reactions	7 NH_4, NO_2, NO_3, Ac, H, O_2, TIC	6 $r_{HET,O2}$, $r_{HET,NO2}$, $r_{HET,NO3}$, r_{AOB}, r_{NOB}, r_{AMX}	4	-
2	Catabolic and anabolic reactions, no kinetics, no bacterial conc.	7 NH_4, NO_2, NO_3, Ac, H, O_2, TIC	12 $\alpha_{HET,O2,growth}$, $\alpha_{HET,O2,end.resp}$, $\alpha_{HET,NO2,growth}$, $\alpha_{HET,NO2,end.resp}$, $\alpha_{HET,NO3,growth}$, $\alpha_{HET,NO3,end.resp}$, $\alpha_{AOB,growth}$, $\alpha_{AOB,end.resp}$, $\alpha_{NOB,growth}$, $\alpha_{NOB,end.resp}$, $\alpha_{AMX,growth}$, $\alpha_{AMX,end.resp}$	6	-
3	Catabolic and anabolic reactions, no kinetics, 1 type of HET	12 NH_4, NO_2, NO_3, Ac, H, O_2, TIC, X_I, X_{HET}, X_{AOB}, X_{NOB}, X_{AMX}	12 $\alpha_{HET,O2,growth}$, $\alpha_{HET,O2,end.resp}$, $\alpha_{HET,NO2,growth}$, $\alpha_{HET,NO2,end.resp}$, $\alpha_{HET,NO3,growth}$, $\alpha_{HET,NO3,end.resp}$, $\alpha_{AOB,growth}$, $\alpha_{AOB,end.resp}$, $\alpha_{NOB,growth}$, $\alpha_{NOB,end.resp}$, $\alpha_{AMX,growth}$, $\alpha_{AMX,end.resp}$	9	-
4	Catabolic and anabolic reactions, no kinetics, 3 types of HET	14 NH_4, NO_2, NO_3, Ac, H, O_2, TIC, X_I, $X_{HET,O2}$, $X_{HET,NO2}$, $X_{HET,NO3}$, X_{AOB}, X_{NOB}, X_{AMX}	12 $\alpha_{HET,O2,growth}$, $\alpha_{HET,O2,end.resp}$, $\alpha_{HET,NO2,growth}$, $\alpha_{HET,NO2,end.resp}$, $\alpha_{HET,NO3,growth}$, $\alpha_{HET,NO3,end.resp}$, $\alpha_{AOB,growth}$, $\alpha_{AOB,end.resp}$, $\alpha_{NOB,growth}$, $\alpha_{NOB,end.resp}$, $\alpha_{AMX,growth}$, $\alpha_{AMX,end.resp}$	11	-
5	Catabolic and anabolic reactions, with kinetics, 3 types of HET	7 NH_4, NO_2, NO_3, Ac, H, O_2, TIC	6 $X_{HET,O2}$, $X_{HET,NO2}$, $X_{HET,NO3}$, X_{AOB}, X_{NOB}, X_{AMX}	6	5280%
6	Catabolic and anabolic reactions, with kinetics, without heterotrophic activity	6 NH_4, NO_2, NO_3, H, O_2, TIC	3 X_{AOB}, X_{NOB}, X_{AMX}	3	5%
7	Catabolic and anabolic reactions, with kinetics and sludge loss, 3 types of HET	8 NH_4, NO_2, NO_3, Ac, H, O_2, TIC, X_{tot}	6 $X_{HET,O2}$, $X_{HET,NO2}$, $X_{HET,NO3}$, X_{AOB}, X_{NOB}, X_{AMX}	6	1210%

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652 **Table 2** Median, minimum and maximum values for stoichiometric parameters of the
653 combined catabolic and anabolic reaction equations. The values are compiled from Alpkvist *et*
654 *al.*,[21] Fang *et al.*,[22] Gujer *et al.*,[23] Henze *et al.*,[19] Kampschreur *et al.*,[24] Koch *et al.*,[25] Koch *et*
655 *al.*,[26] Moussa *et al.*,[27] Strous *et al.*,[20] Vangsgaard *et al.*,[28] Wiesmann[29] and Wyffels et al.[30]

