Stellingen

behorende bij het proefschrift
Biological Anaerobic Ammonium Oxidation
van A.A. van de Graaf

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1. Het totaal aantal vislarven per jaar is geen goede parameter voor de verandering in de levensgemeenschap waar ze deel van uitmaken.
   Greve et al., 1996, J. Marine Science, 53:951-956.

2. De interpretatie van de resultaten van het experiment in Jetten *et al.*, waarin de invloed van zuurstof op de Anammoxactiviteit onderzocht wordt, gaat ten onrechte voorbij aan hun eerdere conclusie dat voor anaërobe oxidatie van ammonium nitriet noodzakelijk is.
   Jetten et al., 1997, Antonie van Leeuwenhoek 71: 75-93 versus dit proefschrift, hoofdstuk 5.

3. Ondanks de vele experimentele bewijzen die Oelze en medewerkers in een periode van vijftien jaar hebben aangedragen ten nadele van het ademhalings-beschermingsconcept, blijft deze hypothese aangaande het zuurstofdilemma in aërobe stikstoffixatie ten onrechte als een algemeen aanvaard 'feit' onder de wetenschappelijke gemeenschap circuleren.
   K. Linkerhägner and J. Oelze, 1997, J. Bacteriol. 179, 1362-1367: het 'laatste' artikel in de serie.

4. Het meten van alle enzymaktiviteiten bij een zelfde pH houdt geen rekening met de compartimentalisatie binnen de eukaryotische cel en leidt derhalve tot verkeerde conclusies omtrent de werkelijke *in vivo* fysiologische processen.
   J. Neermann and R. Wagner, 1996, J. Cell. Physiol. 166, 152-169.

5. Studie naar het DMS-metabolisme in een microbiële mat, met een op thiosulfaat geïsoleerd organisme, is zonder verdere verklaring, geen juiste aanpak voor het leren begrijpen van DMS-afbraak in ecosystemen.
   P.T. Visscher, P. Quist, and H. van Gemerden, 1990, Appl. Env. Microbiol., 57, 1758-1763.

6. Het gebruik van slecht geaëreerde cultures en/of het gebruik van niet gebalanceerde media bij het kweken van *Kluyveromyces lactis* leidt ten onrechte tot conclusies aangaande het vermogen tot 'aërobe fermentatie' van dit organisme.
Kiers, J. et al. (1997) Submitted for publication.

7. Het niet-bestaande verschil tussen anaëroob en anoxisch vertegenwoordigt het wél-bestaande verschil tussen microbiologen en afvalwaterzuiveraars.

8. Het is frappant dat juist degenen die *niet* in een "kantoortuin"-situatie zitten zich lovend uitlaten over de openheid ervan en precies weten hoe deze ingericht hoort te worden.

9. Door de sterk toenemende beschikbaarheid van mobiele telefoons wordt onbereikbaarheid een economische factor.

10. De citatie-index als maat voor de waarde(ring) van onderzoek geeft aanleiding tot publicatie van veel geactualiseerde overzichtsartikelen en analysemethodes.

11. Het bestaan van de mogelijkheid in Groot-Brittannië om zich te verzekeren tegen onbevlekte ontvangenis en ontvoering door buitenaardse wezens onderschrijft de opmerking van John Major dat Groot-Brittannië maatschappelijk nog niet toe is aan de Europese Unie.

12. Tabaksaccijns is verslavend.

13. De door de overheid nagestreefde flexibilisering van de arbeid kan pas werkelijk succesvol zijn als de aanwezigheidsduur op kantoor als maatstaf voor de kwaliteit en waardering van arbeid heeft afgedaan.

14. Wetenschapspopularisering heeft, gezien het vertoon van excentrieke en verstrooide types in populaire lachshows, nog een lange weg te gaan.

Biological

Anaerobic Ammonium Oxidation

Cover: Micro-organism from a slib recycle chemostat culture on waste water.
Electron micrograph by Wilma Batenburg-van de Vegte (TUD)

Biological

Anaerobic Ammonium Oxidation

PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Technische Universiteit Delft,

op gezag van de Rector Magnificus Prof. dr. ir. J. Blaauwendraad,

in het openbaar te verdedigen ten overstaan van een commissie,

door het College van Dekanen aangewezen,

op dinsdag 16 september te 16.00 uur

door

Astrid Alexandra VAN DE GRAAF

scheikundig ingenieur

geboren te 's Gravenhage

Dit proefschrift is goedgekeurd door de promotor:
Prof. dr. J.G. Kuenen

Samenstelling promotiecommissie:

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This study was carried out at the Department of Microbiology and Enzymology, Kluyver Laboratory for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands. The research as described in this thesis was sponsored by the Foundation for Applied Research (STW, Stichting voor de Technische Wetenschappen), project number DST 88-1506, and Gist-brocades N.V.

Published and distributed by:
Delft University Press
Mekelweg 4
2628 CD Delft
The Netherlands
Telephone: +31 15 2783254
Fax: +31 15 2781661
E-mail: DUP@DUP.TUDelft.NL

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Graaf, A.A. van de

Biological Anaerobic Ammonium Oxidation / A.A. van de Graaf. - Delft:Delft University Press. - Illustrations.
Thesis Delft University of Technology. - With ref. - With summary in Dutch.
ISBN 90-407-1518-1
NUGI 841
Subject headings: ammonium oxidation, denitrification, nitrification, Anammox.

"Quod scripsi, scripsi"

Voor pa en ma

Contents

Chapter 1

General introduction

The Role of Nitrogen in Nature

Nitrogen is an essential element for living organisms occurring in compounds such as amino acids, proteins, and nucleic acids. The atmosphere of the earth contains 80 % of molecular nitrogen, which equals 75.10^6 kg N above each hectare of the earth's surface. Molecular nitrogen is extremely inert, chemically, and cannot be used directly as a nitrogen source, except by a few specialist bacterial species. All other plants, animals and microorganisms depend on a fixed form of nitrogen in their nutrition. Under natural conditions, a small fraction (1-15 kg N $ha^{-1}yr^{-1}$) is converted by N_2 fixing microorganisms to biologically more active forms of nitrogen: for example ammonium and nitrate. About 1-30 kg N $ha^{-1}yr^{-1}$ is recycled from the earth to the atmosphere by denitrification. The earth itself contains 5 times more nitrogen than the atmosphere, but weathering of solid is a generally negligible source of biologically active nitrogen.

The processes that play a important role in the conversion of inorganic and organic nitrogen to nitrogen gas are nitrification, denitrification and dissimilatory nitrate reduction. Cyclic transformations of nitrogen compounds are therefore of great significance in the turnover of nitrogen in the biosphere. The bacteria that carry out these reactions must interact, since they either cross-feed or compete for their various electron donors and acceptors. However, the global element cycles are influenced more and more by the agro-chemical industry, as well as other human activities which cause extra input. Nitrogen compounds accumulate and the interacting nitrogen flows are inbalanced.

The main features of the nitrogen cycle are illustrated schematically in Figure 1, and will be briefly described below. More detailed information about the ecology, the physiology and the biochemistry of nitrification and denitrification is provided. For further information, the reader is referred to reviews (e.g. Alexander et al. 1960, Bock *et al.*, 1991; Wood, 1988; Prosser, 1989; Abeliovich, 1992; Focht and Verstraete, 1977; Kuenen and Robertson, 1987; Knowles, 1982; Zumft, 1992)

Nitrogen fixation to ammonium

As already mentioned, atmospheric nitrogen is unavailable to most living organisms, except for a few highly specialized nitrogen-fixing organisms (e.g. *Rhizobium*, *Azotobacter*, *Azospirillum* species). The ability to reduce molecular nitrogen to ammonium is dependent on the presence of the enzyme nitrogenase. This enzyme has even been found in a limited number of Archaea. The ammonium formed is either built into more complex organic compounds such as proteins and nucleic acids, or may be excreted as ammonium, to be assimilated by other bacteria, and plants.

Globally, biological nitrogen fixation is an important process. Without it, atmospheric nitrogen would not be available to living organisms. As agriculture has become

increasingly intensive, the chemical fixation of dinitrogen in the form of ammonium and nitrate by the fertilizer industry has become a very significant part of the total input of nitrogen into the biosphere.

Nitrogen assimiliation

Once nitrogen is fixed in the form of ammonium, most bacteria can use this compound as their source of nitrogen. It can be assimilated via the glutamate or alanine dehydrogenase pathways when sufficient ammonium is present. Another pathway involving two enzymes, glutamine synthetase and glutamate synthase, is used when the ammonium concentration is low. This pathway requires the input of energy in the form of ATP (i.e. 1 ATP per extra ammonium fixed).

Many bacteria and plants can use nitrate or nitrite as a nitrogen source instead of ammonium, at the expense of reducing equivalents. Nitrate is reduced to ammonium by means of the assimilative nitrate reduction pathway.

Ammonification

Dead organic material contains nitrogen in a fixed form, especially as protein. Ammonium can be released by decomposition by a wide variety of micro-organisms. It can then either be assimilated by other organisms, or converted to more oxidized forms of nitrogen.

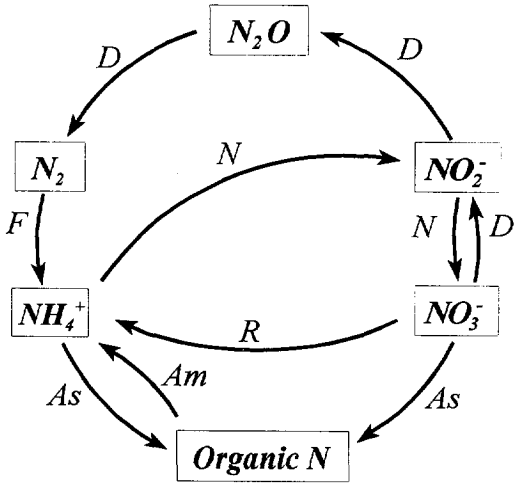

Figure 1 The Nitrogen Cycle (Kuenen & Robertson, 1987). For simplicity, many intermediates have been omitted. D = Denitrification; N = Nitrification; Am = Ammonification; As = Assimilation Nitrogen; F = Fixation; R = Dissimilatory Nitrate Reduction.

Nitrification

Nitrification is that part of the cycle where reduced nitrogenous compounds (e.g. ammonium, hydroxylamine and nitrite) are aerobically converted to more oxidized products. This will be discussed in more detail below.

Nitrate reduction

In the nitrogen cycle, three different nitrate reduction pathways are known: reduction of nitrate to ammonium (assimilative and dissimilatory nitrate reduction) and reduction of nitrate to nitrogen gas (denitrification).

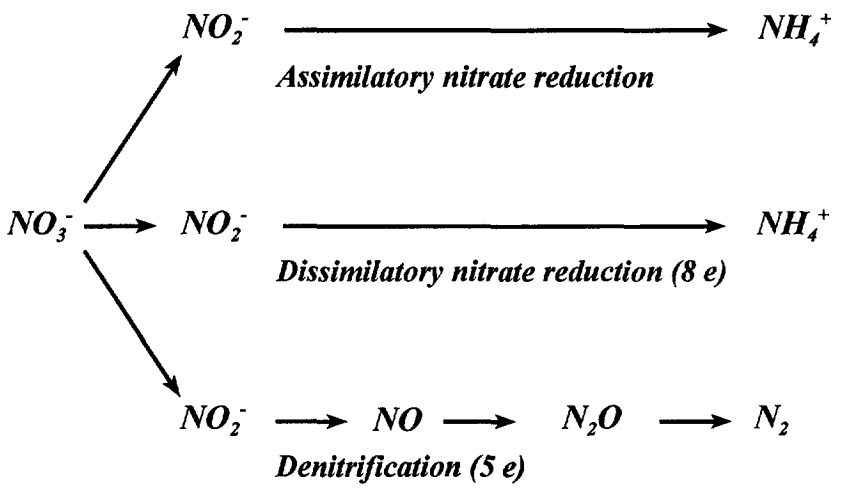

Figure 2 Three pathways of nitrate reduction available to bacteria.

○ Assimilatory and dissimilatory nitrate reduction

Nitrate reduced to ammonium by these two processes is conserved within the ecosystem. Although the assimilation and dissimilation nitrate reduction pathways are superficially similar, their enzymes and functions are not. In assimilatory nitrate reduction, the ammonium produced remains within the biomass as protein and other organic nitrogen compounds. Dissimilatory nitrate reduction, in contrast, appears to serve different purposes in different bacteria. In *Clostridia*, for example, nitrate serves as an electron sink, allowing additional substrate-level phosphorylation to occur when the reduction of organic substrates to fermentation end-products is not possible (Cole, 1987). Sulphate-reducing bacteria reduce nitrate to ammonium in a true respiratory process coupled to electron transport phosphorylation (Dalsgaard en Bak, 1994). Dissimilatory

nitrate reduction may be favoured over denitrificaton in environments where organic carbon is available in excess, and growth is limited by electron acceptor availability (Tiedje *et al.*, 1982).

○ *Denitrification*

In denitrification, nitrate and other nitrogen oxides are converted to nitrogen gas via such intermediates as nitrite, nitrous oxide and nitric oxide (Knowles, 1982; Payne, 1981; Zumft 1992). Denitrification will be discussed below.

Nitrification

Reduced nitrogenous compounds, such as ammonium, are aerobically converted to more oxidized products. Different forms of nitrification occur in autotrophic and heterotrophic bacteria.

Autotrophic nitrification

Ammonium is oxidized to nitrate in two separate steps by chemolithoautotrophic bacteria, examples of which are the genera *Nitrosomonas* spp. and *Nitrobacter* spp., respectively (Wood, 1988, Hooper, 1989, Bock *et al.*, 1992). In the oxidation of ammonium, reaction (1) is catalyzed by ammonium monooxygenase (AMO). This enzyme is dependent on a reduced co-enzyme such as NAD(P)H, as second electron donor. The second, energy generating step (reaction 2) is carried out by hydroxylamine oxidoreductase (HAO) (Hooper *et al.*, 1984), which probably delivers the reducing equivalents needed for the first step. The source of the second oxygen atom in nitrite is H_2O (Anderson and Hooper, 1983).

$NH_3 + O_2 + 2\,[H] \rightarrow NH_2OH + H_2O \quad + 17$ kJ/mol (1)

$NH_2OH + H_2O \rightarrow NO_2^- + 4\,[H] + H^+$ (2)

$0.5\,O_2 + 2[H] \rightarrow H_2O$

$NH_3 + 1.5\,O_2 \rightarrow NO_2^- + 2\,H_2O + 2\,H^+ - 273$ kJ/mol

Nitrite is subsequently oxidized to nitrate by the second group of autotrophic nitrifiers. Nitrite oxidoreductase catalyzes this reaction, and is coupled to the electron transport chain in order to generate energy for growth.

$NO_2^- + H_2O \rightarrow NO_3^- + 2\,[H] \quad - 84$ kJ/mol

The ammonium monooxygenase of *Nitrosomonas europaea* is non-specific. It can catalyze the oxidation of alkanes (up to C_8), alcohols and alkenes (up to C_5) to epoxides and alcohols in the presence of ammonium ions (Hyman *et al.* 1988). Autotrophic nitrifying bacteria can even (co-)oxidize methane, but gain no energy for growth from this reaction (Jones and Morita, 1983). AMO has been found to be a very labile membrane protein, which makes it very difficult to assay and purify (Juliette *et al.*, 1995). The ammonium oxidation is very effectively inhibited by low concentrations of acetylene (Hyman *et al.*, 1988).

Hydroxylamine oxidation involves a four-electron transfer which is still not completely understood. It has been suggested that, during ammonium oxidation, 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 would then be utilized for the generation of ATP and NAD(P)H. When hydroxylamine itself is a substrate, all four electrons can be used for energy production and molar growth yields on hydroxylamine should, therefore, be twice those on ammonium. Although it has been known for some time that *Nitrosomonas* spp. cells can oxidize hydroxylamine to nitrite, growth on hydroxylamine has only been recently reported. By careful, repetitive additions of small amounts of hydroxylamine (0.4 mM), it was possible to grow *Nitrosomonas* mixotrophically on ammonium and hydroxylamine in batch cultures (Chapter 4, this thesis). In line with predicted values, the molar growth yields on hydroxylamine (0.5 mg protein/mM) have been reported to be approximately twice those on ammonium (0.2 mg protein/mM) alone (Boettcher and Koops, 1994).

HAO has been purified and characterized in detail (Arciero and Hooper, 1993; Arciero *et al.*, 1993; Hendrich *et al.*, 1994). The enzyme is located in the periplasm and contains at least 7 heme-c and one heme P_{460}. HAO shares many properties with the "cytochrome *c*-552" dissimilatory nitrite reductases from *Escherichia coli*, *Wolinella* spp., *Desulfovibrio* spp. and *Vibrio* spp. These nitrite reductases catalyze the reduction of nitrite to ammonium, the reaction catalyzed by HAO have been mentioned to be a shortened version of the reversal of that reaction. The various similar properties have let to the speculation that the two enzymes might have a common evolutionary ancestor (Hooper *et al.*, 1991).

The effect of environmental factors on autotrophic nitrification has been studied extensively (e.g. Strenstrøm and Poduska, 1980, Painter and Loveless, 1983; Helder and de Vries, 1983; MacFarlane and Herbert, 1984) and dissolved oxygen has been identified as being of great importance. However, Abelovich has shown that some autotrophic nitrifiers can produce N_2O from NO_2^-. This will be discussed further below.

Carbon dioxide is the primary carbon source for these bacteria, and is assimilated via the Calvin cycle, also known as the ribulose bisphosphate carboxylase pathway (Nicholas, 1978). The fixation of CO_2 costs autotrophic bacteria about 80% of the energy generated by substrate oxidation (Kelly, 1978). It can be calculated (for growth yields 0.42 to 1.4 g dry weight per mol ammonium, and C-content of the biomass of 44%) that *Nitrosomonas* spp. must oxidize about 20-65 molecules of ammonium to provide sufficient energy for the fixation of one carbon atom (Drozd, 1980). Apart from their low growth yield, nitrifiers are also slow growers. The maximum growth rate in the exponential phase of *Nitrosomonas* spp. has been reported to be 0.08 h^{-1}.

Ammonia, rather than ammonium, is transported through the cell membrane and used by nitrifiers (Suzuki *et al.*, 1974). Consequently, at low pH values, less ammonia is available for growth. However, autotrophic bacteria are able to nitrify even below pH 4, and are found in high numbers in some forest soils. Heterotrophic bacteria isolated from the same acid soil showed no nitrifying ability (Stam *et al.* 1990), although nitrifying fungi have been suggested to be responsible for nitrite production in acid forest soils (Killham, 1986).

Heterotrophic nitrification

Heterotrophic nitrifiers oxidize a range of reduced nitrogen compounds, apparently without gaining energy for growth from the reaction (Focht & Verstraete 1977). They require an additional organic source and energy in order to do so. Oxidation of nitrogen compounds to hydroxylamine, hydroxamates, oximes, nitrite and nitrate is carried out by a variety of heterotrophic prokaryotes and eukaryotes, and probably serves different functions. (Verstraete and Alexander, 1972; Robertson and Kuenen, 1988). It has been observed in bacteria, fungi and algae, and even in cells from rat livers. There are at least two distinct pathways involved (Figure 3).

Inorganic pathway

$$NH_4^+ \longrightarrow NH_2OH \longrightarrow NOH \longrightarrow NO_2^- \longrightarrow NO_3^-$$

Inorganic pathway

$$R\text{-}NH_2 \longrightarrow R\text{-}NHOH \longrightarrow R\text{-}NO \longrightarrow R\text{-}NO_2 \longrightarrow NO_3^-$$

Figure 3 Organic and inorganic oxidation routes in heterotrophic nitrifiers

Three heterotrophic nitrifiers, *Alcaligenes faecalis*, *Arthrobacter* sp. and *Thiosphaera pantotropha* (closely related to *Paracoccus denitrificans*, Ludwig *et al.* 1993, and recently suggested to be transferred to *Pa. pantotropha*, Baker *et al.*, 1995) have been examined for their ability to generate energy when oxidizing nitrogenous compounds (Castignetti et al., 1990; Castignetti, 1990, Robertson and Kuenen, 1988). None of these bacteria were observed to translocate protons in a manner consistent with energy conservation.

Since energy appears to not be generated during heterotrophic nitrification, there must be another reason for the oxidation of nitrogen compounds. Verstraete (1975) suggested that heterotrophic nitrification is mainly involved in the conversion of organic nitrogen compounds into compounds with a specific function: iron-chelators, biocidal products or particular nitrogen compounds necessary for growth. *Arthrobacter* spp., for example (Verstraete and Alexander, 1973), produced chelating hydroxamates when iron concentrations were limiting for growth. Baghi and Kleiner (1991) found a novel enzyme, hydroxylamine dismutase, which generated ammonium and nitrite from hydroxylamine and hydrogen peroxide in the cyanobacterium *Phormidium uncinatum*. They suggested that this was part of a minor pathway and used for detoxification. In *Thiosphaera pantotropha* and a few other species, it seems that heterotrophic nitrification serves as a means of dumping excess reducing power (Robertson and Kuenen, 1990). The addition of hydroxylamine to nitrifying heterotrophs stimulates nitrification rates, and induces the expression of HAO. In some, it inhibits ammonium oxidation. The lower growth yields observed in nitrifying cultures could be explained by a mechanism which dissipated excess reducing equivalents via uncoupled electron transport, in order to generate $NAD(P)^+$ more rapidly. (Robertson and Kuenen, 1992; Wehrfritz *et al.*, 1993).

TABLE 1 Autotrophic and heterotrophic nitrification rates.

species	(nmol $N \cdot min^{-1} \cdot mg^{-1}$ dry weight)	component
Pseudomonas sp.	40-450	hydroxylamine
Thiosphaera pantotropha	35	ammonium
Alcaligenes faecalis	17-22	ammonium
Pseudomonas sp.	24	ammonium
Nitrosomonas sp.	130-1200	ammonium and hydroxylamine

Numbers taken from Jetten *et al.* 1997

Heterotrophic nitrification has been known for a long time, but was considered to be of little significance (Meiklejohn, 1940; Verstraete, 1975). The main reason was that there was no large accumulation of oxidation products (nitrite or nitrate). The observation that *T. pantotropha* was not only a heterotrophic nitrifier but also a aerobic denitrifer, which converted most of its oxidation product (nitrite) directly to gaseous nitrogen products, caused a reevaluation of the importance of heterotrophic nitrification (Robertson *et al.*, 1988, 1989, 1995).

Most of the heterotrophic nitrifying bacteria are also denitrifiers (Castignetti and Hollocher, 1984). When denitrification is taken into account (Table 1), heterotrophic nitrification levels can actually be much higher then previously believed. Although, heterotrophic nitrifiers generally oxidize nitrogenous compounds at rates substantially below those of the autotrophs (Focht & Verstraete, 1977; Van Niel *et al.*, 1993), with a few exceptions (Castignetti and Hollocher, 1984; Kuenen and Robertson, 1987), they can predominate in natural environments with abundant organic carbon. Their contribution to overall nitrification can in this case be significant. Other environmental conditions which are known to reduce the rate of nitrification by autotrophs, such as low of high pH (Focht & Verstraete, 1977) or low dissolved oxygen concentrations (Van Niel *et al.*, 1993) may also favour the less sensitive heterotrophic nitrifiers (Verhagen *et al.*, 1992)

A special group among the heterotrophic nitrifiers are the methanotrophic bacteria. They can oxidize a variety of substances in addition to their primary substrate CH_4, without being able to grow on them (Dalton, 1977; Bosse *et al.*, 1993). Among these substances is NH_4^+, which is oxidized by methane monooxygenase, the same enzyme that catalyses the first step in CH_4 oxidation (Bédard and Knowles 1989). In pure cultures of methanotrophs, NH_4^+ inhibited CH_4 oxidation (Whittenbury *et al.*, 1970; Bosse *et al.*, 1993).

In contrast, energy is conserved during nitrification by some, but not all, methylotrophs when oxidizing NH_3 to NO_2^- (Malashenko *et al.*, 1979 and Romanovskaya *et al.*, 1985). Why this group apparently differs from the rest of the heterotrophic nitrifiers in this respect has yet to be examined.

Anaerobic metabolism of aerobic nitrifiers

As mentioned above, both the ammonium oxidizers and the nitrite-oxidizing bacteria appear to have unexpected properties that have changed the classical picture of nitrification. It has been known for some time that during autotrophic nitrification, nitric and nitrous oxides can be released. As early as 1968, Hooper isolated a nitrite reductase

from *N. europaea* that produced NO from NO_2^-. Studies with labelled nitrogen have confirmed that *Nitrosomonas* spp. are able to use nitrite as an alternative electron acceptor to oxygen while producing NO, N_2O (Remde and Conrad, 1990; Poth and Focht, 1985) and N_2 (Poth 1986) *in vivo*. Although NO was generated by *N. europaea* and *Nitrosovibrio* sp. under aerobic conditions, N_2O was only produced in significant amounts after the culture became anaerobic.

Abeliovich (1987), found that both *Nitrosomonas* and *Nitrobacter* species are common in the anaerobic areas of waste water reservoirs, as well as in anaerobic settling ponds. It has since been shown that *N. europaea* was able to use nitrite as its electron acceptor under strictly anaerobic conditions, if pyruvate was provided as an energy source. The utilization of nitrite depended upon the supply of both pyruvate and ammonium. Pyruvate was essential for the incorporation of $^{14}CO_2$ into cell material, but was not itself used for cell material. It has been speculated that pyruvate might provide the energy needed by *Nitrosomonas* to survive anaerobic conditions (Abelovich and Vonshak, 1992)

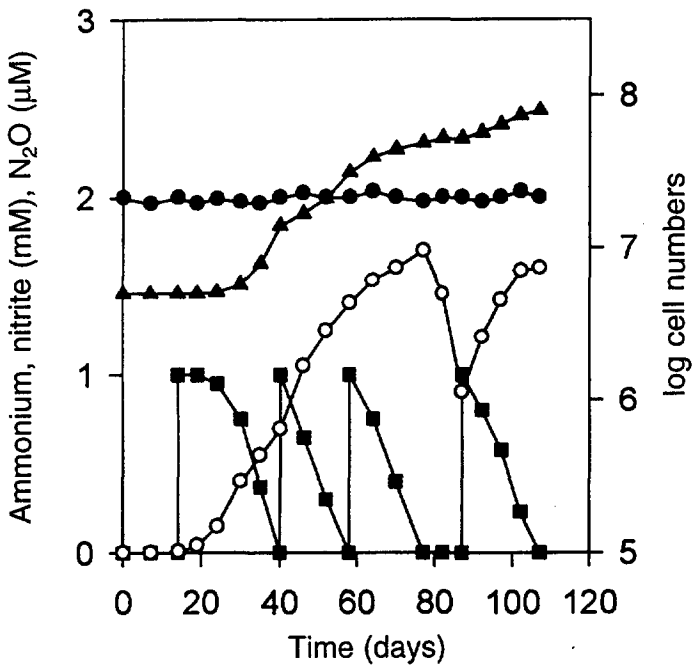

Figure 4 Anaerobic growth of Nitrosomonas on H_2 and NO_2^-
Symbols: ●, NH_4^+; ■, NO_2^-; ○, N_2O; ▲, cell numbers; hydrogen was supplemented together with each new nitrite addition; From Bock et al. (1995) Archives for Microbiology 163:16-20.

Recently, Bock *et al.* (1995) showed that pure and mixed cultures with *N. eutropha* were able to denitrify nitrite using hydrogen and ammonium as electron donors. Molecular hydrogen could serve as an electron donor for nitrite reduction by *N. eutropha* under anoxic conditions. Growth 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, even ammonium could serve as 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 growth was observed (Bock *et al.*, 1995).

Production of dinitrogen gas from ammonium at low oxygen concentrations 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 completely anaerobic conditions, ammonium conversion was not observed.

Furthermore, it has been shown that some nitrite oxidizers are not obligate autotrophs, but mixotrophs (Bock, 1976). Nitrite oxidizers are also capable of reducing nitrite and nitrate (denitrification) to dinitrogen. Some *Nitrobacter* spp. produced NO when grown mixotrophically under anaerobic conditions (Freitag *et al.*, 1987; Bock *et al.*, 1988) or at low oxygen tension (Bock *et al.*, 1992).

Denitrification

Denitrification is the process whereby nitrate and other nitrogen oxides are reduced to nitrogen gas (Knowles, 1982; Payne, 1981; Zumft 1992). The complete pathway follows several steps:

NO_3^- -> NO_2^- -> NO -> N_2O -> N_2

Although there is no evidence for other intermediates, hyponitrous acid (HON=NOH), nitramide (H_2NNO_2), imidonitric acid (HN=NOOH), and nitroxyl (NO^-), all at a formal oxidation state of +1, have been occasionally discussed. The N-N bond must be formed between NO and nitrous oxide, which might involve one of these compounds as an enzyme-bound intermediate (Zumft, 1979).

Denitrification is a facultative trait, in all of the bacteria thus far studied. For a time, it was believed that *Thiomicrospira denitrificans* was an obligate denitrifier, but it has now been shown to grow with oxygen as its electron acceptor, provided that the oxygen concentration is so low as to be limiting. Higher O_2 concentrations are inhibitory (Timmer

ten Hoor, 1975). Denitrification is carried out by a variety of respiratory bacteria that can use oxidized nitrogen compounds as electron acceptor instead of oxygen. It appears to proceed along more or less the universal biochemical pathways (Knowles, 1982). Interestingly, denitrification is even found among Archaea, as some of the halobacteria can denitrify. Denitrification has not been observed among the extreme thermophiles or the obligate anaerobic methanogenic bacteria (Zumft, 1992).

Denitrifying bacteria can use a wide range of organic compounds as their electron donor for energy generation. Some can also use sulfur compounds or H_2 (Zumft, 1992, Table 2). Reduced organic compounds generally serve as a source of carbon for the formation of biomass. During autotrophic growth on inorganic electron donors, inorganic carbon (CO_2) serves as the carbon source, usually via the Calvin cycle (Kuenen and Beudeker, 1982).

