The Use of a Metabolically Structured Model in the Study of Growth, Nitrification, and Denitrification by *Thiosphaera pantotropha*

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Thiosphaera pantotropha is capable of aerobic heterotrophic nitrification and both aerobic and anaerobic denitrification. These phenomena have been studied in acetate-limited aerobic and anaerobic continuous cultures supplied with ammonia and nitrate. The internal reaction rates were defined, based on biochemical knowledge. The observable external conversion rates are related through a linear equation on the basis of the specified internal reaction rates. The linear equation is a Pirt relation extended for microbial systems with multiple electron donors (acetate and ammonia) and electron acceptors (oxygen and nitrate). The coefficients in this equation were estimated from the continuous culture measurements, and are composed of parameters involved in ATP production and consumption by the microorganism. It is shown that with realistic values for these parameters, the metabolically structured model describes the aerobic as well as the anaerobic experiments.

INTRODUCTION

In various types of waste water, nitrogen compounds such as ammonia and nitrate can be an environmental problem. These compounds are normally removed microbiologically in two steps, exploiting two different types of microorganisms. The first step is aerobic, and involves the oxidation of ammonia to nitrate by autotrophic bacteria which derive energy from the reaction (nitrification). In the second step, the nitrate is reduced anaerobically to nitrogen gas, generally by heterotrophs or methylotrophs (denitrification). The emphasis of studies on nitrification has mainly been concentrated on the autotrophic organisms. However, many heterotrophs are also capable of nitrification.[1,2] The importance of heterotrophic nitrification is still a matter of debate, especially as heterotrophic nitrification does not generate any energy. The specific nitrifying activity of the heterotrophs is said to be 10^3–10^4 times lower than that of the autotrophs, and therefore heterotrophic nitrification is often considered to be of minor ecological significance.[1,3,4] However, this activity was measured by the accumulation of nitrite or nitrate. Many heterotrophic nitrifiers are able to denitrify aerobically[5] as well as anaerobically.[6] In this way, ammonia is directly converted to nitrogen gas and nitrite or nitrate will not accumulate. When making mass balances for continuous cultures, it was found that the nitrification activity (in terms of ammonia oxidized) of at least one heterotrophic nitrifier/aerobic denitrifier, *Thiosphaera pantotropha*, is only 10–10^3 times lower than the autotrophs.[5] While growing as heterotrophs (during which heterotrophic nitrification takes place) the growth rate of it *T. pantotropha* is much higher than when it would grow autotrophically. It seems likely that, as other bacteria of this physiological type are studied, it will be found that most nitrification rates have been underestimated because of the simultaneous nitrite reduction. Thus, in view of the fact that heterotrophs generally outnumber autotrophs in the bacteria communities found in most waste water treatment systems, sediments and soils, heterotrophic nitrifying organisms might well be of greater significance than previously thought.

In wastewater treatment, the nitrification step is often a bottleneck. The residence time in the nitrification reactor is mainly determined by the maximum growth rate of the autotrophs, which is relatively low. In view of their higher growth rates and their ability to convert ammonia to nitrogen gas in one step, heterotrophic nitrifiers/denitrifiers might be an attractive alternative for waste water treatment. Additionally, a pretreatment step, where organic material is removed prior to autotrophic nitrification, would not be necessary if heterotrophs were used. However, before these organisms can successfully be applied, the heterotrophic nitrification/denitrification processes must be fully understood.

To evaluate the potential application of such microorganisms, mathematical modelling of their performance is

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advisable. These mathematical models can also be used, when extended, to predict the behavior of immobilized organisms.[7]

In this paper growth and simultaneous nitrification and denitrification by homogeneous, continuous cultures of *T. pantotropha* is described mathematically. Hereby it is assumed that the aerobic nitrification/denitrification is due to two reactions, in which ammonia is first oxidized to nitrite, and subsequently reduced to nitrogen gas, and that the anaerobic denitrification proceeds according to the "conventional" reaction in which nitrate is reduced to nitrite and consequently to nitrogen gas.

MATERIALS AND METHODS

The model is based on the results of experiments using chemostat cultures of *Thiosphaera pantotropha* LMD 82.5. This organism was originally isolated from a sulfide-oxidizing, denitrifying industrial wastewater treatment system in which it was one of the dominant mixotrophs.[8] The growth media and analytical techniques, together with the full experimental results, can be found in ref. 9. All of the cultures were acetate limited and supplied with ammonia as nitrogen source (32 mol m^{-3}). Aerobic cultures were maintained at 80% air saturation, and nitrate was supplied to some cultures, as mentioned in the text.

METABOLICALLY STRUCTURED MODELLING

On the basis of biochemical knowledge and stoichiometry, the reactions which play a role in the metabolism of a heterotrophic microorganism can be formulated and are called internal reaction rates. The rates of these reactions can be related to the substrate consumption and product formation rates (observable conversion rates), as described by Roels et al.,[10-13] whose approach and notation is followed here. First, the principles and the assumptions for the metabolically structured model will be given and applied to aerobic nitrification/denitrification of ammonia and denitrification of nitrate. Second, the model will be applied to the anaerobic denitrification of nitrate. For ease of notation, $NADH_2$ instead of $NADH + H^+ + e^-$ will be used in the text and formulae.

The assumptions necessary for a metabolically structured model are as follows:

- Biomass is in pseudo-steady state with respect to all compounds being considered in the metabolism. Consequently, there is no net accumulation of ATP, $NADH_2$, and precursors.
- Compounds such as $NADH_2$ and ATP do not leave the cell in significant amounts. There is no net transport to the environment.
- For the compounds for which a pseudo-steady-state hypothesis is justified, and which are not transported to the environment, generation during metabolism must match consumption. This can be applied to $NADH_2$, ATP, and precursors. Their net conversion rate is therefore zero.
- Stoichiometric coefficients used in the metabolic reactions related to energy conversion are independent of the experimental growth conditions used (type of substrate, high or low oxygen concentration, etc.).

