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TECHNICAL NOTE 45.2

Behaviour of the nitrifying compartment fed with organic substrates

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CONTENT

T.N. 45.2: Behaviour of the nitrifying compartment fed with organic substrates

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Introduction

The purpose of this technical note was to complete and update the technical note 32.1 "nitrification and organic carbon sources".
The first part of this note is an update of the analysis of the energetic metabolism of the nitrifiers *Nitrosomonas* and *Nitrobacter*. The stoichiometric equations obtained from the biochemically structured model of the metabolism of the nitrifiers were established and validated with experimental results from literature.

In a second part, the kinetic model proposed in TN 32.1 for the growth of nitrifiers in mixotrophic condition (presence of organic matter in compartment III), associated to the proposed stoichiometric equations was introduced in the NitriSim software (v 4.0).
The software was also adapted to account for the increase of the biofilm, and then for the decrease of the voidage (fill in of the bed).

Simulations in autotrophic and mixotrophic operating conditions were performed and compared in terms of biomass distribution, nitrifying efficiencies and voidage of the column in the last part.

1 Biochemistry of nitrifiers and stoichiometric equations

1.1 Biochemistry and energetic metabolism

Since the last model established for the energetic metabolism of *Nitrosomonas* and *Nitrobacter* (TN 32.1), some changes have been made. If these changes have relatively low effect on the final stoichiometric expression of the nitrification, they give a better understanding of the phenomenon. In mixotrophic conditions the energetic metabolism is the same as in autotrophic growth. The models briefly detailed below are thus valid for the aerobic growth with ammonia and nitrite as electron donor and CO_2 or/and CO_2+organic matter as carbon sources. A more complete description of the energetic metabolism of the aerobic micro-organism *Nitrosomonas* and *Nitrobacter* can be found in TN 23.2 and TN 32.1.

Nitrosomonas

Ammonia oxidation in oxygenic conditions is detailed in figure 1. Compared to the previous description of the phenomenon, changes concern the position of the Ammonia Mono Oxygenase (AMO), situated at the periplasmic side of the membrane, and concern also the oxydoreductive reactions carried out by Hydroxylamine Oxydase (HAO).

The cyctochrome c_{554} is the electron acceptor coming from reactions catalysed by HAO. The cytochrome c_{554} can release its electrons at the UQ pool level (at least 2 electrons in order to enable the oxidation of NH_3 by AMO), but also directly to the mobile one electron carrier cytochrome c_{552} (which seems to be the case when hydroxylamine is the substrate and when ammonia oxidation is not necessary). It is not known up to date how the reactions NOH→NO→NO_2^- are carried out by HAO, but the electrons coming from these reactions always reach cyt c_{552}.

The production of nitrous oxide gases (N_2O, NO) is low in autotrophic conditions, and higher in hetrotrophic and anoxygenic conditions. NO can be produced from NO_2^- reduction, via a nitrite reductase (if HAO drives the reaction NO→NO_2^-, it is not known if this reaction is reversible). N_2O results in figure 1 from a chemical reaction from NOH, but some authors consider also a reaction between nitrite and hydroxylamine:

$$NH_2OH + HNO_2 \longrightarrow N_2O + 2H_2O$$

At the present time the assumptions chosen are that HAO can drive all the oxidation steps from hydroxylamine to nitrite, and that the only way for the production of N_2O is a chemical reaction from NOH.

A validation of the theoretical energetic model can be made using the H^+/O yields reported by Hollocher et al. (1982), reported in Table 1.

Table 1 : Experimental (Hollocher et al., 1982) and theoretical H^+/O yields for *Nitrosomonas*

	Experimental	Theoretical
Net H^+/O yield for NH_4^+ oxidation	3.4	3.33
Net H^+/O yield for hydroxylamine oxidation	4.4	4.5

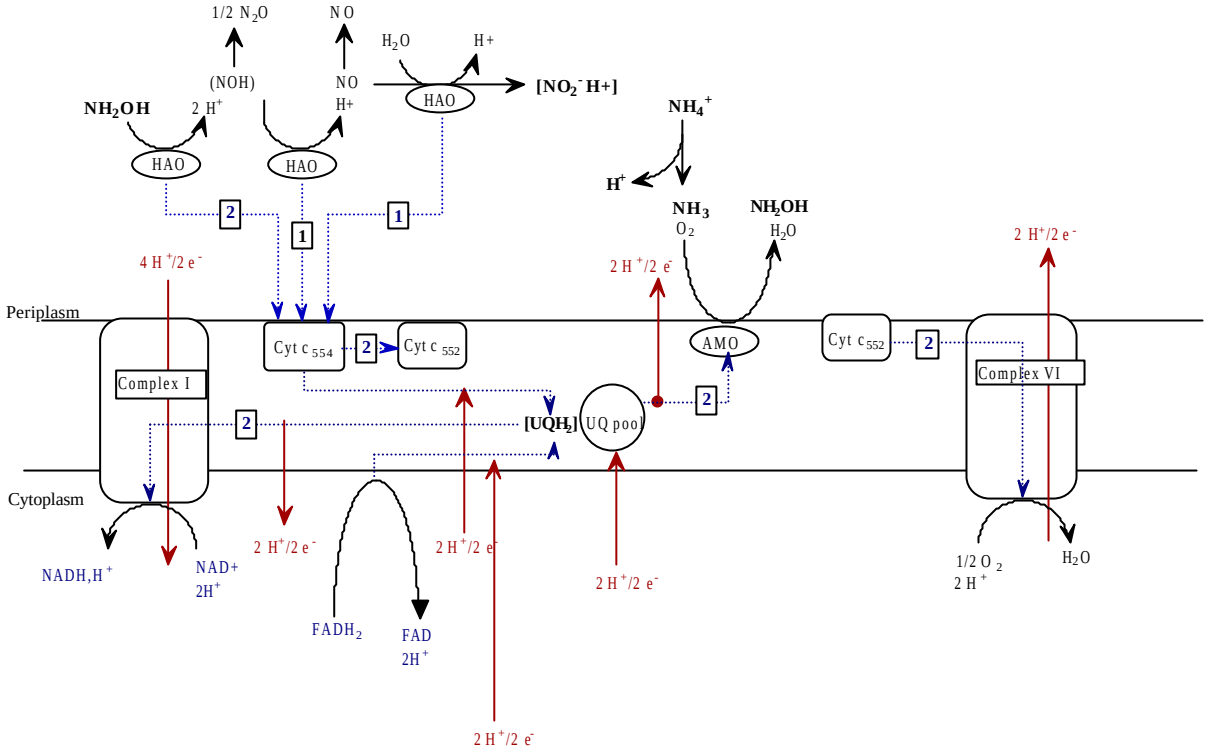


Figure 1 : Oxidoreductive pathways in the oxidation of ammonia by *Nitrosomonas*.

Nitrobacter

Taken from the previous description (TN 37.1), the energetic model proposed here (Figure 2) involves NO as an important intermediate for the reverse electron chain. This intermediate reported by Bock et al. (1991) allows to explain how electrons can reach the UQ pool from cyt c_{552} driven by the membrane potential. The reactions NO_2^- → NO, and NO → NO_2^- at the opposite sides of the membrane are an important node in the energetic metabolism of *Nitrobacter* allowing a H^+/O yield ranging from 0 to 2. This can explain the different yields reported : 0 (Hollocher et al, 1982) and 1 (Cobley et al, 1976). This futile cycle in the electron transport chain introduces a degree of freedom allowing important variations of the growth yields and an interesting flexibility in the model.

