NITROGEN REMOVAL FROM WATER AND WASTE

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INTRODUCTION

The catalogue of environmental problems associated with inorganic nitrogen compounds is impressive, and growing. It is ironic that enormous amounts of money are invested every year in synthesizing fixed nitrogen fertilizers for agriculture, while further sums are spent reducing the nitrogen content of wastes. Considerable progress has been made in devising systems for removing dissolved nitrogen compounds, giving improved water quality, but as yet little attention has been paid to the quality of effluent gases. It is even possible that the increase in numbers of nitrification and denitrification plants has actually boosted the escape of nitrogen oxides into the atmosphere.

Atmospheric nitrogen pollution is increasing, to the extent that a steady increase in the nitrate level in Greenland ice cores has been measured (Stauffer & Neftal, 1988; Mayewski et al., 1990). A similar increase in nitrate in Antarctic ice cores has not been detected (Legrand & Delmas, 1987), indicating the Greenland nitrate is mainly due to anthropogenic emissions from the more heavily industrialized northern hemisphere. The primary sources of NOx (i.e. all N-oxide gases including N2O) in the troposphere are summarized in Table 1 together with rough estimates of their individual contributions (Logan, 1983). Because of its intensive animal farming activities, the Netherlands has a more localized nitrogen-related problem (ammonia as well as NOx) which does not show on this global budget which was extrapolated mainly from American data. Not included in Table 1 is a recent estimate that as much as 25% of the NOx in the atmosphere comes from sub-optimally managed waste treatment systems.

Human activities are directly or indirectly responsible for the generation of the various polluting nitrogen compounds. As will be shown from the examples discussed below, increased concentrations of nitrogen compounds in the air, water and even soil can result in a multiplicity of problems, some of which may have far-reaching consequences. We can no longer risk waiting until the problem has been accurately quantified, but must improve existing systems and design new processes to control, and preferably prevent further pollution.
Table 1. Global source inventory for NO$_x$ in the troposphere (data from Logan, 1983). The numbers given are best estimates, with possible ranges in brackets

<table>
<thead>
<tr>
<th>Source</th>
<th>Tg N year$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fossil fuel combustion</td>
<td>21 (14–28)</td>
</tr>
<tr>
<td>Biomass burning</td>
<td>12 (4–24)</td>
</tr>
<tr>
<td>Soil emission</td>
<td>8 (4–16)</td>
</tr>
<tr>
<td>Lightning</td>
<td>8 (2–20)</td>
</tr>
<tr>
<td>Atmospheric oxidation of NH$_3$</td>
<td>1–10</td>
</tr>
<tr>
<td>Oceans</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Stratosphere input</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Total</td>
<td>25–29</td>
</tr>
</tbody>
</table>

*Teragram (Tg) = 10$^{12}$ grams.

This chapter will aim to review the sources and effects of dissolved and gaseous nitrogen pollutants, with an emphasis on the microbiology involved, and consider traditional and novel microbiological means of treating them.

ENVIRONMENTAL PROBLEMS ASSOCIATED WITH NITROGEN POLLUTION

This section will review the consequences of nitrogen pollution, and emphasize the need to control both gaseous and liquid emissions of nitrogen compounds other than N$_2$.

*Eutrophication, ground water contamination and unwanted fertilization*

Contamination of freshwater with nitrogen compounds is associated with atmospheric deposition and, more importantly, with run-off from agricultural areas and leaching of nitrate and nitrite from soil, often after nitrification of deposited ammonia. It can lead to two major problems – eutrophication and ground water pollution. This last is of special concern as ground water serves as a major source of drinking water. Nitrifying and denitrifying activity has even been observed in subterranean aquifers, indicating deep penetration of leaching mineral nitrogen compounds.

Eutrophication was probably one of the earliest environmental problems associated with boosting nutrient cycles to be recognized, and there is therefore little new to be said. While phosphates are the primary cause of problems associated with freshwater, nitrogen compounds, especially ammonia, are often also associated with algal blooms.
A less obvious, related problem is the unwanted fertilization of nutrient-poor heathland. For example, most Dutch heathland has been converted to arable fields or forestry plantations (de Boer, 1989), and the remains are generally within nature reserves. Here the vegetation is subjected to a regime of traditional techniques including grazing and sod-cutting which are designed to maintain the original flora (i.e. predominantly Calluna vulgaris and Erica tetralix). However, there have been a number of reports that this vegetation is being supplanted by grass species (e.g. Deschampsia flexuosa and Molina caerula). This type of succession is associated with an increasing availability of mineral nitrogen (Heil & Bruggink, 1987), the main source of which is atmospheric deposition of ammonia and nitrogen oxides. As already mentioned, atmospheric nitrogen pollution, and hence deposition, is increasing. Thus it has been estimated that pre-industrial deposition in the Netherlands was equivalent to 0.15–4 kg N ha\(^{-1}\) year\(^{-1}\). Estimates made during the period 1980–1987 showed that it had increased to about 42 kg N ha\(^{-1}\) year\(^{-1}\) (Schneider & Bresser, 1987, 1988). In areas of intensive animal husbandry, additional ammonia deposition can more than double this value (de Boer, 1989).

The greenhouse effect

Over the last few years, there has been a great deal of attention paid to the probability and repercussions of global warming, especially as a consequence of the steady increase in CO\(_2\) levels since the industrial revolution. Even today, insufficient action is being taken to reduce CO\(_2\) emissions. For example, it has been estimated that biomass containing 2000–5000 Tg (1 Tg = 10\(^{12}\) grams) is burnt each year (Crutzen & Andreae, 1990), and carbon from fossil fuels is not included in this figure. However, progress is being made in some areas. For example, much recent attention has been paid, with some political results, to the repercussions of continued CFC release into the atmosphere because of the involvement of these compounds in the ‘dirty window’ effect. Because of the properties of the constituents of the unpolluted atmosphere, especially water and CO\(_2\), the loss of heat to space from the earth by radiation normally takes place through a narrow band, or window, of wavelengths between 8–13 \(\mu\)m. If cooling was not limited, the surface of the earth would be frozen, and life as we know it could not exist. Thus a limited greenhouse effect is essential, and promoted by the naturally occurring CO\(_2\) and water. However, the gases associated with the greenhouse effect absorb heat within the atmospheric window (hence the term ‘dirty window’), reducing heat loss and inducing a net warming effect. Anthropogenic pollutants such as the CFCs are very efficient heat retaining agents as they absorb in the centre of the band (Ramanathan, 1988), but naturally occurring molecules are also implicated in the ‘dirty window’ effect. Table 2 gives some examples, together with the warming to
Table 2. Atmospheric trace gases, their assumed concentrations in 1975, and the probable greenhouse effect of a doubling in their concentration. Major climatic changes are predicted to result from an increase in average temperature of 1 K (from Wang et al., 1976)

<table>
<thead>
<tr>
<th>Gas</th>
<th>Assumed concentration (ppmv)*</th>
<th>Predicted greenhouse effect (K)</th>
<th>Trace gas lifetime (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂O</td>
<td>0.28</td>
<td>0.68</td>
<td>130 years</td>
</tr>
<tr>
<td>NH₃</td>
<td>6 × 10⁻³</td>
<td>0.12</td>
<td>10 days</td>
</tr>
<tr>
<td>HNO₃</td>
<td></td>
<td>0.08</td>
<td>10 days</td>
</tr>
<tr>
<td>Total K</td>
<td></td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>SO₂</td>
<td>2 × 10⁻³</td>
<td>0.03</td>
<td>10 days</td>
</tr>
<tr>
<td>CH₄</td>
<td>1.6</td>
<td>0.28</td>
<td>3–7 years</td>
</tr>
<tr>
<td>CO₂</td>
<td>330</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>O₃</td>
<td></td>
<td>1.25</td>
<td></td>
</tr>
</tbody>
</table>

*ppmv = parts per million volume.

be expected from a doubling of their concentrations. It is expected that major climatic changes will occur after an average increase of 1 K. At first glance, the concentration and effect of N₂O appears low in comparison with that of CO₂, but its lifetime of 130 years should be noted, and it should be remembered that these are figures from 1976 – the most recent estimates suggest that N₂O concentrations have already risen to 0.35 ppmv (parts per million volume).

The ozone layer

Atmospheric chemistry is complex, and a detailed review is not appropriate here. However, it has already become clear that NOₓ concentrations critically affect ozone levels. A model put forward at a recent (1988) Dahlem Conference, and based on measurements made in the USA, indicates that ozone levels increase with increasing NOₓ concentrations until an NOₓ concentration between 0.1–2 ppbv. Above this level, ozone loss occurs because of competing reactions with the free radicals necessary for the production of ozone. In the dark, NO₂ reacts with ozone to form NO₃, and then with the NO₃ to form N₂O₅. The formation of HNO₃, and its loss in rainfall, thus also leads to reduction in O₃. Interested readers are referred to the report on the Dahlem Workshop in the Changing Atmosphere (Rowland & Isaksen, 1988).
Table 3. Nitrification and NO production (ng N h⁻¹ g⁻¹ dry weight) associated with samples of stone from various sources (from Baumgartner et al., 1991)

<table>
<thead>
<tr>
<th>Building</th>
<th>pH</th>
<th>Nitrification</th>
<th>NO production</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAPAO building, Hamburg</td>
<td>4.8</td>
<td>&lt;5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Regensburg Cathedral</td>
<td>7.6</td>
<td>517</td>
<td>24.2</td>
</tr>
<tr>
<td>Cologne Cathedral</td>
<td>7.6</td>
<td>66</td>
<td>2.2</td>
</tr>
<tr>
<td>Alte Pinakotheke, Munich</td>
<td>7.7</td>
<td>151</td>
<td>7.5</td>
</tr>
<tr>
<td>Birkentield Convent</td>
<td>8.3</td>
<td>&lt;5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**Acid rain, acidification**

Acid rain has been found to be a world-wide problem (Crutzen & Andreae, 1990) which has also received a great deal of public and media attention over the last decade. In addition to the acid rain of the industrialized zones, which is predominantly sulphuric and nitric acids, acid rain in the tropics contains formic and acetic acids, as well as nitric acid. It is believed that the organic acids are due to biomass burning. Nitric acid is a common factor in both areas, however, and recent models indicate that a pH of around 4.2 can be expected in the tropics in the dry season as a consequence of nitric acid formation, alone (Crutzen & Andreae, 1990).

