

# Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor

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Received 25 July 1994; revised 24 October 1994; accepted 24 October 1994

## Abstract

Until now, oxidation of ammonium has only been known to proceed under aerobic conditions. Recently, we observed that  $\text{NH}_4^+$  was disappearing from a denitrifying fluidized bed reactor treating effluent from a methanogenic reactor. Both nitrate and ammonium consumption increased with concomitant gas production. A maximum ammonium removal rate of  $0.4 \text{ kg N} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$  ( $1.2 \text{ mM/h}$ ) was observed. The evidence for this anaerobic ammonium oxidation was based on nitrogen and redox balances in continuous-flow experiments. It was shown that for the oxidation of 5 mol ammonium, 3 mol nitrate were required, resulting in the formation of 4 mol dinitrogen gas. Subsequent batch experiments confirmed that the  $\text{NH}_4^+$  conversion was nitrate dependent. It was concluded that anaerobic ammonium oxidation is a new process in which ammonium is oxidized with nitrate serving as the electron acceptor under anaerobic conditions, producing dinitrogen gas. This biological process has been given the name 'Anammox' (anaerobic ammonium oxidation), and has been patented.

**Keywords:** Ammonium removal; Anaerobiosis; Denitrification; Waste water; Nitrification

## 1. Introduction

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

prove the classical nitrification and denitrification processes.

Nitrification, frequently combined with denitrification, is the most widely used method for nitrogen control in waste water treatment [1–3]. Nitrification is generally carried out by aerobic, autotrophic bacteria that oxidize  $\text{NH}_4^+$  to  $\text{NO}_2^-$ , and  $\text{NO}_2^-$  to  $\text{NO}_3^-$ , with molecular oxygen as electron acceptor. To be fully effective, these bacterial conversions require a very efficient oxygen supply [4].  $\text{NO}_2^-$  and  $\text{NO}_3^-$  are subsequently reduced to  $\text{N}_2$  by denitrifying bacteria that use the  $\text{NO}_x^-$  as alternative electron acceptors to oxygen, and are most effective in the absence of oxygen [5–7]. The situation is further complicated,

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because the autotrophic nitrifiers cannot compete with aerobic heterotrophs for oxygen and other nutrients in the presence of substantial amounts of organic compounds [8] and can therefore easily be overgrown by the heterotrophs. An additional complication is that the denitrifying bacteria must be provided with a suitable electron donor, usually organic compounds.

The different requirements of nitrifiers and denitrifiers have led to a number of reactor combinations for the removal of nitrogen from waste water. The combined nitrification/denitrification process (single-sludge system) can be distinguished from a system where nitrification and denitrification are carried out by two separated sludges (dual-sludge system) [9]. In the single-sludge system, nitrification and denitrification are achieved by alternating aerobic and anaerobic zones. The dual-sludge system uses separate nitrification and denitrification reactors. If the nitrification stage follows denitrification, recirculation of nitrified waste water is required [10]. When nitrification precedes denitrification, the addition of an external electron donor (such as hydrogen, methanol, sulphur and sulfide, see Table 1) is necessary [3,12,13].

In theory, ammonium can also be used as an inorganic electron donor for denitrification (Table 1, equation 3). The free energy balance for this reaction is nearly as favourable as in the aerobic nitrification process (Table 1, equation 4). In 1977, Broda [14] published a theoretical paper entitled ‘‘Two kinds of lithotrophs missing in nature’’ describing the potential existence of chemolithotrophic bacteria able to oxidize ammonia to dinitrogen with nitrate, carbon dioxide or oxygen as oxidant. These predictions were based on thermodynamic calculations, but the existence of these microorganisms has never been demonstrated.

This paper describes the discovery of an anaerobic process in which ammonium was used as electron donor for denitrification, following an earlier preliminary report [15].