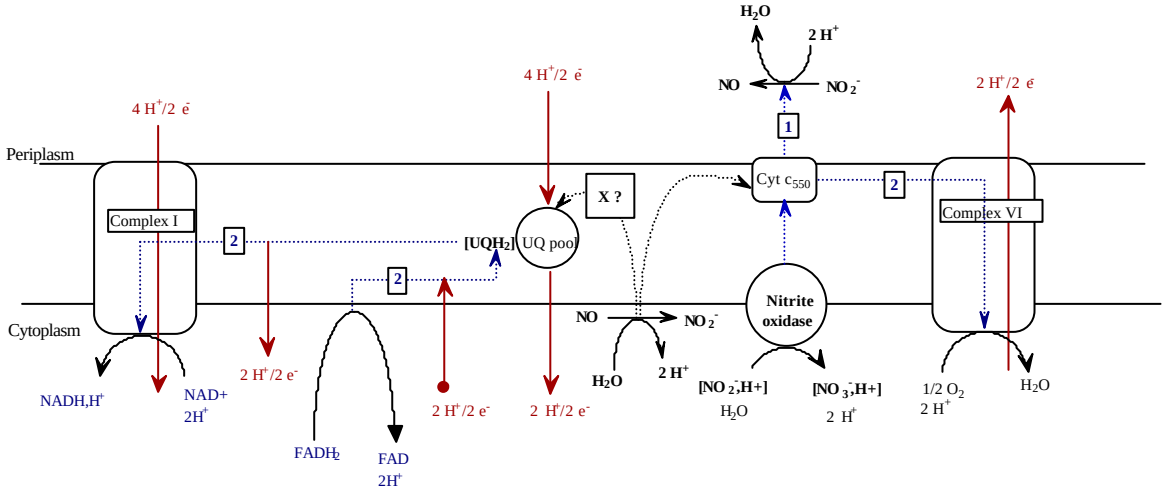


Figure 2 : Oxidoreductive pathways in the oxidation of nitrite by *Nitrobacter*.

NO intermediate can also be an electron sink in case of oxygen limitation, leading to the production of nitrous oxide gas.

1.2 Maintenance and nitrification

Maintenance is considered as the key driver for autotrophic nitrification. In the structured models, maintenance is classically expressed as hydrolysis of ATP to ADP. Rather than this reaction, we can consider a proton gradient dissipation flux, as the energetic model developed is based upon the proton gradient generation ($H^{+}_{Periplasmic} \rightarrow H^{+}_{Cytoplasmic}$).

Using this gradient dissipation yield for *Nitrosomonas* and *Nitrobacter* maintenance calculation gives an interesting result. It appears that the maintenance for *Nitrosomonas* and *Nitrobacter* is similar to that of other aerobic organisms (56% of the proton motive force). The computations have shown that this assumption is compatible with the experimental yields, and that in fact maintenance is not much higher in nitrifiers than for other aerobes. But the effect of the maintenance on the growth yield of nitrifiers is much higher; this leads to a value of 76% of NH3 oxidised for maintenance by *Nitrosomonas* and at least 53% of NO_2^- oxidised for *Nitrobacter*.

1.3 Nitrifiers and organic compounds

Nitrifiers are usually called obligate autotrophs, but some of them , especially *Nitrobacter* species are able to growth in presence of organic matter. The mixotrophic and heterotrophic growth of *Nitrosomonas* and *Nitrobacter*, were already detailed in TN 32.1. The carbon sources cited in literature and the ability of the organism to grow on it are reported in table 2.
If the mixotrophic growth is possible for both *Nitrosomonas* and *Nitrobacter*, it must be kept in mind that organic substrate are often inhibiting at increasing concentration for *Nitrosomonas* and that only *Nitrobacter* was found to grow hetrotrophically.

From the previous TN 32.1, no further informations were found concerning the growth of pure cultures of *Nitrosomonas* and/or *Nitrobacter* in mixotrophic or heterotrophic conditions. As previously noticed, the growth of nitrifiers in presence of organic matter is always accompanied with the growth of heterotrophic bacteria which eliminate the organic load, while nitrifiers eliminate the ammonia load (it is the waste-water treatment principle). But these data can not be extended to the mixotrophic growth of nitrifiers.

We will restrict this study to the mixotrophic growth of nitrifiers, *e.g*. the growth with organic matter as the main carbon source, and oxygen and N-inorganic as energy sources.

1.4 Stoichiometric equations (mass balance models)

For all the operating conditions, the stoichiometric equations were established assuming that maintenance can be represented by a proton gradient dissipation of 56%. This homogenous description for the maintenance is easy to use in the metabolic network description of the two

organisms whatever are the operating conditions or the substrates used, because the energetic metabolism has always the same driving force: a proton gradient.

The overall mass balance equations obtained for both autotrophic and mixotrophic operating conditions are reported in table 2. The assumptions for the metabolic behaviour description and the biomass composition were detailed in TN 23.2 and 32.1. At the opposite of TN 32.1, the stoichiometric relations proposed in Table 2 take into account the N-oxidation due to maintenance phenomenon, enabling to estimate growth yields for the mixotrophic growth of pure cultures (Table 3). Nevertheless it is important to keep in mind that the reserve biosynthesis (probably PHB) is not taken into account in these calculations as we do not know exactly what are the relationships between the growth condition and the synthesis of PHB in nitrifiers. The synthesis of reserve material can have significant influence on the carbon yields.

***Nitrosomonas* in autotrophic operating conditions** $CO_2 + 25.1142\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 17.6726\ NH_3$ $\downarrow$ $CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 16.9975\ H_2O + 17.4619\ [NO_2^- + H^+]$
***Nitrosomonas* in mixotrophic operating conditions** $1.0374\ CH_3COOH + 3.9844\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 2.2029\ NH_3$ $\downarrow$ $CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 1.0748\ CO_2 + 3.6026\ H_2O + 1.9922\ [NO_2^- + H^+]$
***Nitrobacter* in autotrophic operating conditions** $CO_2 + 35.8834\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 73.9242\ [NO_2^- + H^+]$ $+ 0.2107\ NH_3 + 0.4643\ H_2O$ $\downarrow$ $CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 73.9242\ [NO_3^- + H^+]$
***Nitrobacter* in mixotrophic operating conditions** $0.2551\ CH_3COOH + 23.0823\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4$ $47.3135\ [NO_2^- + H^+] + 0.2107\ NH_3 + 0.4959\ CO_2$ $\downarrow$ $CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 0.0398\ H2O + 47.3135\ [NO_3^- + H^+]$

Table 2 : Overall mass balance equations for the growth of nitrifiers in autototrophic and mixotrophic operating conditions.

	Nitrosomonas		*Nitrobacter*	
	Autotrophy	Mixotrophy	Autotrophy	Mixotrophy
Biomass/N oxid. (g/mol)	1.28	11.24	0.30	0.47
O_2 / N oxid. (mol/mol)	1.38	2	0.48	0.49
CO_2 / N oxid. (mol/mol)	0.057	**-1.85**	0.013	0.021
Acetate / N oxid. (mol/mol)	-	0.52	-	0.005
Biomass / Acetate (g/g)	-	0.36	-	1.48
CO_2 fixed by Calvin / C total fixed	88%	26%	88%	50%
Maintenance				
as proton gradient dissipation	58%	56%	56%	56%
as N-oxidised	75%	50%	76%	73%

Table 3 : Nitrification calculated yields in autotrophy and mixotrophy

It can be noted that the growth of the two microorganisms in presence of organic matter is very different. For *Nitrosomonas*, the N-oxidation yields decrease tenfold, while for *Nitrobacter* the differences for NO_2^- oxidation between autotrophic growth and mixtrotrophic growth are small. The stoichiometric biomass/NO_2^- yield calculated is 57% higher for mixotrophic growth and can be compared to the 11% to 48% measured for mixotrophic growth of *Nitrobacter winogradskyi* on various culture filtrates of heterotrophic bacteria (Steinmüller and Bock, 1976).

The metabolic assumptions used for the modelling of the growth of *Nitrobacter* were checked and validated from experiments of Smith and Hoare (1968), on the basis of the carbon distribution from acetate. But the data found for the mixotrophic growth of *Nitrosomonas* are insufficient for a validation of the assumptions used for this organism.

The mass balance equations proposed above can be used in steady-state process modelling and simulation without kinetics laws. These equations are a representation of the whole nitrification process and enable to compare the yields calculated with the experimental ones.

In the case of the use of mass balance equations with kinetic rate equations, as previously developed in NitriSim (TN 27.1, 27.2), each overall stoichiometric equation was split into 2 equations : one characterising anabolic metabolism and the other characterising the maintenance reactions. At each of these stoichiometric equations was associated a kinetic equation. The dissociation of the metabolism into several equations introduces a greater flexibility, but also a higher complexity of the model and requires to determine much more parameters.
For both *Nitrosomonas* and *Nitrobacter*, the mass balance model is constituted of 4 groups equations (Table 4a and 4b), for:

- anabolism
- maintenance
- reserve synthesis
- NOx by-products production

The reserve synthesis (synthesis of PHB) as well as the production of NOx are very dependant on the operating conditions, and especially on the limitations and inhibitions by substrates and products.

