Anaerobic Ammonia Oxidation in the Presence of Nitrogen Oxides (NO$_x$) by Two Different Lithotrophs

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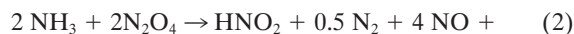
The anaerobic ammonia-oxidizing activity of the planctomycete *Candidatus* "Brocadia anammoxidans" was not inhibited by NO concentrations up to 600 ppm and NO$_2$ concentrations up to 100 ppm. *B. anammoxidans* was able to convert (detoxify) NO, which might explain the high NO tolerance of this organism. In the presence of NO$_2$, the specific ammonia oxidation activity of *B. anammoxidans* increased, and *Nitrosomonas*-like microorganisms recovered an NO$_2$-dependent anaerobic ammonia oxidation activity. Addition of NO$_2$ to a mixed population of *B. anammoxidans* and *Nitrosomonas* induced simultaneous specific anaerobic ammonia oxidation activities of up to 5.5 mmol of NH$_4^+$ g of protein^{-1} h^{-1} by *B. anammoxidans* and up to 1.5 mmol of NH$_4^+$ g of protein^{-1} h^{-1} by *Nitrosomonas*. The stoichiometry of the converted N compounds (NO$_2^-$/NH$_3$ ratio) and the microbial community structure were strongly influenced by NO$_2$. The combined activity of *B. anammoxidans* and *Nitrosomonas*-like ammonia oxidizers might be of relevance in natural environments and for technical applications.

Two different metabolic pathways for anaerobic ammonia oxidation by lithotrophic microorganisms have been described (8, 14). The anaerobic ammonia oxidation by the planctomycete *Candidatus* "Brocadia anammoxidans" is a nitrite-dependent reaction. The reaction mechanism of this anammox process was examined in a laboratory-scale fluidized bed reactor by using ^{15}N-labeled compounds. Ammonia is oxidized with hydroxylamine as the most probable electron acceptor, and hydrazine is the first detectable intermediate (22). Subsequently, the hydrazine is oxidized to dinitrogen gas. The four reducing equivalents are transferred to nitrite, forming hydroxylamine. During the conversion of ammonia, some nitrate is formed from nitrite. This reaction could provide reducing equivalents necessary for the fixation of carbon dioxide (22). The overall nitrogen balance shows a ratio of about 1:1.32:0.26 for the conversion of ammonia, nitrite, and nitrate (18). The overall anammox reaction is presented in equation 1.

$$NH_3 + 1.3\ HNO_2 \rightarrow 0.3\ HNO_3 + 1\ N_2 + \qquad (1)$$

$$1.7\ H_2O + 0.6H^+ + 0.6\ e^-$$
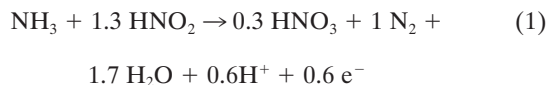
Under optimal growth conditions the specific anammox activity is about 3 mmol of NH$_4^+$ g of protein^{-1} h^{-1}. The affinity for the substrates ammonia and nitrite is high, and the K_s values are less than 10 μM (5, 19). Interestingly, nitrite concentrations of 5 mM or higher and oxygen concentrations as low as 2 μM completely, but reversibly, inhibit the anammox activity (17). During the conversion of ammonia and nitrite no other intermediates or products, such as NO or N$_2$O, could be detected.

The obligately anaerobic nature of *B. anammoxidans* is in sharp contrast with the more versatile metabolism of *Nitrosomonas* strains (1, 2, 10). The latter organisms are able to gain energy by aerobic or anaerobic ammonia oxidation or by denitrification by using hydrogen or organic compounds as the electron donor (2). The anaerobic ammonia oxidation by *Nitrosomonas* is a nitrogen dioxide-dependent reaction (12). It has been shown that N$_2$O$_4$, the dimeric form of NO$_2$, is used as an oxidant (13). About 50% of the nitrite produced is used as a terminal electron acceptor and is converted to N$_2$ (equation 2).

$$2\ NH_3 + 2N_2O_4 \rightarrow HNO_2 + 0.5\ N_2 + 4\ NO + \qquad (2)$$

$$1\ H^+ + 1\ e^- + 2\ H_2O$$

In a pure culture, the specific anaerobic ammonia oxidation activity of *Nitrosomonas eutropha* is about 0.15 mmol of NH$_4^+$ g of protein^{-1} h^{-1}. In contrast to *B. anammoxidans*, *Nitrosomonas* produces significant amounts of nitric oxide and nitrous oxide during ammonia oxidation. The nitrogen oxides, NO and NO$_2$, are obligatory intermediates in the oxidation of ammonia (14), and they have regulatory effects on the metabolism of the nitrifiers (16, 23).

NO has toxic effects on many microorganisms (4, 6, 15, 24), but the effect on the anammox activity of planctomycetes is still unknown. In natural and man-made environments (e.g., wastewater treatment plants) these organisms may be exposed to high NO concentrations and frequently also to high NO$_2$ concentrations The aim of this study was to investigate the effect of nitrogen oxides on the ammonia oxidation of *B. anammoxidans*. Furthermore, the influence of NO and NO$_2$ on the microbial community structure and the specific ammonia oxidation activities of *B. anammoxidans* and *Nitrosomonas* were evaluated.