Acid rain can be damaging in terms of leaf damage, but also in its contribution to soil and water acidity. Another cause of acidification is nitrification of available ammonia to nitrous or nitric acids. In freshwater, this can prove lethal for the fauna and flora, and in soils it mobilizes toxic aluminium ions and promotes the leaching of other, nutritive, ions including calcium and magnesium (Crutzen & Andreae, 1990).

**Building corrosion**

Nitrifiers colonizing building surfaces are responsible for corrosion and the gradual destruction of the concrete or stone (Kaltwasser, 1976; Baumgartner et al., 1989; Meincke, Krieg & Bock, 1989). The corrosion is due to the production of HNO₂ or HNO₃, which reacts with constituents in the building materials. Baumgartner et al. (1991) have recently reported that NO was produced when samples of corroding sandstone from churches in Germany were incubated with NH₃, and that this was inhibited by the inclusion of nitrpyrin (an inhibitor of nitrification) or by autoclaving. As can be seen from Table 3, only stone samples with a relatively neutral pH gave significant HNO₃ accumulation (a measure of nitrification) and NO production.
Table 4, Summary of total N$_2$O emissions from University of Wisconsin Arboreium sites over two sampling periods (data from Goodroad & Keeney, 1984)

<table>
<thead>
<tr>
<th>Site</th>
<th>Period</th>
<th>1979</th>
<th></th>
<th>1980</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period</td>
<td>N$_2$O</td>
<td></td>
<td>Period</td>
<td>N$_2$O</td>
</tr>
<tr>
<td></td>
<td>11/6-5/11</td>
<td>0.19</td>
<td>28/3-10/11</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Forest</td>
<td>Coniferous</td>
<td>0.99</td>
<td>28/3-15/12</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Prairie</td>
<td>Burned</td>
<td>0.09</td>
<td>9/4-10/11</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unburned</td>
<td>0.10</td>
<td>9/4-10/11</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Organic soils</td>
<td>Drained marsh</td>
<td>2.3</td>
<td>28/3-10/11</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Undrained marsh</td>
<td>0.02</td>
<td>9/4-10/11</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wet meadow</td>
<td>1.1</td>
<td>24/4-10/11</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

SOURCES OF NITROGEN POLLUTANTS

Non-anthropogenic sources

Naturally occurring bogs and sediments are known to be a source of NO and N$_2$O. However, as can be seen from Table 4, undisturbed marshes release far less N$_2$O than those which have been drained. Indeed, most of the NO and N$_2$O emissions into the atmosphere can be attributed to anthropogenic sources, either because of agricultural practices, or industry.

Agriculture-related sources

Fertilizers

Agriculture is relevant to a paper on waste treatment for several reasons. First, the addition of some types of fertilizers to soil appears to favour N$_2$O production, adding to air pollution. Only in recent years has the impact of nitrogen supplements on agricultural land been appreciated. Table 5 shows the results of supplementing two different types of soil with different nitrogen compounds. It appears that N$_2$O emission was at its lowest when nitrate was the fertilizer supplied (Brenner & Blackmer, 1981). Secondly, fertilizers are being leached out of the soil (generally after nitrification, negatively charged nitrate and nitrite ions leach out more rapidly than positively charged ammonium), and accumulating in ground water. This is a primary source of drinking water which must then be treated before it can be used. The extent of the problem may be massive, and reflects not only a
Table 5. Effect of various additives on N₂O emission (ng g⁻¹ soil) from well-aerated soil samples after 7 days' incubation at 30°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Harps soil (ng N (g soil)⁻¹ week⁻¹)</th>
<th>Webster soil (ng N (g soil)⁻¹ week⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>246</td>
<td>50</td>
</tr>
<tr>
<td>Urea</td>
<td>202</td>
<td>75</td>
</tr>
<tr>
<td>Alanine</td>
<td>218</td>
<td>81</td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Glucose</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Nitrate + glucose</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

All nitrogen compounds were supplied at a concentration giving 100 μg N g⁻¹ soil. Glucose, when added, was at a concentration of 0.25 mg g⁻¹ soil. The Harps soil contained 7.1% organic material and had a pH of 7.9. The organic content of the Webster soil was 10.2%, with a pH of 6.2 (data from Brenner & Blackmer, 1981).

Nitrates and nitrates are a major environmental hazard, but also extensive financial waste on the part of the farmers. For example, nitrogen balances made in Iowa, USA (Brenner & Blackmer, 1981) revealed that of the 1 million tonnes of N (10% of the USA total) used per year as NH₄⁺, there was complete conversion to NO₃⁻ in 2-3 weeks. The fate of the nitrogen was then as follows.

N-balance:
20% removed in the harvest
20-25% remains in the soil
55% either leached or denitrified

Obviously, this type of balance will be different for different types of soil and crop, and precise figures are impossible to obtain. Rosswall & Paustian (1984) surveyed agricultural practices worldwide, and estimated that in 1980 the amount of fertilizers used was equivalent to 40% of the total biological nitrogen fixation, and 36% more than is fixed in the fields. Of the 60 Tg N estimated to have been used as fertilizer during 1980/81, about 30 Tg was taken up in the harvest. Leaching, erosion and denitrification were responsible for the loss of 2, 2-20 and 1-44 Tg, respectively, while ammonia volatilization was estimated at 13-23 Tg.

It is clear that the use of nitrogen fertilizers is seriously in need of reconsideration. The possible use of other nitrogen compounds which would
not enrich for nitrifiers and denitrifiers (e.g. organic nitrogen, slow release compounds) would be of benefit to everybody.

The ‘manure mountain’

The use of intensive ‘factory’ farming techniques has resulted in animal wastes becoming a significant problem. The old solution, of spreading the slurry on the land, is no longer adequate. There is insufficient land available, and nitrogen run-off from agricultural lands is also causing problems (see above). Moreover, Goodroad & Keeney (1985) showed that manuring field soil resulted in three times more N$_2$O emission than when ammonium nitrate was used on its own. Another major environmental problem associated with the accumulation, collection and spraying of slurry is the emission of ammonia. Demmers (1989) estimated that in the Netherlands, ammonia emission in a year from these sources reaches 227 900 tonnes. Of this, cattle (132 900 tonnes) contribute the most, with pigs (66 700 tonnes) and chickens (29 200) producing lesser amounts. Processes for nitrifying the ammonia from cattle and swine manure (after degradation of organic carbon constituents) are relatively simple (see, for example, Blouin et al., 1990). Chicken wastes are another matter. Firstly, they are much drier, and are not easily collected in drainage channels. Secondly, they contain a lot of uric acid (Holthuizen & van Beek, 1990; Holthuizen, 1991). At neutral to slightly alkaline pH values and temperatures above 25°C, uric acid is readily broken down to ammonia by bacteria from a number of genera including Rhodococcus, Pseudomonas, Corynebacterium, Mycobacterium and Alcaligenes. As chicken manure has a natural pH around 7, it presents a double problem: breakdown in situ results in high ammonia concentrations in the air within the chicken house and high emissions into the atmosphere, and the accumulation of high-nitrogen waste. Although filters or scrubbers on the air vents would alleviate the emission problem, they would not do much to help the situation inside the battery house, and treatment in situ is therefore being investigated. Holthuizen & van Beek (1990) have described two potential solutions – the addition of lactic acid bacteria to the manure to reduce the pH, thereby cutting ammonia emission, and the use of bacteria capable of heterotrophic nitrification and aerobic denitrification to give direct conversion to N$_2$.

Deforestation

Deforestation, especially of the tropical rain forests, can radically affect the amount of N$_2$O released from the soil. Up to three times more N$_2$O was observed in emissions from cleared soil, than from neighbouring forests (Keller et al., 1986). Studies have even revealed that the change in temperate climates from natural deciduous forests to plantations of conifers appears to have increased N$_2$O emission (Table 4). If it is assumed that the majority of the N$_2$O emission is coming from the top 10–20 cm of the soil, the
Table 6. Estimates of annual nitrogen and other emissions due to pyrodenitrification (data from Crutzen & Andreae, 1990)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Emission from biomass burning (Tg of each element year⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOx</td>
<td>15-46</td>
</tr>
<tr>
<td>RNC</td>
<td>2.1-5.5</td>
</tr>
<tr>
<td>NH₃</td>
<td>0.5-2.0</td>
</tr>
<tr>
<td>N₂O</td>
<td>0.1-0.3</td>
</tr>
<tr>
<td>NO</td>
<td>2-6</td>
</tr>
<tr>
<td>N₂</td>
<td>&lt;11-19</td>
</tr>
<tr>
<td>CO₂</td>
<td>1600-4000</td>
</tr>
<tr>
<td>CO</td>
<td>120-510</td>
</tr>
<tr>
<td>CH₄</td>
<td>11-53</td>
</tr>
<tr>
<td>SO₂</td>
<td>1.0-4.0</td>
</tr>
</tbody>
</table>

ranges of emissions shown in Tables 4 and 5 are in the same order of magnitude.