For NOx production by *Nitrosomonas* (Table 4a) the pathways involved are closely linked to the energetic metabolism of the organism (Figure 1) and would then probably interact with maintenance and anabolic reactions. It is impossible (at the present time) to model the synthesis of PHB by *Nitrosomonas*. This result is not inconsistent with experimental results as the PHB synthesis was not reported in this organism (TN 32.1); that was interpreted as a possible reason of the high sensibility of this organism to organic matter (TN 32.1)

Nitrosomonas - anabolism in autotrophic operating conditions

$$CO_2 + 5.4696\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 4.5762\ NH_3 \downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 3.9011\ H_2O + 4.5762\ [NO_2^- + H^+]$$

Nitrosomonas - anabolism in mixotrophic operating conditions

$$1.0374\ CH_3CCOH + 3.9844\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 2.2029\ NH_3 \downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 1.0748\ CO_2 + 3.6026\ H_2O + 1.9922\ [NO_2^- + H^+]$$

Nitrosomonas - maintenance mass balance equation

$$NH_3 + 1.5\ O_2 \longrightarrow [NO_2^- + H^+] + H_2O$$

Nitrosomonas - reserve mass balance equation

[Non solvable]

Nitrosomonas - NOx production

$$NH_3 + O_2 \longrightarrow 0.5\ N_2O + 1.5\ H_2O$$

(A proton gradient is generated. Then the reaction can interact with maintenance reaction). N_2O is a sink on the oxido-reductive electron transport chain when O_2 is limiting.

$$[NO_2^- + H^+] + NH_2OH \longrightarrow N_2O + 2\ H_2O$$

(Chemical reaction (?))

$$[NO_2^- + H^+] + H^+ + e^- \longrightarrow NO + H_2O$$

(Competition with O_2 as terminal e^- acceptor). This reaction can not occur alone and requires a coupling with a reaction producing H^+ and releasing one electron.

Table 4a: Mass balance equation for a complete biological model of *Nitrosomonas* in autotrophic and mixotrophic growth.

Nitrobacter - anabolism in autotrophic operating conditions

$$CO_2 + 7.6268\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 0.2107\ NH_3 + 17.4109\ [NO_2^- + H^+] + 0.4643\ H_2O \downarrow CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 17.4109\ [NO_3^- + H^+]$$

Nitrobacter - anabolism in mixotrophic operating conditions

$$0.2521\ CH_3CCOH + 5.8475\ O_2 + 0.0041\ H_2SO_4 + 0.0136\ H_3PO_4 + 0.2107\ NH_3 + 12.8440\ [NO_2^- + H_+] + 0.4959\ CO_2 \downarrow CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 0.0398\ H_2O + 12.8440\ [NO_3^- + H^+]$$

Nitrobacter - maintenance mass balance equation

$$[NO_2^- + H^+] + 0.5\ O_2 \longrightarrow [NO_3^- + H^+]$$

Nitrobacter - reserve mass balance equation

$$0.5\ CH_3COOH + 1.7083\ O_2 + 3.6667\ [NO_2^- + H^+] \downarrow CH_{1.505}O_{0.503} + 0.2475\ H_2O + 3.6667\ [NO_3^- + H^+]$$

Nitrobacter - NO production

$$[NO_2^- + H^+] + H^+ + e^- \longrightarrow NO + H_2O$$

(Competition with O_2 as terminal e^- acceptor - Also intermediate in the energetic metabolism). The reaction is coupled with nitrite oxidation, and when growing heterotrophically (*i.e*. the Q cycle is functional) with the $NADH,H^+$ oxidation.

Table 4b: Mass balance equation for a complete biological model of *Nitrobacter* in autotrophic and mixotrophic growth.

2 Mixotrophic kinetic model

The metabolic behaviour of nitrifiers and the products formed are dependant of the growth conditions and of the substrates available (Figure 3). Even if nitrifiers are considered as important micro-organisms in water-treatment, their behaviour as pure culture and pure coculture has not been extensively studied.

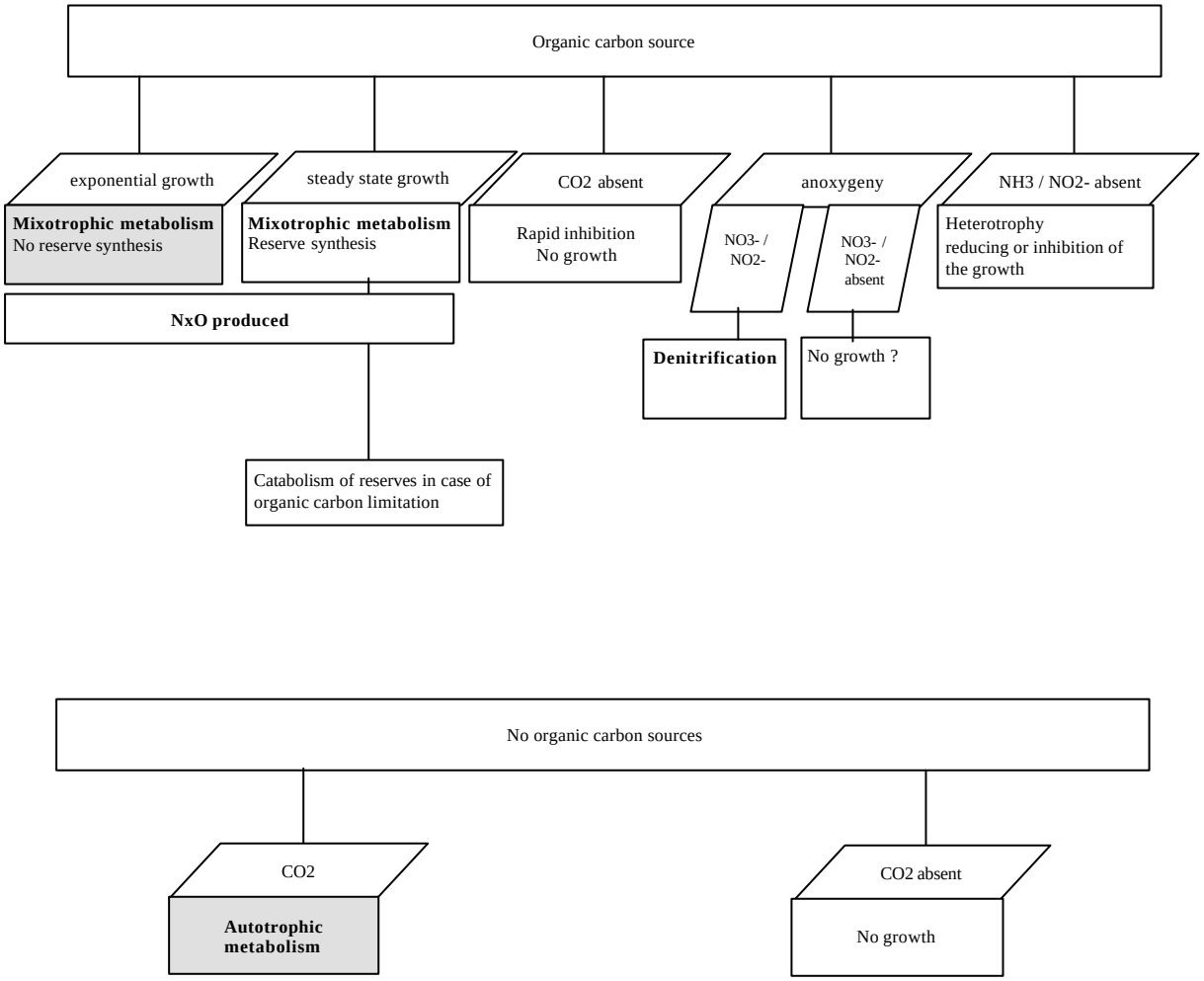


Figure 3 : Overview of the variability of the metabolic behaviour of nitrifiers.

As it is difficult to find kinetic parameters values for the growth of pure cultures in controlled conditions, it was decided in a first approach **to limit the study to a situation where carbon storage (reserve synthesis) and NOx gas production are neglected**.