## 2. Materials and methods

### 2.1. Operating conditions of the denitrifying fluidized bed reactor

A denitrifying microbial population was grown in a glass, 23 l, fluidized bed reactor, at 36°C and pH 7. Degassed anoxic liquid from the top of the reactor was recirculated to boost the flow to approximately 255 l/h in order to keep the bed fluidized at a superficial liquid velocity of 30–34 m/h. The hydraulic retention time was 4.2 h. The influent of the denitrifying reactor, supplied at a rate of 5–6 l/h, came from a methanogenic reactor that was operated with waste water from a bakers yeast production plant, and contained (mg/l): COD, 550–750; TOC, 165–190;  $\text{SO}_4^{2-}$ , 25–150;  $\text{S}^{2-}$ , 90–130;  $\text{NH}_4^+ - \text{N}$ , 90–130. Nitrate solution (75 g  $\text{NaNO}_3/\text{l}$ ) was separately supplied at a rate of about 450 ml/h. The redox-potential was measured continuously with a platinum electrode and an Ag/AgCl reference electrode ( $E_{\text{ref}} = +230 \text{ mV}$  at 36°C; Elektrofact SR20/AP24, Elscolap, Maarsenbroek, the Netherlands). The redox potential of the process water was maintained at 150–250 mV by controlling the waste water supply.

All tubing and connectors were of butyl rubber or polyvinylchloride (PVC) to limit oxygen diffusion. For the same reason, the 5 l settler at the top of the reactor was flushed with  $\text{N}_2$ .

Sand particles (diameter 0.3–0.6 mm) were the carrier material of the fluidized bed on which bacte-

Table 1  
Gibbs free energy of some reactions involved in denitrification and ammonium oxidation (from [11])

Equation no.	Reaction	$\Delta G^\circ$
1	$2 \text{NO}_3^- + 5 \text{H}_2 + 2 \text{H}^+ \rightarrow \text{N}_2 + 6 \text{H}_2\text{O}$	– 1121 kJ/reaction
2	$8 \text{NO}_3^- + 5 \text{HS}^- + 3 \text{H}^+ \rightarrow 4 \text{N}_2 + 5 \text{SO}_4^{2-} + 4 \text{H}_2\text{O}$	– 3721 kJ/reaction
3	$3 \text{NO}_3^- + 5 \text{NH}_4^+ \rightarrow 4 \text{N}_2 + 9 \text{H}_2\text{O} + 2 \text{H}^+$	– 297 kJ/mol $\text{NH}_4^+$
4	$\text{NH}_4^+ + 2 \text{O}_2 \rightarrow \text{NO}_3^- + \text{H}_2\text{O} + 2 \text{H}^+$	– 349 kJ/mol $\text{NH}_4^+$
5	$8 \text{NH}_4^+ + 6 \text{O}_2 \rightarrow 4 \text{N}_2 + 12 \text{H}_2\text{O} + 8 \text{H}^+$	– 315 kJ/mol $\text{NH}_4^+$

ria grew as a biofilm at a biomass concentration of 150–300 mg Volatile Suspended Solids (VSS)/g<sub>carrier</sub> (equivalent to 14 g VSS/l). The reactor was inoculated with a small amount of sand covered with denitrifying biofilms, originating from previous experiments. This resulted in fast growth of the fluidized bed, which reached a volume of 13 l after a period of four weeks. The fluidized bed height was maintained at a constant level by the removal of biofilm-covered sand from the top of the bed and the addition of clean sand.

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

## 2.2. Fed-batch experiments

Biomass from the denitrifying fluidized bed reactor was transferred to a temperature-controlled (36°C), intermittently-stirred reactor with a volume of 2.4 l for fed-batch experiments. This biomass sample contained 252.6 g sand particles and 40.1 g biomass (VSS), corresponding to a biofilm coverage of 159 mg VSS/g<sub>carrier</sub>. The experiment was started by the addition of 4.0 g NH<sub>4</sub>NO<sub>3</sub>, followed by three successive additions of 5.3, 3.3, and 2.3 g NaNO<sub>3</sub> after 123, 341 and 557 h, respectively. The concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup>, and the gas production, and SO<sub>4</sub><sup>2-</sup> reduction levels were all monitored.