**Burning of woodlands, etc**

The burning of biologically-produced material (e.g. wood, vegetation, waste) returns between 5 and 50% of fixed nitrogen to the atmosphere (Kuhlbusch et al., 1991). Although the greater part of this is N₂, other nitrogen compounds including NO₂, N₂O, HCN, nitriles and higher organic nitrogen compounds have been detected in smoke and fumes. Table 6 shows estimates of nitrogen emissions produced by burning which were collected by Crutzen & Andreae (1990). Data for a few other compounds have been added for comparison. The emission of NO is especially high, being equivalent to that from natural sources (2-10 and 5-15 Tg N year⁻¹ from lightning and soil, respectively), but it should be remembered that N₂O, especially, has a very long atmospheric lifetime (130 years) and the contribution from this gas represents a slow but steady build up. Since the carbon to nitrogen ratio of the material being burnt appears to have an effect (Kuhlbusch et al., 1991), this information must be relevant for the current practice of burning domestic garbage, as well as forest clearance and agricultural burning (e.g. stubble).

**Industrially related nitrogen pollution**

As yet, it is unknown how much industry contributes to nitrogen-related pollution. Most European factories generating high ammonia wastes have
associated treatment plants where the ammonia is (at least) converted to nitrate (e.g. after methanogenesis). The petrochemical industry, including motorized transport, is another well-known source of nitrogen pollution. However, new sources are coming to light, and a recent paper (Thiemans & Troger, 1991) has described the production of N₂O during the manufacture of adipic acid, a precursor of nylon. The reaction proceeds as follows:

\[ C_6H_{11}OH/C_6H_{10}O + HNO_3 \]

\[ \xrightarrow{cyclohexane} \]

\[ \xrightarrow{cyclohexanone} \]

\[ \rightarrow HOOC(CH_2)_4COOH + aN_2 + bNO + cNO_2 + dN_2O \]

The values for a, b, c and d were previously unknown, but it is now estimated that adipic acid and N₂O are produced in equimolar amounts. The global adipic acid production is about 2.2 × 10⁸ kg year⁻¹, equivalent to an N₂O production rate of 1.5 × 10¹⁰ mol N₂O (about 10% of the observed annual N₂O increase).

Photographic waste

The photographic industry is making great efforts to cut the environmental hazards posed by photographic waste. However, currently used techniques result in a very complex effluent. Silver and cadmium can be (and are) recovered commercially, but this leaves a wastewater which can contain up to 150 g l⁻¹ ammonium thiourea as well as 10–20 g l⁻¹ aromatics, 50 g l⁻¹ sulphide and 1–3 g l⁻¹ cyanide and thiocyanate (Kuenen et al., 1984). Denitrification (using the thiourea as electron donor) with this effluent proved impractical. Qualitative data indicated that the nitrate reduction was terminating at N₂O, presumably because of the cyanide. Aerobic cultures of diluted effluent also converted thiourea to tetrathionate. Cyanide has been shown to inhibit respiration through cytochrome c (Southamer, 1980), and, since the pathway of thiourea oxidation in many bacteria proceeds through cytochrome c, it seems likely that the cyanide was also the problem here, especially after further dilution of the effluent to a level where cyanide should theoretically not be inhibitory resulted in complete conversion of the thiourea to sulphate (Kuenen et al., 1984). Cyanide can be metabolized (generally as a nitrogen source) by a number of pseudomonads and *Bacillus megaterium* (Castrie & Strobel, 1969). A possible solution to this problem would thus be to strip the ammonia by physico-chemical methods (providing a relatively ‘clean’ ammonia solution which could be treated by conventional nitrification techniques). The reduced sulphur compounds could then be treated by cyanide-N assimilating bacteria. Such a system would require a great deal of research before implementation, especially as the aromatics would also require treatment. A potential alternative system using an immobilized biocatalyst (called Cyanidase by its patentees) has recently been described (Basheer,
Kut & Premosil, 1991). This system breaks cyanide down to formate and ammonia, both of which can be readily utilized in a conventional wastewater treatment plant. The authors say that common ions occurring at low concentrations in wastewaters do not interfere with the enzymatic reaction, but it is not clear what effect the complex 'cocktail' of compounds present in photographic wastes would have.

**Aromatic nitrogen compounds**

Because of the toxicity and persistence of halogenated organic compounds in nature, especially the chloroaromatics, their industrial use has recently been reduced. Industry has been switching, where possible, to organonitrogen compounds. Nitroaromatics are important building blocks in the chemosynthesis of aromatic amines including all kinds of acyl derivatives, isocyanates, benzidine compounds and azocompounds. In addition, considerable amounts of nitroaromatics are used in pesticides, explosives, etc. It is thus inevitable that a new class of compounds, seven of which have been listed by the US Environmental Protection Agency as ‘organic priority pollutants’, are beginning to be discharged into the environment. Many of these compounds are mutagenic (and carcinogenic), but can be co-metabolized. Since this can give rise to incomplete or unproductive breakdown, the bacterial strains tend to occur as minor members of microbial communities. As with other xenobiotic compounds, conventional, empirically designed wastewater treatment plants can thus not efficiently handle nitroaromatics. Advanced biotechnological techniques are needed, these include genetic modification (see, for example, Bruhn, Bayly & Knackmuss, 1988; Bruhn, Lenke & Knackmuss, 1987), to provide the required metabolic pathways, and improved reactor design, to retain the modified bacteria.

**THE MICROORGANISMS OF THE NITROGEN CYCLE**

From pp. 228–32 it is clear that nitrogen-related pollution problems can be extremely damaging. Although (micro)biological activity is not primarily responsible for the problems associated with nitrogen pollution, the catalytic role of microorganisms in the conversion of nitrogen compounds may, in a number of cases, aggravate nitrogen-related pollution (e.g. by converting relatively innocuous compounds into more damaging ones). Indeed, if undisturbed, the nitrogen cycle tends to be in balance, but anthropogenic and in rare cases natural factors, are seriously upsetting this balance, resulting in the accumulation of various intermediates, many of which are pollutants.

Mineral nitrogen conversions are generally due to bacteria from diverse physiological groups, all of which have been extensively reviewed (e.g. Payne, 1981; Yordy & Ruoff, 1981; Wood, 1986; Cole, 1987; Kuenen &
Robertson, 1987; Stouthamer, 1988; Bock et al., 1991). Eukaryotes are involved in some processes (e.g. many fungi are heterotrophic nitrifiers, a protozoan has been reported to denitrify (Finlay, Span & Harman, 1983; Finlay, 1985; Killham, 1986), but this review will concentrate exclusively on the bacteria as they are most relevant to the treatment of waste, effluent and water. Of course, the requirements of these bacteria must be understood as sub-optimal conditions in waste treatment reactors will result in incomplete processes and the accumulation of undesirable intermediates (e.g. N₂O).

The main processes of the nitrogen cycle are nitrogen fixation, assimilation of fixed nitrogen, ammonification, nitrification, denitrification and the dissimilatory reduction of oxidized nitrogen compounds. Assimilation is universal, and will not be discussed here. The bacteria carrying out the other reactions fall into five categories. The nitrifiers and denitrifiers will be discussed in greater detail than the others because denitrification and, especially, nitrification, have often been bottlenecks in the design of bioreactors, and in the daily operation of wastewater treatment plants.

*The nitrogen fixing bacteria*

These are the specialized bacteria which transform atmospheric N₂ to biologically available NH₃. They are not especially relevant in pollution studies, except for the observation that some of them (e.g. *Rhizobium 'hedyseri', Rhizobium japonicum*) can also produce N₂O or N₂ (Anderson & Levine, 1986; Casella et al., 1988).

*The ammonifying bacteria*

This is a very heterogenous group of bacteria which carry out the breakdown of organic nitrogen compounds to ammonia. It includes such dissimilar species as the anaerobes responsible for acidification prior to methanogenesis, and freshwater aerobes which break down amino acids (Seper's, 1979). These bacteria are essential to the overall functioning of the nitrogen cycle, but their main importance to this review is their part in the return of organic nitrogen to the inorganic state, making it available for the activities of the nitrifiers.

*The nitrifying bacteria*

Nitrification may be defined as the oxidation of reduced nitrogen compounds. Although Pasteur (1862) first suggested that nitrification in soil was probably biological, it was not experimentally identified as a biological process until the work of Schloesing & Muntz (1877a,b). The earliest claim
Table 7. Examples of autotrophic nitrifying bacteria

<table>
<thead>
<tr>
<th>Ammonia to nitrite</th>
<th>Nitrite to nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitrosonomas europae</em></td>
<td><em>Nitrobacter winogradskyi</em></td>
</tr>
<tr>
<td><em>Nitrosocystis javanensis</em></td>
<td><em>Nitrobacter agilis</em></td>
</tr>
<tr>
<td><em>Nitrosospira brieni</em></td>
<td><em>Nitrococcus mobilis</em></td>
</tr>
<tr>
<td><em>Nitrosolobus multiformis</em></td>
<td><em>Nitrospina gracilis</em></td>
</tr>
</tbody>
</table>

for the isolation of nitrifying bacteria was made by Heraeus (1886) but other workers were unable to repeat his work. There were other claims, but it is the work of Winogradsky (1890a,b,c) which is generally regarded as providing the foundation for the modern study of nitrification. Reviews of this very early work have been published by Nelson (1929), Kingma-Boltjes (1934) and Macdonald (1986).