2.1 Principles

The principles for the construction of a kinetic model for nitrifiers, taking into account both autotrophic and mixotrophic growth have been already detailed in TN 32.1.
It must be kept in mind that here is presented the overall expression of the model, which can be simplified by the fact that most of the parameters are set to zero, as the inhibition or saturation constants for phosphorous and sulphur sources for example.

2.1.1 Kinetic model for autotrophic growth

$r^{Ns}_{Xact\text{-}auto}$, the autotrophic growth rate (active biomass production) is expressed as a combination of the anabolic growth rate $r^{Ns}_{gr-auto}$ and of the maintenance rate $r^{Ns}_{m\text{-}auto}$. For Nitrosomonas, this growth rate ($r^{Ns}_{Xact\text{-}auto}$) can be written:

$$r^{Ns}_{Xact\text{-}auto} = r^{Ns}_{gr-auto} + \left(\frac{\mu^{Ns}}{\mu^{Ns}_{max-auto}} - 1 \right) Y^{Ns}_{Xact/NH3}\Big|_{auto}\, r^{Ns}_{m-auto}$$

with

$$r^{Ns}_{gr-auto} = m^{Ns}_{auto}\, C_{Xact-Ns}\Big|_B$$

$$r^{Ns}_{m\text{-}auto} = m^{Ns}_{auto}\, C_{Xact-Ns}\Big|_B$$

$$m^{Ns}_{auto} = m^{Ns}_{max-auto} \prod_k \frac{C_{Si}\Big|_B}{\left(K_{Si} + C_{Si}\Big|_B\right) \dfrac{1}{1 + \dfrac{C_{Si}\Big|_B}{I_i}}}$$

m^{Ns}_{auto} is the maintenance coefficient
$C_{Xact-Ns}\Big|_B$ the concentration of the active biomass of *Nitrosomonas* in the biofilm
Si: substrate i
C_{si}: concentration of substrate i
K_{si}: saturation constant for substrate i
I_i: inhibition constant for substrate i

The production and consumption rates of other compounds are expressed by:

$$r^{Ns}_{Si\text{-}auto} = \frac{1}{Y^{Ns}_{Xac/Si}\Big|_{auto}} r^{Ns}_{Xact-auto} + \frac{1}{Y^{Ns}_{Smt/Si}\Big|_{auto}} r^{Ns}_{m-auto}$$

2.1.2 Kinetic model for mixotrophic growth.

The kinetic model for mixotrophic growth has been developed on the same basis as the autotrophic growth model (TN 32.1), i.e. :

$$r_{Xact\text{-}mixo}^{Ns} = r_{gr-mixo}^{Ns} + \left(\frac{m^{Ns}}{m_{max-mixo}^{Ns}} - 1 \right) Y_{Xact/NH3}^{Ns}\Big|_{mixo} r_{m-mixo}^{Ns}$$

$$r_{Si\text{-}mixo}^{Ns} = \frac{1}{Y_{Xac/Si}^{Ns}\Big|_{mixo}} r_{Xact-mixo}^{Ns} + \frac{1}{Y_{Smt/Si}^{Ns}\Big|_{mixo}} r_{m-mixo}^{Ns}$$

2.1.3 Combined model for autotrophic/mixotrophic growth

The combination of the rates previously established for the different possible kinds of growth gives a general expression for the kinetic law for the 2 different growth conditions: autotrophy (when CO_2 is the sole carbon source) and mixotrophy (when an organic carbon source is added to the autotrophic medium).

The growth rate for the active biomass is given as the sum of the rates in the different growth conditions, and the consumption and production of other substrates are given by:

$$r_{Xac}^{Ns} = \sum \begin{vmatrix} r_{Xact-auto}^{Ns} \left[1 - \frac{K}{K + C_{Sorga}} \right] \\ r_{Xact-mixo}^{Ns} \left[\frac{K}{K + C_{Sorga}} \right] \end{vmatrix} \qquad r_{Si}^{Ns} = \sum \begin{vmatrix} r_{Si-auto}^{Ns} \left[1 - \frac{K}{K + C_{Sorga}} \right] \\ r_{Si-mixo}^{Ns} \left[\frac{K}{K + C_{Sorga}} \right] \end{vmatrix}$$

K is an arbitrary constant which determines when the metabolism shifts from autotrophic to heterotrophic behaviour. This shift is here dependant on the concentration of organic matter. In this model, for very low values of K, the metabolism shifts immediately from an autotrophic to an heterotrophic growth.

$K/[K+C_{sorga}]$ can also be interpreted as the ratio of cells which have a mixotrophic growth behaviour to the cells which have an autotrophic growth behaviour.

2.2 Kinetic parameters

The kinetic parameter values of the biological model for *Nitrosomonas* and *Nitrobacter* in autotrophic growth are reported in Table 5. It must be kept in mind that the maintenance coefficients presented are those calculated in TN 39.2 for the bench column at UAB, in order to have the best fitting of these experiments.

For the mixotrophic growth, the kinetic parameter values are unknown at the present time. The generation time reported in the literature are higher in mixotrophic growth than in autotrophic growth, e.g. for *Nitrobacter* they range between 30 h and 150 h in mixotrophy (Bock, 1991), while the value is about 28 h in autotrophy (Table 5). Despite these observations, as no detailed values were found for the maximum growth rate in mixotrophic growth, the values used in autotrophy will be conserved.

For the maintenance coefficient, the problem is a little more complicated in mixotrophy.
The maintenance reaction in mixotrophy is the oxidation of the N substrate (NH_3 or NO_2^-), but in mixotrophy, the organic substrate can have a non negligible effect on the energetic metabolism of the organism and then also on the maintenance yields (Table 2). The value used for the maintenance coefficient in autotrophic growth can not be used in mixotrophic growth. As for the maximum growth rate, there is no value found in the literature that can be used. Nevertheless it is possible to estimate a value for the maintenance coefficient in mixotrophic growth from the autotrophic maintenance.

Considering that equation 1.1 and 1.2 are respectively a representation of the steady-state for *Nitrosomona*s for autotrophy and mixotrophy, the N-oxidation yields for maintenance are calculated as :

$$m_{auto} = 75\%.\frac{1}{1.27} = 0.586 \text{ mol NH}_3 \text{ / g active biomass}$$
$$m_{mixo} = 50\%.\frac{1}{11.24} = 0.044 \text{ mol NH}_3 \text{ / g active biomass}$$

Using the kinetic equations, the NH_3 consumption in steady-state (*i.e.* $r^{Ns}_{Xact-auto} = 0$ assuming no input nor output of biomass) can be written respectively for autotrophic and mixotrophic growth :

$$r^{Ns}_{NH3-auto} = \frac{1}{Y^{Ns}_{NH3/NH3}\Big|_{auto}} r^{Ns}_{m-auto}$$
$$r^{Ns}_{NH3-mixo} = \frac{1}{Y^{Ns}_{NH3/NH3}\Big|_{mixo}} r^{Ns}_{m-mixo}$$

As the two expressions (mass balance and kinetic equations) are the representation of the same situation, their ratio can be compared :

$$\frac{m_{auto}}{m_{mixo}} = \frac{r^{Ns}_{NH3-auto}}{r^{Ns}_{NH3-mixo}}$$
$$m^{Ns}_{mixo} = m^{Ns}_{auto}.\frac{Y^{Ns}_{NH3/NH3}\Big|_{mixo}}{Y^{Ns}_{NH3/NH3}\Big|_{auto}}\frac{m_{mixo}}{m_{auto}}$$

