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Dynamic investigation and modeling of the nitrogen co-metabolism in *Methylococcus capsulatus* (Bath)

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Abstract

The methanotrophic bacterium *Methylococcus capsulatus* is capable of assimilating methane and oxygen into a protein rich biomass, however the diverse metabolism of the microorganism also allows for several undesired co-metabolic side-reactions to occur. In this study, the ammonia co-metabolism in *Methylococcus capsulatus* is investigated using pulse experiments. Surprisingly *Methylococcus capsulatus* oxidizes ammonia to nitrate through a yet unknown mechanism, and fixes molecular nitrogen even at a high dissolved oxygen tension. The observed phenomena can be modeled using 14 ordinary differential equations and 18 kinetic parameters, of which 6 were revealed by Morris screening to be identifiable from the experimental data. Monte Carlo simulations showed that the model was robust and accurate even with uncertainty in the parameter values as confirmed by a statistical error analysis.

Graphical Abstract

The methanotrophic bacterium *Methylococcus capsulatus* is capable of assimilating methane and oxygen into a protein rich biomass, however the diverse metabolism of the microorganism also allows for several undesired co-metabolic side-reactions to occur. In this study, the ammonia co-metabolism in *Methylococcus capsulatus* is investigated using pulse experiments.

Keywords: Methylococcus; Nitrogen metabolism; Methanotrophs; SCP; Dynamic Model; Pulse experiment; Sensitivity; Uncertainty

Introduction

The methanotrophic bacterium *Methylococcus capsulatus* is capable of assimilating methane and oxygen into a protein rich biomass (up to 70% of the total dry weight content).

The process has historically been described by a combination of steady state stoichiometric coefficients (Eq. 1) and unstructured Monod-like kinetics (Eq. 2) (Villadsen et al., 2011):

$$q_x = \mu_{max} \frac{[CH_4]}{K_{CH_4} + [CH_4]} \cdot \frac{[O_2]}{K_{O_2} + [O_2]} C_X \quad (2)$$

While this approach is not without merit for steady state operation, it offers little to describe and enable prediction of deviations from ideal growth conditions.

The very group of enzymes that renders methanotrophy a possibility, the methane monooxygenases, enable oxidation of a multitude of carbon and nitrogen compounds, leading to numerous co-metabolic byproducts (Dalton et al., 1982).

The formation of carbon source related co-metabolic byproducts can easily be avoided, by using a methane source of high quality and by the inclusion of a small population of heterotrophic bacteria (Bothe et al., 2002), but the nitrogen related co-metabolic activity is a different story entirely. Nitrogen is the fourth most abundant compound in biological lifeforms; unlike higher hydrocarbons, it has to be supplied to the methanotrophic bacteria in one form or the other. In living organisms, nitrogen is

present in the redox state of minus three, making ammonia (NH_3) the least energy expensive inorganic nitrogen source to assimilate.

Like the higher hydrocarbons ammonia is readily oxidized by the methane monooxygenases of *Methylococcus capsulatus* even at low extracellular concentration, unless methane is in large excess (Carlsen et al., 1991; Dalton, 1977).

To make the most cost competitive single cell protein (SCP) product by *Methylococcus capsulatus*, ammonia must be the nitrogen source in the fermentation. The solubility of ammonia in aqueous fermentation broth is many orders of magnitude larger than the solubility of methane, making ammonia oxidation a real problem, even if the obvious immediate issue of gas to liquid mass transfer is addressed by the use of an appropriate reactor design (Petersen et al., 2017).

To address the ammonia oxidation problem, a better understanding of the phenomenon itself is needed. While the inhibitory effects of ammonia on methane uptake as well as the downstream fate of the oxidised ammonia in cell free extracts have been studied in great detail (Carlsen et al., 1991; Dalton, 1977), little research has discussed what happens to the ammonia in living cells. It is also apparent, that the process description presented in Eq. 1 and 2 needs to be expanded. Even if Eq. 1 is extended to include ammonia, one reaction will never be able to capture that the ammonia oxidation rate fluctuates dependent on the concentration of dissolved methane in the liquid.

The present paper experimentally investigates the nitrogen co-metabolism in *Methylococcus capsulatus* and presents a simple first bioengineering principles model, which extends Eq. 1 and 2. The model is intended to be slightly more complex than traditional black box models, but a simplification of the recently published genome

scale metabolic model for *Methylococcus capsulatus* (Lieven et al., 2018b; Lieven et al., 2018a) based on the full genome sequence of the microorganism (Ward et al., 2004). The experimental data is used to estimate sensitive model parameters and the uncertainty of the model prediction is evaluated.