The reactions and the procedure to make a metabolic structured model for *T. pantotropha* are outlined below.

AEROBIC NITRIFICATION/DENITRIFICATION OF NH_3 AND HNO_3

The Internal Reaction Rates

The behavior of aerobic cultures which have either been supplied with ammonia alone or ammonia and nitrate, can be described as follows.

Biomass Formation Reaction, Rate r_1

Two overall reactions are involved in the synthesis of biomass. The first, anabolism, involves the biomass. The formation of biomass follows a specified stoichiometry which only depends on the nature of the substrate used. The nitrogen source is assumed to be ammonia:

$$\begin{aligned}(1 + \alpha_{11})CH_{a2}O_{b2} + c1NH_3 \xrightarrow{r_1} (\alpha_{12} + K)ATP \\ + CH_{a1}O_{b1}N_{c1} + \alpha_{11}CO_2 \\ + [b_2(1 + \alpha_{11}) - 2\alpha_{11} - b1]H_2O \\ + [(0.5a2 - b2)(1 + \alpha_{11}) \\ + b1 - 0.5a1 + 2\alpha_{11} + 1.5c1]NADH_2 \quad (1)\end{aligned}$$

In this reaction it is assumed that $NADPH_2$ is equivalent to $NADH_2$. The extra energy content of $NADPH_2$ is implicitly present in the amount of ATP necessary for biomass synthesis. The amount of ATP involved in the anabolism is divided in the amount necessary for the formation of biomass precursors (α_{12}) and the amount necessary for the polymerization of precursors to biomass (K). Moreover, as can be seen in eq. (1) the amount of biomass and substrate are expressed in carbon-mole, being the amount containing one mole of the element carbon.

The second reaction involves ATP used in maintenance processes. The ATP requirement for maintenance is first order in the amount of biomass:

$$r_{ATP} = -m_{ATP}C_X = \frac{-m_{ATP}}{\mu}r_1 \quad (2)$$

The amount of ATP required for maintenance generally ranges from 0.005 to 0.05 mol/C mol h.[10]

The biomass composition determined from element analysis is $CH_{1.9}O_{0.5}N_{0.25}$.[9] Equations (1) and (2) can be combined to give an equation for biomass formation with an ATP requirement composed of maintenance, synthesis of precursors, and precursor polymerization yielding the following balance equation for the biomass formation rate, r_1:

$$\begin{aligned}(1 + \alpha_{11})CH_2O + 0.25NH_3 \xrightarrow{r_1} \left(\alpha_{11} + K + \frac{m_{ATP}}{\mu}\right)ATP \\ + CH_{1.9}O_{0.5}N_{0.25} + \alpha_{11}CO_2 + (0.5 - \alpha_{11})H_2O \\ + (2\alpha_{11} - 0.075)NADH_2 \quad (3)\end{aligned}$$

The amount of ATP required for the synthesis of biomass from acetate ($K + \alpha_{12}$) generally ranges from 2 to 3 mol/C mol h.[14] Furthermore, it is assumed that in the assimilation reaction, about 0.3 mol carbon dioxide is produced per C mol biomass,[15] $\alpha_{11} = 0.3$.

Substrate Catabolism Reaction, Rate r_2

Part of the substrate is catabolized to produce ATP and reducing equivalents:

$$CH_{a2}O_{b2} \xrightarrow{r_2} CO_2 + \alpha_{21}ATP + a2NADH_2 + (b2 - 2)H_2O \quad (4)$$

For acetate this gives the following balance equation for the catabolism reaction rate, r_2.

$$CH_2O + H_2O \xrightarrow{r_2} CO_2 + \alpha_{21}ATP + 2NADH_2 \quad (5)$$

To find a value for α_{21}, information is required about the dissimilation of acetate. Acetate is taken up by the cell by means of active transport and must be activated to form acetyl-COA. Both processes theoretically require, in total, 2 ATP per mol acetate.[16] The citric acid (TCA) cycle contains one substrate phosphorylation step, yielding 1 ATP. It is assumed that in the conversion of isocitrate to ketogluterate, 1 $NADPH_2$ is produced, 1 $FADH_2$ is generated by the conversion of succinate to fumarate, and 2 $NADH_2$ are produced during the two subsequent dehydrogenation steps. The subsequent oxidation of $FADH_2$ in the electron transfer chain involves one less proton translocation step than $NADH_2$. In the model, only $NADH_2$ is considered, therefore a correction must be made for $FADH_2$ by subtracting 1 ATP. Thus there is a net consumption of 2 ATP per dissimilation of one molecule of acetate,[16] or 1 ATP per C mol acetate, $\alpha_{21} = -1$.

Oxidative Phosphorylation

T. pantotropha possesses both cytochrome aa_3 and cytochrome o as terminal oxidases.[5] The available literature[17] indicates that there are fewer proton translocation steps involved in the reduction of oxygen via cytochrome o than when aa_3 is involved. However, the model cannot discriminate between the use of two terminal oxidases, thus the overall value for the ATP produced per two electrons for cytochrome aa_3 and o is given by δ. From experiments with other organisms, it was found that the ATP/2*e* will range from 1.5 to 3.[10,17,18]

$$0.5O_2 + NADH_2 \xrightarrow{r_3} H_2O + \delta ATP \quad (6)$$

Nitrification/Denitrification

For a particular microorganism, specific biochemical reactions must be added to reactions r_1 to r_3. For *T. pantotropha* these are the nitrification and denitrification reactions. The basis of the modelling described in this article is the electron transfer chain of *T. pantotropha*.[5] Figure 1 shows a working model of the transport chain of *T. pantotropha*