## 2.3. Analytical methods

Nitrate, nitrite and sulphate were determined by HPLC with a conductance detector (Millipore Waters Model 430, Millipore Waters, Ettenleur, the Netherlands). The ammonium content was measured by distillation of ammonia, followed by absorption and titration [16]. The COD of untreated samples and supernatant of centrifuged samples were determined by titration of the amount of potassium dichromate oxidized in two hours [16]. The TOC was determined with a TOC-sin II aqueous carbon analyzer (Phase Separations). The volatile fatty acids were determined with a GC (Perkin-Elmer 1B, Perkin-Elmer,

Gouda, the Netherlands) equipped with a 1.8 m × 2 mm glass column (15% SP 1220/1% H<sub>3</sub>PO<sub>4</sub> on Chromsorb WAW 100–120 mesh, Supelchem, Leusden, the Netherlands) and a FID-detector. The attached biomass was analyzed for the content of volatile suspended solids [16].

The gas produced in the reactor was collected by a funnel at the top, and measured with a wet-type laboratory gas meter (Schlumberger, type 1, Schlumberger, Dordrecht, the Netherlands). The gas was sampled weekly, using 60 ml polypropylene syringes. CH<sub>4</sub>, N<sub>2</sub>, CO<sub>2</sub>, N<sub>2</sub>O (detection limit 65 ppm) and O<sub>2</sub> were measured with a GC equipped with a 500 × 0.3 cm glass column (Porapak Q, 80–100 mesh, Chrompack, Rozendaal, the Netherlands) and a thermal conductivity detector, operated at 0°C and 110°C, respectively. Gas production during the fed-batch experiments was measured by the liquid displacement method (Mariotte bottle). At the pH values used in this work, both ammonium and ammonia would be present. Ammonium will therefore be used to represent both forms.

## 3. Results and discussion

### 3.1. Performance of the denitrifying fluidized bed reactor

The steady-state data for the influent, effluent and reactor performance are summarized in Table 2. Sulfide and organic acids were the major electron donors. The gas production rates are directly related

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

	Before	After
Gas production (ml/1 <sub>feed</sub> )	80	200
Sulphate production (mg/l)	400	375
VFA oxidation (mg COD/l)	20–40	25–45
TOC removal (mg TOC/l)	15–40	20–50
NO <sub>3</sub> <sup>-</sup> in effluent (mg N/l)	160–200	40–120
NO <sub>2</sub> <sup>-</sup> in effluent (mg N/l)	25	0–12
NH <sub>4</sub> <sup>+</sup> 'lost' (mg N/l)	0	60–100
N-removal rate (kg N.m <sup>-3</sup> .d <sup>-1</sup> )	0.7	1.5
pH	7.2	6.9

VFA = volatile fatty acids; TOC = total organic carbon.

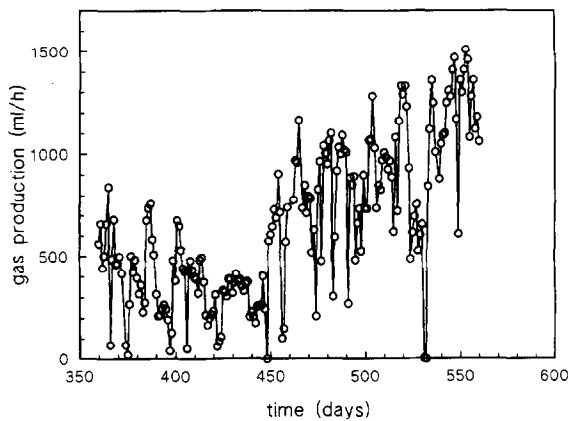


Fig. 1. Effect of the occurrence of anaerobic ammonium oxidation on day 420 on the gas production by the denitrifying fluidized bed reactor. Day 350–420: the last part of the first period. Day 420–560: the second period of the run, when ammonium conversion and additional nitrate reduction increased simultaneously,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

to the conversion rates in the reactor, and can be used as a parameter for the control of the process performance. The sharp declines in the gas production rates (Fig. 1) were caused by regular interruptions in the flow of the feed.

The average amount of biomass leaving the reactor after the bed had fully grown was only  $10 \pm 5$  mg VSS/l<sub>waste water</sub>. This corresponds to a biomass yield of approximately 0.04 g  $COD_{biomass}/g$   $COD_{removed}$ . The redox balances (Table 3) show that the ratio between electron donor and electron acceptor consumed was 1.1, indicating that only 10% of the electron donor had been used for biomass production. This relatively low biomass production in sulfide-containing waste water has been previously described [10,17] and is three times lower than commonly observed for organic media [3].

