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Novel principles in the microbial conversion of nitrogen compounds

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Abstract

Some aspects of inorganic nitrogen conversion by microorganisms like N_2O emission and hydroxylamine metabolism studied by Beijerinck and Kluyver, founders of the Delft School of Microbiology, are still actual today. In the Kluyver Laboratory for Biotechnology, microbial conversion of nitrogen compounds is still a central research theme. In recent years a range of new microbial processes and process technological applications have been studied. This paper gives a review of these developments including, aerobic denitrification, anaerobic ammonium oxidation, heterotrophic nitrification, and formation of intermediates (NO_2^-, NO, N_2O), as well as the way these processes are controlled at the genetic and enzyme level.

Abbreviations: AMO – ammonium monooxygenase; Anammox – anaerobic ammonium oxidation; BAS – biofilm airlift suspension reactor; FISH – fluorescent in situ hybridisation; HAO – hydroxylamine oxidoreductase; NAR – nitrate reductase.

Introduction

During the last 100 years conversion of inorganic nitrogen compounds by micro organisms has received considerable attention. Many studies of early members of the Delft School of Microbiology were devoted to the elucidation of the principles of these conversions. Beijerinck described pure cultures of *Nitrosomonas* and *Nitrobacter* in his work on soil fertility (Beijerinck 1904) after aerobic oxidation of ammonium and nitrite by bacterial isolates was reported by Winogradsky (1890). Later on Kluyver was the first to postulate hydroxylamine as an intermediate in ammonium oxidation, based on inhibitor studies with hydrazine (Kluyver & Donker 1926). During his PhD study with Kluyver, Kingma-Boltjes investigated many aspects of nitrification (Kingma-Boltjes 1934). He described an improved and fast method (less than 5 weeks) for the isolation of pure strains and the influence of organic matter on ammonium oxidation (Kingma-Boltjes 1936).

The production of gaseous nitrogen compounds from nitrate in garden soil was studied by Beijerinck and coworkers (Beijerinck & Minkman 1910) after publications on reduction of nitrate (Breal 1892; Gayon & Dupetit 1886). Beijerinck was the first to recognize nitrous oxide as an intermediate in denitrification. Among the bacteria capable of complete conversion of nitrate to dinitrogen gas, isolated by Beijerinck, was *Micrococcus* (now *Paracoccus*) *denitrificans*. This bacterium has become the model organism in studies on denitrification. Kluyver and Verhoeven studied many aspects of 'true' dissimilatory nitrate reduction, including the adaptation process, the mechanism, aerobic denitrification, and the possible involvement of nitric oxide (Kluyver 1936, Kluyver 1953, Kluyver & Verhoeven 1954a,b , Verhoeven 1956a,b).

The studies of van Niel and co-workers did not concentrate on conversion of inorganic compounds, but were devoted to the elucidation of autotrophic and photosynthetic growth of bacteria (van Niel 1949, 1954,

1967). However, many organisms converting inorganic nitrogen compounds are (facultative) autotrophs.

Several topics addressed by the early members of the Delft School of Microbiology are still actual these days. In this overview, some of the topics, including autotrophic nitrification, anaerobic ammonium oxidation, molecular characterization of nitrifiers, heterotrophic nitrification, aerobic denitrification, and combined nitrification / denitrification processes will be discussed in relation to the removal of nitrogen from waste water.

Autotrophic nitrification

Ammonium oxidation

Chemolithoautotrophic ammonium-oxidizing bacteria catalyze the conversion of ammonium to nitrite, which is assumed to be a two step process (Bock et al. 1992). The first reaction is catalyzed by the enzyme ammonium monooxygenase (AMO). The second, energy generating step is performed by hydroxylamine oxidoreductase (HAO) (Hooper et al. 1984).

$$NH_3 + O_2 + 2[H] \xrightarrow{AMO} NH_2OH + H_2O$$

$$NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + H^+ + 4[H]$$

The AMO enzyme has turned out to be a very labile membrane protein, which is very difficult to assay and purify (Juliette et al. 1995). The ammonium oxidation is very effectively inhibited by acetylene (Hyman et al. 1988). This property was used to isolate the acetylene binding protein, supposedly being the 27 kDa membrane polypeptide subunit of AMO (McTavish et al. 1993a). On the basis of the N-terminal amino acid sequence a degenerated oligonucleotide was designed and used as a probe in Southern hybridization. This oligonucleotide was found to hybridize to two different bands, indicating the presence of two *amo* copies in the genome. The probes were also used to identify clones derived from both a *Sau*A3 and a size fractionated *Kpn*I genomic library. Sequence analysis showed that the *amo* gene from *Nitrosomonas europaea* is organized in an *amo*A-*amo*B operon (McTavish et al. 1993a, Bergmann & Hooper 1994). Comparison of the deduced amino acid sequence from overlapping clones with a quantative amino acids composition analysis of purified AMO indicated that the *amo*A gene had been cloned. The *amo*B gene codes for a 40 kDa polypeptide, which function in the ammonium oxidation is not yet known. Recently also the *amo* genes of *Nitrosolobus* and *Nitrosospira* have been sequenced (Klotz & Norton 1995, Rotthauwe et al. 1995). Sequence alignment revealed a similar organization of the *amo*A-*amo*B operons. Alignment of the deduced amino acid sequences showed a high (>90%) degree of homology between the *Nitrosospira* and *Nitrosolobus* AMO polypeptide, the AMO from *Nitrosomonas* has 82% homology (Figure 1) . Since all the function specific target genes investigated so far, turned out to be highly conserved, deduced oligonucleotides can be used to characterize nitrifying populations in soil and marine sediments by PCR techniques (Sinigalliano et al., 1995, see also section on molecular identification).

```
      1                                                            50
NssA  MS..RTDEIL KAAKMPPESV KMSRMIDAIY FPILCILLVG TYHMHFMLLA
NssB  .......... .......... .......... .......... ..........
Nlm   --IF-TE--- --------A- -------V-- -------V-- --------V-
NlmC  .......... .......... .......... .......... ..........
NsmE  V-IF-TE--- --------A- H---L---V- ----I----- ----------

      51                                                           100
NssA  GDWDFWLDWK DRQWWPVVTP IVGITYCATI MYDLWVNYRL PFGATLCIVC
NssB  ---------- ---------- ---------- --Y------- ----------
Nlm   ---------- ---------- --------E- --Y------- AG-D------
NlmC  ---------- ---------- --------A- --Y------- ----------
NsmE  ------M--- ---------- -------SA- --Y------Q -------V--

      101                                                          150
NssA  LLVGEWLTRF WGPYWWSHYP INFVLPSTMI PGALIMDTVM LLTRNWMITA
NssB  ---------- ---------- ---------- ---------- ----------
Nlm   ---------- ---------- M---F----- ----V----L ----------
NlmC  ---------- ---------- M---F----- ----V----L ----------
NsmE  --I------Y ---------- ----T-GI-L ----ML-FTL Y-----LV--

      151                                                          200
NssA  LVGGGARGLL FYPGNWPIFG PTHLPLVAEG VLLSLADYTG FLYVRTGTPE
NssB  ------F--- ---------- ---------- ---------- ----------
Nlm   ------F--- ------T--- ---------- ----V----- ----------
NlmC  ------F--- ------T--- ---------- ----V----- ----------
NsmE  -----FF--- ---------- -----I-V-- T---M---M- H---------

      201                                                          250
NssA  YVRLIEQGSL RTFGGHTTVI AGFFSAFVSM LMFCVWWYFG KLYCTAFYYV
NssB  ---------- ---------- -A-------- ---------- ----------
Nlm   ---------- ---------- -S-------- ---T------ -V--------
NlmC  ---------- ---------- -S-------- ---T------ -V--------
NsmE  ---H------ ---------- -A-------- ---T----L- -V-----F--

      251                          277     % Homolgy
NssA  KGPRGRVTMK NDVTAYGEEG FPEGIK*     100      90
NssB  ---------- ---------- ------*     99       93
Nlm   --A----S-- -----F---- -G----*     90       100
NlmC  --A----S-- -----F---- -A----*     93       98
NsmE  --K---IVHR -----F---- ------*     82       83
```

Figure 1. Alignment of deduced amino acid sequences of the ammonia monooxygenase from different ammonium oxidizing bacteria. NssA = *Nitrosospira* sp. NpAV (U20644); NssB = *Nitrosospira* AHB1 (X90821); Nlm = *Nitrosolobus multiformis* (U15733); NlmC = *Nitrosolobus multiformis* C–71 (X90822); NsmE = *Nitrosomonas europaea* (L08050). Percentage homology compared to NssA or Nlm is indicated at the end of the sequence.

