

Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor

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An autotrophic, synthetic medium for the enrichment of anaerobic ammonium-oxidizing (Anammox) micro-organisms was developed. This medium contained ammonium and nitrite, as the only electron donor and electron acceptor, respectively, while carbonate was the only carbon source provided. Preliminary studies showed that the presence of nitrite and the absence of organic electron donors were essential for Anammox activity. The conversion rate of the enrichment culture in a fluidized bed reactor was 3 kg NH₄⁺ m⁻³ d⁻¹ when fed with 30 mM NH₄⁺. This is equivalent to a specific anaerobic ammonium oxidation rate of 1000–1100 nmol NH₄⁺ h⁻¹ (mg volatile solids)⁻¹. The maximum specific oxidation rate obtained was 1500 nmol NH₄⁺ h⁻¹ (mg volatile solids)⁻¹. Per mol NH₄⁺ oxidized, 0.041 mol CO₂ were incorporated, resulting in a estimated growth rate of 0.001 h⁻¹. The main product of the Anammox reaction is N₂, but about 10% of the N-feed is converted to NO₃⁻. The overall nitrogen balance gave a ratio of NH₄⁺-conversion to NO₂⁻-conversion and NO₃⁻-production of 1:1.31 ± 0.06:0.22 ± 0.02. During the conversion of NH₄⁺ with NO₂⁻, no other intermediates or end-products such as hydroxylamine, NO and N₂O could be detected. Acetylene, phosphate and oxygen were shown to be strong inhibitors of the Anammox activity. The dominant type of micro-organism in the enrichment culture was an irregularly shaped cell with an unusual morphology. During the enrichment for Anammox micro-organisms on synthetic medium, an increase in ether lipids was observed. The colour of the biomass changed from brownish to red, which was accompanied by an increase in the cytochrome content. Cytochrome spectra showed a peak at 470 nm gradually increasing in intensity during enrichment.

Keywords: ammonium, nitrite, dinitrogen gas, nitrification, denitrification

INTRODUCTION

Ammonium is a common pollutant which is normally eliminated from waste water by a combination of two processes, nitrification and denitrification. Nitrification is the biological formation of nitrate or nitrite from compounds containing reduced nitrogen with O₂ as the terminal electron acceptor. Two separate and distinct

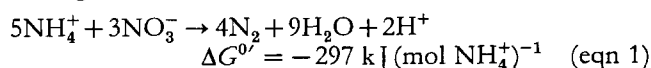
steps are involved in nitrification. First, the oxidation of NH₄⁺ to NO₂⁻ is carried out by ammonium-oxidizing bacteria such as *Nitrosomonas* or *Nitrosospira* (Koops & Möller, 1992). This is followed by the oxidation of NO₂⁻ to NO₃⁻ by nitrite-oxidizing bacteria such as *Nitrobacter*-like organisms (Abeliovich, 1992). During the subsequent denitrification step, NO₃⁻ or NO₂⁻ are reduced to N₂ by denitrifying bacteria. Denitrification is carried out by a wide spectrum of respiratory bacteria representing many genera and physiological types (Kuenen & Robertson, 1994; Zumft, 1992).

Recently, a novel anaerobic process in which ammonium was used as electron donor for denitrification was discovered in a laboratory-scale fluidized bed reactor

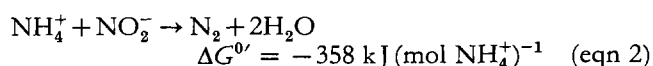
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Abbreviations: Anammox, ANaerobic AMMonium OXidation/-OXidizing; FBR, fluidized bed reactor; RuBPCase, ribulose-1,5-bisphosphate carboxylase; VS, volatile solids.

(FBR) (Mulder *et al.*, 1995). It was demonstrated that in this ANaerobic AMMonium OXidation/-OXidizing (Anammox) process, nitrate was used as an electron acceptor. Redox balance calculations showed the following stoichiometry:



During further examination of this new process, which is catalysed by an as yet unidentified mixed microbial population, indications were obtained that nitrite could also serve as a suitable electron acceptor for the Anammox process (van de Graaf *et al.*, 1995).



To obtain a more fundamental understanding of this new process, we developed a synthetic inorganic mineral medium for the enrichment of the micro-organisms capable of Anammox in an FBR. This paper reports on the characteristics and properties of this enrichment culture.

METHODS

Origin of biomass. Sludge from a 27 l denitrifying FBR in which the Anammox process occurred was used as the source for biomass (Mulder *et al.*, 1995). When used in batch experiments, the sludge was homogenized by passing it several times through a 60 ml syringe. For continuous experiments, a small denitrifying FBR (2.5 l) was directly fed with the effluent from a methanogenic reactor. Details of the system are described by Mulder *et al.* (1995).

Mineral medium. For the start-up of the small FBR, an anaerobic synthetic medium was used. This medium contained (per l demineralized water): $(\text{NH}_4)_2\text{SO}_4$, 330 mg (5 mM); NaNO_2 , 345 mg (5 mM); KHCO_3 , 500 mg; KH_2PO_4 , 27.2 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 300 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 180 mg, and 1 ml trace element solutions I and II. Trace element solution I contained (per l demineralized water): EDTA, 5 g; FeSO_4 , 5 g; and trace element solution II contained (per l demineralized water): EDTA, 15 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.43 g; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.24 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.99 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25 g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.22 g; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.19 g; $\text{NaSeO}_4 \cdot 10\text{H}_2\text{O}$, 0.21 g; H_3BO_3 , 0.014 g. The mineral medium was autoclaved at 120 °C. Solutions of trace elements, CaCl_2 and MgSO_4 were sterilized separately at 120 °C and added aseptically to the autoclaved medium. After cooling, the medium was flushed with argon for at least 30 min to achieve anaerobic conditions. After the start-up, ammonium and nitrite concentrations were increased by steps of 5 mM.

