

## Metabolic pathway of anaerobic ammonium oxidation on the basis of $^{15}\text{N}$ studies in a fluidized bed reactor

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**A novel metabolic pathway for anaerobic ammonium oxidation with nitrite as the electron acceptor has been elucidated using  $^{15}\text{N}$ -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  $\text{NH}_2\text{OH}$  to an operating fluidized bed system caused a stoichiometric increase in the ammonium conversion rate ( $1 \text{ mmol l}^{-1} \text{ h}^{-1}$ ) and a decrease in the nitrate production rate ( $0.5 \text{ mmol l}^{-1} \text{ h}^{-1}$ ). 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 could provide the anaerobic ammonium-oxidizing bacteria with the reducing equivalents necessary for  $\text{CO}_2$  fixation.**

Keywords: ammonium oxidation, hydroxylamine, hydrazine, mass spectrometry

### 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).

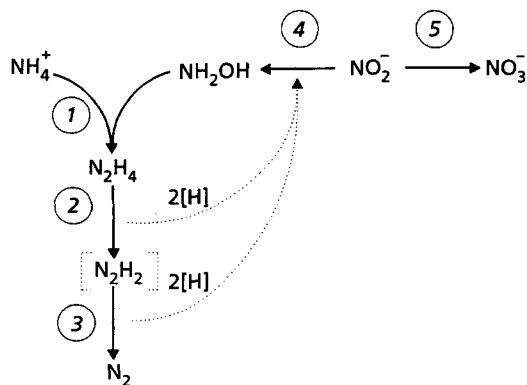
Although nitrification and denitrification reactions are often represented by rather simple equations, it is recognized that they may be more complex and may involve the formation of various intermediates (Bock *et al.*, 1992; Jetten *et al.*, 1997; Kuenen & Robertson, 1994). The use of  $^{15}\text{N}$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

the complex reaction pathways by providing information about processes involving gaseous nitrogen compounds.

Previous experiments using  $^{15}\text{N}$ -labelled ammonium and  $^{14}\text{NO}_3^-$  in an Anammox pilot plant reactor resulted in the production of  $^{14-15}\text{N}_2$  (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 ( $\Delta G^{0'}$  =  $-358 \text{ kJ mol}^{-1}$ ) 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  $\text{N}_2\text{O}$  ( $\Delta G^{0'}$  =  $-270 \text{ kJ mol}^{-1}$ ). The  $\text{N}_2\text{O}$  could thereafter be converted to dinitrogen gas, while ammonium is oxidized to hydroxylamine ( $\Delta G^{0'}$  =  $-87 \text{ kJ mol}^{-1}$ ). Alternatively, one could envisage a pathway (Fig. 1) which involves the reaction between ammonium and hydroxylamine to form hydrazine ( $\Delta G^{0'}$  =  $-47 \text{ kJ mol}^{-1}$ ). The hydrazine could be converted to dinitrogen gas and four reducing equi-

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Abbreviations: Anammox, ANAerobic AMMonium OXidaton; FBR, fluidized bed reactor.



**Fig. 1.** Possible metabolic pathway for anaerobic ammonium oxidation. Consumption and production of  $\text{H}_2\text{O}$  or  $\text{H}^+$  is not indicated. Ammonium is oxidized by hydroxylamine to form hydrazine (step 1). Reducing equivalents derived from  $\text{N}_2\text{H}_4$  then reduce nitrite to form even more hydroxylamine and  $\text{N}_2$  (steps 2, 3 and 4). Nitrate formation could generate reducing equivalents for biomass growth (step 5).

valents ( $\Delta G^0 = -271 \text{ kJ mol}^{-1}$ ) 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.

## METHODS

**Biomass and mineral medium.** An Anammox enrichment culture, grown in a fluidized bed reactor (FBR) 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.