Symbol	Parameter	Median	Min	Max	Unit
$Y_{HET,O2}$	Yield for growth of X_{HET} with oxygen	0.630 [19,23]	0.609 [29]	0.800 [26]	g COD·g COD^{-1}
$Y_{HET,NO2}$	Yield for growth of X_{HET} with nitrite	0.540 [19,23]	0.540 [19,23]	0.650 [26]	g COD·g COD^{-1}
$Y_{HET,NO3}$	Yield for growth of X_{HET} with nitrate	0.540 [19,23]	0.540 [19,23]	0.650 [26]	g COD·g COD^{-1}
Y_{AOB}	Yield for growth of X_{AOB}	0.210 [22,25]	0.150 [21]	0.292 [28]	g COD·g N^{-1}
Y_{NOB}	Yield for growth of X_{NOB}	0.046 [21,22]	0.030 [25]	0.059 [29]	g COD·g N^{-1}
Y_{AMX}	Yield for growth of X_{AMX}	0.150 [25]	0.124 [28]	0.159 [20]	g COD·g N^{-1}
$i_{N,XI}$	Nitrogen content of inorganic biomass X_I	0.04 [26]	0.02 [19]	0.06 [19]	g N·g COD^{-1}
f_{XI}	Fraction of biomass converted into X_I during endogenous respiration	0.18 [24,25, 26,27]	0.08 [19]	0.20 [25, 26]	-

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Table 3 Process rates ρ_j for a nitritation/anammox system with high HET activity based on Kaelin *et al*.[31] in case of AOB, NOB and HET, and Lackner *et al*.[33] in case of AMX. The parameter values are given in Table 4 and the reference concentrations in Table 5.

	Process	Process rate
HET,O2	Growth	$\mu_{max,HET} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,HET}} \cdot \frac{S_{Ac}}{S_{Ac} + K_{Ac,HET}}$
	Endogenous respiration	$b_{max,HET} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,HET}}$
HET,NO2	Growth	$\mu_{max,HET} \cdot \eta_{NOX} \cdot \frac{K_{I,O2,HET}}{K_{I,O2,HET} + S_{O2}} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2,HET}} \cdot \frac{S_{Ac}}{S_{Ac} + K_{Ac,HET}}$
	Endogenous respiration	$b_{max,HET} \cdot \eta_{NOX} \cdot \frac{K_{I,O2,HET}}{K_{I,O2,HET} + S_{O2}} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2,HET}}$
HET,NO3	Growth	$\mu_{max,HET} \cdot \eta_{NO3} \cdot \frac{K_{I,O2,HET}}{K_{I,O2,HET} + S_{O2}} \cdot \frac{S_{NO3}}{S_{NO3} + K_{NO3,HET}} \cdot \frac{S_{Ac}}{S_{Ac} + K_{Ac,HET}}$
	Endogenous respiration	$b_{max,HET} \cdot \eta_{NOX} \cdot \frac{K_{I,O2,HET}}{K_{I,O2,HET} + S_{O2}} \cdot \frac{S_{NO3}}{S_{NO3} + K_{NO3,HET}}$
AOB	Growth	$\mu_{max,AOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,AOB}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4,AOB}}$
	Endogenous respiration	$b_{max,AOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,AOB}}$
NOB	Growth	$\mu_{max,NOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,NOB}} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2,NOB}}$
	Endogenous respiration	$b_{max,NOB} \cdot \frac{S_{O2}}{S_{O2} + K_{O2,NOB}}$
AMX	Growth	$\mu_{max,AMX} \cdot \frac{K_{O2,AMX}}{K_{O2,AMX} + S_{O2}} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4,AMX}} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2,AMX}}$
	Endogenous respiration	$b_{max,AMX} \cdot \frac{S_{NO2}}{S_{NO2} + K_{NO2,AMX}}$