Operation and continuous experiments with the FBR run with waste water. The glass FBR of 2.5 l was operated at 36 °C and pH 7. Batch experiments with the reactor had confirmed that these were the preferred temperature and pH. The pH was adjusted with 0.5 M H_2SO_4 or 0.5 M NaOH. Anoxic liquid from the top of the reactor was recirculated to boost the flow to approximately 47 l h⁻¹ in order to keep the bed fluidized at a superficial liquid velocity of 24 m h⁻¹. The hydraulic retention time was 4.2 h. The influent (see below) of the reactor was supplied at a rate of 600 ml h⁻¹. A nitrate solution (60 g NaNO_3 l⁻¹) was supplied at a rate of about 13 ml h⁻¹. Sand particles (diameter 0.3–0.6 mm) were the carrier material of the fluidized bed on which bacteria grew as a biolayer. The reactor

was inoculated with a large amount of sand covered with denitrifying and Anammox biofilms (400 ml), originating from the 27 l installation (Mulder *et al.*, 1995). This resulted in fast start-up of the experiments.

Samples of the influent and effluent of the reactor were taken once or twice a day and analysed for ammonium, nitrate, nitrite, sulfide, sulfate and sometimes for dissolved organic carbon. Gas production was monitored continuously. All tubing and connectors were of butyl rubber, norprene or polyvinylchloride to limit oxygen diffusion. For the same reason, the settler at the top of the reactor was flushed with argon.

Pulse experiments were conducted to measure the effect of different electron donors, electron acceptors and other medium components on the Anammox activity. A component was supplied to the reactor for 1 d and the effect was measured by following the effluent ammonium concentration. At least 1 week was allowed for the biomass to regain its original activity. During these experiments, the FBR was run continuously, or on a few occasions, in an intermittent feeding mode. For these experiments, ammonium removal was controlled at 50 % of the influent concentration by removal or addition of biomass, allowing the determination of positive or negative effects.

Operation and continuous experiments with the FBR run with synthetic medium. After the pulse experiments were finished, the influent of the FBR was changed to synthetic medium. The temperature was decreased to 30 °C. For practical reasons, concentrated synthetic medium was supplied at a rate of 200 ml h⁻¹ and diluted with tap water at a rate of 400 ml h⁻¹. Both the medium and the tap water were continuously flushed with argon to maintain anaerobic conditions.

The experiments with synthetic medium were started in batch mode using the original waste water, to ensure that the sludge was actively oxidizing ammonium anaerobically. Continuous feeding of the synthetic medium (5 mM NH_4^+ , 5 mM NO_2^-) to the reactor was started after the ammonium concentration reached zero. The influent concentration was raised by steps of 5 mM over a period of 3 months until 30 mM was reached.

Anaerobic batch culture experiments with waste water or synthetic medium. Anaerobic batch experiments with waste water were conducted in 500 ml serum bottles under static anaerobic incubation in the dark at 37 °C, as described previously by van de Graaf *et al.* (1995). For every type of addition, an independent batch culture was used. Separately, a control batch with ammonium and nitrate was monitored over the whole course of the experiment.

Anaerobic batch experiments with synthetic medium were conducted in 30 ml serum bottles with static incubation in the dark at 30 °C. Each bottle contained 24 ml mineral medium (pH 7) and 1 ml biomass suspension obtained from the enrichment culture grown on synthetic medium.

Ribulose-1,5-bisphosphate carboxylase (RuBPCase) assay. Sludge was harvested from the FBR, separated from the sand particles, and, after centrifuging (10000 g, 10 min), washed with a buffer containing 100 mM Tris/HCl, 20 mM MgCl_2 , 2.0 mM NaHCO_3 (pH 8.2). For preparation of cell-free extracts, the cells were resuspended in the same buffer with 5 mM DTT to a biomass concentration of approximately 30 mg dry weight ml⁻¹ and were disrupted by sonication at 4 °C in an MSE 150 W sonifier (eight bursts of 30 s with intermittent cooling). Intact cells and debris were removed by centrifuging (20000 g, 20 min). RuBPCase was assayed as described by Beudeker *et al.* (1980).

¹⁴CO₂-fixation assay. Sludge was harvested from the FBR. After settling, the sludge was rinsed four times with anaerobic

tap water. The same volume of medium without substrate was added to the biomass. The cells were separated from the sand particles by passing it several times through a 60 ml syringe. Sludge suspension (1 ml) and 9 ml medium containing 2 mM KHCO_3 (pH 7) was incubated in 25 ml flasks with butyl rubber septa. After flushing the flasks with argon for 10 min, and pre-incubation at 30 °C, 100 μl ^{14}C -labelled bicarbonate solution (stock 50 mM $\text{NaH}^{14}\text{CO}_3$, 31.45 GBq mol^{-1}) was introduced. The flasks were incubated in the dark at 30 °C. At predetermined time intervals, 0.5 ml samples (in duplicate) were taken to measure $^{14}\text{CO}_2$ incorporation. At the same time, samples for ammonium and nitrite determinations were taken from separately incubated flasks without labelled CO_2 . The samples for $^{14}\text{CO}_2$ measurements were washed three times with 25 mM KH_2PO_4 buffer (pH 8) and transferred to scintillation bottles for counting.