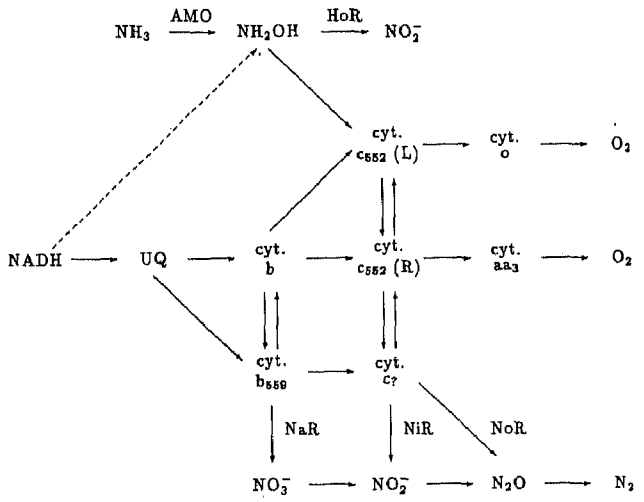

Figure 1. Schematic representation of the cytochrome chains devised to explain electron tranport in *Thiosphaera pantotropha*: (Cyt.) cytochromes; (L and R) arbitrary designations to distinguish 2 cyts. c_{552}; (cyt. $c_?$) indicates one or more additional cytochrome c; (UQ) ubiquinone; (NaR) nitrate reductase; (NiR) nitrite reductase; (Nor) nitrous oxide reductase; (AMO) ammonia monooxygenase; (HoR) hydroxylamine oxidoreductase. The dashed line indicates uptake of $NADH_2$.

based on physiological measurements and preliminary cytochrome spectra of cells grown under various conditions[5] and on previous experiments with *Paracoccus denitrificans*.[17] There are some obvious differences from the cytochrome chain of *P. denitrificans* including the nitrite reductase of *T. pantotropha* being a copper enzyme, and that of *P. denitrificans* being a cytochrome cd. The branch points of the cytochrome chain of *T. pantotropha* have not yet been determined, and they have therefore been assumed to be similar to those of *P. denitrificans*. Nitrification and aerobic denitrification by *T. pantotropha* may be explained by a bottleneck in the electron transfer somewhere between cytochrome c and oxygen via cytochrome aa_3, resulting in a greater degree of reduction in the electron transport chain, allowing electrons to flow to the reductases of the denitrifrication.

Nitrification of Ammonia to Nitrite

Nitrification of ammonia to nitrate involves the following:

$$NH_4 + 2O_2 \xrightarrow{r_4} NADH_2 + HNO_2 + 2H_2O + \alpha_{41}ATP \quad (7)$$

The involvement of ATP in heterotrophic nitrification is not known. ATP is not necessary for the in vitro oxidation of ammonia or hydroxylamine by crude cell free extracts. However, $NADPH_2$ rather than $NADH_2$ is required by the ammonia monooxygenase of *T. pantotropha*.[19] In the TCA-cycle, 1 $NADPH_2$ can be produced per molecule of acetate, which can be used as a reductor in the synthesis of biomass. If this production route is not able to cope with the demand of the cell, $NADPH_2$ can also be produced from $NADH_2$ by a transhydrogenase which may require an amount of ATP ranging between 0 and 1 per $NADPH_2$.[16] It may well be that due to nitrification, the cell

is forced to use this energy expensive route. It is assumed that, as with the autotrophic ammonia oxidases, the two enzymes needed for heterotrophic nitrification, ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HOR) are located at the periplasmic side of the cell membrane,[5] making active transport of ammonia and/or hydroxylamine unnecessary. The overall reaction including production of $NADPH_2$ is assumed to require a minimum of 1 ATP. Hence the value for α_{41} will be at least -1.

Nitrate Reduction to Nitrite

Nitrate reduction to nitrate involves

$$HNO_3 + NADH_2 \xrightarrow{r_5} HNO_2 + H_2O + \alpha_{51}ATP \quad (8)$$

Denitrification of Nitrite

Denitrification of nitrate involves

$$HNO_2 + 1.5NADH_2 \xrightarrow{r_6} 0.5N_2 + 2H_2O + \alpha_{61}ATP \quad (9)$$

Stouthamer et al.[17] found that in *P. denitrificans* the reductions of nitrate to nitrite, nitrite to nitrous oxide, and nitrous oxide to nitrogen all yield the same maximum ATP/2*e*, and that the value for each step is lower than that for oxygen. In analogy with these findings, the maximum theoretical values for α_{51} and α_{61} will be 1.67 and 2.51, respectively. However, *T. pantotropha* has a lower efficiency in proton translocation or oxygen and all the denitrifying steps (D. Castignetti, personal communication). On the basis of these results the value of α_{61} and α_{51} should be approximately half of these values. It may thus be concluded that $\alpha_{51} + \alpha_{61}$ will be between 1.0 and 1.5, and $\alpha_{41} + \alpha_{61}$ will be about -0.2 to -1.2.

Linear Equation between Observable Conversion Rates

The reactions r_1 to r_6 are termed "internal reactions," and are based on biochemical knowledge and stoichiometry. These reactions occur within the cell and cannot be observed directly. However, the internal reaction rates can be related to the observable conversion rates outside of the cell. If, for *T. pantotropha*, the cell is represented as a box, the flows of substrate and product to and from the cell can be schematically given as shown by Figure 2. Due to growth, nitrification, and denitrification, ammonia, nitrite, nitrate, oxygen, and acetate are consumed and biomass, nitrogen, and carbon dioxide are produced. These conversion rates are related to the internal reaction rates. Knowledge of the internal reactions gives a direct relation between the observable conversion rates.