**$^{15}\text{N}$  experiments with the FBR.** The 2.5 l glass FBR was operated at  $30^\circ\text{C}$  and pH 7. The pH was adjusted with 0.25 M  $\text{H}_2\text{SO}_4$  or 0.5 M 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, noreprene or 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 FBR 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 FBR contained 80–85% dinitrogen, 15–10% argon, 1–2% carbon dioxide and water vapour. Steady-state values of input and output of nitrogen compounds from one representative experiment were as follows ( $\text{mmol l}^{-1}$ ):  $\text{NH}_4^+_{\text{in}} = 29.7$ ,  $\text{NH}_4^+_{\text{out}} = 2.2$ ;  $\text{NO}_2^-_{\text{in}} = 34.7$ ,  $\text{NO}_2^-_{\text{out}} = 0.25$ ;  $\text{NO}_3^-_{\text{in}} < 0.01$ ,  $\text{NO}_3^-_{\text{out}} = 6.0$ ; inlet and outlet concentrations of  $\text{NH}_2\text{OH}$  and  $\text{N}_2\text{H}_4$  were below detection level ( $< 0.001 \text{ mM}$ ).

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

ammonium or 35 mM  $^{15}\text{N}$ -nitrite had replaced the  $^{14}\text{N}$ -labelled equivalents. The  $^{15}\text{N}$ -hydroxylamine and  $^{15}\text{N}$ -nitrate solutions were supplied separately giving inlet concentrations of 5 mM each.  $\text{N}_2\text{O}$  gas (100%) and/or NO (5% NO and 95% helium) were added by pumping them ( $2 \text{ mmol h}^{-1}$  and  $0.1 \text{ mmol h}^{-1}$ ) 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  $^{15}\text{N}$  addition, and analysed for ammonium, nitrate, nitrite and hydroxylamine. After the addition of each  $^{15}\text{N}$ -labelled compound to the fluidized bed system, the effluent was collected for 0.5 h and stored at  $-18^\circ\text{C}$ . This effluent was used for batch experiments with *Paracoccus denitrificans* (see below). At the end of each experiment, the influent to the FBR 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 experiment with  $^{15}\text{N}$ -labelled compounds, the gas composition was also monitored with a gas chromatograph to confirm whether  $\text{N}_2\text{O}$  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) kindly provided by Gist-brocades (Delft, The Netherlands). The sampling capillary from the mass spectrometer to the Anammox reactor was maintained at  $80^\circ\text{C}$ . This capillary was connected to the gas collector at the top of the FBR. The levels of  $^{14-15}\text{N}_2$ ,  $^{15-15}\text{N}_2$ ,  $^{15}\text{NO}$ ,  $^{14-15}\text{N}_2\text{O}$  and  $^{15-15}\text{N}_2\text{O}$  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  $^{15}\text{N}$ -labelled compounds with an NMR spectrometer (Varian VXR-400S) prepared for nitrogen measurements by Dr A. Sinnema, Department of Organic Chemistry, Delft University of Technology, The Netherlands.

**Batch experiments with effluent from the FBR.** The  $^{14}\text{N}/^{15}\text{N}$  composition of nitrate remaining in the effluent from the FBR was determined with batch experiments. Serum bottles (40 ml) were filled with 25 ml effluent containing  $^{14}\text{NO}_3^-$  and/or  $^{15}\text{NO}_3^-$ , 5 ml *P. denitrificans* suspension and 10 mM acetate. After flushing for 10 min with argon gas, cultures were incubated at  $30^\circ\text{C}$ . 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  $^{14}\text{NO}_3^-$  and  $^{15}\text{NO}_3^-$  was also made.

**Block pulse experiments in the FBR.** The effects of hydroxylamine, nitrite and hydrazine on the conversion rate of ammonium in the FBR were 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.

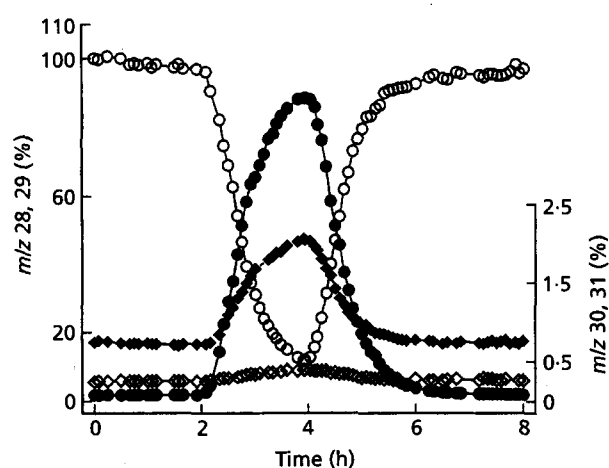
**Analytical methods and chemicals.** Nitrate, nitrite, ammonium, hydroxylamine and dry weight were determined as previously described (van de Graaf *et al.*, 1996). Hydrazine was determined colorimetrically by the method described by Watt & Crisp (1952). Absorbance was measured at 460 nm after 10 min incubation at room temperature. N<sub>2</sub>O formation was measured using a GC (Hewlett Packard model 428) with thermal conductivity detection.