Determination of aerobic nitrifiers. Estimates of ammonium and nitrite-oxidizing populations were made by the most probable number (MPN) method according to Alexander (1982). Six-week incubations were done at 30 and 26 °C in the dark for ammonium and nitrite oxidizers, respectively. Petri dishes (Sterilin) of 100 × 100 mm with 25 square holes filled with 5 ml medium (Schmidt & Belser, 1982) were used to incubate the MPN dilutions. The result of an MPN test was considered to be positive when either nitrite formation could be detected, or when nitrite had disappeared and nitrate had been formed. Every incubation series was done in duplicate and several times a pure culture of *Nitrosomonas europaea* LMD 86.25 (culture collection of the Department of Microbiology and Enzymology, Delft, The Netherlands) and *Nitrobacter winogradskyi* (kindly provided by Drs W. de Boer & H. J. Laanbroek, Heteren, The Netherlands) were used as controls.

TLC. Cells were harvested from the FBR and, after removal of the sand particles, freeze-dried and stored before use. TLC analysis of whole-cell methanolysates was assayed as described by Ross *et al.* (1981).

Electron microscopy. Cells were fixed in glutaraldehyde, embedded in Spurr and stained with osmium tetroxide and ruthenium red. Ultra-thin sections were studied in a Philips EM201.

Cytochrome spectra. The spectra were measured with an Aminco DW-2a dual-wavelength spectrophotometer (American Instruments) which was equipped with computer data acquisition (kindly made available by Dr A. H. Stouthamer of the Free University of Amsterdam). The cell suspensions in 50 mM phosphate buffer were measured at room temperature and at 77 K. The protein content of the suspensions were measured and the spectra were all normalized at 1 mg protein ml^{-1} . The spectrophotometer was equipped with a magnetic stirrer and the cuvettes were closed by a lid fitted to allow continuous flushing of gas and making of additions. The cytochromes were reduced with 60 μl dithionite solution (0.1 g ml^{-1}). For CO spectra, this gas was allowed to react for 15 min with the cytochromes. To remove the CO ligands, the frozen suspension was exposed to an intense light source for five intervals of 10 s (Intralux 5000, 185 W). All spectra were measured at a reference wavelength of 540 nm. For further handling of the spectral data, the methods and software described by van Wielink *et al.* (1982) were used.

Analytical procedures. Nitrate, nitrite, ammonium, sulfide and sulfate were determined as previously described (van de Graaf *et al.*, 1995). Hydroxylamine was determined colorimetrically (Frear & Burrell, 1955). Nitrous oxide formation was checked using a GC (Hewlett-Packard model 428) with catharometric detection. 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 700 °C for 1 h. The dry weight minus the ashed weight is hereafter termed volatile solids (VSs). Protein for the cytochrome spectra was determined according to Herbert *et al.* (1971).

Chemicals. Ribulose 1,5-bisphosphate was obtained from Sigma, and ^{14}C -NaHCO₃ (2.11 TBq mol^{-1}) was from Amersham International. All other chemicals used were reagent grade and obtained from commercial sources.

RESULTS

The Anammox process using waste water

Batch experiments. The first attempts to run Anammox on a simple synthetic denitrification (mineral) medium with sulfide and acetate as electron donors failed. Analysis of the influent and effluent of the original denitrification reactor revealed no unusual or unexpected (changes of) inorganic components. To obtain more information about specific requirements for Anammox, batch experiments were carried out with waste water supplemented with various chemicals (Table 1). The addition of 5 or 50 mM

Table 1. Effect of additions of various compounds on the Anammox activity in batch experiments with waste water or in continuous experiments with an FBR

Compound	Concentration	Effect on Anammox activity
Batch expts		
Acetate	1 or 5 mM	Increase, nitrite formation
Propionate	1 mM	No effect
Glucose	1 mM	Increase, nitrite formation
Fructose	1 mM	Increase, nitrite formation
Lactate	1 mM	No effect
Casamino acids	50 mg l^{-1}	No effect
Sulfide	1 or 5 mM	Increase
Sulfite	1 mM	Increase
Thiosulfate	1 mM	Increase, nitrite formation
KCl	50 mM	No effect
KHCO_3	20 or 40 mM	No effect
EDTA	100 mg l^{-1}	No effect
KH_2PO_4	5 or 50 mM	Loss of activity
KH_2PO_4	1 mM	No effect
Continuous expts		
Acetate	2 mM	−28 %
Glucose	1 mM	−12 %
Pyruvate	1 mM	−20 %
Formate	5 mM	−10 %
Cysteine	0.8 mM	−11 %
Sulfide	2 mM	+20 %*
Sulfide	2 mM	+60 %†
Thiosulfate	2 mM	+47 %
Hydroxylamine	2 mM	−28 %
N_2O	0.7 mM	No effect
Nitrite	2, 4 or 6 mM	+11, 24, or 54 %

* Nitrite formation during a pulse feed.

† Addition during intermittent feeding of the reactor.