then

$$m^{Ns}_{mixo} = 6.46\ 10^{-4} \text{ mol / g biomass.h}$$

			Reference	Remarks
m_{max}^{Ns}	$5.7\ 10^{-2} h^{-1}$		Hunik et al (1994)	mean values calculated from several continuous cultures
m_{max}^{Nb}	$3.6\ 10^{-2} h^{-1}$		Hunik et al (1994)	
m^{Ns}	8.6 10^{-3} mol/g biomass.h		TN 39.2	
m^{Nb}	5.1 10^{-3} mol/g biomass.h		TN 39.2	
Limiting substrate	K^{Ns}	K^{Nb}		
NH_3	6.625 10^{-5} mol/l	-	Hunik et al (1994)	Model parameter
NO_2^-	-	3.6 10^{-4} mol/l	Hunik et al (1994)	values for a fixed bed
O_2	5.05 10^{-6}mol/l	1.7 10^{-5} mol/l	Hunik et al (1994)	of carragenan beads
HCO_3^-	10^{-6} mol/l	10^{-6} mol/l		No carbon limitation
Inhibiting substrate	I^{Ns}	I^{Nb}		
NO_3^-		0.188 mol/h	Hunik et al (1992)	
NO_2^-	-	-0.159 mol/l	Hunik et al (1992)	
Substrate	$Y_{X/Si}^{Ns}$	$Y_{X/Si}^{Nb}$		g biomass / mol Si
NH_3	-5.0600	-109.8980		
NO_2^-	5.3042	-1.3299		
NO_3^-		1.3299		
O_2	-4.2335	-3.0361		
HCO_3^-	-23.1555	-23.1555		
HPO_4^{2-}	-1702.6103	-1702.6103		
SO_4^{2-}	-5647.6826	-5647.6826		
H^+	-5.3476	654.1102		
OH^-	23.1555	23.1555		
H2O	5.9356	-49.8719		
	$Y_{Smt/Si}^{Ns}$	$Y_{Smt/Si}^{Nb}$		mol maintenance substrate/mol Si Algebraic value
NH_3	-1			
NO_2^-	1	-1		
NO_3^-		1		
H_2O	1			
H^+	1			
O_2	-0.5	-1.5		

Table 5a : Kinetic parameter values for autotrophic growth of nitrifiers.

			Reference	Remarks
m_{max}^{Ns}	$5.7\ 10^{-2}\ h^{-1}$		Hunik et al (1994)	mean values calculated from several continuous cultures
m_{max}^{Nb}	$3.6\ 10^{-2}\ h^{-1}$		Hunik et al (1994)	
m^{Ns}	0.64 10^{-3} mol/g biomass.h		**ESTIMATED**	
m^{Nb}	8.32 10^{-3} mol/g biomass.h		**ESTIMATED**	
Limiting substrate	K^{Ns}	K^{Nb}		
NH_3	$6.625\ 10^{-5}$ mol/l	-	Hunik et al (1994)	Model parameter
NO_2^-	-	$3.6\ 10^{-4}$ mol/l	Hunik et al (1994)	values for a fixed bed
O_2	$5.05\ 10^{-6}$mol/l	$1.7\ 10^{-5}$ mol/l	Hunik et al (1994)	of carragenan beads
Acetic acid	10^{-6} mol/l	10^{-6} mol/l		
HCO_3^-	10^{-6} mol/l	10^{-6} mol/l		No carbon limitation
Inhibiting substrate	I^{Ns}	I^{Nb}		
NO_3^-		0.188 mol/h	Hunik et al (1992)	
NO_2^-	-	-0.159 mol/l	Hunik et al (1992)	
Substrate	$Y_{X/Si}^{Ns}$	$Y_{X/Si}^{Nb}$		g biomass / mol Si
NH_3	-21.0447	-109.8980		
NO_2^-	26.0291	-1.8028		
NO_3^-		1.8028		
O_2	-13.0153	-3.9599		
Acetic acid	-204.3733	-91.8505		
HCO_3^-	44.2405	-46.6939		
HPO_4^{2-}	-1702.6103	-1702.6103		
SO_4^{2-}	-5647.6829	-5647.6829		
H^+	-27.1078	654.1102		
OH^-	-44.2405	46.6939		
H2O	11.8825	581.7965		
	$Y_{Smt/Si}^{Ns}$	$Y_{Smt/Si}^{Nb}$		mol maintenance substrate/mol Si Agebraic value
NH_3	-1			
NO_2^-	1	-1		
NO_3^-		1		
H_2O	1			
H^+	1			
O_2	-0.5	-1.5		

Table 5b : Kinetic parameters values for mixotrophic growth of nitrifiers.
The constant K was currently set to Ks of acetate, *i.e.* the limitation of organic matter (acetic acid) shifts the metabolism to an autotrophic behaviour.

2.3 NitriSim software v4.0

2.3.1 Changes in the software - Version 4.0

The software developed for the simulation of the fixed bed nitrification process (NitriSim) is upgraded in order to be enable to simulate the mixotrophic growth of nitrifiers.
The changes concern :

a) the addition of compounds not considered previously :

- Acetic acid, and its ionic form, CH_3COO^-. The physico-chemical constants (pK, gas/liquid partition coefficient) of acetic acid have been detailed in TN 23.1.
- An inert biomass (defined as dead biomass), fixed or free (in case of biofilm detachment), satisfying the mass balance conservation for the process and entailing the growth of the biofilm thickness by accumulation of the "inert biomass", while the growth of active biomass (*Nitrosomonas* and *Nitrobacter*) is assumed occurring only at the surface of the biofilm.

b) the insertion of the mixotrophic model previously detailed, including the stoichiometries for anabolic growth and maintenance for *Nitrosomonas* and *Nitrobacter*, and the kinetic model for the growth with organic matter.

c) the calculation of the voidage of the bed as a function of the biofilm thickness. This is interesting for long term operation as the voidage affects both the liquid and gas flows and the K_La value.

2.3.2 Definition of the so-called "inert biomass"

The inert biomass is defined as the "dead biomass". Then its production rate r_{Xinert} is defined as the lethality term of the growth rate for the active biomass of both *Nitrosomonas* and *Nitrobacter*. For autotrophy this equation is written as follows:

$$r^{Ns}_{Inert\text{-}auto} = -\left(\frac{\mu^{Ns}}{\mu^{Ns}_{max-auto}} - 1\right) Y^{Ns}_{Xact/NH3}\Big|_{auto} r^{Ns}_{m-auto} - \left(\frac{\mu^{Nb}}{\mu^{Nb}_{max-auto}} - 1\right) Y^{Ns}_{Xact/NO2}\Big|_{auto} r^{Nb}_{m-auto}$$

2.3.3 Biofilm thickness and voidage of the bed

The problem of the clogging of the column can be taken into account by calculating the reduction of the voidage in each part of the column as a function of the biofilm thickness. Assuming a mean geometry of a cell of 1 x 1 x 1 µm (i.e. 1 μm^3) and considering that 1 mg of dry biomass is equivalent to an average of $3.7\ 10^9$ cells (Hunik et al., 1994) (Cox et al., 1980, proposed a value of $5\ 10^9$), a relation can be established giving the average thickness of the biofilm as a function of the quantity of bacteria (active and inert) fixed. The voidage of each part of the column ε^n is defined as :

$$\varepsilon^n = \frac{\varepsilon^2}{\varepsilon + \varepsilon_L \cdot \left[C^n_{Ns}\Big|_B + C^n_{Nb}\Big|_B + C^n_{Inert}\Big|_B\right] 3.7\ 10^3}$$

Note : it was supposed for this formula that the ratio $\frac{e_L}{e_G}$ *was constant in the whole column whatever the time. This assumption implies also :* $\frac{e_L}{e_G} = \frac{V_{Liq}^{Beb}}{V_{Gas}^{Bed}} = \frac{U_{Liq}^{Bed}}{U_{Gas}^{Bed}} = \text{constant}$.

The clogging of the column occurs when ε^n reaches zero.

It is important to notice that this calculation must begin with the start-up of the column, as the calculated voidage depend on the whole "history" of the column. When performing steady state simulations, the change in the voidage can not be calculated as it is not a steady-state value (e.g. the active biomass can be in steady-state for defined operating conditions, but the total biomass including fixed inert biomass increases continuously)

2.3.4 Voidage of the bed and K_La

The variation of the voidage in the fixed bed can also affects the k_La value which can explain the problems encountered in TN 39.2 for the bench columns. The correction of k_La as a function of the gas superficial velocity (TN 39.2) is significantly dependent on the voidage of the bed. The correction proposed by Perez et al. (TN 43.410) is then:

$$k_L a\big|_{Bed} = \frac{Section\ for\ the\ gas\ at\ the\ top\ (measurement)}{Section\ for\ the\ gas\ in\ the\ fixed\ bed} k_L a\big|_{measured}$$

This gives if the column diameter is constant (e.g. pilot column) :

$$k_L a\big|_{Bed} = \frac{1}{e} k_L a\big|_{measured}$$

3 Simulation of UAB columns with/without organic matter

The simulations performed can only give an overview of the behaviour of the nitrifying columns when organic matter is present. If stoichiometric equations and growth yields are well established, the kinetic parameter values remain uncertain as they are taken from autotrophic parameters.
In order to observe the effects of organic matter on the growth and the nitrification yields a mixotrophic and an autotrophic growth in the same operating conditions were compared. The experiment chosen is the so-called M5 experiment of TN 39.2.
For the simulation of the bench column, a 15-tanks in series model was chosen with a liquid back-mixing of 9 ml/min (TN39.2). The K_La is corrected from the measured value considering the change in the superficial gas velocity in the different parts of the column (section 2.3.4).

