MELiSSA

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TECHNICAL NOTE 32.1

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Modelling of the Nitrifying compartment of MELiSSA

Metabolism and growth of Nitrosomonas and Nitrobacter in presence of organic matter

- Carbon metabolism: autotrophy; mixotrophy; heterotrophy
- Energetic metabolism: oxygeny and anoxygeny
- Stoichiometries for mixotrophic growth
- Basis for kinetics in various growth conditions

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Content

T.N. 32.1: Nitrification and organic carbon sources

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Introduction

In the MELiSSA loop, the nitrifiers are pure cultures of *Nitrosomonas europea* and *Nitrobacter winogradsky*. At the present time, these nitrifiers are considered to growth lithoautotrophically (e.g. the carbon source source is CO_2, the nitrogen source is NH_3 and the energy source is NH_3 or NO_2^-). In the MELiSSA loop, the nitrifying compartment is linked to the previous compartment II (photohetrotrophic compartment), which is in charge of the assimilation of AGV. In order to have a lithoautotrophically growth in the nitrifying compartment, the organic load (AGV) must be completely assimilated by the phototrophs of compartment II. In fact that will probably not be the case; as seen in TN 17.2, organic matter can enter the nitrifying compartment. Then, the influence (enhancement/inhibition of the pure nitrification) of the organic load on the nitrifiers must be considered.

The purpose of this technical note, is the study of the effect of organic carbon on the metabolism and on the growth of nitrifiers.

Usually, the growth of an organism is characterized by its energetic metabolism and its carbon metabolism. The following classical definitions will be used:

If **inorganic substrates** oxidation or reduction serves as energy source, the growth is called **lithotrophic**.

If **organic substrates** oxidation or reduction serves as energy source, the growth is called **organotrophic**.

If **light** serves as energy source, the growth is called **phototrophic**.

If **organic** substrates serves as carbon source, the growth is called **heterotrophic**.

If **inorganic** substrates serves as carbon source (CO_2), the growth is called **autotrophic**.

By combination of the classification of energy source and carbon source, appropriate adjectives can be constructed which are descriptive of the metabolism of an organism. Other adjectives can also be used for special growth, as "mixotrophic" which describes a growth where carbon source is organic and energy source is inorganic, or "diauxic" which describes the growth on two carbon sources. Often, an heterotrophic growth is relevant of a growth where both carbon and energy source are organic.

Two types of nitrification are observed:

- **lithotrophic nitrification**, which is characterised by the fact that the inorganic substrates NH_3 or NO_2^- serve as energy sources for growth. Two groups of bacteria are involved: the ammonia and the nitrite oxidisers.
- **heterotrophic nitrification**, which is a cooxidation not coupled to energy generation. It is carried out by diverse groups of bacteria, fungi and even some algae. The heterotrophic nitrifiers predominantly use organic nitrogen sources and produce only a small amount of nitrite and nitrate.

In natural environment, the lithotrophic nitrifiers are the only group of organisms producing considerably high amount of nitrite and nitrate from ammonia.

In waste water treatment, heterotrophs and autotrophs don't act simultaneously. Until the organic load is sufficiently low, the growth rate of heterotrophs is greater than that of autotrophs. Both the decay of heterotrophs and the increase in ammonia (from the effluent and released by heterotrophs), give the start up of the nitrification process by autotrophs nitrifiers.

As well, in the fixed bed growth nitrification systems developed for waste water treatment, nitrification occurs after the organic load falls low enough to limit the growth of heterotrophs, so that the nitrifiers can compete for space in the oxygen zone of the biofilm (Strand, 1986).

Even if nitrifiers are lithoautotrophs, using ammonia or nitrite as the energy source and carbon dioxide as carbon source, they can grow mixotrophically and heterotrophically. For nitrification they are obligate aerobes. But in absence of oxygen and with organic carbon, their ability to reduce nitrate and nitrite to nitrous oxide (NxO) is used in waste treatment for nitrification-denitrification processes, in association to other bacteria. This anoxygenic behaviour is not wanted in the MELiSSA loop concept, and then the process conditions must be taken in order to avoid it.

The purpose of this work is to adapt the previous analysis of Nitrosomonas/Nitrobacter cultures in lithotrophic conditions, as presented in TN27.1, to heterotrophic conditions.

I Bibliography

This part will give an overview of the ability or the inability of nitrifiers to growth on organic carbon sources. It must be outlined that the heterotrophic growth of nitrifying bacteria generally concerns mixed cultures of autotrophs nitrifiers (*Nitrosomonas*, *Nitrobacter*) and heterotrophs, the first species oxidising ammonia to nitrate and other species insuring organic load reduction and ammonia assimilation into biomass. As noticed by Hanaki et al.(1990), the effects of organic load on nitrifiers can be difficult to analyse because of the presence of other species such as heterotrophs.

1.1 - Carbon metabolism of ammonia oxidisers [Nitrosomonas]

The ammonia oxidisers are autotrophs. Although organic compounds can be assimilated to a limited extend to mixotrophic growth. Heterotrophic growth has not been observed (Bock,1991).

1.1.1 - Autotrophic growth

Ammonia oxidisers have the capacity of using the CO_2 as the main carbon source via the Calvin cycle (Glover, 1983, TN 23.2).

1.1.2 - Mixotrophic growth

Bock (1991) reports that all ammonia oxidisers studied are able to metabolise organic compounds to a limited extend, in presence of the inorganic energy source of NH_3 (mixotrophic growth). In general the most suitable organic compounds are acetate and formate, and to a lesser extent pyruvate. But in details, the influence of organics on growth depends both on the substrate and on the strain used. However, at increasing concentrations, organic substrates are often inhibitory (Bock,1991 ; Hanaki et al.,1990 ; Clark and Schmidt, 1966). Most of the the experiments were leaded on mixed culture for waste sewage treatment, thus it is difficult to used the results for the pure cultures involved in the MELiSSA nitrifying process. Further studies will be needed for a better comprehension of the effect of organic matter on amonia oxidizers.

1.1.3 - Carbon metabolic pathways

Detailed investigation on the central metabolic pathways of ammonia oxidising bacteria has been carried out with only very few strains (Bock, 1991).

The distribution of ^{14}C from pyruvate and acetate (mixotrophic growth) into cell amino acid was restricted to the pyruvate and glutamate family, and to leucine and glutamate family respectively (Bock, 1991). That supposes an incomplete TCA cycle (Tri-carboxilic Acids) and corresponds to the observations of Hooper and Watson that *N. europea* and *Nitrosolobus*

multiformis are both lacking of an α-oxoglutarate dehydrogenase. The distribution pattern also indicates the absence of pyruvate carboxylase and of phosphoenol pyruvate synthetase.

The growth of ammonia oxidisers on glucose supposes the presence of the Embden-Meyerhoff-Parnas (glycolysis) pathway. Nevertheless, it is generally observed that organic carbon sources were incorporated at relatively small amount compared to the main carbon source CO_2

The NADH-oxidase activity coupled with ATP synthesis is reported for several strains, but is absent for some of others.

We have summarised these informations by representing the central metabolic pathways and the carbon partition, considering the different carbon source (CO_2, acetate, pyruvate). It helps to explain the inhability of Nitrosomonas to grow on organic substrates.

In lithoautotrophic growth, the CO_2 assimilation via the Calvin cycle, used reduced cofactors supplied by NH_3 oxidation. It is sufficient to feed the totality of the central metabolic pathways in carbon precursors for the synthesis of all molecules needed for the growth (RNA, DNA, amino acids, carbohydrates, lipids) (figure 1-a).

In mixotrophic growth, the carbon source results in acetate or pyruvate assimilation. CO_2 assimilation via the Calvin cycle is probably partially repressed, even if there is a supply in reduced cofactors by NH_3 oxidation. The carbon assimilation from acetate or pyruvate provides only a part of the central metabolic pathways. The limited ability of Nitrosomonas to growth in mixotrophic condition can be due to the limited ability to fix CO2 via the Calvin-benson cycle, because of the repression of RubiscCo enzymes by organic matter. In addition, it seems that the enzymes capable to feed the upper part of glycolysis (glucose 6P to phosphoenol pyruvate) from the lower part (pyruvate and TCA intermediates) are not operatives in Nitrosomonas species. These enzymes are respectively the malic enzyme and the PEP synthase.

Therefore acetate or pyruvate are not sufficient to feed the totality of the central metabolic pathway, and must be completed by the assimilation of CO_2 via the Calvin cycle; but this last pathway is partly repressed. (figure 1-b).

In heterotrophic growth, the CO_2 fixation by Calvin cycle is completely repressed and/or insufficiently supplied in reduced cofactors (there is no more NH_3 oxidation, and the oxidation of organic substrate is not sufficient to produce the required reduced cofactors).

The reality is probably a combination of these two assumptions. The result is that only a part of the metabolites needed for the growth can be synthetised, and then no growth can occur (figure 1-c).

1.1.4 - Nitrite reduction ability of ammonia oxidisers

At low oxygen concentration, N_2O and NO are produced by cells or cell free extracts of *Nitrosomonas*. Bock (1991) reports an evolution of N_2O from 0.3 to 10% (molN/mol NO_2^- produced) at decreasing dissolved oxygen concentration from 7 to 0.18 mg/l.

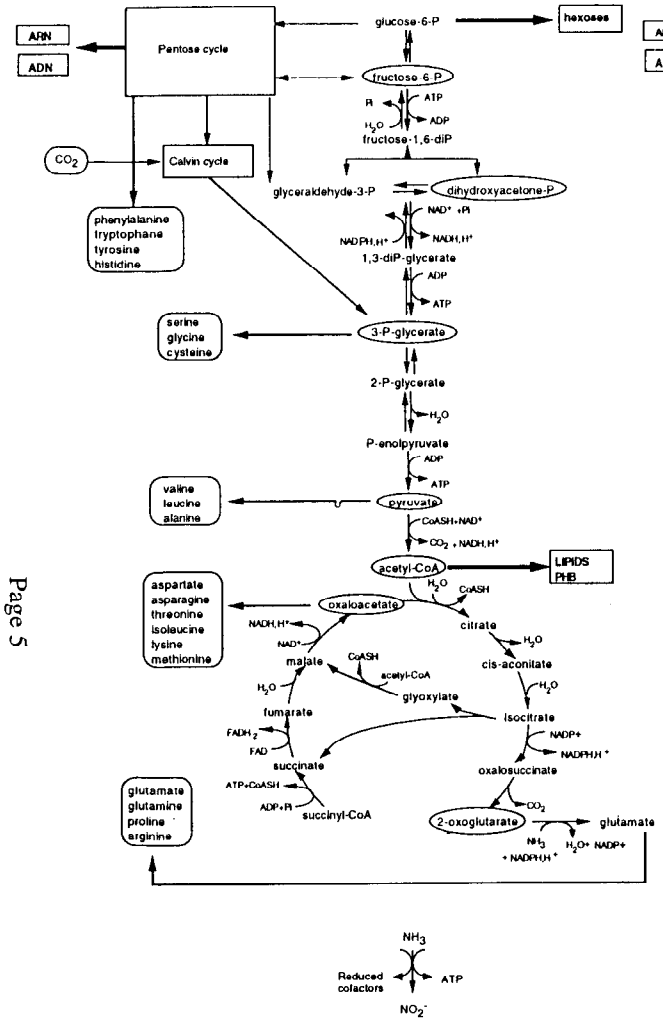


Figure 1-a: Central metabolic pathway of Nitrosomonas in autotrophic growth. all precursors can be synthesised.