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MATERIALS AND METHODS

Organisms. An anammox enrichment culture containing about 80% *B. anammoxidans* (5×10^8 cells ml^{-1}) was grown in a 2-liter sequencing batch reactor (18). Cells of *N. eutropha* strain N904 (isolated from cattle manure) were grown as described previously (12).

Medium and growth conditions. Precultures of the anammox biomass were grown anaerobically at 28°C and pH 7.5. The mineral medium contained (per liter of demineralized water) 2.02 g of NaNO$_2$, 1.96 g of (NH$_4$)$_2$SO$_4$, 1.25 g of KHCO$_3$, 25 mg of KH$_2$PO$_4$, 300 mg of CaCl$_2 \cdot$2H$_2$O, 200 mg of MgSO$_4 \cdot$7H$_2$O, 6.25 mg of FeSO$_4$, 6.25 mg of EDTA, 25 ml of HCl (1 M), and 25 ml of a trace element solution. The trace element solution contained (per liter of demineralized water) 15 g of EDTA, 430 mg of ZnSO$_4 \cdot$7H$_2$O, 240 mg of CoCl$_2 \cdot$6H$_2$O, 990 mg of MnCl$_2 \cdot$4H$_2$O, 250 mg of CuSO$_4 \cdot$5H$_2$O, 220 mg of NaMoO$_4 \cdot$2H$_2$O, 190 mg of NiCl$_2 \cdot$6H$_2$O, 210 mg of NaSeO$_4 \cdot$10H$_2$O, and 14 mg of H$_3$BO$_4$. The influx of fresh medium into the 2-liter reactor was adjusted to 170 ml h^{-1}.

Precultures of *N. eutropha* were grown aerobically in a 10-liter sequencing batch reactor in mineral medium (12) aerated with 2 liters of air min^{-1}. The pH was kept at 7.5 by using a 20% Na$_2$CO$_3$ solution.

Experimental design. Experiments were performed in a 2-liter laboratory-scale reactor with 1.7 liters of medium; 170 ml of fresh medium was added per hour, and the same volume was removed from the reactor. The effluent was filtered by using a hollow fiber membrane module (0.4 m^2) with membranes consisting of polysulfone and a cutoff of 6 kDa (SPS 6005-6; Fresenius, St. Wendel, Germany) to achieve complete biomass retention (cross-flow filtration). While the cell-free medium (170 ml h^{-1}) was removed, the biomass was routed back into the reactor. Depending on the experiment the concentrations of substrates (ammonia and nitrite) in the fresh medium varied between 0 and 100 mM. The reactor was gassed with a 95% argon–5% CO$_2$ gas mixture supplemented with 0 to 1,000 ppm of NO or NO$_2$ at a rate of 0.5 to 1 liter min^{-1} by using mass flow controllers. The level, temperature, dissolved oxygen content, and pH were permanently controlled (Applikon, Schliedam, The Netherlands). The temperature was maintained at 28°C. The pH was kept at 7.5. Due to cell growth the biomass concentration in the reactor increased. Every 3 days the biomass concentration was measured by protein determination, and the biomass concentration was adjusted to about 80 μg of protein ml^{-1} (5×10^8 cells ml^{-1}) by removing small amounts of biomass from the reactor.

Batch experiments were carried out in 100-ml glass bottles that were sealed with butyl rubber stoppers and contained 20 ml of mineral medium. The bottles were flushed with a 95% argon–5% CO$_2$ gas mixture supplemented with 0 to 1,000 ppm of NO. The anammox biomass (80% *B. anammoxidans*) was purified by density gradient centrifugation (99.6% ± 0.2% *B. anammoxidans*) as described previously (20). During the experiments (8 h) the cell suspension (5×10^9 cells ml^{-1}) was stirred (800 rpm) to ensure efficient gas transfer. NO consumption was calculated on the basis of the difference in the NO concentrations at the beginning and at the end of an experiment. Control experiments were carried out with cell-free medium and heat-sterilized cell suspensions. The chemical conversion rates were low (less than 3% of the biological conversion rates).

Analytical procedures. Measurements were carried out as described by Schmidt and Bock (12) (NH$_4^+$, NO, and NO$_2$), Van de Graaf et al. (21) (NO$_2^-$ and NO$_3^-$), and Bradford (3) (protein concentration). Most-probable-number (MPN) analysis was performed as described by Mansch and Bock (7), and fluorescent in situ hybridization (FISH) was performed as described by Neef et al. (9). The following probes were used: NEU 653 (specific for *Nitrosomonas*-like microorganisms) and Amx 820 (specific for planctomycete-like bacteria). The FISH and MPN methods were used to determine the numbers of planctomycete and *Nitrosomonas* cells in the reactor. 4′,6′-Diamidino-2-phenylindole (DAPI) staining was used to determine the total cell number.