<sup>15</sup>N-labelled sodium nitrite (99%) and nitrate (99%) were obtained from MSD Isotopes, <sup>15</sup>N-labelled ammonium sulfate (98%) from Sigma and <sup>15</sup>N-labelled hydroxylamine.HCl (99%) from Cambridge Isotope Laboratories. All other chemicals used were reagent grade and obtained from commercial sources.

## RESULTS

### <sup>15</sup>N-labelling experiments

Three combinations of N isotopes are possible for N<sub>2</sub> produced from unlabelled <sup>14</sup>N and <sup>15</sup>N-labelled precursors: <sup>14-14</sup>N<sub>2</sub> (*m/z* 28), <sup>14-15</sup>N<sub>2</sub> (*m/z* 29) and <sup>15-15</sup>N<sub>2</sub> (*m/z* 30). If other labelled gases are formed, this can be observed by changes in *m/z* 31, 45 and 46, representing <sup>15</sup>NO, <sup>14-15</sup>N<sub>2</sub>O and <sup>15-15</sup>N<sub>2</sub>O production, respectively. The results of the <sup>15</sup>N-labelling experiments are summarized in Table 1. During control experiments using unlabelled additions, none of the measured *m/z* ratios changed.



**Fig. 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 <sup>15</sup>NH<sub>4</sub><sup>+</sup> in a continuously operated FBR running on synthetic medium. The <sup>15</sup>NH<sub>4</sub><sup>+</sup> was supplied 2 h after the start of the measurements. ○, *m/z* 28; ●, *m/z* 29; ◆, *m/z* 30; ◇, *m/z* 31.

### <sup>15</sup>N-Ammonium or <sup>15</sup>N-nitrite addition

The addition of <sup>15</sup>NH<sub>4</sub><sup>+</sup> for 2 h to a FBR in the presence of <sup>14</sup>NO<sub>2</sub><sup>-</sup>, resulted in the changes in the *m/z* 28, 29, 30 and 31 values shown in Fig. 2. The largest increase (87%) was observed at *m/z* 29 (<sup>14-15</sup>N<sub>2</sub>), while *m/z* 28 (<sup>14-14</sup>N<sub>2</sub>) simultaneously declined by the same amount. Apparently the dinitrogen gas formed contained one <sup>15</sup>N from ammonium and one <sup>14</sup>N from nitrite. The dominant product, <sup>14-15</sup>N<sub>2</sub>, made up 98.3% of the total

**Table 1.** Effect of addition of <sup>15</sup>N-labelled compounds on the composition of gas from an Anammox culture grown in a FBR, 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 Methods).