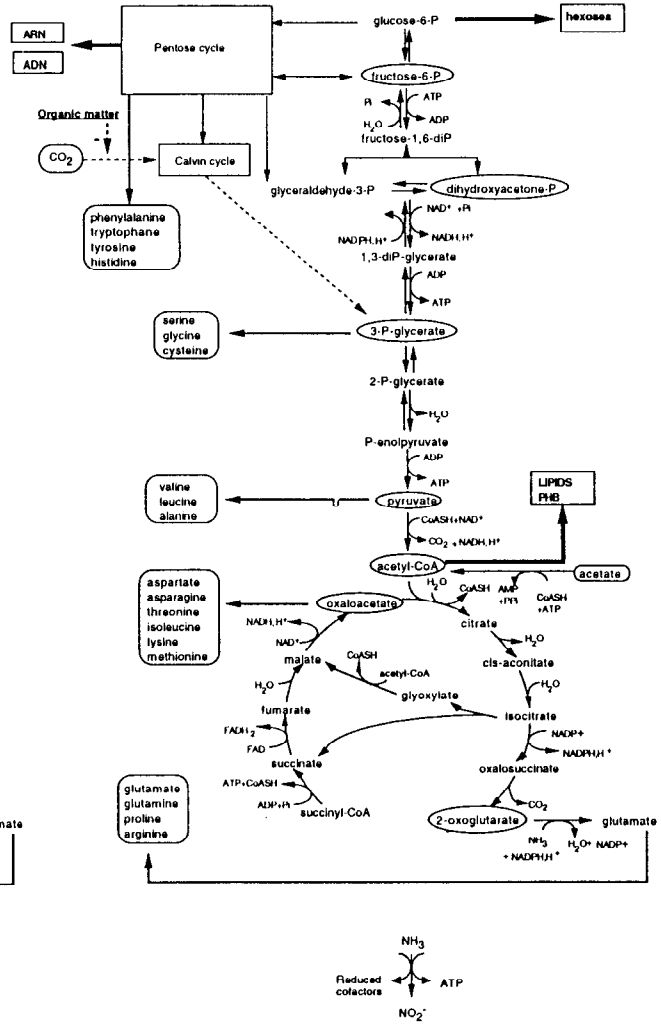


Figure 1-b: Central metabolic pathway of Nitrosomonas in mixotrophic growth. All precursor can be synthesused. There is a partial repression of CO_2 fixation

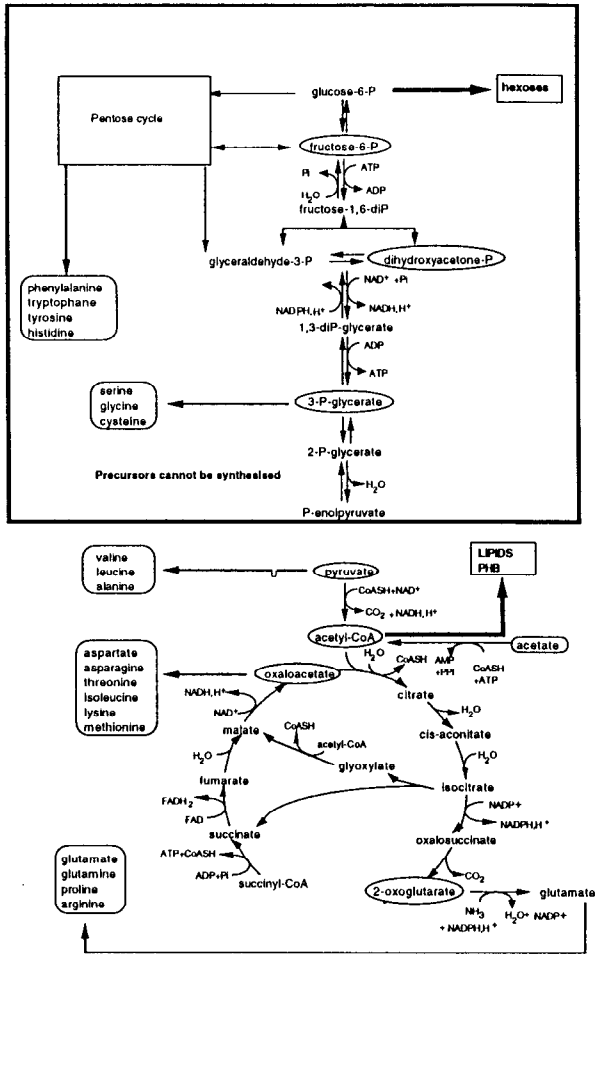


Figure 1-c: Central metabolic pathway of Nitrosomonas in heterotrophic growth. The precursors synthesised from the upper part of the carbon metabolic patway can not be synthesised.

Blackmer et al. (1980) report that *Nitrobacter winogradsky* does not have the capacity to convert NH_3 or nitrite to N_2O.

Ritchie and Nicholas (1971) studied the nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europea.* They concluded that N_2O is produced during the oxidation of NH_3 and hydroxylamine, and using N-labelled nitrogen, they show that in fact the end product of ammonia oxidation (nitrite) is the real source of N_2O.

Under anaerobic conditions, cells also produce N_2O by the reduction of nitrite.

The scheme of inorganic nitrogen metabolism proposed by Ritchie and Nicholas (1971) is reported in figure 2.

1.2 - Carbon metabolism of nitrite oxidisers [Nitrobacter]

The nitrite oxidisers are autotrophs and CO_2 is the main carbon source. All nitrite oxidisers can grow mixotrophically. Only some strains of *Nitrobacter* are able to grow heterotrophically (Bock, 1991).

1.2.1 - Autotrophic growth

In autotrophically grown cells, nitrite serves both as energy and nitrogen sources. Wallace and Nicholas (1969) demonstrated that the ^{15}N-labelled nitrogen atom of nitrate or nitrite was incorporated after 24h. Nitrobacter contains soluble enzymes (reductases) for the reduction of nitrate, nitrite and hydroxylamine (Bock,1991). Nevertheless, in presence of NH_3, nitrite is still the energy source, while ammonia is easier to use as nitrogen source.

Using 2 types of RubisCo enzymes, bacteria of the Nitrobacter genus are able to fix CO_2 via the Calvin cycle (Bock, 1991; TN 23.2). The RubisCo enzymes are soluble or bound to greater structures (carboxysomes).

1.2.2 - Mixotrophic growth

Nitrite oxidising bacteria can grow mixotrophically when nitrite is the only energy source and CO_2 together with organic compounds are the carbon sources. Although nitrite oxidisers of the genera Nitrospira and Nitrobacter assimilate limited amount of organic compounds, normally the growth rate increases (Watson et al., 1986).

Steinmuller et al. (1976) reported that nitrite oxidation is stimulated by low concentrations of culture filtrate of *Pseudomonas fluorescens* grown on yeast extract, and that there is inhibition at high concentrations (medium with 50% of filtrate). In presence of 10% of culture filtrate, the cells yield (N-cell/N oxidised) of *Nb. agilis* grown mixotrophically is about 11% higher than in autotrophic control.

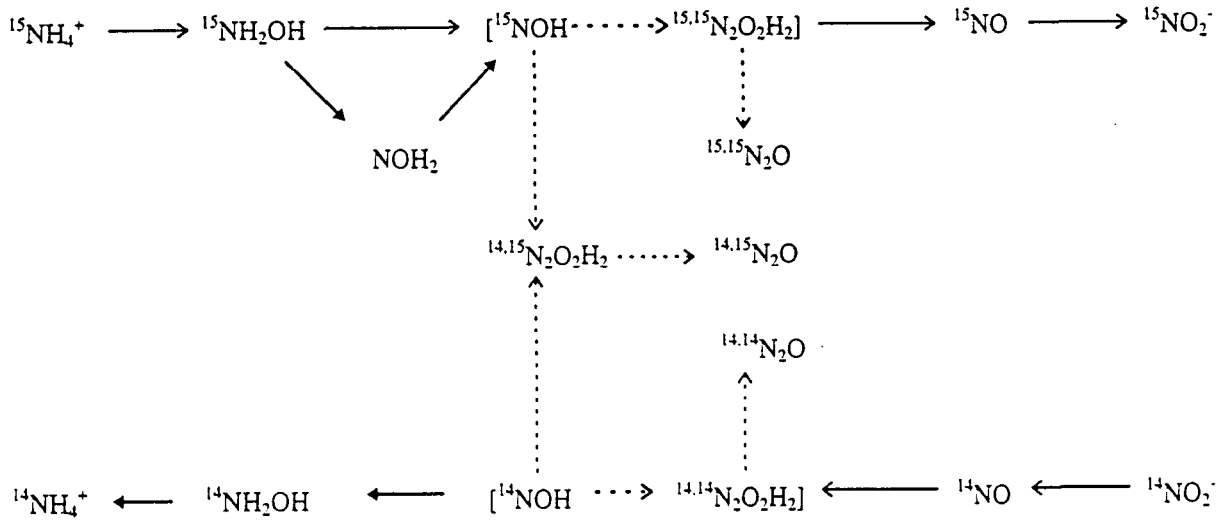


Figure 2: Inorganic nitrogen metabolism in *Nitrosomonas europea*. Ritchie and Nicholas (1971). ^{15}N-labeled amonium. - - - » Chemical reaction; ⟶ Enzymatic reaction; ⟶ Possible enzymatic reaction.

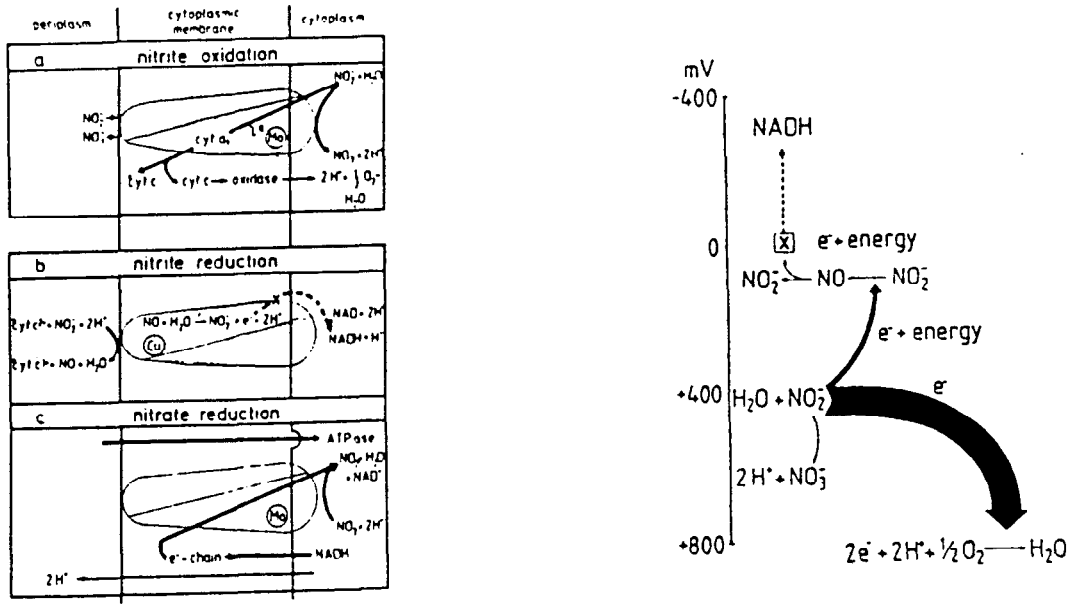


Figure 4: Inorganic nitrogen metabolism in Nitrobacter and energy generation. (Bock, 1991). X: reducing equivalent (redox potential unknown).

The growth of *Nitrobacter agilis* in presence of organic matter (Smith and Hoare, 1968) has shown that with concentrations of acetate in the range of 1 to 10mM supplemented to an "autotrophic medium", the growth is neither stimulated nor inhibited. Similarly, the organic supplement do not affect the nitrite oxidation rate. Carbon from acetate contribute to 33 to 39% of newly synthesized cell carbon under exponential growth (assuming that cell carbon represents 50% of dry weight). Even at lower acetate concentration more than half of the added acetate remains unchanged at the end of the growth experiments. Radioactive carbon from ^{14}C acetate is present in fraction containing lipids, nucleic acids, proteins (37.2% of total ^{14}C) and PHB (4.6% of total ^{14}C)

1.2.3 - Heterotrophic growth

Numerous strains of *Nb. winogradskyi*, *Nb. hamburgersis* and *Nitrobacter ssp.* (Bock et al, 1991) have been grown heterotrophically. *Nb winogradskyi* is able to grow in medium containing acetate as energy source and casein hydrolysates as nitrogen source. Pyruvate, glycerol and less favourable formate and α-oxoglutarate can also support heterotrophic growth (Bock, 1976).

Heterotrophic growth is slow under aerobic as well under anaerobic conditions. The generation times between 30 and 150h are generally higher than those of lithoautotrophically grown cells but there is a 10 - to - 50 fold increase in cell number per millilitre at the end of growth.