The autotrophic nitrifiers

The autotrophic nitrifying bacteria can be divided into two groups. Those in the first group obtain energy for growth from the oxidation of ammonia to nitrite. The bacteria from the second group derive energy from the oxidation of nitrite to nitrate. In both cases, CO₂ serves as the carbon source. Examples of the species in the two groups are shown in Table 7. The physiology of the autotrophic nitrifying bacteria has been relatively well studied (see, for example, reviews by Hooper, 1984; Bock, Koops & Harms, 1986; Wood, 1986), but one subject relevant to wastewater treatment which remains a matter of discussion is their sensitivity or insensitivity to organic compounds. On one hand, it has been known for a long time that *Nitrobacter* species can grow chemoorganoheterotrophically on acetate, formate and pyruvate (Bock, 1976), and recent work (Bock *et al.*, 1991) has shown that many *Nitrobacter* species can be grown as heterotrophs while denitrifying. On the other hand, it has been claimed that even small amounts of organic chemicals are sufficient to inhibit ammonia oxidation. Indeed, it has been shown that organic solvents such as acetone or ethanol (which are used to dissolve the nitrification inhibitor, N-serve) have almost as much effect without the inhibitor (Hall, 1984). However, extensive studies have revealed that low concentrations of organic compounds do not inhibit nitrification (and can even be assimilated; van Niel, 1991), although higher concentrations of common organic compounds such as organic acids may indeed be inhibitory.

The enzymology of autotrophic ammonia and nitrite oxidation has recently been reviewed (Bock *et al.*, 1986, 1991; Wood, 1986). The autotrophic nitrifiers oxidize ammonia via hydroxylamine to nitrite. Ammonia oxidation is carried out by means of an ammonia monooxygenase which
probably reacts with NH₃ rather than NH₄⁺ (Suzuki, Dular & Kwok, 1974). Experiments with stable isotopes have shown that the oxygen comes from molecular oxygen rather than water (Dua, Bhandari & Nicholas, 1979). The reaction is energetically unfavourable, and it is the oxidation of hydroxylamine to nitrite by means of hydroxylamine oxidoreductase which actually generates energy for growth. In addition to the usual production of nitrite, hydroxylamine oxidoreductase can also form N₂O from hydroxylamine and nitrite (Hooper, 1984). Nitrite oxidation proceeds to nitrate by means of nitrite oxidoreductase without any detectable intermediates (Bock et al., 1986). The oxygen involved has been shown to come from water.

Nitrification is, of course, linked to the cytochrome chain. The terminal oxidases in the obligate autotrophs appear to be cytochrome a₁ in *N. europaea* (Erikson & Hooper, 1972; Yamazaki, Fukumori & Yamanaka, 1985) and cytochrome aa₃ in *Nitrobacter agilis* (Yamanaka, Kamita & Fukumori, 1981). Cytochromes o and a₁ have also been found in *Nitrobiacter* (Wood, 1986). NADH is generated by reversed electron flow in which the proton motive force drives electrons ‘backwards’ along the cytochrome chain, generating NAD⁺ (Wood, 1986). However, Frietag & Bock (1990) concluded from their investigations with *Nitrobacter winogradskyi* that the oxidation of nitrite or nitric oxide by this strain resulted in the generation of NADH, which was then used for ATP synthesis.

### Heterotrophic nitrification

The heterotrophic nitrifiers are less well understood. Some of them (including fungi) oxidize the amino- and nitro- groups of inorganic nitrogen compounds, releasing the nitrogen as nitrate or nitrite (Verstraete, 1975). Another (overlapping) group oxidizes ammonia to nitrite or nitrate. They only nitrate if supplied with an external, organic substrate, and have not been shown to gain any energy from the reaction. Examples of their potential substrates and products are shown in Table 8. The ecological significance of this phenomenon has been a subject of discussion and controversy since the time of Winogradsky. Doubts expressed about the purity of cultures led Kingma-Boljes (1934) to dismiss the description of a heterotrophic nitrifier by Stutzer & Hartleb (1894) as valueless. Indeed, occasional spurious claims, the use of cultures which were subsequently found to be communities, poor experimental methods and insufficient experimental documentation over-shadowed the early literature and makes it very difficult to assess the validity of results. Kingma-Boljes (1934) extensively reviewed the published work on heterotrophic nitrification and concluded that the only authentic nitrifiers which had been isolated up until the time of his writing were the obligate autotrophs isolated by Winogradsky.

The more modern reviews such as those by Verstraete (1975) and Killham (1986) recognise not only the existence of heterotrophic nitrification, but also the surprisingly heterogenous group of prokaryotes and eukaryotes
Table 8. Examples of prokaryotic and eukaryotic heterotrophs which nitrify (data mainly from Verstraete, 1975)*

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthrobacter globiformis</td>
<td>ammonium</td>
<td>hydroxylamine</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>ammonium</td>
<td>monohydroxamic a.</td>
</tr>
<tr>
<td>Streptomycetes sp.</td>
<td>ammonium</td>
<td>monohydroxamic a.</td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>ammonium</td>
<td>dihydroxamic a.</td>
</tr>
<tr>
<td>Aerobacter aerogenes</td>
<td>ammonium</td>
<td>dihydroxamic a.</td>
</tr>
<tr>
<td>Rhodotorula sp.</td>
<td>ammonium</td>
<td>dihydroxamic a.</td>
</tr>
<tr>
<td>Ustilago sphaerogena</td>
<td>ammonium</td>
<td>trihydroxamic a.</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>ammonium</td>
<td>trihydroxamic a.</td>
</tr>
<tr>
<td>Streptomycetes griseus</td>
<td>ammonium</td>
<td>trihydroxamic a.</td>
</tr>
<tr>
<td>Thiophaera pantotropha</td>
<td>ammonium</td>
<td>nitrite*</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>hydroxylamine</td>
<td>nitrite</td>
</tr>
<tr>
<td>Alcaligenes sp.</td>
<td>oximes</td>
<td>nitrite*</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>aromatic nitro comps.</td>
<td>nitrite</td>
</tr>
<tr>
<td>Flavobacterium sp.</td>
<td>aromatic nitro comps.</td>
<td>nitrite</td>
</tr>
<tr>
<td>Nocardia sp.</td>
<td>aromatic nitro comps.</td>
<td>nitrite</td>
</tr>
<tr>
<td>Chlorocella sp.</td>
<td>ammonium</td>
<td>nitrate</td>
</tr>
<tr>
<td>Aspergillus parasticus</td>
<td>ammonium</td>
<td>nitrate</td>
</tr>
<tr>
<td>Aspergillus wentii</td>
<td>nitrite</td>
<td>nitrate</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>aliphatic nitro comps.</td>
<td>nitrate</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>aromatic nitro comps.</td>
<td>nitrate</td>
</tr>
</tbody>
</table>

* indicates bacteria known to simultaneously denitrify to N₂O or N₂.  
comp. = compounds. a. = acids.

which are involved. Representatives of this group are shown in Table 8, but for an exhaustive list, the reader is referred to Verstraete (1975). The reasons for heterotrophic nitrification may be as diverse as the bacteria which do it. For example, heterotrophic nitrification by an Arthrobacter species may be related to the production of chelating agents (Verstraete, 1975). Other species, including Thiophaera pantotropha, Pseudomonas 'determinants', and an Alcaligenes species are thought to use the ammonia oxidizing step (which requires NADPH) as a dump for excess reducing power, possibly because of a rate-limiting step in the electron transport chain to oxygen (Robertson et al., 1988, 1989a; Robertson & Kuenen, 1990; van Niel, 1991). In other cases, heterotrophic nitrification may be due to co-metabolism, when the only reason that the reactions occur is a biochemical accident rather than a physiological trait which is important to the survival of the species. Of course, with such a diverse group of organisms ranging from bacteria to rat liver cells, it is very likely that apparently similar reactions are used for a multiplicity of reasons.

For a long time, it was considered that as the apparent rates of nitrification by heterotrophic bacteria appeared to be very low compared with those of the autotrophs, the phenomenon was of little significance outside the laboratory. However, some researchers have claimed that in certain types of
soil (e.g. very acid soils) where autotrophic nitrifiers were unable to grow, the heterotrophs were responsible for the bulk of nitrification taking place (for example, Strayer, Lin & Alexander, 1981). Additionally, many common soil denitrifiers have been shown to be heterotrophic nitrifiers (Castignetti & Hollocher, 1984). Nitrification has traditionally been evaluated in terms of oxidation product (i.e. nitrite or nitrate) accumulation, and the observation that many heterotrophic nitrifiers are simultaneously denitrifying, with little or no accumulation of dissolved oxidation products, means that many heterotrophic nitrification rates have probably been underestimated, and will have to be reevaluated in terms of complete nitrogen balances (Robertson & Kuenen, 1990). From the pure culture studies done thus far, it appears that when the combination of denitrification with nitrification is taken into account, heterotrophic nitrification rates are only one to two orders of magnitude lower than those of the autotrophs. Given the dominance of heterotrophs over autotrophs in most ecosystems, this implies that heterotrophic nitrifiers could significantly contribute to nitrification in nature. For the potential of this type of organism in wastewater treatment, see pp. 254-5.

The biochemistry of heterotrophic nitrification has been studied by several research teams (e.g. Doxtader, 1965; Aleem, 1975) and there is evidence that at least two different (organic and inorganic) pathways exist (Killham, 1986). It seems likely that most of the fungi use an organic route as follows:

\[
\text{RNH}_2 \rightarrow \text{RNOH} \rightarrow \text{R-NO} \rightarrow \text{RNO}_2 \rightarrow \text{NO}_3^-
\]

However, evidence for an inorganic pathway has also been found (e.g. Aleem, 1975):

\[
\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NOH} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-
\]

Recent work in Delft has indicated that a well-documented heterotrophic nitrifier/denitrifier, \textit{Tsa. pantotropha}, also uses the inorganic pathway with a NADPH dependent, light sensitive monooxygenase as the first enzyme of the pathway (Robertson & Kuenen, 1988). It is, of course, also possible that some organisms use combinations of these pathways, as proposed by Verstraelen (1975). As yet, it is not clear if, or how, nitrification by heterotrophs relates to the cytochrome chain as the reaction does not generate energy.

