APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1246–1251 Vol. 61, No. 4
0099-2240/95/$04.00+0

Anaerobic Oxidation of Ammonium Is a Biologically Mediated Process

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Received 25 July 1994/Accepted 9 January 1995

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

The removal of ammonium is an important problem in modern wastewater treatment systems. It is generally achieved by a combination of two processes, nitrification and denitrification. Ammonium is oxidized first to nitrite and then to nitrate by nitrifying bacteria. Nitrification is an O_2-requiring process and therefore requires an aerobic environment (1, 7). During the subsequent denitrification step, nitrate or nitrite is converted to dinitrogen gas (11). Denitrification is carried out by a wide spectrum of respiratory bacteria representing most genera and physiological types (27). Most denitrifying bacteria carry out these reactions only under anaerobic conditions (20).

Very recently, however, ammonium losses under anaerobic conditions were discovered to occur in a laboratory-scale denitrification reactor (12). Increased removal of ammonium was paralleled by increased disappearance of nitrate. This suggested that the following reaction was taking place (4):

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+ \qquad (1)$$

$$\Delta G^{\circ\prime} = -1{,}483.5 \text{ kJ per reaction}$$

The overall reaction for this anaerobic ammonium oxidation (Anammox) process is exergonic and thus could, in theory, supply energy for growth. It was therefore postulated that the removal of ammonium observed to occur in the denitrifying reactor was carried out by bacteria using ammonium as an electron donor for nitrate reduction (12). The aim of the research described here was threefold: first, to confirm that the observed ammonium removal (12) was mediated by (micro)biological activity; second, to establish that oxidation of ammonium took place under fully anaerobic conditions; and finally, to demonstrate that ammonium was converted to dinitrogen gas by [^{15}N]ammonium.

MATERIALS AND METHODS

Origin of biomass and preparation. Sludge from the denitrifying fluidized bed reactor in which anaerobic ammonium oxidation occurred was used as a source for biomass (12). The sludge from the reactor was either used immediately or else stored at 4°C until needed. Before use, the sludge was homogenized by passing it several times through a 60-ml syringe. For experiments with [^{15}N]ammonium, the sludge in the reactor was used.

Medium. Effluent from the denitrifying fluidized bed reactor, supplemented with various concentrations of ammonium and nitrate, was used as the medium for all batch experiments. The effluent contained the following (per liter): total organic carbon, 130 to 155 mg; SO_4^{2-}-S, 80 to 130 mg; NH_4^+-N, 10 to 70 mg; NO_3^--N, 40 to 120 mg. Details of the denitrifying reactor, which was being fed with effluent from a methanogenic reactor, were as described by Mulder et al. (12).

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

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

For experiments with killed cells, sludge was autoclaved for 30 min at 120°C. This was repeated after 60 h. A single treatment was insufficient to stop denitrification and sulfate reduction completely. The medium was sterilized for 40 min at 120°C. For gamma irradiation of the sludge, 40-ml sludge samples were exposed to 25 kGy of radiation in 7 h by using a ^{57}Co source (Gammaster, Ede, The Netherlands). In the inhibition experiments, different amounts (see Table 1)

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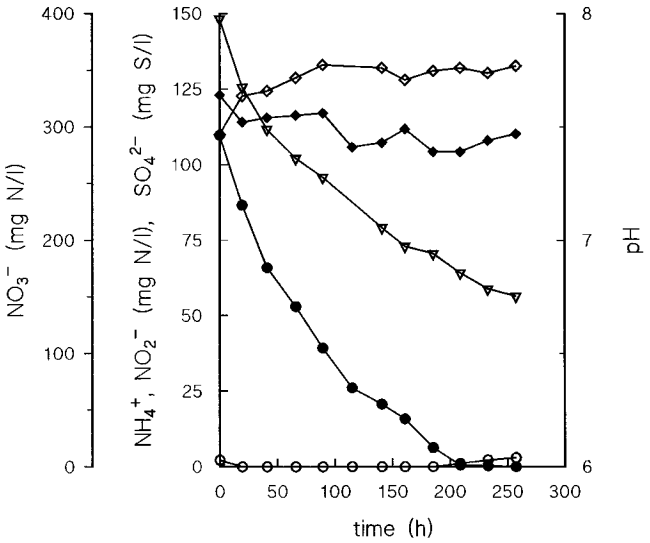