The amino acid were stimulatory to pyruvate+NH_3 or pyruvate+NO_3^- growing cells *of Nb. agilis* and *Nb. winogradskyi*..

When Nitrobacter is grown on pyruvate +yeast extract, less than 1% of the cell contain polyhedral inclusion (i.e. carboxysomes), whereas lithoautrophic bacteria possess 5 to 10 particles per cell. In cells grown on pyruvate+ammonia or pyruvate+nitrate, these particles can not be detected.

In absence of nitrite, *Nitrobacter agilis* (Smith and Hoare, 1968) is able to assimilate acetate carbon into cell material (proteins and PHB). But the acetate assimilation is enhanced by the nitrite oxidation. In absence of nitrite, the energy necessary for the assimilation of acetate and biosynthesis could have come from endogenous reserves (PHB, glycogen like compounds) or from the oxidation of acetate itself. In absence of nitrite, *Nitrobacter agilis* growing with acetate produces CO_2 (the moles of CO_2 produced equals the moles of ^{14}C-acetate incorporated in cell material). In presence of nitrite, the CO_2 production is suppressed and the acetate assimilation is almost doubled. By the way the cells growing with nitrite+acetate did not fix significant amounts of CO_2.

1.2.4 - Carbon metabolic pathways

In Nitrobacter cells, all enzymes of the TCA cycle can be detected, but some as the succinate dehydrogenase are at very low concentrations. Generally they have higher activities in heterotrophic growth. The isocitrate dehydrogenase is shown to be NADH dependent. The activity of the pyruvate dehydrogenase is doubled when pyruvate is the only source of energy. NADH but no NADPH oxidase is present. NADH oxidase, phosphoenol pyruvate carboxylase

and RubisCo are partially repressed in heterotrophically grown cells, with nitrate or ammonia as nitrogen source.

The distribution of ^{14}C in cell compounds from acetate-1-14C in *Nitrobacter agilis* (Smith and Hoare, 1968), reflects the normal metabolism of acetate via the tricarboxylic cycle. *Nitrobacter agilis* uses acetate carbon for the synthesis of a variety of cell constituents without any obvious metabolic restriction. The authors were not certain of the presence of a functional glyoxylate shunt in *Nb. Agilis* when growing in presence of acetate.

Nitrobacter agilis growing with acetate+nitrite stores PHB (Bock, 1976; Smith and Hoare, 1968). Cells growing with yeast extract-peptone store large quantities of PHB, polyphosphate and possibly glycogen like material, and in cell growing from α-ketoglutaric acid, formate and pyruvate, PHB granules prevails (Bock, 1976). Bock (1976) reports that in culture supplied with pyruvate, some bacteria were motiles. Bock et al. (1988) report that Nitrobacter growing under anoxic conditions contains up to 10 mg PHB per mg of cell protein. The increase in reserve material (mainly PHB), lead Smith and Hoare (1968) to note the danger of interpreting the increase in turbidity in terms of growth.

Clarck and Schmidt (1966), reported for *Nitrobacter sp.*, that neither acetate nor other organic amendments allow any growth or nitrification in absence of carbon dioxide.

Table 1: Protein to dry weight ratios of *Nitrobacter agilis* grown under various conditions (Smith and Hoare, 1968).

Medium	Phase of growth [a]	
	Exponential	Stationary
Autotrophic	0.55	0.54
Autotrophic + 1 mM acetate	0.47	-
Autotrophic + 5 mM acetate	0.44	0.26

a: organisms were harvested from cultures in the exponential phase of growth or from cultures containing limiting nitrite, after 332h.

We have summarised these informations by representing the central metabolic pathways and the carbon partition, considering these different carbon source (CO_2, acetate, pyruvate) (figures 3).

In lithoautotrophic growth, the CO_2 assimilation via the Calvin cycle, supplied in reduced cofactors by NO_2^- oxidation, is sufficient to feed the totality of the central metabolic pathway and all molecules needed for the growth (RNA, DNA, amino acid, carbohydrates, lipids) can be synthesized (figure 3-a). The phosphoenol pyruvate carboxylase exists (it is repressed in heterotrophic cells). The succinate deshydrogenase is present but is one of the less active enzyme of the TCA cycle (Smith and Hoare, 1968), and is then assumed to be absent in the central carbon pathway. This is in accordance with the assumption of the reduced activity of the span oxoglutarate -> succinate when CO_2 is fixed via the Calvin cycle (TN 23.2). The second anaplerotic pathway of the TCA cycle, the glyoxylate shunt, is supposed to be inactive, what can be related to the low activities of the fumarase and of the malic dehydrogenase (Smith and Hoare, 1968).

In mixotrophic growth, the carbon source results in acetate and CO_2 assimilation. The CO_2 fixation is confirmed by the observations of Clark and Schmidt (1966), who report for *Nitrobacter sp.* growing on acetate, the inability to grow without CO_2 on organic matter, and by the fact that, only 33 to 39% of the new synthesized carbon comes from acetate. This could be the result of a lower repression of Rubisco by organic matter than on *Nitrosomonas sp.*.

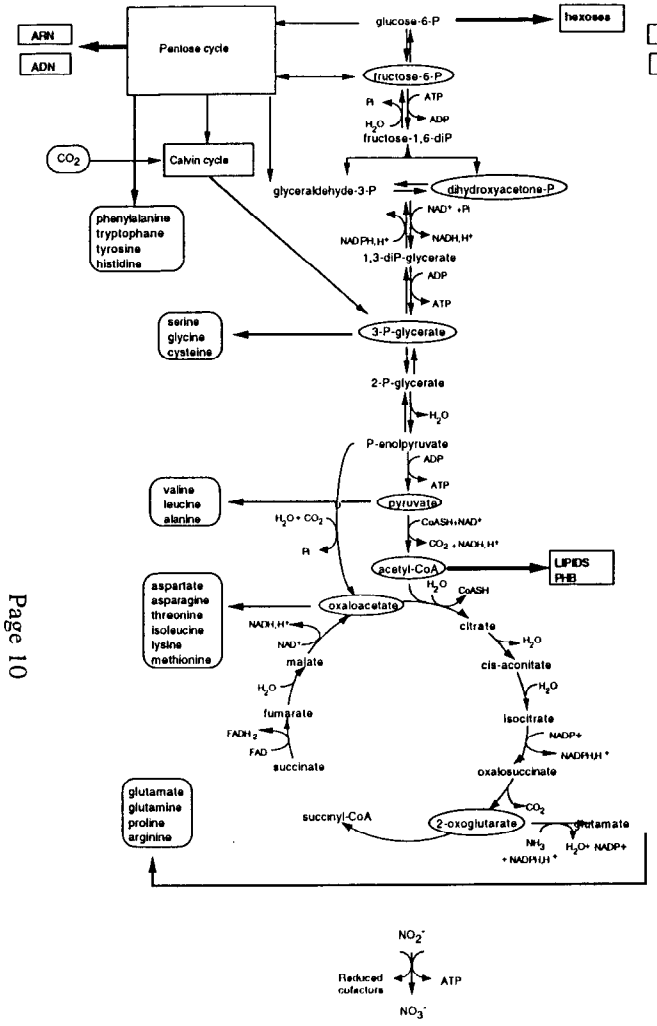


Figure 3-a: Central metabolic pathway of Nitrobacter in autotrophic growth. All precursors can be synthesised.

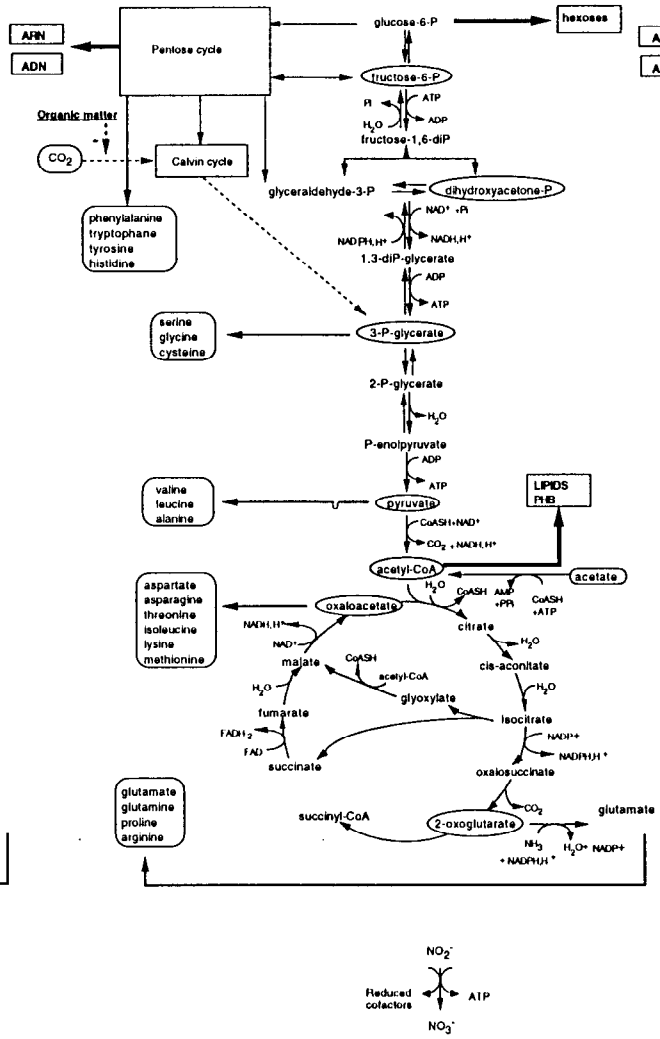


Figure 3-b: Central metabolic pathway of Nitrobacter in mixotrophic growth. all precursors can be synthesised. CO_2 fixation is partially repressed.

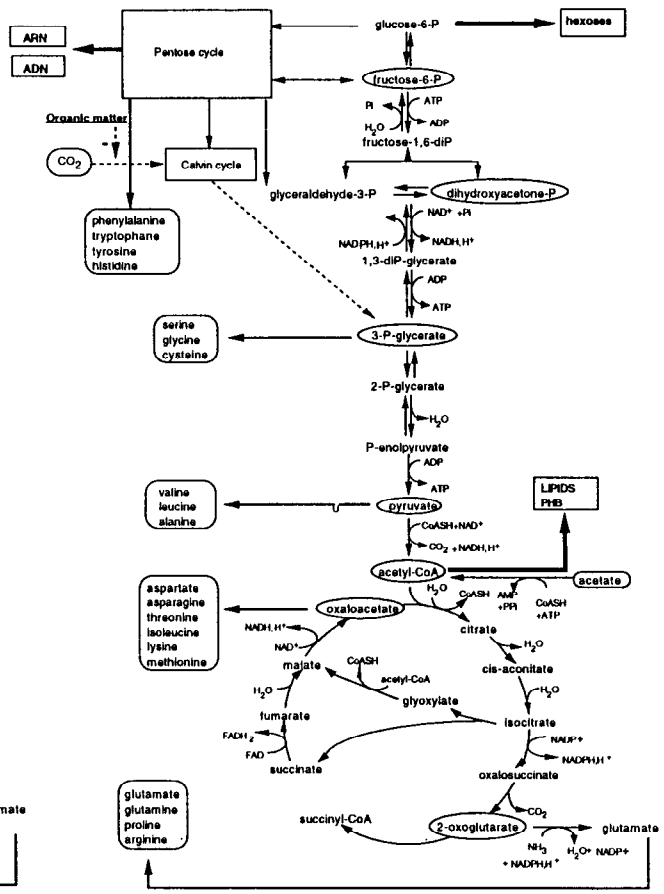


Figure 3-c: Central metabolic pathway of Nitrobacter in mixotrophic growth. all precursors can be synthesised. CO_2 fixation is partially repressed.

For the TCA cycle, the anaplerotic pathway chosen is the glyoxylate shunt (phosphoenol pyruvate carboxylase is repressed in heterotrophic growth) (figure 3-b).

In heterotrophic growth, the ability to fix CO_2 via the Calvin-Benson pathway is conserved. The cells can grow because all components of the cell can be synthetised. It must be noted that this assumption is not in accordance with the observation that carboxysomes are absent in heterotrophically growing Nitrobacter cells, but because no pathway between acetate or TCA cycle intermediate, and phosphoenol pyruvate (or other intermediate of the glycolysis) are cited, the Calvin-Benson pathway must be conserved. Excepted for the energy production, the central metabolic pathways in heterotrophic growth are then the same as in mixotrophic growth.