Determination of ammonia oxidation activity. The aerobic ammonia oxidation activity of *N. eutropha* was determined by short-term activity tests (2 h). The tests were performed in 100-ml Erlenmeyer flasks containing 10 ml of mineral medium (12). The protein concentration was measured, and the time-dependent consumption of ammonia was determined. The aerobic ammonia oxidation activity was used to estimate the numbers of *Nitrosomonas* cells in the anammox systems. The method was calibrated with anaerobically grown *N. eutropha* cells (13), and the correlation between cell number and the amount of ammonia converted was determined; 10^7 cells oxidized 40 ± 3.3 nmol of NH$_4^+$ h^{-1}. This method was used to confirm the results of the FISH and MPN analyses. Due to the presence of colonies the MPN method underestimates the number of cells (7); the activity method tends to overestimate the number of cells.
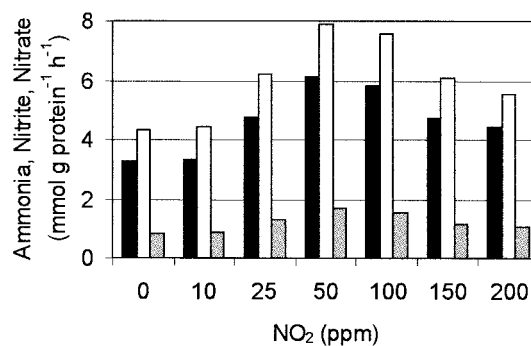

FIG. 1. Specific ammonia (solid bars) and nitrite (open bars) consumption rates and nitrate production rates (shaded bars) in an anammox system supplemented with different concentrations of NO$_2$. According to the Mann-Whitney U test, the increases in activity as the NO$_2$ concentration was increased up to 50 ppm and the decreases in activity at NO$_2$ concentrations between 50 and 200 ppm are highly significant (error rate, 0.005). The experiments were repeated three times.

RESULTS

Effects of NO$_2$ and NO on anammox reactor systems. The initial experiments were designed to investigate the influence of nitrogen oxides on the nitrogen conversion activities in anammox systems. For these experiments, anammox biomass precultured for 3 months in the absence of NO$_x$ was supplemented with increasing amounts of NO$_2$ or NO. The NO$_x$ concentration was increased every 2 weeks. Throughout the experiments the ammonia and nitrite concentrations were adjusted to about 4 and 0.5 mM, respectively. After the NO$_x$ concentration was increased, the system adapted within 1 week to the new conditions. In the second week the nitrogen conversion activities were measured every day, and the mean values were calculated (the standard deviation was ±6.5%). The results of the experiments which were performed in the presence of NO$_2$ are shown in Fig. 1.

When the NO$_2$ concentration in the gas mixture used for gassing was increased, ammonia and nitrite consumption, as well as nitrate production, increased. The highest ammonia and nitrite conversion rates observed in the presence of 50 ppm of NO$_2$ were about two times higher than the conversion rates in the absence of NO$_2$. A further increase in the NO$_2$ concentration resulted in slowly decreasing anaerobic ammonia oxidation activities. In the absence of NO$_2$ the growth rate of *B. anammoxidans* was about 0.003 h^{-1}, in the presence of 50 ppm of NO$_2$ the growth rate was about 0.004 h^{-1}, and in the presence of 200 ppm of NO$_2$ the growth rate was about 0.0028 h^{-1}. According to a Mann-Whitney U test the differences in the growth rates are significant (error rate, 0.05).

To enlarge the statistical basis for the observed effects, short-term batch experiments were performed to examine the effect of NO$_2$ (0 to 200 ppm) on the anammox biomass (10 replicate experiments). Again, the highest activities were measured in the presence of 50 ppm of NO$_2$ (the activities were 1.5 times higher than those in an NO$_2$-free control). Interestingly, the ratio between ammonia and nitrite consumption changed depending on the NO$_2$ concentration (Table 1). When no gaseous NO$_2$ was added, the NO$_2^-$/NH$_3$ ratio was about 1.3, as previously determined by Strous et al. (18). When the NO$_2$

TABLE 1. NO_2^-/NH_3 consumption ratios in anammox reactors in the presence of NO_2 or NO

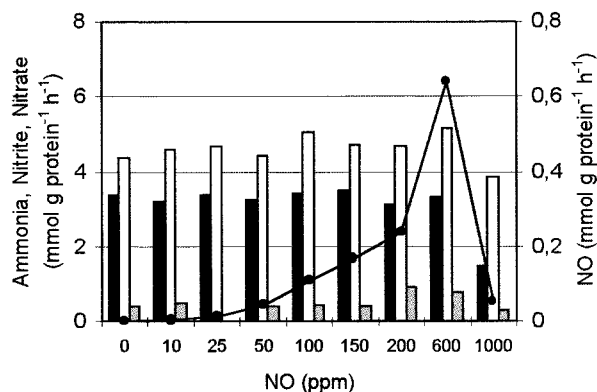| NO_x concn (ppm)	NO_2^-/NH_3 ratio in the presence of NO_2	NO_2^-/NH_3 ratio in the presence of NO
0	1.33	1.30
10	1.32	1.44
25	1.30	1.39
50	1.28	1.37
100	1.29	1.49
150	1.28	1.34
200	1.25	1.50
600	ND^a	1.53
1,000	ND	2.58

[a] ND, not determined.