TABLE 2 Denitrification reactions with different electron acceptors.

organic C:	$2\ NO_3^- + CH_2O + 2\ H^+ \rightarrow N_2 + CO_2 + 2\ H_2O$
inorganic H:	$2\ NO_3^- + 5\ H_2 + 2\ H^+ \rightarrow N_2 + 6\ H_2O$
inorganic S:	$8\ NO_3^- + 5\ HS^- + 3\ H^+ \rightarrow 4\ N_2 + 5\ SO_4^{2-} + 4\ H_2O$

Denitrification often encompasses the complete set of reactions, starting with nitrate and terminating with N_2, but this coupling can be interrupted, as is evident from numerous observations of nitrite accumulation or the liberation of NO and N_2O from denitrifying cultures, and anaerobic growth on different nitrogen oxides, since some of the denitrifiers seem not to have all of enzymes (Zumft, 1992; Arts, 1995). The pathway is more an assembly of several, more or less independent processes, each converting an oxidized nitrogen compound into a more reduced form. Each reaction has a sufficiently large change of free energy to allow the build-up of a proton motive force and subsequent phosphorylation (Zumft, 1992). The yields obtained with denitrifying bacteria are generally at least 60 % lower than those found when the same species is grown aerobically. In many denitrifying bacteria, the presence of oxygen inhibits denitrification, but this is not always the case (Robertson and Kuenen, 1984).

Two types of denitrifying nitrate reductases are known. One is membrane-bound and the other is a soluble periplasmic enzyme. The presence of both denitrifying nitrate

reductases under similar conditions in one and the same organism raised questions about their respective roles, but it seems that the periplasmic enzyme is associated with aerobic denitrification (Bell *et al.,* 1990; Craske and Ferguson, 1986; Siddiqui *et al.*, 1993).
In its orginal meaning, the process of denitrification is the reduction of oxidized nitrogen species whereby dinitrogen is formed. In this way true denitrification, loss of nitrogen, occurs. Some nitrate reducers only produce nitrite (e.g. *Thiobacillus thioparus*) and hence are not strictly denitrifiers. Some of these may require re-checking as it has recently been found that a new isolate *Burkholderia pickettii* G9 has nitrate and N_2O reductases, but not the enzymes in between (Arts, 1995).

A key intermediate in the denitrification process is nitrite, which can react readily with secondary amines to form carcinogenic nitrosamines. Now two different types of denitrifying nitrite reductase have been found. Approximately one third of know denitrifiers possess a copper-containing nitrite reductase, others have a haem cd_1-containing enzyme. These enzymes appear to be mutually exclusive. The product of nitrite reduction is NO.

For long time, the role of NO as an intermediate in the formation of N-N bond of nitrous oxide by reducing two nitrite ions has been questioned (Averill and Tiedje, 1982). However, there is now direct evidence that NO is a kinetically-competent intermediate. NO is probably strongly bound to the enzyme, and only small amounts are freely detectable. Studies involving NO are not helped by its chemical reactivity. Its steady-state concentration varies between 1-65 nM (Goretski et al. 1990), depending on the species.

Several denitrifiers are able to grow anaerobically with N_2O as the sole electron acceptor as a consequence of the coupling to oxidative phosphorylation (Stouthamer 1980). N_2O reductase is generally O_2, H_2S and temperature sensitive, and like the other nitrogen oxide reductases is derepressed in some denitrifiers simply by anoxia (Knowles, 1982). Of the N_2O reduction inhibitors, only acetylene is considered specific for the activity of this enzyme during denitrification (Yoshinari and Knowles, 1976), while sulphide inhibits both NO and N_2O reductase (Sørensen *et al.*, 1980). N_2O reductase differs from nitrogenase (which can also reduce N_2O to N_2) in that it cannot reduce acetylene.

Aerobic denitrification

Molecular oxygen has been known to repress denitrification enzymes in many bacteria. However, over the last 100 years, there have been sporadic reports of aerobic denitrification. Because of technical problems (e.g. accurate O_2 and N_2 measurements and mixing requirements), it has only recently been possible to confirm using *T. pantotropha* in continuous cultures, that aerobic denitrification occurs. *T. pantotropha* has been observed to produce gas from nitrate or nitrite, even in the presence of high dissolved

oxygen concentrations (Robertson & Kuenen 1983, 1984, 1990).

As mentioned above, *T. pantotropha* combines heterotrophic nitrification and aerobic denitrification, converting NH_4^+ directly to gaseous nitrogen products in the presence of an organic substrate (Robertson et al., 1988, 1989). Other aerobic denitrifiers have been shown to be common in natural samples (e.g. Carter *et al.*, 1995).

Over the years, it has became apparent that *T. pantotropha* has gradually lost must of its aerobic denitrifying activity (Kuenen and Robertson, 1994; Ferguson, 1994; Richardson and Ferguson, 1992). Tests in batch culture with ^{15}N-labelled compounds confirmed that current *T. pantotropha* cultures made N_2 and N_2O from ammonium and/or nitrite and nitrate, although at a rate about 10% of the originally reported values (Robertson *et al.* 1988, 1995). Other heterotrophic nitrifiers produced N_2 under the same conditions (van Niel *et al.*, 1992, Robertson *et al.*, 1995). Continuous culture experiments with ^{15}N-compounds and on-line mass spectrometry confirmed that *T. pantotropha* generates N_2 as well as N_2O in well-mixed fully aerobic chemostat cultures (Arts *et al.*, 1995).

Nitrogen removal from waste water

Human activities, both industrial and agricultural, have strongly increased the amount of biologically-active nitrogen compounds in the environment, thereby disturbing the balance of the natural nitrogen cycle. A major environmental problem is the removal of nitrogen from waste water, dominated by two molecular species: ammonium and nitrate (Robertson and Kuenen, 1992; Cole 1993). The removal of nitrogen is accomplished by making use of the processes of the natural nitrogen cycle (see Figure 1). A nitrification step where ammonium is oxidixed to nitrate in an aerated reactor, followed by (post-) denitrification of the nitrate. In this second step, the addition of an electron donor, for example methanol, is necessary. If the waste water contains a high amount of organic material, this must be removed before the nitrification, otherwise the nitrifiers will be overgrown.

Traditional waste water treatment systems by means of actived sludge are not ideal for nitrification. Autotrophic nitrifying bacteria grow slowly, and retention times must therefore be long. Biomass wash-out must be small compared to the biomass concentration in the reactor, and this can only be achieved in large volume reactors. Active sludge processes produce a large amount of surplus sludge. Since intensive cattle farming has produced excess manure, and use of chemical fertilizers has also increased, agricultural land is no longer an instant distrubution point for sludge disposal. It is clear

that minimization of sludge production is necessary. This demand is naturally met in anaerobic waste water treatment systems (Mergaert *et al.*, 1992).

In practice for aerobic processes, minimization of sludge production means an increase in reactor volume and required settler area. Another possibility is lower sludge loading so that more energy is dissipated for maintenance of the microorganisms. The major drawbacks of the activated sludge process are the low biomass concentration and the need for large reactors. Consequently, a number of reactor systems have been developed to deal with these disadvantages (Table 3). These systems involve the use of new reactor configurations, special microbial processes or a combination of the two.

TABLE 3 Biomass concentration and conversion capacities for different types of reactors.

	Biomass g/l	Area	Nitrification capacity kg $N \cdot m^{-3} \cdot d^{-1}$	Limitations
Active sludge system	2-5	-	0.1	Biomass concentration
Trickling filter	5	100	0.1	Specific Area
Fluidized bed reactor	30	3000	3.5	Water circulation
BAS reactor[1]	30	3000	6.0	Oxygen transfer

[1] BAS Biofim airlift suspension reactor is essentially a fluidized bed reactor that can be aerated directly. The addition of a draft tube created a three-phase airlift system (Heijnen, 1990; Tijhuis *et al.*, 1995).

Industrial waste water treatment

A major effort has been spent on increasing the conversion capacity, decreasing the area/volume ratio and reducting excess sludge production in waste water treatment processes by using immobilised biomass. Some research has been focussed on combining nitrification and denitrification in a single reactor, others on linking the conventional processes in a more efficient way, or optimilizing reactor performance.

Immobilizing bacteria in biofilms results in increased sludge concentrations in the reactor, and consequently a significant reduction of the reactor necessary volume. This advantage can only be obtained for aerobic processes if the biofilm surface area-volume ratio is large enough to prevent oxygen limitation in the biofilm (Table 3).

In biofilms, the different physiological groups can be separated in layers around the carrier material, although these layers usually overlap and interact. If waste water is rich in organic material, slow-growing organisms such as nitrifiers cannot compete with the faster-growing heterotrophs for surface area and other nutrients such as oxygen (van Loosdrecht, 1995; Ohashi *et al.*, 1996). Treatment of this type of waste water thus requires two separate reactors, one to remove the organic material and one for the nitrification step.

In a fluidized bed system, the biofilms (biocatalyts) are grown on a carrier. This can be sand or basalt. The reactors are tall cylinders in which the upward flow of liquid (recycle stream) keeps the biocatalyst suspended and mixed. For aerobic processes, the reactor contents can be aerated by oxygenation of the recycle stream.

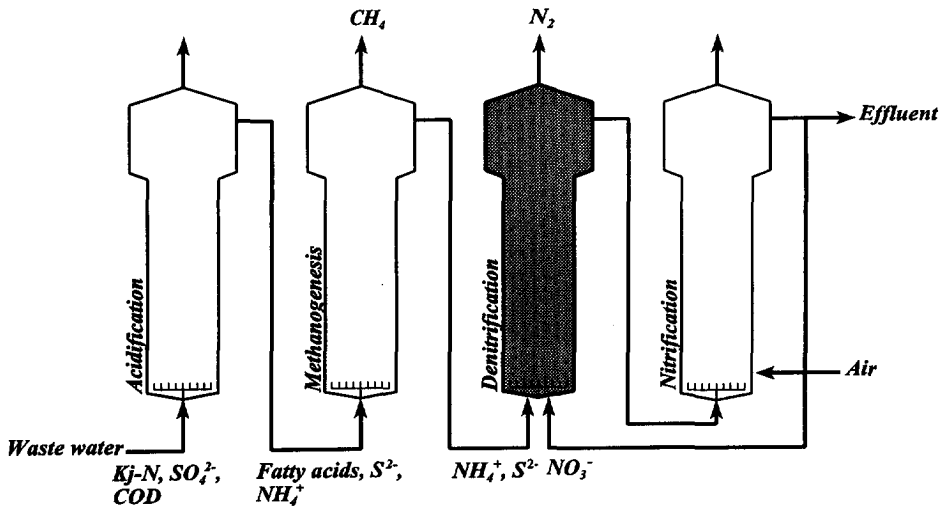

Figure 5. Multi-stage system for treatment of waste water as described in EPA 0051.888.

A multi-stage system, developed at Gist-brocades in Delft (patented EPA 0051 888, Mulder 1982) is schematically shown in Figure 5. This system involves two UASB reactors, a fluidized bed and an airlift reactor. In the first reactor organic material in the waste water is acidified by anaerobic, acetogenic bacteria to produce simple organic compounds, e.g. acetate. In the second anaerobic reactor, these simple compounds are

utilized by methanogens and sulphate-reducing bacteria, and converted to sulphide, methane and carbon dioxide. The sulphide and surplus organic material is used as the electron donor for denitrification in the third reactor. Nitrogen compounds, almost exclusively ammonium, are biologically converted to nitrate in the final, aerobic, nitrification reactor. Most of the nitrate-containing water is then recirculated, mixed with the sulphide-containing effluent from the methanogenic reactor, and treated in the denitrification reactor. It is clear that such a process has the advantages of its simultaneous removal of nitrate (denitrified to nitrogen gas) and conversion of sulphide to sulphate, as well as the fact that the four tubular reactors take up much less land.

Anaerobic processes with methane

Under aerobic conditions ammonium and methane oxidation are similar to the extent that the enzyme responsible for the oxidation of ammonium can oxidize methane, and *visa versa* (Bédard and Knowles 1989). Furthermore, methanotrophic bacteria can also fix dinitrogen (Davis *et al.*, 1964; De Bont, 1976). Methylotrophs, such as *Hyphomicrobium spp.*, are able to denitrify with methanol as their substrate (Sperl and Hoare, 1971). *Methylosinus trichosporium* produces N_2O but at lower rates than in *Nitrosomonas* (Yoshinari, 1985).

Anaerobic methane oxidation

Theoretically, nitrate is a possible oxidant of methane (Table 4). The change in free energy with nitrate as an oxidant is almost equivalent to the change in free energy with oxygen as an oxidant. The energy yield for sulphate is less favorable than for oxygen and nitrate (Thauer, 1977).

TABLE 4 Methane oxidation reactions with different electron acceptors.

Reaction	ΔG^{o1} (kJ/mol CH_4)
$5\ CH_4 + 8\ NO_3^- + 8\ H^+ \rightarrow 5\ CO_2 + 14\ H_2O + 4\ N_2$	- 701.1
$CH_4 + 2\ O_2 \rightarrow CO_2 + 2\ H_2O$	- 778.9
$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + 2\ H_2O + HS^-$	- 13.0

○ *Nitrate as electron acceptor*

At first glance, methane should be an attractive substrate for denitrification (Harremoes and Henze-Christensen, 1971), because it is produced by the anaerobic digestion processes in many treatment plants, and hence is available on-site. There have been reports of anaerobic methane oxidation. Pretorius (1972) and Davies (1973) reported that pure cultures of methanotrophic bacteria were able to denitrify, while utilizing methane as sole carbon energy substrate, but this work was very largely discredited by Mason (1977). There is currently no evidence that methane can be oxidized by nitrate (Mason 1977, Panganiban 1979), leave alone that it would be a practical subtrate for denitrification in waste water treatment (Hamer and Mechsner, 1984).

○ *Sulphate as electron acceptor*

The involvement of sulphate in anaerobic methane oxidation is strongly suggested by coinciding peaks of sulphate reduction and methane oxidation in saltwater environments (Iversen and Jørgensen, 1985; Devol and Ahmed, 1981; Iversen and Blackburn, 1981). In the sulphate-methane transition zone, methane could account for as much as 89% of the total electron donor requirement for sulphate reduction, and could be the primary electron donor in this zone (Iversen and Jørgensen, 1985). Direct evidence for the involvement of sulphate in anaerobic methane oxidation, in the form of a pure culture of sulphate-reducing methanotrophs is not yet available. *Desulfovibrio desulfuricans* can co-oxidize small amounts of methane when grown on lactate and sulphate(Davis and Yarborough, 1966), but cannot grow with methane as its sole carbon and energy source. Methane was oxidized to CO_2, rather than being assimilated, in anoxic lake water samples (Panganiban *et al.*, 1979).

In freshwater environments, anaerobic methane oxidation seems to play a minor role. Methanogens have been shown to reoxidize a small amount (0.001%) of the methane they have produced (Zehnder and Brock, 1979). Zehnder and Brock (1980) showed that eight different strains of methanogenic bacteria could oxidize methane anoxically, and suggested that these bacteria are responsible for methane oxidation in anoxic sediments and sewage sludge. The addition of different electron acceptors (e.g. sulphate or nitrate) did not stimulate the reaction, but on the other hand, enrichment cultures did not oxidize methane without added sulphate.

○ *Carbon dioxide as electron acceptor*

Another example that methane is not completely inert under anaerobic conditions, and that some biochemical mechanism for anaerobic methane oxidation might exist, was shown by Zehnder and Brock (1979). Another electron acceptor in quantities available

under methanogenic conditions is CO_2. Acetate formation from bicarbonate and methane is, under standard conditions at pH 7, an endergonic process.

$CH_4 + HCO_3^- \rightarrow CH_3COO^- + H_2O$ $\Delta G^{o1} = +28.0$ kJ/mol CH_4

However, in natural habitats such as anaerobic sediments, the high concentrations of bicarbonate and methane and the low concentration of acetate, shift this reaction energetically into a more favorable range. A process similar to that demonstrated for interspecies hydrogen transfer can be postulated.

Anaerobic and aerobic processes with ammonium

As mentioned, the removal of ammonium-N from waste water is conventionally a two step process, with nitrification and denitrification in separate reactors, but new combinations of nitrogen oxidation and reduction reactions are being investigated.

Aerobic denitrification and heterotrophic nitrification

Heterotrophic nitrification rates of several species (Robertson *et al.*, 1988, 1989) have been shown to be considerably higher than previously believed. Combined with the ability to denitrify aerobically, this seemed a promising concept for single-stage nitrogen removal. The heterotrophic nitrifier *T. pantotropha*, was able to outcompete the autotrophic nitrifier, *N. europaea*, for ammonium at low dissolved oxygen concentrations and at high C:N ratios (van Niel *et al.*, 1993). However, a substantial amount of the ammonium was assimilated, reflecting the fact that heterotrophs give much higher biomass yields. Higher nitrogen assimilation corresponds with higher sludge production, making this process less attractive for the use in the new generation of waste water treatment systems.

Incomplete nitrification

Complete nitrification requires a large amount of oxygen, which requires energy, and denitrification requires the use of additional electron donor. If the end-product of nitrification was nitrite rather than nitrate, this would reduce both the oxygen demand and the amount of substrate needed for denitrification (Rahmani et al., 1995).

Nitrite does often accumulate in bioreactors in significant amounts, but a stable nitrification process terminating in nitrite has thus far been difficult to achieve. Only at higher temperatures, nitrite-oxidizing bacteria appear to be at disadvantage (Hunik, 1994), but heating large volumes of waste water is impractical. At elevated temperatures (30-35°C), waste water with a relatively high nitrogen content can be operated without sludge retention. In a stirred tank reactor, nitrite-oxidizing bacteria can be washed out,

by adjusting the dilution rate to values less than 1 to 1.2 per day. The SHARON process is based on these principles (Brouwer, 1995). This process appears to be attractive for the treatment of sludge digestion (rejection) water which is low in organics, relatively rich in ammonium, and is already at the appropriate temperature. Nitrite produced in the SHARON process is subsequently denitrified with added methanol.

Anaerobic ammonium oxidation

On the basis of thermodynamic calculations, Broda (1977) predicted that two lithoautotrophic (chemo- and photo-) bacteria capable of generating dinitrogen directly from ammonium should exist (Table 5). For autotrophic growth, the production of ATP and consequently a negative free energy change is a precondition.

TABLE 5 Ammonium oxidation reactions with different electron acceptors.

Reaction		ΔG^{o1} (kJ/mol NH_4^+)
$NH_4^+ + NO_2^-$	$\rightarrow N_2 + 2\ H_2O$	- 358
$5\ NH_4^+ + 3\ NO_3^-$	$\rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+$	- 297 chemotroph
$8\ NH_4^+ + 6\ O_2$	$\rightarrow 4\ N_2 + 12\ H_2O + 8\ H^+$	- 315
$1.3\ NH_4^+ + CO_2$	$\rightarrow 0.65\ N_2 + CH2O + H_2O + 1.3\ H^+$	+ 39 phototroph

○ Carbon dioxide as electron acceptor

In addition to the "missing" organisms predicted by Broda which should be able to oxidize ammonium with CO_2 phototrophically, Bhadra *et al.* (1987) claimed to have found another possible form of anaerobic ammonium oxidation. From nitrogen balances in their reactor, the authors deduced that ammonium supplied hydrogen for the reduction of carbon dioxide, resulting in increased methane generation. The reaction was postulated to be as follows:

$8\ NH_4^+ + 3\ HCO_3^- \text{--> } 4\ N_2 + 3\ CH_4 + 9\ H_2O + 5\ H^+ \quad \Delta G^o = -75.3$ kJ/mol.

As far as we know, further evidence regarding this hypothesis has not yet been published.

○ Nitrate, nitrite as electron acceptor

In 1932, it was reported (Allgeier *et al.*) that nitrogen gas generated during fermentation in Lake Mendota sediments must be produced by an unknown mechanism, but this was never verified. Brezonik (1966) rejected the idea that ammonium was a

potential source for nitrogen gas production, and emphasised that no other mechanisms than bacterial denitrification provided dinitrogen gas. However, several earlier workers had reported the concept that denitrification was not able to account for all the nitrogen gas formed during sludge digestion. Although, Malina's observations in 1961 (mentioned in Brezonik 1966) could be ascribed to small amounts of air entering the digestion apparatus, Koyama (1965) found indications of the direct formation of dinitrogen gas from ammonium in Lake Kizaki-ko in Japan (on the basis of missing carbon dioxide), and proposed that the reaction shown in Table 4 (reaction 2 or 3) took place. However, none of these findings were considered to be strong evidence for the direct conversion of ammonium into nitrogen gas.

Apart from these reports of unexplainable N_2 production, no other evidence on the anaerobic removal of ammonium by nitrate reduction became available for some time. It has recently been reported (Tanimoto *et al.*, 1992) that under denitrifying conditions the fungus *Fusarium oxysporum* could combine nitrogen compounds such as azide, salicylhydroxamic acid, and possibly ammonium ions with another nitrogen atom from nitrite to form the hybrid N_2O. Although the results with ammonium ions were not conclusive. Others (Jørgensen *et al.*, 1991, Murray *et al.*, 1989) reported that the chemical zonation of the upper few hundred meters of the central Black Sea waters has changed significantly over the last decades. There is now a 20-30 m deep intermediate layer of anoxic and H_2S free water overlying the sulphide zone. In this intermediate layer, the NO_3^- and NH_4^+ profiles overlapped, suggesting that anaerobic ammonium oxidation might take place. It is not surprising that, if anaerobic ammonium oxidation exists, it would be observed in sediments or lakes. Two giga-years ago when oxygen began to appear in the biosphere on earth, as a result of photosynthesis, nitrifiers were able to develop and to produce nitrite and nitrate. Sediments or lakes are typical surroundings where oxygen diffusion limits nitrification. At the aerobic - anaerobic interface, the ideal niche for the existence of anaerobic ammonium oxidizers would exist.

Outline of this thesis

During experiments on the pilot plant of the multi-stage system at Gist-brocades (Figure 5), a new process for nitrogen removal was discovered and patented (EP 0 327 184 A1, Mulder, 1989). Ammonium disappeared at the expense of nitrate. The aim of the studies presented in this thesis was to increase the understanding of this process, in which ammonium is oxidized under anaerobic conditions.

Chapter 2 describes the first observations of ammonium conversion under anaerobic

conditions. Redox balances and batch experiments were conducted to confirm the reactions. That a biological process, rather than a chemical one, was responsible for the reaction is addresed in Chapter 3. Different ways to inhibit the biological activity were investigated. Special care was taken to exclude the role of oxygen in the conversion reaction. ^{15}N-labelled ammonium was used to establish the major end product of anaerobic ammonium oxidation.

It has been known for sometime that aerobic nitrifiers were capable of existence in anaerobic environments, and that *Nitrosomonas* cells are able to denitrify under oxygen limited conditions, although growth without oxygen was never measured. Since aerobic nitrifying potential was detected in the sludge from the anaerobic ammonium oxidation reactor, it was decided to examine this capability in some detail, using hydroxylamine, rather than ammonium as substrate. To this end, a well-characterized ammonium oxidizer, *Nitrosomonas europaea,* was used as model organism. Continuous cultures of *Nitrosomonas* on hydroxylamine and ammonium were grown aerobically and then made anaerobic to examine their denitrification potential. The results are discussed in Chapter 4.

Chapter 5 describes the development of autotrophic mineral medium which was used to grow and enrich for the microorganisms capable of converting ammonium under anaerobic conditions. The enrichment culture obtained on this medium has been characterised using different techniques.

Finally, in Chapter 6, a hypothetical metabolic pathway for anaerobic ammonium oxidation is proposed. Making use of continuous experiments in the fluidized bed system, the enrichment culture was investigated using ^{15}N mass spectrometry. These results have provided the basis for a new hypothetical metabolic pathway for anaerobic ammonium oxidation.

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Chapter 2

Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor

Abstract

Until now, oxidation of ammonium has only been known to proceed under aerobic conditions. Recently, we observed that NH_4^+ was disappearing from a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor. Both nitrate and ammonium consumption increased with concomitant gas production. A maximum ammonium removal rate of 0.4 kg N m^{-3} d^{-1} (1.2 mM h^{-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 mol ammonium, 3 mol nitrate were required, resulting in the formation of 4 mol dinitrogen gas. Subsequent batch experiments confirmed that the NH_4^+ conversion was nitrate dependent. 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), and has been patented.

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FEMS Microbiology Ecology **16** (1995) 177-184

Introduction

As nitrogen pollution has become a greater cause for concern in recent years, techniques for reducing the nitrogen content of both drinking water and waste water have attracted a great deal of attention. Most research has concentrated on attempts to improve the classical nitrification and denitrification processes.

Nitrification, frequently combined with denitrification, is the most widely used method for nitrogen control in waste water treatment (Johnson and Schroepfer, 1964; Henze Christensen and Harremoes, 1978; McCarty *et al.*, 1969). Nitrification is generally carried out by aerobic, autotrophic bacteria that oxidize NH_4^+ to NO_2^-, and NO_2^- to NO_3^-, with molecular oxygen as electron acceptor. To be fully effective, these bacterial conversions require a very efficient oxygen supply (Helder en de Vries, 1983). NO_2^- and NO_3^- are subsequently reduced to N_2 by denitrifying bacteria that use the NO_x^- as alternative electron acceptors to oxygen, and are most effective in the absence of oxygen (Knowles, 1982; Stouthamer, 1988; Zumft, 1992). The situation is further complicated, because the autotrophic nitrifiers cannot compete with aerobic heterotrophs for oxygen and other nutrients in the presence of substantial amounts of organic compounds (van Niel *et al.*, 1993) and can therefore easily be overgrown by the heterotrophs. An additional complication is that the denitrifying bacteria must be provided with a suitable electron donor, usually organic compounds.

The different requirements of nitrifiers and denitrifiers have led to a number of reactor combinations for the removal of nitrogen from waste water. The combined nitrification/denitrification process (single-sludge system) can be distinguished from a system where nitrification and denitrification are carried out by two separated sludges (dual-sludge system) (Böhnke and Pinnekamp, 1986). In the single-sludge system, nitrification and denitrification are achieved by alternating aerobic and anaerobic zones. The dual-sludge system uses separate nitrification and denitrification reactors. If the nitrification stage follows denitrification, recirculation of nitrified waste water is required (Mulder *et al.*, 1986). When nitrification precedes denitrification, the addition of an external electron donor (such as hydrogen, methanol, sulphur and sulphide, see Table 1) is necessary (Driscoll and Bisogni, 1978; Kurt *et al.*, 1987; McCarty *et al.*, 1969).

In theory, ammonium can also be used as an inorganic electron donor for denitrification (Table 1, equation 3). The free energy balance for this reaction is nearly as favourable as in the aerobic nitrification process (Table 1, equation 4). 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 ammonium to dinitrogen with nitrate, carbon dioxide or oxygen as oxidant. These

predictions were based on thermodynamic calculations, but the existence of these micro-organisms has never been demonstrated.

TABLE 1 Gibbs free energy of some reactions involved in denitrification and ammonium oxidation (from Thauer *et al.*, 1977).

equation no.	reaction	$\Delta G^{o'}$
1	$2\ NO_3^- + 5\ H_2 + 2\ H^+ \rightarrow N_2 + 6\ H_2O$	- 1121 kJ/reaction
2	$8\ NO_3^- + 5\ HS^- + 3\ H^+ \rightarrow 4\ N_2 + 5\ SO_4^{2-} + 4\ H_2O$	- 3721 kJ/reaction
3	$3\ NO_3^- + 5\ NH_4^+ \rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+$	- 297 kJ/mol NH_4^+
4	$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2\ H^+$	- 349 kJ/mol NH_4^+
5	$8\ NH_4^+ + 6\ O_2 \rightarrow 4\ N_2 + 12\ H_2O + 8\ H^+$	- 315 kJ/mol NH_4^+

This paper describes the discovery of an anaerobic process in which ammonium was used as electron donor for denitrification, following an earlier preliminary report (van de Graaf *et al.*, 1990).

Materials and Methods

Operating conditions of the denitrifying fluidized bed reactor

A denitrifying microbial population was grown in a glass, 23 l, fluidized bed reactor, at 36°C and pH 7. Degassed anoxic liquid from the top of the reactor was recirculated to boost the flow to approximately 255 l h^{-1} in order to keep the bed fluidized at a superficial liquid velocity of 30 - 34 m h^{-1}. The hydraulic retention time was 4.2 h. The influent of the denitrifying reactor, supplied at a rate of 5 - 6 l h^{-1}, came from a methanogenic reactor that was operated with waste water from a bakers yeast production plant, and contained (mg l^{-1}): COD, 550 - 750; TOC, 165 -190; SO_4^{2-}, 25 - 150; S^{2-}, 90 - 130; NH_4^+-N, 90 - 130. Nitrate solution (75 g $NaNO_3$ l^{-1}) was separately supplied at a rate of about 450 ml h^{-1}. The redox-potential was measured continuously with a platinum electrode and an Ag/AgCl reference electrode (E ref = +230 mV at 36°C; Elektrofact SR20/AP24, Elscolap, Maarsenbroek, The Netherlands). The redox-potential of the process water was maintained at 150 - 250 mV by controlling the waste water supply. All tubing and connectors were of butyl rubber or polyvinylchloride (PVC) to limit oxygen diffusion. For the same reason, the 5 l settler at the top of the reactor was flushed with N_2.

Sand particles (diameter 0.3-0.6 mm) were the carrier material of the fluidized bed on which bacteria grew as a biofilm at a biomass concentration of 150-300 mg Volatile Suspended Solids (VSS) $(g_{carrier})^{-1}$ (equivalent to 14 g VSS l^{-1}). The reactor was inoculated with a small amount of

sand covered with denitrifying biofilms, originating from previous experiments. This resulted in fast growth of the fluidized bed, which reached a volume of 13 l after a period of four weeks. The fluidized bed height was maintained at a constant level by the removal of biofilm-covered sand from the top of the bed and the addition of clean sand.

Samples of the influent and effluent of the denitrifying reactor were taken under refrigeration over a period of 18 h, twice a week, and analyzed for ammonium, nitrate, nitrite and sulphate. The influent samples were also analyzed for COD, TOC and volatile fatty acids.

Fed-batch experiments

Biomass from the denitrifying fluidized bed reactor was transferred to a temperature-controlled (36° C), intermittently-stirred reactor with a volume of 2.4 l for fed-batch experiments. This biomass sample contained 252.6 g sand particles and 40.1 g biomass (VSS), corresponding to a biofilm coverage of 159 mg VSS $(g_{carrier})^{-1}$. The experiment was started by the addition of 4.0 g NH_4NO_3, followed by three successive additions of 5.3, 3.3, and 2.3 g $NaNO_3$ after 123, 341 and 557 hours, respectively. The concentrations of NH_4^+, NO_3^-, and NO_2^-, and the gas production, and SO_4^{2-} reduction levels were all monitored.