1.2.5 - Nitrite and nitrate reduction ability of nitrite oxidisers

Heterotrophic denitrifying cells of Nitrobacter are inhibited by nitrite concentrations above 0.5 mM (Freitag et all, 1987). Probably the nitrate reductase is inhibited by high concentrations of nitrite. The nitrite oxidase is an inductible enzyme. Cytochrome a_1 is present at high concentrations in lithoautotrophic and at lower concentrations in chemoorganotrophic cells grown on pyruvate+nitrite or yeast extract. Heterotrophic cells grown on ammonia or Casamino acid as N-source possess hardly any nitrite oxidase activity. The nitrite oxidase is induced by nitrite as an energy source as well as by substrate like yeast extract or nitrate when used as N-sources.

Under anaerobic conditions, cells are able to reduce nitrate to nitrite. Therefore, nitrite oxidation is a reversible process. Several authors report that nitrate reduction is coupled to ATP generation. Under anaerobic condition, Nitrobacter could work with pyruvate as electron donor and nitrate as electron acceptor. In the nitrite reduction, it can not be found any ATP production, but a NO production and an increase in the NADH pool.

NO appears as an important intermediate in the nitrifying cells. NO is produced under anaerobic conditions, but at low oxygen partial pressure (5-8% air saturation) in mixotrophic growth, a NO production can be obtained (Bock, 1991).

The energetic metabolism model proposed by Nicholls and Ferguson (TN 23.2) does not include the NO intermediate. From Bock (1991), the NO formation is energy dependent and represents the second electron sink in the oxido-reductive chain. The reduction of NO_2^- to NO occurs at the cyt c level. The two possible reactions are:

$$NO_2^- + 2H^+ + e^- \longrightarrow NO + H_2O \qquad E'^0 = 0.36V$$

and

$$NO_2^- + H_2O + e^- \longrightarrow NO + 2\,OH^-$$

but the first one is the most probable.

NO oxidation seems coupled with NADH generation; when *Nb. winogradskyi* oxidises 2 molecules of NO via nitrite to nitrate, 1 molecule of NADH is synthesised (Bock, 1991).

The different aspect of inorganic nitrogen metabolism and energy generation in Nitrobacter are summarised in figure 4.

1.2.6 - Possible origin of the difference in the inhibition of the RubisCo in Nitrosomonas species and Nitrobacter species

It seems strange that, as can be seen in the metabolic description reported in figures 1 and 3, with the same carbon pathways the 2 organisms (Nitrosomonas and Nitrobacter) have very different behaviours in presence of organic matter. To explain the ability of N

itrobacter species to grow in heterorotrophic conditions, while Nitrosomonas species can not grow, it was supposed that the sensibility of RubisCo enzymes to organic acid (i.e. the ability to fix HCO_3^- via the Calvin cycle) is different for the two organisms.

At high pH value, organic acids are under an ionised form (i.e. acetate under the form $CH3COO^-$), which interact with the catalytic site of RubisCo enzyme.

It seems that both for Nitrosomonas and Nitrobacter not growth nor nitrification can occur in absence of carbon dioxide (Clark and Schmidt, 1966). CO_2 fixation is needed to supply the cell in precursors synthesised by the upper part of the central metabolic pathways (Glucose 6P - PEP). This is in accordance with the observation that Nitrobacter in mixotrophic growth on acetate assimilate only 33 to 39% of carbon from acetate (the other part comes from CO_2).

In order to reduce the effect of organic acid on the RubisCo, the organic load in the cell must be reduced. The synthesis of PHB could be the path through which the acetate is removed from the intracellular medium. The key enzyme for the PHB synthesis (and all other Poly-hydroxy alkanoate) is the β-cethiolase (acetylCoA ←→ acetoacetylCoA). The synthesis of PHB exits in Nitrobacter species species, and PHB can represent up to 90% of the cell mass. In Nitrosomonas species, the presence of PHB is not reported in literature, the absence of a β-cethiolase, and then the inability to make PHB could be the reason why the organism is more sensible to organic acid than Nitrobacter. The inability of Nitrosomonas to synthesise PHB can be deduced from experiments leaded by Clark and Schimdt (1966): they observed that in mixed culture, Nitrosomonas growing with pyruvate 2-C^{14} labeled does not assimilate more than 1% of C^{14}, and that more than 95% of the marked carbon is found under a carbonate form in the medium. It seems then that in Nitrosomonas species the organic acid are oxidised in CO_2 rather than stored in PHB.

II - Structured stoichiometries for nitrifiers in mixotrophic or heterotrophic growth

2.1 - Structured stoichiometries

The building of structured stoichiometries was developed in TN 23.2 and 23.3. The energetic metabolism of Nitrosomonas and Nitrobacter was studied in TN 23.2.

The previous structured stoichiometries established for Nitrosomonas and Nitrobacter involved a metabolic matrix of 114 compounds and 104 reactions, solved by using the following assumptions for the establishement of an anabolic stoichiometry (without maintenance) (TN 23.2 and 27.2) :

- biomass composition (table 2) taken from *Rs. rubrum* (TN 23.3)
- absence of α-ketoglutarate deshydrogenase
- absence of the glyoxylate shunt
- absence of maintenance
- the span glucose 6P ->gluconolactone 6P is inactive

These assumptions previously used must be modified, according to the results presented in the first part of this TN. These modifications concern :

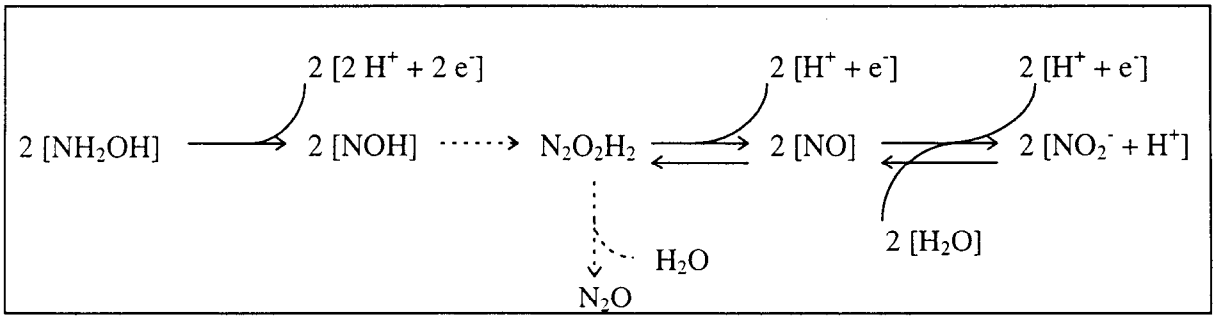
- the description of the oxido-reductive chain both of Nitrosomonas and Nitrobacter, providing reduced cofactors and energy (ATP)
- the assumptions on the metabolic pathways used to solved the metabolic matrix
- the assumptions on the biomass composition.

2.1.1 - Oxido-reductive chain - energy metabolism

As shown in figure 2 (Nitrosomonas) and in figure 4 (Nitrobacter), some intermediates of the oxidation (or reduction) of inorganic compounds by nitrifiers were not taken into account in the previous description of their oxido-reductive chain (figures 5 and 6).

Nitrosomonas

Nitrosomonas is able to produce N_2O (involving a chemical reaction from $N_2O_2H_2$) and NO (an intermediate of the oxidation of hydroxylamine to nitrite). The reaction paths proposed by Ritchie and Nicholas (1971) can be summarised by the following chain of reactions :



Including this chain of reactions in the previous oxido-reductive chain of Nitrosomonas (figure 7) leads to a representation similar to that reported by Bock (TN 23.2).

The span NH_2OH -> NOH is driven by the hydroxylamine oxidase complex (HAO).

The chemical span NOH->$N_2O_2H_2$ and the enzymatic span $N_2O_2H_2$->NO_2^- are still unclear. Nevertheless, there is evidence that in the Nitrobacter oxido-reductive chain, the electron reduces nitrite to NO at the Cytochrome c level. Perhaps the last span NO->nitrite in Nitrosomonas is located at the Cyt_{552} level, what could be in accordance with the remark of Wood (TN23.2) that one of the 4 electrons released by hydroxylamine oxidation reaches directly the Cytochrome c, at lower redox potential than the others.

The updated theoretical oxido-reductive chain and energetic metabolism of Nitrosomonas are reported in figure 7.

The stoichiometries for the production of reduced cofactors and of ATP production (electrons transfer and protons translocation) can be represented by the following group of reactions :

$$NH_3 + 4H^+_{in} + O_2 \longrightarrow 4H^+_{out} + \frac{1}{2}N_2O_2H_2 + H_2O$$

$$\frac{1}{2}N_2O_2H_2 + \frac{1}{2}UQ \longrightarrow NO + \frac{1}{2}UQH_2$$

$$\frac{1}{2}N_2O_2H_2 \longrightarrow \frac{1}{2}N_2O + \frac{1}{2}H_2O$$

$$NO \longrightarrow NOgas$$

$$NO + \frac{1}{2}H_2O + 2H^+_{in} + \frac{1}{4}O_2 \longrightarrow \left[NO_2^- + H^+\right] + 2H^+$$

$$UQH_2 + 6H^+_{in} + \frac{1}{2}O_2 \longrightarrow UQ + 6H^+_{out} + H_2O$$

$$NAD^+ + UQH_2 + 4H^+_{out} \longrightarrow NADH,H^+ + UQ + 4H^+_{in}$$

$$3H^+_{out} + ADP + Pi \longrightarrow 3H^+_{in} + ATP + H_2O$$

The redox potentials of the couples involved in the oxido-reductive chain are:

$E'^\circ_{NH3/NH2OH}$=1.133 V

$E'^\circ_{NH2OH/N2O2H2}$=0.148 V

$E'^\circ_{NH2OH/NO}$=0.270 V

$E'^\circ_{NO/NO2^-}$=0.3673 V

This new description of the energetic metabolism introduces new compounds : NO, $N_2O_2H_2$ and N_2O, which will require more information than in the previous description for the solving of the metabolic matrix.

Nitrobacter

Nitrobacter is able to produce NO gas but not N_2O. Bock (1991) reports that the NO production is the result of the reduction of nitrite at the Cyt c level. Moreover, NO seems linked to $NADH,H^+$ production and must then be considered as a possible intermediate of the

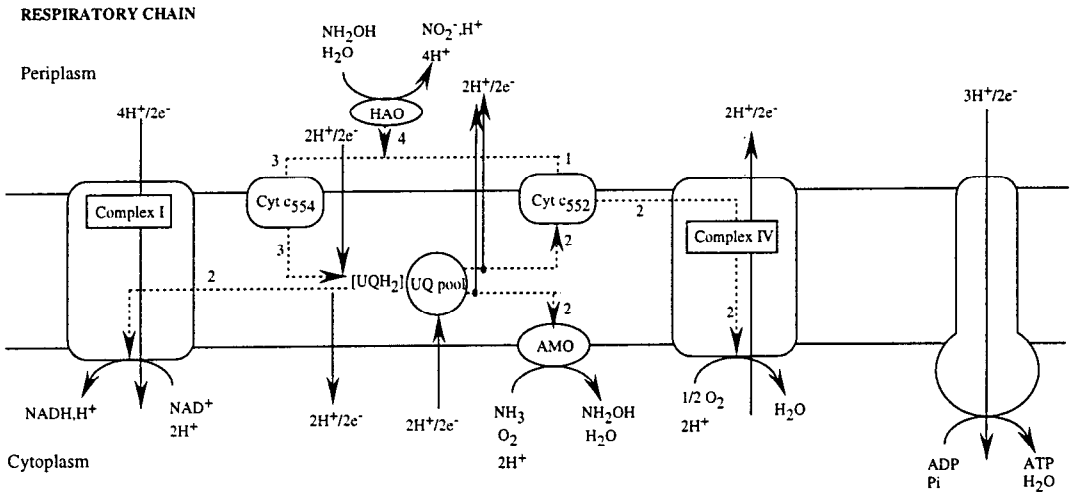


Figure 5: Oxydo-reductive chain of Nitrosomonas (from TN 23-2)