Materials and methods

Methylococcus capsulatus strain Bath purchased from NCIMB (NCIMB 11132) was used in all experiments. Procedure for preparing seed cultures for the actual experiments can be found in the appendix.

Media

The same two substrates were used in all experiments, a standard nitrate mineral salt medium (NMS) and an ammonia mineral salt medium (AMS). Both substrates had an equimolar nitrogen content of 0.02molN.

- *NMS* (g/L): NaNO_3 (1.7), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (7.16), KH_2PO_4 (2.6), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05), $\text{NaFeEDTA} \cdot 3\text{H}_2\text{O}$ (0.038), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ($4.8 \cdot 10^{-4}$), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($1 \cdot 10^{-3}$), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($2.5 \cdot 10^{-3}$), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ($2 \cdot 10^{-3}$), H_3BO_3 ($7.5 \cdot 10^{-5}$), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ($2.5 \cdot 10^{-4}$), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ($1.25 \cdot 10^{-3}$), $\text{MnCl}_4 \cdot 4\text{H}_2\text{O}$ ($1 \cdot 10^{-4}$), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ($5 \cdot 10^{-5}$)
- *AMS* (g/L): NH_4Cl (1.07), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (7.16), KH_2PO_4 (2.6), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05), $\text{NaFeEDTA} \cdot 3\text{H}_2\text{O}$ (0.038), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ($4.8 \cdot 10^{-4}$), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($1 \cdot 10^{-3}$), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($2.5 \cdot 10^{-3}$), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ($2 \cdot 10^{-3}$), H_3BO_3 ($7.5 \cdot 10^{-5}$), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ($2.5 \cdot 10^{-4}$), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ($1.25 \cdot 10^{-3}$), $\text{MnCl}_4 \cdot 4\text{H}_2\text{O}$ ($1 \cdot 10^{-4}$), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ($5 \cdot 10^{-5}$)

Fermentation setup

Cells of *Methylococcus capsulatus* were cultivated in 1 L BIOSAT® B plus bioreactors (Sartorius, DK) retrofitted with two six-bladed Rushton turbines of $2 \cdot 10^{-2}$ m and $5 \cdot 10^{-2}$ m respectively. Temperature was maintained at 42°C, agitation at 10 s^{-1} and pH at 6.7 ± 0.05 by internal control loops adjusting cooling jacket water flow, motor frequency and dosing of 2M H₂SO₄ or 2M NaOH. pH was monitored using an EasyFerm PHI K8 120 electrode (Hamilton, USA). Dissolved oxygen (DO) was monitored using a VisiFerm DO ECS 120 H2 optical DO electrode (Hamilton, USA). The bioreactors were continuously sparged with $96.81 \text{ g} \cdot \text{h}^{-1}$ sterile air and $4.95 \text{ g} \cdot \text{h}^{-1}$ of sterile methane (Instrument methane 3.5, AGA, DK). Gasses were mixed under ATEX conditions prior to contact with the liquid phase to ensure that the assumption of a well-mixed gas phase was not violated. The off-gas concentrations of oxygen, carbon dioxide, nitrogen, methane and traces of inert gas species were measured with a Prima Pro Process Mass Spectrometer (Thermo-Fisher Scientific, USA). The mass spectrometer was calibrated monthly, and all gasses were dried before measurement. Dissolved oxygen and pH electrodes were calibrated prior to use according to the manufacturers standard procedures.

All cultures were initiated as a batch process on NMS medium, and then switched to continuous cultures on AMS medium once nitrate was depleted. The feed flow rate during continuous cultivations was $48.95 \cdot 10^{-3} \text{ L} \cdot \text{h}^{-1}$. Cultures were brought to steady state after several residence times (>5) before any attempt to induce co-metabolism was initiated.

Several different approaches to induce ammonia co-metabolism in a reproducible manner were attempted, and after testing both feed step up, feed step

down and pulse experiments of different ammonia concentrations ranging from 0.5 g·L⁻¹ – 50 g·L⁻¹ , a pulse size of 2 ml of a 30 g·L⁻¹ ammonia stock solution was selected. All experiments were carried out in duplicate.

Mass transfer

The gas to liquid mass transfer coefficient of oxygen in air ($k_L a^{Air}$) was determined in a prior experiment in an abiotic system aerated at an equivalent superficial gas velocity, using the hydrogen peroxide method (originally by Hickman, 1988; discussed in Villadsen et al., 2011). The mass transfer coefficient was determined to $371 \pm 5 \text{ h}^{-1}$.