Effect of oxygen concentration on nitrification

It is a generally held view that nitrification does not proceed efficiently at low dissolved oxygen concentrations, but the reports of the actual concentrations are variable. As will be discussed on pp. 256-7, there have been reports of oxygen-limited ammonia oxidizers denitrifying, producing N\textsubscript{2}O or even N\textsubscript{2}. Values for \(K_c\) (the dissolved oxygen concentration at which the
specific growth rate is half that obtained when oxygen is not limiting) vary from 0.5 μm to 6.25 μm (Winkler, 1981). This variability can be attributed to many factors including oxygen diffusion through biolayers, competition for oxygen by other organisms and various physical parameters. Species or even strain differences among the nitrifiers present may also be a factor. Gundersen, Carlucci & Bostrum (1966) showed that *Nitrosomonas oceana*us lost 25% of its nitrifying capacity when the oxygen concentration of cultivation was reduced from 100% to about 10% of air saturation. However, under the same conditions *Nitrosomonas europaea* greatly increased its nitrification rate. The amount of nitrite oxidized by *Nitrobacter agilis* dropped by 50% when cultivated at 10% rather than 100% air saturation. Chemostat cultures of *Nitrosomonas* and *Nitrobacter* isolates have been used to show that the two types of nitrifier have different responses to low oxygen (Helder & de Vries, 1983). Steady state cultures of the two organisms gave complete conversion of ammonia to nitrate at dissolved oxygen concentrations down to about 100 μmol l⁻¹. Below this, the nitrate concentration fell, and nitrite accumulated, indicating that the nitrite oxidizer was in trouble. At dissolved oxygen concentrations below 50 μmol l⁻¹, ammonia also began to accumulate and the culture washed out. This, and other work on the effect of dissolved oxygen concentrations in natural systems, was extensively reviewed by Kuenen & Robertson, (1987).

As has already been pointed out, some heterotrophic nitrifiers nitrify most efficiently at low dissolved oxygen concentrations, and this might indicate a possible niche for them. Oxygen limitation in wastewater treatment systems (e.g. within thick biofilms) could therefore favour such organisms.

**Nitrification-related pollution**

As mentioned above, the nitrifiers can potentially contribute to pollution in a number of ways. They are responsible for a great deal of soil and water acidification as they produce HNO₂ and HNO₃. They have been shown to generate NO and N₂O themselves and, as will be discussed in the section on agriculture, also make fixed nitrogen available for denitrification, providing a second possible source for nitrogenous gases. Moreover, the heterotrophic nitrifiers are not alone in their ability to nitrify and denitrify simultaneously. NH₃ oxidizing bacteria from the genera *Nitrosomonas* and *Nitrososybrio* have been found to produce NO, N₂O and N₂ under oxygen stress (Poth & Focht, 1985; Poth, 1986; see pp. 256–7). Various studies of soil activity using organic and inhibitor additions have indicated that the autotrophic ammonia oxidizers are a source of NO and N₂O (Tortoso & Hutchinson, 1990). Moreover, recent findings (see pp. 256–8) have demonstrated that anoxic ammonia oxidation (to N₂) can take place, indicating that even ammonia oxidation is not absolutely dependent on molecular oxygen (van de Graaf et al., 1990).
The dissimilatory nitrate reducers

These are generally fermentative bacteria (e.g. *Escherichia coli*) which reduce nitrate to nitrite probably as an electron acceptor (Cole, 1990). As with aerobic denitrification, this had been believed to be an anaerobic phenomenon, but a *Bacillus licheniformis* strain has recently been reported to carry out the reactions aerobically (Brons & Zehnder, 1991). The second phase, from nitrite to ammonia, appears to be a means of dumping excess reducing power, but may have other functions (Cole, 1987). Kaspar & Tiedje (1981) showed that these bacteria were more likely than denitrifiers to occur when the concentrations of organic compounds were high in relation to the amount of available nitrate or nitrite. At such times they might be responsible for localized nitrite accumulation, or even ammonia build up. Dissimilatory nitrate reduction has been observed in freshwater, estuarine and marine deep sediments where the C:N ratio was high (Cole & Brown, 1980; Sorensen, 1987), and dissimilatory nitrate reducing bacteria have been isolated from a wide variety of sources, including marine and fresh water as well as from samples from the rumen and human stomach (Jones, 1972; Kuenen & Robertson, 1987; Forsythe *et al.*, 1988). At least two of these bacteria, *E. coli* and a *Citrobacter* species, have been shown to also reduce nitrite to N₂O (Smith, 1982; 1983).

The denitrifiers

The denitrifiers are the bacteria generally, but not solely (see above) responsible for returning fixed nitrogen to the atmosphere. In the right place, they are therefore very valuable in efforts to reduce nitrogen pollution. Most denitrifiers can reduce nitrate via nitrite, NO and N₂O to N₂ without the appearance of intermediates. However, some species lack key enzymes (e.g. *Pseudomonas aureofaciens* has no N₂O reductase, some *Alcaligenes* strains do not have a nitrate reductase). The environmental conditions also play a role in defining the extent and efficiency of denitrification. For example, sulphide often inhibits N₂O reductase, and high nitrate concentrations can result in nitrite accumulation. Sub-optimal pH and/or temperature can result in N₂O or NO generation. Denitrification is at its most efficient under anaerobic conditions, but many bacteria can denitrify at significant oxygen concentrations, and the entire pathway is not always shut down at a single critical oxygen concentration. NO and N₂O can thus be the result of aerobic denitrification (Hochstein, Betlach & Kritikos, 1984; Davies, Lloyd & Boddy, 1989), and even ‘classical’ denitrifiers such as *Paracoccus denitrificans* may accumulate nitrite or N₂O after exposure to relatively short anoxic periods (Kawakami, Pacaud & Nishimura, 1985; Robertson & Kuenen, 1990).

Denitrification is not a phenomenon limited to a few special genera, but
occurs in some (but not all) members of apparently unrelated genera. Examples of bacteria which denitrify are shown in Table 9. Such a wide spread in the types of bacteria able to denitrify implies either converging evolution or, alternatively, that denitrification is a very old phenomenon in evolutionary terms. Indeed, it has been suggested (Mancinelli & McKay, 1988) that denitrification actually evolved before oxygen respiration. This suggestion is based on the idea that lightning in the CO₂/N₂ atmosphere of the early earth would have generated NO. Deposition of this in the oceans would lead to a series of chemical reactions culminating in NO₃, and providing a potential electron acceptor before O₂ became available. The evolutionary sequence proposed is:

Ammonification → Denitrification → Nitrification → Nitrogen fixation

In addition, speculation that the denitrifying bacteria (such as Pa. denitrificans) and the eukaryotic mitochondrion are descended from a common ancestor has been fuelled by the finding that members of at least one freshwater protozoan genus, Leoxodes, are capable of denitrification, and that the denitrifying enzymes seem to be associated with their mitochondria (Finlay, Span & Harman, 1983; Finlay, 1985).

Gayon & Dupetit (1986) first introduced the term 'denitrification' for a phenomenon which had, by then, been known for some years. There has since been an enormous number of papers published on the subject (for an extensive review see Payne, 1981).

Occurrence

The opportunities for survival and/or occurrence (under anoxic conditions) of denitrifying and other bacteria which use nitrate reduction for dissimilatory purposes are limited by the amount of nitrate or nitrite available, and thus, to some extent, by the activity of the nitrifiers. As pointed out on
p. 244, dissimilatory nitrate reduction is the preferred route for nitrate reduction when the ratio of electron donor to electron acceptor is high (Kaspar & Tiedje, 1981; Tiedje et al., 1982). When the reverse is true, denitrification is favoured. This model appears to hold particularly well for sediments (MacFarlane & Herbert, 1984; Kuenen & Robertson, 1987).

The wide range of conditions favouring denitrifying bacteria is illustrated by the results of a study of bacteria found in agricultural soils made by Gamble, Betlach & Tiedje, (1977). Denitrifiers were found to occur in all of the soil types studied, at a wide range of temperatures (5–30 °C), pH values (4.4–7.8) and moisture levels. There was no clear correlation between the size of the denitrifying population and either the amount of organic material present or the number of different isolates which were found. Most of the isolates appeared to be members of the genus Pseudomonas.

Physiology and electron transport
As already mentioned, nitrate reduction in denitrifying bacteria proceeds via nitrite, nitric oxide and nitrous oxide to N₂. Nitric oxide is also generated in some cases. The enzymology of denitrification has been reviewed in detail (Payne, 1981; Stouthamer, 1988).

The nitrate reductases from the denitrifying and dissimilatory nitrate reducing bacteria appear to be surprisingly similar (Stouthamer, 1988). These nitrate reductases are molybdoenzymes located on the cytoplasmic side of the cell membrane. In Escherichia coli, one of the subunits has been found to be a cytochrome b which is necessary not only for electron transport, but also for the association of the enzyme with the membrane. A second, periplasmic, denitrifying nitrate reductase has been reported for Rhodospirillum sphaeroides and aerobically denitrifying Tsa. pantotropha (Ferguson, Jackson & McEwan, 1987; Bell, Richardson & Ferguson, 1990). In addition to its location outside the cell membrane, this enzyme differs from the better-known enzyme in its resistance to low concentrations of azide (20 μM).