In the second step of autotrophic nitrification, hydroxylamine is oxidized by hydroxylamine oxidoreductase to nitrite with H_2O as the source for the second oxygen atom in nitrite (Anderson & Hooper 1983). This reaction involves a four electron transfer, which is not yet completely understood. The HAO enzyme

has been purified and characterized in detail (Arciero & Hooper 1993; Aciero et al. 1993; Hendrich et al. 1994). The enzyme is located in the periplasm, contains at least 7 heme-c and one heme P_{460}, and is composed of two to three subunits of 62 kDa. So far considerable knowledge of the HAO biochemistry has accumulated but the available genetic information is still limited to the *hao* gene of *Nitrosomonas* (Sayaverde et al. 1993). In order to clone the *hao* gene, a genomic library in phage lambda was constructed. *Hao* encoding clones were identified using a comparable approach as for the *amo* gene. (Arciero et al. 1993, Sayaverde et al. 1993). Three *hao* and two tetra heme c cytochrome gene copies have so far been identified (McTavish et al. 1993b; Bergman et al. 1994).

It has been suggested that two of the four reducing equivalents generated by HAO are used for the initial hydroxylation of ammonium via cytochrome C_{554}. The remaining two reducing equivalents are believed to be utilized for the generation of ATP and NAD(P)H. Molar growth yields on hydroxylamine should therefore be twice those on ammonium. Although it has been known for sometime that *Nitrosomonas* cells can oxidize hydroxylamine to nitrite, growth on hydroxylamine has only been reported recently (Boettcher & Koops 1994; de Bruijn et al. 1995).

Hydroxylamine conversion

By careful, repetitive additions of small amounts of hydroxylamine (0.4 mM) it was possible to grow *Nitrosomonas* mixotrophically on ammonia and hydroxylamine in batch cultures (Boettcher & Koops 1994). Molar growth yields on hydroxylamine (0.5 mg protein/mM) were reported to be approximately twice those on ammonia (0.2 mg protein/mM) alone. Similar results were obtained when chemostat cultures instead of batch cultures were used (de Bruijn et al. 1995). Growth on mixtures of 20 mM ammonia and 10 mM hydroxylamine was possible by stepwise increasing the hydroxylamine concentration in the medium. The increased biomass yield due to hydroxylamine (4.7 mg dry weight/mmol) was higher than predicted from the growth on ammonia (1.4 mg dry weight/mmol) alone. This showed the high potential of electrons derived from hydroxylamine. During the growth on mixtures of hydroxylamine and ammonia, nitrogen recovery in the form of nitrite was always lower than expected, indicating the formation gaseous NO_x compounds.

Anaerobic activity experiments were performed to further investigate this N-loss. These experiments

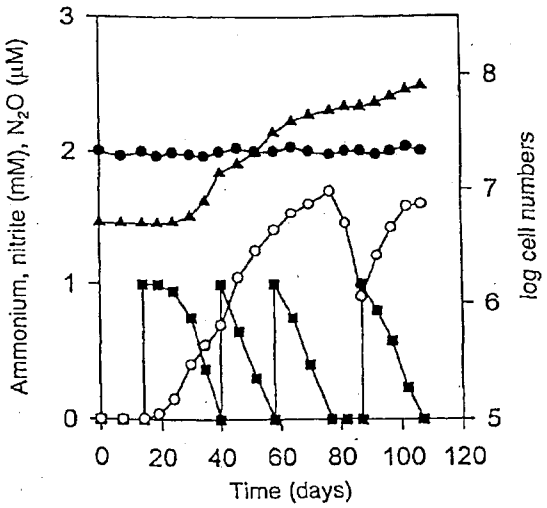

Figure 2. Anaerobic growth of *Nitrosomonas eutropha* with hydrogen and nitrite. Filled circles, ammonium; filled squares nitrite, hydrogen was supllemented together with each new nitrite addition; open circles nitrous oxide; filled triangles, cell numbers (from Bock et al. 1995 Arch. Microbiol. 163:16–20)

showed that under anoxic conditions,*Nitrosomonas* was able to reduce nitrite in the presence of hydroxylamine, thereby producing N_2O. Also ammonium could serve as an electron donor for the reduction of nitrite to N_2O. Repetitive attempts to grow *Nitrosomonas* anaerobically in continuous cultures on mixtures of hydroxylamine, ammonia, and nitrite were not successful (de Bruijn et al. 1995).

Nitrifier denitrification

High N-losses (16–100%) have been reported for both pure and mixed cultures of *Nitrosomonas* strains grown under oxygen limitations (Bock et al. 1995). Molecular hydrogen could serve as an electron donor for nitrite reduction by *N. eutropha* under anoxic condition (Figure 2). The cell growth in these experiments was directly coupled to nitrite reduction. The main end product was dinitrogen gas, but small amounts of NH_2OH and N_2O were also detected. Under anoxic conditions achieved by suffocation, even ammonium could serve as a suitable electron donor for nitrite reduction in mixed cultures of *N. eutropha* and *Enterobacter aerogenes*. In 44 days 2.2 mM ammonium and nitrite were consumed, but no cell growth was observed (Bock et al. 1995). Production of dinitrogen gas from ammonium at low oxygen concentration was also observed

in gas-tight recycling reactors inoculated with ammonium oxidizing sludge (Muller et al. 1995). At oxygen concentrations above 0.15 %, oxygen consumption was accompanied by dinitrogen gas production. At 0.3% dissolved oxygen, 58% of converted ammonium could be recovered as dinitrogen gas. However, under complete anaerobic conditions no ammonium conversion was observed at all. Three possible explanations for this nitrogen production were discussed: conventional denitrification by heterotrophs on endogenous substrate, denitrification from nitrite in which the electrons were supplied by either ammonium- or thirdly nitrite-oxidation. The dinitrogen gas evolution only occurred in the presence of oxygen, after addition of ammonium, and ceased while sufficient nitrite was still present; therefore both, conventional denitrification and electrons supplied via nitrite oxidation were ruled out as possiblities. On the basis of reaction stoichiometries and nitrogen balances, it could be shown that the dinitrogen was derived form ammonium.

From all these reports and previous studies, it seems possible that nitrifiers can contribute significantly to the production of nitrous oxide and dinitrogen gas at low oxygen concentrations in soil and waste water treatment systems.

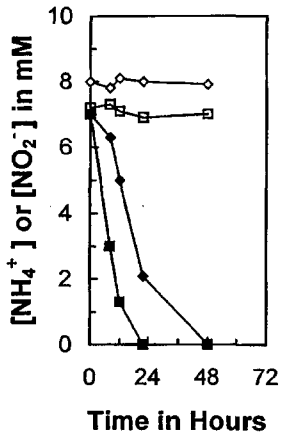

Figure 3. Influence of oxygen on the Anammox activity in batch cultures. Squares, nitrite; diamonds ammonium; closed symbols anaerobic conditions; open symbols aerobic conditions.

Anaerobic ammonium oxidation

Discovery

Thus far, only aerobic and oxygen limited systems have been considered for the oxidation of ammonium. In theory, however, ammonium can also be used as an inorganic electron donor for denitrification. The free energy for this reaction (-297 kJ/mol) is nearly as favourable as the aerobic nitrification process (-315 kJ/mol). In 1977, Broda published a theoretical paper entitled 'Two kinds of lithotrophs missing in nature' describing the potential existence of chemolithotrophic bacteria able to oxidize ammonia to dinitrogen gas with nitrate as electron acceptor. Recently, it was observed that ammonium was disappearing from a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor (van de Graaf et al. 1990, Mulder et al. 1995, European Patent 0327184A1). Ammonium conversion was associated with nitrate consumption and concomitant gas production. A maximum ammonium removal rate of 0.4 kg $N \cdot m^{-3} \cdot d^{-1}$ was observed. The evidence for this anaerobic ammonium oxidation was based on nitrogen and redox balances in continuous-flow experiments. It was shown that for the oxidation of 5 moles ammonium, 3 moles nitrate were required, resulting in the formation of 4 moles dinitrogen gas. It was concluded that anaerobic ammonium oxidation is a new process in which ammonium is oxidized with nitrate serving as the electron acceptor under anaerobic conditions, producing dinitrogen gas. This biological process has been given the name 'Anammox' (*an*aerobic *amm*onium *ox*idation) (Mulder et al 1995). Experiments with ^{15}N-labelled NH_4^+ and $^{14}NO_3^-$ were used to confirm that the end product of the Anammox reaction was dinitrogen gas. However, comparison of the labelling pattern of the formed $^{14,15}N_2$ indicated that nitrite, rather than nitrate might be the preferred electron acceptor of the process (van de Graaf et al. 1995).