FIG. 1. Concentration curves and pH course of a typical anaerobic ammonium oxidation batch culture experiment using 500-ml serum bottles at 37°C with nitrate as the electron acceptor and a 30-ml inoculum. The specific anaerobic ammonium oxidation activity of the biomass used was 66 ng of NH_4^+-N · h^{-1}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

of antibiotics or inhibitors were added to the bottles before incubation. When different volumes (0, 10, 20, 30, and 40 ml) of sludge were used for inoculation, the final volume of the batch cultures was always adjusted to 500 ml with medium.

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

Tracer studies. Studies with [^{15}N]ammonium were done by using the 23-liter denitrifying fluidized bed reactor in which the Anammox reaction was occurring (12). During the tracer experiments, the fluidized bed reactor was operated in batch mode, with liquid recycle but without its usual feed from the methanogenic reactor. At the beginning of the experiment, 4.5 g of $(^{15}NH_4)_2SO_4$ was supplied to the culture in the reactor, increasing the ammonium concentration by 45 mg of N per liter. Control experiments were done either by adding unlabelled ammonium or without any addition. Samples for analysis of ammonium, nitrate, and nitrite were taken. Gas production was monitored during the experiment. During the days between successive experiments, the reactor was operated as usual.

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

Analytical procedures. Nitrate, nitrite, and sulfate were determined with an ion-exchange high-pressure liquid chromatograph fitted with an ionospher-A column (Chrompack, Middelburg, The Netherlands), a mobile phase of 0.04 M sodium salicylate (pH 4.0), and a refractive index detection system (Waters, Milford, Mass.). When needed, nitrite was determined colorimetrically with Griess-Romijn reagent (8) or was assayed semiquantitatively by using test strips from Merck (detection range, 0 to 80 mg/liter). Ammonium was determined colorimetrically (6). The term ammonium will be used to indicate both the protonated and the unprotonated forms, since, at the pH values used in these experiments, ammonium and ammonia would both be present. Sulfide was determined according to the method of Trüper and Schlegel (21). Total organic carbon was determined with a Tocamaster model 915-B instrument (Beckmann Industrial Corp., La Habra, Calif.). Biomass dry weight was determined by drying the sample at 65°C for at least 24 h. The quantity of sand in the dried sample was measured after ashing at 650°C for 1 h. The dry weight minus the sand is hereafter termed volatile solids (VS).

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

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

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

[b] NA, not applicable.

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

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

RESULTS

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

Sterilization experiments. As can be seen from Table 1, sterilization of the sludge or wastewater by gamma irradiation or autoclaving prevented ammonium removal during the experiments. Control bottles (with untreated sludge) showed a