Table 3

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

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

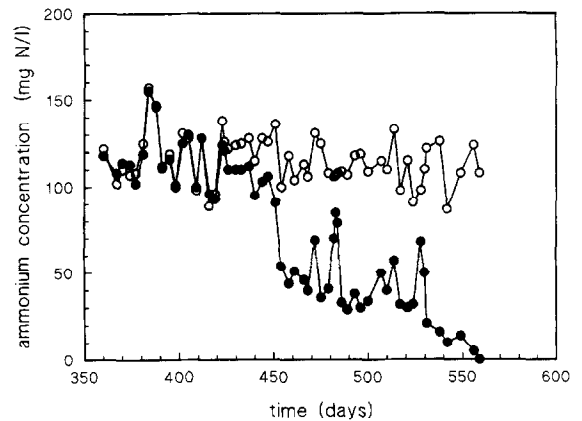


Fig. 2. Ammonium concentration in the influent (○) and the effluent (●) of the denitrifying fluidized bed reactor before and after the onset of anaerobic ammonium disappearance on day 420.

### 3.2. Occurrence of ammonium removal

During the first period (0–420 days) of the run, the ammonium concentration in the influent of the denitrifying fluidized bed reactor was the same as in the effluent. In the second period (420–560 days), it became apparent that the ammonium concentration in the effluent was steadily decreasing (Fig. 2). By the end of the experiment, almost all of the ammonium was converted. The most obvious explanation for this ammonium removal, the occurrence of aerobic nitrification resulting in nitrate or nitrite production, seemed unlikely because these concentrations in the effluent had also decreased (Table 2). In addition, the observed ammonium removal rate of  $0.4 \text{ kg } N \cdot m^{-3} \cdot d^{-1}$  would have required an oxygen supply corresponding to  $1.8 \text{ kg } O_2 \cdot m^{-3} \cdot d^{-1}$ . This was very unlikely in view of the precautions taken (see Materials and methods). Moreover, oxygen could not be detected in the gas samples. During the second

period, the gas production and nitrate consumption rates also increased, and the pH fell (Table 2). The  $\text{NH}_4^+$  deficit cannot simply be explained by incorporation in biomass, because biomass production remained stable throughout the experiment (Table 2). These factors, taken together, all indicate that the most likely explanation of the ammonium conversion is the reaction given in Table 1, equation 3: anaerobic ammonium oxidation to  $\text{N}_2$ .

The redox balances shown in Table 3 provided further support for the occurrence of anaerobic denitrification with ammonium as the electron donor. When a comparison was made for nitrate consumption before and after ammonium conversion began, it was clear that in the first period, there was good agreement between the calculated and measured amounts of nitrate utilization. Once the  $\text{NH}_4^+$  concentration began falling, the calculated and measured  $\text{NO}_3^-$  consumptions could only be reconciled if the stoichiometric amount of  $\text{NO}_3^-$  required to oxidize the  $\text{NH}_4^+$  was included.

### 3.3. Fed-batch experiments

In order to substantiate this hypothesis, and to confirm the correlation between  $\text{NH}_4^+$  conversion and increased  $\text{NO}_3^-$  utilization, batch experiments were carried out with biomass-covered sand originating from the fluidized bed reactor. As shown in Fig. 3A,  $\text{NH}_4^+$  removal coincided with  $\text{NO}_3^-$  removal, stopped when the  $\text{NO}_3^-$  became exhausted, and subsequently started again when more  $\text{NO}_3^-$  was supplied. Throughout the experiment,  $\text{NO}_2^-$  concentrations were below the detection limit (Fig. 3A). Gas production during this experiment also corresponded to the periods of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  conversion (Fig. 3C). In contrast, sulphate reduction began when the  $\text{NO}_3^-$  was exhausted, and stopped as soon as fresh  $\text{NO}_3^-$  was added (Fig. 3B), confirming that denitrifying bacteria were active and maintaining the redox at a level suitable for denitrification rather than  $\text{SO}_4^{2-}$  reduction [18,19]. The observed ammonium conversion capacity of  $2.7 \text{ mg NH}_4^+ - \text{N} \cdot \text{g}^{-1} \text{VSS} \cdot \text{day}^{-1}$  in this fed-batch experiment was approximately 15 times lower than the conversion rate found in the continuous-flow denitrifying reactor.