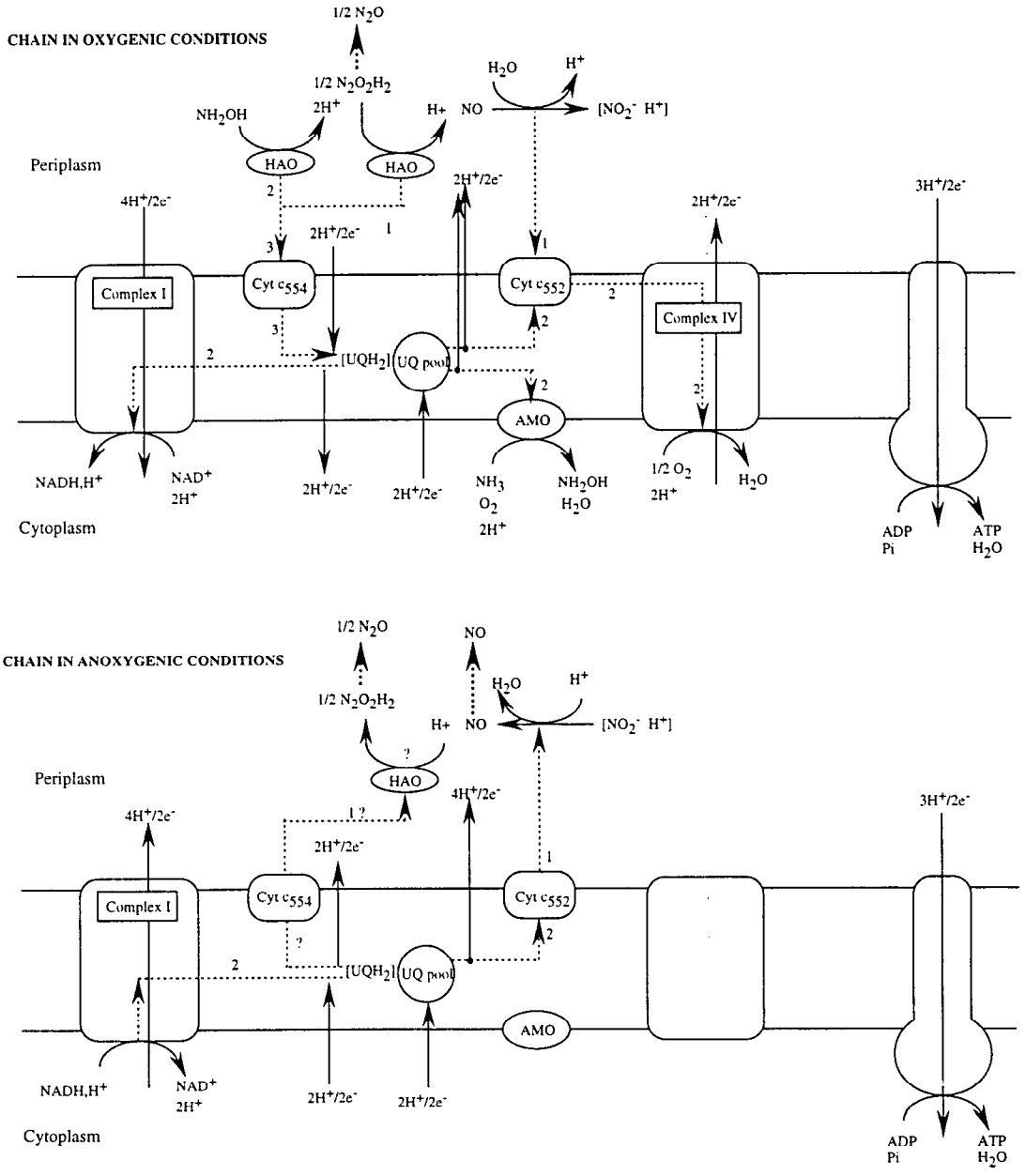


Figure 7: Oxydo-reductive chain of Nitrosomonas

oxido-reductive chain. Considering the proposed scheme for Nitrosomonas, it can be assumed that the reaction nitrite -> NO is reversible. The ability of Nitrobacter to reduce nitrate to nitrite supposed the reversibility of the reaction catalysed by the nitrite oxidase (figure 4).

The following reaction is added to the previous description of the oxido-reductive chain of Nitrobacter :

$$\left[NO_2^- + H^+\right] + H^+ + e^- \rightleftarrows NO + H_2O$$

As for Nitrosomonas, it is supposed that the reaction is driven by the Cyt c which provides electron for the reduction of nitrite or takes the electron when NO is oxidised (figure 5).

The updated theoretical oxido-reductive chain and energetic metabolism of Nitrobacter are reported in figure 8. There is no informations about the origin of the protons used in the reaction ; considering that Cyt c is usually located on the periplasmic side of the membrane, then it can be supposed that the protons involved are periplasmic.

The stoichiometries for the production of reduced cofactors and of ATP production (electrons transfer and protons translocation) can be represented by the following group of reactions :

$$\left[NO_2^- + H^+\right] + HO + Cytc_{ox} \rightleftarrows \left[NO_3^- + H^+\right] + Cytc_{red} + 2H^+$$

$$Cytc_{red} + 8H^+_{out} + NAD^+ \longrightarrow Cytc_{ox} + NADH, H^+ + 6H^+ + H_2O$$

$$Cytc_{red} + \frac{1}{2}O_2 + 4H^+_{in} \rightleftarrows Cytc_{ox} + H_2O + 2H^+_{in}$$

$$\frac{1}{2}Cytc_{red} + \left[NO_2^- + H^+\right] + H^+_{out} \longrightarrow NO + H_2O + \frac{1}{2}Cytc_{ox}$$

$$3H^+_{out} + ADP + Pi \rightleftarrows 3H^+_{in} + ATP + H_2O$$

The redox potentials of the couples involved in the oxido-reductive chain are:
$E'^{\circ}_{NO/NO2-} = 1.133$ V
$E'^{\circ}_{NO2-/NO3-} = 0.148$ V

2.1.2 - Carbon metabolism - Catabolism of organic substrates

The most usual organic carbon sources used in growth experiments of nitrifiers are acetate, pyruvate and glucose. We will focus on the organic carbon assimilation by Nitrosomonas and Nitrobacter on acetate and pyruvate, because these products are well representative of the carbon sources which may reach the nitrifying compartment.

The biosynthesis of reserve material (PHB, glycogen) is taken into account.

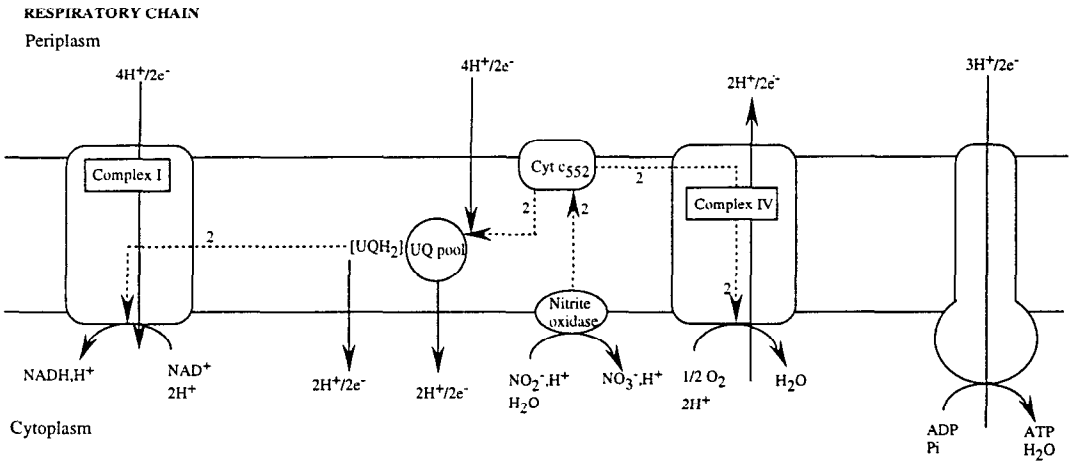


Figure 6: Oxydo-reductive chain of Nitrobacter (TN 23-2)

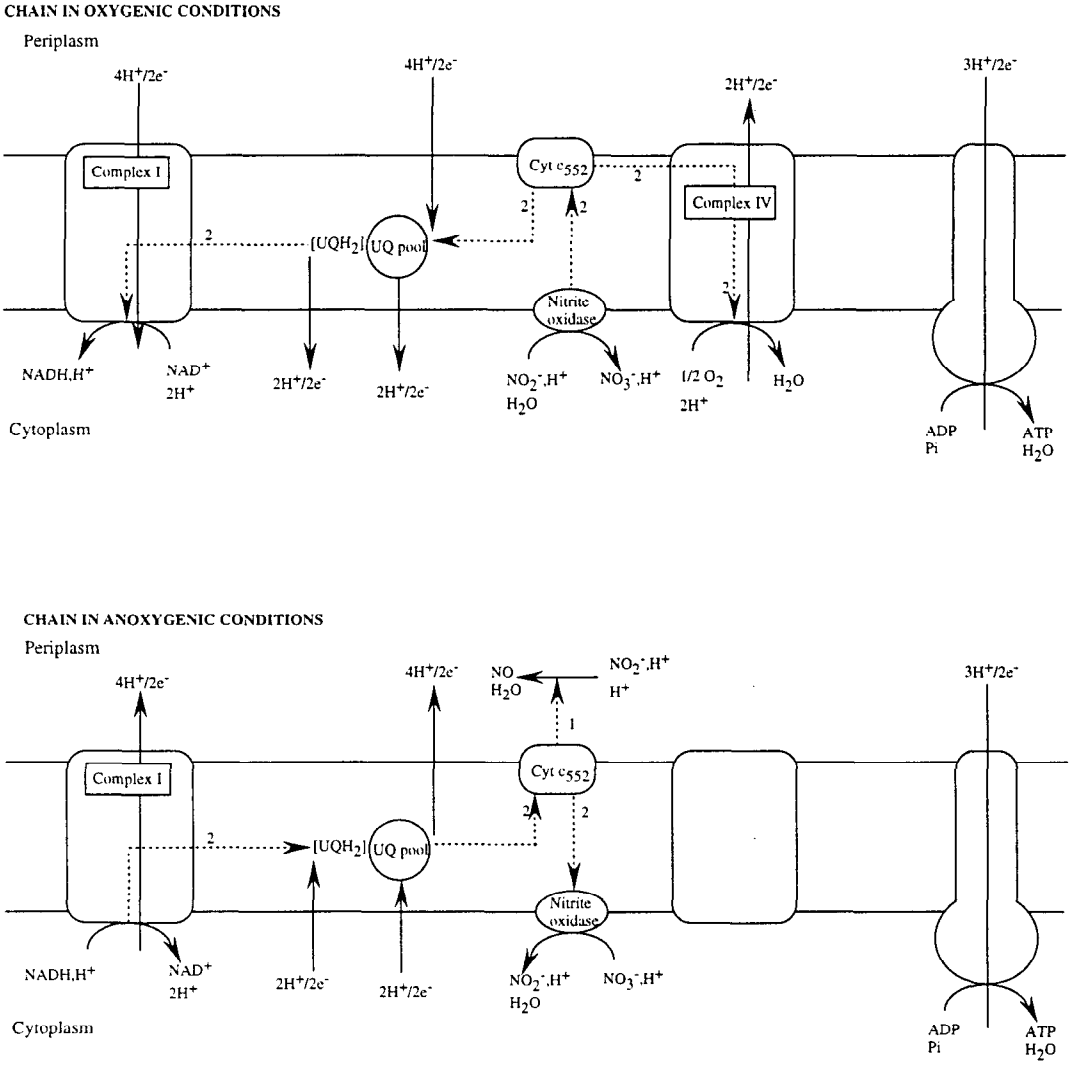


Figure 8: Oxydo-reductive chain of Nitrobacter

Pathways for C-substrates assimilation

The central pathway of carbon metabolism was studied for *Rb. Capsulatus* and *Rs. Rubrum* (TN 23.3). For acetic acid, and pyruvic acid, the metabolic pathways are quite "classical", and those described for Rhodobacteraceae can be used.