For steady-state conditions in a fermentor, the conversion rate r_i is given by the product of the dilution rate and the concentration difference between the incoming and outgoing streams to the fermentor:

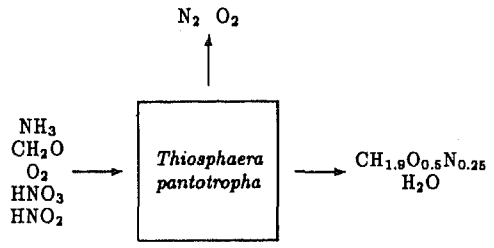

Figure 2. Black box model of *T. pantotropha* showing the relevant components entering and leaving the cell.

$$r_i = D(C_{i,\text{out}} - C_{i,\text{in}}) \quad (10)$$

where r_i is positive for production and negative for consumption of components. In the case of *T. pantotropha*, r_S, r_{NH_3}, r_{O_2}, and r_{HNO_3} are negative, and r_X, r_{N_2}, and r_{HNO_3}, are positive. The conversion rates are related by a linear equation with coefficients composed of the parameters (stoichiometric coefficients) of the internal reactions. The observable conversion rates are determined by the internal reaction rates. If the conversion rates are expressed as a function of the internal reaction rates it follows that:

$$r_S = -(1 + \alpha_{11})r_1 - r_2 \quad (11)$$

$$r_{NH_3} = -0.25r_1 - r_4 \quad (12)$$

$$r_{HNO_2} = r_4 + r_5 - r_6 \quad (13)$$

$$r_{N_2} = 0.5r_6 \quad (14)$$

$$r_X = r_1 \quad (15)$$

$$r_{O_2} = -0.5r_3 + r_2 \quad (16)$$

$$r_{CO_2} = \alpha_{11}r_1 + r_2 \quad (17)$$

$$r_{H_2O} = (0.5 - \alpha_{11})r_1 - r_2 + r_3 + 2r_4 + r_5 + 2r_6 \quad (18)$$

$$r_{HNO_3} = -r_5 \quad (19)$$

As it is assumed that there is no net accumulation of $NADH_2$ and ATP, $r_{NADH_2} = 0$; $r_{ATP} = 0$:

$$r_{NADH_2} = (2\alpha_{11} - 0.075)r_1 + 2r_2 - r_3 - r_4 - r_5 - 1.5r_6 = 0 \quad (20)$$

$$r_{ATP} = \left(\alpha_{12} + K + \frac{m_{ATP}}{\mu}\right)r_1 + \alpha_{21}r_2 + \delta r_3 + \alpha_{41}r_4 + \alpha_{51}r_5 + \alpha_{61}r_6 = 0 \quad (21)$$

As there are 15 reaction rates available (r_1 to r_6 and r_S to r_{HNO_3} and 11 equations (r_S to r_{ATP}), the system can be described with four conversion rates. This results in a linear relation between the five conversion rates necessary to calculate the coefficients in the linear relation. The following conversion rates determined during the continuous culture experiments were selected: r_X, r_S, r_{NH_3}, r_{HNO_2}, and r_{HNO_3}. If the internal reaction rates are expressed as functions of the

conversion rates, it follows that:

$$r_1 = r_X \tag{22}$$

$$r_2 = -(1 + \alpha_{11})r_X - r_S \tag{23}$$

$$r_3 = -1.45r_X - 2r_S + 2.5r_{NH_3} + 1.5r_{HNO_2} + 2.5r_{HNO_3} \tag{24}$$

$$r_4 = -0.25r_X - r_{NH_3} \tag{25}$$

$$r_5 = -r_{HNO_3} \tag{26}$$

$$r_6 = -0.25r_X - r_{NH_3} - r_{HNO_2} - r_{HNO_3} \tag{27}$$

Substitution in the ATP balance and recalculation ($r_X/C_X = \mu$) finally gives:

$$\left(\frac{-r_S}{C_X}\right) = \left[\frac{-(\alpha_{12} + K) + \alpha_{21} + \alpha_{21}\alpha_{11} + 0.25\alpha_{41} + 0.25\alpha_{61} + 1.45\delta}{\alpha_{21} + 2\delta}\right]\mu + \left[\frac{2.5\delta - \alpha_{41} - \alpha_{61}}{\alpha_{21} + 2\delta}\right]\left(\frac{-r_{NH_3}}{C_X}\right) + \left[\frac{2.5\delta - \alpha_{61} - \alpha_{51}}{\alpha_{21} + 2\delta}\right] \times \left(\frac{-r_{NHO_3}}{C_X}\right) + \left[\frac{1.5\delta - \alpha_{61}}{\alpha_{21} + 2\delta}\right]\left(\frac{-r_{HNO_2}}{C_X}\right) - \left[\frac{m_{ATP}}{\alpha_{21} + 2\delta}\right] \tag{28}$$

The specific acetate conversion rate ($-r_S/C_X$) is a linear function of the specific growth rate, μ, the specific ammonia conversion rate ($-r_{NH_3}/C_X$), the specific nitrate conversion rate ($-r_{HNO_3}/C_X$), and the specific nitrite conversion rate ($-r_{HNO_2}/C_X$). We can write eq. (28) as:

$$y = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + b \tag{29}$$

The minus sign is incorporated in x_1, x_2, x_3, and x_4 because conversion rates for consumed components are negative. In this way y, x_1, x_2, x_3, and x_4 are positive numbers. The results of the experiments are also given as positive numbers. By using a linear regression procedure, it is possible to calculate the coefficients a_1, a_2, a_3, and a_4 which are composed of parameters involved in the ATP conversion of the internal reactions. In this case, Marquardt's compromise method was used.[20] From these coefficients a number of stoichiometric coefficients [α_{61}, ($\alpha_{12} + K$), etc.] can be determined. In this way, the ATP consumption or production during the specific reactions can be calculated and compared with biochemical data. It should be noted that the simple Pirt equation is no longer applicable.