<sup>15</sup> N-labelled compound added	Percentage change in component with <i>m/z</i> :					
	28 ( <sup>14-14</sup> N <sub>2</sub> )	29 ( <sup>14-15</sup> N <sub>2</sub> )	30 ( <sup>15-15</sup> N <sub>2</sub> )	31 ( <sup>15</sup> NO)	45 ( <sup>14-15</sup> N <sub>2</sub> O)	46 ( <sup>15-15</sup> N <sub>2</sub> O)
<sup>15</sup> NH <sub>4</sub> <sup>+</sup>	-87.0	+87.0	+1.4	+0.11	-	-
<sup>15</sup> NO <sub>2</sub> <sup>-</sup>	-86.0	+85.0	+3.2	+0.04	-	-
<sup>15</sup> NO <sub>3</sub> <sup>-</sup>	-1.3	+2.0	+0.04	-	-	-
<sup>15</sup> NH <sub>2</sub> OH	-13.0	+12.0	+0.4	+0.04	+0.08	-
<sup>15</sup> NH <sub>4</sub> <sup>+</sup> / <sup>14-14</sup> N <sub>2</sub> O	-84.0	+88.0	+1.2	+0.14	+0.02	-
<sup>15</sup> NO <sub>2</sub> <sup>-</sup> / <sup>14-14</sup> N <sub>2</sub> O	-85.0	+88.0	+2.3	+0.15	+0.02	+0.10
<sup>15</sup> NH <sub>4</sub> <sup>+</sup> / <sup>14</sup> NO	-86.0	+84.0	+1.1	+0.10	-	-
<sup>15</sup> NO <sub>2</sub> <sup>-</sup> / <sup>14</sup> NO	-85.0	+87.0	+2.9	+0.16	+0.02	+0.05
<sup>16</sup> NH <sub>4</sub> <sup>+</sup> / <sup>15</sup> NH <sub>2</sub> OH	-85.0	+76.0	+13.8	+0.18	+0.04	-
<sup>15</sup> NO <sub>2</sub> <sup>-</sup> / <sup>15</sup> NH <sub>2</sub> OH	-95.0*	+95.0	+5.5	-	-	-

-, Not observed.

\* After correction for changes in mass 40 of argon.

labelled dinitrogen gas produced. Furthermore, a small amount of  $^{15-15}\text{N}_2$  ( $m/z$  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  $\text{N}_2\text{O}$  was not produced. The very small increase of 0.1% at  $m/z$  31 was therefore probably due to the formation of traces of  $^{15}\text{NO}$ .

The addition of  $^{15}\text{NO}_2^-$  in the presence of  $^{14}\text{NH}_4^+$  gave similar  $m/z$  changes (Table 1). Only the increase of  $^{15-15}\text{N}_2$  ( $m/z$  30) was slightly higher, probably because of some background denitrification in the mixed culture.

### $^{15}\text{N}$ -Hydroxylamine addition

Hydroxylamine was a potential intermediate in the metabolic pathway proposed for anaerobic ammonium oxidation (Fig. 1). To test whether  $^{14}\text{N}$  from ammonium or from nitrite would be coupled to the  $^{15}\text{N}$  of hydroxylamine during the formation of the N-N bond, experiments using combinations of either  $^{15}\text{NH}_2\text{OH}$  and  $^{15}\text{NH}_4^+$  or  $^{15}\text{NH}_2\text{OH}$  and  $^{15}\text{NO}_2^-$ , were carried out. As shown in Table 1, the addition of  $^{14}\text{NH}_4^+$ ,  $^{15}\text{NH}_2\text{OH}$  and  $^{15}\text{NO}_2^-$  resulted in  $^{14-15}\text{N}_2$  being the dominant gas. This was also true when  $^{15}\text{NH}_4^+$ ,  $^{15}\text{NH}_2\text{OH}$  and  $^{14}\text{NO}_2^-$  were used, but the proportion of  $^{15-15}\text{N}_2$  rose sharply, indicating a reaction between  $^{15}\text{NH}_4^+$  and  $^{15}\text{NH}_2\text{OH}$  in a ratio of 1:1.

### $^{15}\text{N}$ -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  $^{15}\text{N}$ -nitrate to the 6 mM  $^{14}\text{N}$ -nitrate already present in the reactor only resulted in a small increase in  $^{14-15}\text{N}_2$  ( $m/z$  29, Table 1).  $^{15}\text{N}$ -NMR analysis of the effluent samples showed that  $^{15}\text{NO}_3^-$  was only formed from  $^{15}\text{NO}_2^-$ . When  $^{15}\text{NH}_4^+$  was used,  $^{15}\text{NO}_3^-$  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  $\text{N}_2$  was examined with the mass spectrometer.  $^{15}\text{N}$ -labelled  $\text{N}_2$  was only obtained when  $^{15}\text{NO}_2^-$  had been used during the experiments. This is in agreement with the NMR findings.