3.1 Bench experiment chosen for testing the mixotrophic growth model

The bench experiment M5 was chosen because the identified K_La value (e.g. value required to obtain simulations results in steady-state consistent with experiments) is close to the corrected value (e.g. calculated considering the effect of the increase of gas superficial velocity inside the fixed bed) (TN 39.2). Nevertheless it must be kept in mind, as the corrected value is about 19 h^{-1} and the identified value in TN 39.2 is 24 h^{-1}, it remains a little discrepancy between efficiencies predicted by the simulation (Table 7) and the experimental ones (Table 6).

The operating conditions of the experiments used for the simulations are reported in Table 5a. Both for simulation of mixotrophy and autotrophy growth, the columns were stated assuming at t=0 a fixation of an homogenous population of *Nitrosomonas* and *Nitrobacter* on the beads, equivalent to 0.1g dry biomass/ L liquid for the both nitrifiers.

Operating conditions	Input (g N-NH_3/l)	Input kg N-NH_3/L.h	NH_3 (g N/l)	Nitrite (g N/l)	Nitrate (g N/l)	Total nitrogen
DR=0.075h^{-1}	0.3	0.0225	0.25 (± 0.006)	0.5 (± 0.003)	0.287 (± 0.010)	0.317 (± 0.019)
RT=13 h						
15 ml air/min			**7.9%**	**1.6%**	**90.5%**	
			8.3% *	1.7% *	95.7% *	105.7% *

Table 6 : Bench columns in steady-state (Perez et al. TN 37.520; TN 43.410) - Details of experiment M5.(TN 39.2). * Calculation based on input concentration.

3.1.1 Autotrophic growth simulation for M5 operating conditions

A steady state can be observed after 600 h. Output concentration of N-compounds are reported in Figure 4, and the efficiencies predicted at steady state are reported in Table 7.

Input (g N-NH_3/l)	NH_3 (g N/l)	Nitrite (g N/l)	Nitrate (g N/l)
0.3	0.059	0.01	0.224
	20%	**3%**	**75%**

Table 7 : Simulation of the UAB bench column for M5 operating conditions in steady-state

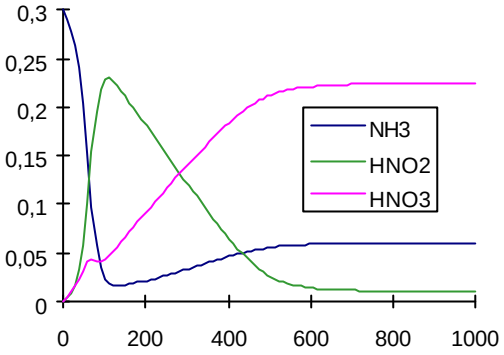


Figure 4 : Output concentrations of N-compounds for **autotrophic** growth in M5 operating conditions. Concentration in g N/l. Time in hours.

The concentrations and biomass profiles are reported in Figures 6. The oxygen limitation appears clearly (Figure 5a) and drives the distribution of the active biomass of *Nitrosomonas* (Figure 5b) and *Nitrobacter* (Figure 5c). Biomass concentrates at the top and at the bottom of the bed where liquid back-mixing with the top and the bottom parts of the column enables higher oxygen availability. The inert biomass (Figure 6d), after 1000 hours, constitutes the major part of the total biofilm: it can represent up to 10 times the active biomass.

The accumulation of biomass increases the biofilm thickness and then reduces the voidage of the bed (Figure 5e). The voidage at the bottom of the bed was set to 0.55 at the start of the simulation and fall to 0.5 after 1000 hour operation.

3.1.2 Mixotrophic growth simulation for M5 operating conditions

The mixotrophic growth is obtained by the addition of 10 mM of acetic acid in the input medium. After 600h the steady-state is not completely reached. The nitrification efficiencies calculated are reported in Table 8 and the output concentrations of N-compounds are reported in Figure 6.
As for autotrotrophic growth, the process is limited by oxygen transfer. The limitation is more important in mixotrophic condition as nitrite is the main product obtained at the output of the column. The oxygen limitation affects also the acetic acid consumption so that less than 13% of the substrate is consumed.

Input ($g\ N\text{-}NH_3/l$)	Input acetic ac. g/l	NH_3 (g N/l)	Nitrite (g N/l)	Nitrate (g N/l)	Acetic ac. g/l
0.3	0.6	0.015	0.23	0.058	0.53
		6%	**78%**	**14%**	

Table 8 : Simulation of bench column for M5 operating conditions in steady-state with organic matter (acetate).

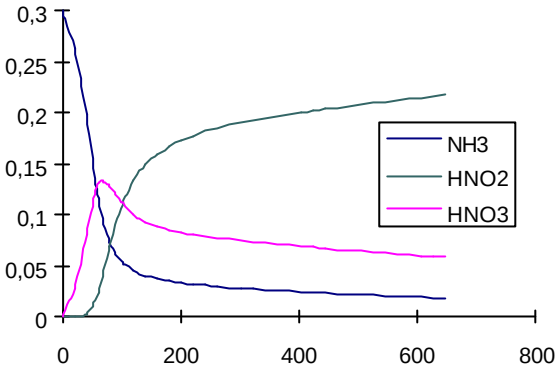
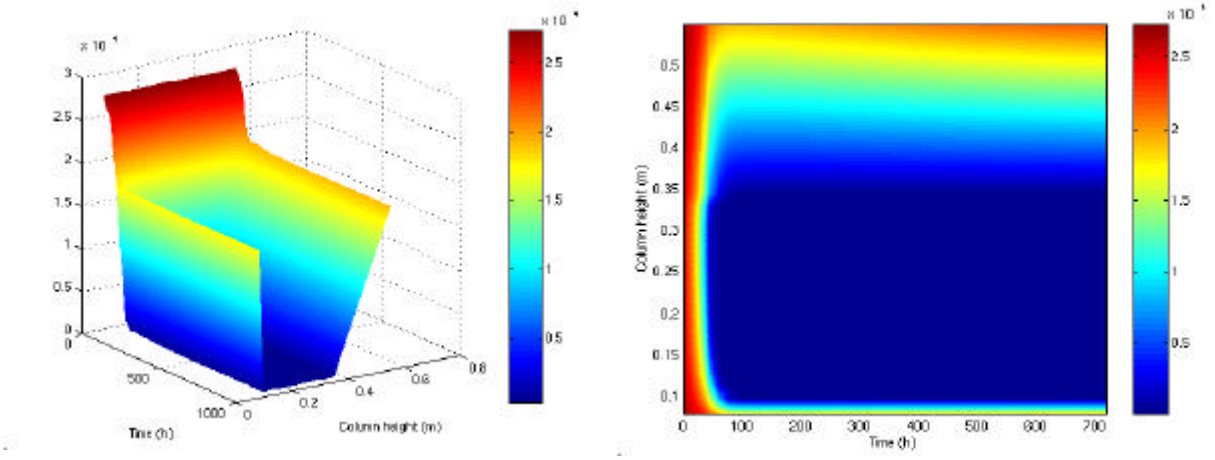


Figure 6 : Output concentrations of N-compounds for **mixotrophic** growth in M5 operating conditions. Concentration in g N/l. Time in hours.

Because of oxygen limitation, the concentration profiles inside the column are similar for autotrophic and mixotrophic growths (Figures 5). But for total biomass (active or "dead" *Nitrosomonas* and *Nitrobacter*) the quantity produced in mixotrophic growth is much higher.