The amount of nitrate and ammonium consumed during the experiment was equivalent to  $2.55 \text{ l N}_2$ .

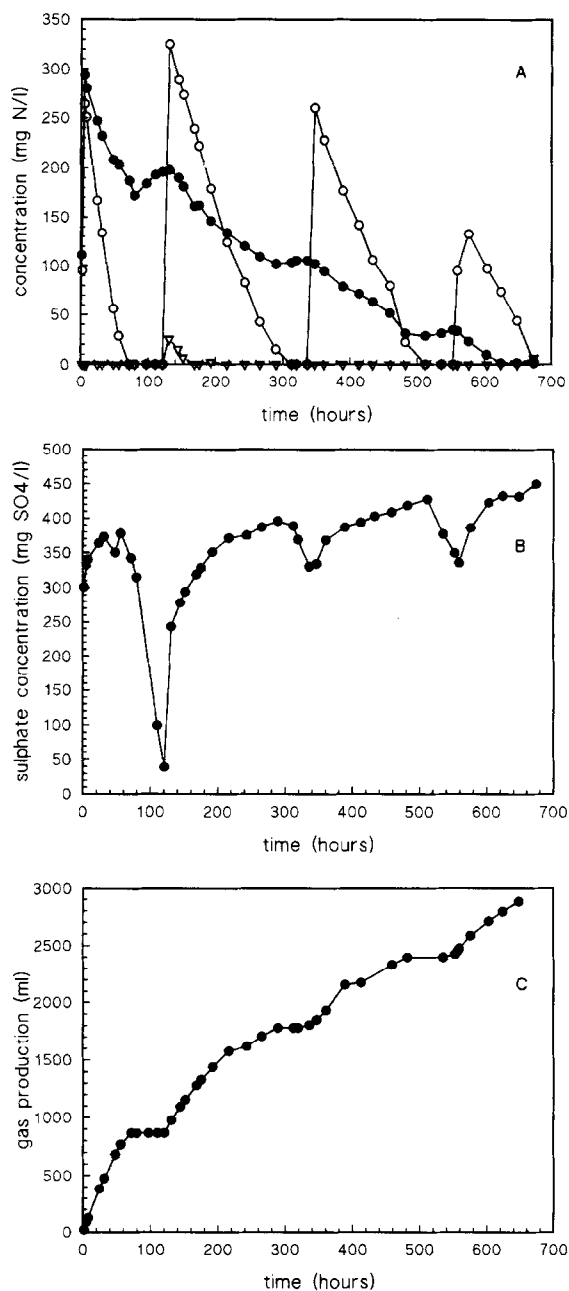


Fig. 3. Nitrate-dependent conversion of ammonium during a batch experiment with a biomass sample taken from the denitrifying fluidized bed reactor 140 days after the onset of anaerobic ammonium oxidation. (A) Simultaneous decrease of ammonium (●), nitrate (○) and nitrite (▽). (B) Changes in the sulphate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

Gas production measurements gave a similar value of 2.80 l N<sub>2</sub> (Fig. 3C), supporting the hypothesis that the ammonium and nitrate were being used to make N<sub>2</sub> (equation 3).

### 3.4. Concluding remarks

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

A precondition for the production of ATP and, consequently, for the reduction of CO<sub>2</sub> to biomass, is a negative free energy change. As shown in the equations 3, 4 and 5 in Table 1, anaerobic ammonium oxidation is almost as energetically favourable as the aerobic reaction. Previous reports of N<sub>2</sub> production from ammonium in water, sludge and sediments e.g. [20,21] were inconclusive, for various reasons. To our knowledge this paper presents the first description of the occurrence of anaerobic ammonium oxidation. Hence, it seems that at least one of the 'missing links in nature' [14] has now been discovered.

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

### Acknowledgements

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

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