The main discrimination between denitrification and dissimilatory nitrate reduction appears at the level of nitrite reduction. In denitrification, two types of nitrite reductase are known to occur. The best-known is cytochrome cd which occurs in, among others, Pa. denitrificans, Ps. aeruginosa, Ps. stutzeri and Thiobacillus denitrificans (Payne, 1981; Stouthamer, 1980, 1988). Purified cytochrome cd produces a mixture of NO and N₂O. The second nitrite reductase is a soluble, copper-containing enzyme which occurs in the periplasm. It has been found in, among others Rhodopseudomonas sphaeroides var denitrificans, Corynebacterium niphridii, Azchromobacter cycloclastes, Alcaligenes sp. and Tsa. pantotropha (Iwasaki et al., 1963; Reuner & Becker, 1970; Iwasaki & Matsubara, 1972; Iwasaki, Noji & Shidara, 1975; Sawada, Sato & Kitamura, 1978; Robertson et al., 1989a).
Nitric oxide is the product of the purified enzyme. The distribution and characteristics of the two nitrite reductases have been reviewed by Coyne & Tiedje (1990). Both nitrite reductases display cytochrome oxidase activity, but have a considerably lower $K_m$ for nitrite than for oxygen.

$\text{N}_2\text{O}$ reductase is not very stable. Like one of the nitrite reductases, it is a copper-containing enzyme with a periplasmic location. Various molecular weights and subunit combinations have been reported (Stouthamer, 1988), but it is not clear whether this is due to a range of different enzymes or the effect of different preparatory methods on a relatively labile enzyme. It can be inhibited by a number of factors (Kuenen & Robertson, 1987) including oxygen, sulphide and acetylene, and tends to be produced when environmental conditions are sub-optimal (e.g. low pH or temperature) for the denitrifier involved.

As with oxygen respiration, denitrification proceeds via the cytochrome chain. Only a few cytochrome chains have been completely mapped (see, for example, Stouthamer, 1988). In Pa. denitrificans, the presence or absence of the various cytochromes appears to be controlled by the redox levels within the cytochrome chain. For example, when the dissolved oxygen concentrations are high, cytochrome $a_a$ is the dominant terminal oxidase. As the dissolved oxygen falls (or during growth on very rich media), cytochrome $c$ becomes more important, and as the dissolved oxygen concentration approaches $0$, cytochrome $c_d$ is synthesized (Sapshead & Wimpenny, 1972).

**Aerobic denitrification**

As has been discussed elsewhere (Robertson & Kuenen, 1984a, b; 1990), it was believed for many years that denitrifying bacteria would invariably shut off their denitrifying capacity whenever significant quantities of oxygen were available. The experimental evidence for this was based on studies of a limited number of species. Because of the difficulty of measuring low oxygen concentrations and some of the intermediates involved, claims that denitrification could occur under aerobic conditions were sometimes ignored. Some reports could be dismissed on the basis of insufficient controls or poor methodology. Recent work in a number of laboratories (see Robertson & Kuenen, 1990 for extensive review) has now shown that the picture is more complex, and the ‘black or white’ response to oxygen is true for many, but not all species. A number of species from different genera have now been shown to be capable of simultaneous oxygen respiration and denitrification (see, for example, Robertson & Kuenen, 1984a, b, 1990; Trevors & Starodub, 1987; Lloyd, Boddy & Davies, 1987; Strand, McDonnell & Unz, 1988; Robertson et al., 1989a, b). These bacteria do not all behave in the same way, and there appears to be a ‘spectrum’ of responses to the dissolved oxygen concentration. Thus *Tsa. pantotropha* continues to denitrify at dissolved oxygen concentrations above 80% air saturation, an *Alcaligenes* species
begins to accumulate intermediates at around 50% air saturation, and *Hyphomicrobium X* can only continue to denitrify at low oxygen concentrations (Meiberg, Bruinenberg & Harder, 1980; Robertson et al., 1988; van Niel, 1991). *Aquilspirillum magnetoaeticum* cannot grow under fully anoxic conditions, but can denitrify microaerophilically (Bazylinski & Blakemore, 1983). Even aerobic cultures of the 'classical' denitrifier *Pseudomonas stutzeri* have been shown to have constitutive N₂O reductase, and to be more responsive to the type of nitrogen compound available in the medium than to anoxia (Korner & Zumft, 1989).

It must be emphasized that aerobic denitrification tends to be slower than anoxic denitrification. All of the aerobic denitrifiers thus far studied increased their rate of denitrification as the dissolved oxygen concentration in the culture fell, with *Tsa. pantotropha* reaching its maximum rate at about 30% air saturation.

*Effect of oxygen on denitrification*

The mechanism (or mechanisms) by which oxygen affects denitrification in many bacteria is not yet fully understood. Anoxia is sufficient to induce nitrate reductase in some strains, others require the presence of nitrate. Similarly, the denitrifying enzymes of some bacteria appear to be inactivated by oxygen, whereas in others their synthesis is repressed but the existing enzymes only gradually disappear (Payne, 1981; Knowles, 1982). Much of the work on oxygen inhibition has been done with *Pa. denitrificans* (Alefounder & Ferguson, 1981; Alefounder et al., 1983, 1984; Boogerd, 1984; Stouthamer, 1988) and it appears that two factors, the redox level of the cytochrome chain and the permeability of the cell membrane to nitrate, may be interacting in the inhibition of nitrate denitrification by oxygen in this species (Kucera & Dadak, 1983; Alefounder et al., 1984). Since the denitrifying nitrate reductase is generally located on the inside of the cell membrane (Stouthamer, 1980), a permeability barrier between the enzyme and its substrate would be a very effective controlling factor. The observation that anaerobically grown cells of the aerobic denitrifiers *Tsa. pantotropha* possess the cytoplasmic nitrate reductase, but aerobically denitrifying cultures contain the periplasmic enzyme (Ferguson et al., 1987; Bell et al., 1990) tends to support this hypothesis. However, inhibition of nitrate transport cannot be the only explanation, since most strains of *Pa. denitrificans* are also unable to reduce nitrite aerobically. As with other denitrifiers, the nitrite reductase is located in the periplasm and thus would be easily accessible to nitrite. Given the fact that nitrite has been shown to inhibit oxygen uptake, affecting the redox level of the electron transport chain, electrons should be able to branch off to nitrite under aerobic conditions. As this apparently does not happen, another reason must be found to account for the inability of *Pa. denitrificans* to denitrify nitrite aerobically.
The aim of this section is to review the microbiological aspects of nitrogenous pollution and its treatment in (waste)water treatment plants. Physico-chemical methods (e.g. stripping, precipitation) will not be discussed.

*Drinking water*

Although a significant amount of nitrate may be ingested in the diet (especially in vegetables such as lettuce, cabbage and spinach as well as some meats and cheeses), the levels of nitrate consumed in drinking water is causing concern in some areas, particularly where the water supply is dependent on ground water or rivers accumulating run-off from agricultural land. Current guidelines suggest that nitrate levels below 10 mg l$^{-1}$ are safe (WHO, 1984). Nitrate is mainly a problem because it is readily reduced to nitrite in the mouth and digestive tract, and nitrite is associated with several health hazards. For example, nitrite reacts with haemoglobin to produce methaemoglobin. This naturally only makes up 1–2% of the total haemoglobin content, but if it reaches 10%, clinical symptoms occur, whilst 30–40% results in anoxia. Increased concentrations of methaemoglobin in blood have been associated with water concentrations of nitrate around 20 mg l$^{-1}$, and infant methaemoglobinemia (blue baby syndrome) can occur when babies are bottle fed with water containing higher levels. At high pH, nitrite can be converted to nitrosamines, some of which may be carcinogenic. Evidence for a connection between nitrate concentrations and cancer is still indirect, but epidemiological studies have revealed a potential link (WHO, 1984).

Major problems associated with the denitrification of drinking water include the selection of a suitable electron donor for the denitrifying bacteria, and the prevention of excessively high bacterial contamination of the water. This latter problem can be overcome by the use of fixed film reactors such as fluidized beds. The former is more difficult to solve. Methanol has been a popular choice (Payne, 1981) as it allows high denitrification rates, but methanol is itself toxic, and it must obviously be completely removed before the water can be consumed. In addition, the first product of the oxidation of methanol by bacteria, formaldehyde, is toxic to both humans and the microorganisms themselves. Indeed, experience gained during research into the production of single cell protein from methanol has shown that formaldehyde can, in some circumstances, accumulate to toxic levels. A semi-industrial drinking water treatment system which was supplemented with methanol removed >97% of the nitrate in the influent water, provided that methanol was not limiting (Table 10). If neither methanol nor nitrate was limiting, nitrate removal was still effective, and the methanol concentration in the effluent water was also low.
Table 10. Denitrification of drinking water in a fluidized bed column at 9°C with methanol as the electron donor (data from Germonpre et al., 1991)

<table>
<thead>
<tr>
<th>Limiting substrate</th>
<th>Influent concentration (mg l⁻¹)</th>
<th>Effluent concentration (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO₃⁻</td>
<td>CH₃OH</td>
</tr>
<tr>
<td>CH₃OH</td>
<td>66.0</td>
<td>29.31</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>65.5</td>
<td>52.97</td>
</tr>
<tr>
<td>Neither</td>
<td>66.5</td>
<td>50.07</td>
</tr>
</tbody>
</table>

(Germonpre, Liessens & Verstraete, 1991). The Flemish Executive government has authorized the addition of 25 mg l⁻¹ methanol to the system for a test period, but has set a maximum effluent concentration of 0.25 mg methanol l⁻¹, even though the subsequent treatment of water in this particular plant ensures that no methanol reaches the distribution system.

As a consequence of methanol toxicity, other electron donors might be attractive, if cheap enough. Ethanol has been used successfully in a French system (Ravarini, Coutteisle & Damez, 1988), but was associated with higher sludge production, lower denitrification rates than achieved with methanol, and higher costs. Sulphur was proposed by van de Hoek (1989), among others, as it could provide a solid substratum for the bacteria as well. This has the added advantage that autotrophic sulphur bacteria do not present a health hazard. However, for every six molecules nitrate reduced, five molecules of sulphate are formed, thus increasing the sulphate level in the water substantially. Unfortunately, high concentrations of sulphate are also undesirable because sulphate can act as a laxative, especially in combination with magnesium, and sensitive people can be affected by as little as 400 mg l⁻¹ (WHO, 1984). Others may be affected at concentrations around 700 mg l⁻¹, although the body gradually adapts after prolonged exposure.