Off-line Sample analysis

Off-line samples were taken from the effluent during continuous culture. Samples were taken every twenty minutes but with a ten minute offset between each duplicate, effectively giving one dataset with a ten-minute sample frequency for parameter estimation. Biomass was quantified by passing a known amount of effluent cell culture (typically around 2 mL) through a pre-dried and weighted Polyethersulfone (PES) filter with a pore size of 0.22 μm (Frisenette, DK). The filter was dried in a microwave at 150 W for 20 min. The dry weight was measured after a cooling period of approximately 30 min in a desiccator. All samples were measured as duplicates. The supernatant from the biomass measurement was assayed for nitrite, nitrate, and ammonia using LCK342, LCK339 and LCK303 colorimetric kits respectively (Hach Lange, UK) and for hydroxylamine using a 5,8-quinolinequinone-5-(8-hydroxy-5-quinolyimide) spectrophotometric assay (Frear and Burrell, 1955). No hydroxylamine could be detected in any of the samples, despite a reported detection limit of the method in the 10^{-9} gram range. To ensure that the detection

range specified by the manufacturer of the colorimetric kits was always met, 50% of the sample volume specified by the manufacturer was substituted with liquid containing a small known concentration of the chemical compound of interest. All measurements were carried out as duplicates.

Methods used to ensure measurement and sample integrity

Supernatant samples were frozen and stored at -20°C for 24 hours before they were analyzed. Nitrite is notoriously volatile and unstable in aqueous solutions, especially if samples are frozen (Goyal and Hafez, 1995; Takenaka et al., 1998). To ensure that abiotic conversion of nitrite was not compromising the samples, a known concentration of sodium nitrite was added to a 42°C nitrite free supernatant sample from the fermenter effluent as a control, with or without addition of a known amount of ammonium chloride, sodium nitrate or both. The concentrations of nitrite, nitrate and ammonium in these samples were measured after repeated freezing and thawing at room temperature over a five-day period, and concentrations of all three species in all samples were found to be unchanged throughout the experiment. According to manufacturer specifications, nitrite in the samples could interfere with the nitrate measurement. To avoid this interaction, all samples containing nitrite were treated with $40 \cdot 10^{-3}$ g sulfamidic acid before assaying for nitrate (manufacturer specification). To ensure that nitrite was effectively removed through this approach and that the sulfamidic acid did not interfere with the measurement either by itself or through interaction with other nitrogen components, a known concentration of sodium nitrite, sodium nitrate, ammonium chloride or a combination of all three was added to a nitrite free supernatant sample as a control. The control sample was afterwards treated with sulfamidic acid. Through this analysis, it was found that the addition of $40 \cdot 10^{-3}$ g

sulfamidic acid did successfully remove nitrite from the sample; sulfamidic acid did not interact with the nitrate measurement by itself, and did not form new nitrogen species with the other nitrogen compounds that caused interference in the nitrate measurements. Addition of a known concentration of nitrite, nitrate or ammonia to the liquid substrate itself or to supernatant samples was also found not to interfere (with the exception of nitrite on measurements of nitrate) with the measurements of nitrite, nitrate, or ammonia respectively. The importance of this rigorous integrity analysis of the collected data will be highlighted in the results section.

Modeling

The overall structure of the model used in this work considered whether a reaction, both of primary or co-metabolic nature, contributed to either the catabolic (energy generating) or anabolic (synthesis of new cells) pathways of the cell. The structure was then transformed into a series of global stoichiometric equations using redox balances based on an extensive literature study (Anthony, 1978; Leak and Dalton, 1986a; Leak and Dalton, 1986b; Lieven et al., 2018b). The overall model structure is shown in Figure 1, and the stoichiometric equations are provided in Table 1.

The stoichiometric equations were used to construct nine non-linear ordinary differential equations (ODEs) describing the liquid phase mass balances, four non-linear ODEs describing the gas phase mass balance, and one ODE describing the volume balance of the bioreactor. The general form of these ODEs can be seen in Eq. 3-5 respectively in Table 2. A full description of all the abbreviations can be found in the nomenclature list of the paper, and a detailed list of all the differential equations,

rate equations and parameters used in the model, can be found in the supplementary material.

Eq. 3-5 of Table 2 are fundamental balance equations describing the change of concentration of the i 'th component in the liquid phase (c_i), the liquid volume (V), and the mass of the i 'th component in the gas phase (m_i) over time in a well-mixed stirred tank reactor.