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663 **Table 4** Median, minimum and maximum values of the parameters that are required for the
664 calculation of the relevant process rates in a nitritation/anammox system with high HET activity
665 (Table 2) at 20°C according Dapena-Mora *et al.*,[34] Guisasola *et al.*,[35] Gujer *et al.*,[23] Hao *et al.*,[36]
666 Henze *et al.*,[19] Hunik et al.,[37] Jayamohan *et al.*,[38] Kaelin *et al.*,[31] Kampschreur *et al.*,[24] Koch *et*
667 *al.*,[25] Koch *et al.*,[26] Manser *et al.*,[39] Moussa *et al.*,[27] Sánchez *et al.*,[40] Vangsgaard *et al.*,[28] Wett
668 and Rauch,[41] Wiesmann[29] and Wyffels *et al.*[30] Temperature dependency was considered as
669 follows: $\mu(20°C) = \mu(T) \cdot \exp(\Theta_T \cdot (20-T))$. The absolute values of the maximum endogenous
670 respiration rates were assumed to be 10% of the maximum growth rates.

Symbol	Parameter	Median	Min	Max	Unit
$\mu_{max,HET}$	Maximum growth rate of X_{HET}	3.0 [26]	2.0 [19,23]	7.2 [29]	d^{-1}
$b_{max,HET}$	Max. endogenous respiration rate of X_{HET}	-0.3			d^{-1}
$\Theta_{T,HET}$	Temperature dependency of X_{HET} rates	0.07 [19,26]			$°C^{-1}$
η_{NOX}	Anoxic reduction factor for nitrite and nitrate	0.7 [25]	0.6 [23]	0.8 [19]	-
$K_{Ac,HET}$	Saturation coefficient of X_{HET} for S_{Ac}	4.0 [19]	2.0 [19,23]	20 [19]	g COD·m^{-3}
$K_{O2,HET}$	Saturation coefficient of X_{HET} for O_2	0.20 [19,23,26,41]	0.08 [29]		g O_2·m^{-3}
$K_{NO2,HET}$	Saturation coefficient of X_{HET} for nitrite	0.50 [23,26,41]	0.14 [29]	8.0 [24]	g N·m^{-3}
$K_{NO3,HET}$	Saturation coefficient of X_{HET} for nitrate	0.50 [19,23,24,26,41]	0.12 [29]		g N·m^{-3}
$K_{I,O2,HET}$	Inhibition coefficient of X_{HET} for O_2	2.0 [24]			g O_2·m^{-3}
$\mu_{max,AOB}$	Maximum growth rate of X_{AOB}	0.770 [29]	0.481 [37]	1.0 [25]	d^{-1}
$b_{max,AOB}$	Max. endogenous respiration rate of X_{AOB}	-0.077			d^{-1}
$\Theta_{T,AOB}$	Temperature dependency of X_{AOB} rates	0.105 [25]	0.094 [36]	0.120 [31]	$°C^{-1}$
$K_{NH4,AOB}$	Saturation coefficient of X_{AOB} for ammonium	1.00 [24]	0.14 [39]	5.00 [27]	g N·m^{-3}
$K_{O2,AOB}$	Saturation coefficient of X_{AOB} for O_2	0.685 [35,38]	0.300 [29]	1.66 [40]	g O_2·m^{-3}
$\mu_{max,NOB}$	Maximum growth rate of X_{NOB}	0.720 [31,36]	0.341 [28]	1.338 [41]	d^{-1}
$b_{max,NOB}$	Max. endogenous respiration rate of X_{NOB}	-0.072			d^{-1}

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$\Theta_{T,NOB}$	Temperature dependency of X_{NOB} rates	0.070 [25]	0.061 [36]	0.078 [31]	$°C^{-1}$
$K_{NO2,NOB}$	Saturation coefficient of X_{NOB} for nitrite	1.55 [29,38]	0.280 [39]	3.00 [24]	g $N \cdot m^{-3}$
$K_{O2,NOB}$	Saturation coefficient of X_{NOB} for O_2	1.05 [24]	0.470 [39]	3.00 [40]	g $O_2 \cdot m^{-3}$
$\mu_{max,AMX}$	Maximum growth rate of X_{AMX}	0.029 [28,36]	0.020 [34]	0.080 [25]	d^{-1}
$b_{max,AMX}$	Max. endogenous respiration rate of X_{AMX}	-0.0029			d^{-1}
$\Theta_{T,AMX}$	Temperature dependency of X_{AMX} rates	0.093 [36]	0.090 [36]	0.096 [25]	$°C^{-1}$

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DOI: 10.1039/C5EW00045A

673 **Table 5** With Aquasim simulated net conversion rates, biomass concentrations and

674 compound concentrations in the reactor at steady state, with and without heterotrophic activity.