FIG. 2. Specific ammonia (solid bars), nitrite (open bars), and NO (●) consumption rates and nitrate production rates (shaded bars) in a reactor supplemented with different concentrations of NO. According to the Mann-Whitney U test, the specific ammonia consumption activity was constant as the NO concentration was increased up to 600 ppm (error rate, 0.05), and the specific nitrite consumption activity increased (error rate, 0.1). Two replicated experiments were performed.

concentration was increased to 200 ppm, the NO_2^-/NH_3 ratio decreased to about 1.25.

The influence of NO_2 addition on the composition of the microbial community in the reactor was evaluated by FISH, MPN, and activity tests (Table 2). Throughout the experiment small amounts of the biomass were removed regularly (every 3 days) to keep the biomass content of the reactor constant. Therefore, the number of *B. anammoxidans* cells remained unchanged (about 5×10^8 cells ml^{-1}). In contrast, the number of *Nitrosomonas* cells increased significantly in the presence of NO_2. The ratio of *B. anammoxidans* cells to *Nitrosomonas* cells changed from about 2×10^6:1 to about 30:1.

The effect of nitric oxide was investigated by using a similar experimental design, but the reactors were gassed with a gas mixture that consisted of argon (95%), carbon dioxide (5%), and NO. The NO concentration was increased every 2 weeks until the final value of 1,000 ppm was reached after 16 weeks (Fig. 2). The specific ammonia consumption activity remained unchanged in the presence of concentrations of NO between 0 and 600 ppm. In contrast, the specific nitrite consumption activity increased significantly as the NO concentration was increased. As a consequence, the NO_2^-/NH_3 ratio increased (Table 1). Furthermore, increasing amounts of NO were consumed (Fig. 2). Strong inhibition of the specific ammonia, nitrite, and NO consumption activities was observed with 1,000

ppm of NO (Fig. 2). The growth rate remained unchanged at $0.003 \ h^{-1}$ in the presence of 0 to 600 ppm of NO but decreased to about $0.001 \ h^{-1}$ in the presence of 1,000 ppm of NO. Throughout the experiments the number of *B. anammoxidans* cells was controlled and remained 5×10^8 cells ml^{-1} (Table 2). The number of active *Nitrosomonas* cells decreased about fivefold (from 5.9×10^2 to 1.1×10^2 cells ml^{-1}). NO consumption (detoxification) by *B. anammoxidans* was also examined in short-term batch experiments with purified biomass (purity, $99.6\% \pm 0.2\%$; concentration, 5×10^8 cells ml^{-1}). In three replicate experiments NO consumption rates of 0, 0.1, and 0.4 mmol of NO g of $protein^{-1} \ h^{-1}$ and ammonia oxidation activities of 2.3, 2.4, and 1.4 mmol g of $protein^{-1} \ h^{-1}$ were measured in the presence of 0, 100, and 1,000 ppm of NO, respectively. Interestingly, the NO consumption rate increased as the NO concentration increased.

Influence of *Nitrosomonas* on anammox reactor systems. The experiments in which NO_2 was added showed that high num-

TABLE 2. Numbers of *B. anammoxidans* and *Nitrosomonas* cells in reactors supplemented with NO_2 or NO^a

NO_x (ppm)	No. of cells (ml^{-1}) in the presence of:							
	NO_2				NO			
	B. anammoxidans (FISH analysis)[b]	*Nitrosomonas* (FISH analysis)[c,d]	*Nitrosomonas* (MPN analysis)[d]	*Nitrosomonas* (activity test)[e,f]	*B. anammoxidans* (FISH analysis)[b]	*Nitrosomonas* (FISH analysis)	*Nitrosomonas* (MPN analysis)[g]	*Nitrosomonas* (activity test)[e,g]
0	5.1×10^8	ND^h	1.7×10^3	2.3×10^2	6.9×10^8	ND	2.6×10^3	5.9×10^2
10	5.2×10^8	ND	6.3×10^3	6.1×10^4	7.2×10^8	ND	2.7×10^3	5.3×10^2
25	5.0×10^8	ND	1.3×10^4	4.7×10^5	6.5×10^8	ND	2.0×10^3	5.7×10^2
50	5.0×10^8	4.5×10^5	3.3×10^4	1.6×10^6	6.9×10^8	ND	2.2×10^3	5.0×10^2
100	5.1×10^8	1.2×10^6	9.1×10^4	4.7×10^6	6.5×10^8	ND	1.6×10^3	3.9×10^2
150	5.1×10^8	2.7×10^5	2.9×10^5	1.2×10^7	7.6×10^8	ND	8.4×10^2	1.9×10^2
200	5.2×10^8	6.4×10^6	7.6×10^5	1.8×10^7	6.9×10^8	ND	4.9×10^2	1.1×10^2

[a] The number of *B. anammoxidans* cells was controlled by biomass removal.
[b] The standard deviation was 7%. According to the Mann-Whitney U test the constant cell number is highly significant (error rate, 0.005).
[c] The standard deviation was 18%.
[d] According to the Mann-Whitney U test the increasing cell number with increasing NO_2 concentration is significant (error rate, 0.05).
[e] The standard deviation was 9%.
[f] According to the Mann-Whitney U test the increasing cell number with increasing NO_2 concentration is highly significant (error rate, 0.005).
[g] According to the Mann-Whitney U test the decreasing cell number with increasing NO concentration is significant (error rate, 0.05).
[h] ND, not detectable.