Measurements Aerobic Experiments

The results of the aerobic chemostat experiments are given in Tables I and II. In all cases, the dissolved oxygen concentration was controlled at 80% air saturation. Gas analysis was not performed, so there was no check of mass balances. There was no accumulation of nitrite, so the linear relation between the conversion rates (29) reduces to

$$y = a_1x_1 + a_2x_2 + a_3x_3 + b \tag{30}$$

in which $x_1 = \mu$; $x_2 = -r_{NH_3/C_X}$, and $x_3 = -r_{HNO_3/C_X}$.

Results of Fitting the Aerobic Experiments

Fitting of the coefficients in the linear relationship eq. (30) gives $a_1 = 1.83 \pm 0.09$; $a_2 = 3.61 \pm 0.25$; $a_3 = 1.18 \pm$

Table I. Analytical data for the aerobic, acetate-limited continuous culture (data are from refs. 5 and 9). Present was AC, NH_3, and O_2.

D $\left(\frac{1}{h}\right)$	ΔNH_3 $\left(\frac{mol}{m^3}\right)$	ΔHNO_3 $\left(\frac{mol}{m^3}\right)$	C_X $\left(\frac{Cmol}{m^3}\right)$	$C_{S,in}$ $\left(\frac{Cmol}{m^3}\right)$	$\frac{-r_{HN_3}}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$	$\frac{-r_{HNO_3}}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$	$\frac{-r_S}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$
0.017	6.20	–	7.55	50.6	0.014	–	0.114
0.046	6.20	–	6.42	37.2	0.044	–	0.267
0.063	4.54	–	9.29	40.0	0.031	–	0.271
0.10	6.90	–	7.13	40.0	0.097	–	0.561
0.20	5.22	–	11.6	40.0	0.090	–	0.691
0.41	4.91	–	11.7	40.0	0.173	–	1.41

Table II. Analytical data for the aerobic, acetate-limited continuous cultures (data are from refs. 5 and 9). Present was Ac, NH_3, HNO_3, and O_2.

D $\left(\frac{1}{h}\right)$	ΔNH_3 $\left(\frac{mol}{m^3}\right)$	ΔHNO_3 $\left(\frac{mol}{m^3}\right)$	C_X $\left(\frac{Cmol}{m^3}\right)$	$C_{S,in}$ $\left(\frac{Cmol}{m^3}\right)$	$\frac{-r_{HN_3}}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$	$\frac{-r_{HNO_3}}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$	$\frac{-r_S}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$
0.017	2.25	8.60	6.54	40.0	0.006	0.022	0.104
0.044	1.67	13.4	8.11	40.0	0.009	0.073	0.217
0.086	1.25	15.7	8.19	40.0	0.013	0.165	0.420
0.14	0.95	16.6	8.11	40.0	0.016	0.286	0.691
0.17	0.95	17.3	8.27	40.0	0.020	0.356	0.822

0.06; and $b = 0.026 \pm 0.007$, resulting in:

$$\left(\frac{-r_S}{C_X}\right) = 1.83\mu + 3.61\left(\frac{-r_{NH_3}}{C_X}\right) + 1.18\left(\frac{-r_{HNO_3}}{C_X}\right) + 0.026 \quad (31)$$

In Tables III and IV, the measured specific conversion rates and the calculated specific acetate conversion rate are given. The dissolved oxygen concentration was controlled at 80% air saturation. It can be seen that the measured specific acetate conversion rate can be described with a linear relationship between the measured conversion rate. The coefficients in the linear relationship eq. (31) are composed of the parameters involved in the internal reaction rates as expressed by eq. (28). From the results of the estimation and eq. (28), the following relations can be calculated.
Using a_1,

$$(\alpha_{12} + K) = -0.83\alpha_{21} + \alpha_{21}\alpha_{11} + 0.25\alpha_{41} + 0.25\alpha_{61} - 2.21\delta \quad (32)$$

Using a_2,

$$\alpha_{41} + \alpha_{61} = -3.61\alpha_{21} - 4.72\delta \quad (33)$$

Using a_3,

$$\alpha_{51} + \alpha_{61} = -1.18\alpha_{12} + 0.14\delta \quad (34)$$

Using b,

$$m_{ATP} = -0.026(\alpha_{12} + 2\delta) \quad (35)$$

Table III. Measured and calculated specific substrate utilization for the aerobic, acetate-limited continuous cultures. Present was Ac, NH_3, and O_2.

D $\left(\frac{1}{h}\right)$	Measured $\frac{-r_S}{C_X}$ $\left(\frac{\text{mol}}{\text{C mol h}}\right)$	Calculated $\frac{-r_S}{C_X}$ $\left(\frac{\text{mol}}{\text{C mol h}}\right)$
0.017	0.114	0.108
0.046	0.267	0.270
0.063	0.271	0.252
0.10	0.561	0.558
0.20	0.691	0.717
0.41	1.41	1.40

Table IV. Measured and calculated specific substrate utilization for the aerobic, acetate-limited continuous cultures. Present was Ac, NH_3, HNO_3, and O_2.

D $\left(\frac{1}{h}\right)$	Measured $\frac{-r_S}{C_X}$ $\left(\frac{\text{mol}}{\text{C mol h}}\right)$	Calculated $\frac{-r_S}{C_X}$ $\left(\frac{\text{mol}}{\text{C mol h}}\right)$
0.017	0.104	0.105
0.044	0.217	0.225
0.086	0.420	0.425
0.14	0.691	0.679
0.17	0.822	0.827

With eqs. (32), (33), (34), and (35), the aerobic experiments can be described. However, there is not enough information to calculate all of the stoichiometric coefficients. Besides α_{11}, three stoichiometric coefficients, for example δ, α_{41}, and α_{51}, still have to be chosen. With the chosen stoichiometric coefficients and eqs. (32), (33), (34), and (35), the other stoichiometric coefficients can be calculated. The results of the estimation will be evaluated after anaerobic denitrification has been dealt with.