Characterization

For the enrichment of anaerobic ammonium oxidizing microorganisms, a synthetic medium containing ammonium, nitrite, bicarbonate, minerals and trace elements was fed to a small scale fluidized bed reactor (Jetten et al. 1995, van de Graaf et al. 1996). Within three months of the start-up, the biomass in this reactor reached conversion rates of 3 kg NH_4^+-$N \cdot m^{-3} \cdot$ day^{-1} on a feed of 30 mM ammonium and nitrite. Maximum specific conversion rates obtained were 1300–1500 nmol $NH_4^+ \cdot h^{-1} \cdot mg^{-1}$VS. Labelling experiments with $^{14}CO_2$ confirmed that the Anammox process is indeed autotrophic, and that incorporation

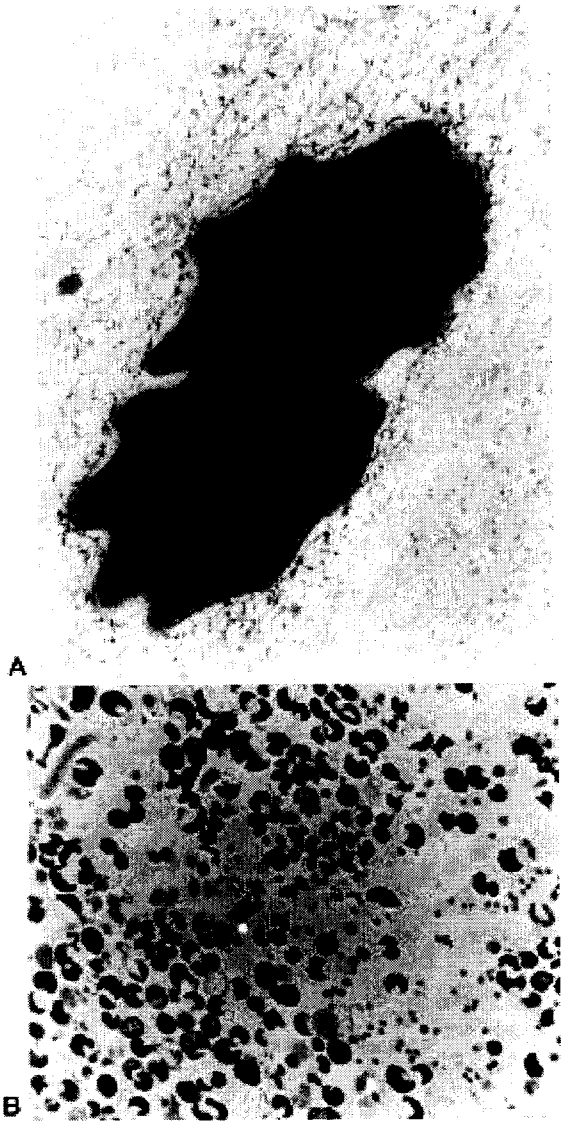

Figure 4. Electron micrograph from the dominant species (close-up, section A) in the Anammox enrichment culture (Overview, section B).

of CO_2 is dependent on the presence of both ammonium and nitrite. Since extreme care was taken to ensure that the experiments were carried out in an oxygen-free atmosphere, the conversion of ammonium to dinitrogen gas did not require oxygen. Indeed, the deliberate addition of O_2 showed that it is inhibitory (Figure 3). The dominant type of microorganism in the enrichment culture was an irregularly shaped cell with an unusual morphology (Figure 4A). In order to assess the amount of this cell type in the enrichment culture, a large series of electron micrographs (Figure 4B) were taken and cell numbers counted. After 177 days of enrichment 64 % of all cells counted (n = 11,433) were of the dominant type (Figure 4B). During the enrichment for anaerobic ammonium-oxidizing microorganisms on synthetic medium, an increase in ether lipids was observed. The color of the biomass changed from brownish to red, which was accompanied by an increase in the cytochrome content (van de Graaf et al. 1996).

Molecular characterization of nitrifying bacteria

Identification, enumeration and characterization of ammonia- and nitrite-oxidizing bacteria in environmental samples by traditional microscopical and microbiological methods are difficult, because of the limited species specific morphological variety, and because of their slow growth rate (Watson et al. 1989), and their low growth yields (Gay & Corman 1984; Wood 1986). The use of molecular techniques to study nitrifying bacteria now enables us to circumvent these limitations, and to obtain useful information on their phylogenetic relationships, and their ecological significance.

Immunological approach

The oldest among the molecular approaches to detect and identify ammonia- and nitrite-oxidizing bacteria is the immunological approach. Specific antibodies have been used in immunofluorescence assays to study nitrifiers in soils (Belser & Schmidt 1978; Fliermans et al. 1974: Josserand & Cleyet-Marel 1979), wastewater (Yoshioka et al. 1982), and seawater (Ward & Perry 1980; Ward 1982; Ward & Carlucci 1985). In a recent paper by Sanden et al. (1994) monoclonal antibodies specific for *Nitrosomonas* and *Nitrobacter* were used in a competitive enzyme-linked immunosorbent assay (ELISA) to characterize and quantify these bacteria in activated sludge from wastewater treatment plants. However, although successful, the immunological approach is hampered by the need of pure strains to produce antisera. The ribosomal RNA approach, initiated by Pace and coworkers (Olsen et al. 1986), and the application of new molecular biological techniques, such as PCR (Saiki et al. 1988), and fluorescence in situ hybridization (FISH; Amann et al. 1995), are nowadays more and more used to study the struc-

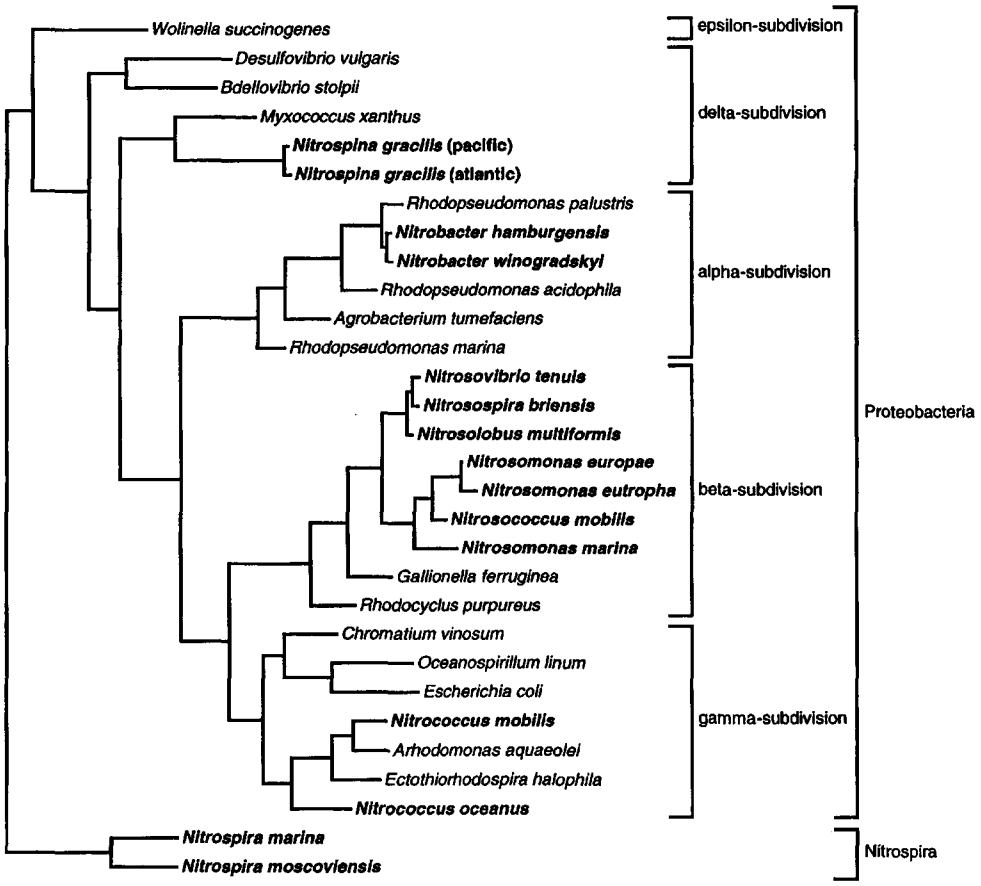

Figure 5. Phylogenetic tree showing relationship between the nitrifying bacteria. [nitrifying bacteria printed bold] (adapted from Teske et al. 1994 J. Bacteriol 176: 6623–6630).

ture and function of microbial communities, and the role of individual inhabitants, such as the nitrifiers, as these techniques do not directly depend on the need of isolated strains (Muyzer & Ramsing 1996).