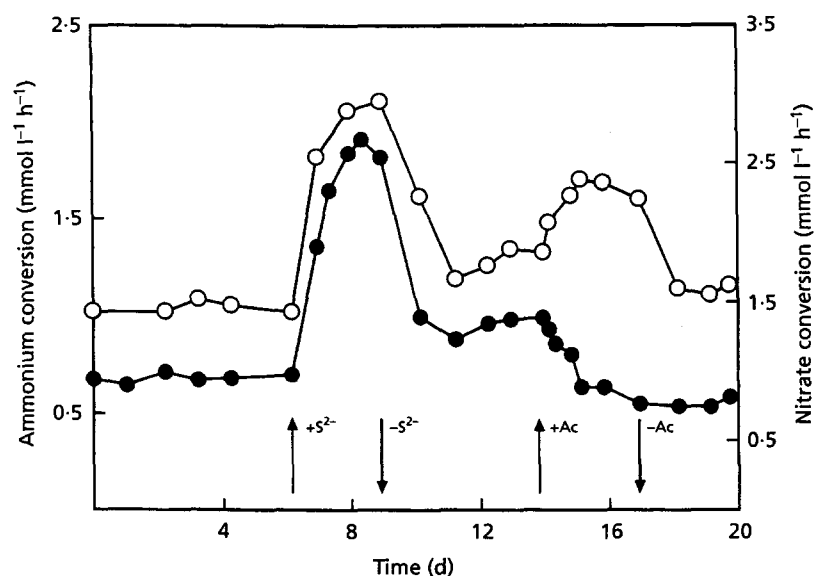


Fig. 1. Effect of sulfide and acetate pulses on the Anammox activity in an FBR intermittently fed with waste water. S^{2-} , pulse of 2 mM sulfide; Ac, pulse of 2 mM acetate. ●, NH_4^+ ; ○, NO_3^- .

phosphate, commonly used as a pH buffer in batch experiments, caused complete loss of the ability to oxidize ammonium anaerobically, but 1 mM phosphate was tolerated. Addition of 50 mM KCl or 40 mM $KHCO_3$ had no effect. Phosphate was thus the cause of inhibition. Electron donors, both organic and inorganic increased the ammonium oxidation rate. However, in almost all cases, this positive effect was accompanied by transient nitrite formation. At the end of a typical batch experiment, nitrite became detectable at the moment that all ammonium was used. When excess nitrate (5 mM) was present, the addition of nitrite accelerated the ammonium oxidation rate.

Continuous experiments. In batch experiments carried out with a large amount of biomass, the precise influence of added components on the growth of anaerobic ammonium oxidizers could not be measured. These experiments only provided insight into the inhibitory or stimulatory potential of certain components. Continuous runs and a few intermittent feeding experiments were carried out to investigate the effect of adding electron donors and electron acceptors on the Anammox activity. Organic electron donors (Table 1) decreased Anammox activity in all cases, while inorganic sulfur-based electron donors such as thiosulfate and sulfide had a positive effect. Fig. 1 shows the changes in the ammonium and nitrate conversion rates in the reactor during a sulfide and acetate pulse. Adding trace elements had no effect, indicating that no trace elements were missing in the original waste water. The provision of nitrite as an extra electron acceptor increased the ammonium conversion. This indicated that nitrite was necessary for Anammox activity, and that the stimulation of the additional sulfur sources might be due to nitrite formation from nitrate. During intermittent medium supply, the effect of increasing the inlet sulfide concentration was even more pronounced. This could imply that pulse-like sulfide additions caused more nitrite formation per molecule supplied. By in-

creasing the nitrite concentration to 6 mM, an almost complete conversion of ammonium (6 mM) could be accomplished. Light had a negative effect (30–50%) on the ammonium removal rate. During all following experiments the equipment was always covered with black plastic and paper to eliminate this effect.

The Anammox process on synthetic medium

Start-up and operation with 'autotrophic' synthetic medium. It was possible to start-up and maintain an Anammox fluidized bed culture using an 'autotrophic' mineral medium with ammonium, nitrite and carbonate (see Methods). Inoculation with the original biofilm still attached to the sand particles facilitated the start-up considerably. Characteristic start-up results are shown in Fig. 2. The feed of ammonium was increased stepwise up to 25 mM, with a concomitant stepwise increase in nitrite. The highest feed level tested was 35 mM nitrite and 30 mM ammonium. Dinitrogen gas production increased with increasing feed load. A conversion of $3.1 \text{ kg } NH_4^+ \text{ m}^{-3} \text{ d}^{-1}$ was achieved with a feed of 30 mM NH_4^+ . A total of 15 runs have been carried out with synthetic medium, the longest lasting over 7 months. During the runs the colour of the culture changed slowly from brown to red. Two to three months were required to obtain completely red biolayers and formation of new biofilm on the sand.

General observations. When cysteine (to reduce the redox potential) or pyruvate [to supply electron donor (Abeliovich & Vonshak, 1992)] were added to the medium, a steady reduction of the Anammox activity (20% activity loss per 6 d; feed 5 mM NH_4^+) was observed. If a large excess of nitrite (8 mM) and ammonium (8 mM) was suddenly supplied, all activity was lost. However, the Anammox activity could be recovered by flushing the column with anoxic tap water until nitrite concentrations decreased below 10 mg l^{-1} , resulting in a steep