### Effect of nitrous oxide or nitric oxide

The addition of  $^{14-14}\text{N}_2\text{O}$  or  $^{14}\text{NO}$  during experiments with  $^{15}\text{NH}_4^+$  or  $^{15}\text{NO}_2^-$  did not change the labelling pattern of the  $\text{N}_2$  (Table 1). The chemical analysis of the effluent from the reactor also confirmed that  $\text{N}_2\text{O}$  or  $\text{NO}$  did not influence the overall biochemical reactions in the culture.

### Batch experiments with hydrazine and hydroxylamine

The results described above indicated that the pathway (Fig. 1) combining  $\text{NH}_2\text{OH}$  and  $\text{NH}_4^+$  to give the N-N bond was probably operative. The first product expected

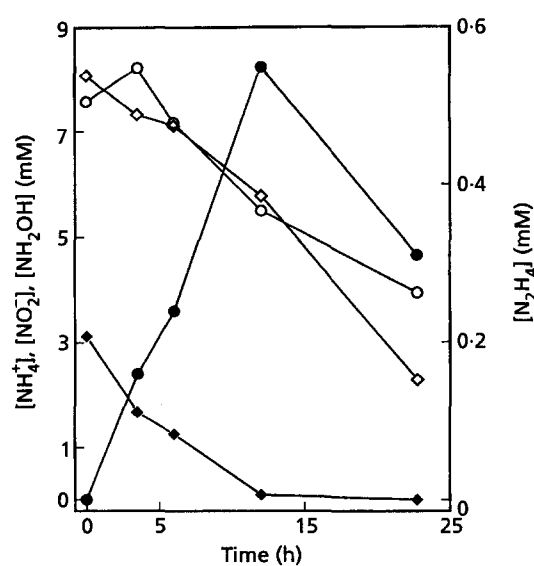
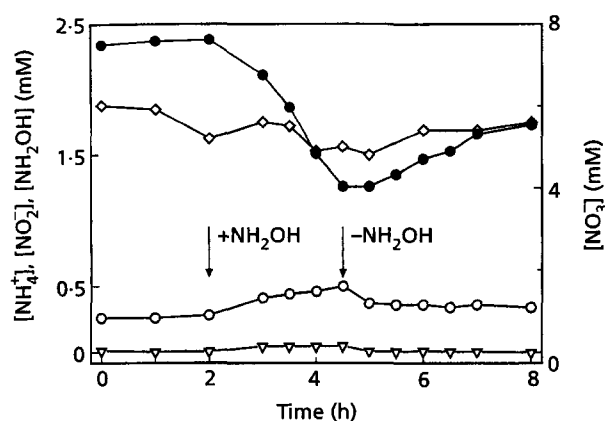


Fig. 3. Concentration profiles during batch experiments with 3 mM hydroxylamine.  $\circ$ ,  $\text{NH}_4^+$ ;  $\diamond$ ,  $\text{NO}_2^-$ ;  $\bullet$ ,  $\text{N}_2\text{H}_4$ ;  $\blacklozenge$ ,  $\text{NH}_2\text{OH}$ .

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 below).

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.

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 Fig. 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 the pathway in Fig. 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  $\text{N}_2\text{H}_4$  formation was only 20% of that observed when  $\text{NH}_4^+$  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



**Fig. 4.** Effect of hydroxylamine on the anaerobic ammonium oxidation in a continuously fed fluidized bed system.  $\text{NH}_2\text{OH}$  was added for 2 h. ●,  $\text{NH}_4^+$ ; ○,  $\text{NO}_2^-$ ; ◇,  $\text{NO}_3^-$ ; ▽,  $\text{NH}_2\text{OH}$ .

lowered after all the hydroxylamine had disappeared. In cultures where hydroxylamine and nitrite were both 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.

#### Effect of hydroxylamine, nitrite or hydrazine block pulse additions on the activity of an operating FBR

When  $^{15}\text{NH}_2\text{OH}$  was supplied during experiments with the FBR (Table 1), there was a significant decrease in the ammonium and a slight increase in the nitrite concentrations in the effluent (Fig. 4). To quantify this observation, hydroxylamine, nitrate and hydrazine were separately supplied to the FBR for longer periods. The changes in ammonium conversion and nitrate formation after each experiment are summarized in Table 2.