Net conversion rates		with HET	without HET	**Biomass conc.** [g COD·m^{-3}]	with HET	without HET
$r_{S,O2}$	[g O_2·m^{-3}·d^{-1}]	-507	-500	$X_{HET,O2}$	3550	0
$r_{S,Ac}$	[g COD·m^{-3}·d^{-1}]	-266	0	$X_{HET,NO3}$	1340	0
$r_{S,NH4}$	[g N·m^{-3}·d^{-1}]	-209	-209	$X_{HET,NO2}$	334	0
$r_{S,NO3}$	[g N·m^{-3}·d^{-1}]	$7.76\cdot10^{-3}$	56.9	X_{AOB}	2030	684
$r_{S,NO2}$	[g N·m^{-3}·d^{-1}]	$6.58\cdot10^{-3}$	$9.49\cdot10^{-2}$	X_{NOB}	0	74.2
$r_{S,H}$	[g H·m^{-3}·d^{-1}]	10.8	19.0	X_{AMX}	1100	691
$r_{S,TIC}$	[g C·m^{-3}·d^{-1}]	61.6	-6.93			
$r_{X,tot}$	[g COD·m^{-3}·d^{-1}]	104	19.3			

Comp. conc. in reactor		with HET	without HET
S_{NH4}	[g N·m^{-3}]	1.18	$1.15\cdot10^{-1}$
S_{NO2}	[g N·m^{-3}]	$2.19\cdot10^{-2}$	$3.16\cdot10^{-1}$
S_{NO3}	[g N·m^{-3}]	$2.59\cdot10^{-2}$	190
S_{O2}	[g O_2·m^{-3}]	$7.11\cdot10^{-3}$	$1.16\cdot10^{-1}$
S_{Ac}	[g COD·m^{-3}]	$6.99\cdot10^{-1}$	890

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677 **Table 6** Calculated concentrations and standard deviations of the main types of bacteria in a
678 nitritation/anammox reactor with heterotrophic activity (mass balances based on the activated
679 sludge model including kinetics (system 5) and including both kinetics and a biomass balance
680 (system 7)) and without heterotrophic activity (mass balances based on the activated sludge
681 model including kinetics; system 6).

	with HET				**without HET**	
	including kinetics		**including kinetics and biomass balance**		**including kinetics**	
	mg COD·L^{-1} **concentration**	% **% st.dev.**	mg COD·L^{-1} **concentration**	% **% st.dev.**	mg COD·L^{-1} **concentration**	% **% st.dev.**
$\mathbf{X_{HET,O2}}$	3470	9420	3510	2120		
$\mathbf{X_{HET,NO3}}$	1290	14900	1310	3590		
$\mathbf{X_{HET,NO2}}$	483	115000	413	31600		
$\mathbf{X_{AOB}}$	2040	3620	2030	846	684	2.4
$\mathbf{X_{NOB}}$	-181	345000	-103	154000	74.0	8.4
$\mathbf{X_{AMX}}$	1080	5280	1090	1210	694	4.9

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684 **Table 7** Required number of measurements and the corresponding standard deviation of the
685 net conversion rate to reach a certain standard deviation for the calculated biomass
686 concentration of anammox bacteria with mass balances based on the activated sludge model
687 including both kinetics and a biomass balance.

Desired % st.dev. of X_{AMX}	Required % st.dev. of the net conversion rates	Required # of measurements if % st.dev. of one measurement is 5 %
50	0.205	595
40	0.164	929
30	0.123	1651
20	0.082	3713
10	0.041	14851

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