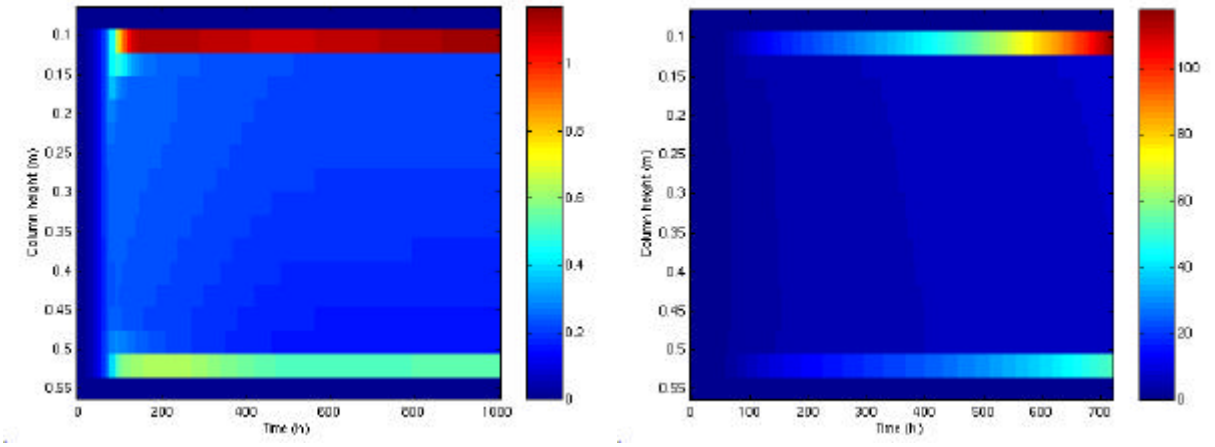
For *Nitrosomonas*, for example, the biomass in mixotrophy is up to 100 times greater than in autotrophy (Figure 4b). The effect of biomass accumulation has important effects on the voidage inside the bed (Figure 5e). After 600h the bottom of the fixed bed has a voidage of only 0.18, instead of the initial value of 0.55 (e.g. 32% of initial value).

The biofilm thickness is of course also increased and biofilm transfer limitation problems must occur. But it must be noted that these limitations phenomena are not taken into account in the model developed.



Autotrophy **Mixotrophy**

Figure 4a : Simulation of dissolved oxygen profile in bench columns for operating conditions of experiment M5. Mixotrophy is represented by a plane projection of a 3D profile (as this is reported for autotrophy).



Autotrophy **Mixotrophy**

Figure 4b : Simulation of the *Nitrosomonas* biomass in bench column for autotrophic and mixotrophic growth. In these representations each band corresponds to one of the tanks of the model. Note that the top of the figure corresponds to the bottom of the column.

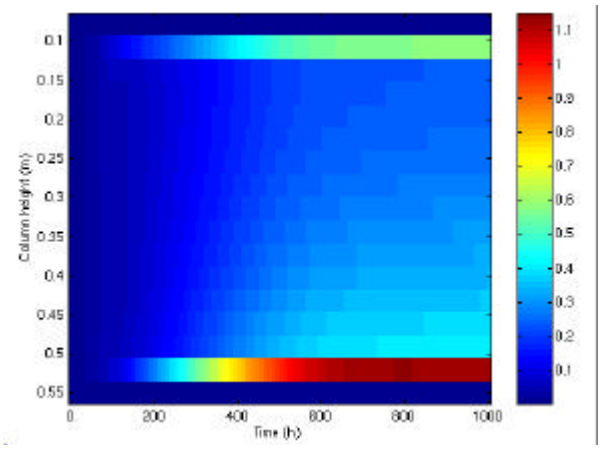


Figure 4c : *Nitrobacter* biomass distribution in autotrophic growth simulation.

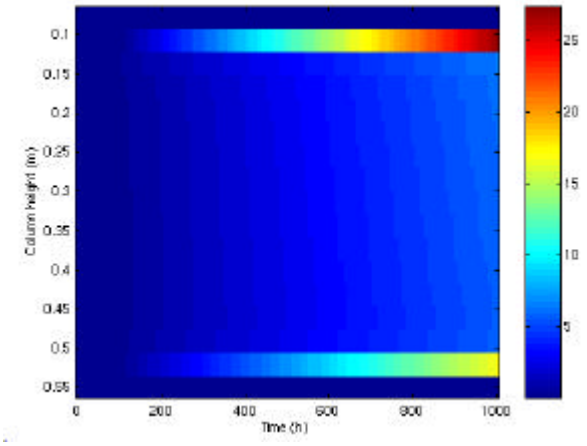
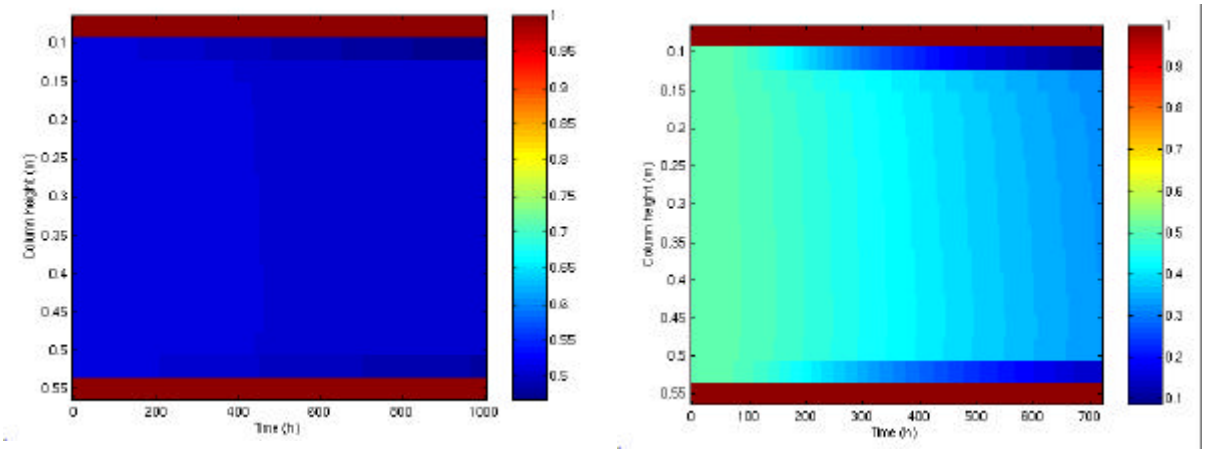


Figure 4d : Inert biomass distribution in autotrophic growth simulation.



Autotrophy **Mixotrophy**

Figure 4e : Evolution of the voidage inside bench column for autotrophic and mixotrophic growths.

3.2 An example of non oxygen limited mixotrophic growth : the UAB pilot column

As it is difficult to operate bench columns without oxygen gas-liquid transfer problems (TN 39.2), it can be interesting to simulate the mixotrophic growth without such oxygen limitations problems. Then a simulation of a mixotrophic growth was performed with the pilot column, with the characteristics presented in TN39.2. A 15-tank column without back-mixing was used for the simulations.

In order to be consistent with the simulations performed for the bench column, the concentrations of input substrate are those used in the M5 experiment operating conditions (Table 8). The flow rates used for the pilot column are also reported in Table 8. It can be outlined that in order to be certain of the absence of oxygen limitation, there is no gas recirculation.

The steady-state for output compounds is quickly reached (about 200h) and the results are reported in Table 9. Nitrification is total (about 100% of nitrate produced from ammonia), but acetic acid is not completely exhausted (52% remaining)). The N-source is limiting for both energetic metabolism and growth.

Operating conditions	Liquid : 0.18 10^{-3} m^3/h Liq. Recycling. : 0.108 10^{-2} $m^{3/}$h Gas : 0.18 m^3/h Gas Recycling. 0. m^3/h K_La measured (Top of the column): 50 h^{-1}

	NH3 (g N/l)	Ac. Acid (g N/l)	Nitrite (g N/l)	Nitrate (g N/l)
Input	0.3	0.6		
Output (Steady-state)	0. 0.%	0.31	#0.3 0.02%	#0.3 99.98%

Table 9 : Operating conditions for the simulation of the pilot column and steady-state output concentrations.

The concentrations and voidage profiles inside the fixed bed are reported in Figures 7.
Figure 7a shows that oxygen is used only at the bottom of the bed, and is a good indicator of the localisation of the biological (i.e. nitrification) activity. This is confirmed by figure 7b which shows that *Nitrosomonas* biomass is concentrated at the bottom of the column. It is worth to note that if the output concentrations have reached a steady-state in about 200h, the biomass is not in steady-state.