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 lowered 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). 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. Hydrazine also lowered the level of nitrate production in the FBR. Some  $\text{N}_2\text{O}$  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.

## 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 (Fig. 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 occurrence 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

**Table 2.** Effect of 24 h supply of hydroxylamine, nitrite or hydrazine on ammonium conversion and nitrate production in a continuously operated FBR for anaerobic ammonium oxidation

The reactor was running on synthetic medium.  $\Delta$  indicates the difference between the concentrations before the experiment started and after 24 h.

Additive	$\text{NH}_4^+/\text{NO}_2^-$ feed load (mM)	$\Delta\text{NH}_4^+$ (mM)	$\Delta\text{NO}_3^-$ (mM)	$\Delta\text{NH}_4^+/\Delta\text{NO}_3^-$
$\text{NH}_2\text{OH}$ (1 mM)	15/15	-0.86	-0.41	2.1
$\text{NH}_2\text{OH}$ (2 mM)	15/15	-2.17	-1.03	2.1
$\text{NH}_2\text{OH}$ (3 mM)	25/25*	-3.19	-1.63	2.0
$\text{NO}_2^-$ (3 mM)	25/25	-2.35	+0.73	
$\text{N}_2\text{H}_4$ (3 mM)	25/25	+0.76	-1.75	
$\text{N}_2\text{H}_4$ (6 mM)	25/25	+0.33	-2.51	

\* During this experiment,  $\text{H}_2$  was supplied in addition to  $\text{NH}_2\text{OH}$  to the FBR. This component had no additional effect on the anaerobic ammonium oxidation, there being no significant change in the ratio of  $\Delta\text{NH}_4^+/\Delta\text{NO}_3^-$ .

reduced to ammonium. The conversion of externally added hydrazine to dinitrogen gas has also been documented for *Nitrosomonas europaea* (Hyman & Arp, 1995; Keener & Arp, 1994). In studies on the *de novo* synthesis of polypeptides in *N. europaea* cells, hydrazine could serve as an energy source for nitrite reduction under anaerobic conditions (Hyman & Arp, 1995). In addition it has been shown that the purified hydroxylamine oxidoreductase from *N. europaea* is capable of catalysing the conversion of hydrazine to dinitrogen gas (Hooper & Terry, 1977). The conversion rate for hydrazine by this enzyme was in the same range as that for hydroxylamine itself [approximately  $40 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ ]. 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 medium 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 centre 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 normally requires molecular oxygen and two electrons from the ubiquinol pool (Keener & Arp, 1993; Ensign *et al.*, 1993). Since the Anammox process is strongly but reversibly 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 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  $^{15}\text{N}$ -labelled hydroxylamine from externally added  $^{15}\text{NO}_2^-$  in 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 FBR, where the consumption of ammonium increased by 3.2 mM; the supply of both  $^{15}\text{NH}_4^+$  and  $^{15}\text{NH}_2\text{OH}$  to a reactor, which resulted in a sharp increase in the  $^{15-15}\text{N}_2$  emission; various batch experiments, where the simultaneous addition 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 catalyse such a reaction: cytochrome *aa*<sub>3</sub> oxidase, haemocyanin or NO reductase are known to form an N=N bond by coupling two molecules of NO to  $\text{N}_2\text{O}$  (Berks *et al.*, 1995; Jetten *et al.*, 1997). However, the addition of NO or  $\text{N}_2\text{O}$  to the fluidized bed system did not influence the labelling patterns, indicating that NO or  $\text{N}_2\text{O}$  reductase does not play an important role in the Anammox process.

### Nitrite and nitrate

Production of  $^{15}\text{NO}_3^-$  was only observed when  $^{15}\text{NO}_2^-$  was supplied. The anaerobic formation of nitrate from nitrite might be catalysed by an enzyme similar to the nitrite oxidoreductase from *Nitrobacter* spp. (Meinke *et al.*, 1992; Sundermeyer-Klinger *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  $\text{CO}_2$  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 nitrate and 20 mg biomass are produced in the Anammox process (van de Graaf *et al.*, 1996). These values are 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.

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