TABLE 3. N conversion rates, NO_2^-/NH_3 ratios, and N loss in 2-liter reactors[a]

No. of N. eutropha cells (ml^{-1})	Conversion rates (μmol reactor^{-1} h^{-1})					NO_2^-/NH_3 ratio	Sp act (μmol of NH_4^+ g of protein^{-1} h^{-1})	
	Ammonia	Nitrite	NO_2[b]	NO	Nitrate		B. anammoxidans[c]	N. eutropha[d]
0[e]	141.4	187.9	0	0.01	36.8	1.33	4,423	
10^7	149.9	191.4	14.4	13.5	38.1	1.28	4,579	1,080
5 $\times$ 10^7	183.8	183.2	70.6	71.2	41.3	1.00	4,963	1,462
10^8	226.2	185.8	129.4	119.1	49.3	0.82	5,525	1,545

[a] The reactor with the anammox biomass was supplemented with 0, 10^7, 5 $\times$ 10^7, or 10^8 N. eutropha cells ml^{-1}. The consumption and production rates were calculated on the basis of the substrates added to the reactor and the products leaving the reactor. The standard deviation for five replicate experiments was less than $\pm$ 5%.
[b] In sterile control experiments, the NO_2 consumption resulting from the reaction of NO_2 with water was determined. The values obtained were subtracted from the data obtained with cells.
[c] The specific ammonia oxidation activity of B. anammoxidans was calculated on the basis of nitrate production.
[d] The specific ammonia oxidation activity of N. eutropha was calculated on the basis of NO_2 consumption and NO production.
[e] The anammox biomass contained about 2 $\times$ 10^3 Nitrosomonas cells ml^{-1}.

bers of active *Nitrosomonas* cells can have a strong influence on the N conversion rates in anammox reactors. This influence was studied in laboratory-scale reactors inoculated with a mixed population of *B. anammoxidans* (about 10^8 cells ml^{-1}) and *N. eutropha* (0, 10^7, 5 $\times$ 10^7, or 10^8 cells ml^{-1}). The experiments were performed for 2 days in the presence of 200 ppm of NO_2 and an excess of ammonia; nitrite was limiting. The ammonia, nitrite, and NO_2 consumption rates, as well as the nitrate and NO production rates, were determined, and the NO_2^-/NH_3 ratio was calculated. The results are shown in Table 3. When the anammox system was supplemented with *N. eutropha*, the conversion of nitrogen compounds changed significantly. Ammonia and NO_2 consumption and NO production increased. Nitrite consumption remained unchanged. The NO_2^-/NH_3 ratio decreased from 1.3 to 0.82, indicating that *Nitrosomonas* produced nitrite, which was directly consumed by *B. anammoxidans*.

The anoxic activity of *N. eutropha* resulted in levels of ammonia and NO_2 consumption and NO production similar to the expected 1:2:1 ratio shown in equation 2 (13). About 50% of the ammonia consumed should have been converted to nitrite. However, in the presence of excess ammonia *B. anammoxidans* immediately converted this nitrite to N_2.

Only when ammonia was the limiting substrate did *B. anammoxidans* and *N. eutropha* have to compete for ammonia. Due to the ammonia limitation, the additional nitrite produced by *N. eutropha* could not be converted by *B. anammoxidans* and therefore should have accumulated in the reactor. The N conversion in an ammonia-limited anammox reactor (10^8 cells ml^{-1}) supplemented with nitrite and 250 ppm of NO_2 is shown in Fig. 3. After 120 h *N. eutropha* (10^8 cells ml^{-1}) was added. Before *N. eutropha* was present, *B. anammoxidans* consumed all of the ammonia and nitrite added with the fresh medium; the concentrations of these compounds in the reactor were low, and increasing concentrations of nitrate were detected. After *N. eutropha* was added, the concentration of nitrite increased rapidly (Fig. 3), NO_2 was consumed, and NO was produced (data not shown), indicating an NO_2-dependent ammonia oxidation activity of *N. eutropha*. The decreasing nitrate concentration indicated that the activity of *B. anammoxidans* was reduced, which was most likely due to competition with *N. eutropha* for ammonia. When the ammonia limitation in the reactor was eliminated, the nitrite concentration decreased to

less than 10 μM and the nitrate concentration reached about 850 μM (data not shown).