Phylogenetic relationships of nitrifiers

The 16S rRNA sequences of several different ammonia- and nitrite-oxidizing bacteria were determined to infer their phylogenetic relationship (Head et al. 1993; Teske et al. 1994, Stehr et al. 1995). From these phylogenetic studies it was concluded that all known nitrifiers were affiliated with the different subdivisions of the proteobacteria (Figure 5). The *Nitrobacter* strains were belonging to the α-subdivision; the genera *Nitrosomonas*, *Nitrosolobus*, *Nitrosospira*, *Nitrosococcus*, and *Nitrosovibrio* to the β-subdivision, *Nitrosococcus*, and *Nitrococcus* to the τ-subdivision, and *Nitrospina* and *Nitrospira* to the δ-subdivision. However, this conclusion was recently rejected after the isolation and characterization of a new nitrite-oxidizing bacterium, *Nitrospira moscoviensis*, (Ehrich et al. 1995). On the basis of 16S rRNA sequence analysis this species is phylogenetically affiliated to a new bacterial phylum, consisting of leptospirilla, 'Magnetobacterium bavaricum', *T. yellowstonii*, and the bacterial isolate OPI-2. The phylogenetic analysis also revealed that the nitrite-oxidizer *Nitrospira marina*, which originally was grouped by Teske et al. (1994) in the δ-subdivision of the proteobacteria, belongs to this new phylum.

PCR amplification of rDNA and functional genes

By using comparative analysis of the determined 16S rRNA sequences, McCaig et al. (1994), and Voytek and Ward (1995) were able to design specific primers for amplification of the 16S rDNA of ammonia-oxidizers belonging to the ß-subgroup of Proteobacteria. McCaig et al. (1994) used this specific amplification approach as an early molecular characterization of ammonia oxidizers in enrichment cultures obtained from surface seawater. After sequence analysis of the PCR fragments 3 new lineages of beta ammonium-oxidizing bacteria were obtained. Voytek and Ward (1995) used their specific primer set for the detection of ammonium-oxidizing bacteria in aquatic samples without any prior cultivation step. However, this was only possible after re-amplification of PCR products obtained with primers specific for all eubacteria. No PCR products were found when the primers for ammonium-oxidizing bacteria were used directly on the environmental DNA. The authors suggested that the presence of large amounts of non-target DNA reduced the annealing efficiency of the specific primers. Hiorns et al. (1995) used specific probes and primers to establish the presence of either *Nitrosospira* or *Nitrosomonas* -like nitrifiers in soil and active sludge. In a nested PCR approach similar to Voytek & Ward (1995) it was demonstrated that *Nitrosospira* species were predominant in the environment. However, only after two weeks of enrichment in autotrophic medium both *Nitrosospira* and *Nitrosomonas* 16 S rDNA were observed, indicating the low abundance of *Nitrosomonas* in natural sites.

Polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) analysis of the ribosomal intergenic spacer was used to differentiate *Nitrobacter* strains isolated from various soils and from a lake (Navarro et al. 1992, Degrange & Bardin 1995). This approach is especially useful as an easy and rapid genotypically characterization of large numbers of pure strains. The approach is not suited for characterization of bacteria from mixed cultures or environmental samples, because the banding patterns might be too complex to interpret. Apart from using ribosomal RNA or their encoding genes as molecular marker, also functional genes have been used to detect nitrifiers in their habitat. Sinigalliano et al. (1995) designed a primer pair for amplifying the ammonia monooxygenase (AMO) gene from ammonium-oxidizing bacteria. By using this PCR they were able to detect natural populations of ammonium-oxidizing bacteria in seawater samples.

Detection of nitrifiers using oligonucleotide probes

Comparative analysis of 16S rRNA sequences was also used to design probes for in situ detection of ammonia-oxidizing bacteria (Wagner et al. 1995), and for the study of nitrifiers in multi-species biofilms (Ohashi et al. 1996). By the combined use of a fluorescent-labelled oligonucleotide probe specific for ammonia-oxidizing bacteria and confocal scanning laser microscopy, Wagner et al. (1995) were able to detect dense cell clusters in samples of sewage treatment plants with stable nitrification. No fluorescent cells were detected in samples without nitrification. The objective of a further study was to investigate the influence of substrate C/N ratio on the species composition of a bacterial biofilm consisting of nitrifiers and heterotrophs. For this purpose RNA samples isolated from the biofilms grown at different substrate loads were spotted onto membrane filters and hybridized with different ^{32}P-labelled oligonucleotide probes specific for the 16S rRNA of ammonia- and nitrite-oxidizing bacteria, as well as with probes for eubacteria, archaebacteria and all life forms. One of the observations was that the percentage of ammonia- and nitrite-oxidizing bacteria decreased with increased substrate C/N ratio, while the heterotrophs became more dominant (Ohashi et al. 1996). It has been suggested (van Niel et al. 1992) that this is due to the competition for the nitrogen source (see also section on heterotrophic nitrification).

Perspectives

New molecular approaches, such as denaturing gradient gel electrophoresis of PCR-amplified DNA fragments (Muyzer et al. 1993), will be used to study the ecological importance of nitrifiers, to detect and identify new nitrifiers, such as the organism responsible for the nitrification under anaerobic conditions (van de Graaf et al. 1995, see previous section) and to monitor the success of isolation of these new species. Other oligonucleotide probes will be designed and used together with advanced microscopical techniques, such as confocal scanning laser microscopy, to enumerate different nitrifiers and to determine their spatial distribution in natural samples, such as soils, aquatic samples and bacterial biofilms. These molecular studies will be complemented by the use of micro-electrodes to pro-

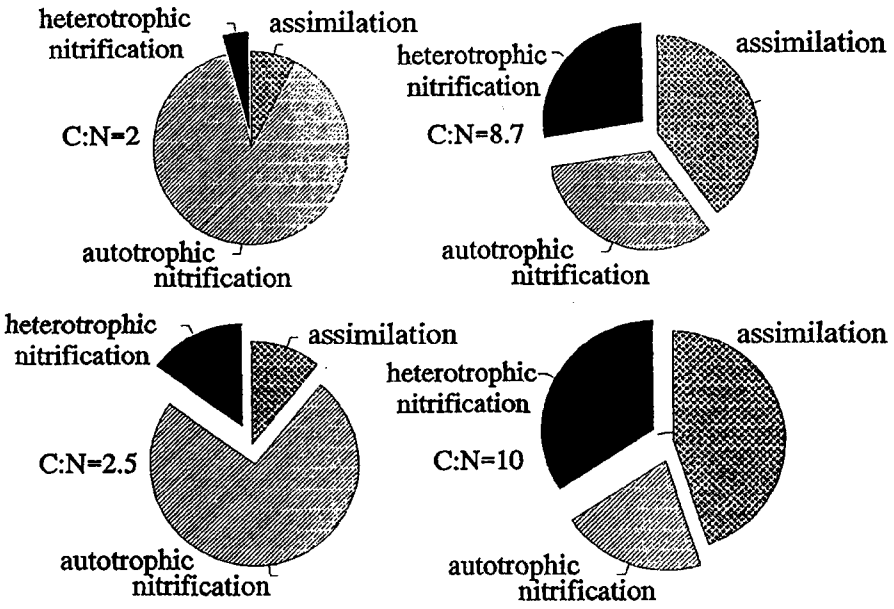

Figure 6. Fate of ammnium in co-cultures of *Thiosphaera pantotropha* and *Nitrosomonas europaea* at different C:N ratios (from Kuenen & Robertson 1994).

file environmental parameters at the microscale, and to determine bacterial activity. Furthermore, amplification of the mRNA of functional genes, such as the AMO-gene (Sinigalliano et al., 1995) by reverse transcriptase (RT)-PCR, or in situ hybridization of the mRNA will be used to detect gene expression and as an indicator for metabolic activity of individual bacterial species.

Heterotrophic nitrification and aerobic denitrification

Heterotrophic nitrification has been known for a long time, but considered of little significance (Meiklejohn 1940, Verstraete 1975). The main reason for this was that the common way of measuring nitrification was to determine the oxidation products (nitrite or nitrate), and heterotrophic nitrifiers do not accumulate large amounts of these compounds. The observation that *Thiosphaera pantotropha* (closely related to *Paracoccus denitrificans*; Baker et al. 1995, Ludwig et al. 1993) was not only a heterotrophic nitrifier, but also an aerobic denitrifier, forced a reevaluation of this approach as this organism converted most of its oxidation products (nitrite) directly to gaseous nitrogen products. Nitrogen balances on various pure cultures (Robertson et al. 1988, 1989) showed that the heterotrophic nitrification rates of several species was considerably higher than previously believed, and raised hopes of single stage nitrogen removal for wastewater treatment systems.