The volumetric rate of the i 'th metabolic reaction (q_i) was modeled using a series of extended empirical hyperbolic Monod specific rate functions (q_i) (Monod, 1949). The original Monod kinetic model was extended to include multiple substrates, competing substrates, and an “on/off” function was also added to simulate catabolite repression. The cell biomass (c_X) was assumed to act as one homogeneous catalyst of all reactions. To allow multiple substrates to be rate limiting and act as potential catabolite repressors, the overall specific rate function (r_i) of each Monod expression (q_i) was subject to a weight function (w_i) and/or (ω_N). A general form of these expressions is given in Eq. 6-10 in Table 2.

Applying the weight function (w_i) achieves the same as letting a simple computer algorithm search for the smallest Monod expression for each reaction participant in a row vector (Roels, 1983; Villadsen et al., 2011), without the drawbacks of loss of continuity and differentiability, as long as the tuning parameter (α) is carefully chosen. The drawbacks of Eq. 2, which tends to underestimate the growth rate at high or at similar concentrations of each individual substrate, are also avoided using this approach. The catabolic volumetric rates (q_s) and (q_{ATP}) were estimated, assuming pseudo-steady state of the concentrations of reducing equivalents and ATP respectively.

Transfer of mass of the i 'th component in the gas phase to the liquid phase (J_i) was modeled assuming complete back-mixing of the gasses, ideal gas behavior and following Henry's law. A general form of these expressions can be seen in Eq. 11-13 in Table 2.

The Henry's coefficient for the i 'th component (H_i) was adjusted for individual dependence of each H_i on system temperature (Sander, 2015). The mass transfer coefficient of the i 'th component ($k_L a^i$) was approximated by adjusting the reference mass transfer coefficient (Air to liquid) with the difference in diffusion coefficient at the system temperature of each component, respectively (Wise and Houghton, 1966). At $\text{pH} \approx 6.7$ a significant quantity of CO_2 produced by the microorganism will be present in the liquid phase as hydrogen carbonate (HCO_3^-). A simple equilibrium model was included to account for this (Royce, 1992). It was assumed that the gas phase in contact with the liquid film was saturated with water vapor, lowering the driving force according to Eq. 13. (Buck, 1981).

The resulting system of fourteen coupled non-linear ODEs with eighteen kinetic parameters was solved numerically using the implicit Runge-Kutta solver ODE15s in Matlab[®] (Mathworks, USA). This solver was chosen after an eigenvalue analysis of the system Jacobi matrix showed a large difference in magnitude between the time constants related to mass transfer rate and the microbial metabolic rate.

Sensitivity analysis, parameter estimation, uncertainty analysis and model accuracy

Given the number of unknown parameters in the model, it was necessary to investigate which parameters were sensitive to the experimental conditions, and thereby identifiable from the experimental data used in this study. To this end Morris screening was employed (Morris, 1991; Sin et al., 2009). The three free parameters in

this method, perturbation factor, number of levels and number of sample points, were selected to be 0.57, 8 and 50 respectively.

To estimate sensitive model parameters and a parameter distribution for uncertainty analysis, a slightly modified Bayesian Markov chain Monte Carlo algorithm (Hastings, 1970; Metropolis et al., 1953) was used. Due to the different orders of magnitude of the different types of measured experimental data, the error term in the likelihood function was scaled according to the standard deviation of each measurement. The algorithm was set to iterate until the mean of each parameter was stable.

The absolute error (AE) and percent average mean absolute error (MAE(%)) were calculated using Eq. 14-15

$$AE = |y(t) - \hat{y}(t)| \quad (14)$$

$$MAE(\%) = \frac{\sum_1^N |y(t) - \hat{y}(t)|}{\sum_1^N y(t)} \quad (15)$$

AE and MAE(%) were chosen, because they are conceptually easier to interpret than the root square error (RSE) and the root mean squared error (RMSE), since these overemphasize large errors compared to smaller ones due to squaring.