B. anammoxidans-N. eutropha community with ammonia and gaseous NO_2 as substrates. On the basis of equations 1 and 2, we speculated that ammonia and gaseous NO_2 are suitable substrates for a combined *Brocadia-Nitrosomonas* community. *Nitrosomonas* seems to be able to produce nitrite, N_2, and NO by combined ammonia oxidation and denitrification (equation 2). Ammonia and the nitrite produced may be further converted to nitrate and N_2 by *B. anammoxidans* (equation 1). The specific ammonia oxidation activity of *B. anammoxidans* is dependent on the rate of nitrite production by *N. eutropha*. Such a coculture was examined in a reactor containing mineral medium (10 mM ammonia). The reactor was inoculated with 10^8 cells of *B. anammoxidans* ml^{-1} and 10^8 cells of *N. eutropha* ml^{-1}. The reactor was gassed at a rate of 0.5 liter min^{-1} with a 95% argon–5% carbon dioxide gas mixture supplemented with 250 ppm of NO_2. Immediately after inoculation ammonia and NO_2 consumption started (Fig. 4). Throughout the experiment nitrate and NO were produced, but nitrite did not accumulate (the concentration was always less than 20 μM). On the basis of NO_2 consumption, the specific anaerobic ammonia
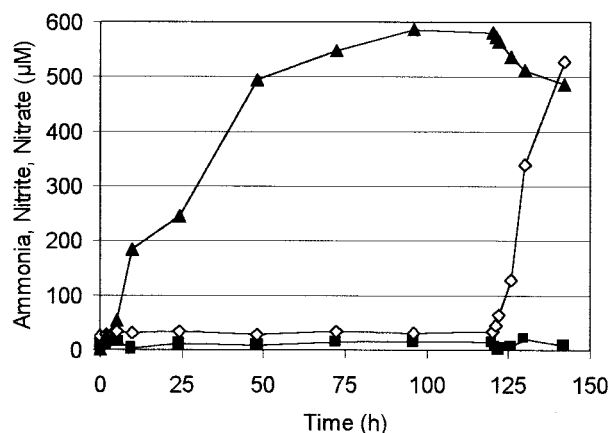
FIG. 3. Ammonia (■), nitrite (◇), and nitrate (▲) concentrations in a reactor before (0 to 120 h) and after (120 to 142 h) the addition of *N. eutropha*. The NO_2 concentration was adjusted to 200 ppm. The cell concentrations were adjusted to 10^8 *B. anammoxidans* cells ml^{-1} and 10^8 *N. eutropha* cells ml^{-1}.
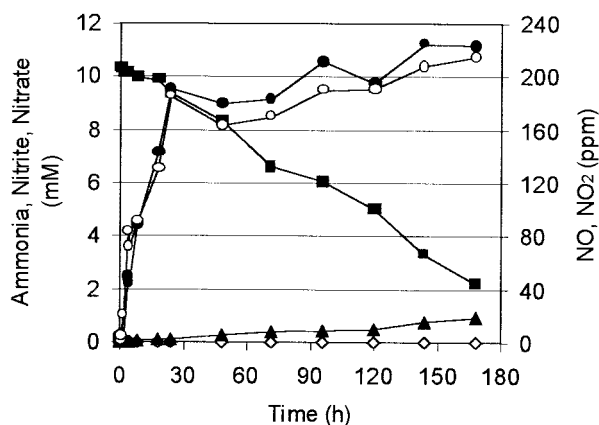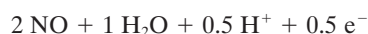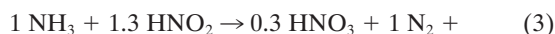
FIG. 4. Ammonia (■), nitrite (◇), and nitrate (▲) concentrations, NO_2 consumption (●) (NO_2 fresh gas minus NO_2 off gas), and NO production (○) (NO concentration in the off gas) in a *B. anammoxidans-N. eutropha* system with ammonia and NO_2 as the only substrates. The cell concentrations were adjusted to 10^8 *B. anammoxidans* cells ml^{-1} and 10^8 *N. eutropha* cells ml^{-1}.

oxidation activity of *N. eutropha* was calculated to be about 1.6 mmol g of protein^{-1} h^{-1}. The production of nitrate was used to calculate the specific activity of *B. anammoxidans* (about 0.9 mmol of NH_4^+ g of protein^{-1} h^{-1}). In sterile control experiments the chemical formation of nitrite and nitrate by reaction of NO_2 with water was determined. The values obtained in the control experiments were taken into account when the specific activity of *B. anammoxidans* was calculated.

DISCUSSION

The results presented here show that *Brocadia* and *Nitrosomonas* are able to coexist under anoxic conditions. While *B. anammoxidans* uses nitrite as a substrate and *Nitrosomonas* uses gaseous NO_2 as a substrate, these organisms compete for ammonia. Key elements used to analyze the results of this study are shown in Fig. 5 and in equations 1 and 2 describing anaerobic ammonia oxidation by *Brocadia* and *Nitrosomonas*. It is important to note that *Nitrosomonas* produces nitrite during anaerobic ammonia oxidation, which can serve as a substrate for *Brocadia* (Fig. 5). The assumption that this additional nitrite is consumed by *Brocadia* when ammonia is not the limiting substrate was confirmed in this study. As a consequence, the NO_2^-/NH_3 ratio in such a mixed population changes depending on the metabolic activities of the two ammonia oxidizers. Since this is important for interpretation of the results, it is illustrated below with a theoretical example (equations 3 to 5 [equation 5 is the sum of equations 3 and 4]).