To this end, competition experiments between the well-know autotrophic nitrifier, *Nitrosomonas europaea* and *T. pantotropha* were undertaken at various dissolved oxygen concentrations and carbon:nitrogen (C:N) ratios (van Niel et al. 1993). It was found that *T. pantotropha* can indeed successfully out-compete *N. europaea* for ammonium at low dissolved oxygen concentrations, and at high C:N ratios. However, as can be seen from Figure 6, although a substantial amount of the ammonium was removed by the heterotroph, more was assimilated, reflecting the fact that heterotrophic biomass yields are much higher than autotrophic ones. This higher nitrogen assimilation can be extrapolated to higher sludge production, making the process unsuitable for most wastewater treatment applications.

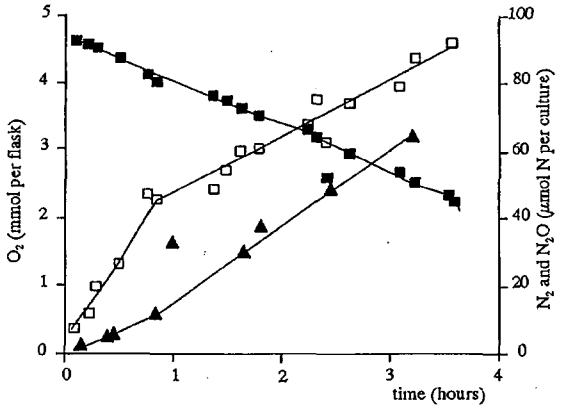

Figure 7. Oxygen consumption (closed squares), nitrogen gas (open squares) and nitrous oxide production (triangles) by batch cultures of *Alcaligenes faecalis* (from Robertson et al. 1995)

Reevaluation

Over the years, it has become apparent that the aerobic denitrifying activity of *T. pantotropha* has been falling. Results in our own laboratory, as well as those of others, have shown varying results (Kuenen & Robertson 1994, Ferguson 1994, Richardson & Ferguson 1992). It therefore became necessary to use ^{15}N-labelled compounds to check the fate of ammonium, nitrate and nitrite in aerobic *T. pantotropha* cultures. Batch culture experiments using gas chromatography and mass spectrometry confirmed that *T. pantotropha* made N_2 from ammonium and/or nitrite and nitrate in well-mixed, aerobic suspensions, although at a rate about 10% of originally reported values (Robertson et al. 1988, 1995). Moreover, N_2 production by other heterotrophic nitrifiers under the same conditions was also confirmed (van Niel et al. 1992, Robertson et al. 1995). One strain, *Alcaligenes faecalis* TUD, produced almost equivalent amounts of N_2 and N_2O (Figure 7). ^{15}N-labelling experiments with *Alcaligenes faecalis* showed that this strain produced $^{14,15}N_2O$ and $^{15,15}N_2O$ from $^{14}NH_4^+$ and $^{15}NO_2^-$ under fully aerobic conditions. Transition experiments and experiments with alternating aerobic/anaerobic periods in acetate limited chemostat cultures with *A. faecalis* revealed that 25% of the NO_2^--N was lost as N_2O (Otte et al. 1996).

Confirmation that *T. pantotropha* generates N_2 in well-mixed chemostat cultures was obtained using acetate-limited continuous cultures linked to an on-line mass spectrometer and supplied with ^{15}N labelled ammonium or nitrite. The main difference between the results obtained from these cultures and the batch experiments mentioned above was that the continuous cultures produced N_2O as well as N_2, possibly because of the substrate limitation. The proportion of N_2O in the gas stream increased as the dissolved oxygen increased until, at dissolved oxygen concentrations above 95% air saturation, only N_2O was produced (Arts et al. 1995). These experiments also revealed that if NH_2OH was added to cultures containing ammonium, the N_2 and N_2O originated exclusively from the NH_2OH (Figure 8), confirming earlier observations (Robertson & Kuenen 1992) that hydroxylamine inhibits ammonium oxidation (without influencing the total nitrification/denitrification rate) by *T. pantotropha*.

It can be concluded that currently-available *T. pantotropha* are still capable of heterotrophic nitrification and simultaneous aerobic denitrification, but that the activities are considerably lower than previously measured. However, it should not be assumed that this returns the heterotrophic nitrifier/denitrifiers to the level of 'insignificant activity'. Not only have other bacteria retained their earlier activity (Robertson et al. 1995), but aerobic denitrifiers have been shown to be common in natural samples (Carter et al. 1995). Even with low specific activity, these bacteria are present and grow at rates sufficiently high to allow them contribute significantly to the turnover of compounds in the nitrogen cycle.

Mechanism

As with the autotrophic nitrifiers, nitrification starting from hydroxylamine has been used to elucidate the underlying mechanism of heterotrophic nitrification (Wehrfritz et al. 1993). Addition of hydroxylamine to actively nitrifying heterotrophs stimulates the nitrification rates and induces the expression of a hydroxylamine oxidoreductase. The HAO enzyme from *T. pantotropha*, like its autotrophic counterpart, is located in the periplasm. The 20 kDa protein does not contain any heme prosthetic groups, but has been suggested to contain non-heme iron. Both cytochrome c_{551} and pseudoazurin could serve as an electron acceptor for the HAO of *T. pantotropha*. Since cytochrome c_{551} and pseudoazurin are soluble redox proteins that can be reduced by HAO and can donate electrons to the denitrification enzymes, but are unable to mediate electron transfer to the quinone pool, they form a link between heterotrophic nitrification and (aerobic) denitrification. The model depicted by Wehrfritz et al. (1993) provides a mechanism for excess reductant dis-

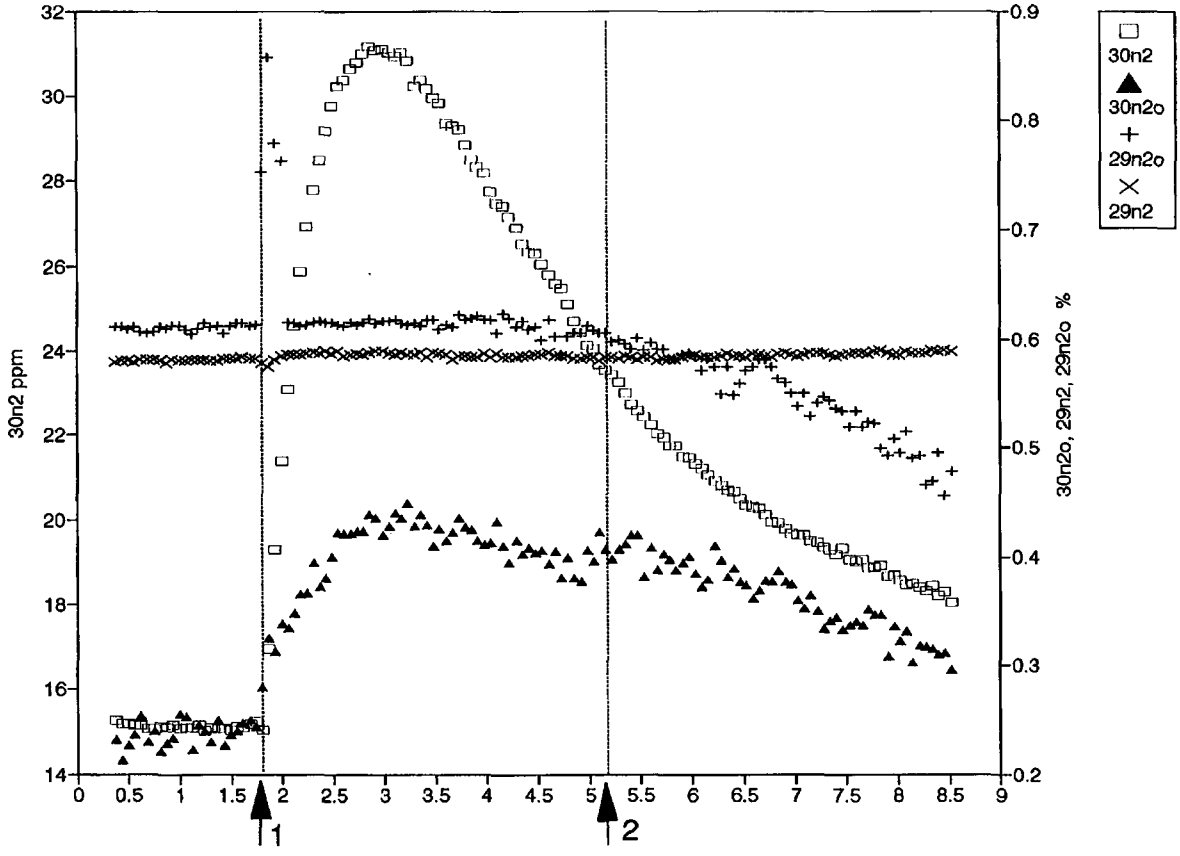

Figure 8. $^{15,15}N_2$ (▼), $^{14,15}N_2$ (x) Dinitrogen gas and $^{15,15}N_2O$ (▲), $^{14,15}N_2O$ (+) production from $^{15}NH_2OH$ in heterotrophically nitrifying *Thiosphaera pantotropha* (Robertson, unpublished results). Arrow 1 indicates addition of $^{15}NH_2OH$, arrow 2 indicates addition of $^{15}NO_2^-$.

sipation (as proposed by Robertson et al. 1988) via an uncoupled electron transport, explaining the lower growth yields of nitrifying cultures.