Results and discussion

Experimental results

The results from the two ammonia pulse experiments are shown in Figure 2. Data from each experiment have been given open square and solid square markers respectively. It is evident in Figure 2 that there was an extremely good agreement

between the data in the two experiments. An interesting observation was that the biomass concentration at steady state was approximately twice of what was expected according to Table 2 given the quantity of ammonia in the liquid substrate. Analysis of the biomass composition revealed no deficiency in nitrogen content, and when the mass balance of the molecular nitrogen (N₂) during the steady state, before pulsing with ammonia, was investigated in detail, it turned out that less molecular nitrogen left the fermenter, compared to the amount that entered. This difference was large enough to account for the elevated levels of biomass, and it was concluded that even at a dissolved oxygen tension of $\sim 2 \cdot 10^{-3} \text{ g} \cdot \text{L}^{-1}$ (equivalent to an oxygen tension of $\sim 22\%$ saturation at atmospheric pressure and 25°C) nitrogen fixation occurred. Nitrogen fixation in *Methylococcus capsulatus* has been reported in literature (Murrell and Dalton, 1983; Zhivotchenko et al., 1995), and while it is surprising that it occurs at such high oxygen tensions, the model was updated accordingly by the inclusion of Eq. 16

The derivation of Eq. 16 is given in the supplementary material. It was assumed that nitrogen was fixed without the formation of hydrogen due to the ability of *Methylococcus capsulatus* to express hydrogenases and utilize hydrogen (Hanczár et al., 2002). The most surprising result, and the sole reason for the rigorous data integrity analysis described in the Materials and Methods section can be seen in Figure 2 when observing the dynamics of the nitrate concentration over time. Nitrate was at no point in time added to the fermentation, but shortly after the addition of the ammonium pulse, comparatively large concentrations could be measured in the fermentation broth. This result was puzzling to say the least, as many (excellent)

scientific works that preceded the present reported nitrite to be the terminal metabolite of the co-metabolic pathway in methanotrophs (Dalton, 1977; DiSpirito et al., 2004; Zahn et al., 1994). The integrity analysis was performed out of sheer respect for the preexisting work, and it left us with no other choice but to conclude, that not only does *Methylococcus capsulatus* oxidize ammonia to nitrite; but also that the oxidation proceeds all the way to nitrate. This realization led to more questions than answers; it is one thing to show that nitrate is produced, but an entirely different thing is to explain *how*. We found the better model fit to data if this phenomenon was explained as a direct fusion of nitrite and oxygen catalyzed by the presence of microbial cells. The model was updated accordingly by the inclusion of Eq. 17 and 18.

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One can only speculate if the physical phenomenon described by Eq. 17 is correct as numerous reactions of both biotic (Hink et al., 2017; Prosser, 1990) and abiotic (Damschen and Martin, 1983; Ignarro et al., 1993; Park and Lee, 1988) nature, could account for the observed production of nitrate. A small experiment where a 2 mL pulse of a 10 g·L⁻¹ NaNO₂ stock solution was added to the steady state culture resulted in no observable production of nitrate, suggesting that the intracellular matrix may play a role in the nitrate production, but further research is clearly necessary. The result from the nitrite pulse experiment can be seen in figure 3.

Sensitivity analysis

The Morris method calculates a number of effects of one parameter on one output (elementary effects) based on the number of levels and number of sample points. A distribution function can then be approximated by calculating the mean and standard deviation of all elementary effects on one output. The parameters that are identifiable from the experimental data can be determined visually by selecting parameters whose elementary effect on a given output has a larger impact than the mean and standard deviation of the distribution function. In Figure 4, the mean of the distribution function is centered on zero, and the standard deviation is indicated by two black lines.

By visual inspection of Figure 4, the six parameters $\alpha, \mu_{max}, \delta, V_{NO2}, K_{S,CH4}, V_{NO3}$ were deemed identifiable from the experimental data.

Parameter Estimation, Uncertainty analysis and model accuracy

The estimated parameter values, their standard deviation and correlation matrix are given in Table 3. The effect of how the correlation coefficient affected each parameter can be seen in Figure 5. As evident from Table 3, accurate estimates of most of the parameters could be obtained from the experimental data. A maximum steady state growth rate (μ_{max}) and methane affinity ($K_{S,CH4}$) value of 0.228 h^{-1} and $7.5 \cdot 10^{-5} \text{ M}$, respectively, is strikingly similar to the values reported in literature when *Methylococcus capsulatus* expresses the soluble form of methane monooxygenase (sMMO) (Joergensen and Degn, 1987). Since no additional copper was added to the original NMS medium (Whittenbury et al., 1970), this is exactly as expected. Since the other parameters are estimated for the first time, it is difficult to compare them to literature values. If one looks at other microorganisms, the amount of ATP produced

by shuffling electrons through the electron transport chain as well as the amount of ATP required to form biomass, (δ) and (α) respectively, appears to be rather low (Roels, 1983). By looking at Table 3 and Figure 5, it becomes evident, that no unique estimate for (δ) and (α) can be obtained from the data, and that the two are linearly correlated. It would therefore appear that any computational method trying to estimate the value of (δ) and (α) would struggle to find an accurate estimate.