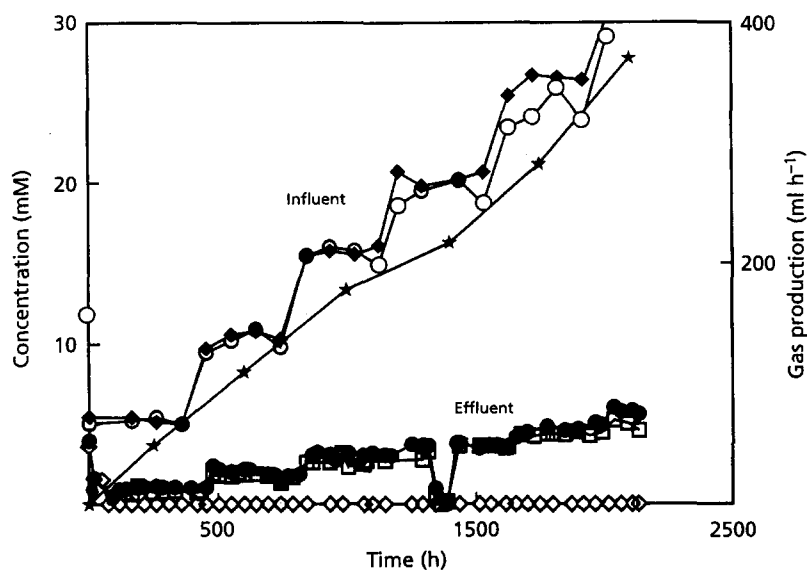


Fig. 2. Start-up and continuous operation of the FBR on synthetic medium, and gas production during increase of the ammonium and nitrite load. Influent: \circ , NH_4^+ ; \diamond , NO_2^- . Effluent: \bullet , NH_4^+ ; \square , NO_3^- ; \star , gas production.

(-350 mV) decline in the redox potential. When the feed was restarted (sometimes at a lower feed load), gas bubbles appeared within 1 h and the nitrite and ammonium levels fell. During the first runs a calcium concentration of 6 mg l^{-1} was used. This concentration was increased to 50 mg l^{-1} since it was shown by Austerman-Haun *et al.* (1993) to improve biofilm formation. Visible growth of red biomass on the sand particles was then observed.

Nitrogen balance. Instead of the expected 1:1 removal of ammonium and nitrite predicted from the equation of Broda (1977), a 1:3:1 ratio for the $\text{NO}_2^-:\text{NH}_4^+:\text{NO}_3^-$ removal was found. This appeared to be due to the formation of nitrate. During the first experiments on synthetic medium, 2 mM nitrate had been added to the feed in order to keep the redox potential at approximately 150 mV. This was also the case when waste water with excess nitrate was used. Once it was discovered that nitrate was formed during the Anammox process, it was omitted from the feed. Nitrate production accounted for 10% of the total nitrogen feed. From four independent runs, 31 nitrogen balances were made. This gave a ratio of $\text{NH}_4^+:\text{NO}_2^-:\text{NO}_3^-$ of $1:1:31 \pm 0.06:0.22 \pm 0.02$. Hydroxylamine, an intermediate of aerobic ammonium oxidation, was not detected in the effluent, and N_2O was not detected in the gas produced. However, if the system was disturbed (e.g. by failure of pH regulation), N_2O was formed in small amounts (0.3% of total gas production).

Specific Anammox activity. The specific Anammox activity of the biomass in the FBR being fed with 25 mM ammonium and nitrite was $700\text{--}800 \text{ nmol NH}_4^+ \text{ h}^{-1} (\text{mg VS})^{-1}$. The feeding rate of this reactor could be doubled instantaneously without causing problems, implying that the maximum specific activity of the biomass in the reactor was $1300\text{--}1500 \text{ nmol NH}_4^+ \text{ h}^{-1} (\text{mg VS})^{-1}$. However, when fresh samples from this reactor were tested for their Anammox activity in batch culture, the activity was

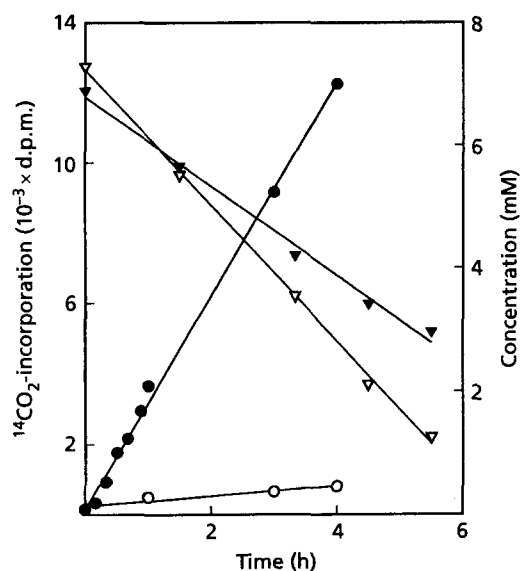


Fig. 3. Incorporation of $^{14}\text{CO}_2$ in a sample of an enrichment culture of Anammox micro-organisms during oxidation of ammonium with nitrite. \bullet , d.p.m. $^{14}\text{CO}_2$ incorporated when incubated with NH_4^+ and NO_2^- ; \circ , d.p.m. $^{14}\text{CO}_2$ incorporated in controls with neither NH_4^+ nor NO_2^- ; \blacktriangledown , NH_4^+ concentration and \triangledown , NO_2^- concentration in control incubations without labelled CO_2 .

only half this level [i.e. $300\text{--}400 \text{ nmol NH}_4^+ \text{ h}^{-1} (\text{mg VS})^{-1}$]. This was most probably due to inhibition by the higher nitrite or ammonium concentrations initially present in the batch experiments.