Since molecular oxygen has been shown to repress denitrification enzymes, the mechanism of aerobic denitrification has received considerable attention. Elucidation of this process started with the discovery that in *T. pantotropha* two different nitrate reductases (NAR) were present. Fractionation of cells grown aerobically or anaerobically in the presence of nitrate revealed an anaerobically active membrane bound NAR and an aerobically active periplasmic NAR. The catalytic properties of the two NARs were clearly distinct. The periplasmic NAR was not able to use chlorate as an alternative electron acceptor and was not sensitive to azide. Only the membrane bound NAR could be coupled to NADH dehydrogenase. It was shown that aerobic expression of the periplasmic NAR by *T. pantotropha* was not dependent on the presence or absence of nitrate, but that its expression was largely influenced by the kind of carbon source used (Richardson & Ferguson 1992). The more reduced the C-source (butyrate or caproate) the higher the periplasmic NAR activity, confirming its possible function as a redox valve during aerobic growth (Robertson et al 1988).

Recently, the periplasmic NAR from *T. pantotropha* was isolated and the genes encoding the enzyme (*nap*EDABC) were characterized (Berks et al. 1994,1995). Furthermore mutants defective in membrane bound NAR were generated by Tn5 insertion in the NAR gene (Bell et al. 1990, 1993). Comparison of growth rates of wild-type and Nar^- mutant M6 clearly showed that the mutant is able to grow in the presence of 50 mM chlorate, in contrast to the wild type, but

that anaerobic growth in the presence of nitrate is 3-fold reduced in M6, due to the absence of membrane bound NAR. The nitrate respiration rate of M6 was comparable to wild type levels, but the rate was not depressed either by azide or oxygen. This is characteristic of periplasmic NAR, and suggests that mutant M6 overexpresses this enzyme. Localization studies showed also that, in M6, 100% of the NAR could be recovered from the periplasm. The lower growth rates and yields of mutant M6 are plausibly a consequence of reduced energy conservation by the periplasmic NAR, compared with the membrane bound NAR in the span from ubiquinol to nitrate. Such difference in energetic coupling is in accord with current ideas about the electron transport mediated phosphorylation during denitrification (Ferguson 1994).

The enzymes of denitrification

Denitrification is the process in which nitrate is converted into dinitrogen via the intermediates nitrite, nitric oxide and nitrous oxide according to the following reactions:

$NO_3^- + UQH_2 \rightarrow NO_2^- + UQ + H_2O$	NAR, nitrate reductase
$NO_2^- + Cu^{1+} + 2H^+ \rightarrow NO + H_2O + Cu^{2+}$	NIR, Cu-nitrite reductase
$NO_2^- + c^{2+} + 2H^+ \rightarrow NO + H_2O + c^{3+}$	NIR, cd_1-nitrite reductase
$2NO + 2c^{2+} + 2H^+ \rightarrow N_2O + H_2O + 2c^{3+}$	NOR, NO reductase
$N_2O + 2c^{2+} + 2H^+ \rightarrow N_2 + H_2O + 2c^{3+}$	NOS, N_2O reductase

Although the presence of NO as a free intermediate has been questioned for some time there is strong direct evidence that NO is a kinetically competent intermediate, Its steady-state concentration varies between 1–65 nM (Goretski et al. 1990), dependent on the species. The process of denitrification is best characterized in Gram-negative bacteria and takes place both in the periplasm and the cytoplasmic membrane. In the reduction of nitrate to nitrite, the reducing equivalents are directly derived from ubi- or menahydroquinone. In the other steps they are indirectly derived via the membrane-bound bc_1-complex which reduces c-cytochromes and/or possibly blue-copper proteins like (pseudo)azurin. Accordingly, the overall reaction of denitrification can be written as:

$$5UQH_2 + 2NO_3^- + 2H^+ \rightarrow N_2 + 6H_2O + 5UQ$$

Part of the free energy of this reaction is converted into a proton-motive force across the cytoplasmic membrane. The driving force is the electrogenic reduction of nitrate by the membrane-bound nitrate reductase and the electrogenic action of the bc_1-complex. Whether the reaction catalysed by NO reductase is also electrogenic, is under debate (see also below).

The primary sequences of the enzymes involved in denitrification from various sources have been determined from the DNA sequences of the corresponding structural genes [see Zumft (1994) and Ferguson (1994) and references therein]. In total, approximately 40 genes are necessary to carry out denitrification. These include the structural and regulatory genes and genes for the biosynthesis of specific prosthetic groups.

Nitrate reductase

Two types of nitrate reductase have been identified, a membrane-bound enzyme consisting of three subunits (120, 60 and 20 kDa) using ubihydroquinone as electron donor and a soluble periplasmic enzyme, consisting of two subunits (94 and 19 kDa) (Bell et al. 1990,1993, Berks et al. 1994,1995, Craske & Ferguson 1986, Siddiqui et al. 1993). The physiological electron donor of the latter is unknown. Both enzymes contain a molybdopterin cofactor. The membrane-bound enzyme has an $\alpha\beta\tau_2$ structure; both α and β subunits are hydrophilic; the α subunit contains the molybdenum pterin cofactor and four [3Fe-4S] or [4Fe-4S] clusters, the two gamma-subunits serve as a membrane anchor and contain two β-type hemes with different redox potentials (E_{m7}= 95 and 210 mV). The large subunit of the soluble periplasmic enzyme contains at least one low-potential [4Fe-4S] cluster in addition to the molybdopterin and the small subunit contains two c-type cytochromes with E_{m7}= −15 and +80 mV.

Although the mechanism of action of the two enzymes is unknown, it is generally assumed that reduction of nitrate to nitrite takes place at the molybdenum center, in which the molybdenum atom shuttles between the Mo(IV) and Mo(VI) redox states with Mo(V) as intermediate. Since the membrane-bound nitrate reductase is able to generate a membrane potential and since the reduction of nitrate occurs in the cytoplasm, it seems most likely that ubihydroquinone is being oxidized at the periplasmic side of the membrane by one of the b hemes. Furthermore the two hemes are arranged in a transmembrane orientation, as in the bc_1-complex, to allow transmembrane electron transfer to the molybdenum center. The role of the var-

ious Fe-S clusters in the enzyme remains unclear in particular because their standard reduction potential is by far too low to be reduced by ubi- or menahydroquinone.

The expression of two nitrate reductases under similar conditions in one and the same organism raises questions about their respective roles. In a mutant of *T. pantotropha* from which the membrane-bound enzyme was absent, the soluble periplasmic enzyme was (over) expressed (Bell et al. 1993) and able to perform the first step in anaerobic denitrification (see previous section). In *Alcaligenes eutrophus* H16 in which the periplasmic enzyme was inactivated, the only apparent phenotype was a delayed growth, compared to the wild type, after transition from aerobic to anaerobic respiration (Siddiqui et al. 1993).

Nitrite reductase

Two different types of nitrite reductase have been found in denitrifiers; approximately one third possess the copper-containing nitrite reductase, the others the heme cd_1-containing enzyme, i.e. their presence is mutually exclusive for reasons not known. The two enzymes are functionally and physiologically equivalent as indicated by the finding that the copper-containing nitrite reductase from *Pseudomonas aureofaciens* can be cloned in an active form in *Pseudomonas stutzeri* in which the gene encoding the heme-containing enzyme had been disrupted (Glockner et al. 1993).

Both nitrite reductases have been crystallized and high resolution structures are available. The copper-containing nitrite reductase is a homotrimer, with subunit of 36 kDa, and two copper atoms per monomer. Each monomer contains a type I copper site with 2 histidines, one cysteine and one methionine as ligands to the copper. The trimer further contains three type II copper sites, one at each of the three subunit interfaces. The type II copper is ligated by three histidines, two from one monomer, one from the other. The distance between the two copper centers is about 12.5 Å (Godden et al. 1994). The type I site most likely accepts the electrons from the physiological donor ((pseudo)azurin and/or cytochrome c). The electrons are subsequently transferred rapidly ($\sim$1400 s^{-1}) to the type II copper site. This latter copper binds the nitrite and reduces it to nitric oxide.