$$1\ NH_3 + 1.3\ HNO_2 \rightarrow 0.3\ HNO_3 + 1\ N_2 + \qquad (3)$$

$$1.7\ H_2O + 0.6\ H^+ + 0.6\ e^-$$

$$1\ NH_3 + 1\ N_2O_4 \rightarrow 0.5\ HNO_2 + 0.25\ N_2 + \qquad (4)$$

$$2\ NO + 1\ H_2O + 0.5\ H^+ + 0.5\ e^-$$

$$2\ NH_3 + 0.8\ HNO_2 + 1\ N_2O_4 \rightarrow 0.3\ HNO_3 + \qquad (5)$$

$$1.25\ N_2 + 2\ NO + 2.7\ H_2O + 1.1\ H^+ + 1.1\ e^-$$

In an anammox reactor system without active *Nitrosomonas* cells (equation 3), the NO_2^-/NH_3 ratio is about 1.3. If actively ammonia-oxidizing *Nitrosomonas* cells are present (equation 4), the NO_2^-/NH_3 ratio of the system decreases to about 0.4 (equation 5) when the two organisms contribute equally to ammonia consumption. According to this calculation the presence of ammonia-oxidizing *Nitrosomonas* cells should be indicated by a decrease in the NO_2^-/NH_3 ratio. Hence, on the basis of this ratio the contributions of *B. anammoxidans* and *Nitrosomonas* to the total ammonia conversion in a mixed population can be calculated. Our results (Table 3) indicate that the NO_2^-/NH_3 ratio was 0.82 in the presence of 10^8 cells of *B. anammoxidans* ml^{-1} and 10^8 cells of *N. eutropha* ml^{-1}. According to equations 3 to 5 *B. anammoxidans* accounts for about 75% and *N. eutropha* accounts for about 25% of the total anaerobic ammonia oxidation. The NO_2^-/NH_3 ratios presented in Tables 1 and 3 provide a strong indication that there is simultaneous anaerobic ammonia oxidation by *B. anammoxidans* and *Nitrosomonas*.

When the number of *N. eutropha* cells in the mixed population supplied with NO_2, ammonium, and nitrite was increased, the total ammonia consumption of the system was stimulated and the NO_2^-/NH_3 ratio decreased significantly (from 1.33 to 0.82). Furthermore, both NO_2 consumption and NO production were detectable, indicating that anaerobically ammonia-oxidizing *N. eutropha* cells were present. When *N. eutropha* oxidized ammonia, nitrite was produced, but nitrite did not accumulate in the reactor. It is very likely that *B. anammoxidans* consumed the additional nitrite, resulting in a higher specific ammonia oxidation activity, which was also indicated by an increased rate of production of nitrate (Table 3 and Fig. 5). When ammonia was the limiting substrate (Fig. 3), *B. anammoxidans* was not able to adapt its activity to the increased nitrite supply (nitrite in the fresh medium plus nitrite produced by *Nitrosomonas*), and therefore, nitrite accumulated in the system.

The results shown in Table 3 were used to calculate the anaerobic ammonia oxidation activities of *B. anammoxidans* and *N. eutropha*. Nitrate production is directly correlated to the activity of *B. anammoxidans* (equation 1), because *N. eutropha* is unable to produce or consume nitrate. According to the nitrate production data (Table 3), the specific anaerobic
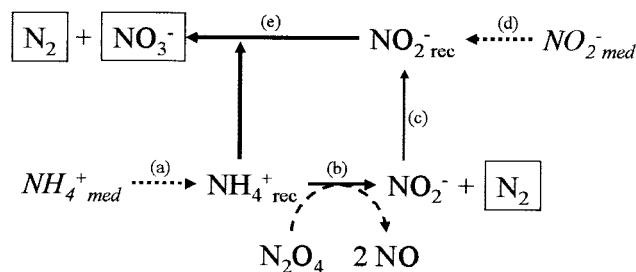

FIG. 5. Schematic model describing the competition and cooperation between *B. anammoxidans* and *Nitrosomonas* under anoxic conditions. (a), ammonia addition (fresh medium); (b), ammonia oxidation by *Nitrosomonas*; (c), additional nitrite in the reactor due to ammonia oxidation; (d), nitrite addition (fresh medium); (e), anammox reaction; *med*, medium; *rec*, reactor. End products are enclosed in boxes.

ammonia oxidation activity of *B. anammoxidans* ranged from 4.4 to 5.4 mmol of NH_4^+ g of protein^{-1} h^{-1}. On the basis of NO_2 consumption and NO production, a specific activity for *N. eutropha* of between 1.1 and 1.5 mmol of NH_4^+ g of protein^{-1} h^{-1} was calculated. Interestingly, the specific anaerobic ammonia oxidation activity of *N. eutropha* in this mixed culture was about 10 times higher than that measured in a pure culture (12). In pure cultures, NO_2 concentrations of more than 50 ppm were toxic for *N. eutropha*. In anammox systems NO_2 concentrations as high as 250 ppm were not inhibitory. The possibility that *N. eutropha* cells in a mixed population are supplied with higher NO_2 concentrations might explain the high anaerobic ammonia oxidation activity of such cells.

Simultaneous anaerobic ammonia oxidation activities of *B. anammoxidans* and *N. eutropha* were also detectable when ammonia was the limiting substrate (Fig. 3). An anammox system was grown with low ammonium and nitrite concentrations in the reactor. Nitrate was produced and accumulated in the medium. When *N. eutropha* (10^8 cells ml^{-1}) was added, the consumption of NO_2 and the production of nitrite and NO indicated that there was anaerobic ammonia oxidation activity by *N. eutropha*. Since the ammonia supply was not increased, *B. anammoxidans* and *N. eutropha* had to compete for this limited resource. As a result of the competition, the specific activity of *B. anammoxidans* decreased, as indicated by a decreasing nitrate concentration.