Sulphate also affects the taste of water, the effective threshold being different for different salts. For the sodium, calcium and magnesium sulphates, the thresholds are 200–500, 250–900 and 400–600 mg l⁻¹ respectively (WHO, 1984). A maximum of 400 mg sulphate l⁻¹ is therefore recommended, indicating that the autotrophic sulphur bacteria may not be the most suitable denitrifiers for drinking water.

Perhaps the most attractive substrate of all for drinking water denitrification is hydrogen. It is not toxic, and the oxidation product is water. A hydrogen-based system employing biomass attached to polyurethane matrices in two reactor columns has been described by researchers at the University of Gent (Dries et al., 1988). The nitrate-containing water flows down the first column against a stream of H₂. It then passes into an up-flow reactor where the remaining hydrogen is oxidized, and the last of the nitrate
reduced. The upper part of this reactor is aerated, so that nitrifiers attached to the polyurethane can remove any remaining intermediates such as nitrite, by re-oxidizing them back to nitrate. Optimization of the system has resulted in 80–100% nitrate removal from drinking water containing less than 50 mg N L⁻¹. The maximum denitrification rate achieved was 0.2 g N L⁻¹ total reactor with 80% hydrogen in the gas phase, which is low compared with more traditional systems. Perhaps the most obvious disadvantage is that hydrogen easily forms explosive mixtures with air, and therefore its use requires careful plant design and safety measures.

A recent paper (Heinemann & Muller, 1991) has suggested that poly β-hydroxybutyrate (PHB) would be a suitable substrate for denitrifiers. The proposed process, which is at an early stage of development, involves the co-entrainment of PHB and denitrifying bacteria in a biopolymer matrix such as alginates or chitosan. Initial results seem promising, although the authors report that nitrite accumulation remains a problem to be solved.

Conventional systems in wastewater treatment

From the discussion of the bacterial physiology above, it is clear that the design of the majority of conventional wastewater treatment systems is based on the concept that nitrification and denitrification are mutually exclusive. The reasoning is that (autotrophic) nitrifiers require a relatively high dissolved oxygen concentration, and little or no competition from rapidly-growing heterotrophs. Equally, the denitrifiers require anoxic conditions and, with a few exceptions, organic substrates. Although the existence of ‘aerobic denitrifiers’ is now recognised, it must be emphasized that all of the species thus far studied perform more efficiently at low dissolved oxygen concentrations. Designers and operators of most wastewater treatment plants have, until now, been compelled to take these opposing requirements into account.

The different systems in use for maintaining aerobic nitrification reactors include trickling filters where the water percolates through a bed with growing biomass (Focht & Chang, 1975), rotating discs to which the biomass is attached (Antonie, 1978), and fluidized beds in which the bacteria form pellets around a carrier material (often sand) and are kept in suspension by the high flow rates used (Andrews, 1982; Heijnen, 1984). The activated sludge process is not always ideal for nitrification because the abundant heterotrophic growth present in the reactor tends to out-grow, and thus out-compete for oxygen, the autotrophic nitrifiers. Perhaps, more importantly, the rate of growth of the sludge defines the rate at which it must be harvested. As the growth rate of the autotrophic nitrifiers tends to be much slower than those of most heterotrophs, they will gradually be ‘diluted out’ by the harvesting process.

In many cases, the effluent from nitrification reactors is passed through a
denitrification step to remove nitrogen as N₂. These denitrification systems, because of the requirement for anoxic conditions, tend to be enclosed. Bioreactors currently in use include fluidized beds, anaerobic activated sludge and packed bed reactors (Francis & Callahan, 1975; Heijnen, 1984; Gommers, 1987). Denitrifying bacteria are fairly catholic in their range of usable substrates, but many of these substrates are too expensive to be economic choices for wastewater treatment. In addition to methanol, acetate and sulphur have been used, the latter relying on Thiobacillus denitrificans and related bacteria (Batchelor & Lawrence, 1978). The prime, and obviously cheaper, source of energy is, of course, the wastewater itself. Although this is only a satisfactory solution if the proportion of usable carbon sources is high enough for the amount of nitrate to be removed, many treatment plants are designed in such a way as to allow this to be done.

Over the years, many integrated nitrification and denitrification systems have been developed (see Winkler, 1981). Of course, nitrogen removal is not always the only concern of wastewater treatment and, in most cases, carbon removal has priority. This has led to more and more sophisticated designs in which biotechnological expertise based on fermentation technology has played a role. Sequential carbon removal and nitrification reactors allow the operation of the different reactors under the optimal conditions for each individual process involved. As already mentioned, a multiple stage system which also includes a denitrification stage has been patented (E.P.A. 0051 888) by Gist Brocades, in Delft (Fig. 1). This system recovers energy from the effluent in the form of methane, and then uses the sulphide generated by sulphate reducing bacteria during methanogenesis, together with any organic material passing through the methanogenic reactors, as the energy sources for denitrification. The reactor configuration avoids the problems associated with nitrifying bacteria in a high BOD effluent by placing the nitrification reactor last in the sequence, and then recirculating the nitrate generated therein to the denitrification reactor.

Some nitrogen removing plant designs have attempted to avoid the requirements for two bioreactors by either incorporating aerobic and anoxic zones within a single vessel, or by aerobic/anaerobic cycles. The best-known systems of this type are the 'Carousel' and the Pasveer ditch, both of which depend on recirculation of the water through channels incorporating aerobic and anoxic zones. The structure and kinetics of such reactors have been described extensively by Winkler (1981).

Use of naturally or artificially immobilized microorganisms in waste water treatment

Biofilms

As mentioned above, many reactors employ immobilized bacteria (e.g. in air lift or fluidized bed reactors) in order to retain slow-growing bacteria in
Fig. 1. Outline of a sequential wastewater treatment system which employs fluidized bed reactors for carbon and nitrogen removal. The nitrogen content of the wastewater is reduced by denitrification of recirculated effluent after nitrification. Reduced sulphur compounds generated during acetogenesis and methanogenesis, together with residual organic carbon, provide the electron donors for denitrification (from Kuenen & Robertson, 1987).

high flow systems or to provide dense biomass in a limited space. In the past, a number of immobilization methods have been tried. These include attachment to a carrier such as sand (Gommers, 1987), cells trapped in liquid surfactant membranes (Mohan & Li, 1975), rotating discs and anaerobic filters (Winkler, 1981). In most of these systems, the natural abilities of microorganisms to attach to a variety of interfaces, supports and surfaces are exploited. Spontaneous biofilm formation often takes place, but selective pressure is sometimes required. For example, in the immobilization of bacteria for methanogenesis in fluidized beds, it has been shown that the dilution rate through the reactor had to exceed the \( \mu_{\text{max}} \) of the bacteria (Heijn, 1984) to select against suspended growth and promote biofilm formation. Even after a (pseudo)steady state had been reached, with profuse growth on the carrier, the high flow rate had to be maintained to prevent breakup of the biomass pellets. It has been postulated that this rapid breakdown may be due to the dynamic nature of these semi-natural biofilms (i.e. growth is continuously balanced by degradation of the biofilm). Any reduction in the dilution rate might result in a decrease in fresh growth, permitting degradation to out-pace formation. In a denitrifying reactor, the need for a high growth rate might be an advantage because the concentration of nitrate could be kept relatively low by means of dilution or recirculation, avoiding the formation of gas bubbles within the film. This can also lead to the destruction of biofilms (Henze & Harremoes, 1983).
As already mentioned, autotrophic nitrifiers tend to require relatively high dissolved oxygen concentrations, and gas transport into aerobic biofilms can become a problem if they are allowed to become too thick. Using micro-electrodes, it has been shown that aerobic biofilms can become oxygen limited very rapidly, and that an anaerobic zone can develop in biofilms more than 100 μm thick (Kuenen, Jorgensen & Revsbech, 1986). A biofilm airlift suspension reactor which uses very fine support material and very thin biolayers (thickness 50–100 μm) for effective nitrification has recently been described (Gjaltema, Loosdrecht & Heijnen, 1991; Tijhuis, van Loosdrecht & Heijnen, 1991). The proceedings of a recent Dahlem Conference on Biofilms have now been published (Characklis & Wilderer, 1989).

**Entrapment in gels**

In 1980, Nilsson et al. (1980) proposed the use of *Pseudomonas denitrificans* trapped in a gel matrix (calcium alginate) for the denitrification of high nitrate drinking water, and demonstrated the effectiveness of the system on the laboratory scale. Using an 80 ml column containing 45 g bacteria-containing alginate, water containing 100 mg nitrate l\(^{-1}\) and potassium aspartate as the substrate, they achieved a denitrification rate of 1.35 g nitrate day\(^{-1}\) (i.e. 13.5 litres of water). Subsequent experiments (Nilsson & Olsen, 1982) revealed leakage of cells from the alginate into the water, an undesirable phenomenon in drinking water treatment. The stability of the alginate improved considerably after the inclusion of 0.37 g CaCl\(_2\) \cdot 2H\(_2\)O l\(^{-1}\) (a level permissible in drinking water) to the water, but the authors also described a simpler method of minimizing cell leakage into the water supply by the use of a filter unit.