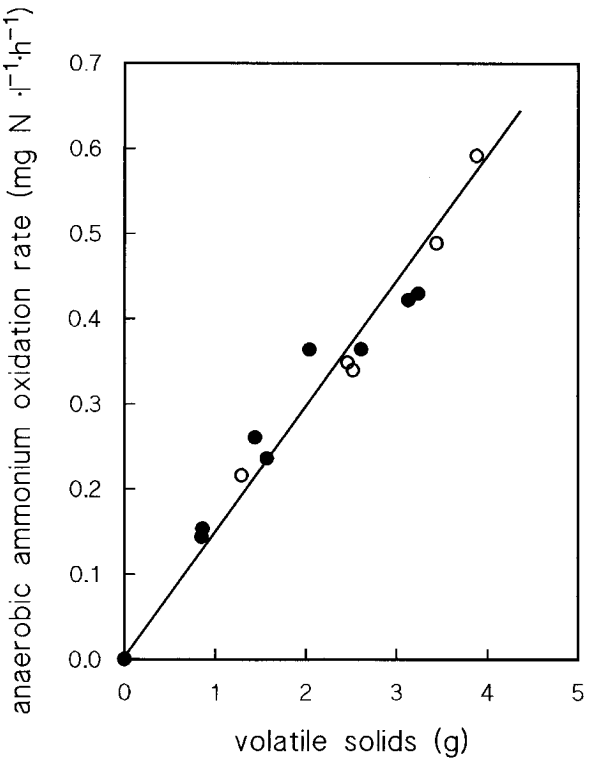

FIG. 2. Correlation between anaerobic ammonium oxidation rate and amount of biomass used in batch cultures. The specific anaerobic ammonium oxidation activity of the biomass used was 67 ng of NH_4^+-N · h^{-1} · mg of VS^{-1}. ●, static incubation; ○, shaken incubation.

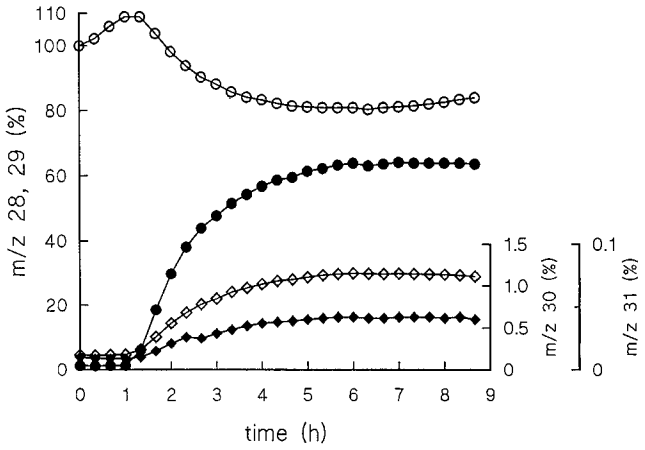

FIG. 3. Changes in *m/z* 28, 29, 30, and 31 as a percentage of the initial partial pressure of *m/z* 28 and as a function of time during a batch experiment with the denitrifying fluidized bed reactor after the addition of labelled NH_4^+ and during control experiments. The $^{15}NH_4^+$ pulse was supplied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

decrease of 110 mg of NH_4^+-N per liter during the same period. Without the addition of the sludge, and even with nonsterile wastewater, the ammonium concentration did not decrease. In addition, the nitrate, nitrite, and sulfate concentrations did not change in any of the experiments using sterilized sludge samples and/or wastewater.

Inhibition experiments. To investigate whether inhibitors or antibiotics inhibited anaerobic ammonium oxidation, chloramphenicol, penicillin V, ampicillin, 2,4-dinitrophenol, CCCP, and $Hg^{II}Cl_2$ were added separately to batch cultures (3). The effect of these compounds on ammonium oxidation is shown in Table 1. The anaerobic ammonium oxidation activity of the control flasks (104 ng of NH_4^+-N · h^{-1} · mg of VS^{-1}) was considered to be 100%. When 200 mg of chloramphenicol per ml was added to batch cultures, 3 days elapsed before significant ammonium conversion was observed. The addition of 20 mg of chloramphenicol per ml caused partial inhibition of ammonium removal, reducing the rate to 64% of the control level. This is in agreement with observations that chloramphenicol can inhibit the activity of existing denitrification enzymes (5). Ampicillin (800 mg/liter) inhibited ammonium removal almost completely. At 400 mg/liter, it reduced the activity by 71%. Penicillin V was less inhibitory. This could be due to the low concentrations of antibiotic used, or to some form of resistance to penicillin V. Known inhibitors of oxidative phosphorylation, 2,4-dinitrophenol (2 mM), mercuric chloride (1 mM), and CCCP (0.2 mM), completely inhibited anaerobic ammonium removal, indicating that the ammonium conversion is due to a metabolic activity.