Autotrophic growth of anaerobic ammonium-oxidizers

CO_2 -fixation. CO_2 -fixation was dependent on Anammox activity as shown in Fig. 3. There was no incorporation of $^{14}\text{CO}_2$ in the control experiments without NH_4^+ or NO_2^- .

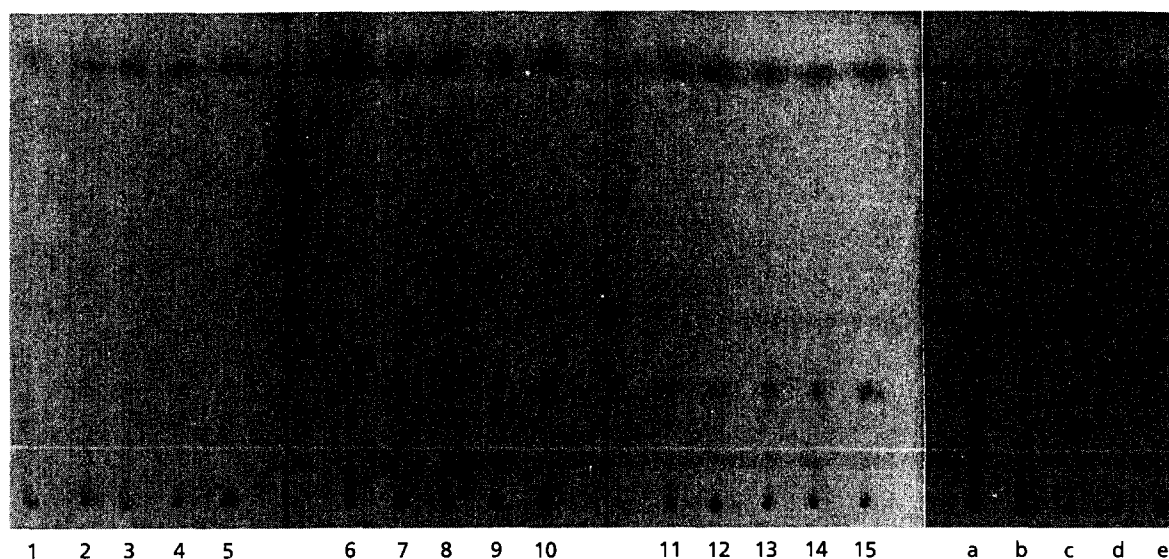


Fig. 4. Increase in the ether lipid content of whole-cell methanolysates during the enrichment for Anammox micro-organisms compared with other cultures. Time after start of enrichment: lane 1, 0 d; lane 2, 0 d; lane 3, 6 d; lane 4, 14 d; lane 5, 22 d; lane 6, 31 d; lane 7, 40 d; lane 8, 48 d; lane 9, 54 d; lane 10, 61 d; lane 11, 69 d; lane 12, 76 d; lane 13, 90 d; lane 14, 181 d; lane 15, 198 d. Lane a, methanogenic sludge; lane b, pilot plant Anammox sludge; lane c, Anammox enrichment culture; lane d, *Nitrosomonas europaea*; lane e, *Methanothrix soehngenii*.

The CO_2 incorporation rate was $12.5 \text{ nmol C h}^{-1} (\text{mg VS})^{-1}$ and the NH_4^+ conversion rate was $307 \text{ nmol NH}_4^+ \text{ h}^{-1} (\text{mg VS})^{-1}$. Hence, the biomass yield of Anammox was $0.041 \text{ mol C incorporated per mol NH}_4^+$ oxidized. Conversion of 20 mmol NH_4^+ will give a biomass formation of 20 mg dry weight (assuming a biomass carbon content of 50%).

Other biomass measurements. When the biomass in the FBR had reached the level of the overflow, a kind of pseudo-steady situation was obtained. The amount of biomass which left the reactor at this time was therefore a measure of the biomass production. During a period of 16 d, the dry weight of the settled and suspended material was estimated to be $18\text{--}34 \text{ mg VS (l feed)}^{-1}$. This is of the same order as the 20 mg calculated from the CO_2 fixation.

RuBPCase. Since HCO_3^- was the only carbon source provided, the Anammox micro-organisms are presumably autotrophs. Measurements to demonstrate an increase of the activity of RuBPCase, the key enzyme of the Calvin cycle, between the original sludge and the enrichment were done. Although the cell-free extract of the original sludge grown on waste water possessed a low RuBPCase-dependent CO_2 -fixation activity of $1.02 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, the cell-free extract of the enrichment had an activity of only $0.28 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. The activity of the enrichment was just above the detection limit (i.e. only twofold higher than the control). However, for the (extremely slow) growth of the Anammox sludge, CO_2 fixation at a rate of only $0.8 \text{ nmol C min}^{-1} (\text{mg protein})^{-1}$ would be required. Sulfide, one of the major electron donors for the original sludge (Mulder *et al.*, 1995), might explain the presence of some autotrophic metabolism in the original sample. Additional tests were

done to examine whether some components in the cell-free extract had an inhibitory effect on the enzyme activity. Mixing on a 1:1 basis with cell-free extract of *Nitrosomonas europaea* gave the expected dilution of activity from 12.1 to $6.15 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Effect of dioxygen

The amount of O_2 leaking through the tubing and connections of the fluidized bed system was measured with a Clark-type oxygen electrode in a separate reactor without biomass. The reactor was made anaerobic by flushing with dinitrogen gas through the bottom and an argon flush through the head space of the column for 30 min. Argon was continuously used in the reactor during operation, but was discontinued for the measurement of potential oxygen leakage. The initial rate of oxygen leakage was $20 \mu\text{mol O}_2 \text{ h}^{-1}$. When the argon flush was maintained during the measurements, oxygen leakage was not detected. Even in the worst-case situation, the amount of oxygen would only be enough to oxidize 0.2% of the total amount of 12 mM ammonium and 15 mM nitrite converted per h.