Acetate

$$\text{Acetic acid} + \text{ATP} + \text{CoA} - \text{SH} \longrightarrow \text{Acetyl} - \text{CoA} + \text{AMP} + \text{PPi}$$

Pyruvate

Pyruvate is directly assimilated in the central carbon metabolism pathway (figures 1 and 3).

Pathways for the reserves metabolism

The type of reserve considered are polyhydroxybutyrate (considered as the principal form of reserve for nitrifiers in mixotrophic or heterotrophic growth), and glycogen (or equivalent). The fact that PHB is often cited as the reserve material in nitrifiers is probably due to the fact that the main organic substrates used are acetate or pyruvate, which lead to PHB storage (TN 23.3).

The pathways for the synthesis and the breakdown of PHB and of glycogen were described in TN 23.3. The stoichiometries representing these pathway are reported below.

Glycogen

The biosynthesis can be represented by the following equation:

$$\text{ATP} + \text{Glucose-1P} + (\alpha\text{-1,4-glucosyl})_n \longleftrightarrow ADP + PPi + (\alpha\text{-1,4-glucosyl})_{n+1}$$

where $(\alpha\text{-1,4-glucosyl})_n$ can be represented by the formula $[C_6H_{11}O_5]_n\,[H_2O]$

The breakdown can be described by the following equation:

$$(\text{Glucosy})_{n+1} + \text{Pi} \longrightarrow \text{Glucose-1P} + (\text{Glucosyl})_n$$

Poly-β-hydroxybutyric acid (PHB)

The stoichiometry of the PHB biosynthesis is:

$$D(-)\text{-}\beta\text{-Hydroxybutyrate-CoA} + PHB_n \longrightarrow \text{CoA-SH} + PHB_{n+1}$$

where PHBn is $[C_4H_6O_2]_n\,[H_2O]$

The biosynthesis of D(-)-b-Hydroxybutyric acid from acetic acid can be described by:

$$2\ \text{acetyl-CoA} + \text{NADH,H}^+ \longrightarrow D(-)\text{-}\beta\text{-Hydroxybutyryl-CoA} + \text{NAD}^+ + \text{CoA-SH}$$

The breakdown of PHB can be described by the following equation:

$$PHB_n + H_2O + \text{succinyl-CoA} + \text{NAD}^+ + \text{CoA-SH}$$
$$\downarrow$$
$$PHB_{n-1} + \text{NADH,H}^+ + \text{Succinic acid} + 2\ \text{acetyl-CoA}$$

2.1.3 - Biomass composition

In TN 23.2, the macro-composition of the biomass (and as a consequence its chemical composition) was chosen identical to that of *Rs. Rubrum*. It must be noticed that the chemical composition obtained from the metabolic matrix and the stoichiometries was different of the one experimentally analysed (Forler, 1994):

from stoichiometries: $CH_{1.6147}O_{0.3906}N_{0.1994}S_{0.0035}P_{0.0089}$
from analysis: $CH_{1.9168}O_{0.4055}N_{0.1553}S_{0.0034}P_{0.0072}$

Walter (1993) reported a biomass analysis of the coculture *Nitrosomonas europea-Nitrobacter winogradskyi*, growing in autotrophic conditions. A dry weight ratio of 24% for the proteins and of 21% for the carbohydrates was obtained. The protein content compared with those reported in table 1 suggests the presence of reserve material and the content of carbohydrate appears quite high compared to the protein content. This could indicate that these reserves are carbohydrate or glycogen like material, but there is no evidence of that, then the composition given by Walter must be carefully considered. Nevertheless, it must be noted that Walter worked in limiting conditions with a high concentration of bicarbonate (4g/l $NaHCO_3$), and it was noticed in TN 23.3 that the reserve material stored from the assimilation of CO_2 was mainly glycogen. In chemical biomass analysis, Walter found a high content in phosphorus element. That could be the result of a polyphosphate accumulation (Hooper, 1987) in limiting growth conditions.

UAB laboratory has determined a macro-component composition of autotrophically growing pure cultures of *Nitrobacter winogradsky* (TN 25.6):

Proteins	62%
Carbohydrates	12%
Lipids	13%
DNA	5.7%
RNA	5.6%

The protein content is quite higher than this reported for *Nitrobacter agilis* (table 1), and is characteristic of an autorophic growth. It can be noted that this composition is close to the previously assumed, excepted for the DNA content.

This composition will be used to define the macro composition of both autotrophic biomass of Nitrosomonas and Nitrobacter (table 2).

The reserve content depends on the growth conditions (type of substrate, limitations, growth phase) and is difficult to estimate. From the results of table 1 and of Walter (1993), it seems that proteins contents could fall to 25%, which corresponds, if we assume that the ratio between the macro-components remains identical, to a reserve content (PHB, glycogen and/or polyphosphate) of 55% of the total biomass dry weight.

2.2 - Stoichiometries for autotrophic and mixotrophic/heterotrophic growth

The biomass composition takes into account the observations made in section 2.1.3. The macro component composition previously used (TN 23.2) is conserved. The complete description of the biomass composition used in the present work is reported in table 2.

Table 2: biomass composition chosen for *Nitrosomonas europea* and *Nitrobacter winogradskyi.*

Biomass composition (mass percentage)					
Proteins	lipids	Carbohydrates	RNA	DNA	Ash
62	13	12	5.6	5.7	-

Amino acids composition of proteins (molar percentage) from *Rs. Rubrum*									
glutamate	glutamine	proline	arginine	aspartate	asparagine	threonine	isoleucine	lysine	methionine
8.835	3.943	5.745	4.688	8.238	3.943	5.339	4.797	4.688	2.574
valine	leucine	alanine	serine	glycine	cysteine	phenylala.	tyrosine	tryptophane	histidine
7.046	8.184	11.787	3.713	7.019	0	3.848	2.330	1.328	1.951

Lipids composition (molar percentage) from *E.coli*					
Phosphatidic acid composition (ph)			Phospholipid composition		
palmitic acid	palmitoleic acid	vaccenic acid	Ph-ethanolamine	Ph-glycerol	diPh-glycerol
43.9	33.9	22.2	77.78	20.1	2.12

RNA	DNA CG%	Carbohydrates
same as E. coli	51% (from Nitrosomonas sp.)	Glucose-polymer

The sum of all equation describing the metabolism is a complex linear system of 107 equations and 124 compounds. The resolution of the metabolic matrix requires to fix several variables (TN 23.3) , *i.e.* the biomass composition (table 2), and to fix some metabolic fluxes. According to the observation reported in chapter I, the metabolic assumptions for the two nitrifiers (reported in chapter 2.1) were slightly modified.

The stoichiometries for the growth in mixotrophic and/or heterotrophic conditions were established. New stoichiometries for the autotrophic growth of Nitrosomonas and Nitrobacter were obtained, taking into account the changes in the biomass composition, in the energetic metabolism and in the metabolic assumptions.

2.2.1 - Nitrosomonas

Autotrophy

Taking into account the metabolic observations made on Nitrosomonas carbon metabolism, and the biomass composition proposed in table 2, a stoichiometry for the

autotrophic growth of *Nitrosomonas europea*, quite differrent from the previous one (TN 23.2, TN 27.1) can be established.

The metabolic matrix, involving the updated description of the oxido-reductive chain is solved using the following metabolic assumptions (figure 1-a):

- the phosphoenol pyruvate carboxylase is absent, and the glyoxylate shunt is the anaplerotic pathway for the TCA cycle.
- there is no maintenance (stoichiometry for anabolism only)
- the succinic dehydrogenase is inactive
- there is no NADPH transhydrogenase
- there is no nitrous oxide gas accumulation

For the pure anabolic autotrophic growth of Nitrosomonas, the stoichiometry is:

$$CO_2 + 4.3652\,O_2 + 0.0041\,H_2SO_4 + 0.0136\,H_3PO_4 + 3.8400\,NH_3 \downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 3.1649\,H_2O + 3.6292\,[NO_2^- + H^+]$$

This stoichiometry is slightly different of the previous one, but the yields always stays in the range of the experimentaly observed yields (table 3).

It must be noted that the biomass chemical composition, $CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136}$, is not in better agreement with the experimental one, determined by Forler. But as this stoichiomettric chemical composition is the result of calculations from an experimental biomass macro-components composition, it can be considered to be correct.

Mixotrophy - Acetate as carbon source and NH_3 as N and energy source

A theoretical stoichiometry for the mixotrophic growth of Nitrosomonas on acetic acid can be established. The resolution of the metabolic matrix is based on the following hypothesis (figure 1-b):

- the biomass composition is assumed to be that presented in table 2
- there is no maintenance (building of a pure anabolic stoichiometry)
- the phosphoenol pyruvate carboxylase is absent, and the glyoxylate shunt is the anaplerotic pathway for the TCA cycle.
- the succinic dehydrogenase is inactive
- there is no NADPH transhydrogenase
- there is no nitrous oxide gases accumulation
- the acetic acid is assimilated via the Tricarboxylic acid cycle, and there is no return of acetate in the glycolysis pathway.

For the anabolic mixotrophic growth of Nitrosomonas, the stoichiometry is:

$$0.2521\,CH_3COOH + 0.4958\,CO_2 + 2.8108\,O_2 + 0.0041\,H_2SO_4 + 0.0136\,H_3PO_4 + 2.4676\,NH_3 \downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0046}P_{0.0136} + 2.2967\,H_2O + 2.2569\,[NO_2^- + H^+]$$

Table 3: Nitritation yields of Nitrosomonas calculated from the stoichiometries established in various growth conditions.

	Autotrophic (anabolism)	Mixotrophic (anabolism without reseve)	Autotrophic (anabolism with 76% of substrate oxidised for maintenance)	Autotrophic (anabolism with 76% of substrate oxidised for maintenance). Previous results (TN 23.2)
NH3 oxidised/CO2 assimilated (mol/mol)	3.6292	4.5515	15.1218	18.061
NH3 oxidised/Acetate assimilated (mol/mol)	-	8.9531	-	-
CO2 assimilated/NH3 oxidised (mol/mol)	0.2755	0.2197	0.066	0.055
Acetate assimilated/NH3 oxidised (mol/mol)	-	0.1117	-	-
N-NH3 oxidised/C assimilated (g/g)	4.2341	2.6329	17.6422	21.0711
N-NH3 oxidised/Acetate assimilated (g/g)	-	2.0891	-	-
O2 assimilated /NH3 oxidised (mol/mol)	1.2028	1.2454	1.4287	1.441
Biomass/ NH3 oxidised (g/mol)	6.3801	10.2599	1.5312	1.276
Biomass/CO2 assimilated (g/mol)	23.1551	46.6976	23.1551	-
Biomass/Acetate (g/mol)	-	91.8584	-	-
Acetate/CO2 (mol/mol)	-	0.5084	-	-
CO2 assimilated by calvin/CO2 tot	95%	90%	96%	93%
XTP/reduced Coefficients	1.6907	2.0208	12.7022	-
NADH synthetised/reduced cofactors	71%	65%	71%	-
ATP synthetised/XTP	68%	75%	96%	-
electron in reverse transport chain	31%	27%	7%	6%

The stoichiometry proposed above does not include the reserves that would be synthesised in presence of organic matter. In presence of acetate or pyruvate, the synthesis of PHB must be considered. This synthesis is variable, depending on the growth conditions (the synthesis mainly occurs in the steady-state growth phase).

Even if the biosynthesis of PHB by Nitrosomonas has not been reported (see 1.2.5), the synthesis of PHB from acetate, using ammonia oxidation and acetate as energy and reduced cofactors sources can be represented by the following stoichiometry

$$CH_3COOH + 1.5833\,O_2 + 1.2222\,NH_3 \downarrow 2\,CH_{1.5050}O_{0.5025} + 1.7172\,H_2O + 1.2222\left[NO_2^- + H^+\right]$$

In the case of there is in fact an oxidation of organic matter (acetate), instead of a synthesis of PHB (see section 1.2.5), the following stoichiometry could represent this oxidation:

$$CH_3COOH + 2\,O_2 \downarrow 2\,CO_2 + 2\,H_2O$$

At the present time, the building of a stoichiometry for anoxygenic growth in presence of acetate has failed.