A propagation of the uncertainty of the parameters to the model predictions is shown in Figure 6. It is evident from Figure 6 that the uncertainties in the estimated parameters have little effect on the predictive ability of the model as long as the parameter covariance is considered, and visually the concentrations and quantities predicted by the model seem to be sound when compared to the experimental data. The exceptions are the predicted carbon dioxide in the exhaust gas, and the predicted nitrite in the liquid phase. A better fit of the nitrite data could be achieved, but this comes at the cost of a poorer fit of the dissolved oxygen data. Were the model ever to be converted into a soft sensor, it would be important to have a good agreement between model and state variables that are easily measured on-line. Therefore, the model fit that better predicts the dissolved oxygen is chosen. The discrepancy between modeled and measured exhaust carbon dioxide, was most likely due to an unmodifiable dead band in the pH control algorithm of the BIOSAT® B control tower. If one inspects the nitrite producing reaction in Table 1, it is seen that this reaction is associated with quite a significant production of acid. This was also observed during the cultivation, and the controller dead band resulted in a slight oscillatory behavior of the pH when co-metabolism was occurring. This undoubtedly disturbed the $\text{CO}_2/\text{HCO}_3^-$ equilibrium, which could lead to the error observed in Figure 6.

A quantitative display of the error of the model prediction with the added parameter uncertainty is shown in Figure 7. Assessing the total error of a model should never be done without looking at individual state variable error, and vice versa, which is why we choose a metric for both. It is apparent that the absolute error (AE) for each state variable is very low compared to the magnitude of each state variable. This is also seen in the percentage mean absolute error (MAE(%)) which never exceeds 1% of the total mass in the system, even if parameter uncertainty is taken into account.

Conclusion

The Nitrogen co-metabolism in living cells of *Methylococcus capsulatus* was investigated through pulse experiments. It was discovered that *Methylococcus capsulatus* oxidizes ammonia all the way to nitrate, and not just nitrite as previously reported in literature. The exact mechanism of nitrate formation could not be determined from data. It was also discovered that *Methylococcus capsulatus* fixes molecular nitrogen even at a dissolved oxygen tension of 22%. A first principles model consisting of 14 ordinary differential equations and 18 kinetic parameters was formulated to describe the observed phenomena. A sensitivity analysis revealed that out of the 18 kinetic parameters, α , μ_{max} , δ , V_{NO2} , $K_{s,CH4}$, V_{NO3} (see the list of Nomenclature) were identifiable from the experimental data. After estimating the parameters, an inspection of the covariance matrix revealed that the parameters α and δ were highly correlated, which affected their accuracy. Good estimates of the remaining parameters could be obtained, and the estimated values for μ_{max} and $K_{s,CH4}$ (0.228 h^{-1} and $7.5 \cdot 10^{-5}\text{ M}$ respectively) were similar to literature values. An uncertainty analysis showed that uncertainty in the parameter estimates had little

effect on the predictive accuracy of the model as long as the covariance matrix was taken into account. Evaluating the model error using the absolute error (AE) and percentage mean absolute error (MAE(%)) agreed with the result of the uncertainty analysis. MAE(%) never exceeded 1% of the total mass in the system. AE was always low compared to the magnitude of each state variable.

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Nomenclature

SymbolName (unit)

c_i	Concentration of the i'th component in the liquid phase ($\text{mol}\cdot\text{L}^{-1}$)
$c_{i,f}$	Concentration of the i'th component in the liquid substrate feed ($\text{mol}\cdot\text{L}^{-1}$)
c_i^*	Saturation concentration of i'th component in the liquid phase ($\text{mol}\cdot\text{L}^{-1}$)
F	Feed flow rate of liquid substrate ($\text{L}\cdot\text{h}^{-1}$)
F_{out}	Effluent flow rate ($\text{L}\cdot\text{h}^{-1}$)
F_{evap}	Evaporation rate ($\text{L}\cdot\text{h}^{-1}$)
H_i	Henry's coefficient for the i'th component in the liquid phase ($\text{atm}\cdot\text{L}\cdot\text{mol}^{-1}$)
I	Concentration of the inhibitory component in the liquid phase ($\text{mol}\cdot\text{L}^{-1}$)
J_i	Mass transfer flux between the gas and liquid phase ($\text{mol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$)