Analytical methods

Nitrate, nitrite and sulphate were determined by HPLC with a conductance detector (Millipore Waters Model 430, Millipore Waters, Ettenleur, The Netherlands). The ammonium content was measured by distillation of ammonia, followed by absorption and titration (*Standard Methods* APHA, 1981). The COD of untreated samples and supernatant of centrifuged samples were determined by titration of the amount of potassium dichromate oxidized in two hours (*Standard Methods* APHA, 1981). The TOC was determined with a TOC-sin II aqueous carbon analyzer (Phase Separations). The volatile fatty acids were determined with a GC (Perkin-Elmer 1B,Perkin-Elmer, Gouda, The Netherlands) equipped with a 1.8 m x 2 mm glass column (15% SP 1220/ 1% H_3PO_4 on Chromsorb WAW 100-120 mesh, Supelchem, Leusden, The Netherlands) and a FID-detector. The attached biomass was analyzed for the content of volatile suspended solids (*Standard Methods* APHA, 1981).

The gas produced in the reactor was collected by a funnel at the top, and measured with a wet-type laboratory gas meter (Schlumberger, type 1, Schlumberger, Dordrecht, The Netherlands). The gas was sampled weekly, using 60 ml polypropylene syringes. CH_4, N_2, CO_2, N_2O (detection limit 65 ppm) and O_2 were measured with a GC equipped with a 500 x 0.3 cm glass column (Porapak Q, 80-100 mesh, Chrompack, Rozendaal, The Netherlands) and a thermal conductivity detector, operated at 0° C and 110° C, respectively. Gas production during the fed-batch experiments was measured by the liquid displacement method (Mariotte bottle). At the pH values used in this work, both ammonium and ammonia would be present. Ammonium will therefore be used to represent both forms.

Results & Discussion

Performance of the denitrifying fluidized bed reactor

The steady-state data for the influent, effluent and reactor performance are summarized in Table 2. Sulphide and organic acids were the major electron donors. The gas production rates are directly related to the conversion rates in the reactor, and can be used as a parameter for the control of the process performance. The sharp declines in the gas production rates (Figure 1) were caused by regular interruptions in the flow of the feed.

TABLE 2 Performance of the denitrifying reactor before and after the onset of anaerobic ammonium oxidation.

	Before	After
Gas production (ml $(l_{feed})^{-1}$)	80	200
Sulphate production (mg l^{-1})	400	375
VFA oxidation (mg COD l^{-1})	20-40	25-45
TOC removal (mg TOC l^{-1})	15-40	20-50
NO_3^- in effluent (mg N l^{-1})	160-200	40-120
NO_2^- in effluent (mg N l^{-1})	25	0-12
NH_4^+ "lost" (mg N l^{-1})	0	60-100
N-removal rate (kg N m^{-3} d^{-1})	0.7	1.5
pH	7.2	6.9

VFA = volatile fatty acids; TOC = total organic carbon

The average amount of biomass leaving the reactor after the bed had fully grown was only 10 ± 5 mg VSS $(l_{waste\ water})^{-1}$. This corresponds to a biomass yield of approximately 0.04 g $COD_{biomass}$ (g $COD_{removed})^{-1}$. The redox balances (Table 3) show that the ratio between electron donor and electron acceptor consumed was 1.1, indicating that only 10% of the electron donor had been used for biomass production. This relatively low biomass production in sulphide-containing waste water has been previously described (Mulder *et al.*, 1986; Gommers *et al.*, 1988) and is three times lower than commonly observed for organic media (McCarty *et al.*, 1969).

Occurrence of ammonium removal

During the first period (0 - 420 days) of the run, the ammonium concentration in the influent of the denitrifying fluidized bed reactor was the same as in the effluent. In the second period (420 - 560 days), it became apparent that the ammonium concentration in the effluent was steadily decreasing (Figure 2). By the end of the experiment, almost all of the ammonium was converted. The most obvious explanation for this ammonium removal, the occurrence of aerobic nitrification resulting in nitrate or nitrite production, seemed unlikely because these concentrations in the effluent had also decreased (Table

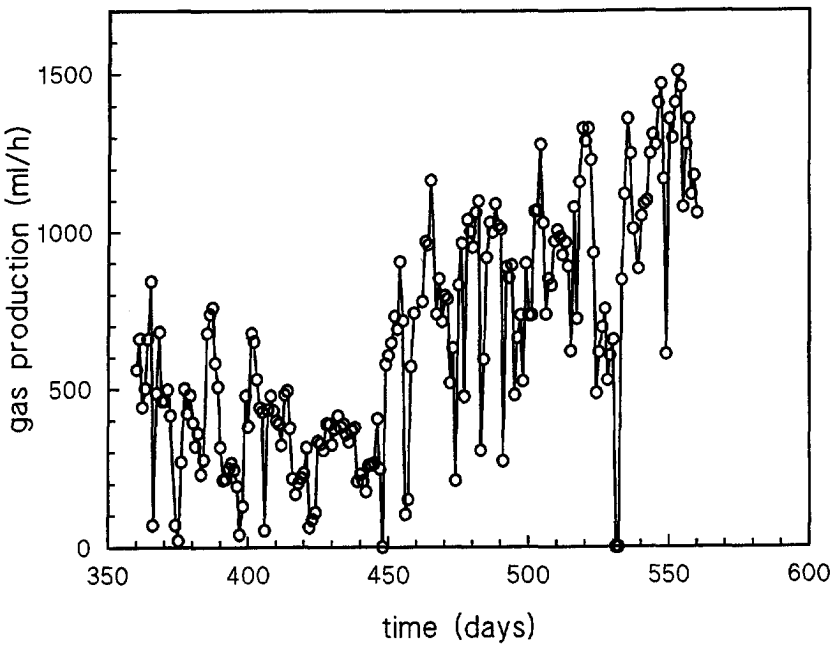

Figure 1 Effect of the occurrence of anaerobic ammonium oxidation on day 420 on the gas production by the denitrifying fluidized bed reactor.
Day 350 - 420: the last part of the first period. Day 420 - 560: the second period of the run, when ammonium conversion and additional nitrate reduction increased simultaneously, causing increase in gas production. The average gas composition was (v/v) 68 - 72% N_2, 15 - 18% CH_4, 13-18% CO_2. N_2O was below the detection limit of 65 ppm.

2). In addition, the observed ammonium removal rate of 0.4 kg N m^{-3} d^{-1} would have required an oxygen supply corresponding to 1.8 kg O_2 m^{-3} d^{-1}. This was very unlikely in view of the precautions taken (see Material and Methods). Moreover, oxygen could not be detected in the gas samples. During the second period, the gas production and nitrate consumption rates also increased, and the pH fell (Table 2). The NH_4^+ deficit cannot simply be explained by incorporation in biomass, because biomass production remained stable throughout the experiment (Table 2). These factors, taken together, all indicate that the most likely explanation of the ammonium conversion is the reaction given in Table 1, equation 3: anaerobic ammonium oxidation to N_2.

The redox balances shown in Table 3 provided further support for the occurrence of anaerobic denitrification with ammonium as the electron donor. When a comparison was made for nitrate consumption before and after ammonium conversion began, it was clear that in the first period, there was good agreement between the calculated and measured amounts of nitrate utilization. Once the NH_4^+ concentration began falling, the calculated

and measured NO_3^- consumptions could only be reconciled if the stoichiometric amount of NO_3^- required to oxidize the NH_4^+ was included.

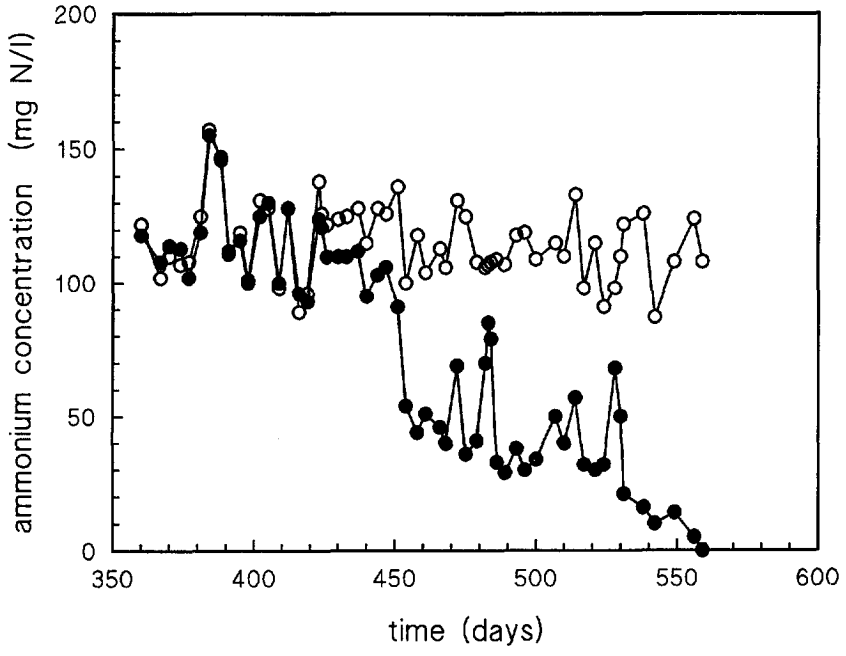

Figure 2 Ammonium concentration in the influent (-o-) and the effluent (-●-) of the denitrifying fluidized bed reactor before and after the onset of anaerobic ammonium disappearance on day 420.

TABLE 3 Nitrogen and redox balances for the denitrifying fluidized bed reactor in which Anammox occurred. (I) before Anammox, (II) after Anammox appeared (all concentrations as mg N per litre). The redox reaction were assumed to proceed according to the equation 1, 2, and 3 in Table 1.

	Amount of NO_3^- required for			Total NO_3^- required		Measured NO_3^-
	SO_4^{2-} formation	Organic carbon removal	NH_4^+ oxidation	without	with Anammox	consumption
(I)	92	21	0	113	113	100
(II)	80	27	48	107	155	150

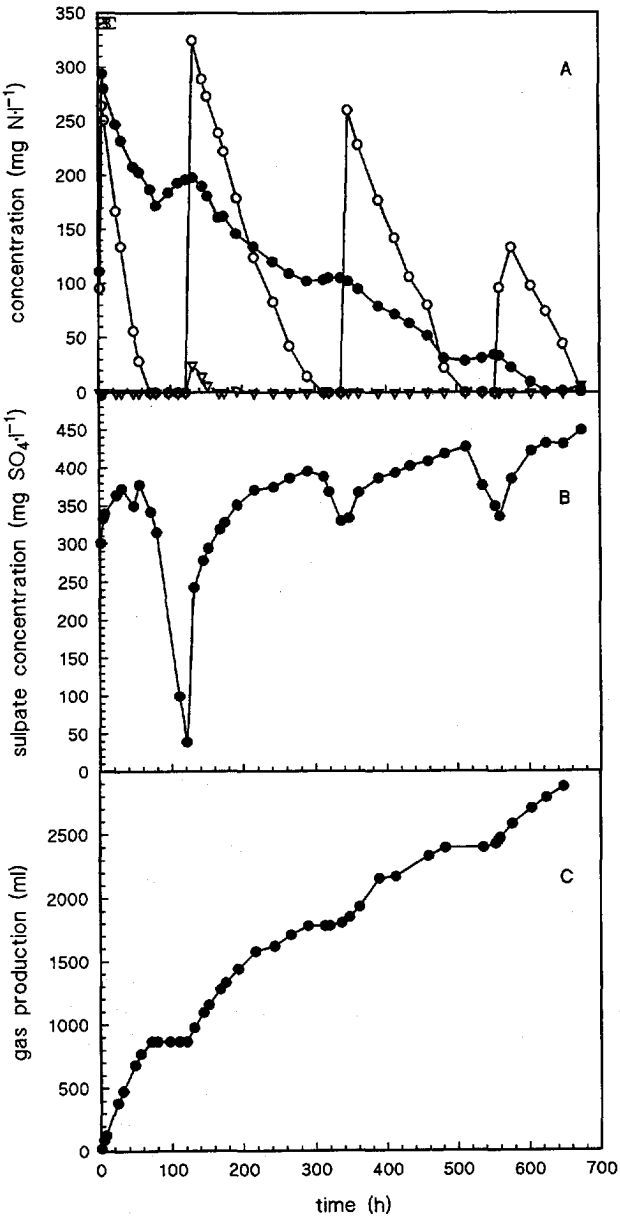

Figure 3 Nitrate-dependent conversion of ammonium during a batch experiment with a biomass sample taken from the denitrifying fluidized bed reactor 140 days after the onset of anaerobic ammonium oxidation. A. Simultaneous decrease of ammonium (●), nitrate (○) and nitrite (▽). B. Changes in the sulphate concentration during periods when nitrate had become exhausted. C. Cumulative gas production during the course of the experiment.

Fed-batch experiments

In order to substantiate this hypothesis, and to confirm the correlation between NH_4^+ conversion and increased NO_3^- utilization, batch experiments were carried out with biomass-covered sand originating from the fluidized bed reactor. As shown in Figure 3A, NH_4^+ removal coincided with NO_3^- removal, stopped when the NO_3^- became exhausted, and subsequently started again when more NO_3^- was supplied. Throughout the experiment, NO_2^- concentrations were below the detection limit (Figure 3A). Gas production during this experiment also corresponded to the periods of NH_4^+ and NO_3^- conversion (Figure 3C). In contrast, sulphate reduction began when the NO_3^- was exhausted, and stopped as soon as fresh NO_3^- was added (Figure 3B), confirming that denitrifying bacteria were active and maintaining the redox at a level suitable for denitrification rather than SO_4^- reduction (Elema, 1934; Golterman, 1975). The observed ammonium conversion capacity of 2.7 mg NH_4^+-N g^{-1}VSS day^{-1} in this fed-batch experiment was approximately 15 times lower than the conversion rate found in the continuous-flow denitrifying reactor.

The amount of nitrate and ammonium consumed during the experiment was equivalent to 2.55 l N_2. Gas production measurements gave a similar value of 2.80 l N_2 (Figure 3C), supporting the hypothesis that the ammonium and nitrate were being used to make N_2 (equation 3).

Concluding remarks

From the results reported here, it is apparent that the observed ammonium loss can be explained by anaerobic ammonium oxidation (equation 3). This occurrence of *an*aerobic *amm*onium *ox*idation has been named the "Anammox" process. Although the process was running at a redox level of 200 mV, and in waste water treatment terms might be called "anoxic" rather than "anaerobic", the generic term is preferred.

A precondition for the production of ATP and, consequently, for the reduction of CO_2 to biomass, is a negative free energy change. As shown in the equations 3,4 and 5 in Table 1, anaerobic ammonium oxidation is almost as energetically favourable as the aerobic reaction. Previous reports of N_2 production from ammonium in water, sludge and sediments (e.g. Allgeier *et al.*, 1932; Koyama, 1965) were inconclusive, for various reasons. To our knowledge this paper presents the first description of the occurrence of anaerobic ammonium oxidation. Hence, it seems that at least one of the "missing links in nature" (Broda, 1977) has now been discovered.

The Anammox process has been patented under the number EP 0 327 184 A1. The isolation of the responsible micro-organisms and the application of this process for waste water treatment will be part of future studies.

Acknowledgements
The authors are grateful to Paul P.M. van der Zanden for his practical assistance and Herman Slijkhuis for his enthusiastic discussions. This work was supported by Gist-brocades (Delft) and the Foundation of Applied Research (STW).

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Chapter 3

Anaerobic oxidation of ammonium is a biologically mediated process

Abstract

A newly discovered process by which ammonium is converted to dinitrogen gas under anaerobic conditions (the Anammox process) has now been examined in detail. In order to confirm the biological nature of this process, anaerobic batch culture experiments were used. All of the ammonium provided in the medium was oxidized within 9 days. In control experiments with autoclaved or raw waste water, without added sludge or with added sterilized (either autoclaved or gamma irradiated) sludge, no changes in the ammonium and nitrate concentrations were observed. Chemical reactions could therefore not be responsible for the ammonium conversion. The addition of chloramphenicol, ampicillin, 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and mercuric chloride ($Hg^{II}Cl_2$) completely inhibited the activity of the ammonium-oxidizing sludge. Furthermore, the rate of ammonium oxidation was proportional to the initial amount of sludge used. It was therefore concluded that anaerobic ammonium oxidation was a microbiological process. As the experiments were carried out in an oxygen-free atmosphere, the conversion of ammonium to dinitrogen gas did not even require a trace of O_2. That the end product of the reaction was nitrogen gas has been confirmed by using $^{15}NH_4^+$ and $^{14}NO_3^-$. The dominant product was $^{14-15}N_2$. Only 1.7% of the total labelled nitrogen gas produced was $^{15-15}N_2$. It is therefore proposed that the N_2 produced by the Anammox process is formed from equimolar amounts of NH_4^+ and NO_3^-.

A.A. van de Graaf, A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson, and J.G. Kuenen. *Appl. Environ. Microbiol.* **61** (1995) 1246-1251.

Introduction

The removal of ammonium is an important problem in modern waste water treatment systems. It is generally achieved by a combination of two processes, nitrification and denitrification. Ammonium is oxidized first to nitrite and then to nitrate by nitrifying bacteria. Nitrification is an O_2-requiring process and therefore requires an aerobic environment (Abeliovich, 1992; Focht and Verstraete, 1977). During the subsequent denitrification step, nitrate or nitrite is converted to dinitrogen gas (Kuenen and Robertson, 1987). Denitrification is carried out by a wide spectrum of respiratory bacteria representing most genera and physiological types (Zumft, 1992). Most denitrifying bacteria carry out these reactions only under anaerobic conditions (Tiedje, 1988).

Very recently, however, ammonium losses under anaerobic conditions were discovered to occur in a laboratory-scale denitrification reactor (Mulder *et al.*, 1995). Increased removal of ammonium was paralleled by increased disappearance of nitrate. This suggested that the following reaction was taking place (Broda, 1977):

$$5\ NH_4^+ + 3\ NO_3^- \dashrightarrow 4\ N_2 + 9\ H_2O + 2\ H^+ \quad \Delta G^{o\prime} = -\ 1483.5\ kJ/reaction \qquad (1)$$

The overall reaction for this anaerobic ammonium oxidation (Anammox) process is exergonic and thus could, in theory, supply energy for growth. It was therefore postulated that the removal of ammonium observed in the denitrifying reactor was carried out by bacteria using ammonium as an electron donor for nitrate reduction (Mulder *et al.*, 1995). The aim of the research described here was threefold: first, to confirm that the observed ammonium removal (Mulder *et al.*, 1995) was mediated by (micro)biological activity; second, to establish that oxidation of ammonium took place under fully anaerobic conditions; and finally, to demonstrate that ammonium was converted to dinitrogen gas by using ^{15}N-labelled ammonium.

Materials and Methods

Origin of biomass and preparation

Sludge from the denitrifying fluidized bed reactor in which anaerobic ammonium oxidation occurred was used as a source for biomass (Mulder *et al.*, 1995). The sludge from the reactor was either used immediately or else stored at 4°C until needed. Before use, the sludge was homogenized by passing it several times through a 60 ml syringe. For experiments with ^{15}N-labelled ammonium, the sludge in the reactor was used.

Medium

Effluent from the denitrifying fluidized bed reactor, supplemented with varying concentrations of ammonium and nitrate, was used as medium for all batch experiments. The effluent contained the following (per litre): total organic carbon (TOC), 130 to 155 mg C; SO_4^{2-}, 80 to 130 mg S; NH_4^+-N, 10 to 70 mg N; NO_3^-, 40 to 120 mg N. Details of the denitrifying reactor, which was being fed with effluent from a methanogenic reactor, were as described by Mulder *et al.* (1995).

Anaerobic batch culture experiments

Serum bottles (500-ml volume) were statically incubated in the dark at 37°C. Each bottle contained 460 ml of treated waste water (pH 7.0) and 40 ml (approximately 60 mg dry weight per ml) of sludge inoculum. The initial ammonium and nitrate concentrations were adjusted by adding NH_4NO_3, $(NH_4)_2SO_4$ or $NaNO_3$. The initial NH_4^+-N and NO_3^--N concentrations were 75 to 115 and 360 to 430 mg/litre, respectively. The sulphate-S concentration was between 80 and 130 mg/litre, depending on the performance of the waste water treatment system.

To prevent O_2 contamination, the bottles were firmly closed with 4-mm-thick butyl rubber septa. Anaerobic conditions were established in each bottle by flushing with pure nitrogen gas for 15 min or more. Samples were taken daily through in situ long sampling needles. During sampling, N_2 was supplied by using a separate sample port. An additional syringe served to indicate and control overpressure. Before being sampled, the bottles were shaken vigorously. After the sludge had settled, the sample was drawn off and centrifuged. Ammonium, nitrite, nitrate, sulphate, and pH were determined. All experiments were performed in duplicate.

For experiments with killed cells, sludge was autoclaved for 30 min at 120°C. This was repeated after 60 h. A single treatment was insufficient to stop denitrification and sulphate reduction completely. The medium was sterilized for 40 min at 120°C. For gamma irradiation of the sludge, 40 ml sludge samples were exposed to 25 kGy of radiation in 7 h using a ^{57}Co source (Gammaster, Ede, The Netherlands). In the inhibition experiments, different amounts (see Table 1) of antibiotics or inhibitors were added to the bottles before incubation. When different volumes (0, 10, 20, 30, and 40 ml) of sludge were used for inoculation, the final volume of the batch cultures was always adjusted to 500 ml with medium.

In order to ensure anaerobiosis, some cultures were incubated in anaerobic jars. In these experiments, 100-ml serum bottles were filled with 10 ml sludge and 90 ml effluent. Five bottles were inoculated with sludge of the same origin and incubated in separate anaerobic jars. The jars were provided with BBL GasPack anaerobic system (Becton Dickinson and Co., Cockeysville, Md.), a palladium catalyst, and an indicator strip (O_2 detection level, 0.05%). At a predetermined time, the first jar was opened for sampling. Cultures were not reincubated after sampling. Control experiment cultures were incubated as normal and were sampled at the same time as the cultures in the jars.

Tracer studies

Studies with ^{15}N-labelled ammonium were done by using the 23-litre denitrifying fluidized bed reactor in which the Anammox reaction was occurring (Mulder *et al.*, 1995). During the tracer experiments, the fluidized bed reactor was operated in batch mode, with liquid recycle but

without its usual feed from the methanogenic reactor. At the beginning of the experiment, 4.5 g $(^{15}NH_4)_2SO_4$ was supplied to the culture in the reactor, increasing the ammonium concentration by 45 mg N per litre. Control experiments were done either by adding unlabelled ammonium or without any addition. Samples for analysis of ammonium, nitrate, and nitrite were taken. Gas production was monitored during the experiment. During the days between successive experiments, the reactor was operated as usual.

Mass spectrometry

Use was made of an on-line quadrupole mass spectrometer (Hal Quadrupole Gas Analyzer and Faraday Cup; Hiden Analytical LTD, Warrington, England) kindly provided by Gist-brocades (Delft, The Netherlands). The sampling capillary from the mass spectrometer to the denitrification reactor was maintained at 80° C. This capillary was connected to the gas collector at the top of the fluidized bed reactor. The levels of $^{14\text{-}15}N_2$, $^{15\text{-}15}N_2$, ^{15}NO, $^{14\text{-}15}N_2O$, and $^{15\text{-}15}N_2O$ were monitored at m/z values (mass/charge ratios) of 29, 30, 31, 45, and 46, respectively.

Analytical procedures

Nitrate, nitrite, and sulphate were determined with an ion-exchange high-pressure liquid chromatograph fitted with an ionospher-A column (Chrompack, Middelburg, The Netherlands), a mobile phase of 0.04 M sodium salicylate (pH 4.0), and refractive index detection system (Waters, Milford, Mass.). When needed, nitrite was determined colorimetrically with the Griess-Romijn reagent (Griess-Romijn van Eck, 1966) or was assayed semiquantitatively using test strips from Merck (detection range, 0 to 80 mg/l). Ammonium was determined colorimetrically (Fawcett and Scott, 1960). The term ammonium will be used to indicate both the protonated and the unprotonated forms, since, at the pH values used in these experiments, ammonium and ammonia would both be present. Sulphide was determined according to the method of Trüper and Schlegel (1964). Total organic carbon was determined with a Tocamaster model 915-B instrument (Beckmann Industrial Corp., La Harba, Calif.). Biomass dry weight was determined by drying the sample at 65° C for at least 24 h. The quantity of sand in the dried sample was measured after ashing at 650° C for 1 h. The dry weight minus the sand is hereafter termed volatile solids (VS).

Chemicals

All chemicals used were reagent grade and obtained from commercial sources. Chloramphenicol was obtained from Serva (Feinbiochemica, Heidelberg, Germany); penicillin V from Gist-brocades, ampicillin, 2,4-dinitrophenol, and mercuric chloride ($Hg^{II}Cl_2$) were obtained from Merck (Darmstadt, Germany); and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Sigma (St. Louis, Mo.).

Results

Microbial activity studies

Results from an anaerobic batch culture experiment are shown in Figure 1. Ammonium and nitrate were converted simultaneously. A small amount of sulphate was formed during the first 2 days. This was probably due to the oxidation of residual elemental sulphur or sulphide in the inoculum. The pH was 7.5 ± 0.1 throughout the experiment. All ammonium was used within 9 days, with a specific ammonium oxidation activity of 66 ng NH_4^+-N h^{-1} mg VS^{-1}. After this, a small amount of nitrite accumulated in the medium, reaching a value of 3 mg N/l after 3 days. The initial total organic carbon content of the supernatant was 115 ± 8 mg C/l, and did not change during the 15-day incubation period. Soluble, degradable organic carbon was therefore probably not available in the waste water during the batch culture experiments. According to equation 1, only 30% of the nitrate converted could be accounted to ammonium oxidation. The remaining 70% was assumed to be denitrified during biomass degradation or utilization of storage material.

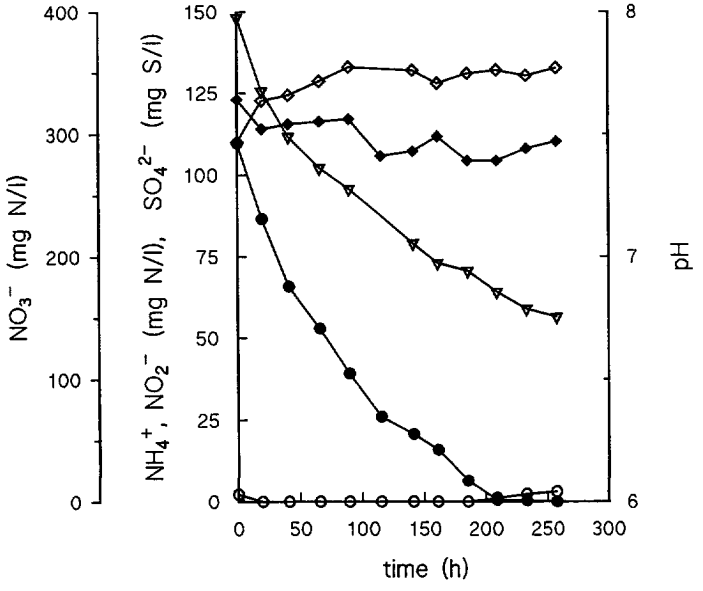

Figure 1 Concentration curves and pH course of a typical anaerobic ammonium oxidation batch culture experiment using 500-ml serum bottles at 37°C with nitrate as the electron acceptor and a 30-ml inoculum. The specific anaerobic ammonium oxidation activity of the biomass used was 66 ng N-NH_4^+ h^{-1} mg VS^{-1}. Symbols: ●, NH_4^+; ▽, NO_3^-; ○, NO_2^-; ◇, SO_4^{2-}; ◆, pH.

TABLE 1 Rate of ammonium removal by oxidation with nitrate at 37°C in the presence of antibiotics or inhibitors, and during inactivation experiments[a]

Sample incubated	Inhibitor concentration (mg/l)	Ammonium removal (% of control)
Inoculated control	NA[b]	100
Noninoculated control	NA	0
Noninoculated sterile effluent	NA	0
Waste water inoculated with sterilized sludge in sterile effluent	NA	0
Waste water inoculated with gamma-irradiated sludge	NA	0
Culture inoculated with:		
Penicillin	0	100 ± 13
	1	83 ± 13
	100	64 ± 10
Chloramphenicol	0	100 ± 5
	20	64 ± 5
	200	2[c] ± 2
Ampicillin	0	100 ± 3
	400	29 ± 3
	800	6 ± 4
$Hg^{II}Cl_2$	0	100 ± 4
	271	0
2,4-Dinitrophenol	0	100 ± 5
	37	47 ± 7
	368	1 ± 2
CCCP	0	100 ± 3
	41	0

[a] The specific anaerobic ammonium oxidation activity of the sludge used was 104 ng NH_4^+-N h^{-1} mg VS^{-1} (100 %).
Data are the means (± standard deviations) for duplicate experiments.

[b] NA, not applicable

[c] For the first 3 days of incubation. After this period the ammonium removal was 32 ± 10% of the control.

Sterilization experiments

As can be seen from Table 1, sterilization of the sludge or waste water by gamma irradiation or autoclaving prevented ammonium removal during the experiments. Control bottles (with untreated sludge) showed a decrease of 110 mg NH_4^+-N per litre during the same period. Without the addition of the sludge, and even with nonsterile waste water, the ammonium concentration did not decrease. In addition, the nitrate, nitrite, and sulphate concentrations did not change in any of the experiments using sterilized sludge samples and/or waste water.