The yields calculated from the stoichiometries are reported in table 3. The yield of ammonia oxidation in mixotrophic condition is reduced of 38 % compared to autotrophic growth (oxidation due to the maintenance is not considered). This yield must be coupled with kinetics in order to know if the ammonia oxidation process is reduced or enhanced (influence of maintenance and growth rates).

2.2.2 - Nitrobacter

Autotrophy

Taking into account the metabolic observations made on Nitrobacter carbon metabolism, and the biomass composition proposed in table 2, a stoichiometry for the autotrophic growth of *Nitrobacter winogradskyi*, different from the previous one (TN 23.2, TN 27.1) is established.

The metabolic matrix, involving the updated description of the oxido-reductive chain (figure 7) is solved using the following metabolic assumptions (figure 3-a):

- the glyoxylate shunt is absent, and the anaplerotic pathway for the TCA cycle is the
- phosphoenol pyruvate carboxylase.
- there is no maintenance (stoichiometry for anabolism only)
- the succinate dehydrogenase is inactive
- there is no NADPH transhydrogenase
- there is no nitrous oxide gas accumulation

For the pure anabolic autotrophic growth of Nitrobacter, the stoichiometry is:

$$CO_2 + 6.8413\,O_2 + 0.0041\,H_2SO_4 + 0.0136\,H_3PO_4 + 0.2107\,NH_3 + 15.8398\,[NO_2^- + H^+]$$
$$\downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0041}P_{0.0136} + 0.4643\,H_2O + 15.8398\,[NO_3^- + H^+]$$

Mixotrophy - Acetate as carbon source and NO_2^- as energy source

A theoretical stoichiometry for the mixotrophic growth of Nitrobacter on acetic acid can be established. The resolution of the metabolic matrix is based on the following hypotheses:

- the biomass composition is assumed to be this presented in table 2
- there is no maintenance
- the phosphoenol pyruvate carboxylase is absent and the glyoxylate shunt is the anaplerotic pathway for the TCA cycle
- there is no NADPH transhydrogenase
- there is no nitrous oxide gas accumulation

For the anabolic mixotrophic growth of Nitrobacter, the stoichiometry is:

$$0.2618\,CH_3COOH + 0.4764\,CO_2 + 4.4644\,O_2 + 0.0046\,H_2SO_4$$
$$+ 0.0136\,H_3PO_4 + 10.0388\,[NO_2^- + H^+] + 0.2107\,NH_3$$
$$\downarrow$$
$$CH_{1.6097}O_{0.3777}N_{0.2107}S_{0.0046}P_{0.0136} + 0.2265\,H_2O + 10.0388\,[NO_3^- + H^+]$$

The stoichiometry proposed above does not include the reserves that would be synthesised in presence of organic matter. In presence of acetate or pyruvate, the synthesis of PHB must be considered. This synthesis is variable, depending on the growth condition (the synthesis mainly occurs in the steady-state growth phase).

The synthesis of PHB from acetate, using nitrite oxidation and acetate as energy and reduced cofactors sources, is represented by the following stoichiometry

$$CH_3COOH + 2.5\,O_2$$
$$5.5\,[NO_2^- + H^+]_3$$
$$\downarrow$$
$$CH_{1.5050}O_{0.5025} + 0.495\,H_2O + 5.5\,[NO_3^- + H^+]$$

The yields of the nitratation by Nitrobacter are reported in table 5.

Smith and Hoare (1968) have measured the nitrite oxidised and the cell yield in the exponential growth of *Nitrobacter agilis*, in presence of 1mM and 5mM of acetate. The

concentrations of acetate used did not affect significantly the nitrite oxidation. The mean value calculated from the results reported by Smith and Hoare (1968) are listed in table 4.

Tableau 4: Mean yield of the growth of *Nitrobacter agilis* in presence of acetate. Calculated from Smith and Hoare (1968)

Nitrite oxidised (mmole/l)	Cell yield (mg protein/l)	Acetate incorporated (µmol/mg protein)	g protein / Cmol biomass **	moles nitrite oxidised / Cmol biomass	moles nitrite oxidised / Cmol anabolic biomass *
83.333	24.5	16.667	14.214	35.15	6.87
+/- 7.75%	+/- 6.12%	+/- 6.0%		+/- 2.37%	+/- 2.37%

* Caculated assuming that 81% of nitrite are oxidised for the maintenance (TN 23.2)

* *Calculated assuming proteins represent 46% (from table 1) of the dry biomass and that the Cmol biomass weight is 23g. It must be noted that with 61.8% of proteins, the yield becomes 9.8 NO_2^- oxidized/Cmol anabolic biomass.

The increase of 48 % in the cell yield reported by Steinmuller et al. (1976) for the growth of *Nitrobacter agilis* in mixotrophic medium was given for the end of the growth. It appears in their results that in the exponential phase of the mixotrophic growth phase, the yield N-oxidized/biomass is lower than in the autotrophic control. This yield varies from 50% to 75% of the autotrophic yield. The stoichiometry for the mixotrophy is based on the same biomass composition of the autotrophic growth, and is then most representative of the exponential growth phase than of the steady state where reserves are synthesized (table 1). With a N-oxidized/biomass mixotrophic yield (10.0388 mol/Cmol) of 63% of the autotrophic one, the yield calculated from the mixotrophic stoichiometry is in accordance with the experimental results reported by Smith and Hoare (1968), and Steinmuller et al. (1976).

Smith and Hoare (1968), report that acetate represents 33 to 39% of the newly synthetised C-material in *Nitrobacter agilis* in the mixotrophic exponential growth. The solution of the metabolic network (table 5) indicates that the acetate contributes to 38% of the carbon assimilated.

At the present time, the building of a stoichiometry for anoxygenic growth in presence of acetate has failed.

Table 5: Nitratation yields of Nitrobacter calculated from the stoichiometries established in various growth conditions.

	Autotrophic (anabolism)	Mixotrophic (anabolism without reserve)	Autotrophic (anabolism with 81% of substrate oxidised for maintenance)	Autotrophic (anabolism with 81% of substrate oxidised for maintenance). Previous results (TN 23.2)
NO_2^- oxidised/CO2 assimilated (mol/mol)	15.8398	21.0733	83.3673	79.8949
NO_2^- oxidised/Acetate assimilated (mol/mol)	-	38.3433	-	-
CO2 assimilated/ NO_2^- oxidised (mol/mol)	0.0631	0.04745	0.0120	0.0125
Acetate assimilated/ NO_2^- oxidised (mol/mol)	-	0.0261	-	-
N- NO_2^- oxidised/C assimilated (g/g)	18.4797	11.7119	97.2619	93.2107
N- NO_2^- oxidised/Acetate assimilated (g/g)	-	2.3423	-	-
O2 / NO_2^- oxidised (mol/mol)	0.4319	0.4447	0.4871	0.486
Biomass/ NO_2^- oxidised (g/mol)	1.4618	2.3065	0.2777	0.288
Biomass/CO2 assimilated (g/mol)	23.15513	48.6070	23.1551	-
Biomass/Acetate (g/mol)	-	88.4415	-	-
Acetate/CO2 (mol/mol)	-	0.5496	-	-
CO2 assimilated by calvin/CO2 tot	90%	90%	90%	92.6%
C-Acetate / C-total fixé	-	38.4%	-	-
XTP/reduced Coefficients	1.733	0.1282	18.025	-
NADH synthetised/reduced coefficients	81%	2.5%	78%	-
ATP synthetised/XTP	70%	48%	97%	93.6%
electron in reverse transport chain	14%	12%	3%	2.6%

III -Proposed kinetics rates for the mixotrophic or hetrotrophic growth of nitrifiers

Few data exist on the heterotrophically and mixotrophically growth of nitrifiers. Models for nitrification-denitrification in waste water treatment can be found, but they concern mixed cultures of nitrifiers and heterotrophs, and cannot be used to represent the heterotrophic or mixotrophic growth of pure culture of nitrifiers (Chen et al., 1989; Strand, 1986, Bonhomme et al., 1990).

For ammonia oxidisers, Bock (1991) reports that the growth of Nitrospira mixotrophically growing on acetate and formate was enhanced up to 130%, compared with autotrophic growth. The pyruvate effect on *Ns. europea* growing on inorganic medium did not result in a marked growth response. Control cultures progressed either at the same rate or only slightly slower than pyruvate treated cultures.

The heterotrophically and mixotrophically growth of nitrite oxidisers have been studied to a greater extend. Normally, in mixotrophic growth, the growth increases. In mixotrophic growth on YP (yeast extract peptone), the nitrite oxidation rate of *Nitrobacter agilis* is 3.38 time-fold of the autotrophic control, in the exponential growth phase (calculated from Steinmuller and Bock, 1976). The cell yield (N-cell/N-oxidised) of *Nb. agilis* on YP supplemented medium is increased up to 48% of the autotrophic yield at the end of the growth.

Cells of nitrite oxidizers growing on pyruvate and ammonia or nitrate as N-sources are characterized by doubling times of 120 to 150 hours. The growth was inhibited most before the organic substrate was oxidized quantitatively (Bock, 1991).

When nitrite is completly exhausted, *Nitrobacter agilis* is able to continue to growth without nitrite oxidation (Steinmuller and Bock, 1976; Smith and Hoare, 1968), indicating the shift of the energetic metabolism from mixotrophy to organotrophy.

In figure 9 are summarized the different metabolisms of nitrifiers for various growth conditions. This figure is helpful to establish the structure of a kinetic representation of the growth of nitrifiers.

Each metabolism is described by a specific set of stoichiometries. Changes in the growth conditions lead to changes in the metabolism, and then in the stoichiometric description of the growth. These variations of the stoichiometric description of the growth (i.e in the growth yield) must be included in the kinetic model.

Bellgardt (1991) proposed a general form for the specific growth rate with p alternative growth substrates:

$$\mu(S_1,...S_n) = \mu_1(S_1,...S_n) + \mu_2(S_1;...S_n) + ... + \mu_p(S_1,...S_n)$$

For each organism (Nitrosomonas or Nitrobacter), the same structure for the construction of the kinetic model can be used. First each metabolism is described by a set of stoichiometric equation associated to kinetic laws (such a representation has been made for the autrophic growth in TN 27.1, 27.2 and 27.3). The combination of the kinetics for each growth conditions on the model given by Bellgardt leads to a global kinetic law valid for the various growth conditions.