In a few batch experiments, oxygen was deliberately introduced by shaking cultures with an aerobic head space. This caused complete inhibition of the anaerobic ammonium conversion (Jetten *et al.*, 1995). In static cultures exposed to air (through a cotton plug), the anaerobic ammonium oxidation and nitrite reduction started after a delay, presumably after facultative anaerobic bacteria removed the oxygen dissolved in the medium and a O_2 -gradient had been established, thus providing an anaerobic environment at the bottom of the flasks (Kato *et al.*, 1993).

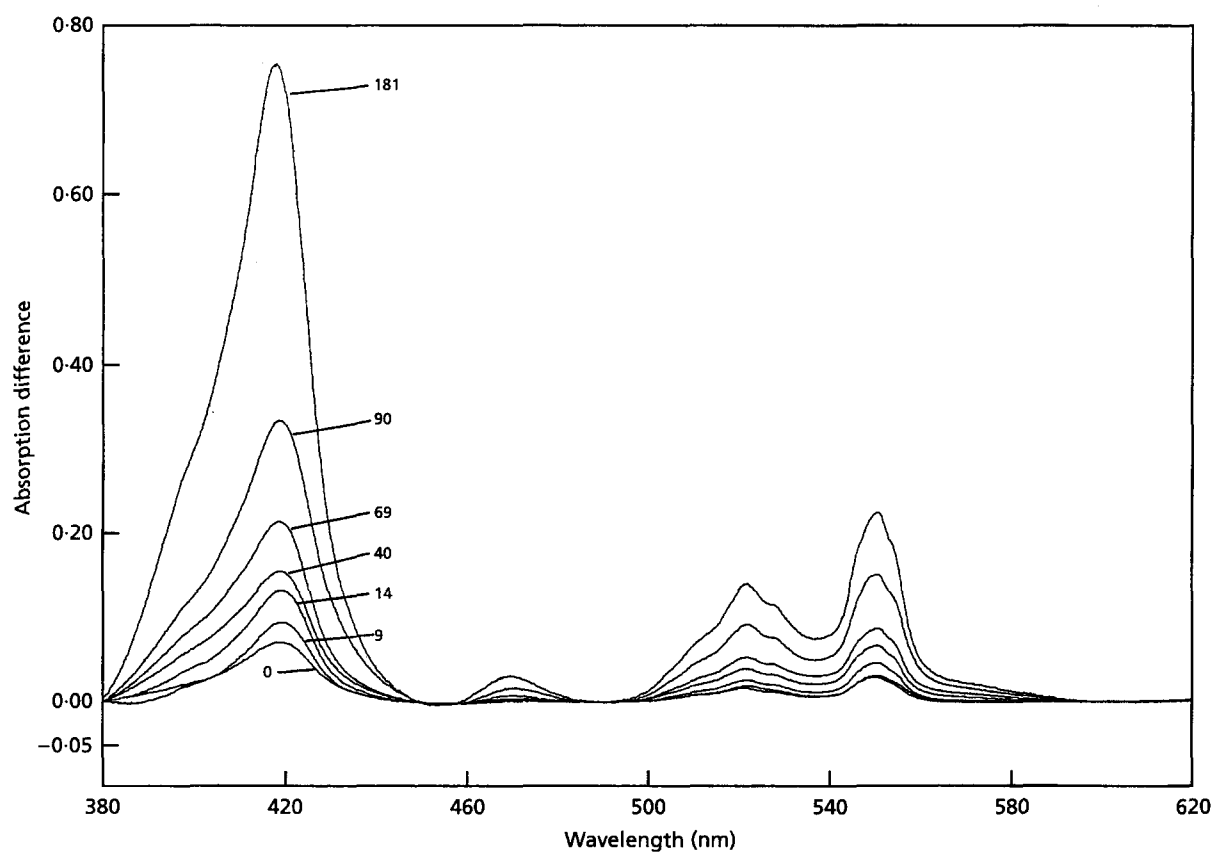


Fig. 5. Increase in the cytochrome content during enrichment for Anammox micro-organisms. Cytochrome spectra were measured at 77 K. The cells were reduced by dithionite in an argon atmosphere. The measured spectra were all normalized to 1 mg protein ml⁻¹. The numbers on the curves represent days after the start-up. Note typical peak at 470 nm.

Aerobic nitrifiers

The standard, aerobic MPN method showed that aerobic nitrifiers were present in the sludge at all times. During the enrichment for Anammox bacteria on synthetic medium, the number of nitrifiers present in the culture did not increase: ammonium oxidizers stayed at about $9 \pm 5 \times 10^3$ cells (mg VS)⁻¹ and nitrite oxidizers at $1.0 \pm 0.9 \times 10^3$ cells (mg VS)⁻¹.