$k_L a^i$	Gas-liquid mass transfer coefficient of the i'th compound (h^{-1})
$K_{s,i}$	Monod constant for the i'th compound in the liquid phase ($\text{mol}\cdot\text{L}^{-1}$)
K_I	Inhibitor affinity constant ($\text{mol}\cdot\text{L}^{-1}$)
m_i	Mass of the i'th component in the gas phase (mol)
$m_{i,f}$	Mass of the i'th component in the gas feed (mol)
$P_{ambient}$	Total system pressure (atm)
P_i^{exit}	Partial pressure of the i'th component in the dried exhaust gas (atm)
$P_{H_2O}^{sat}$	Saturated water vapor pressure at operating temperature (atm)
q_i	Volumetric rate of the i'th metabolic reaction ($\text{mol}\cdot\text{L}^{-1}\text{ h}^{-1}$)
r_i	Overall specific rate function of the i'th metabolic reaction ($\text{mol}\cdot(\text{mol } c_X)^{-1}\cdot\text{h}^{-1}$)
t	Time (h)
T	Temperature ($^{\circ}\text{C}$)
V	Total liquid volume (L)
V_i	Maximum specific rate of non-growth reactions ($\text{mol}\cdot(\text{mol } c_X)^{-1}\cdot\text{h}^{-1}$)
w_i	Weight function applied to the i'th individual specific rate function
$y(t)$	Vector of all measurements of c_i and m_i at time t ($\text{mol}\cdot\text{L}^{-1}$ or mol)
$\hat{y}(t)$	Vector of all model predicted values of c_i and m_i at time t ($\text{mol}\cdot\text{L}^{-1}$ or mol)

α Tuning parameter

Greek Name (unit)

α ATP demand in biomass forming reactions ($\text{mol ATP} \cdot (\text{mol } c_X)^{-1}$)

δ ATP yield from the electron transport chain ($\text{mol ATP} \cdot (\text{mol } 2e^-)^{-1}$)

μ_{max} Maximum specific growth rate at steady state (h^{-1})

q_i Individual specific rate function of the i'th metabolic reaction ($\text{mol} \cdot (\text{mol X})^{-1} \cdot \text{h}^{-1}$)

ω_N Weight function to induce catabolite repression expression

Abbreviations Name (unit)

AE Absolute error ($\text{g} \cdot \text{L}^{-1}$ or g)

MAE(%) Average mean absolute error

RSE Root squared error ($\text{g} \cdot \text{L}^{-1}$ or g)

RMSE Root mean squared error

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Figures

Figure 1: Overall structure of the model used in this study. Top: Catabolic reactions where the dashed line indicates the flow of electrons from substrates to the electron transport chain to generate ATP. Bottom: Anabolic reactions where carbon and nitrogen are assimilated into biomass. Flow of electrons is indicated by the dashed line, and the dotted line indicates that ATP is consumed.

Figure 2: Data from the ammonia pulse experiment. White square and black square markers are used for each individual experiment respectively. The x-axis shows the time in hours, the y-axis shows the concentration in g·L⁻¹ (for liquid phase components) or the mass in g (for gas phase components) for the respective component (also listed on the y-axis).

Figure 3: Data from the liquid phase of the nitrite pulse experiment. Different markers are used for each component, and the explanation about which marker relates to what component is shown on the legend of each graph. The x-axis shows the time in hours. The y-axis shows the concentration in $\text{g}\cdot\text{L}^{-1}$

Figure 4: Results from the Morris screening experiment. The black lines show the standard deviation of the average effect of all parameters on the given output. Parameters (o) that are identifiable from the experimental data are found outside the area covered in between the black lines. The mean of the average effect is shown on the x-axis, the standard deviation of the average effect is shown on the y-axis. The specific output is specified in the title of each plot.

Figure 5: Parameter distribution generated by the Markov chain Monte Carlo algorithm. The histograms in the diagonal show the parameter distribution, where the neighboring graphs shows a visualization of the correlation matrix found in Table 3. Strong correlation between α and δ is clearly observed.

Figure 6: Effects of parameter uncertainty on model prediction accuracy. The black line shows mean predicted value; the dash-dotted line shows the 95% confidence interval. The experimental data points are shown as the black squares. The x-axis shows the time in hours, the y-axis shows the concentration in $\text{g}\cdot\text{L}^{-1}$ (for liquid phase components) or the mass in g (for gas phase components) for the respective component. Component and units on the y-axis are shown in the title of each graph.