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

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

Tracer studies. For N_2 produced from unlabelled and ^{15}N-labelled precursors, three combinations of N isotopes are possible: $^{14-14}N_2$ (*m/z* 28), $^{14-15}N_2$ (*m/z* 29), and $^{15-15}N_2$ (*m/z* 30). After the addition of $^{15}NH_4^+$, the resulting labelled N_2 should cause a change in the *m/z* 28, 29, and 30 values, depending on how the N-N bond was formed. After $^{15}NH_4^+$ was added, a large increase of *m/z* 29 $^{14-15}N_2$ was observed (Fig. 3). Furthermore, small amounts of $^{15-15}N_2$ (*m/z* 30) were also formed. *m/z* 45 and 46 did not increase during these experiments, indicating that labelled N_2O was not formed. The small increase at *m/z* 31 (Fig. 3) was therefore due to the formation of labelled nitric oxide (^{15}NO). This represented only a trace amount of the total labelled end products. During control experiments with no or unlabelled ammonium, *m/z* 29, 30, and 31 did not change. It can thus be concluded that the main product of anaerobic ammonium oxidation is dinitrogen gas.

Ammonium and nitrate consumption and the cumulative gas production during the ^{15}N labelling and control experiments

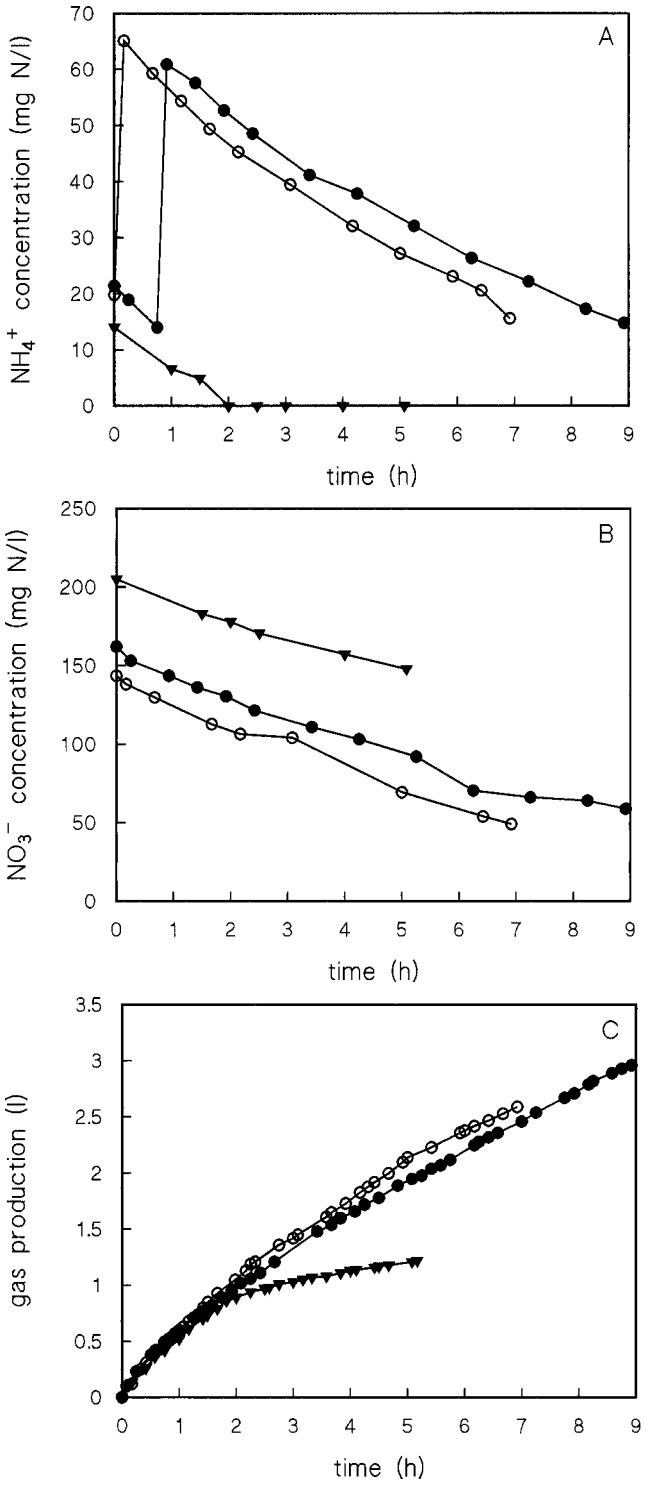

FIG. 4. Consumption of ammonium (A) and nitrate (B) and cumulative production of gas (C) during a batch experiment using a fluidized bed reactor with $^{15}NH_4^+$ addition and controls. Symbols: ●, $^{15}NH_4^+$ addition; ○, NH_4^+ addition; ▼, no addition.

are presented in Fig. 4. The ammonium concentration in the control experiment (without the addition of extra ammonium) reached zero after 2 h. Simultaneously, the gas production rate decreased. This emphasizes the correlation between ammonium conversion and gas production.

DISCUSSION

The results presented here clearly demonstrate that anaerobic ammonium oxidation is a biological process that terminates in N_2. It is dependent on NO_3^- and does not require traces of O_2. The fact that ammonium did not disappear when heat-inactivated or gamma-irradiated sludge was used, or in cultures that were not inoculated, indicates that the well-known chemical Van Slyke reaction (24) between ammonium or amino groups and nitrite, forming dinitrogen gas, did not occur. Nitrite was not present in the medium during the batch experiments and was found only after the ammonium had become depleted. Moreover, the