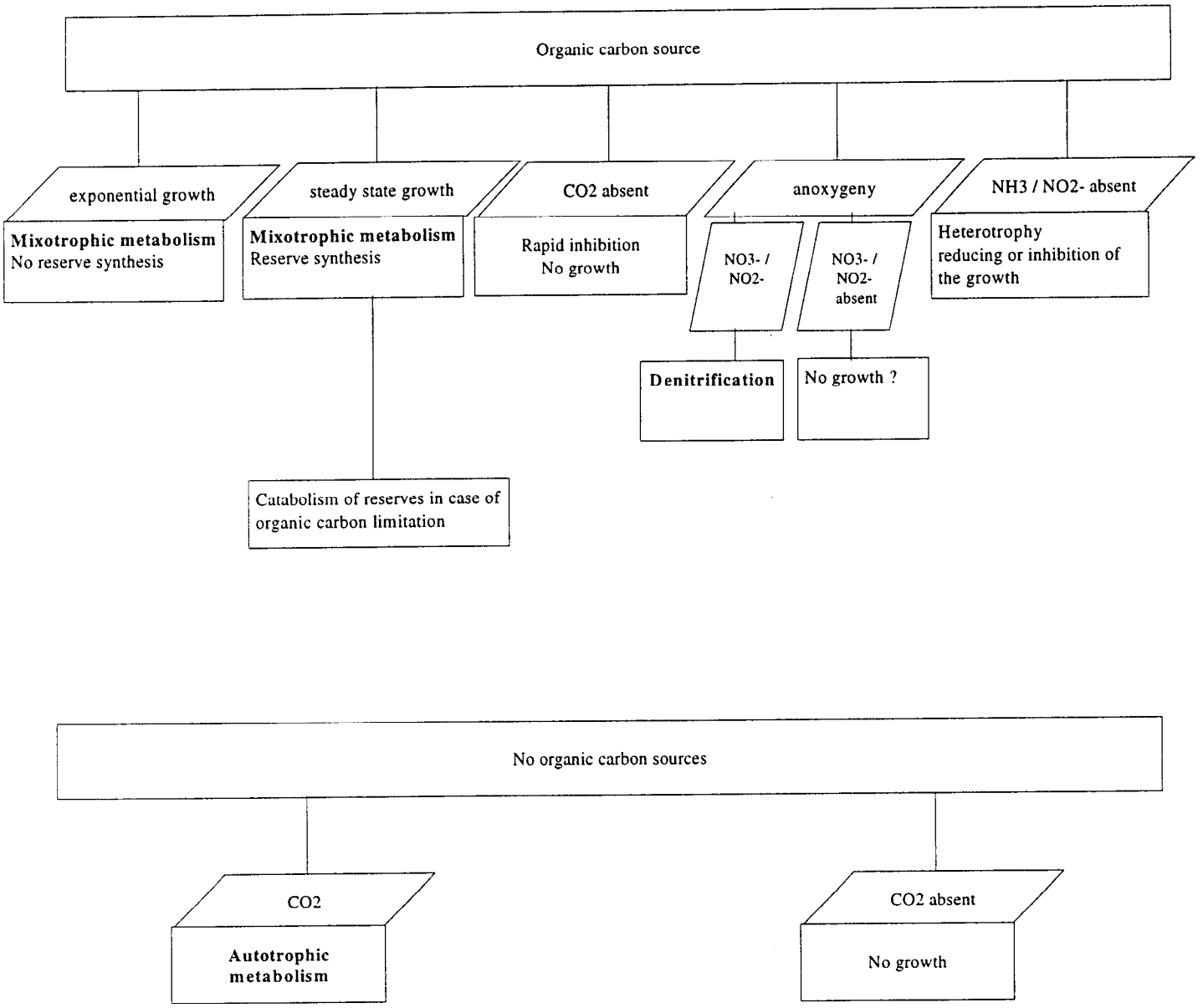


Figure 9: Metabolism of nitrifiers for various growth conditions

3.1 - Dynamic representation of the autotrophic growth

Anabolism

The stoichiometryof the anabolic growth, leading to the biosynthesis of an active biomass (noted Xact), has been established in the previous section for Nitrosomonas and Nitrobacter. It can be represented for Nitrosomonas by the following reaction:

$$(CO_2; NH_3; H_2SO_4: H_3PO_4; O_2) \longrightarrow (Xact; H_2O; HNO_2)$$

$r_{gr-auto}^{Ns}$ is the anabolic growth rate associated to this reaction. It takes the form (TN 27-1):

$$r_{gr-auto}^{Ns} = \mu_{auto}^{Ns} C_{Xact-Ns}\big|_B$$

where $C_{Xact-Ns}\big|_B$ is the concentration of the active biomass of Nitrosomonas in the biofilm.

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

Si: substrate i
C_{si}: concentration of substrate i
K_{si}: saturation constant for substrate i
I_i: inhibition constant for substrate i

Maintenance for autotrophic growth

The maintenance is classicaly represented by the hydrolysis of ATP. In lithoautotrophic growth, ATP production is coupled to NH_3 (Nitrosomonas) or NO_2^- (Nitrobacter) oxidation. For Nitrosomonas, the maintenance is described by a reaction of the form:

$$(NH_3; O_2) \longrightarrow (H_2O; HNO_2)$$

r_{m-auto}^{Ns} is the maintenance rate associated to this reaction. It is related to the active biomass concentration, and takes the form (TN 27-1):

$$r_{m-auto}^{Ns} = m_{auto}^{Ns} C_{Xact-Ns}\big|_B$$

where m_{auto}^{Ns} is the maintenance coefficient

Autotrophic production and consumption rates

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

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

The production and consumption rate of other substrates are expressed by:

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

3.2 - Mixotrophic growth

In mixotrophic growth, the biosynthesis of reserves must be considered. This synthesis depends on the growth phase (exponential, steady-state). The biomass X is composed of an active part, Xact, and of an inactive or reserve part,Xres :
X=Xact+Xres

Anabolism in mixotrophic growth

Anabolism leads to the synthesis of active biomass. In the previous section, the stoichiometry for anabolism in a mixotrophic growth was established, assuming that the organic carbon source was acetate. As a consequence of this source, the reserve material was PHB.

For Nitrosomonas, the anabolic stoichiometry in mixotrophic condition can be summarized by the reaction:

$$(CO_2; Acetate; NH_3; H_2SO_4 : H_3PO_4; O_2) \longrightarrow (Xact; H_2O; HNO_2)$$

$r^{Ns}_{gr-mixo}$ is the anabolic growth rate associated to this reaction. As for autotrophic metabolism, it can be represented by the expression:

$$r^{Ns}_{gr-mixo} = \mu^{Ns}_{mixo} C_{Xact-Ns}\Big|_B$$

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

Maintenance for mixotrophic growth

The maintenance in mixotrophic growth of Nitrosomonas is described as in the autotrophic growth by the following set of reaction and kinetic rates:

$$(NH_3;O_2)\longrightarrow(H_2O;HNO_2)$$

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

Reserve synthesis

The stoichiometries for the synthesis of PHB from acetate, by Nitrosomonas and Nitrobacter were established in the previous section.

For Nitrosomonas, a reserve synthesis rate, r^{Ns}_{Res} is associated to the stoichiometry :

$$(NH_3;Acetate;O_2)\longrightarrow(PHB;H_2O;HNO_2)$$

In this expression of the reserve synthesis rates, it must be taken into account that the synthesis depends on the active biomass growth.

$$r^{Ns}_{Res} = R^{NS}.\left[\prod_{Substrate\ k}\frac{C_{Si}}{K_{Si}+C_{Si}}\right].\left(1-\frac{m^{Ns}_{mixo}}{m^{Ns}_{max-mixo}}\right).\left(1-\frac{\frac{C_{XREs}}{C_X}}{Xres-Max}\right).C_{Xact}$$

where R^{NS} is the reserve synthesis coefficient rate and Xres-Max is the maximal mass fraction of reserve in the biomass.

NOTE: It is not established if the PHB synthesis occurs or not in Nitrosomonas species. If there is no synthesis, then $r^{Ns}_{Res}=0$.

Mixotrophic production and consumption rates

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

$$r^{Ns}_{Xact\text{-}mixo} = r^{Ns}_{gr-mixoo} + \left(\frac{m^{Ns}}{m^{Ns}_{max-amixo}} - 1\right) Y^{Ns}_{Xact/NH3}\big|_{mixo}\, r^{Ns}_{m-mixo}$$

The second part of the biomass (reserves) is expressed by the reserve synthesis rate r^{Ns}_{Res}.

The production and consumption rate of other substrate are expressed by:

$$r^{Ns}_{Si\text{-}mixo} = \frac{1}{Y^{Ns}_{Xac/Si}\big|_{mixo}} r^{Ns}_{Xact-mixo} + \frac{1}{Y^{Ns}_{Smt/Si}\big|_{mixo}} r^{Ns}_{m-mixo} + \frac{1}{Y^{Ns}_{Xres/Si}\big|_{mixo}} r^{Ns}_{Res}$$

3.3 - Denitrification

Denitrification occurs in anoxygenic conditions and in presence of organic matter. The stoichiometries, both for Nitrosomonas and Nitrobacter, were yet unable to established.We are unable to build a metabolic matrix which could be correctly solved.

At least 2 stoichiometries would exist for each microorganism: one for the anabolic growth and one for the maintenance.

The anabolic stoichiometry for denitrifying growth will have the form (for Nitrosomonas):

$$(CO_2; Acetate; NH_3; HNO_2; H_2SO_4 : H_3PO_4) \longrightarrow (Xact; H_2O; NxO\ gases)$$

$r_{gr-denit}^{Ns}$ is the anabolic growth rate associated to this reaction. It will have an expression similar to that used for autotrophic and mixotrophic growth:

$$r_{gr-denit}^{Ns} = m_{denit}^{Ns} C_{Xact-Ns}\Big|_B$$

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

In the same way, the maintenance will be described by:

$$(NH_3; Acetate; HNO_2) \longrightarrow (H_2O; NxO\ gases)$$

$$r_{m-denit}^{Ns} = m_{denit}^{Ns} C_{Xact-Ns}\Big|_B$$

3.4 - Combination of kinetics allowing growth under autotrophic, mixotrophic and denitrifying conditions

The combination of the rates previously established for the different possible types of growth gives a global kinetic law for three various growth conditions: autotrophy (when CO_2 is the sole carbon source), mixotrophy (when an organic carbon source is added to the autotrophic medium) and denitrification (in oxygen limitation, when an organic source is present).

The growth rate for the active biomass is given by the sum of the rates in the diffrent growth conditions:

$$r_{Xac}^{Ns} = \sum \begin{vmatrix} r_{Xact-auto}^{Ns} \left[\dfrac{K}{K + C_{Sorga}}\right]\left[\dfrac{K'}{K' + X_{res}}\right] \\ r_{Xact-mixo}^{Ns} \\ r_{Xact-denit}^{Ns} \left[\dfrac{K''}{K'' + C_{O2}}\right] \end{vmatrix}$$

The reserve production rate is given by r_{Xres}^{ns}

The consumption and production of other substrates is given by:

$$r_{Si}^{Ns} = \sum \begin{vmatrix} r_{Si-auto}^{Ns} \left[\frac{K}{K + C_{Sorga}} \right] \left[\frac{K'}{K' + X_{res}} \right] \\ r_{Si-mixo}^{Ns} \\ r_{Si-denit}^{Ns} \left[\frac{K''}{K'' + C_{O2}} \right] \end{vmatrix}$$

3.5 - Kinetic parameters

The parameters K, K' and K" introduced above represent the shift from one metabolism to one other. High values for this constants are characteristics of a rapid shift between each metabolism. Low values allows the presence of the different metabolisms at the same time. At the present time, there is no value to set these constants.

Kinetic parameters for autotrophic growth.

Values for the kinetic parameters involved in the autotrophic model were obtained from litterature data, and are reported in TN 27-3.

Kinetic parameters for mixotrophic growth.

At the present time, insufficient data relative to the growth of pures culture were found. The only avalaible data found concern the generation time of the microorganisms. The generation time of Nitrobacter is generally higher in mixotrophic and heterotrophic growth (in the range of 30 to 150h) than in autotrophic growth (i.e $\mu_{max-mixo} < \mu_{max-auto}$) (Bock, 1991). For Nitrosomonas, growing on a medium supplemented with pyruvate, the nitrite oxidation rate is unchanged, but the lag phase is generally reduced (Clark and Schmidt, 1966).

Kinetic parameters for denitrifying growth

The denitrification is a process intensively used in waste water treatment. Nevertheless, at the present time, only data and models concerning denitrification in waste water were found, and as these processes involved mixed cultures of heterotrophs and nitrifiers; it is then difficult to use these data for a denitrifying process with pure culture of nitrifiers.

CONCLUSION

The oxido-reductive chain of Nitrosomonas species and Nitrobacter species built in TN 23.2 have been completed and detailed in order to take into account the ability of nitrifiers to produce nitrogen gases.

The ability of nitrifiers to grow with organic substrates (organic acids and VFA), has been studied and discussed.

The stoichiometries in mixotrophic conditions for the nitrifiers Nitrosomonas and Nitrobacter have been established. In heterotrophic conditions (both carbon and nitrogen sources are organic), Nitrosomonas species are unable to grow. Moreover, as the heterotrophic conditions will not be the normal conditions in the MELiSSA nitrifying compartment design, the stoichiometries for the growth of nitrifiers in such condition was not established.

The stoichiometries describing the growth in anoxygenic conditions, linked to denitrification, have yet not be established due to problems in the resolution of the metabolic matrix of Nitrosomonas and Nitrobacter.

The reserves (mainly PHB) synthesis and catabolism is an important aspect of the growth in presence of organic matter for Nitrobacter. Stoichiometries describing the biosynthesis of PHB associated to NH_3 and NO_2^- oxidation has been established.

The basis of a kinetic model of the nitrification which applies for various growth condition (autotrophy, mixotrophy and anoxygeny) has been build. If the kinetics parameters for the autotrophic growth can be found in the literature (TN 27), it is difficult to find parameters for pure cocultures of Nitrosomonas and Nitrobacter growing in anoxygenic and mixotrophic conditions. Further studies will be necessary.

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