In an anammox reactor system without added *N. eutropha* cells (Fig. 1 and 2) the total activity of the system increased significantly when up to 50 ppm of NO_2 was added (Fig. 1). A further increase in the NO_2 concentration led to decreases in activity. The growth rate of *B. anammoxidans* increased only 1.4-fold, while the specific ammonia oxidation activity increased about 2-fold (Fig. 1) when the NO_2 concentration was increased from 0 to 50 ppm. Perhaps parts of the reducing equivalents generated during ammonia oxidation are necessary for the conversion (reduction) of NO_2. In contrast to the stable number of *B. anammoxidans* cells (the cell number was adjusted to about 5×10^8 cells ml^{-1} every 3 days), the number of *Nitrosomonas* cells increased from about 10^3 to 10^7 cells ml^{-1} (Table 2) (the growth rate was about 0.005 h^{-1}). The ammonia oxidation activity of *Nitrosomonas* provided the system with additional nitrite, which was consumed by *B. anammoxidans*. Obviously, the increased specific activities of both *B. anammoxidans* (more nitrite available) and *Nitrosomonas* (NO_2 available) led to higher N conversion activities in the reactor (Fig. 1) (0 to 50 ppm of NO_2). Apparently, concentrations of NO_2 higher than 50 ppm were inhibitory for *B. anammoxidans* in this system (Fig. 1). The cell number (Table 2) and the specific activity (Table 3) of *Nitrosomonas* were too low to compensate for the NO_2-inhibited activity of *B. anammoxidans* (NO_2 concentrations greater than 50 ppm). When the reactor system was incubated without supplemental NO_2, *Nitrosomonas* was not able to oxidize ammonia (12, 14), and the conversion rates of the N compounds were dependent only on the activity of *B. anammoxidans*.

The situation was less complex when NO was added. The activity of the anammox system was stable at NO concentrations between 0 and 600 ppm. At higher NO concentrations the activity decreased. Under anoxic conditions in the presence of NO, *Nitrosomonas* was not able to oxidize ammonia (12). As a

consequence, *Nitrosomonas* did not contribute to the ammonia oxidation activity of the system. Therefore, the N conversion rates were dependent only on the activity of *B. anammoxidans*. It is interesting that higher NO concentrations added to the anammox system led to increased specific NO consumption activity of *B. anammoxidans* (Fig. 2). The specific nitrate and NO production activities increased almost parallel to the specific nitrite consumption activity (Fig. 2). Since part of the nitrite oxidation activity provides *B. anammoxidans* with reducing equivalents (22), it seems likely that the electrons derived from nitrite were used to reduce NO. This might have been possible via the hydroxylamine oxidoreductase, which has some NO-reducing capacity, or via the cytochrome *c* nitrite reductase, producing ammonia (11). Another possibility is disproportion of NO to N_2O and nitrite.

The final experiment in this study showed that *B. anammoxidans* and *Nitrosomonas* formed stable cocultures under anoxic conditions when they were supplemented with a surplus of ammonium and gaseous NO_2 (Fig. 4). Under these conditions, ammonia was oxidized by *N. eutropha* to dinitrogen and nitrite. The nitrite produced was further converted by *B. anammoxidans* with ammonia as an electron donor. The model for this process is shown in Fig. 5. The ammonia oxidation activity of *N. eutropha* was indicated by the NO_2 consumption and NO production profiles. The activity of *B. anammoxidans*, which was dependent on the nitrite production of *N. eutropha*, was indicated by the nitrate production rate. Under anoxic conditions *N. eutropha* converts only 50 to 60% of the ammonia consumed to nitrite (12). Because of this nitrite limitation the specific ammonia oxidation activity of *B. anammoxidans* (0.9 mmol g of protein^{-1} h^{-1}) was lower than the specific ammonia oxidation activity of *N. eutropha* (1.6 mmol g of protein^{-1} h^{-1}).

The results of this study provide strong indications that the anaerobic ammonia-oxidizing planctomycetes (*B. anammoxidans*) are not sensitive to NO concentrations up to 600 ppm and that the N conversion rates of an anammox reactor system increase about twofold in the presence of 50 ppm of NO_2. Further experiments demonstrated that addition of NO_2 leads to increases in the number of cells and the specific ammonia oxidation activity of *Nitrosomonas* under anoxic conditions. Although the two groups of ammonia oxidizers compete for ammonia, cooperation also seems to be possible when the NO_2-dependent ammonia oxidation of *Nitrosomonas* supplies *B. anammoxidans* with nitrite as an oxidant. This might have ecological relevance. On the one hand, the ammonia oxidation of *B. anammoxidans* is restricted to anoxic environments. On the other hand, *Nitrosomonas*-like microorganisms need an oxidizing agent (O_2 or NO_2) that is available only under oxic conditions. The oxic-anoxic interface might be a suitable environment for both groups of ammonia oxidizers. The availability of ammonia and nitrite is decisive for coexistence. At the oxic-anoxic interface the ammonia oxidation of *Nitrosomonas*-like microorganisms might be the major source of the nitrite necessary for the nitrite-dependent anammox metabolism. The products of this cooperation are mainly N_2 and small amounts of nitrate. When ammonia is the limiting substrate, the two groups of ammonia oxidizers compete for ammonia (Fig. 3), and the specific affinities for ammonia might be decisive for the outcome of the competition.

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