pH in these experiments remained far above 3, the required pH for the Van Slyke reaction. Additional tests carried out during the current study (data not shown) gave no evidence for spontaneous chemical reactions even when relatively high concentrations of NH_4^+, NO_3^-, or NO_2^- (30 mM, 420 mg of N per liter) were used. Only at very high NH_4^+ and NO_2^- concentrations (40 g of N per liter), with a low pH and under an NO atmosphere (to prevent decomposition of nitrite), has the chemical production of N_2 from ammonium and nitrite been confirmed (18, 22). Interactions between nitrite and ammonium ions have been reported (25) to occur in alkaline soils (pH 8.5), but only after desiccation. At low concentrations, loss due to chemical reaction was not observed.

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

As the cultures incubated in anaerobic jars gave the same results as similar cultures incubated normally, O_2 was evidently not needed for the Anammox reaction. The known NH_4^+-oxidizing nitrifiers, such as *Nitrosomonas europaea*, are thought to use molecular O_2 for at least the first oxidation step of ammonium to hydroxylamine, carried out by an ammonium monooxygenase (26). This implies that the bacteria able to carry out anaerobic ammonium oxidation have a novel nitrifying enzyme system. It is known, however, that some nitrifiers are capable of partial denitrification (1, 17). It was demonstrated that *N. europaea*, under conditions of O_2 stress, could use nitrite as the terminal electron acceptor, producing nitrous oxide (15). A new isolate identified as a *Nitrosomonas* species was able to produce, under similar conditions, N_2 (14). Labelled-nitrogen experiments, however, demonstrated that the N_2O and N_2 were produced by nitrite reduction rather than by ammonium oxidation. Remde and Conrad (16) also concluded that nitrite was the main source of the NO and N_2O produced by *N. europaea*. O_2 was still required for the initial oxidation of ammonium to NH_2OH, although the O_2 for the oxidation of hydroxylamine might come from H_2O. The known autotrophic NH_4^+-oxidizing nitrifiers can consequently not be grown under anaerobic denitrifying conditions. Indeed, *N. europaea* washed out of continuous cultures once the dissolved O_2 had dropped below the critical level of 8% air saturation (23). The Anammox reaction is therefore not likely to be due to the activity of known ammonium-oxidizing bacteria.