Inhibition experiments

To investigate whether inhibitors or antibiotics inhibited anaerobic ammonium oxidation, chloramphenicol, penicillin V, ampicillin, 2,4-dinitrophenol, CCCP, and $Hg^{II}Cl_2$ were added separately to batch cultures (Betina, 1983). The effect of these compounds on ammonium oxidation is shown in Table 1. The anaerobic ammonium oxidation activity of the control flasks (104 ng NH_4^+-N h^{-1} mg VS^{-1}) was considered to be 100%. When 200 mg of chloramphenicol per litre was added to batch cultures, 3 days elapsed before significant ammonium conversion was observed. The addition of 20 mg/l chloramphenicol caused partial inhibition of ammonium removal, reducing the rate to 64% of the control level. This is in agreement with observations that chloramphenicol can inhibit the activity of existing denitrification enzymes (Brooks *et al.*, 1992). Ampicillin (800 mg/l) inhibited ammonium removal almost completely. At 400 mg/l, it reduced the activity by 71%. Penicillin V was less inhibitory. This could be due to the low concentrations of antibiotic used, or to some form of resistance to penicillin V. Known inhibitors of oxidative phosphorylation, 2,4-dinitrophenol (2 mM), mercuric chloride (1 mM), and CCCP (0.2 mM), completely inhibited anaerobic ammonium removal, indicating that the ammonium conversion is due to a metabolic activity.

Activity experiments

Figure 2 shows the ammonium removal rates when different amounts of sludge were used in batch culture experiments. During one series of experiments, the cultures were statically incubated and were only shaken once a day, before sampling. In the second series, the cultures were shaken throughout the experimental period. The ammonium oxidation rates were determined by linear regression, calculated from seven and nine time points for the shaken and static cultures, respectively. The specific ammonium removal rate was not affected by the shaking. Hence, the cultures were not limited by diffusion of nutrients through the sludge clumps. As shown in Figure 2, the ammonium removal rate was directly proportional to the amount of sludge used. This linear relationship between activity and the amount of sludge employed confirms the biological nature of

anaerobic ammonium oxidation.

Anaerobic jar experiments

In order to exclude the possibility that O_2 leaks might be contributing to ammonium oxidation during experiments with closed screw-cap bottles exposed to air, experiments were done in anaerobic jars under an O_2-free atmosphere. Ammonium conversion in control experiments was monitored at the same time. There was no significant difference between ammonium removal rates in the control cultures and in those incubated in anaerobic jars. Anammox activity is therefore not dependent on the presence of traces of O_2.

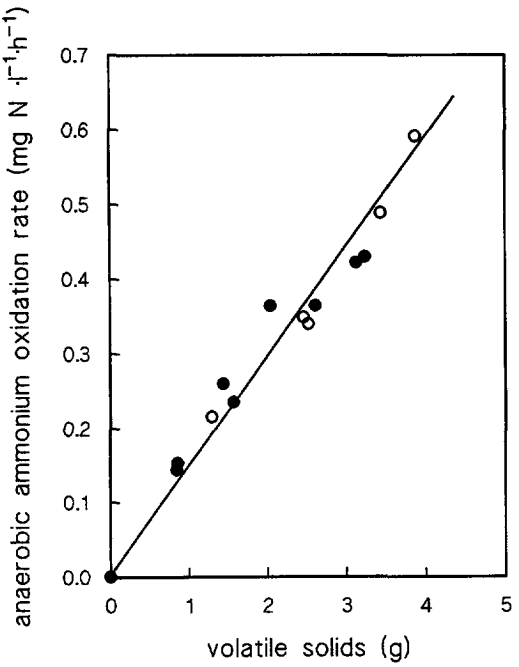

Figure 2 Correlation between anaerobic ammonium oxidation rate and amount of biomass used in batch cultures. The specific anaerobic ammonium oxidation activity of the biomass used was 67 ng N-NH_4^+ h^{-1} mg VS^{-1}. Symbols: ●, static incubation; ○, shaken incubation.

Tracer studies

For N_2 produced from unlabelled and ^{15}N-labelled precursors, three combinations of N isotopes are possible: $^{14-14}N_2$ (*m/z* 28), $^{14-15}N_2$ (*m/z* 29), and $^{15-15}N_2$ (*m/z* 30). After the addition of $^{15}NH_4^+$, the resulting labelled N_2 should cause a change in the *m/z* 28, 29 and

30 values, depending on how the N-N bond was formed. After $^{15}NH_4^+$ was added, a large increase of *m/z* 29 $^{14\text{-}15}N_2$ was observed (Figure 3). Furthermore, small amounts of $^{15\text{-}15}N_2$ (*m/z* 30) were also formed. *m/z* 45 and 46 did not increase during these experiments, indicating that labelled N_2O was not formed. The small increase at *m/z* 31 (Figure 3) was therefore due to the formation of labelled nitric oxide (^{15}NO). This represented only a trace amount of the total labelled end products. During control experiments with no or unlabelled ammonium, *m/z* 29, 30, and 31 did not change. It can thus be concluded that the main product of anaerobic ammonium oxidation is dinitrogen gas.

Ammonium and nitrate consumption and the cumulative gas production during the ^{15}N-labelling and control experiments are presented in Figure 4. The ammonium concentration in the control experiment (without the addition of extra ammonium), reached zero after 2 h. Simultaneously, the gas production rate decreased. This emphasizes the correlation between ammonium conversion and gas production.

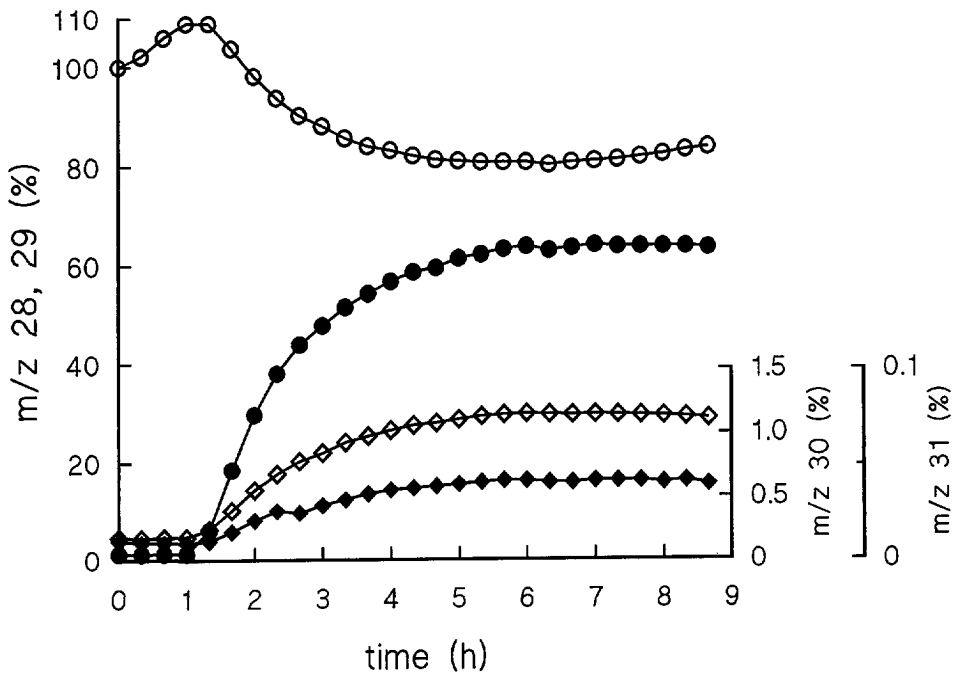

Figure 3 Changes in m/z 28, 29, 30 and 31 as a percentage of the initial partial pressure of m/z 28 and as a function of time during a batch experiment with the denitrifying fluidized bed reactor after the addition of labelled NH_4^+ and during control experiments. The $^{15}NH_4^+$ pulse was supplied after the start of the experiment at 0.75 h. Symbols: ●, m/z 29; ○, m/z 28; ◇, m/z 30; ◆, m/z 31.

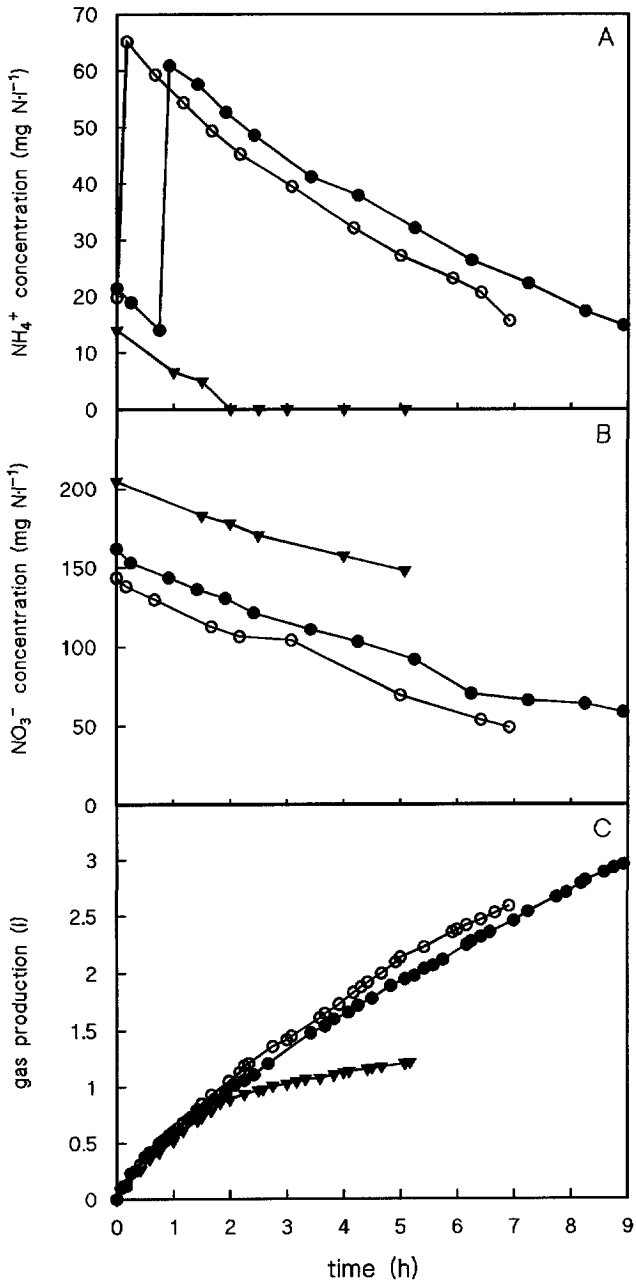

Figure 4 Consumption of ammonium (A) and nitrate (B), and cumulative production of gas (C) during a batch experiment using a fluidized bed reactor with $^{15}NH_4^+$ addition and controls. Symbols: ●, $^{15}NH_4^+$ addition; ○, NH_4^+ addition; ▼, no addition.

Discussion

The results presented here clearly demonstrate that anaerobic ammonium oxidation is a biological process that terminates in N_2. It is dependent on NO_3^-, and does not require traces of O_2. The fact that ammonium did not disappear when heat-inactivated or gamma-irradiated sludge was used, or in cultures that were not inoculated, indicates that the well-known chemical van Slyke reaction (van Slyke, 1912) between ammonium or amino groups and nitrite, forming dinitrogen gas, did not occur. Nitrite was not present in the medium during the batch experiments and was found only after the ammonium had become depleted. Moreover, the pH in these experiments remained far above 3, the required pH for the van Slyke reaction. Additional tests carried out during the current study (data not shown) gave no evidence for spontaneous chemical reactions even when relatively high concentrations of NH_4^+, NO_3^- or NO_2^- (30 mM, 420 mg N/l) were used. Only at very high NH_4^+ and NO_2^- concentrations (40 g N/l), with a low pH and under an NO atmosphere (to prevent decomposition of nitrite) has the chemical production of N_2 from ammonium and nitrite been confirmed (Smith and Clark, 1960; van Cleemput and Baert, 1984). Interactions between nitrite and ammonium ions have been reported (Wahhab and Uddin, 1954) to occur in alkaline soils (pH 8.5), but only after desiccation. At low concentrations, loss due to chemical reaction was not observed.

Further confirmation of the biological nature of the reaction can be derived from the fact that chloramphenicol (200 mg/l), ampicillin (800 mg/l), 2,4-dinitrophenol (1 mM), CCCP (0.2 mM), and $Hg^{II}Cl_2$ (1 mM) inhibited anaerobic ammonium oxidation by more than 95% (Table 1). Moreover, the ammonium oxidation was directly proportional to the amount of biomass in the culture. The Anammox reaction can therefore be due only to microbial activity.

As the cultures incubated in anaerobic jars gave the same results as similar cultures incubated normally, O_2 was evidently not needed for the Anammox reaction. The known NH_4^+-oxidizing nitrifiers, such as *Nitrosomonas europaea*, are thought to use molecular O_2 for at least the first oxidation step of ammonium to hydroxylamine, carried out by an ammonium monooxygenase (Wood, 1987). This implies that the bacteria able to carry out anaerobic ammonium oxidation have a novel nitrifying enzyme system. It is known, however, that some nitrifiers are capable of partial denitrification (Abeliovich, 1992, Ritchie and Nicholas, 1972). It was demonstrated that *N. europaea*, under conditions of O_2 stress, could use nitrite as the terminal electron acceptor, producing nitrous oxide (Poth and Focht, 1985). A new isolate identified as a *Nitrosomonas* species was able to produce, under similar conditions, N_2 (Poth, 1986). Labelled nitrogen experiments, however, demonstrated that the N_2O and N_2 were produced by nitrite reduction rather than by ammonium oxidation. Remde and Conrad (1990) also concluded that nitrite was

TABLE 2 Distribution of ^{15}N label over dinitrogen gas for five hypothetical reactions for the oxidation of labelled ammonium to N_2 and the Gibbs free energy changes.

no	Reaction	$\Delta G^{o\prime}$ [a]	N_2 composition[b] (%)	
		(kJ/molNH_4^+)	$^{14-15}N_2$	$^{15-15}N_2$
1[c]	$5\ NH_4^+ + 3\ NO_3^- \rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+$	- 297	75	25
2[c]	$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	- 358	100	0
3[d]	$5\ NH_4^+ + NO_3^- + 2.5\ O_2 \rightarrow 3\ N_2 + 8\ H_2O + 4\ H^+$	- 310	33	67
4[e]	$2\ NH_4^+ + 2\ O_2 + H_2 \rightarrow N_2 + 4\ H_2O + 2\ H^+$	- 435	0	100
5[f]	$8\ NH_4^+ + 6\ O_2 \rightarrow 4\ N_2 + 12\ H_2O + 8\ H^+$	- 316	0	100
6	Observed		98.2	1.7

[a] Gibbs free energies were calculated on the basis of data from Thauer *et al.* (1977)

[b] Expected composition of labelled dinitrogen gas when ammonium is labelled with ^{15}N, when no exchange between ^{15}N and ^{14}N is assumed.

[c] Anaerobic ammonium oxidation, as proposed by Broda (1977).

[d] A dehydrogenase is involved in the oxidation of ammonium to hydroxylamine, and nitrate and O_2 are both electron acceptors for this process.
$5\ NH_4^+ + 5\ H_2O \rightarrow 5\ NH_2OH + 10\ [H] + 5\ H^+$
$10\ [H] + 2.5\ O_2 \rightarrow 5\ H_2O$
$5\ NH_2OH + NO_3^- + H^+ \rightarrow 3\ N_2 + 8\ H_2O$

[e] A monooxygenase catalyses the first step in the oxidation of ammonium, in which hydroxylamine is formed. No nitrate is used for ammonium oxidation, and there is an overall requirement for additional electron donor (organic material or reduced sulphur compounds) since hydroxylamine does not provide enough reducing equivalents.
$2\ NH_4^+ + 2\ O_2 + 4[H] \rightarrow 2\ NH_2OH + 2\ H_2O + 2\ H^+$
$2\ NH_2OH \rightarrow N_2 + 2\ H_2O + 2[H]$; (2[H] is represented by H_2 in the overall reaction)

[f] Ammonium is directly converted to dinitrogen gas with O_2 as electron acceptor.

the main source of the NO and N_2O produced by *N. europaea*. O_2 was still required for the initial oxidation of ammonium to NH_2OH, although the O_2 for the oxidation of hydroxylamine might come from H_2O. The known autotrophic NH_4^+-oxidizing nitrifiers can consequently not be grown under anaerobic denitrifying conditions. Indeed, *N. europaea* washed out of continuous cultures once the dissolved O_2 had dropped below the critical level of 8% air saturation (van Niel, 1991). The Anammox reaction is therefore not likely to be due to the activity of known ammonium-oxidizing bacteria.

As can be seen from the experiments with ^{15}N-labelled ammonium (Figure 3), NH_4^+-N was incorporated in the end product of the Anammox reaction, N_2. Mixed-labelled nitrogen gas, $^{14-15}N_2$, was the dominant product, making up 98.2% of the total labelled dinitrogen gas produced (Figure 3). Only 1.7% was $^{15-15}N_2$. These findings do not fully

agree with the overall reaction (equation 1) proposed by Mulder *et al.* (1995). From this reaction it was to be expected that 75% of the labelled nitrogen would be $^{14-15}N_2$, and 25% would be $^{15-15}N_2$, because of the 5:3 stoichiometry of ammonium oxidation to nitrate reduction. Because of the very low growth yields (Mulder *et al.*, 1995), ammonium assimilation was assumed not to be significant within the period of the experiment. The stoichiometries of four other potential reactions for the oxidation of ammonium to dinitrogen gas are summarized in Table 2. In all of these reactions, only the oxidation of ammonium with nitrite as electron acceptor predicts a labelling percentage close to the observed data. If O_2, for example, was involved, only $^{15-15}N_2$ would be expected. Thus, instead of nitrate, nitrite appears to be the direct oxidizing agent of ammonium in the Anammox reaction.

Apart from reports of unexplainable N_2 production (e.g. Allgeier *et al.*, 1932; Koyama, 1964), there is no other published evidence for the anaerobic removal of ammonium by biological nitrate reduction. Studies recently done in the Black Sea have indicated that there is also a correlation between the nitrate and ammonium profiles in nature (Jørgensen *et al.*, 1991). The chemical zonation of O_2 and H_2S of the upper few hundred meters of the central Black Sea waters has been shown to have changed significantly over the last decades (Jørgensen *et al.*, 1991; Murray *et al.*, 1989). The O_2-containing layer is separated from the sulphide-rich lower zone by a 30-m-deep layer that is free of O_2 and H_2S. In this layer, opposing gradients of NO_3^- and NH_4^+ were found, suggesting that anaerobic ammonium oxidation might be taking place.

Acknowledgments

We thank H. Slijkhuis and R. Beudeker of Gist-brocades for their stimulating discussions. This work was supported by the Foundation of Applied Research (STW) and Gist-brocades.

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Chapter 4

Growth of *Nitrosomonas europaea* on hydroxylamine

Abstract

Hydroxylamine is an intermediate in the oxidation of ammonium to nitrite, but until now it has not been possible to grow *Nitrosomonas europaea* on hydroxylamine. This study demonstrates that cells of *N. europaea* are capable of growing mixotrophically on ammonium and hydroxylamine. The molar growth yield on hydroxylamine (4.74 $g.mol^{-1}$ at a growth rate of 0.03 h^{-1}) was higher than expected. Aerobically growing cells of *N. europaea* oxidized ammonium to nitrite with little loss of inorganic nitrogen, while significant inorganic nitrogen losses occurred when cells were growing mixotrophically on ammonium and hydroxylamine. In the absence of oxygen, hydroxylamine was oxidized with nitrite as electron acceptor, while nitrous oxide was produced. Anaerobic growth of *N. europaea* on ammonium, hydroxylamine and nitrite could not be observed at growth rates of 0.03 h^{-1} and 0.01 h^{-1}.

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FEMS Microbiology Letters **125** (1995) 179-184.

Introduction

The oxidation of ammonium to nitrate by nitrifying bacteria is considered to be a strictly aerobic process, although there is evidence that autotrophic nitrifiers can survive under oxygen limitation (Abeliovich, 1992; van de Graaf *et al.*, 1990). Under oxygen limitation, significant inorganic nitrogen losses have been observed (Goreau *et al.*, 1980; Poth 1986). These nitrogen losses were due to the production of nitric oxide and nitrous oxide. The formation of NO and N_2O by ammonium oxidizers is attributed to reduction of nitrite by the enzyme nitrite reductase. The oxidation of hydroxylamine or hydrazine was suggested to provide the reduction equivalents (Hooper, 1968; Poth and Focht, 1985; Remde and Conrad, 1990). Organic substances, such as pyruvate or formate are also suitable electron donors for NO and N_2O production (Stüven, 1992). The denitrifying activity of *Nitrosomonas europaea* could not be related to growth, but it may serve as a survival mechanism in anaerobic habitats.

During mixotrophic growth of *N. europaea* on ammonium and pyruvate, hydroxylamine was formed (Stüven, 1992). Although *N. europaea* oxidizes hydroxylamine to nitrite, this substrate does not support growth even when added continuously (Watson *et al.*, 1989). Utilization of hydroxylamine as growth substrate by *N. europaea* under hydroxylamine limitation has not been reported. This paper describes the growth of *N. europaea* on mixtures of hydroxylamine and ammonium under aerobic conditions in chemostat culture. During transition experiments from aerobic to anaerobic conditions, the potential for anaerobic growth of *N. europaea* was investigated. We also show that in batch experiments under anaerobic conditions N_2O is produced from nitrite and hydroxylamine. During the review of this manuscript, Böttcher and Koops (1994) published a paper, in which growth of several ammonium-oxidizing bacteria on mixtures of ammonium and hydroxylamine in batch cultures was described.

Materials and Methods

Organism and cultivation

Nitrosomonas europaea LMD 86.25 was obtained from the culture collection of the Department of Microbiology and Enzymology, Delft, the Netherlands. *N. europaea* was routinely maintained in batch cultures. Phenol red (0.05 mg/l) was included as a pH indicator. Sodium carbonate (7%) was used to adjust the pH to 8. The cultures were incubated at 30 °C in the dark. *N. europaea* was grown in a synthetic medium containing per litre of demineralized water: KH_2PO_4, 0.2 g; $(NH_4)_2SO_4$, 2.64 g; $MgSO_4.7H_2O$, 0.04 g and 2 ml trace element solution. The trace element solution contained per litre of demineralized water: EDTA, 50 g; $ZnSO_4.7H_2O$, 2.2

g; $CoCl_2.6H_2O$, 1.61 g; $MnCl_2.4H_2O$, 5.06 g; $CuSO_4.5H_2O$, 1.57 g; $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.10 g; $CaCl_2.2H_2O$, 5.54 g; $FeSO_4.7H_2O$, 4.99 g. The medium was autoclaved at 120 °C, hydroxylamine was filter-sterilized. Contamination by heterotrophs was monitored by plating onto tryptone and yeast extract agar with subsequent incubation at 30 °C for 2 weeks.

Growth conditions

Continuous cultivation were performed in Applikon laboratory fermenters with a working volume of 10 l. The medium was automatically adjusted to pH 8.0 with 1 M Na_2CO_3. The aerobic cultures were continuously gassed with air and stirred at 400 rpm. During the anaerobic period of the transition experiments from aerobic to anaerobic conditions, the cultures were gassed with 5% CO_2/95% Argon. The medium vessels were kept anaerobic by sparging with Argon. The dissolved oxygen concentration was monitored with a polarographic electrode (Ingold, Urdorf, CH). Ammonium limited chemostat cultures were grown on 20 mM NH_4^+ at a dissolved oxygen concentration of 30% air saturation at 30 °C and at a dilution rate of 0.03 h^{-1}. The chemostats were wrapped in black paper to exclude light.

Anaerobic batch culture experiments

Anaerobic batch culture experiments were done in the dark at 30° C in 30-ml thermostatically controlled reaction chambers which were tightly closed with butyl rubber septa after flushing with argon. The cells for these experiments were obtained by continuous centrifugation of 20 l continuous culture fluid at 10.000 x *g*. The pellet was resuspended in anaerobic mineral medium. Biomass was determined after each experiment. Ammonium, nitrite and hydroxylamine concentrations were determined and rates calculated. All experiments were performed at least three times.

Analytical procedures

Nitrite was determined using the Griess-Romijn reagent (Griess-Romijn van Eck, 1966). Ammonium and hydroxylamine were determined colorimetrically (Frear and Burrell, 1955, Fawcett and Scott, 1960). Nitrous oxide was analyzed with a gas chromatograph (Packard Instrument company INC, USA) equipped with a TCD detector and a 180 cm column of CTR packed with Porous Polymer Mixture (Chromosorb 101). The term "ammonium" will be indicating both the protonated and the unprotonated forms, since, at the pH values used in these experiments, ammonium and ammonia both would be present. Dry weight of the cell suspensions was determined by filtrating aliquots over nitrocellulose filters (pore diameter 0.45 µm, Gelman Sciences, USA). The cells were washed three times with demineralized water and dried to constant weight.

Oxygen uptake experiments

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA). Cells from ammonium-limited chemostat cultures were assayed directly in the culture fluid. Calculations were made on the basis of an oxygen concentration of 0.24 mM in air-saturated mineral medium at 30° C.

Results

Chemostat cultures grown on ammonium and hydroxylamine

Growth of *N. europaea* in ammonium-limited chemostat cultures was studied at a dilution rate of 0.03 h^{-1}, being approximately 30% of its maximum growth rate (Loveless and Alexander, 1968). The molar growth yield of *N. europaea* in these ammonium-limited chemostat cultures was 1.43 g (mol ammonium)$^{-1}$. This yield is similar to values described for other *N. europaea* strains (Drozd, 1980). The cells obtained from a steady state culture were capable of oxidizing hydroxylamine at a rate of 150 nmol min^{-1} (mg dry weight)$^{-1}$. Until now, it has not been possible to grow *N. europaea* on hydroxylamine, because of its toxic nature (Poth and Focht, 1985, Watson *et al.*, 1989). However, the observed capacity to oxidize hydroxylamine suggested that *N. europaea* might be able to grow mixotrophically on a mixture of ammonium and hydroxylamine, provided that both compounds were kept gowth limiting to prevent toxicity problems. Therefore hydroxylamine was included in the medium to a level that would not exceed the observed

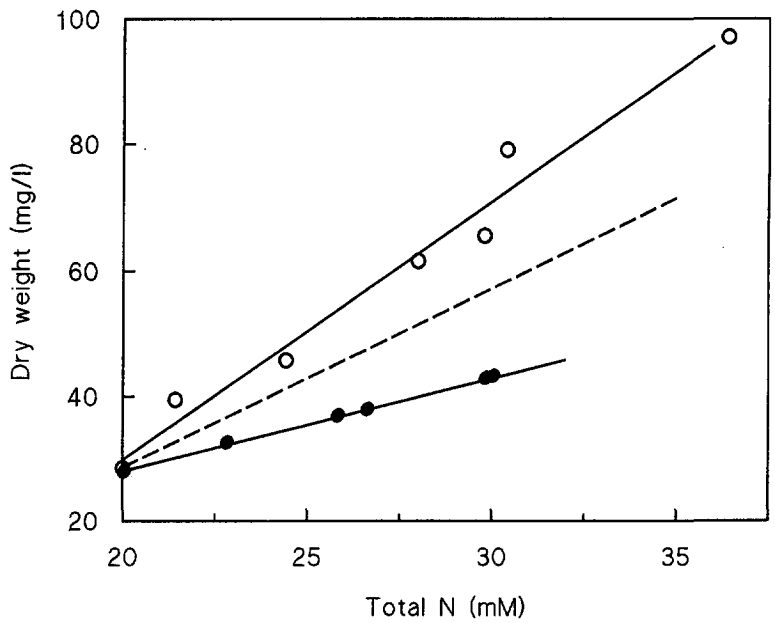

Figure 1 Effect of increasing concentration of hydroxylamine and ammonium in the reservoir medium on the biomass concentration in chemostat cultures of N. europaea. The dotted line indicates the theoretical growth yield on a mixture of hydroxylamine and ammonium on the basis of electron availability. Symbols: ●, dry weight on ammonium alone; ○, mixotrophic growth yields on ammonium and hydroxylamine.

hydroxylamine oxidation capacity. In this way a mixotrophic steady state culture could be established with hydroxylamine at undetectably low concentrations. Following a similar procedure, the hydroxylamine concentration in the medium reservoir could gradually be increased without exceeding the hydroxylamine oxidation capacity of the previous steady state culture.

Addition of hydroxylamine to the reservoir medium of ammonium-limited chemostat cultures resulted in a linear increase of biomass density (Figure 1). This indicated that *N. europaea* was able to grow on hydroxylamine, while simultaneously oxidizing ammonium. The increase in biomass was higher than growth on ammonium alone (Figure 1) and higher than the theoretically calculated growth yield on a mixture of ammonium and hydroxylamine. During growth on ammonium and hydroxylamine the nitrogen recovery in the form of nitrite was lower than expected for biomass formation (Table 1; 0.26 mM $N_{biomass}$ for growth on 20 mM NH_4^+). This gap in the nitrogen balance could be due to formation of nitrous oxide, which was detected in the off gas. Formation of N_2O by *N. europaea* has also been described previously (Poth, 1986; Ritchie and Nicholas, 1972). Measurements of the affinity constants (K_S) for NH_4^+ (0.2 mM) and NH_2OH (130 μM) during growth on a mixture of ammonium and hydroxylamine, or ammonium alone showed no significant difference.

TABLE 1 Nitrogen balances for growth of *N. europaea* on ammonium and hydroxylamine.

$[NH_4^+]$ (mM) Medium	$[NH_2OH]$ (mM) Medium	$[NO_2^-]$ (mM) Culture fluid	N-loss (%)
20.0	0	19.3	3.5
29.8	0	29.5	1.1
26.9	1.4	25.2	10.0
19.8	8.0	23.3	16.2
19.0	9.8	24.4	15.3
21.0	10.4	26.2	16.6

Growth conditions: growth rate, 0.03 h^{-1}; pH, 8.0; temperature, 30 °C.

Anaerobic activity experiments

N. europaea cells incubated under anaerobic conditions in the presence of a combination of nitrite, hydroxylamine and ammonium, converted only nitrite and hydroxylamine (Table 2). Also when cells were incubated in the presence of nitrite and hydroxylamine without ammonium, both nitrite and hydroxylamine were consumed. In both cases nitrous oxide was formed. When cells were incubated with nitrite alone nitrous oxide was also formed, while nitrite was consumed, probably by using reduction equivalents derived from storage compounds as electron donor. Incubation with

hydroxylamine alone showed no consumption of hydroxylamine and no formation of nitrous oxide. This suggests that, under anaerobic conditions, *N. europaea* uses nitrite as electron acceptor and produces nitrous oxide from nitrite with hydroxylamine as electron donor. When the cells were heat-inactivated the concentrations of nitrite, hydroxylamine and ammonium remained unchanged while there was no formation of nitrous oxide. This excludes chemical formation of nitrous oxide.