The cytochrome cd_1-nitrite reductase is a homodimer (64 kDa per subunit), each monomer containing one heme c and one heme d_1. Each monomer consists of two clearly separated domains (Fülöp et al. 1995), one harbouring the heme c and the other containing the heme d_1. The heme c-containing domain serves as the electron entry from (pseudo)azurin and other c-type cytochromes. Although the structure of this domain resembles that of class 1 c-cytochromes, the environment of the heme iron is different, i.e. the iron atom is coordinated to two histidine nitrogens. The heme d_1-containing domain has an eight-bladed b-propeller structure and resembles methylamine dehydrogenase and methanol dehydrogenase. The iron atom of the heme d_1 is coordinated to a histidine nitrogen and a tyrosin oxygen at least in the oxidized enzyme. Since this tyrosin residue is not conserved in all sequences its role in catalysis is probably limited. In fact, Fülöp et al. (1995) propose that this tyrosin is displaced upon reduction so that the sixth ligand position of the heme d_1 iron is able to bind nitrite and subsequently reduce it to NO.

The organization of genes involved in expression of the cytochrome cd_1-nitrite reductase has been described for *Ps. stutzeri* and *Ps. aerigunosa* (Zumft 1994) and for *P. denitrificans* (de Boer et al. 1994). The gene clusters clearly contain so-called *fnr*-boxes and the *nnr*A regulatory protein both factors being important for the expression under anaerobic conditions (see de Boer et al. 1994 for a more detailed discussion). An intriguing observation was made in a *nir*Q disruption strain (a regulatory gene located upstream of *nir*S) in which both nitrite and NO reductase activities in vivo were disrupted (Jungst and Zumft 1992). Both proteins were expressed, however, with only nitrite reductase being active in vitro. These observations may be indicative for a close structural relation between the two enzymes, i.e. forming a functional enzymic unit.

NO reductase

NO reductase is the least characterized enzyme involved in denitrification. The enzyme is membrane-bound and has been purified in the presence of detergent from two sources, *Paracoccus denitrificans* (Carr and Ferguson 1990) and *Pseudomonas stutzeri* (Heiss et al. 1989). The purified enzyme apparently consists of two subunits, one of 16 kDa carrying heme c, one carrying heme b of 53 kDa. The two hemes have been reported to be present in a 1:1 ratio, in addition, the enzyme contains non-heme iron in variable amounts.

Recently, the gene encoding the NO reductase from *P. stutzeri* has been sequenced Zumft et al. (1994). The primary sequence indicates a highly hydrophobic

enzyme with 12 membrane-spanning helices. A structural and evolutionary homology between NO reductase and cytochrome c oxidase has been noted by others (van der Oost et al. 1994, Saraste and Castresana 1994) i.e. both enzymes contain a similar pattern of membrane-spanning helices and further, six histidine residues are conserved between them. In cytochrome c oxidase, three histidine residues serve as ligands to the high-and low-spin heme centers and three are coordinated to Cu_B. Both high-spin (heme b) and low-spin hemes (heme b and c) may actually be present in NO reductase, but copper is not (Kastrau et al. 1994). Perhaps in NO reductase the non-heme iron is coordinated to the histidines that coordinate Cu_B in cytochrome oxidase. Given the structural similarity between NO reductase and cytochrome c oxidase the possibility exists that reduction of NO to N_2O is in fact electrogenic, i.e. two protons are translocated from the cytoplasm to the periplasm forming water (see the reaction above). Apparently, the NO reductase does not function as a proton pump (see Ferguson 1994 for a discussion). Direct information on the mechanism of action of the enzyme is lacking, but suggestions on the mechanism have been made (Ye et al. 1994). The regulation of expression of NO reductase is coupled to that of nitrite reductase (see above). Genetic evidence from *nir*T and *nir*S disruption strains further suggests that NO may be involved directly in the expression of NO reductase (Zumft et al. 1994) although how and where it would act as an effector remains to be elucidated.

N_2O reductase

Several periplasmic N_2O reductases have been purified today but none have been crystallized. Nevertheless detailed information on one of the prosthetic groups is available from various spectroscopic techniques such as Electron Paramagnetic Resonance (EPR), Extended X-ray Absorption Fine Structure (EXAFS) and Resonance Raman spectroscopy.

The purified enzyme is a homodimer, each subunit (molecular mass ~70 kDa) containing four copper atoms. The purified enzyme is very labile with respect to oxygen. The four copper atoms are arranged into two different dinuclear centers, Cu_A(E_m= 260 mV) and Cu_Z. The Cu_A center is structurally similar to Cu_A in cytochrome oxidases (Saraste 1990, Andrew et al. 1994), i.e. a mixed-valence dinuclear center (von Wachenfeldt et al. 1994, Antholine et al. 1992) with a 2.5 Å copper-copper distance (Blackburn et al. 1994), the two copper atoms being bridged by two cysteine sulphurs (Iwata et al. 1995, Tsukihara et al. 1995). Further, each copper atom is ligated to a histidine nitrogen and one to methionine sulphur. The Cu_Acenter is both in cytochrome oxidase and N_2O reductase the site of entry of electrons.

In contrast to Cu_A, not much is known about the other copper center Cu_Z. This center is, like Cu_A, believed to be dinuclear, with both coppers in the cupric state and antiferromagnetically coupled, based on the absence of an EPR signal. Upon reduction with dithionite a blue absorbance appears, probably due to the Cu_Zcenter. The absence of an EPR signal under these conditions suggests that the two copper atoms are in the cuprous state (Farrar et al. 1991, Dooley et al. 1991). The Cu_Zcenter is generally considered as the site of N_2O reduction (and of inhibition acetylene) although no direct experimental evidence is available to substantiate this view.

The inhibition or repression of N_2O reductase by oxygen has been subject of many studies (see Ferguson 1994 for details). Inhibition of N_2O reductase results in unwanted release of N_2O during nitrogen removal. Clearly, more structural and kinetic information must be obtained to understand the mechanism of action of the denitrification enzymes, in particular of NO and N_2O reductase in order to prevent unwanted release of NO_x compounds in the environment.

Applications of (de)nitrification in activated sludge systems

Traditionally nitrogen is removed from waste water by the use of the activated sludge process. This process is well established and a description can be found in textbooks on waste water engineering. Recently more and more industrial applications of nitrogen removal appeared. The different requirements for these kind of processes led to the development of new conversion processes. Innovation was possible either by new microbial processes or new reactor configurations, or by a combination From the previously described 'new' microbial conversion processes especially the application of anaerobic ammonium conversion is highly promising with regard to the potential savings in energy and COD requirement. The application of aerobic denitrification or heterotrophic nitrification is less promising. In general the nitrogen conversion rates are slower then for autotrophic nitrification (Table 1). Moreover these processes will occur mainly in systems with a high COD/N ratio. In practice these types of waste