The concentration profile for ammonia and acetic acid concentrations profiles are reported in Figure 7c and Figure 7d. First, both ammonia and acetic acid are quickly consumed. When ammonia becomes limiting, the acetic acid concentration increases until a steady state value. It can be outlined that the output concentrations are representative of the concentrations inside the bed.

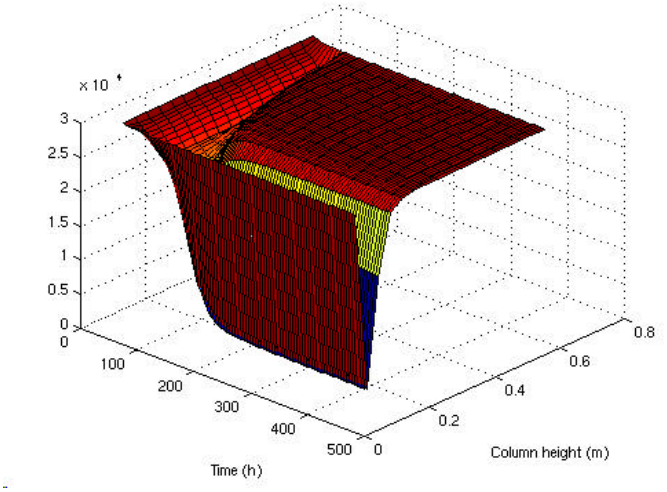


Figure 7a : Dissolved oxygen profile inside the pilot column for mixotrophic growth simulation.

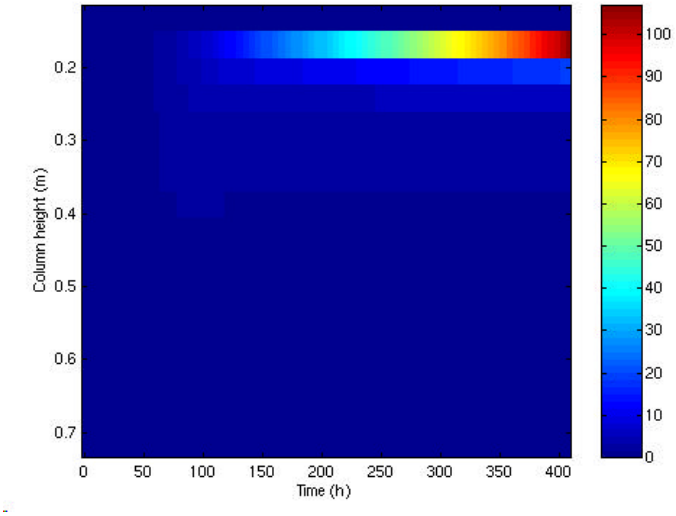


Figure 7b : Active *Nitrosomonas* biomass inside the pilot column for mixotrophic growth.

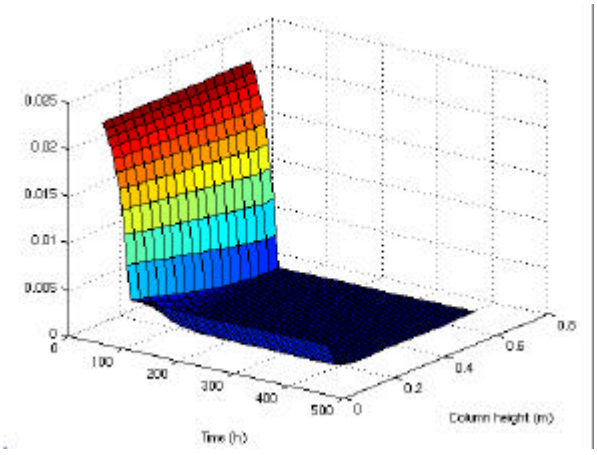


Figure 7c : Ammonia concentration profile inside the pilot column for mixotrophic growth.

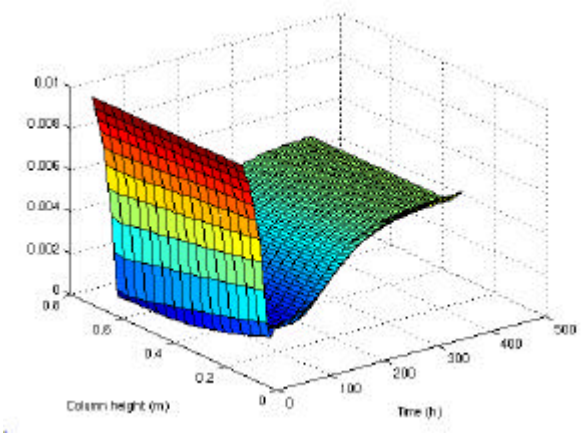


Figure 7d : Acetic acid concentration profile inside the pilot column for mixotrophic growth.

As for the bench column simulations, the biomass produced in mixotrophic growth in the pilot column is very important (Figure 7b). The effect on the voidage of the bed is thus not negligible (Figure 7e). After 400h, the voidage in the first tank of the fixed bed (bottom of the bed) has fallen from 0.37 to 0.11.

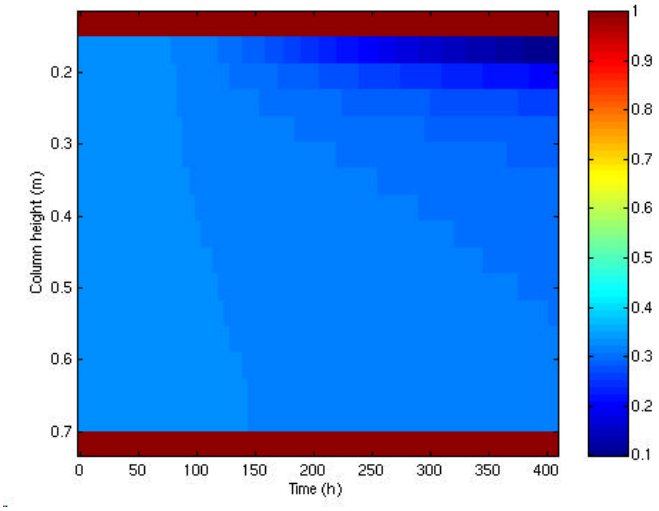


Figure 7e : Evolution of the voidage of the bed during mixotrophic growth in the pilot column.

Conclusion

The overall stoichiometric equations have been established for both autotrophic and mixotrophic growths. The yields calculated for mixotrophic growth are 4.5 10^{-2} moles acetic acid/g dry biomass for *Nitrosomonas*, 1.1 10^{-2} moles acetic acid/g dry biomass for *Nitrobacter* and about 4.4 10^{-2} moles acetic acid/g dry biomass for the co-culture. It must be take in mind that this last yield is calculated assuming that there is no limitation for the mixotrophic growth, and that there is no autotrophic growth. In case of a dual growth autotrophy+mixotrophy, the yield will decrease.
The stoichiometric model enables to take into account compounds such as PHB and NOx gas, but their productions depend on the culture operating conditions (mainly limitations) and are then difficult to introduce in a mass balance model without kinetic relations.

The kinetic model for both autotrophic and mixotrophic growths previously presented in TN 32.1 has been integrated into the NitriSim software, but the estimation of the kinetic parameters for the mixotrophic model remains problematic.
By taking into account the dead biomass in the calculation of the biofilm thickness, the growth of the biofilm during long operations of the column can be determined. This growth of the biofilm leads to a decrease of the voidage of the bed, and can fill in the column.
As attempted, the impact of the organic matter on the growth of the biofilm is very important, and clogging can appear rapidly.

Simulations were performed to test the model and to observe, for similar operating conditions, the effect of the organic matter on the nitrifying performances and on the overall behaviour of the column. The main result is the important impact on the biofilm thickness. The biofilm diffusion limitation can not be neglected, but it must be kept in mind that if biofilm diffusion is taken into account the model complexity and the computation time will be increased.
The oxygen requirements are much higher in presence of organic matter and thus nitrification efficiency is reduced if the operating conditions are close to oxygen limitation conditions.
Acetic acid can be completely consumed if the growth is not limited by another substrate (O_2 or N source). It can be noted that the effect of a limitation by O_2 or N-source will probably lead to the synthesis of PHB, but this phenomenon is not taken into account.
The saturation constant value used is uncertain, mainly for *Nitrosomonas* which is usually more sensible to organic matter than *Nitrobacter*.

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