TABLE 2 Nitrous oxide production during anaerobic incubation in batch cultures of *N. europaea* cells (pH, 8.0; 30 °C)[a].

Addition	Increase N_2O	Decrease NO_2^-	Decrease NH_2OH
5 mM-NO_2^-	1	1	0
2 mM-NH_2OH	0	0	0
1 mM-NH_2OH + 5 mM-NO_2^-	4	5	10
5 mM-NO_2^-, NH_4^+	3	6	0
2 mM-NH_2OH + 5 mM-NH_4^+	0	0	0
2 mM-NH_2OH + 5 mM-NH_4^+,NO_2^-	4	3	5
Sterilized cells	0	0	0

[a] Values are in velocity in nmol min $^{-1}$ (mg dry weight)$^{-1}$

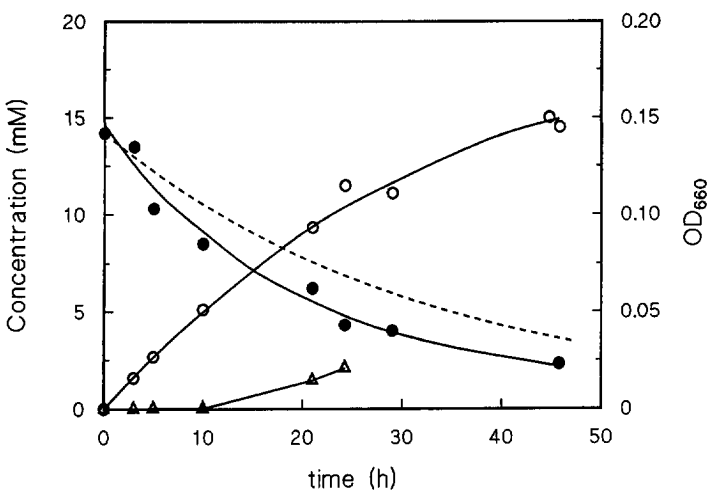

Figure 2 Wash-out curve of N. europaea and accumulation of NH_4^+ and NH_2OH during transition experiments from aerobic to anaerobic conditions at a dilution rate of 0.03 h^{-1}. The dashed line indicates the theoretical wash-out.
Symbols: ●, OD_{660} N. europaea; ○, NH_4^+; △, NH_2OH.

Transition experiments from aerobiosis to anaerobiosis

N. europaea was cultivated in chemostat culture at a dilution rate of 0.03 h^{-1} and at a dissolved oxygen concentration of 30% air saturation under combined ammonium and hydroxylamine limitation with a NH_4^+/NH_2OH ratio of 4. When the culture had reached steady state, the dissolved oxygen concentration was decreased to 0% by gassing the culture with 5% CO_2 and 95% argon. At the moment of the switch to anaerobic conditions, the nitrite concentration was approximately 26 mM (Table 1), thus providing ample electron acceptor for anaerobic metabolism of hydroxylamine. In Figure 2 the density in the culture is presented as a function of time. It can be seen that the density of the culture started to decrease and that it followed the theoretical wash-out line, thus indicating that there was no significant growth. After the shift from aerobic to anaerobic conditions, ammonium and hydroxylamine directly started to accumulate into the medium. Anaerobic growth of *N. europaea* was also not observed during transition experiments at a dilution rate of 0.01 h^{-1} (not shown).

Discussion

Growth of *N. europaea* on hydroxylamine has not been reported previously. Due to the toxicity of hydroxylamine, substrate-limited growth conditions are required for utilization of this compound by *N. europaea*. It is well known that hydroxylamine is toxic at millimolar concentrations (Hyman and Wood, 1983). Cultures growing on ammonium and hydroxylamine could only be obtained by careful manipulation of the influent hydroxylamine concentration. When ammonium and hydroxylamine were present in the culture at growth limiting concentrations, *N. europaea* could use hydroxylamine as a source of energy for growth. The increase in biomass concentration as a result of hydroxylamine addition was 4.74 g mol^{-1} or 1.18 g (mol redox equivalents)$^{-1}$. The molar growth yield of *N. europaea* in ammonium-limited chemostat cultures grown at the same dilution rate was 1.43 g mol^{-1} or 0.72 g (mol redox equivalents)$^{-1}$. Thus, the energetic value of the hydroxylamine redox equivalents is (1.18 : 0.72) x 100% = 164% of that of the redox equivalents from ammonium oxidation. Studies have shown that hydroxylamine and ammonium oxidation are coupled to proton translocation (Hollocher *et al.*, 1982). The H^+/O ratios reported for hydroxylamine are 3.9 and for ammonium ions 2.7 (Hollocher *et al.*, 1982). The energetic value of hydroxylamine redox equivalents should be (3.9 : 2.7) x 100% = 144% of that of the redox equivalents from ammonium oxidation, which is still 20% less than the measured values.

During growth of *N. europaea* on ammonium, a gap of 2% in the nitrogen balance was found (Table 1). This gap could be due to formation of nitrous oxide by a nitrite

reductase. It is well known that ammonium oxidizing bacteria produce small amounts of nitrous and nitric oxides in addition to nitrite. Gorreau *et al.* (1980) found a yield of 2.5% total N as N_2O for *N. europaea*. During growth on ammonium and hydroxylamine, the gap in the nitrogen balance increased to 16% (Table 1), which is comparable to the value observed by Stüven *et al.* (1992). A soluble nitrite reductase has been characterized from *N. europaea* which catalyses the reduction of nitrite to N_2O and of O_2 to water (Miller and Nicholas, 1985). When nitrite is used as electron acceptor instead of oxygen, the limited amount of oxygen present, can be used by the monooxygenase (K_m for O_2 = 15-20 μM). Oxidation of hydroxylamine to nitrite does not require molecular oxygen (Andersson and Hooper, 1983). Cells of *N. europaea* produced N_2O by the reduction of nitrite under anaerobic conditions (Table 2). Production of N_2O increased when cells were incubated with hydroxylamine and nitrite, while hydroxylamine was consumed. This indicates that N_2O is formed by the oxidation of hydroxylamine using nitrite as electron acceptor.

Although it would seem possible for *N. europaea* to grow anaerobically while oxidizing hydroxylamine, shift experiments from aerobic to anaerobic conditions at a dilution rate of 0.03 h^{-1} showed that *N. europaea* was unable to grow under these circumstances. Even when the dilution rate was decreased to 0.01 h^{-1} (Figure 2). During these experiments, ammonium and hydroxylamine accumulated into the medium. The failure to grow *N. europaea* anaerobically is probably the result of inhibition effects of hydroxylamine observed in biochemical reactions involving autotrophic CO_2-fixation (Poth and Focht, 1985). It would be highly interesting to grow *N. europaea* in chemostat cultures under hydroxylamine limitation to enable accurate estimates of growth yield and maintenance substrate consumption.

Acknowledgements

This research was supported by the Foundation of Applied research (STW).

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Chapter 5

Autotrophic growth of anaerobic, ammonium-oxidizing micro-organisms in a fluidized bed reactor

Abstract

An autotrophic, synthetic medium for the enrichment of anaerobic ammonium- oxidizing (Anammox) micro-organisms was developed. This medium contained ammonium and nitrite, as the only electron donor and electron acceptor, respectively, while carbonate was the only carbon source provided. Preliminary studies showed that the presence of nitrite and the absence of organic electron donors were essential for Anammox activity. The conversion rate of the enrichment culture in a fluidized bed reactor was 3 kg NH_4^+ m^{-3} d^{-1} when fed with 30 mM NH_4^+. This is equivalent to a specific anaerobic ammonium oxidation rate of 1000-1100 nmol NH_4^+ h^{-1} (mg volatile solids)$^{-1}$of (VS). The maximum specific oxidation rate obtained was 1500 nmol NH_4^+ h^{-1} (mg volatile solids)$^{-1}$. Per mol of NH_4^+ oxidized, 0.041 mol CO_2 were incorporated, resulting in a estimated growth rate of 0.001 h^{-1}. The main product of anaerobic ammonium oxidation is N_2, but about 10% of the N-feed is converted to NO_3^-. The overall nitrogen balance gave a ratio of NH_4^+-conversion to NO_2^--conversion and NO_3^--production of 1:1.31±0.06:0.22±0.02. During the conversion of NH_4^+ with NO_2^-, no other intermediates or end products such as hydroxylamine, NO and N_2O could be detected. Acetylene, phosphate, and oxygen were shown to be strong inhibitors of the Anammox activity. The dominant type of micro-organism in the enrichment culture was an irregularly shaped cell with an unusual morphology. During the enrichment for anaerobic ammonium-oxidizing micro-organisms on synthetic medium, an increase in ether lipids was observed. The colour of the biomass changed from brownish to red, which was accompanied by an increase in the cytochrome content. Cytochrome spectra showed a peak at 470 nm gradually increasing in intensity during enrichment.

Extended version of: A.A. van de Graaf, P. de Bruijn, L.A. Robertson, M.S.M. Jetten and J.G. Kuenen, *Microbiology* **142** (1996) 2187-2196.

Introduction

Ammonium is a common pollutant which is normally eliminated from waste water by a combination of two processes, nitrification and denitrification. Nitrification is the biological formation of nitrate or nitrite from compounds containing reduced nitrogen with O_2 as the terminal electron acceptor. Two separate and distinct steps are involved in nitrification. First, the oxidation of NH_4^+ to NO_2^- is carried out by the ammonium-oxidizing bacteria such as *Nitrosomonas* or *Nitrosospira* (Koops and Möller, 1992). This is followed by the oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria such as *Nitrobacter*-like organisms (Abelovich, 1992). During the subsequent denitrification step, NO_3^- or NO_2^- are reduced to N_2 by denitrifying bacteria. Denitrification is carried out by a wide spectrum of respiratory bacteria representing many genera and physiological types (Kuenen and Robertson, 1994; Zumft, 1992).

Recently, a novel anaerobic process in which ammonium was used as electron donor for denitrification was discovered in a laboratory-scale fluidized bed reactor (Mulder *et al.*, 1995). It was demonstrated that in this ANaerobic AMMonium OXidizing/OXidation (Anammox) process, nitrate was used as an electron acceptor. Redox balance calculations showed the following stoichiometry:

$$5\ NH_4^+ + 3\ NO_3^- \rightarrow 4\ N_2 + 9\ H_2O + 2\ H^+ \quad \Delta G^{o\prime} = -297\ kJ\ (mol\ NH_4^+)^{-1} \quad (eq.\ 1)$$

During further examination of this new process, which is catalysed by an as yet unidentified mixed microbial population, indications were obtained that also nitrite could serve as a suitable electron acceptor for Anammox (van de Graaf *et al.*, 1995).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O \quad \Delta G^{o\prime} = -358\ kJ\ (mol\ NH_4^+)^{-1} \quad (eq.\ 2)$$

To obtain more fundamental understanding of this new process, we developed a synthetic inorganic mineral medium for the enrichment of the micro-organisms capable of Anammox in a fluidized bed system. This paper reports on the characteristics and properties of this enrichment culture.

Materials and Methods

Origin of biomass and waste water

Sludge from a 27 litre denitrifying fluidized bed reactor in which the Anammox process occurred was used as the source for biomass (Mulder *et al.*, 1995). When used in batch

experiments, the sludge was homogenized by passing it several times through a 60 ml syringe. For continuous experiments, a small denitrifying fluidized bed reactor (2.5 l) was directly fed with the effluent from a methanogenic reactor. This waste water mainly contained: total organic carbon, 150-190 mg C l^{-1}; HS^-, 77-106 mg S l^{-1} (± 2.9 mM); NH_4^+-N, 75-100 mg N l^{-1} (± 6 mM). The effluent from the denitrifying fluidized bed reactor, supplemented with varying concentrations of ammonium and nitrate, was used as medium for batch experiments. The effluent contained: total organic carbon, 130 - 155 mg C l^{-1}; SO_4^{2-}, 80 - 130 mg S l^{-1}; NH_4^+-N, 10 - 70 mg l^{-1}N; NO_3^-, 40-120 mg N l^{-1}. Other compounds in mmol l^{-1}: HCO_3^-, 21; K^+, 13; Na^+, 22; Cl^-, 3.3; Ca^{2+}, 2.2; Mg^{2+}, 0.4; HPO_4^{2-}, 0.01; Fe, 0.005. Details of the system are described by Mulder *et al.* (1995).

Mineral Medium

For the start-up of the small fluidized bed reactor, an anaerobic synthetic medium was used. This medium contained (per l of demineralized water): $(NH_4)_2SO_4$, 330 mg (5 mM); $NaNO_2$, 345 mg (5 mM); $KHCO_3$, 500 mg; KH_2PO_4, 27.2 mg; $MgSO_4 \cdot 7H_2O$, 300 mg; $CaCl_2 \cdot 2H_2O$, 180 mg, and 1 ml of trace element solution I and II. Trace element solution I contained (per l demineralized water): EDTA, 5 g; $FeSO_4$, 5 g; and trace element solution II contained (per l of demineralized water): EDTA, 15 g; $ZnSO_4 \cdot 7H_2O$, 0.43 g; $CoCl_2 \cdot 6H_2O$, 0.24 g; $MnCl_2 \cdot 4H_2O$, 0.99 g; $CuSO_4 \cdot 5H_2O$, 0.25 g; $NaMoO_4 \cdot 2H_2O$, 0.22 g; $NiCl_2 \cdot 6H_2O$, 0.19 g; $NaSeO_4 \cdot 10H_2O$, 0.21 g; H_3BO_4, 0.014 g. The mineral medium was autoclaved at 120°C. Solutions of trace elements, $CaCl_2$ and $MgSO_4$ were sterilized separately at 120°C and added aseptically to the autoclaved medium. After cooling, the medium was flushed with argon for at least 30 minutes to achieve anaerobic conditions. After the start-up ammonium and nitrite concentrations were increased by steps of 5 mM.

Operation and continuous experiments with the fluidized bed reactor run with waste water.

The glass fluidized bed reactor of 2.5 l was operated at 36°C and pH 7. Batch experiments with the reactor had confirmed that these were the preferred temperature and pH. The pH was adjusted with 0.5 N H_2SO_4 or 0.5 N NaOH. Anoxic liquid from the top of the reactor was recirculated to boost the flow to approximately 47 l h^{-1} in order to keep the bed fluidized at a superficial liquid velocity of 24 m h^{-1}. The hydraulic retention time was 4.2 h. The influent (see waste water) of the reactor was supplied at a rate of 600 ml h^{-1}. A nitrate solution (60 g $NaNO_3$ l^{-1}) was supplied at a rate of about 13 ml h^{-1}. Sand particles (diameter 0.3-0.6 mm) were the carrier material of the fluidized bed on which bacteria grew as a biolayer. The reactor was inoculated with a large amount of sand covered with denitrifying and anaerobic ammonium-oxidizing biofilms (400 ml), originating from the 27 l installation (Mulder *et al.*, 1995). This resulted in fast start-up of the experiments.

Samples of the influent and effluent of the reactor were taken once or twice a day and analysed for ammonium, nitrate, nitrite, sulphide, sulphate and sometimes for dissolved organic carbon. Gas production was monitored continuously. All tubing and connectors were of butyl rubber, norprene or polyvinylchloride to limit oxygen diffusion. For the same reason, the settler at the top of the reactor was flushed with argon.

Pulse experiments were conducted to measure the effect of different electron donors, electron acceptors and other medium components on the Anammox activity. A component was supplied to the reactor for 1 d and the effect was measured by following the effluent ammonium concentration. At least 1 week was allowed for the biomass to regain its original activity. During these experiments, the fluidized bed reactor was run continuously or on a few occasions, in an intermittent feeding mode. For these experiments, ammonium removal was controlled at 50% of the influent concentration by removal or addition of biomass, allowing the determination of positive or negative effects.

Operation and continuous experiments with the fluidized bed reactor run with synthetic medium

After the pulse experiments were finished, the influent of the fluidized bed reactor was changed to synthetic medium. The temperature was decreased to 30° C. For practical reasons, concentrated synthetic medium was supplied at a rate of 200 ml h^{-1} and diluted with tap water at a rate of 400 ml h^{-1}. Both the medium and the tap water were continuously flushed with argon to maintain anaerobic conditions.

The experiments with synthetic medium were started in batch mode using the original waste water, to ensure that the sludge was actively oxidizing ammonium anaerobically. Continuous feeding of the synthetic medium (5 mM NH_4^+, 5 mM NO_2^-) to the reactor was started after the ammonium concentration reached zero. The influent concentration was raised by steps of 5 mM over a period of 3 months until 30 mM was reached.

Anaerobic batch culture experiments with waste water or synthetic medium

Anaerobic batch experiments with waste water were conducted in 500-ml serum bottles under static anaerobic incubation in the dark at 37 °C, as described previously by van de Graaf *et al.* (1995). For every type of addition, a independent batch culture was used. Separately, a control batch with ammonium and nitrate was monitored during the whole course of the experiment.

Anaerobic batch experiments with synthetic medium were conducted in 30-ml serum bottles with static incubation in the dark at 30 °C. Each bottle contained 24 ml of mineral medium (pH 7) and 1 ml of a biomass suspension obtained from the enrichment culture grown on synthetic medium.

Ribulose 1,5-bisphosphate carboxylase (RuBPCase) assay

Sludge was harvested from the fluidized bed reactor, separated from the sand particles, and, after centrifugating (10 000 *g*, 10 min), washed with a buffer containing 100 mM Tris HCl, 20 mM $MgCl_2$, 2.0 mM $NaHCO_3$ (pH 8.2). For preparation of cell-free extracts, the cells were resuspended in the same buffer with 5 mM dithiothreitol (DDT) to a biomass concentration of approximately 30 mg dry weight ml^{-1} and were disrupted by sonication at 4° C in an MSE 150 W sonifier (eight burst of 30 s with intermittent cooling). Intact cells and debris were removed by centrifugating (20 000 *g*, 20 min). RuBPCase was assayed as described by Beudeker *et al.* (1980).

$^{14}CO_2$-fixation assay

Sludge was harvested from the fluidized bed reactor. After settling, the sludge was rinsed four times with anaerobic tap water. The same volume of medium without substrate was added to the biomass. The cells were separated from the sand particles by passing it several times through a 60 ml syringe. Sludge suspension (1 ml) and 9 ml medium containing 2 mM $KHCO_3^-$ (pH 7) was incubated in 25 ml flasks with butyl rubber septa. After flushing the flasks with argon for 10 minutes, and preincubation at 30°C, 100 µl of a ^{14}C-labelled bicarbonate solution (stock 50 mM $NaH^{14}CO_3$, 31.45 GBq mol^{-1}) was introduced. The flasks were incubated in the dark at 30°C. At predetermined time intervals, 0.5 ml samples (in duplicate) were taken to measure the $^{14}CO_2$-incorporation. At the same time, samples for ammonium and nitrite determinations were taken from separately incubated flasks without labelled CO_2. The samples for $^{14}CO_2$ measurements were washed three times with 25 mM KH_2PO_4buffer (pH 8) and transferred to scintillation bottles for counting.

Determination of aerobic nitrifiers

Estimates of ammonium and nitrite-oxidizing populations were made by the most-probable-number (MPN) method according to Alexander (1982). The medium (pH 8) used for counting the ammonium-oxidizing bacteria contained (per l demineralized water): $(NH_4)_2SO_4$, 330 mg; KH_2PO_4, 66.7 mg; $MgSO_4.7H_2O$, 40 mg; $CaCl_2.2H_2O$, 15 mg; 1 ml of each trace element solution described by Schmidt and Belser (1982) and 1 ml of phenol red stock solution of (0.5 g/l). The medium (pH 7) used for counting the nitrite oxidizing bacteria contained (per l demineralized water): $NaNO_2$, 69 mg; KH_2PO_4, 13.6 mg; K_2HPO_4, 69.6 mg; $MgSO_4.7H_2O$, 200 mg; $CaCl_2.2H_2O$, 15 mg; and 1 ml of each trace element solution (Schmidt and Belser, 1982). Six-week incubations were done at 30 and 26°C in the dark for ammonium and nitrite oxidizers, respectively. Petri dishes (Sterilin) of 100x100 mm with 25 square holes filled with 5 ml medium were used to incubate the MPN dilutions. The result of a MPN test was considered to be positive when either nitrite formation could be detected, or when nitrite had disappeared and nitrate had been formed. Every incubation series was done in duplicate and several times a pure culture of *Nitrosomonas europaea* LMD 86.25 (Culture collection of the Department of Microbiology and Enzymology, Delft, The Netherlands) and *Nitrobacter winogradskyi* (kindly provided by Drs. W. de Boer & H.J. Laanbroek, Heteren, The Netherlands) were used as controls.

Thin-layer chromatography (TLC)

Cells were harvested from the fluidized bed reactor and, after removal of the sand particles, freeze-dried and stored before use. TLC analysis of whole-cell methanolysates were assayed as described by Ross *et al.* (1981).

Electron microscopy

Cells were fixed in glutaraldehyde, embedded in Spurr and stained with osmium tetroxide and ruthenium red. Ultra-thin sections were studied in a Philips EM 201.

Cytochrome spectra

The spectra were measured with a Aminco DW-2a dual-wavelength spectrophotometer (American Instruments) which was equipped with computer data acquisition (kindly made available by Dr. A.H.Stouthamer of the Free University of Amsterdam). The cell suspensions in 50 mM phosphate buffer were measured at room temperature and 77K. The protein content of the suspensions were measured and the spectra were all normalized at 1 mg protein ml^{-1}. The spectrophotometer was equipped with a magnetic stirrer and the cuvettes were closed by a lid fitted to allow continuous flushing of gas and making of additions. The cytochromes were reduced by 60 µl dithionite solution (0.1 g ml^{-1}). For CO-spectra, this gas was allowed to react for 15 min with the cytochromes. To remove the CO-ligands, the frozen suspension was exposed to an intense light source for five intervals of 10 s (Intralux 5000, 185 W). All spectra were measured at a reference wave length of 540 nm. For further handling of the spectrum data, the methods and software described by van Wielink *et al.* (1982) were used.

Analytical procedures

Nitrate, nitrite, ammonium, sulphide and sulphate were determined as previously described (van de Graaf *et al.*, 1995). Hydroxylamine was determined colorimetrically (Frear and Burell 1955). Nitrous oxide formation was checked using a GC (Hewlett Packard model 428) with catharometeric detection. Dry weight was determined by drying the sample at 65° C for at least 24 h. The quantity of sand in the dried sample was measured after ashing at 700° C for 1 h. The dry weight minus the ashed weight is hereafter termed volatile solids (VS). Protein for the cytochrome spectra was determined according to Herbert *et al.* (1971).

Chemicals

Ribulose 1,5-bisphosphate was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), [^{14}C]$NaHCO_3$ (2.11 TBq mol^{-1}) from Amersham International. All other chemicals used were reagent grade and obtained from commercial sources.

Results

Anaerobic ammonium oxidation using waste water

Batch experiments

The first attempts to run Anammox on a simple synthetic denitrification (mineral) medium with sulphide and acetate as electron donors failed. Analysis of the influent and effluent of the original denitrification reactor revealed no unusual or unexpected (changes of) inorganic components. To obtain more information about specific requirements for Anammox, batch experiments were carried out with waste water supplemented with various chemicals (Table 1). The addition of 5 or 50 mM phosphate, commonly used as

TABLE 1 Effect of additions of various compounds on the Anammox activity in batch experiments with waste water or in continuous experiments with a fluidized bed reactor.

Compound	Concentration	Effect on Anammox activity
Batch experiments		
Acetate	1 or 5 mM	Increase[a]
Propionate	1 mM	No effect
Glucose	1 mM	Increase[a]
Fructose	1 mM	Increase[a]
Lactate	1 mM	No effect
Casamino acids	50 mg/l	No effect
Sulphide	1 or 5 mM	Increase
Sulphur	1 or 5 mM	Increase[a]
Sulphite	1 mM	Increase
Thiosulphate	1 mM	Increase[a]
KCl	50 mM	No effect
$KHCO_3$	20 or 40 mM	No effect
EDTA	100 mg/l	No effect
KH_2PO_4	5 or 50 mM	Loss of activity
KH_2PO_4	1 mM	No effect
Continuous experiments		
Acetate	2 mM	- 28 %
Glucose	1 mM	- 12 %
Pyruvate	1 mM	- 20 %
Formate	5 mM	- 10 %
Cysteine	0.8 mM	- 11 %
Sulphide	2 mM	+ 20 %[a]
Sulphide	2 mM	+ 60 %[a,b]
Thiosulphate	2 mM	+ 47 %[a]
Hydroxylamine	2 mM	+ 28 %
N_2O	0.7 mM	No effect
Nitrite	2, 4 or 6 mM	+ 11, 24, or 51 %

[a] Nitrite formation
[b] Addition during intermittent feeding of the reactor: 0.5 h feeding/ 0.5 h batch.

pH buffer in batch experiments, caused complete loss of the ability to oxidize ammonium anaerobically, but 1 mM phosphate was tolerated. Addition of 50 mM KCl or 40 mM $KHCO_3$ had no effect. Phosphate was thus the cause of inhibition. Electron donors, both organic and inorganic increased the ammonium oxidation rate. However, in almost all cases, this positive effect was accompanied by transient nitrite formation. At the end of a typical batch experiment, nitrite became detectable at the moment that all ammonium was used. When excess nitrate (5 mM) was present, the addition of nitrite accelerated the ammonium oxidation rate.

Continuous experiments

In batch experiments carried out with a large amount of biomass, the precise influence of added components on the growth of anaerobic ammonium oxidizers could not be measured. These experiments only provided insight into the inhibitory or stimulatory potential of certain components. Continuous runs and a few intermittent feeding experiments were carried out to investigate the effect of adding electron donors and electron acceptors on the Anammox activity. Organic electron donors (Table 1)

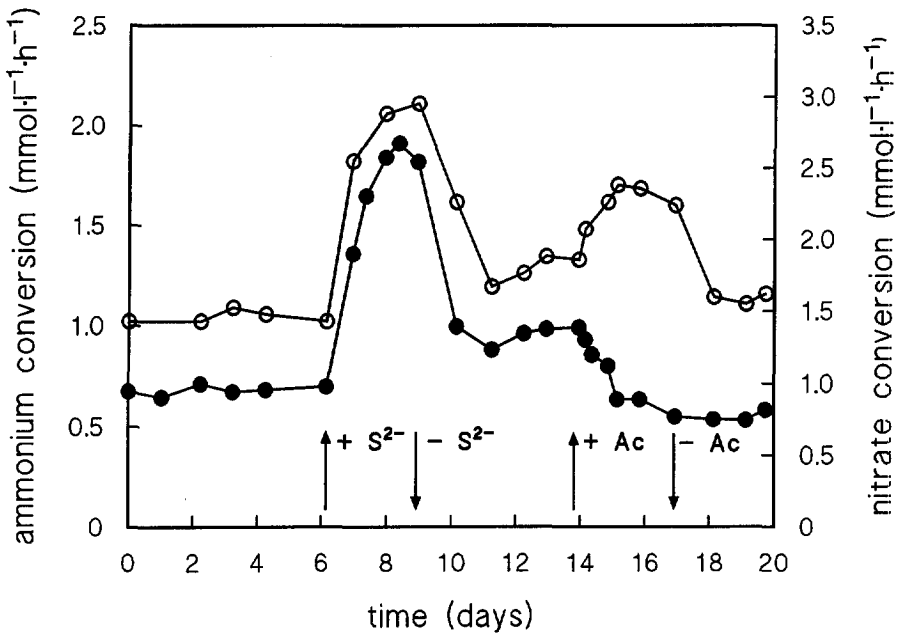

Figure 1 The effect of sulphide and acetate pulses on the Anammox activity in a fluidized bed reactor intermittently fed with waste water. S^{2-}, pulse of 2 mM sulphide; Ac, pulse of 2 mM Acetate. Symbols: ●, NH_4^+; ○, NO_3^-.

decreased the Anammox activity in all cases, while inorganic sulphur-based electron donors as thiosulphate and sulphide had a positive effect. Figure 1 shows the changes in the ammonium and nitrate conversion rates in the reactor during a sulphide or acetate pulse. Adding trace elements had no effect, indicating that no trace elements were missing in the original waste water. The provision of nitrite as an extra electron acceptor increased the ammonium conversion. This indicated that nitrite was necessary for Anammox activity, and that the stimulation of the additional sulphur sources might be due to nitrite formation from nitrate. During intermittent medium supply, the effect of increasing the inlet sulphide concentration was even more pronounced. This could imply that pulse-like sulphide additions caused more nitrite formation per molecule supplied. By increasing the nitrite concentration to 6 mM an almost complete conversion of ammonium (6 mM) could be accomplished. Light had a negative effect (30-50%) on the ammonium removal rate. During all following experiments the equipment was always covered with black plastic and paper to eliminate this effect.

Anaerobic ammonium oxidation on synthetic medium

Start-up and operation with 'autotrophic' synthetic medium

It was possible to start-up and maintain an Anammox fluidized bed culture, using an 'autotrophic' mineral medium with ammonium, nitrite and carbonate (see Material and Methods). Inoculation with the original biofilm still attached to the sand particles facilitated the start-up considerably. Characteristic start-up results are shown in Figure 2. The feed of ammonium was increased stepwise up to 25 mM, with a concomitant stepwise increase in nitrite. The highest feed level tested was 35 mM nitrite and 30 mM ammonium. Dinitrogen gas production increased with increasing feed load. A conversion of 3.1 kg NH_4^+ m^{-3} day^{-1} was achieved with a feed of 30 mM NH_4^+. A total of 15 runs have been carried out with synthetic medium, the longest lasting over 7 months. During the runs the colour of the culture changed slowly from brown to red. Two to three months were required to obtain completely red biolayers and formation of new biofilm on the sand.

General observations

When cysteine (to reduce the redox-potential) or pyruvate (to supply electron donor (Abeliovich and Vonshak, 1992) were added to the medium, a steady reduction of the Anammox activity (20% activity loss per 6 d; feed 5 mM NH_4^+) was observed. If a large excess of nitrite (8 mM) and ammonium (8 mM) was suddenly supplied, all activity was lost. However, the Anammox activity could be recovered by flushing the column with

anoxic tap water until nitrite concentrations decreased below 10 mg l^{-1}, resulting in a steep (-350 mV) decline in the redox potential. When the feed was restarted (sometimes at a lower feed load) gas bubbles appeared within 1 h and the nitrite and ammonium levels fell. During the first runs a calcium concentration of 6 mg l^{-1} was used. This concentration was increased to 50 mg l^{-1}, since it was shown by Austerman-Haun *et al.* (1993) to improve biofilm formation. Visible growth of red biomass on the sand particles was then observed.