In the 10 years since then, variations in these methods have attracted a certain amount of attention. Different gels (e.g. polyelectrolytes, see Kokufuta, Shimohashi & Nakamura, 1987) have been tried, with different denitrifying bacteria. One of the most imaginative ideas has come from the work of Tramper and co-workers who have been investigating the possibilities of gel beads with an outer nitrifying layer, and an inner denitrifying core. Initial studies using *Nitrosomonas europaea*, *Nitrobacter agilis* and an unidentified denitrifier (Tramper & Grootjen, 1986; Tramper & de Man, 1986; Wijffels & Tramper, 1989; Wijffels, Schukking & Tramper, 1990) entrapped in alginate or carrageenan have shown that this may indeed be a viable prospect (Hunik & Tramper, 1991). Experiments using mixed cultures are now in progress.

**Novel concepts in nitrification and denitrification**

*Combined heterotrophic nitrification and denitrification*

With the discovery that single strains were capable of simultaneously nitrifying and denitrifying came the idea that such organisms could be used
Table 11. Fate of ammonia (as % of total) supplied to a mixed culture of N. europaea and Tsa. pantotropha at different C:N ratios. Data from van Niel, 1991

<table>
<thead>
<tr>
<th>C:N ratio</th>
<th>Assimilation</th>
<th>Autotrophic nitrification</th>
<th>Heterotrophic nitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
<td>72</td>
<td>18</td>
</tr>
<tr>
<td>3.8</td>
<td>15</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td>8.7</td>
<td>37</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td>19</td>
<td>38</td>
</tr>
</tbody>
</table>

for single-stage nitrogen removal. While, in principle, this seems a good idea, there are a number of hurdles to be overcome before such a process becomes practical.

The first problem is that, although higher than previously believed (Robertson & Kuenen, 1990), heterotrophic nitrification (and thus the linked denitrification) rates are still lower than those of the autotrophic ammonia oxidizers. For example, using chemostat cultures and similar media, van Niel (1991) found that the nitrification rates for Nitrosomonas europaea were ten times those observed with Tsa. pantotropha (470 and 40.5 nmol min$^{-1}$ mg protein$^{-1}$, respectively). However, it should be remembered that heterotrophic nitrifiers grow much more rapidly than autotrophic nitrifiers (e.g. $\mu_{\text{max}} = 0.08$ and 0.6 h$^{-1}$ for N. europaea and Tsa. pantotropha under relevant conditions, respectively), provided that an organic substrate is available. Van Niel (1991) used substrate-limited chemostat cultures of these two species to show that selection between autotrophic and heterotrophic nitrifiers is controlled by the C:N ratio in the medium. Table 11 shows the fate of nitrogen from ammonia in these cultures. It can be seen that as the C:N ratio increased, and the heterotrophic nitrifier became more important, the amount of N being assimilated also substantially increased, indicating that the use of such cultures would be associated with increased sludge formation (although less than if nonnitrifying heterotrophs were used). Similar chemostat experiments were run to test the effect of dissolved oxygen on the behaviour of the two types of nitrifier (Fig. 2). As expected, N. europaea washed out once the dissolved oxygen had been allowed to fall to less than 40 $\mu$M while Tsa. pantotropha remained unaffected (van Niel, 1991).

Much remains to be discovered about the reasons underlying heterotrophic nitrification as well as the factors controlling it. Research is currently under way in Delft (Pot et al., 1991) to determine the environmental parameters necessary to favour simultaneous nitrification and aerobic denitrification, to design an optimal bioreactor for such a system, and to determine the feasibility of using such a reactor for nitrogen removal.
Anoxic ammonia oxidation (anammox)

Broda (1977) suggested that denitrifying ammonia-oxidizers should exist (on thermodynamic grounds), and called them 'lithotrophs missing in nature'. When considered thermodynamically, the energy to be gained from oxidizing ammonia with nitrate is not very different from that gained when oxygen is the oxidizing agent. The reactions are as follows:

\[
\begin{align*}
\text{NH}_4^+ + \text{NO}_2^- & \rightarrow \text{N}_2 + 2\text{H}_2\text{O} & G^o & = -361 \text{ kJ/mol NH}_4^+ \\
\text{NH}_4^+ + 0.75\text{O}_2 & \rightarrow 0.5\text{N}_2 + 1.5\text{H}_2\text{O} + \text{H}^+ & G^o & = -315 \text{ kJ/mol NH}_4^+
\end{align*}
\]
Table 12. Nitrogen balances for the anammox reactor (I) before anammox, (II) after anammox appeared (all concentrations as mg N l⁻¹). Data from van der Graaf et al., 1990

<table>
<thead>
<tr>
<th>Amount of NO₃⁻ required for</th>
<th>Total NO₃⁻ required</th>
<th>Measured NO₃⁻ consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₂⁻ formed v.f.a. use NH₃ anammox</td>
<td>without anammox with anammox</td>
<td></td>
</tr>
<tr>
<td>(I) 92 18 0</td>
<td>110 110</td>
<td>100</td>
</tr>
<tr>
<td>(II) 80 18 48</td>
<td>98 146</td>
<td>150</td>
</tr>
</tbody>
</table>

v.f.a. = volatile fatty acids.

Moreover, as previously mentioned, autotrophic ammonia oxidizers can produce N₂O or N₂ under ‘oxygen stress’ (Poth, 1986, Poth & Focht, 1985). The main limiting factors would be the need for a new ammonia oxidizing enzyme (not a monooxygenase) and suitable linkage to the cytochrome chain.

It now appears that denitrifying ammonia-oxidizers are missing no longer (van der Graaf et al., 1990). Nitrogen balances made on a sulphide-oxidizing, denitrifying column being fed from a methanogenic reactor revealed that ammonia was disappearing from the system. Table 12 shows nitrogen balances made for the reactor before (I) and after (II) the phenomenon appeared. It can be seen that although the measured nitrate consumption agreed reasonably well with the predicted (on the basis of sulphate produced from sulphide and volatile fatty acids oxidized) nitrate consumption before the ammonia started disappearing, this was not the case afterwards. It was necessary to take into account the stoichiometric requirement for nitrate to oxidize the ammonia before the two figures agreed. Moreover, the ammonia removal rates have proved to be proportional to the amount of (live) biomass present, and to be dependent on the presence of nitrate or nitrite. If nitrate became depleted, both the ammonia disappearance and gas production stopped, and only resumed when more nitrate or nitrite was added (Fig. 3). Ammonia removal continued even under a hydrogen atmosphere provided in order to ensure anaerobiosis (van der Graaf et al., 1991). The observation that a pulse of ¹⁵[NH₃] to the anammox reactor resulted in the production of a peak at mass 29, equivalent to ¹⁴,¹⁵N₂, forced the authors to conclude that the anoxic ammonia oxidation was indeed a form of biological, ammonia-dependent denitrification. Work is now under way to discover the organisms responsible for the reaction, and to optimize the system since an anoxic, single stage nitrogen removing system has obvious attractions for wastewater treatment.
CONCLUSION

Little can be done by the microbiologist or biotechnologist about some forms of N-pollution, especially NO$_3$ due to burning (be it forests, petrochemicals or garbage), except to devise methods of off-gas treatment. However, the obvious involvement of microorganisms, especially bacteria, as catalysts in NO$_3$ emissions from bogs, marshes, the oceans and waste treatment plants, indicates the vast amount of work to be carried out in this field. Microbiological and biotechnological research into the physiology and behaviour of relevant organisms in natural and man-made environments is urgently needed. Until we understand the fundamental processes underlying N$_2$O and NO production during ammonia and NO$_2$ metabolism under naturally occurring conditions, we cannot hope to direct these processes towards desirable products (generally N$_2$) and thus control a significant proportion of NO$_3$ emission. At the Workshop on Microbial Production and Consumption of Radiatively Important Trace Gases, organized in November 1989 by the US Environmental Protection Agency, it was concluded that not enough is known about metabolism involving these gases. The report of this meeting ends ‘Considerable basic research needs to be carried out to elucidate the mechanisms of trace gas production and consumption (especially for NO) at the cellular, mechanistic level. Without a fundamental understanding of the mechanisms producing these gases, we cannot understand what physiological controls exist on their operation.’

From the discussion above, two points are immediately obvious.
1. It is essential that we must continue efforts to keep nitrogen compounds where they are wanted; that is on the fields, and not elsewhere (e.g. in freshwater, marsh or heathlands, or in the sea). Considerable progress in research into nitrification and denitrification has been made. Further work is necessary, but there is some hope of bringing nitrate levels in drinking water and the dumping of NH₃ and NO₃⁻ or NO₂⁻ into the environment under control. However, because wastewater treatment systems tend to be managed in order to control dissolved nitrogen compounds, little or no attention is generally paid to the quality of effluent gases, and atmospheric pollution may increase as a result. The time has now come when attention must now be paid to the optimizing of treatment systems to ensure that only N₂ is produced – after all, with a lifetime of 130 years, N₂O produced today will survive our grandchildren!

2. To meet the demands for removing various components from drinking and wastewater from different sources, the treatment systems must be of ever-increasing complexity. If requirements for phosphate and sulphate removal, for example, are added to the current demands for carbon and nitrogen elimination, extreme creativity will be required to design reactors or reactor combinations which are practical, efficient and cost-effective. This will require close cooperation between microbiologists and (bioprocess) engineers, in the true spirit of biotechnology, rather than the more empirical, traditional forms of reactor development. If the estimate that 25% of all NOₓ in the atmosphere originates from sub-optimal wastewater treatment systems is correct, there is a great deal to be done. Fortunately, as has been shown here, some progress is being made, and new microbiological and (bio)technological principles should help to provide the urgently needed innovations.

REFERENCES


bacteroids of *Rhizobium 'hedysoni'* strain HCNT1. *Archives of Microbiology*, 149, 384–8.


Kucera, I. & Dadak, V. (1983). The effect of uncoupler on the distribution of the electron flow between the terminal acceptors oxygen and nitrite in the cells of


NITROGEN REMOVAL FROM WATER AND WASTE