As can be seen from the experiments with [^{15}N]ammonium (Fig. 3), NH_4^+-N was incorporated in the end product of the Anammox reaction, N_2. Mixed-labelled nitrogen gas, $^{14\text{-}15}N_2$, was the dominant product, making up 98.2% of the total labelled dinitrogen gas produced (Fig. 3). Only 1.7% was $^{15\text{-}15}N_2$.

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

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

[a] Gibbs free energies were calculated on the basis of data from Thauer et al. (19).
[b] Expected composition of labelled dinitrogen gas when ammonium is labelled with ^{15}N, when no exchange between ^{15}N and ^{14}N is assumed.
[c] Anaerobic ammonium oxidation, as proposed by Broda (4).
[d] A dehydrogenase is involved in the oxidation of ammonium to hydroxylamine, and nitrate and O_2 are both electron acceptors for this process.

$$5NH_4^+ + 5H_2O \rightarrow 5NH_2OH + 10[H] + 5H^+$$
$$10[H] + 2.5O_2 \rightarrow 5H_2O$$
$$5NH_2OH + NO_3^- + H^+ \rightarrow 3N_2 + 8H_2O$$

[e] A monooxygenase catalyzes the first step in the oxidation of ammonium, in which hydroxylamine is formed. No nitrate is used for ammonium oxidation, and there is an overall requirement for additional electron donors (organic material or reduced sulfur compounds) since hydroxylamine does not provide enough reducing equivalents.

$$2NH_4^+ + 2O_2 + 4[H] \rightarrow 2NH_2OH + 2H_2O + 2H^+$$
$$2NH_2OH \rightarrow N_2 + 2H_2O + 2[H];\ (2[H] \text{ is represented by } H_2 \text{ in the overall reaction})$$

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

These findings do not fully agree with the overall reaction (equation 1) proposed by Mulder et al. (12). From this reaction, it was to be expected that 75% of the labelled nitrogen would be $^{14-15}N_2$ and 25% would be $^{15-15}N_2$, because of the 5:3 stoichiometry of ammonium oxidation to nitrate reduction. Because of the very low growth yields (12), ammonium assimilation was assumed not to be significant within the period of the experiment. The stoichiometries of four other potential reactions for the oxidation of ammonium to dinitrogen gas are summarized in Table 2. In all of these reactions, only the oxidation of ammonium with nitrite as electron acceptor predicts a labelling percentage close to the observed data. If O_2, for example, were involved, only $^{15-15}N_2$ would be expected. Thus, instead of nitrate, nitrite appears to be the direct oxidizing agent of ammonium in the Anammox reaction.

Apart from reports of unexplainable N_2 production (e.g., references 2 and 10), there is no other published evidence for the anaerobic removal of ammonium by biological nitrate reduction. Studies recently done in the Black Sea have indicated that there is also a correlation between the nitrate and ammonium profiles in nature (9). The chemical zonation of O_2 and H_2S of the upper few hundred meters of the central Black Sea waters has been shown to have changed significantly over the last decades (9, 13). The O_2-containing layer is separated from the sulfide-rich lower zone by a 30-m-deep layer that is free of O_2 and H_2S. In this layer, opposing gradients of NO_3^- and NH_4^+ were found, suggesting that anaerobic ammonium oxidation might be taking place.

ACKNOWLEDGMENTS

We thank H. Slijkhuis and R. Beudeker of Gist-brocades for their stimulating discussions.

This work was supported by the Foundation of Applied Research (S.T.W.) and Gist-brocades.

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