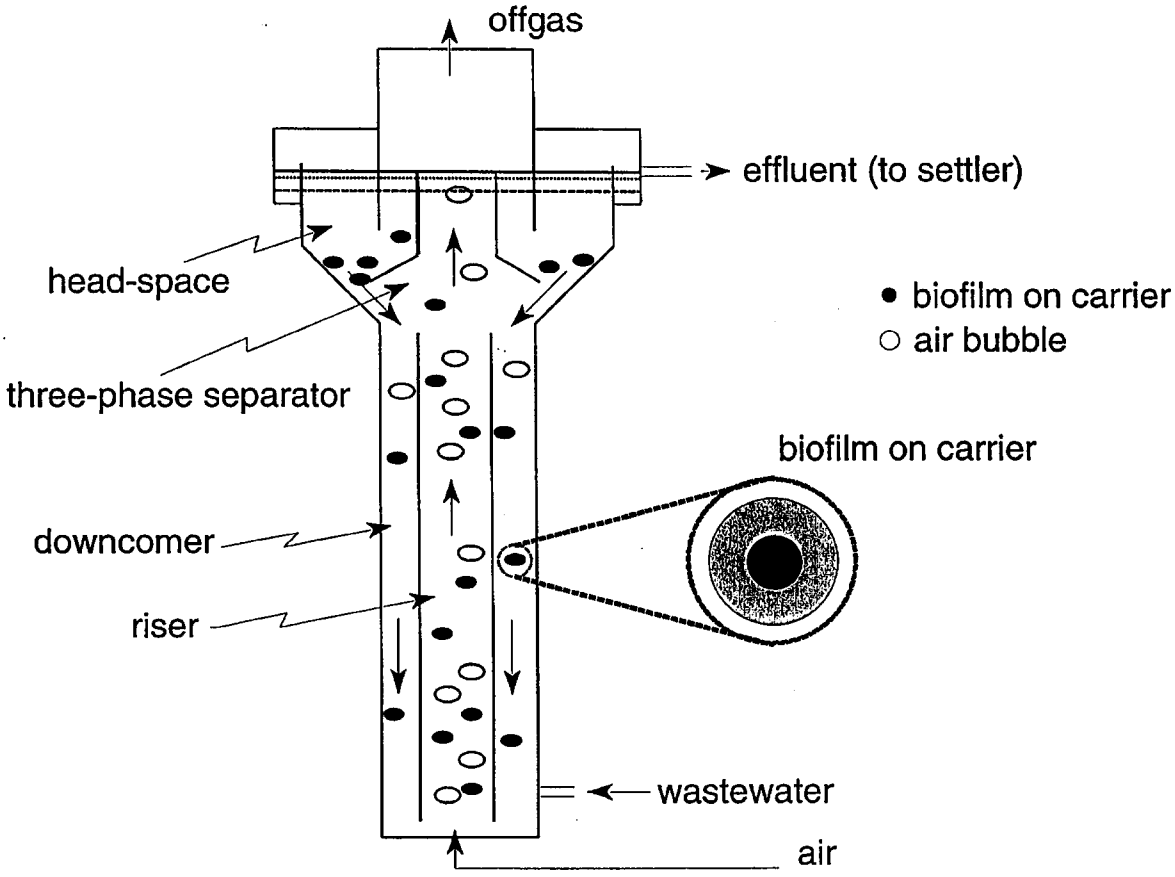

Figure 9. Biofilm airlift suspension reactor with coaxial anoxic zones (adapted from Heijnen et al. 1990).

water form no problem for nitrogen removal, and the majority of the ammonium is removed by assimilation rather then conversion to dinitrogen gas. In the process development two lines of research can be distinguished: (i) intensification of the process by using immobilisation and (ii) decreasing energy and chemicals demand by using nitrite as intermediate.

Immobilization

The main problem for nitrogen removal is caused by the fact that the autotrophic bacteria responsible for nitrification, are slow growing organisms. This means that high sludge ages are required with the consequence that extremely large reactors are needed. Immobilization of bacteria in biofilms can result in increased sludge concentrations and thereby to a significant reduction of reactor volume. This advantage can only be obtained

Table 1. Comparison of autotrophic and heterotrophic nitrification rates in nmol N. min^{-1}. mg^{-1} dry weight

Organism	Activity	N-compound
Pseudomonas aeruginosa	12–28[1]	Hydroxamate
Pseudomonas aeruginosa	70–90	Hydroxylamine
Pseudomonas sp.	40–450	Hydroxylamine
Alcaligenes sp.	33	pyruvic oxime
Pseudomonas sp.	2.6	pyruvic oxime
Thiosphaera pantotropha	35	ammonia
Alcaligenes faecalis	17–22	ammonia
Pseudomonas sp.	24	ammonia
Nitrosomonas sp.	130–1200	ammonia and hydroxylamine

[1]Numbers taken from Kuenen & Robertson 1994 and Jetten et al. 1995

when the biofilm surface area is large enough to prevent limitations due to oxygen mass transfer to the biofilm. In the biofilm airlift suspension (BAS) reactor this is achieved by growing the bacteria on small suspended particles (Figure 9) (Heijnen et al. 1990). Nitrification capacity can be as high as 6 kg N / m^3. day (Tijhuis et al. 1995) in this reactor which is 30 times more than the 0.2 kg N / m^3. day in conventional processes. The research on nitrification in biofilms has led to an increased understanding of the ecology of immobilized bacteria. In general the activity of the nitrifiers in biofilms is comparable to the activity of suspended cells, provided the effects of diffusion are taken into account. In biofilms the different physiological groups are more or less segregated. Slowly growing organisms like nitrifiers are outcompeted from the surface regions by faster growing heterotrophic organisms (Van Loosdrecht et al. 1995, Ohashi et al. 1996). This competition is probably influenced by the relative volumetric biomass accumulation rate of a specific organism in the biofilm in combination with the mechanical removal. The fact that nitrifiers are outcompeted from the biofilm surface layer means that they are more subjected to oxygen limitation. Since their affinity constant for oxygen is relatively high, they will have an even higher competitive disadvantage.

Reduction of energy demand

Nitrogen removal from waste water is a process in which normally ammonium is oxidized to nitrate and then reduced to dinitrogen gas. This means that large amounts of oxygen (i.e. energy) and electron donor (i.e. chemicals) are required. The costs of the process will be strongly reduced if less oxidized intermediates could be used (Rahmani et al. 1995). In principle hydroxylamine would be an ideal intermediate. In practice such a conversion has never been observed in significant amounts. Muller et al. (1995) recently showed in a laboratory set-up that at low oxygen concentrations ammonium could be directly converted to dinitrogen. In practice this conversion might also occur. In activated sludge or biofilm processes diffusion limitations at low DO will always result in anaerobic zones inside the flocs or biofilm. Nitrogen removal under these conditions will then be mainly due to conventional conversions.

The second intermediate of interest is nitrite. Nitrification is a two step process with nitrite as intermediate . In practical systems nitrite can often accumulate in significant amounts, but a stable nitrification with

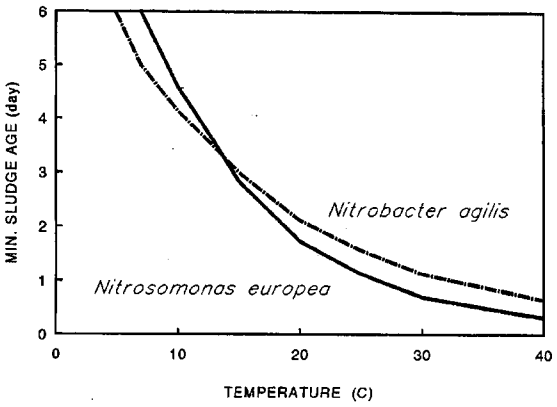

Figure 10. Effect of temperature in °C on the minimal required sludge retention time ($1/\mu_{max}$) for ammonium and nitrite oxidation in a waste water plant. Above 14 °C it is possible to wash out the nitrite oxidizing bacteria, while maintaining ammonium oxidizers. The temperature coefficients are taken from Hunik et al. 1994.

nitrite as end product is difficult to obtain. Anthonissen (1976) showed in a classical paper that at high pH nitrite oxidizers can be inhibited by free ammonia. However in open cultures it seems that this strategy has only temporarily success. The nitrite oxidizers become relatively quickly resistant to high ammonia concentrations and nitrification proceeds onto nitrate (Turk & Mavinic 1989, Brouwer 1995). Only at higher temperatures (as often occur in industrial waste water or effluent from sludge digesters) there seems to be a possibility to effectively outcompete nitrite oxidizing bacteria. Nitrite oxidizing bacteria are less influenced by temperature than ammonium oxidizers (Hunink 1994). This means that at higher temperatures nitrite oxidizing bacteria can be outcompeted based on their lower growth rate (Figure 10). This strategy is difficult to apply in systems with sludge retention. At elevated temperatures (30–35 °C) waste water with relatively high nitrogen content can be operated without the requirement of sludge retention. A chemostat like reactor was successfully applied for the oxidation of ammonium to nitrite from sludge digester effluent. By adjusting the dilution rate to values less than 1 to 1.2 per day, nitrate oxidizing bacteria could be washed out from the process. Based on this finding, the so-called SHARON process was developed (Brouwer 1995). In a reactor operated as chemostat with discontinuous aeration it was possible to obtain a stable nitrification and denitrification. In our laboratory, this process has operated successfully for more than two years now. This process has as advantage that energy and methanol requirements

are strongly reduced. The reactor, however, is approximately three times larger than a nitrification reactor alone. This increased reactor size is compensated by the absence of a settling tank and a sludge recycle.

Conclusions

After 100 years of microbiological and biotechnological research into the physiology and behavior of bacteria in natural and man-made systems, considerable progress has been made in the understanding of inorganic nitrogen conversions. However, until we unravel the fundamental processes underlying nitric and nitrous oxide production during ammonia and NO_x metabolism, we will not be able to direct these processes towards desirable products and thus control a significant portion of NO_x emission. New process technological applications and novel microbial processes like anaerobic ammonium oxidation will further enhance the removal of nitrogen from waste water and reduce the chemical and energy demand for this removal.

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