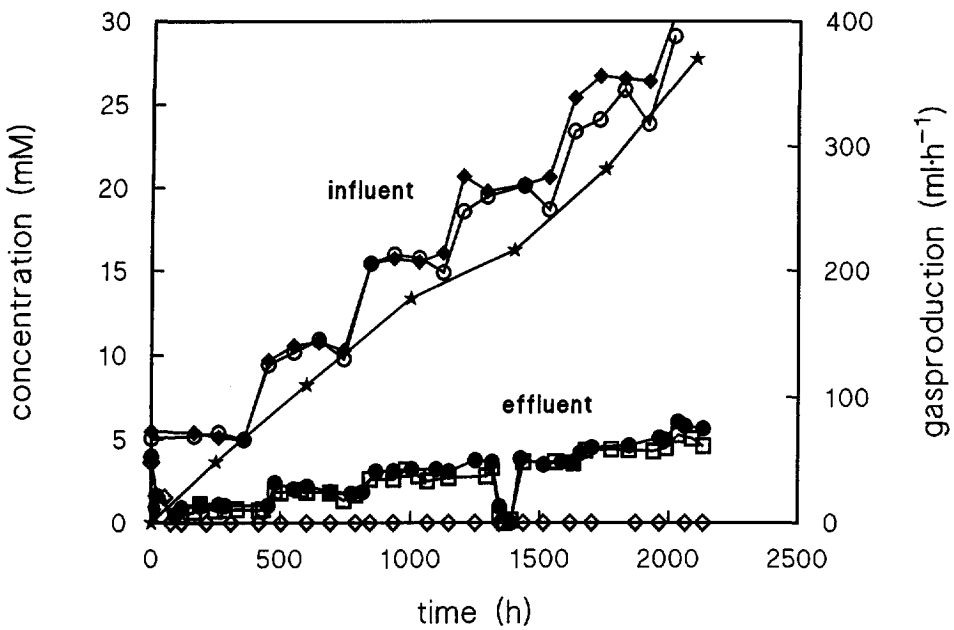

Figure 2 Start-up and continuous operation of the fluidized bed system on synthetic medium and gas production during increase of the ammonium and nitrite load. Symbols: influent: ○, NH_4^+ and ♦ NO_2^-; effluent: ●, NH_4^+; □ NO_3^-; ◇, NO_2^-; ★, gas production.

Nitrogen balance

Instead of the expected 1:1 removal of ammonium and nitrite predicted from the equation of Broda (1977), a 1.3:1 ratio for the NO_2^-:NH_4^+ removal was found. This appeared to be due to the formation of nitrate. During the first experiments on synthetic medium, 2 mM nitrate had been added to the feed in order to keep the redox potential at approximately 150 mV. This was also the case when waste water with excess nitrate

was used. Once it was discovered that nitrate was formed during the Anammox process, it was omitted from the feed. Nitrate production accounted for 10% of the total nitrogen feed. From four independent runs, 31 nitrogen balances were made. This gave a ratio of NH_4^+-conversion:NO_2^--conversion:NO_3^--production of $1:1.31\pm0.06:0.22\pm0.02$. Hydroxylamine, an intermediate of aerobic ammonium oxidation, was not detected in the effluent, and N_2O was not detected in the gas produced. However, if the system was disturbed (e.g. by failure of pH regulation), N_2O was formed in small amounts (0.3% of total gas production).

Specific anaerobic ammonium oxidation activity

The specific Anammox activity of the biomass in the fluidized reactor being fed with 25 mM ammonium and nitrite was 700-800 nmol NH_4^+ h^{-1} (mg VS)$^{-1}$. The feeding rate of this reactor could be doubled instantaneously without causing problems, implying that the maximum specific activity of the biomass in the reactor was 1300-1500 nmol NH_4^+ h^{-1} (mg VS)$^{-1}$. However, when fresh samples from this reactor were tested for their Anammox activity in batch culture, the activity was only half this level (i.e. 300-400 nmol NH_4^+ h^{-1} (mg VS)$^{-1}$. This was most probably due to inhibition by the higher nitrite or ammonium concentration initially present in the batch experiments.

Autotrophic growth of anaerobic ammonium oxidizers

CO_2-fixation

CO_2-fixation was dependent on Anammox activity as shoMw in Figure 3. There was no incorporation of $^{14}CO_2$ in the control experiments without NH_4^+ or NO_2^-. The CO_2 incorporation rate was 12.5 nmol C h^{-1} (mg VS)$^{-1}$ and the NH_4^+ conversion rate was 307 nmol NH_4^+ h^{-1} (mg VS)$^{-1}$. Hence, the biomass yield of anaerobic ammonium oxidation was 0.041 mol C incorporated per mol of NH_4^+ oxidized. Conversion of 20 mmol NH_4^+ will give a biomass formation of 20 mg dry weight (assuming a biomass carbon content of 50%).

Other biomass measurements

When the biomass in the fluidized bed reactor had reached the level of the overflow, a kind of pseudo-steady situation was obtained. The amount of biomass which left the reactor at this time was therefore a measure for the biomass production. During a period of 16 days, the dry weight of the settled and suspended material was estimated to be 18-34 mg VS $(l_{feed})^{-1}$. This is of the same order as the 20 mg calculated from the CO_2 fixation.

RuBPCase

Since HCO_3^- was the only carbon source provided, the Anammox micro-organisms are presumably autotrophs. Measurements to demonstrate an increase of the activity of RuBPCase, the key enzyme of the Calvin Cycle, between the original sludge and the enrichment were done. Although the cell-free extract of the original sludge grown on waste water possessed a low RuBPCase-dependent CO_2-fixation activity of 1.02 nmol min^{-1} (mg protein)$^{-1}$, the cell-free extract of the enrichment had only an activity of 0.28 nmol min^{-1} (mg protein)$^{-1}$. The activity of the enrichment was just above the detection limit (i.e. only twofold higher than the control).

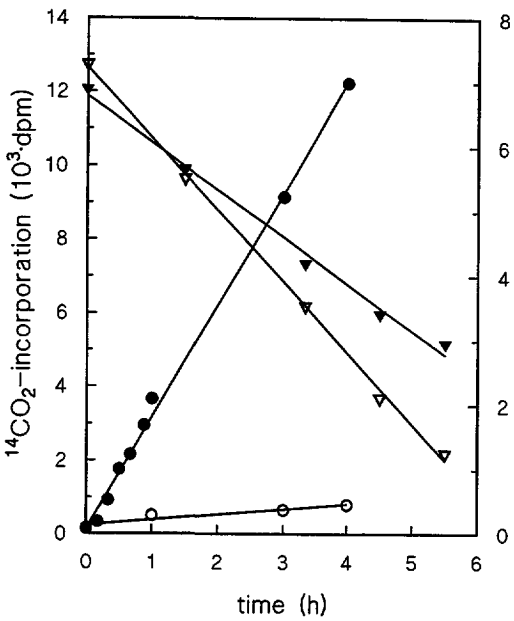

Figure 3 Incorporation of $^{14}CO_2$ in a sample of an enrichment culture of Anammox micro-organisms during oxidation of ammonium with nitrite. Symbols: ●, dpm $^{14}CO_2$ incorporated when incubated with NH_4^+ and NO_2^-; ○, dpm $^{14}CO_2$ incorporated in controls with neither NH_4^+ nor NO_2^-; ▼, NH_4^+ concentration and ▽, NO_2^- concentration in control incubations without labelled CO_2.

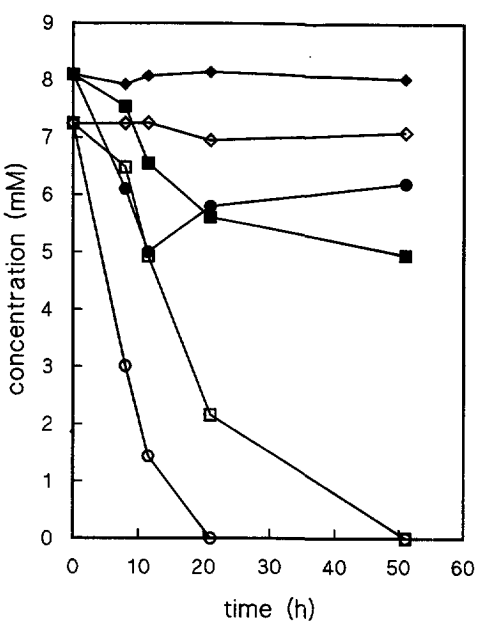

Figure 4 Effect of oxygen on the Anammox activity in batch cultures.
Symbols: anaerobic control: ●, NH_4^+ and ○, NO_2^-; static aerobic culture: ■, NH_4^+ and □, NO_2^-; shaken aerobic culture (100 rpm): ◆, NH_4^+ and ◇, NO_2^-.

However, for the (extremely slow) growth of the Anammox sludge, CO_2 fixation at a rate of only 0.8 nmol C min^{-1} (mg protein)$^{-1}$ would be required. Sulphide, one of the major electron donors for the original sludge (Mulder *et al.*, 1995) might explain the presence of some autotrophic metabolism in the original sample. Additional tests were done to examine whether some components in the cell-free extract had an inhibitory effect on the enzyme activity. Mixing on a 1:1 basis with cell-free extract of *Nitrosomonas europaea* gave the expected dilution of activity from 12.1 to 6.15 nmol min^{-1} (mg protein)$^{-1}$.

Effect of dioxygen

The amount of O_2 leaking through the tubing and connections of the fluidized bed system was measured with a Clark-type oxygen electrode in a separate reactor without biomass. The reactor was made anaerobic by flushing with dinitrogen gas through the bottom and an argon flush through the head space of the column for 30 min. Argon was continuously used in the reactor during operation, but was discontinued for the measurement of potential oxygen leakage. The initial rate of oxygen leakage was 20 µmole O_2 h^{-1}. When the argon flush was maintained during the measurements, oxygen leakage was not detected. Even in the worst-case situation, the amount of oxygen would only be enough to oxidize 0.2% of the total amount of 12 mM ammonium and 15 mM nitrite converted per hour.

In a few batch experiments, oxygen was deliberately introduced by shaking cultures with an aerobic head space. This caused complete inhibition of the anaerobic ammonium conversion (Figure 4). In static cultures exposed to air (through a cotton plug) the anaerobic ammonium oxidation and nitrite reduction started after a delay, presumably after facultative anaerobic bacteria removed the oxygen dissolved in the medium and a O_2-gradient had been established, thus providing an anaerobic environment at the bottom of the flasks (Kato *et al.*, 1993).

Aerobic nitrifiers

The standard, aerobic MPN method showed that aerobic nitrifiers were present in the sludge at all times. During the enrichment for Anammox micro-organisms on synthetic medium, the number of nitrifiers present in the culture did not increase: ammonium oxidizers stayed at about $9\pm5 \times 10^3$ cells (mg VS)$^{-1}$ and nitrite oxidizers at $1.0\pm0.9 \times 10^3$ cells mg VS^{-1}.

Electron micrographs of the aerobic MPN cultures showed the characteristic

membrane structures of *N. europaea*, and immunofluorescence microscopy with antibodies against *N. europaea* gave a positive reaction. Addition of penicillin G (35 mg l^{-1}) completely inhibited growth of the MPN cultures. Attempts to isolate the nitrifiers were unsuccessful, due to persistent contamination by heterotrophs.

Effect of inhibitors on Anammox activity

The specific inhibitors (hydrazine, acetone, N-serve, allylthiourea) for the first step of aerobic ammonium oxidation did not effect the Anammox activity. However, acetylene inhibited the Anammox process by 87% compared with the control. Penicillin G, penicillin V, and specific inhibitors or stimulators for methanogens (bromoethane sulfonic acid) and sulphate reducers ($NaMoO_4/Na_2SO_4$) had no effect. Chloramphenicol, which can have a direct inhibitory effect on denitrification enzymes, did not inhibit the initial rate of anaerobic ammonium oxidation.

TLC of ester and ether lipids

The level of ether lipids increased during the enrichment for Anammox micro-organisms (Figure 5). Control experiments with methanogenic sludge, *N. europaea*, and *Methanothrix soehngenii* served as reference. The ester lipids typical for (eu)bacterial membranes remained present during the enrichment.

Cytochrome spectra

During the enrichment of anaerobic ammonium oxidizers on synthetic medium, the colour of the biomass changed from brownish to red. Spectra taken from whole cells showed a clear increase in typical haem spectra, in particular of the cytochrome *c* (540-554; α-region, Figure 6). Measurements at higher wavelengths (700-1100) showed that the characteristic maxima of bacteriochlorophyll *a* and *b* (Stanier *et al.*, 1986) were absent. The presence of carotenoids of the spheroidene group giving peaks between 450 and 550 nm was also not evident. The spectra of reduced cell suspensions at 77K revealed the absence of cytochromes *a* (600-605; location of terminal oxidase), *b* (554-564 nm) and d_1 (662-665 nm; cd_1-containing nitrite reductase). Spectra recorded in the presence of CO showed that the CO gas was binding to cytochromes *c*. No oxidases of *b* type cytochromes were observed. Interestingly, during the increase in Anammox activity a peak at 470 nm gradually increased in intensity. This spectroscopic feature reacted irreversibly with CO, as indicated by a shift from 470 to 450 nm.

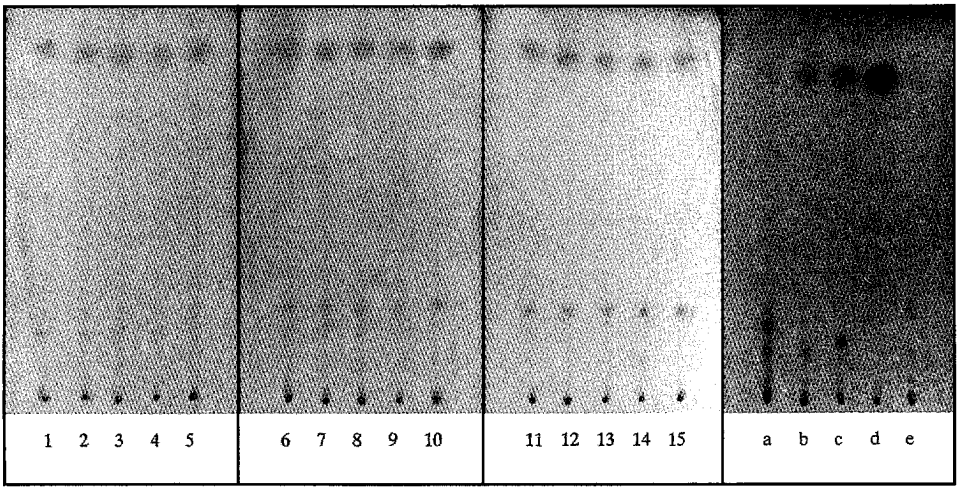

Figure 5 Increase of ether lipid content of whole-cell methanolysates during the enrichment for Anammox micro-organisms compared with other cultures. Time after start of enrichment: lane 1, 0 d; lane 2, 0 d; lane 3, 6 d; lane 4, 14 days; lane 5, 22 d; lane 6, 31 d; lane 7, 40 d; lane 8, 48 d; lane 9, 54 d; lane 10, 61 d; lane 11, 69 d; lane 12, 76 d; lane 13, 90 d; lane 14, 181 d; lane 15, 198 d. Lane a, methanogenic sludge; lane b, pilot plant Anammox sludge; lane c, Anammox enrichment culture; lane d, Nitrosomonas. europaea; lane e, Methanothrix soehngenii.

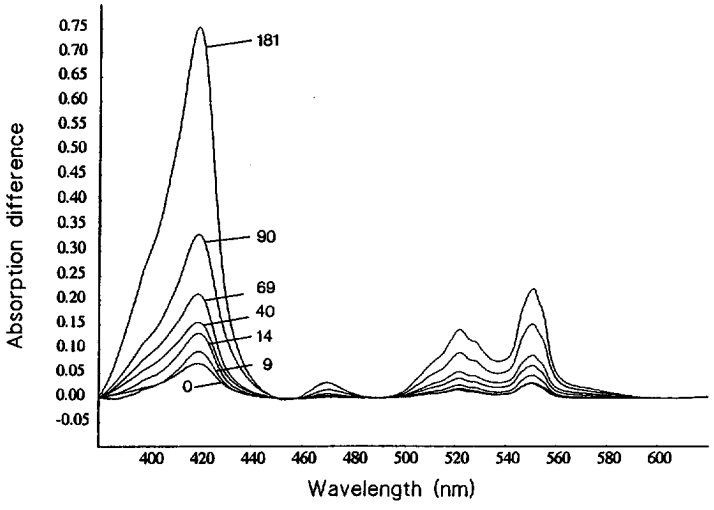

Figure 6 Increase in the cytochrome content during enrichment for Anammox micro-organisms. Cytochrome spectra were measured at 77K. The cells were reduced by dithionite in an argon atmosphere. The measured spectra were all normalized to 1 mg protein ml^{-1}. The numbers on the curves represent the amount of days after the start-up. Note typical peak at 470 nm.

Morphology

The dominant micro-organism of the enrichment culture was Gram-negative, with an unusual and irregular morphology as shown in Figure 7. The cells were usually seen as single cells or in pairs, the latter possibly being a division state. When the biofilm was disrupted and the cells were allowed to settle for two or more days in a centrifuge tube the clear appearance of the red colour was even more pronounced. The organisms from this sediment could easily be collected with a Pasteur pipette. Electron micrographs of this fraction showed an almost pure culture of the already dominant type of cells.

To gain an indication of the amount of the numbers of the apparently dominant type in the enrichment culture, a large series of electron micrographs were taken from different section of imbedded samples. After 177 days of enrichment 64% of all cells counted (total count = 11433) was of the described dominant type (Figure 7). This is a fourfold increase in numbers compared with the inoculum used for the start-up of the process (16%; total count = 10200). The dominant organisms appear to occupy a much larger volume than the other organisms present in the material. In some parts of the biolayers of the enrichment cultures, a few autofluorescent cells reminiscent of methanogens were observed.

Discussion

The continuous experiments with the systematic, stepwise addition of various supplements to the waste water showed that nitrite was the main electron acceptor used in anaerobic ammonium oxidation, as was observed before by van de Graaf *et al.* (1995). It is likely that all other positive effects can be interpreted to the effect that they transiently stimulated (additional) nitrite production. The negative effect of prolonged addition of organic compounds, such as cysteine or pyruvate, may be due to the undesirable increase of a heterotrophic population overgrowing the organisms responsible for the Anammox process. Such a phenomenon is well known for overgrowth of aerobic nitrifiers by heterotrophs in the presence of organic materials (Tijhuis *et al.*, 1994).

The biomass responsible for the anaerobic ammonium oxidation can now be reproducibly grown autotrophically in an inorganic (synthetic) medium, with bicarbonate as the only carbon source. Considering the low growth rate (0.001 h^{-1}), it is not surprising that a biomass retention system, such as a fluidized bed reactor with biofilm attached to solid support, is required. Indeed, enrichment cultures in suspended continuous cultures, even at a dilution rate as low as D = 0.01 h^{-1} failed, and complete conversion of ammonium only occurred in this system when sufficient biomass had accumulated on the fermentor walls.

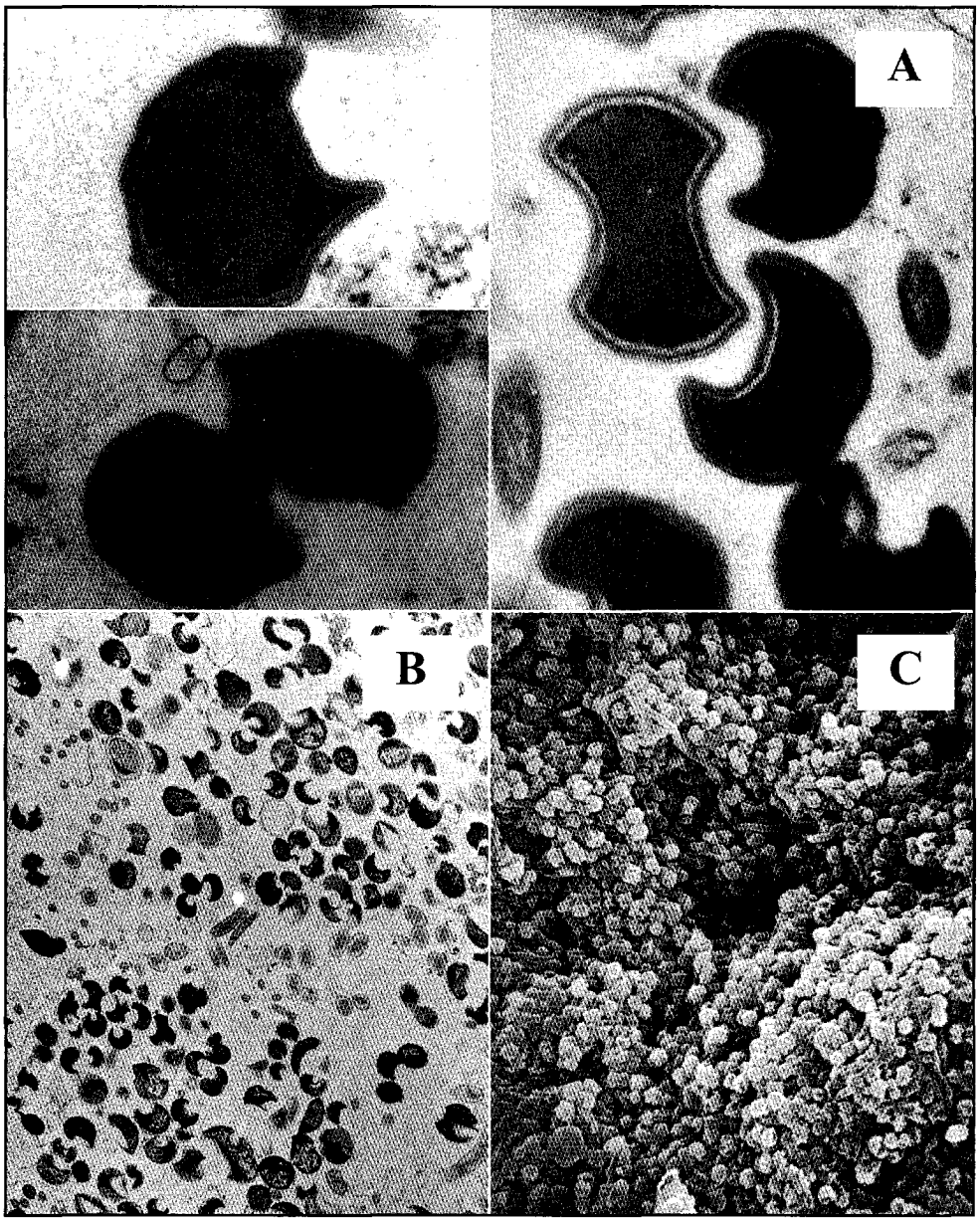

Figure 7 Electron micrograph of resuspended Anammox biofilm grown on synthetic medium in a fluidized bed reactor. The dominant species in the enrichment culture.
(A) The dominant species in the enrichment culture.
(B) An overview of the enrichment culture; 64% of the dominant, irregular species.
(C) SEM photograph (25 keV, 50 pA) of the biofilm surface of the enrichment culture.

It has been shown that nitrite, an intermediate in nitrate reduction, can accumulate in waste-water-treatment systems during changes in the reactor loading (Gommers *et al.*, 1988). It is therefore logical to suggest that the Anammox process with nitrate as electron acceptor could be the overall reaction of at least two types of bacteria, one of which reduces nitrate to nitrite using sulphide or degradable organic material as an electron donor. The second species would be the one that oxidizes ammonium with nitrite as the electron acceptor. Due to the direct supply of nitrite, the nitrite-providing nitrate reducing organism would no longer be necessary in the anaerobic ammonium oxidizing enrichment culture using synthetic medium. The observed difference between the inhibitory effect of chloramphenicol on the original sludge (van de Graaf *et al.*, 1995) and the non-inhibitory effect of chloramphenicol on the enrichment culture could be explained by assuming that the nitrite-producing reaction was the one sensitive to chloramphenicol. Given the extremely low growth rate of the new enrichment, a short term (24 h) effect of chloramphenicol (i.e. inhibition of protein synthesis) is unlikely.

The role of nitrate formation is not yet clear. The most likely explanation is that reducing power is required for CO_2-fixation. When the general formula 'CH_2O' for biomass is used, the production of 0.22 mol of NO_3^- from NO_2^- would allow the fixation of 0.0425 CO_2 according to the reaction:

$$1\ NH_4^+ + 1.31\ NO_2^- + 0.0425\ CO_2 \dashrightarrow 1.045\ N_2 + 0.22\ NO_3^- + 1.87\ H_2O + 0.09\ OH^- + 0.0425\ CH_2O$$

The anaerobic conversion of 20 mM ammonium (with 0.22 mol NO_3^- as a by-product) would thus allow the production of 26 mg dry weight. This is close to the observed 20 mg biomass produced. This is of the same order of magnitude as aerobic ammonium oxidizing bacteria such as *N. europaea*, which produce 25 mg dry weight from the same amount of ammonium (de Bruijn *et al.*, 1995). The possibility that nitrate would be formed by oxidation of NO_2^- to NO_3^- by *Nitrobacter* species is very unlikely in view of the very low quantity of O_2 penetrating into the reactor.

The original sludge had an anaerobic ammonium removal rate of 0.4 kg N m^{-3} d^{-1} (Mulder *et al.*, 1995). After enrichment with synthetic medium the ammonium removal rate was 3 kg NH_4^+ m^{-3} d^{-1} (equivalent to 2.4 kg N m^{-3} d^{-1}). The total nitrogen removal of the Anammox-reactor, including the conversion of nitrite, was 4.8 kg N m^{-3} d^{-1}. In aerobic, nitrifying fluidized bed reactors, ammonium conversion rates of 1.8 - 2.9 kg N m^{-3} d^{-1} have been obtained, depending on the pre-treatment system (Mulder *et al.*, 1986). Higher nitrification rates of 5 kg N m^{-3} d^{-1} were recently reached with a biofilm airlift suspension reactor (Tijhuis *et al.*, 1994). Compared with these aerobic systems, the

Anammox process can contribute significantly to the nitrogen removal from waste water with low carbon content.

The consistent presence of the nitrifiers in the Anammox sludge suggests that they can survive long periods of anaerobiosis, as shown by Abeliovich (1987). Poth (1986) showed that a new isolate, identified as a *Nitrosomonas* species, was able to produce N_2 under anaerobic conditions. More recently, it has been shown that *N. europaea*, under strictly anaerobic conditions, utilized nitrite as electron acceptor, and pyruvate as energy source (Abelovich and Vonshak, 1992), and pure and mixed cultures of *N. eutropha* were able to denitrify with hydrogen and ammonium as electron donor (Bock *et al.*, 1995). If the consistent but low numbers of nitrifiers (10^3-10^4 (mg VS)$^{-1}$) present are compared with the value of $9x10^9$ cells (mg dry wt)$^{-1}$ (biomass of 35 mg dry wt l^{-1} and $3x10^8$ cell ml^{-1}) of a pure culture of *N. europaea*, their contribution to the anaerobic ammonium oxidation is highly unlikely. Even if the nitrification of the aerobic nitrifier was the same under anaerobic conditions as under aerobic conditions, their activity would be three orders of magnitude too low to explain the observed ammonium oxidation rate of 800 nmol NH_4^+ h^{-1} (mg VS)$^{-1}$.

The presence of ether lipids seems to be confined to the most ancient micro-organisms (e.g.the Archaea or the deepest phylogenetic branches within the Bacteria), thus suggesting that the ether linkage in the lipids could have appeared during the evolution of life before the ester types. Membrane lipids of the members of the order *Thermogotales* are based on ester and ether linkage (Gambacorta *et al.*, 1994). Also members of the genus *Aquifex* were found to possess diethers, but not archaeal diethers. Knowledge of the lipids can be used for taxonomic purposes, once catalogues of signature lipids have been developed (Gambacorta *et al.*, 1994).

The findings reported here, show that the observed increase in anaerobic ammonium oxidizing capacity of the enrichment is directly related to an increase in morphologically conspicuous micro-organisms, an increase in ether lipids and an increase in cytochromes. These properties might all be due to one and the same organism, although the possibility remains that two or more micro-organisms are responsible for the Anammox reaction. In the future, molecular techniques, such as 16S RNA analysis, will be carried out to unravel the consortium structure.

Acknowledgements

This research was supported by the Netherlands Foundation of Applied Research (STW), Gist-brocades and the Royal Netherlands Academy of Arts and Sciences. We thank H. Slijkhuis of

Gist-brocades and A. Mulder of TNO-IMW for their stimulating discussions. We thank especially M. Hazelaar for assistance and interpretation of the cytochrome spectra, W. Batenburg-van de Vegte for performing the electron and other microscopical photographs, Brian Jones for advice on the TLC work, and E. Dumoulin and F. van Velzen for their dedicated work on the fluidized bed reactors.

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Chapter 6

Metabolic pathway of anaerobic ammonium oxidation on basis of ^{15}N-studies in a fluidized bed reactor

Abstract

A novel metabolic pathway for anaerobic ammonium oxidation with nitrite as the electron acceptor has been elucidated using ^{15}N-labelled nitrogen compounds. These experiments showed that ammonium was biologically oxidized with hydroxylamine as the most probable electron acceptor. The hydroxylamine itself is most likely derived from nitrite. Batch experiments in which ammonium was oxidized with hydroxylamine transiently accumulated hydrazine. The conversion of hydrazine to dinitrogen gas is postulated as the reaction generating electron equivalents for the reduction of nitrite to hydroxylamine. During the conversion of ammonium, a small amount of nitrate was formed from some of the nitrite. The addition of NH_2OH to an operating fluidized bed system caused a stoichiometric increase in the ammonium conversion rate (1 mmol l^{-1} h^{-1}) and a decrease in the nitrate production rate (0.5 mmol l^{-1} h^{-1}). Addition of hydrazine also caused a decrease in the nitrate production. On the basis of these findings, it is postulated that the oxidation of nitrite to nitrate could provide the anaerobic ammonium-oxidizing bacteria with the reducing equivalents necessary for CO_2 fixation.