Figure 7: Error analysis of the model predictions. **Top:** the gray area shows the absolute error (AE) of the mean model prediction of each measured output at each time point. The dash-dotted line indicates the error if the 95% confidence interval of the model prediction is taken into account. The x-axis shows the time in hours, the y-axis shows the absolute error in $\text{g}\cdot\text{L}^{-1}$ (for liquid phase components) or in g (for gas phase components) for the respective component. The name of each component and its units (on the y-axis) are shown in the title of each graph. **Bottom:** the gray area shows the percentage mean absolute error (MAE(%)) of the mean model prediction of all measured outputs at each time point. the dash-dotted line indicates the error if the 95% confidence interval of the model prediction is taken into account. The x-axis shows the time in hours; the y-axis shows the percentage mean absolute error as percentage of the total mass in the system.

Tables

Table 1: Metabolic reactions included in the model, each reaction is written in terms of C-mol. Volumetric reaction rate of species (q_i) are in C-mol·L⁻¹·h⁻¹ units.

Catabolic reactions

Maintenance

Anabolic reactions

Co-Metabolic reactions

Table 2: Generalised equations used in the model

Mass Balances

$$\frac{dc_i}{dt} = q_i + \frac{F}{V} c_{i,f} + J_i - \left(\frac{F_{out}}{V} + \frac{1}{V} \frac{dV}{dt} \right) c_i \quad (3)$$

$$\frac{dm_i}{dt} = m_{i,f} - J_i V - m_i \quad (4)$$

Volume Balance

$$\frac{dV}{dt} = F - F_{out} - F_{evap} \quad (5)$$

Rate Equations

$$q_i = r_i X \quad (6)$$

$$r_i = \begin{cases} \mu_{max,i} \frac{\sum_1^N w_{i,N} Q_{i,N}}{\sum_1^N w_{i,N}} & q_i = q_{x,i} \\ V_i \frac{\sum_1^N w_{i,N} Q_{i,N}}{\sum_1^N w_{i,N}} & q_i \neq q_{x,i} \end{cases} \quad (7)$$

$$Q_i = \begin{cases} \frac{c_i}{K_{s,i} \left(1 + \frac{1}{K_I} \right) + c_i} & \text{competing substrates} \\ \left(\frac{\sum_1^N \omega_N \frac{1}{1+e^{K_{I,N}-I_N}}}{\sum_1^N \omega_N} \right) \frac{c_i}{K_{s,i} + c_i} & \text{catabolite repressed} \\ \frac{c_i}{K_{s,i} + c_i} & \text{otherwise} \end{cases} \quad (8)$$

$$w_i = (1 + Q_i)^\alpha, \alpha \in \{10; 100\} \quad (9)$$

$$\omega_N = \left(1 + \frac{1}{1 + e^{K_{I,N}-I_N}} \right)^\alpha, \alpha \in \{10; 100\} \quad (10)$$

Mass Transfer

$$J_i = k_L a^i (c_i^* - c_i) \quad (11)$$

$$c_i^* = \frac{P_i^{exit} (P_{ambient} - P_{H_2O}^{sat})}{H_i} \quad (12)$$

$$P_{H_2O}^{sat} = 6.03 \cdot 10^{-3} e^{\left(\left(18.678 - \frac{T}{234.5} \right) \left(\frac{T}{257.14 + T} \right) \right)} \quad (13)$$

Table 3: Values of the parameters that were estimated from the experimental data, their unit, standard deviation and correlation matrix

Parameter	Parameter value	Unit	σ	Correlation matrix					
				μ_{max}	V_{NO2}	K_{SCH4}	α	V_{NO3}	δ
μ_{max}	0.228	h^{-1}	0.002	1	-0.09	0.31	-0.55	0.06	-0.55
V_{NO2}	0.032	$mol \cdot (mol \ c_X)^{-1} \cdot h^{-1}$	0.001		1	-0.14	0.16	-0.32	0.14
K_{SCH4}	$7.50 \cdot 10^{-5}$	$mol \cdot L^{-1}$	$1.24 \cdot 10^{-5}$			1	-0.41	-0.09	-0.12
α	0.020	$Mol \ ATP \cdot (mol \ c_X)^{-1}$	0.009				1	0.01	0.95
V_{NO3}	0.023	$mol \cdot (mol \ c_X)^{-1} \cdot h^{-1}$	0.002					1	0.002
δ	0.020	$Mol \ ATP \cdot (mol \ 2e^{-})^{-1}$	0.003						1