Anaerobic Denitrification of HNO_3

The Internal Reaction Rates

The same strategy as in the aerobic case can be applied to the anaerobic situation in which nitrate is the sole electron acceptor. In this case, nitrification (r_4) and aerobic oxidative phosphorylation (r_3) can be left out. The denitrification of nitrite and the nitrite formation from nitrate can be combined into one reaction rate, r_7, since nitrite formation from ammonia is unlikely to occur in the absence of oxygen. This results in the following balance equations:
For biomass formation,

$$(1 + \alpha_{11})CH_2O + 0.25NH_3 \xrightarrow{r_1} \left(\alpha_{12} + K + \frac{m_{ATP}}{\mu}\right)ATP + CH_{1.9}O_{0.5}N_{0.25} + \alpha_{11}CO_2 + (0.5 - \alpha_{11})H_2O + (2\alpha_{11} - 0.075)NADH_2 \quad (36)$$

For substrate catabolism,

$$CH_2O + H_2O \xrightarrow{r_2} CO_2 + \alpha_{21}ATP + 2NADH_2 \quad (37)$$

Denitrification is as follows

$$HNO_3 + 2.5NADH_2 \xrightarrow{r_7} 0.5N_2 + 3H_2O + (\alpha_{51} + \alpha_{61})ATP \quad (38)$$

Linear Equation between Observable Conversion Rates

If the internal reaction rates are expressed as function of the observable conversion rates, it follows that:

$$r_S = -(1 + \alpha_{11})r_1 - r_2 \quad (39)$$

$$r_{NH_3} = -0.25r_1 \quad (40)$$

$$r_{HNO_3} = -r_7 \quad (41)$$

$$r_{N_2} = 0.5r_7 \quad (42)$$

$$r_X = r_1 \quad (43)$$

$$r_{CO_2} = \alpha_{11}r_1 + r_2 \quad (44)$$

$$r_{H_2O} = (0.5 - \alpha_{11})r_1 - r_2 + 3r_7 \quad (45)$$

No net accumulation of $NADH_2$ and ATP is assumed, $r_{NADH_2} = 0$ and $r_{ATP} = 0$:

$$r_{NADH_2} = (2\alpha_{11} - 0.075)r_1 + 2r_2 - 2.5r_7 = 0 \quad (46)$$

$$r_{ATP} = \left(\alpha_{12} + K + \frac{m_{ATP}}{\mu}\right)r_1 + \alpha_{21}r_2 + (\alpha_{51} + \alpha_{61})r_7 = 0 \quad (47)$$

There are 10 rates and 9 equations. The total system thus can be described with only one conversion rate. Two conversion rates are necessary to estimate the coefficients in the linear relationship between the conversion rates, here r_S and r_X are chosen.

If the internal reaction rates are expressed as functions of the two measured conversion rates, r_S and r_X, it follows that:

$$r_1 = r_X \quad (48)$$

$$r_2 = -(1 + \alpha_{11})r_X - r_S \quad (49)$$

$$r_7 = -0.83r_X - 0.8r_S \quad (50)$$

Substitution in the ATP balance gives:

$$\left(\frac{-r_S}{C_X}\right) = \left[\frac{-(\alpha_{12} + K) + \alpha_{21} + \alpha_{21}\alpha_{11} + 0.83(\alpha_{51} + \alpha_{61})}{0.8(\alpha_{51} + \alpha_{61})\alpha_{21}}\right]\mu - \left[\frac{m_{ATP}}{0.8(\alpha_{51} + \alpha_{61}) + \alpha_{21}}\right] \quad (51)$$

Equation (51) is a relation of the typical Pirt form

$$y = a_1x_1 + b \quad (52)$$

Coefficients a_1 and b can be estimated from the measurements and a number of parameters [$(\alpha_{12} + K)$, $(\alpha_{51} + \alpha_{61})$, etc.] can be determined. In this way, the ATP consumption or production in the specific reactions can be calculated and compared with biochemical knowledge.

Measurements Anaerobic Experiments

The NH_3 concentration was not measured during these experiments but it is assumed that all of the ammonia consumed was incorporated in biomass. Subsequent experiments have confirmed this.[5] With this assumption, the reduction degree balance[1] fits within 10%. The experimental data are given in Table V. There was no accumulation of nitrite.

Results of Fitting of the Anaerobic Experiments

Fitting of only the coefficients in the linear eq. (52) gives $a_1 = 9.15 \pm 0.28$ and $b = 0.07 \pm 0.03$, resulting in

$$\left(\frac{-r_S}{C_X}\right) = 9.15\mu + 0.07 \quad (53)$$

In Table VI, the measured specific reaction rates and the calculated specific acetate reaction rate are given. It can be seen from Table VI that the measured specific acetate reaction rate can be described with a linear relationship between measured conversion rates. From the fitted coefficients and eq. (51), the following can be calculated:

$$m_{ATP} = -0.056(\alpha_{51} + \alpha_{61}) - 0.07\alpha_{21} \quad (54)$$

$$-(\alpha_{12} + K) = -6.49(\alpha_{51} + \alpha_{61}) - 8.15\alpha_{21} + \alpha_{21}\alpha_{11} \quad (55)$$

With eqs. (54) and (55), the anaerobic experiments can be described. Besides α_{11}, three other parameters, for example α_{21}, α_{51}, and m_{ATP}, still have to be estimated. The results of the estimation will be evaluated in the following section.