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Microbiology **143** (1997) 2415-2421.

Introduction

A strictly anaerobic process in which ammonium was used as the electron donor for denitrification has recently been described (Mulder *et al.*, 1995). During further examination of this novel anaerobic ammonium oxidation (Anammox) process, it became clear that nitrite was the most suitable electron acceptor, and that this process appeared to be carried out by autotrophic organisms (van de Graaf *et al.*, 1995, 1996).

Although the nitrification and denitrification reactions are often reported as rather simple equations, it is recognized that they may be more complex, and may involve the formation of various intermediates (Bock *et al.*, 1992; Jetten *et al.*, 1997; Kuenen and Robertson, 1994). The use of ^{15}N-labelled compounds in combination with mass spectrometry can help to elucidate the complex reaction, by providing information about processes involving gaseous N-compounds.

Previous experiments using ^{15}N-ammonium and $^{14}NO_3^-$ in an Anammox pilot plant reactor resulted in the production of $^{14-15}N_2$ (van de Graaf *et al.*, 1995). It was shown that ammonium and nitrate (via nitrite) contributed equally to the end product. Several pathways for these reactions are thermodynamically possible. The overall Gibbs free energy change ($\Delta G^{o'} = -358$ kJ mol^{-1}) for the total conversion and partial reactions were calculated using the values reported by Wood (1986) and Thauer *et al.* (1977). One possible mechanism could involve the reaction between hydroxylamine and nitrite to form N_2O ($\Delta G^{o'} = -270$ kJ mol^{-1}). The N_2O could thereafter be converted to dinitrogen gas, while ammonium is oxidized to hydroxylamine ($\Delta G^{o'} = -87$ kJ mol^{-1}). Alternatively, one could envisage a pathway (Figure 1), which involves the reaction between ammonium and hydroxylamine to form hydrazine ($\Delta G^{o'} = -47$ kJ mol^{-1}). The hydrazine could be converted to dinitrogen gas and four reducing equivalents ($\Delta G^{o'} = -271$ kJ mol^{-1}), which then are transferred to the nitrite reducing system to form hydroxylamine.

The aim of the work described here was to find an experimental basis for the possible metabolic pathways for anaerobic conversion of ammonium.

Materials and Methods

Biomass and mineral medium

An Anammox enrichment culture, grown in a fluidized bed reactor on synthetic medium containing ammonium and nitrite, was used for the labelled nitrogen experiments (van de Graaf *et al.*, 1996). When used in batch experiments, the biomass from the fluidized bed system was homogenized by passing it several times through a 60 ml syringe.

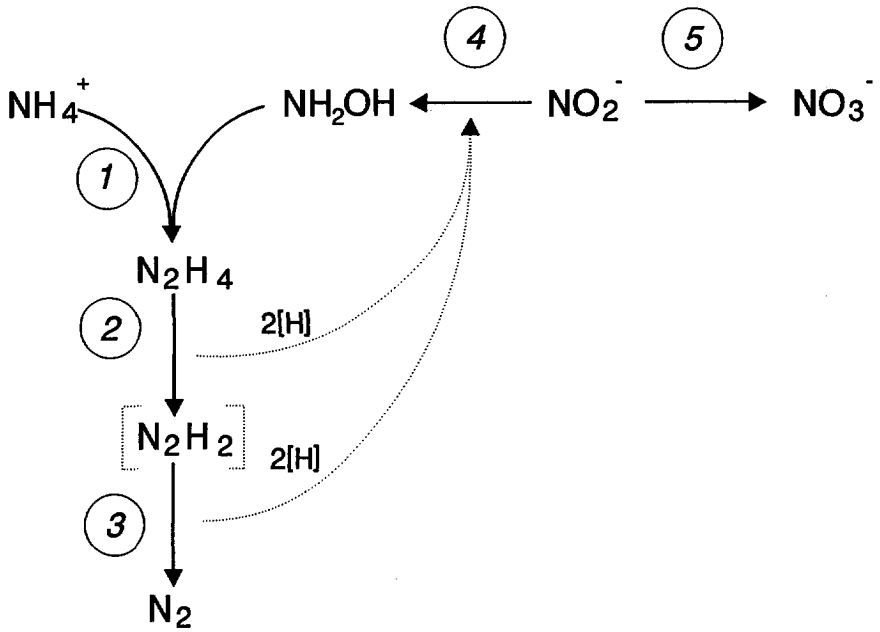

Figure 1 Possible metabolic pathway for anaerobic ammonium oxidation. Consumption and production of H_2O or H^+ is not indicated. Ammonium is oxidized by hydroxylamine to form hydrazine (step 1). Reducing equivalents derived from N_2H_4 then reduce nitrite to form even more hydroxylamine and N_2 (steps 2, 3 and 4). Nitrate formation could generate reducing equivalents for biomass growth (step 5).

^{15}N-experiments with the fluidized bed reactor

The 2.5 l glass fluidized bed reactor was operated at 30°C and pH 7. The pH was adjusted with 0.5 N H_2SO_4 or 0.5 N NaOH. The medium, operation and start-up were as previously described (van de Graaf *et al.*, 1996). All tubing and connectors were of butyl rubber, norprene or polyvinylchloride (PVC) to limit oxygen diffusion. For the same reason, the settler at the top of the reactor was flushed with argon gas. The medium was continuously flushed with argon gas to maintain anaerobic conditions. The fluidized bed reactor was fed with 30 mM ammonium and 35 mM nitrite, except during the last three experiments when 25 mM ammonium and 25 mM nitrite were used. The off-gas of the fluidized bed reactor contained 80-85% dinitrogen, 15-10% argon, 1-2% carbon dioxide and water vapor. Steady state values of in- and output of nitrogen compounds from one representative experiment were as follows (mmol l^{-1}): $NH_4^+{}_{in}$ = 29.7, $NH_4^+{}_{out}$ = 2.2; $NO_2^-{}_{in}$ = 34.7, $NO_2^-{}_{out}$ = 0.25; $NO_3^-{}_{in}$ < 0.01, $NO_3^-{}_{out}$ = 6.0; in- and outlet concentrations of NH_2OH and N_2H_4 were below detection level (<0.001 mM).

Labelled nitrogen compounds were supplied to the reactor for a 2 h period. The ^{15}N-solutions had previously been flushed with argon, and then kept under argon overpressure. When ammonium and nitrite were supplied, the normal feed was replaced by a solution in which 30

mM ^{15}N-ammonium or 35 mM ^{15}N-nitrite had replaced the ^{14}N-equivalents. The ^{15}N-hydroxylamine and ^{15}N-nitrate solutions were supplied separately giving inlet concentrations of 5 mM each. N_2O gas (100%) and/or NO (5% NO and 95% Helium) were added by pumping (2 mmol h^{-1} and 0.1 mmol h^{-1}) them directly into the bottom of the reactor. Control experiments were done by following the same procedure but using unlabelled compounds.

Samples of the effluent from the reactor were taken every 0.5 h before, during and after the ^{15}N-addition, and analyzed for ammonium, nitrate, nitrite, and hydroxylamine. After each ^{15}N-addition to the fluidized bed system, the effluent was collected for 0.5 h and stored at -18° C. This effluent was used for batch experiments with *Paraccocus denitrificans* (see below). At the end of each experiment, the influent to the fluidized bed reactor was changed to unlabelled synthetic medium. Between the different experiments, the composition of the gas from the fluidized bed system was monitored continuously with the mass spectrometer. One hour before the start, and at the end of the experiments with ^{15}N-labelled compounds, the gas composition was also monitored with a gas chromatograph in order to confirm whether N_2O was being produced.

Mass spectrometry and Nuclear Magnetic Resonance measurements

Changes in gas composition were monitored with an on-line quadrupole mass spectrometer (Hal Quadrupole Gas Analyzer; Faraday Cup; Hiden Analytical LTD, Warrington, England) kindly provided by Gist-brocades (Delft, The Netherlands). The sampling capillary from the mass spectrometer to the Anammox reactor was maintained at 80° C. This capillary was connected to the gas collector at the top of the fluidized bed reactor. The levels of $^{14\text{-}15}N_2$, $^{15\text{-}15}N_2$, ^{15}NO, $^{14\text{-}15}N_2O$, and $^{15\text{-}15}N_2O$ were monitored at *m/z* values (mass over charge ratio) of 29, 30, 31, 45 and 46, respectively.

Effluent samples were tested for the presence of dissolved ^{15}N-compounds with a NMR (Varian VXR-400S) prepared for nitrogen measurements by Dr. A. Sinnema, Department of Organic Chemistry, Delft University of Technology. The Netherlands.

Batch experiment with effluent from the fluidized bed reactor

The ^{14}N/^{15}N composition of nitrate remaining in the effluent from the fluidized bed reactor was determined with batch experiments. Serum bottles (40 ml) were filled with 25 ml of effluent containing $^{14}NO_3^-$ and/or $^{15}NO_3^-$, 5 ml of *Paracoccus denitrificans* suspension and 10 mM acetate. After flushing for 10 minutes with argon gas, cultures were incubated at 30° C. After 24 h, all nitrate had been reduced. The gas composition in the headspace of the bottles was then measured with the mass spectrometer. A calibration series with different known ratios of $^{14}NO_3^-$ and $^{15}NO_3^-$ was also made.

Block pulse experiments in fluidized bed reactor

The effects of hydroxylamine, nitrite and hydrazine on the conversion rate of ammonium in the fluidized bed reactor was determined by supplying each compound to the reactor for 24 h (block pulse). After 24 h, the volume had been replaced six times and it was assumed that the system had formed a pseudosteady-state. The effect was measured by following the effluent ammonium, nitrite, nitrate, hydroxylamine and hydrazine concentrations. Samples were taken

every 0.5 h prior to the start of the experiment, during the first 9 h of the addition of the pulse, for 9 h after the feed had returned to normal, and 24 h later.

Anaerobic batch culture experiments

Anaerobic batch experiments were done in 30-ml serum bottles under static incubation in the dark at 30°C (van de Graaf *et al.*, 1996). The concentrations of ammonium, nitrite, hydroxylamine, and hydrazine used were 7, 7, 3, and 5 mM, respectively. For each combination, four bottles were incubated, and a control experiment with ammonium and nitrite was included. Samples of the supernatant were analysed for ammonium, nitrite, hydroxylamine, and hydrazine.

Analytical methods and chemicals

Nitrate, nitrite, ammonium, hydroxylamine and dry weight were determined as previously described (van de Graaf *et al.*, 1996). Hydrazine was determined colorimetrically by means of the method described by Watt and Crisp (1952). Absorption was measured at 460 nm after 10 minutes incubation at room temperature. N_2O formation was measured using a GC (Hewlett Packard model 428) with a thermal conductivity detection.

^{15}N-labelled sodium nitrite (99%) and nitrate (99%) were obtained from MSD Isotopes, ^{15}N-labelled ammonium sulfate (98%) from Sigma and ^{15}N-labelled hydroxylamine.HCL (99%) from Cambridge Isotope Laboratories. All other chemicals used were reagent grade and obtained from commercial sources.

Results

^{15}N-labelling experiments

Three combinations of N isotopes are possible for N_2 produced from unlabelled ^{14}N and ^{15}N-labelled precursors: $^{14-14}N_2$ (*m/z* 28), $^{14-15}N_2$ (*m/z* 29) and $^{15-15}N_2$ (*m/z* 30). If other labelled gases are formed, this can be observed by changes in *m/z* 31, 45 and 46, representing ^{15}NO, $^{14-15}N_2O$ and $^{15-15}N_2O$ production, respectively. The results of the ^{15}N-labelling experiments are summarized in Table 1. During control experiments using unlabelled additions, none of the measured *m/z* ratios changed.

^{15}N-ammonium or ^{15}N-nitrite addition

The addition of $^{15}NH_4^+$ for 2 h to a fluidized bed reactor in the presence of $^{14}NO_2^-$ resulted in the change in the *m/z* 28, 29, 30 and 31 values as shown in Figure 2. The largest increase (87%) was observed at *m/z* 29 ($^{14-15}N_2$), while *m/z* 28 ($^{14-14}N_2$) simultaneously declined by the same amount. Apparently the dinitrogen gas formed contained one ^{15}N from ammonium and one ^{14}N from nitrite. The dominant product, $^{14-15}N_2$, made up 98.3% of the total labelled dinitrogen gas produced. Furthermore, a small amount of $^{15-15}N_2$ (*m/z* 30), in which both nitrogen atoms must have been derived from

ammonium, was also formed. *m/z* 45 and 46 did not increase during this experiment, indicating that labelled N_2O was not produced. The very small increase of 0.1% at *m/z* 31 was therefore probably due to the formation of traces of ^{15}NO.

The addition of $^{15}NO_2^-$ in the presence of $^{14}NH_4^+$ gave similar *m/z* changes (Table 1). Only the increase of $^{15-15}N_2$ (*m/z* 30) was slightly higher, probably because of some background denitrification in the mixed culture.

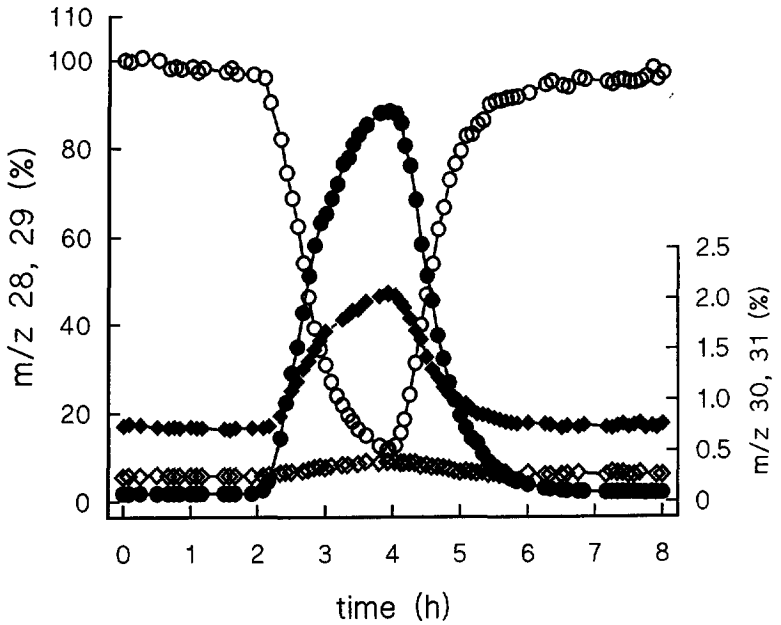

Figure 2 Changes in m/z 28, 29, 30 and 31 are shown as a percentage of the initial partial pressure of m/z 28, and as a function of time during an experiment with labelled $^{15}NH_4^+$ in a continuously-operated fluidized bed reactor running on synthetic medium. The $^{15}NH_4^+$ was supplied 2 h after the start of the measurements. Symbols: ○, m/z 28; ●, m/z 29; ♦, m/z 30; ◇, m/z 31.

^{15}N-hydroxylamine addition

Hydroxylamine was a potential intermediate in the metabolic pathway proposed for anaerobic ammonium oxidation (Figure 1). In order to test whether ^{14}N from ammonium or from nitrite would be coupled to the ^{15}N of hydroxylamine during the formation of the N-N-bond, experiments using combinations of either $^{15}NH_2OH$ and $^{15}NH_4^+$ or $^{15}NH_2OH$ and $^{15}NO_2^-$, were carried out. As shown in Table 1, the addition of $^{14}NH_4^+$, $^{15}NH_2OH$ and $^{15}NO_2^-$ resulted in $^{14-15}N_2$ being the dominant gas. This was also true when $^{15}NH_4^+$,

$^{15}NH_2OH$ and $^{14}NO_2^-$ were used, but the proportion of $^{15\text{-}15}N_2$ rose sharply, indicating a reaction between $^{15}NH_4^+$ and $^{15}NH_2OH$ in a ratio of 1:1.

TABLE 1 The effect of adding of ^{15}N-labelled components on the composition of gas from an Anammox culture grown in a fluidized bed reactor, shown as the abundance of the different atomic masses. The influent was an autotrophic mineral medium containing 30 mM ammonium and 35 mM nitrite. Hydroxylamine was added at 5 mM (see Materials and Methods).

^{15}N-labelled compound added	Percentage change in components with *m/z*:					
	28($^{14\text{-}14}N_2$)	29($^{14\text{-}15}N_2$)	30($^{15\text{-}15}N_2$)	31(^{15}NO)	45($^{14\text{-}15}N_2O$)	46($^{15\text{-}15}N_2O$)
$^{15}NH_4^+$	-87	+87	+1.4	+0.11	-	-
$^{15}NO_2^-$	-86	+85	+3.2	+0.04	-	-
$^{15}NO_3^-$	-1.3	+2.0	+0.04	-	-	-
$^{15}NH_2OH$	-13	+12	+0.4	+0.04	+0.08	-
$^{15}NH_4^+/^{14\text{-}14}N_2O$	-84	+88	+1.2	+0.14	+0.02	-
$^{15}NO_2^-/^{14\text{-}14}N_2O$	-85	+88	+2.3	+0.15	+0.02	+0.10
$^{15}NH_4^+/^{14}NO$	-86	+84	+1.1	+0.10	-	-
$^{15}NO_2^-/^{14}NO$	-85	+87	+2.9	+0.16	+0.02	+0.05
$^{15}NH_4^+/^{15}NH_2OH$	-85	+76	+13.8	+0.18	+0.04	-
$^{15}NO_2^-/^{15}NH_2OH$	-95*	+95	+5.5	-	-	-

-, not observed.
* after correction for changes in mass 40 of argon during these experiments.

^{15}N-nitrate addition and nitrate determination in effluent samples

As reported previously, about 10% of the total added N was oxidized to nitrate during the Anammox process (van de Graaf *et al.*, 1996). The addition of 5 mM ^{15}N-nitrate to the 6 mM ^{14}N-nitrate alr eady present in the reactor only resulted in a small increase in $^{14\text{-}15}N_2$ (*m/z* 29, Table 1). ^{15}N-NMR-analysis of the effluent samples showed that $^{15}NO_3^-$ was only formed from $^{15}NO_2^-$. When $^{15}NH_4^+$ was used, $^{15}NO_3^-$ was not detectable. This was confirmed by incubating the effluent from the Anammox reactor with *P. denitrificans*. After denitrification of any nitrate to dinitrogen gas, the composition of the N_2 was examined with the mass spectrometer. ^{15}N-labelled N_2 was only obtained when $^{15}NO_2^-$ had been used during the experiments. This is in agreement with the NMR-findings.

Effect of nitrous oxide or nitric oxide

The addition of $^{14\text{-}14}N_2O$ or ^{14}NO during experiments with $^{15}NH_4^+$ or $^{15}NO_2^-$ did not change the labelling pattern of the N_2 (Table 1). The chemical analysis of the effluent from the reactor also confirmed that N_2O or NO did not influence the overall

biochemical reactions in the culture. *Batch experiments with N_2H_4 and NH_2OH*

The results described above indicated that the pathway (Figure 1) combining NH_2OH and NH_4^+ to give the N-N-bond was probably operative. The first product expected would be hydrazine. Further experiments to confirm that hydrazine was an intermediate were therefore carried out with batch cultures or by block pulse addition (see next section).

Different combinations of nitrogen components were used to test the formation and consumption of possible intermediates. When hydrazine was added at the start of an experiment, it was used immediately. Control experiments without biomass confirmed that hydrazine was not converted chemically in the medium, but that conversion only occurred after addition of the Anammox sludge. During the conversion of hydrazine, ammonium removal was partly inhibited (results not shown). When the culture was provided with 3 mM hydrazine in the absence of an electron acceptor, 3.9 mM ammonium and 1 mM dinitrogen gas was formed.

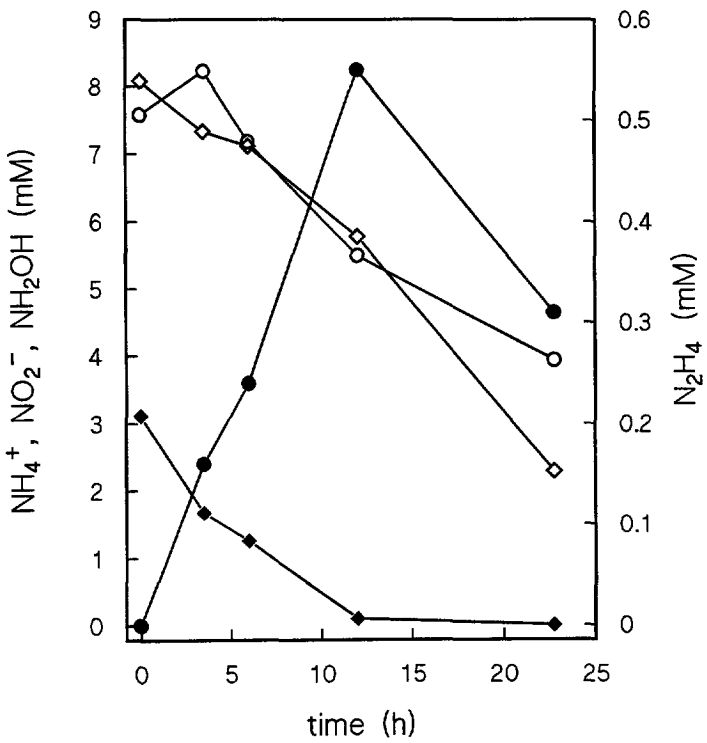

Figure 3 Concentration profiles during batch experiments with 3 mM hydroxylamine. Symbols: ○, NH_4^+; ◇, NO_2^-; ●, N_2H_4; ◆, NH_2OH.

When hydroxylamine was added at the start of a batch experiment, accumulation of hydrazine (up to 0.5 mM) could be determined, as shown in Figure 3. The hydrazine concentration only decreased when the hydroxylamine had been completely converted. When different combinations of ammonium, nitrite and hydroxylamine were tested, hydrazine was always found as an intermediate (not shown). Hydrazine production was most pronounced with the mixture of ammonium and hydroxylamine without nitrite. According to pathway in Figure 1, this would be due to the absence of the electron acceptor, nitrite, for the oxidation of the hydrazine. With the mixture of nitrite and hydroxylamine the N_2H_4 formation was only 20% of that observed when NH_4^+ was present. This is not unexpected as ammonium is required for hydrazine formation. Since a small amount of hydrazine was still formed, ammonium could have been produced from other processes (e.g. endogenous ammonium, or by reduction of nitrite or hydroxylamine). Nitrite was only significantly lowered after all the hydroxylamine had disappeared. In cultures where hydroxylamine and nitrite both were supplied, the hydroxylamine conversion was slower than in the absence of nitrite. Nitrate production was only detected in batch experiments with ammonium and nitrite. If hydroxylamine or hydrazine were added, nitrate did not accumulate.

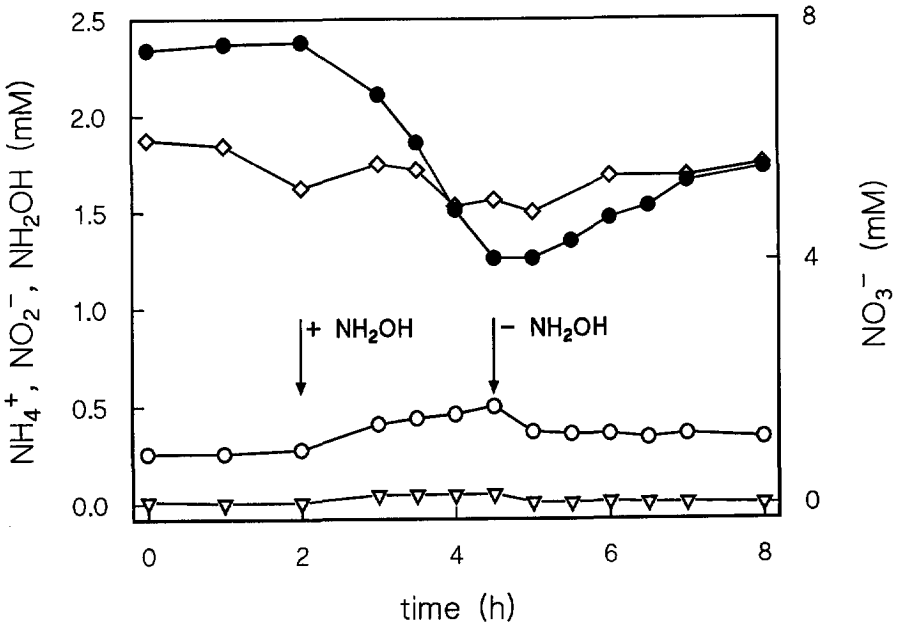

Figure 4 Effect of hydroxylamine on the anaerobic ammonium oxidation in a continuously-fed fluidized bed system. NH_2OH was added for 2 h. Symbols: ●, NH_4^+; ○, NO_2^-; ◇, NO_3^-; ▽, NH_2OH.

Effect of hydroxylamine, nitrite or hydrazine block pulse additions on the activity of an operating fluidized bed reactor

When $^{15}NH_2OH$ was supplied during experiments with the fluidized bed reactor (Table 1), there was a significant decrease in the ammonium and a slight increase in the nitrite concentrations in the effluent (Figure 4). To quantify this observation, hydroxylamine, nitrate and hydrazine were separately supplied to the fluidized bed reactor for longer periods. The changes in ammonium conversion and nitrate formation after each experiment are summarized in Table2.

When hydroxylamine was added to the influent, the ammonium consumption increased by a nearly equivalent amount, and nitrate production fell. For every mmol of hydroxylamine supplied, the nitrate production was reduced by 0.5 mmol. In contrast, the addition of nitrite increased nitrate formation, as has previously been described (van de Graaf *et al.*, 1996). When hydrazine was added, it was used immediately but incompletely, 0.3 mM and 1 mM were left after the addition of 3 and 6 mM, respectively. Hydroxylamine was not observed, and only about 0.04 mM nitrite was detected in the effluent. The provision of 3 mM hydrazine reduced the ammonium oxidation by 0.8 mM, while adding 6 mM hydrazine only caused a small increase in the ammonium concentration in the effluent (0.3 mM). Hydrazine also reduced the level of nitrate production in the fluidized bed reactor. The incomplete oxidation of hydrazine could be due to the absence of sufficient electron acceptor (nitrite), resulting in a decline in nitrate formation and ammonium oxidation. Some N_2O production (< 0.1%) could be detected in the off-gas when hydrazine was supplied to the reactors. Neither hydrogen nor methane (detection limit of 0.003%) were present in the off-gas.

TABLE 2 The effect of 24 hours supply of hydroxylamine, nitrite, or hydrazine on ammonium conversion and nitrate production in a continuously-operated fluidized bed reactor for anaerobic ammonium oxidation. The reactor was running on synthetic medium. Δ indicates the difference between the concentrations before the experiment started and after 24 hours.

Additive	NH_4^+/NO_2^- feed load (mM)	ΔNH_4^+ mM	ΔNO_3^- mM	$\Delta NH_4^+/\Delta NO_3^-$
NH_2OH (1 mM)	15/15	- 0.86	- 0.41	2.1
NH_2OH (2 mM)	15/15	- 2.17	- 1.03	2.1
NH_2OH (3 mM)	25/25*	- 3.19	- 1.63	2.0
NO_2^- (3 mM)	25/25	- 2.35	+0.73	
N_2H_4 (3 mM)	25/25	+0.76	- 1.75	
N_2H_4 (6 mM)	25/25	+0.33	- 2.51	

* During this experiment, H_2 was supplied in addition to NH_2OH to the fluidized bed reactor. This component had no additional effect on the anaerobic ammonium oxidation, as can be noticed from the constant ratio of $\Delta NH_4^+/\Delta NO_3^-$.

Discussion

Hydrazine

On the basis of the experiments with labelled nitrogen compounds described in this paper, a novel pathway for anaerobic ammonium oxidation is proposed (Figure 1). In this pathway, ammonium is biologically oxidized using hydroxylamine as the electron acceptor, resulting in the production of hydrazine. This hydrazine is then oxidized to dinitrogen gas. The occurence of hydrazine as an intermediate in microbial nitrogen conversions is rare. To our knowledge, hydrazine has only been proposed as an enzyme-bound intermediate in the nitrogenase reaction (Dilworth & Eady, 1991), where dinitrogen gas is reduced to ammonium. The conversion of externally added hydrazine to dinitrogen gas has also been documented for *Nitrosomonas europaea* (Hyman and Arp, 1995; Keener and Arp, 1994). In studies on the *de novo* synthesis of polypeptides in *Nitrosomonas* cells, hydrazine could serve as an energy source for nitrite reduction under anaerobic conditions (Hyman and Arp, 1995). In addition it has been shown that the purified hydroxylamine oxidoreductase from *Nitrosomonas* is capable of catalysing the conversion of hydrazine to dinitrogen gas (Hooper and Terry, 1977). The conversion rate for hydrazine by this enzyme was in the same range as that for hydroxylamine itself (approximately 40 μmol min^{-1} (mg protein)$^{-1}$). A similar enzyme system could be operative in the Anammox process.

On the other hand, hydrazine is a relatively reactive compound which can undergo numerous abiological transformations (Coucouvanis *et al.*, 1996, Sykes, 1970). However, in control experiments without any Anammox sludge significant transformation of hydrazine was not observed in the media described here. The accumulation of hydrazine in the batch experiments supplied with hydroxylamine strongly suggests a biological transformation; spontaneous formation of hydrazine from hydroxylamine has not been observed among the many oxidation/reduction reactions in the inorganic chemistry literature (e.g. Sykes, 1970).

Very recently, it was reported that the R2 ribonucleotide reductase from *Escherichia. coli* is capable of catalysing the disproportionation of hydrazine to ammonia and dinitrogen gas (Han *et al.*, 1996). The stoichiometry of the conversion was very similar to one observed in the Anammox batch cultures provided with hydrazine in the absence of an electron acceptor. Whether an enzyme with a dinuclear iron center similar to that observed in the R2 ribonucleotide reductase is operative in the Anammox system remains to be determined.