EVALUATION OF THE RESULTS OF THE METABOLIC MODEL IN THE AEROBIC AND ANAEROBIC CONTINUOUS CULTURE EXPERIMENTS

From the results of the anaerobic and aerobic experiments, the coefficients in the linear relationship between the flows could be estimated. The linear relations satisfactorily describe the measurements. The minimum number of conversion rates needed to describe the system is 1 for the anaerobic and 4 for the aerobic cases. The number of conversion rates needed to describe the system can also be calculated from the number of components involved in the reactions and the element balance. In this case, the cell is considered as a black box (see Fig. 2) and no information about the internal reaction rates is available (unstructured model). For the aerobic situation, nine components are pres-

Table V. Analytical data for the aerobic, acetate-limited continuous cultures (data are from refs. 5 and 9). Present was Ac,HNO_3, and NH_3.

D $\left(\frac{1}{h}\right)$	ΔHNO_3 $\left(\frac{mol}{m^3}\right)$	C_X $\left(\frac{Cmol}{m^3}\right)$	C_S $\left(\frac{Cmol}{m^3}\right)$	$\frac{-r_{HNO_3}}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$	$\frac{-r_S}{C_X}$ $\left(\frac{mol}{Cmol\ h}\right)$
0.040	20.3	3.61	40.0	0.225	0.443
0.075	25.7	4.25	40.0	0.454	0.706
0.090	25.9	3.87	40.0	0.601	0.930
0.108	26.0	4.11	40.0	0.683	1.05
0.163	26.2	4.19	40.0	1.02	1.56

Table VI. Measured and calculated specific substrate utilization for the anaerobic, acetate-limited continuous cultures. Present was Ac, HNO_3, and NH_3.

D $\left(\frac{1}{h}\right)$	Measured $\frac{-r_S}{C_X}$ (mol/C mol h)	Calculated $\frac{-r_S}{C_X}$ (mol/C mol h)
0.040	0.443	0.433
0.075	0.706	0.753
0.090	0.930	0.890
0.108	1.05	1.05
0.163	1.57	1.56

ent and four elements, thus five conversion rates must be known. For the anaerobic case, seven components are present and also four elements, so three conversion rates must be known. It can be seen that by the use of a structured model and definition of the internal reaction rates, the conversion rates necessary to describe the system are reduced by 1 for the aerobic and 2 for the anaerobic situations.

Another aspect of using structured rather than unstructured models is that it helps to attain insight into the metabolism of *T. pantotropha*. Furthermore, the results of the aerobic and anaerobic experiments can be combined by means of the stoichiometric coefficients involved in the ATP conversion.

From the values of the coefficients in the linear relations (31) and (53), the stoichiometric parameters involved in the internal reactions can be determined. However, the values for the different stoichiometric parameters cannot be calculated directly. Choices must be made for a few parameters because the number of eqs. (32), (33), (34), (35), (54), and (55), following from the values of the coefficients of the linear relation between the conversion rates, is less than the number of unknown parameters. For these choices, realistic values, as discussed in the introduction, based on published work can be chosen.

If the model describes the aerobic nitrification/denitrification and anaerobic denitrification by *T. pantotropha* correctly, the stoichiometric parameters must be the same in both the aerobic and anaerobic situations. It must also be mentioned that the standard deviation in the calculated parameters can be high because of the propagation of error and the structure of the equations for the derivation of the parameters. This is especially true for the value of δ in eq. (34) and for the value of $(\alpha_{51} + \alpha_{61})\alpha_{21}$ and consequently m_{ATP} in eq. (54). These equations will not be used in the calculation of the stoichiometric coefficients. The relative errors of the coefficients of the other linear equations turned out to be relatively low, varying between 3 and 10%, so the system can be described by the eqs. (32), (33), (35), and (55). When using $\alpha_{11} = 0.3$, the model is consistent for the aerobic and the anaerobic cultures for $\alpha_{51} + \alpha_{61} = 1.3$, $\alpha_{41} = -2$, $\delta = 0.9$, and $\alpha_{21} = -0.8$, yielding:

$$\alpha_{41} + \alpha_{61} = -1.3 \pm 0.7$$

$$m_{ATP} = -0.03 \pm 0.02$$

$$(\alpha_{12} + K) = -1.9 \pm 0.4 \quad \text{(aerobic condition)}$$

$$(\alpha_{12} + K) = -2.2 \pm 0.4 \quad \text{(anaerobic condition)}$$

For $\alpha_{41} = -2$:

$$\alpha_{61} = 0.7 \pm 0.7$$

$$\alpha_{61} = 0.6 \pm 0.7$$

It can be concluded that the calculated stoichiometric coefficients have realistic values, but that the separate values of, for example, α_{61} and α_{51} cannot be determined accurately. Again, the model is consistent for the aerobic and anaerobic situations. Whether the theoretical values of the stoichiometric coefficients are proven to be correct by experimental verification cannot be concluded at this time. More accurate measurements and complete mass balances for the continuous cultures will be necessary.

An alternative approach which can be used to determine the stoichiometric coefficients involved in the ATP conversion, which has not been mentioned before but was also considered, will be briefly reviewed here. Equations (28) and (51), the relationships between the observable conversion rates, can be rearranged. This results in relations in which the stoichiometric coefficients are separated from each other as much as possible, and the conversion rates combined, yielding the following equations:

For the aerobic case,

$$\begin{aligned}-(\alpha_{12} + K)\mu &+ \alpha_{21}\left[1.3\mu - \left(\frac{-r_S}{C_X}\right)\right] \\ &+ \delta\left[1.45\mu - 2\left(\frac{-r_S}{C_X}\right) + 2.5\left(\frac{-r_{NH_3}}{C_X}\right)\right. \\ &\left. + 2.5\left(\frac{-r_{HNO_3}}{C_X}\right) + 1.5\left(\frac{-r_{HNO_2}}{C_X}\right)\right] \\ &+ \alpha_{41}\left[0.25\mu - \left(\frac{-r_{NH_3}}{C_X}\right)\right] - \alpha_{51}\left(\frac{-r_{HNO_3}}{C_X}\right) \\ &+ \alpha_{61}\left[0.25\mu - \left(\frac{-r_{NH_3}}{C_X}\right) - \left(\frac{-r_{HNO_3}}{C_X}\right) - \left(\frac{-r_{HNO_2}}{C_X}\right)\right] \\ &- m_{ATP} = 0 \quad (56)\end{aligned}$$