Ammonium and hydroxylamine

The oxidation of ammonium to hydroxylamine by ammonium monooxygenase (AMO) normally requires molecular oxygen and two electrons from the ubiquinol pool (Keener

and Arp, 1993; Ensign *et al.,* 1993). Since the Anammox process is strongly but reversible inhibited by oxygen (van de Graaf *et al.,* 1995; Jetten *et al.,* 1997) an ammonium monooxygenase-like hydroxylamine formation seems highly improbable. Other mechanisms for the formation of hydroxylamine, include the incomplete reduction of nitrite to hydroxylamine by a cytochrome *c*-type nitrite reductase (Berks *et al.*, 1995). This enzyme will even catalyse the conversion of hydroxylamine to ammonia under the appropriate conditions. A similar mechanism might be operative in the Anammox process. However, it will be very difficult to obtain direct evidence for this mechanism in Anammox. Of all nitrogen compounds, hydroxylamine is the compound most rapidly metabolized by Anammox, and a selective inhibitor for the hydroxylamine conversion has not been discovered yet. Therefore it has not been possible to accumulate sufficient ^{15}N-labelled hydroxylamine from externally added $^{15}NO_2^-$ in the Anammox cultures.

The stoichiometric coupling of hydroxylamine to ammonium was observed in three independent and different types of experiments: a block pulse addition of 3 mM hydroxylamine to a fluidized bed reactor, where the consumption of ammonium increased by 3.2 mM; the supply of both $^{15}NH_4^+$ and $^{15}NH_2OH$ to a reactor resulted in a sharp increase in the $^{15-15}N_2$ emmission; various batch experiments, where the simultaneous additon of ammonium and hydroxylamine in the absence of an electron acceptor resulted in the transient accumulation of hydrazine.

Taken together, these results indicate the presence of a novel type of enzyme capable of combining two nitrogen atoms. Thus far only a limited number of enzymes has been reported to catalyze such reaction, i.e. cytochrome aa_3 oxidase, haemocyanin or NO reductase are known to form an N=N bond by coupling of two molecules of NO to N_2O (Berks *et al.*, 1995, Jetten *et al.,* 1997). However, the addition of NO or N_2O to the fluidized bed system did not influence the labelling patterns, indicating that NO or N_2O reductase does not play an important role in the Anammox process.

Nitrite and nitrate

Production of $^{15}NO_3^-$ was only observed when $^{15}NO_2^-$ was supplied. The anaerobic formation of nitrate from nitrite might be catalyzed by an enzyme similar to the nitrite oxidoreductase from *Nitrobacter sp.* (Meincke *et al.,* 1992; Sundermeyer *et al.,* 1984). The source of the third oxygen atom would then have to be water, which could be investigated using oxygen isotopes. It should be noted that the nitrite-hydroxylamine-ammonium reactions have a closed redox balance. Any reducing equivalents for CO_2-fixation would therefore have to be generated separately from these reactions. This may be the function of the nitrate production. Per mol of ammonium, 0.2 mol of nitrate and 20 mg of biomass are produced in the Anammox process (van de Graaf *et al.*, 1996). This value is similar to values reported for other autotrophic organisms using reverse electron transport (Bock

et al., 1992).

Concluding remarks

From the results obtained in this study, it is clear that the Anammox process involves the participation of many complex reactions for the conversion of different nitrogen compounds. Future studies will concentrate on the isolation and identification of the possible enzymes which catalyse these reactions. Hydroxylamine oxidoreductase and nitrite reductase are two obvious candidates for these studies, but enzymes similar to the R2 ribonucleotide reductase may play an important role in the disproportionation or formation of hydrazine.

Acknowledgements

This research was supported in by the Foundation of Applied research (STW) and Gist-brocades. The authors thank H. Slijkhuis of Gist-brocades and A. Mulder of TNO-IMW for their stimulating discussions, R. Appelman for his dedicated work on maintaining and operating the fluidized bed reactors, and J. Schalk and H. Oustad for their survey of the literature on hydrazine conversions.

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Summary

Ammonium and nitrate are notorious pollutants in industrial and municipal waste water. In order to prevent damage to the environment (e.g. eutrophication and acid rain), these components should be removed before discharge of the waste water. Conventional methods for the microbiological removal of these compounds involve two discrete steps that are generally carried out in separate bioreactors. Ammonium is oxidized to nitrate in an aerobic nitrifying reactor, and subsequently, nitrate and the other nitrogen oxides are reduced to denitrogen gas (denitrification) in a separate anaerobic reactor. The nitrifying and denitrifying micro-organisms responsible for these conversions do not only play an important role in waste water treatment but they also, in fact, play a key role in maintaining the balance of the nitrogen cycle in the natural environment.

Thus far, only aerobic and oxygen-limited systems based on conventional nitrification processes have been used for the removal of ammonium from waste water. Theoretically, ammonium could also be used as an inorganic electron donor for denitrification. The free energy for this reaction (-297 $kJ \cdot mol^{-1}$) is nearly as favourable as the aerobic nitrification process (-315 $kJ \cdot mol^{-1}$). For waste water treatment, such a system could have considerable advantages. For example, lower oxygen requirements for the oxidation of nitrogen, and lower amounts of organic materials necessary for the achievement of denitrification.

In 1986, it was observed that ammonium was disappearing from a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor (Chapter 2). Ammonium disappearance was associated with nitrate consumption and concomitant dinitrogen gas production. A maximum ammonium removal rate of 0.4 kg $N \cdot m^{-3} \cdot d^{-1}$ was observed. The evidence for the occurence of anaerobic oxidation of ammonium by nitrate was based on nitrogen and redox balances in continuous-flow experiments. It was shown that for the oxidation of 5 moles of ammonium, 3 moles of nitrate were required, resulting in the formation of 4 moles of dinitrogen gas. From these results, 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 process has been given the name "Anammox" (*An*aerobic *amm*onium *ox*idation) and has been patented (EP 0 327 184 A1).

Whether or not the process is of biological nature was established by use of anaerobic batch cultures including a series of inhibitors of biological activity (Chapter 3). Special care was taken to exclude oxygen during the experiments. The deliberate addition of oxygen to the cultures resulted in the total inhibition of the ammonium conversion. Furthermore, in experiments to show the correlation between biomass and the specific

activity of ammonium conversion, the rate of ammonium oxidation was proportional to the initial amount of biomass used. In control experiments without biomass, or with inactivated biomass, changes in the ammonium and nitrate concentrations were not observed. Chemical reactions could therefore not be responsible for the ammonium conversion. It was concluded that anaerobic ammonium oxidation was a microbiological process. 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, the labelling patterns of the $^{14,15}N_2$ formed indicated that nitrite, rather than nitrate, might be the preferred electron acceptor for the process.

Although the Anammox process is inhibited by oxygen, aerobic nitrifiers have been found in these cultures. It is known from literature, that some nitrifiers have shown to be capable of surviving in anaerobic environments. Moreover, some *Nitrosomonas* strains are able to denitrify under oxygen-limited conditions, although growth without oxygen has not been shown. Although the first step of ammonium conversion in these bacteria requires molecular oxygen, it is not actually required for the oxidation of hydroxylamine, the first intermediate of the ammonium oxidation. It was therefore investigated if hydroxylamine was able to support anaerobic growth of *Nitrosomonas* spp. To this end, continuous cultures of *N. europaea* were grown, for the first time, on hydroxylamine but still in the presence of ammonium (Chapter 4). In aerobic cultures, the molar growth yields on hydroxylamine were approximately twice those on ammonium alone. Aerobic growth on mixtures of ammonium and hydroxylamine was possible by step-wise increase of the hydroxylamine concentration in the medium. Anaerobic activity experiments showed that *N. europaea* was able to reduce nitrite anaerobically in the presence of hydroxylamine or even ammonium, thereby producing nitrous oxide. However, repeated attempts to grow *Nitrosomonas* anaerobically in continuous cultures on mixtures of hydroxylamine, ammonium, and nitrite were not successful.

In order to be able to grow and characterize the micro-organisms involved in anaerobic ammonium oxidation, different synthetic media were tested (Chapter 5). Adding different compounds to the incoming waste water of the Anammox reactor showed that organic compounds decreased the rate of ammonium removal, while additional sulfide or nitrite stimulated it. Finally, an autotrophic synthetic medium containing ammonium, nitrite, bicarbonate, minerals and trace elements suitable for growth of the Anammox-biomass in a fluidized bed reactor, was developed. For the enrichment of the anaerobic ammonium-oxidizing micro-organisms, the synthetic medium was fed to a small-scale fluidized bed reactor. Within three months of the start-up, the biomass in the reactor had reached conversion rates of 3 kg $NH_4^+ \cdot m^{-3} \cdot day^{-1}$ on a feed of 30 mM ammonium and

35 mM nitrite. Maximum specific conversion rates obtained were between 1300-1500 nmol $NH_4^+ \cdot h^{-1} \cdot mg\ VS^{-1}$. Surprisingly, small amounts of NO_3^- were produced in this system. The overall nitrogen balance gave a ratio of NH_4^+-conversion to NO_2^--conversion and NO_3^--production of 1 : 1.31 ± 0.06 : 0.22 ± 0.02. Nitrate production accounted for 10 % of the total nitrogen feed. No other intermediates or products (e.g. hydroxylamine, NO, N_2O) could be detected. Acetylene and phosphate were shown to be strong inhibitors of the Anammox activity.

Labelling experiments with $^{14}CO_2$ (Chapter 5) showed that the Anammox process is autotrophic, and that incorporation of CO_2 is dependent on the presence of both ammonium and nitrite. The biomass yield was 0.041 mol CO_2 incorporated per mol of NH_4^+ oxidized, resulting in an estimated growth rate of 0.001 h^{-1}.

The dominant type of micro-organism in the enrichment culture was made up of irregularly-shaped cells with an unusual morphology. During the enrichment for anaerobic ammonium-oxidizing micro-organisms on synthetic medium, an increase in ether lipids was observed. The colour of the biomass changed from brownish to red, which was accompanied by an increase in the cytochrome content, with an unusual peak at 470 nm.

In earlier work with ^{15}N-labelled ammonium and $^{14}NO_3^-$ in anaerobic batch experiments using undefined waste water and original 'Anammox' biomass (see Chapter 3), the main product of anaerobic ammonium oxidation was ^{14}N-^{15}N-dinitrogen gas. On the basis of these results, a hypothetical metabolic pathway was formulated, in which hydroxylamine would serve as an intermediate in the dehydrogenation of ammonium under anaerobic conditions - in analogy to the aerobic pathway - with N_2O as the electron acceptor. The N_2O would be produced by a reaction between hydroxylamine and nitrite. To test this hypothesis, the conversion of various ^{15}N labelled compounds by the Anammox enrichment culture was tested. The results indicated that a different metabolic pathway for anaerobic ammonium oxidation is operating, with hydrazine as the essential intermediate (Chapter 6). According to this pathway, hydroxylamine is the electron acceptor for ammonium oxidation. The two compounds are joined during this oxidation-reduction reaction to form the N-N bond of hydrazine. The hydrazine is subsequently oxidized to dinitrogen gas. This part of the anaerobic ammonium oxidation pathway looks similar to a reversed N_2-fixation step. During the oxidation of hydrazine, the electrons liberated are used to reduce nitrite to hydroxylamine.

Batch experiments in which ammonium was oxidized with hydroxylamine as the electron acceptor transiently accumulated hydrazine (Chapter 6). As already mentioned, during the conversion of ammonium in the fluidized bed system, a small amount of nitrate was formed from some of the nitrite. The addition of NH_2OH to an operating fluidized bed system caused a stoichiometric increase in the ammonium conversion rate,

and a decrease in the nitrate production rate. Addition of hydrazine also caused a decrease in nitrate production. On the basis of these findings, it is postulated that the oxidation of nitrite to nitrate may provide the anaerobic ammonium-oxidizing bacteria with reducing equivalents needed, for example, for CO_2-fixation.

The identification of the anaerobic ammonium-oxidizing bacteria has still not been resolved, as the organism is very difficult to cultivate in axenic cultures. Ribosomal RNA comparison analysis, and other molecular biological techniques, such as PCR and fluorescence *in situ* hybridization, may help to further identify the mixed culture, as these techniques do not depend on the availability of isolated strains.

Energy generation in anaerobic ammonium-oxidizing bacteria, as far as we know, is determined solely by the redox conversion reactions of inorganic nitrogen substrates. Further studies on the enzymes of the proposed new metabolic pathway will be of great interest.

The studies presented in this Thesis have revealed a novel metabolic pathway in which ammonium is oxidized anaerobically. The research has also shown that the mixed culture can be enriched to give high volumetric activity, and that nitrite, rather than nitrate, is the preferred electron acceptor. This knowledge will help to adapt and find the proper process conditions and control parameters to make the process suitable for application in industrial or domestic waste water treatment.

Samenvatting

De stikstofverbindingen ammonium en nitraat zijn belangrijke componenten in industrieel en huishoudelijk afvalwater. Om te voorkomen dat deze stoffen, in de vorm van zure regen of eutrofiëring, schade toe brengen aan het milieu kunnen ze het best verwijderd worden voordat het afvalwater wordt geloosd. De biologische verwijdering van deze componenten vindt gewoonlijk plaats in twee stappen: nitrificatie en denitrificatie. Ammonium wordt eerst in een aërobe reactor met behulp van zuurstof geoxideerd tot nitraat. In een daaropvolgende reactor wordt nitraat samen met andere stikstofoxides gereduceerd tot stikstofgas. De micro-organismen die deze processen kunnen uitvoeren spelen een zeer belangrijke rol in de stikstofcyclus zoals die in de natuur voorkomt. Bij de behandeling van stikstofhoudend afvalwater worden ze specifiek door de mens ingezet om de natuurlijke stikstofcyclus zo goed mogelijk in stand te houden.

In de praktijk zijn voor de verwijdering van ammonium uit afvalwater tot nu toe alleen aërobe of zuurstof-gelimiteerde processen ingezet, gebaseerd op het hierboven beschreven conventionele nitrificatie-denitrificatiemodel. Theoretisch is het echter mogelijk om ammonium ook zonder zuurstof te oxideren. Ammonium fungeert in dat geval als een anorganische electronendonor voor het denitrificatieproces. De vrije Gibbs energie van deze reactie (-297 kH/mol) is bijna even gunstig als voor het klassieke aërobe nitrificatieproces (-315 kJ/mol). Ammonium direct aanwenden als electronendonor kan ook belangrijke voordelen opleveren voor de behandeling van afvalwater. Zo is er bijvoorbeeld minder zuurstof nodig voor de oxidatie van stikstof en is er minder organisch materiaal nodig om de denitrificatie volledig te laten verlopen.

Eind 1986 werd in een proefopstelling van Gist-brocades waargenomen dat ammonium verdween uit een anaërobe afvalwaterstroom afkomstig van een denitrificerende fluïde-bedreactor (Chapter 2). Deze reactor behandelde afvalwater afkomstig uit een methanogene reactor. De verdwijning van ammonium ging gepaard met een verhoogde nitraat-consumptie en stikstofgasproductie. Dit duidde op het optreden van anaerobe oxidatie van ammonium met nitraat. De maximale ammoniumomzettingssnelheid die werd waargenomen bedroeg 0.4 kg $N \cdot m^{-3} \cdot d^{-1}$. De bewijsvoering hiervoor was gebaseerd op stikstof- en redoxbalansen die berekend waren over periodes met een continue bedrijfsvoering. Hieruit bleek, dat voor de oxidatie van 5 mol ammonium 3 mol nitraat nodig was. Deze omzetting resulteerde in de vorming van 4 mol stikstofgas. Deze resultaten leidden tot de volgende conclusie: anaërobe ammonium oxidatie is een nieuw proces waarin ammonium met behulp van nitraat als electronenacceptor omgezet wordt tot stikstofgas. Dit proces heeft de naam "Anammox" (*An*aërobe *amm*onium *ox*idatie) gekregen en is als zodanig gepatenteerd (EP 0 327 184 A1).

Of het nieuw ontdekte proces van biologische aard is werd onderzocht aan de hand van een aantal series van anaërobe batchcultuurexperimenten. Hieraan werden onder

andere verscheidene remmers toegevoegd die de biologische activiteit kunnen stilleggen (Chapter 3). Om te voorkomen dat tijdens de experimenten zuurstof binnenlekte werden speciale voorzorgen genomen. Daarentegen leidde het opzettelijk toevoegen van zuurstof aan een dergelijke cultuur zelfs tot een totale remming van de ammoniumomzetting. In experimenten waarin de relatie tussen de omzetting en de hoeveelheid biomassa werd onderzocht bleek dat de ammoniumoxidatiesnelheid rechtevenredig was met de toegevoegde hoeveelheid biomassa. In controle-experimenten zonder biomassa of met gesteriliseerde biomassa trad geen verandering in de ammonium- of nitraatconcentratie op. Op basis van deze resultaten werd uitgesloten dat de omzetting van ammonium het gevolg was van het optreden van chemische reacties, en kon geconcludeerd worden dat anaërobe ammonium oxidatie een microbiologisch proces is. Aanvullende experimenten met ^{15}N-gelabeld NH_4^+ en $^{14}NO_3^-$ werden uitgevoerd om te bevestigen dat het omzettingsproduct van ammonium stikstofgas was. Het labelingspatroon van het gevormde $^{14,15}N_2$ suggereerde echter dat nitriet in plaats van nitraat bij voorkeur als electronacceptor werd gebruikt.

Alhoewel het Anammoxproces geremd werd door aanwezigheid van zuurstof, konden in de culturen toch aërobe nitrificeerders worden aangetoond. Het is bekend uit de literatuur dat aërobe ammoniumoxideerders anaërobe omstandigheden kunnen overleven. Sommige *Nitrosomonas*-stammen zijn onder zuurstof gelimiteerde condities zelfs in staat tot denitrificatie. Onder deze omstandigheden is echter nog geen groei waargenomen. Alhoewel voor de eerste stap in dit aërobe oxidatieproces van ammonium moleculaire zuurstof nodig is, is dit voor de oxidatie van hydroxylamine, het eerste product van ammoniumoxidatie, niet het geval. Daarom werd onderzocht of Nitrosomonas in staat was te groeien met hydroxylamine als substraat onder aërobe en anaërobe condities. Hiertoe werden culturen met *N. europaea* gekweekt op hydroxylamine in aanwezigheid van ammonium (Chapter 4). De aërobe culturen op hydroxylamine leverde een twee maal grotere molaire groeiopbrengst dan de culturen die alleen op ammonium gekweekt waren. Aërobe groei op een substraatmengsel van ammonium en hydroxylamine was alleen mogelijk door de hydroxylamineconcentratie in het medium stapsgewijs te vergroten. Batchexperimenten met betrekking tot de anaërobe activiteit toonden aan dat *N. europaea* in aanwezigheid van hydroxylamine of ammonium in staat was nitriet te reduceren, waarbij lachgas werd geproduceerd. Herhaalde pogingen om *Nitrosomonas* onder anaërobe omstandigheden in een continu cultuur op een mengsel van hydroxylamine, ammonium en nitriet te laten groeien leveren geen positief resultaat.

Verschillende synthetische media zijn getest teneinde de micro-organismen die verantwoordelijk zijn voor de anaërobe ammoniumoxidatie, te kweken en te

karakteriseren (Chapter 5). Experimenten waarbij verschillende componenten aan het afvalwater werden toegevoegd gaven aan dat organische componenten een afname van de omzettingssnelheid tot gevolg hadden terwijl toevoegingen van sulfide of nitriet de omzetting juist stimuleerden. Uiteindelijk bleek het mogelijk een autotroof synthetisch medium samen te stellen dat ammonium, nitriet, bicarbonaat, mineralen and sporenelementen bevatte. Met dit medium was het mogelijk om de mengcultuur met Anammox-bacteriën te kweken in een fluïde-bedreactor. Voor de ophoping van de anaërobe ammonium-oxiderende micro-organismen werd een fluïde-bedreactor gevoed met het synthetische medium. Na drie maanden had de ophopingscultuur in de reactor een omzettingssnelheid van 3 kg $NH_4^+ \cdot m^{-3} \cdot day^{-1}$ bereikt op een voeding van 30 mM ammonium en 35 mM nitriet. De maximale specifieke omzettingssnelheid, gemeten in batchcultuur experimenten, lag tussen de 1300 en 1500 nmol $NH_4^+ \cdot h^{-1} \cdot mg\ VS^{-1}$. Verrassend was dat er kleine hoeveelheden NO_3^- gevormd werden in dit systeem. De stikstofbalans gaf een verhouding aan van NH_4^+-omzetting tot NO_2^--omzetting tot NO_3^--productie van 1 : 1.31 ± 0.06 : 0.22 ± 0.02. De hoeveelheid nitraat die gevormd werd was ongeveer 10% van de totale stikstoftoevoer aan het systeem. Er konden geen andere intermediaren of producten (bv. hydroxylamine, NO of N_2O) worden waargenomen. Verder bleken acetyleen en fosfaat goede remmers te zijn van de Anammoxactiviteit.

Labellingsexperimenten met $^{14}CO_2$ (Chapter 5) toonden aan dat het Anammoxproces een autotroof proces is en dat incorporatie van CO_2 afhankelijk is van zowel de aanwezigheid van ammonium als nitriet. Per mol geoxideerd NH_4^+ werd 0.041 mol CO_2 ingebouwd. Een dergelijke biomassaopbrengst in dit systeem komt overeen met een geschatte groeisnelheid van 0.001 h^{-1}.

In de ophopingscultuur kwam een dominant micro-organisme voor met een opmerkelijke morfologie van zeer onregelmatig gevormde cellen. Tijdens de ophoping op het synthetisch medium werd een toename van de etherlipiden in de celwand waargenomen. De kleur van de cultuur veranderde hierbij van bruin naar rood. Dit ging gepaard met een toename van de cytochroominhoud met een bijzondere piek bij 470 nm.

Uit eerdere experimenten met ^{15}N-gelabeld ammonium en $^{14}NO_3^-$, uitgevoerd met ongedefineerd afvalwater en de originele "Anammox"-cultuur, bleek dat het eindproduct ^{14}-^{15}N-stikstofgas was (zie Chapter 3). Op basis van deze resultaten werd een hypothetische metabole route geformuleerd. Hydroxylamine zou volgens deze metabolische route het intermediaire product zijn van de dehydrogenering van ammonium onder anaërobe condities - in analogie met de aërobe nitrificatie route - met N_2O als electronenacceptor. N_2O zou dan gevormd worden uit de reactie tussen hydroxylamine en nitriet. Om deze hypothese te verifiëren werd de omzetting van verschillende ^{15}N-gelabelde verbindingen in de Anammoxophopingscultuur uitgetest. De

resultaten gaven echter aanleiding om een geheel andere metabolische route voor anaërobe ammoniumoxidatie op te stellen. In deze route is hydrazine de essentiële verbinding (Chapter 6). Volgens dit mechanisme is hydroxylamine de electronenacceptor voor de ammoniumoxidatie. De twee componenten vormen samen the N-N binding van hydrazine tijdens de oxidatie-reductiereactie. Hydrazine wordt vervolgens geoxideerd tot stikstofgas. Dit gedeelte van de anaërobe ammoniumoxidatie route vertoont enige overeenkomst met een omgekeerde N_2-fixatieroute. De electronen die bij de oxidatie van hydrazine vrijkomen zouden aangewend kunnen worden om nitriet tot hydroxylamine te reduceren. Daarmee is een oxidatie/reductie kringloop beschikbaar die (via electronentransport) kan dienen voor energie-opwekking.

In experimenten met batchcultures waarin ammonium met hydroxylamine als electronenacceptor geoxideerd werd, vond ophoping van hydrazine plaats (Chapter 6). Zoals reeds is vermeld, werd gedurende de anaërobe omzetting van ammonium in een fluïde-bedsysteem nitraatproductie waargenomen. Het geproduceerde nitraat bleek alleen afkomstig te zijn van nitriet. Toevoeging van NH_2OH aan het synthetische medium van een continu fluïde-bedsystem veroorzaakte een stoichiometrische toename van de ammoniumomzettingssnelheid en een afname van de nitraatproductie. Toevoeging van hydrazine had tevens een afname van de nitraatproductie tot gevolg. Aan de hand van deze bevindingen wordt gepostuleerd dat de oxidatie van nitriet naar nitraat de anaërobe ammoniumoxiderende bacteriën waarschijnlijk gereduceerd vermogen levert om bijvoorbeeld CO_2 te fixeren.

De identificatie van de anaërobe ammoniumoxideerders is nog steeds niet opgelost, daar deze organismen nog niet in geïsoleerde cultures gekweekt kunnen worden. Moleculaire biologische technieken zoals ribosomaal RNA analyses, PCR en fluorescentie *in situ* hybridisatie kunnen wellicht worden toegepast om de mengcultuur beter te karakteriseren, daar deze technieken niet afhankelijk zijn van de beschikbaarheid van reinculturen. Voor zover wij weten, zijn het alleen de redoxreacties van de anorganische subtraten die energie voor groei van de anaërobe ammoniumoxideerders leveren. Onderzoek naar de enzymen, die betrokken zijn bij de gepostuleerde metabole route, zullen hier meer inzicht over kunnen verschaffen.

In conclusie kan worden gesteld dat het hier gepresenteerde onderzoek heeft geleid tot de ontdekking van een nieuwe metabole route waarin ammonium anaëroob geoxideerd wordt. Met de ophoping van deze mengcultuur kunnen hoge omzettingssnelheden behaald worden, waarbij nitriet in plaats van nitraat de geprefereerde electronenacceptor is. Deze kennis draagt bij aan het vinden van de juiste procesparameters om het proces geschikt te maken voor verder toepassing bij de behandeling van industrieel en huishoudelijk afvalwater.

Nawoord

Als je iets gedaan hebt is het goed om er een tijdje afstand van te nemen. Misschien is drie jaar iets te lang. Maar gelukkig zijn er vrienden en collega's. Vrienden die vragen. Collega's die vragen. Voortdurend vragen of je al een datum hebt. Vanaf vandaag hoeft dat niet meer. It's now or never.

Net als elk proefschrift kwam dit tot stand met hulp van medewerking van anderen. Hiervoor wil ik graag iedereen in en om het Kluyverlaboratorium bedanken.

Een van de aparte aspecten van dit onderzoek was dat het begon bij Gist-brocades. In een situatie van nog al wat ongeloof ("het is gewoon een artefact") tot neutrale interesse ("oh, heb je hier al naar gekeken") was ik de laatste die het mocht proberen voordat het definitieve doek viel voor iets wat twee jaar daarvoor tot Anammox was gedoopt. Samen met Arnold Mulder en bijgestaan door Jaap van de Burg is het ons toch gelukt om het proces weer aan de gang te krijgen.

Het promotieonderzoek vond plaats bij de TU-Delft. Daar begon het spel met het ongeloof opnieuw. Terecht uiteraard. Als er naast het ongebruikelijke proces van aërobe denitrificatie (dat normaal alleen *zonder* zuurstof plaatsvindt) er nu ineens 'anaërobe nitrificatie' (een proces dat alleen *met* zuurstof hoort te verlopen) bestaat, kan je je voorstellen dat niet iedereen direct eureka roept. Een groot deel van de eerste jaren werd besteed aan het steeds opnieuw bewijzen van het bestaan. De door de jaren heen nimmer aflatende reeks van Gist-brocades mededenkers Herman Slijkhuis, Hans Hollander, Brian Jones, Rob Beudeker, Cees van der Beek waren daarbij een welkome afwisseling; Jan Block en Marcel Tilborg met hun nimmer falende massaspectrometer een uitkomst.

Een jaar na aanvang erfden wij de installatie van Gist-brocades die onze one-and-only-bacteriecultuur in stand hield. Een extra handenbindertje erbij. Ook de verhuizing van de installatie was een interessante operatie. Gelukkig was het Kluyverlab in bezit van een zeer goede werkplaats met heel handige mannen. Met name Sjaak Lispet dank ik voor zijn positieve ondersteuning en het 'alles is te regelen gevoel' dat hij kan overbrengen. De instrumentmakerij, de glasblazerij en de elektronische dienst ontzettend bedankt, ook voor alle andere kleine maakdingetjes en een stuk gezelligheid. En niet te vergeten de dagelijkse ondersteuning van de mediumkeuken, laboratorium- en fermentortoebehoren, en onze echte gassenman.

Peter, als we weer in het weekend mochten dweilen of als we weer aan een ontelbare hoeveelheid ammonium, nitriet en nitraatbepalingen begonnen, dan zijn dat de momenten die zijn bij gebleven. Ongeveer vier jaar lang en passant een eigen slibproductiebedrijfje in stand houden en elk weekend nog *even* naar het lab was best wel

een hele belasting. Het geploeter is in ieder geval niet voor niets geweest, gezien de ruime voortzetting. Ontzettend bedankt voor de trouwe steun tijdens de vijf jaar onderzoek. Onmisbaar voor dit onderzoek waren ook de stagiaires en afstudeerders: Frans van Velzen, Michel Tindal, Eugenie Dumoulin, Rene Appelman en Marco van de Berg.

Gijs, bedankt voor de begeleiding, ondersteuning en de laatste vele besprekingen 's avonds thuis. Lesley, bedankt voor alle steun ook bij de laatste promotielootjes. Jij wist toch altijd weer een vrolijke noot te zingen, wat er ook gebeurde. Mike, waarde eerste opvolger, bedankt voor alle hulp bij het gereed maken van de artikelen voor publicatie. De black hole: Guus, Ed en Henk, in het begin; MartEn, Patries, continu; en Jolyn en Nicole aan het eind. Jullie hebben de herinnering aan het lab en het onderzoek onvergetelijk gemaakt.

Wiebe, mijn destijds anaërobe medelab-maarvandegistgroep-genoot, bedankt voor je continue eigenwijze steun. Voor jou een fragment van de net-niet-winnende compositie van de talentenjacht tijdens het Kluyverlabfeest van 1990: "*Het was op het Kluyverlab dat ik haar ontmoette. Het was voor het fotobord dat zij mij groette. Mijn hartje sprong verblijd, al van verliefdigheid. Het was in de lente, het was voorjaarstijd.*"

@ Astrid

Curriculum vitae

Astrid Alexandra van de Graaf werd geboren op 1 april 1964 te 's Gravenhage. Gedurende de periode 1976-1982 werd voorbereid wetenschappelijk onderwijs genoten aan het Huygens Lyceum te Voorburg. Aansluitend is de studie Scheikundige Technologie aan de Technische Universiteit Delft aangevangen die is afgerond in 1988. Na een vooronderzoek bij Gist-brocades werd in 1989 gestart met het promotieonderzoek, zoals neergelegd in dit proefschrift. Sinds 1994 is zij werkzaam bij newMetropolis science and technology center (opvolger van Technologie Museum NINT) te Amsterdam als programmaontwikkelaar en beleidsmedewerker.