For the anaerobic case,

$$\begin{aligned}-(\alpha_{12} + K)\mu &+ \alpha_{21}\left[1.3\mu - \left(\frac{-r_s}{C_X}\right)\right] \\ &+ \alpha_{51}\left[0.83\mu - 0.8\left(\frac{-r_s}{C_X}\right)\right] \\ &+ \alpha_{61}\left[0.83\mu - 0.8\left(\frac{-r_s}{C_X}\right)\right] - m_{ATP} = 0 \quad (57)\end{aligned}$$

To obtain two linear equations in the form of

$$y = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + b \quad (58)$$

one of the (combinations of) conversion rates on the left-hand side of eqs. (56) and (57) must be written as function of the other (combinations of) conversion rates. It can be seen that $(-r_S/C_X)$ can not be written explicitly, as in the other approach, so μ has been chosen to be written explicitly. The parameters x_1 to x_5 are combinations of measured conversion rates, being different for the aerobic and anaerobic cultures, and a_1 to a_5 and b are the coefficients involved in the ATP conversion, which are equal for the aerobic and anaerobic situation. Coefficient α_{11} was set to 0.3, $a_1 = \alpha_{21}/(\alpha_{12} + K)$; $a_2 = \alpha_{41}/(\alpha_{12} + K)$; $a_3 = \alpha_{51}/(\alpha_{12} + K)$; $a_4 = \alpha_{61}/(\alpha_{12} + K)$; $a_5 = \delta/(\alpha_{12} + K)$; $b = m_{ATP}$.

In this way, the results of the aerobic and anaerobic experiments could be combined and fit together, directly resulting in the values for the stoichiometric coefficients. Moreover, for the anaerobic experiment the nitrate consumption data were also included. However, it appeared that the standard deviation in the resulting parameters was very high. This is due to the propagating error in the measured conversion rates, and also to the larger number of parameters to be fit (six instead of four). When only three stoichiometric coefficients were fit (α_{51}, α_{61}, and m_{ATP}), and the others set to the values previously obtained by the other approach, the results were in agreement with those found by the other approach.

CONCLUSIONS

Aerobic nitrification/denitrification and anaerobic denitrification in an acetate limited continuous culture of *Thiosphaera pantotropha* can be described by linear equations between the various conversion rates. The stoichiometric coefficients concerned with ATP production and consumption can be calculated from the coefficients in the linear equations. For modelling of a steady-state continuous culture, the use of a linear relation between the various conversion rates is sufficient. However, a biochemically structured model gives more insight into the physiology of the cell, and the results of the aerobic and anaerobic experiments can be combined. Choices for a few coeffcients must be made because the number of unknown coefficients is higher than the number of equations derived from the coefficients. For these choices, realistic values obtained from the literature and from biochemical knowledge are used. The standard deviations in the calculated coefficients is high due to the deviation in the estimation of the coefficients in the linear equation, to the structure of the equations to derive the coefficients, and to the propagation of error in the calculations. For further and more accurate experimental validation complete mass balances, including gas analysis, must be made for continuous cultures.

The model is consistent for both the aerobic and the anaerobic conditions. The realistic choices: $\delta = 0.9$, $\alpha_{21} = -0.8$, $\alpha_{41} = -2$, $\alpha_{11} = 0.3$, and $\alpha_{51} + \alpha_{61} = 1.3$ give realistic results for the ATP requirement for biomass production, the ATP yield in the denitrification reactions and for the maintenance on ATP. These results are: $(\alpha_{12} + K) = -2.1 \pm 0.4$, $\alpha_{41} + \alpha_{61} = -1.3 \pm 0.7$, $\alpha_{61} = 0.7 \pm 0.7$, $\alpha_{51} = 0.6 \pm 0.7$, and $\mu_{ATP} = -0.03 \pm 0.02$.

It should be noted that experimental verification of biochemical stoichiometric coefficients has not yet been obtained using the presently available continuous culture data. However, by choosing realistic values for five stoichiometric coefficients acceptable (although inaccurate) values can be estimated for the remaining stoichiometric coefficients. The complete set of stoichiometric coefficients thus determined describes both aerobic as well as anaerobic performance.

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NOMENCLATURE

a1, *a2*, *b1*, *b2*, *c1*	stoichiometric coefficients (mol C mol^{-1})
$a_1, \ldots, a_5$	coefficients in linear equation between conversion rates (mol C mol^{-1})
b	intercept in linear equation between conversion rates (mol C mol^{-1})
C_i	concentration of component i (mol m^{-3})
D	dilution rate (h^{-1})
K	ATP requirement for polymerization of precursors to biomass (mol C mol^{-1})
m_i	maintenance coefficient on component i(mol C mol^{-1} h^{-1})
r_i	conversion rate of component i or reaction rate number i(mol m^{-3} h^{-1})
$x_1, \ldots, x_5, y$	conversion rates in linear equation between conversion rates (mol C mol^{-1} h^{-1})

Greek Symbols

α_{11}	stoichiometric coefficient in biomass formation reaction (mol C mol^{-1})
α_{ij}	stoichiometric coefficient number j for ATP conversion in reaction number i(mol C mol^{-1})
δ	P/O ratio, ATP yield from oxydative phosphorylation (mol)
μ	growth rate (h^{-1})
Δ	difference sign for incoming and outgoing concentration subscripts

Subscripts

in	ingoing
out	outgoing
S	carbon substrate
X	biomass

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