Electron micrographs of the aerobic MPN cultures showed the characteristic membrane structures of *Nitrosomonas europaea*, and immunofluorescence microscopy with antibodies against *Nitrosomonas europaea* gave a positive reaction. Addition of penicillin G (35 mg l⁻¹) completely inhibited growth of the MPN cultures. Attempts to isolate the nitrifiers were unsuccessful, due to persistent contamination by heterotrophs.

Effect of inhibitors on Anammox activity

The specific inhibitors (hydrazine, acetone, N-serve, allylthiourea) for the first step of aerobic ammonium oxidation did not affect the Anammox activity. However, acetylene inhibited the Anammox process by 87 % compared with the control. Penicillin G, penicillin V and

specific inhibitors or stimulators for methanogens (bromoethane sulfonic acid) and sulfate-reducers (NaMoO₄/Na₂SO₄) had no effect. Chloramphenicol, which can have a direct inhibitory effect on denitrification enzymes, did not inhibit the initial rate of Anammox activity.

TLC of ester and ether lipids

The level of ether lipids increased during the enrichment for Anammox micro-organisms (Fig. 4). Control experiments with methanogenic sludge, *Nitrosomonas europaea*, and *Methanobrevibacter smithii* served as reference. The ester lipids typical for (eu)bacterial membranes remained present during the enrichment.

Cytochrome spectra

During the enrichment of anaerobic ammonium oxidizers on synthetic medium, the colour of the biomass changed from brownish to red. Spectra taken from whole cells showed a clear increase in typical haem spectra, in particular of the cytochrome *c* (540–554 nm; α -region, Fig. 5). Measurements at higher wavelengths (700–1100 nm) showed that the characteristic maxima of bacteriochlorophyll *a* and *b* (Stanier *et al.*, 1986) were

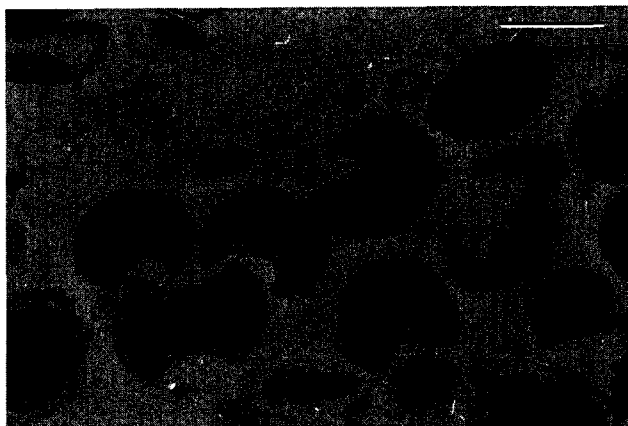


Fig. 6. Electron micrograph of resuspended Anammox biofilm grown on synthetic medium in an FBR. The dominant species in the enrichment culture. Bar, 1 μ m.

absent. The presence of carotenoids of the spheroidene group giving peaks between 450 and 550 nm was also not evident. The spectra of reduced cell suspensions at 77 K revealed the absence of cytochromes *a* (600–605 nm; location of terminal oxidase), *b* (554–564 nm) and *d*₁ (662–665 nm; *cd*₁-containing nitrite reductase). Spectra recorded in the presence of CO showed that the CO gas was binding to cytochromes *c*. No oxidases of *b*-type cytochromes were observed. Interestingly, during the increase in Anammox activity, a peak at 470 nm gradually increased in intensity. This spectroscopic feature reacted irreversibly with CO, as indicated by a shift from 470 to 450 nm.

Morphology

The dominant micro-organism of the enrichment culture was Gram-negative, with an unusual and irregular morphology as shown in Fig. 6. The cells were usually seen as single cells or in pairs, the latter possibly being a division state. When the biofilm was disrupted and the cells were allowed to settle for two or more days in a centrifuge tube, the clear appearance of the red colour was even more pronounced. The organisms from this sediment could easily be collected with a Pasteur pipette. Electron micrographs of this fraction showed an almost pure culture of the already dominant type of cells.

To gain an indication of the numbers of the apparently dominant type in the enrichment culture, a large series of electron micrographs were taken from different sections of imbedded samples. After 177 d enrichment, 64 % of all cells counted (total count = 11 433) were of the described dominant type (Fig. 6). This is a fourfold increase in numbers compared with the inoculum used for the start-up of the process (16 %; total count = 10 200). The dominant organisms appear to occupy a much larger volume than the other organisms present in the material. In some parts of the biolayers of the enrichment cultures, a few autofluorescent cells reminiscent of methanogens were observed.

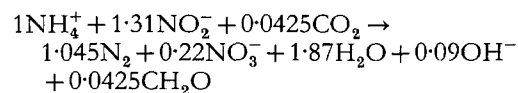
DISCUSSION

The continuous experiments with the systematic, step-wise addition of various supplements to the waste water showed that nitrite was the main electron acceptor used in Anammox, as was observed before by van de Graaf *et al.* (1995). It is likely that all other positive effects can be interpreted to the effect that they transiently stimulated (additional) nitrite production. The negative effect of prolonged addition of organic compounds, such as cysteine or pyruvate, may be due to the undesirable increase of a heterotrophic population overgrowing the organisms responsible for the Anammox process. Such a phenomenon is well known for overgrowth of aerobic nitrifiers by heterotrophs in the presence of organic materials (Tijhuis *et al.*, 1994).

The biomass responsible for the Anammox process can now be reproducibly grown autotrophically in an inorganic (synthetic) medium, with bicarbonate as the only carbon source. Considering the low growth rate (0.001 h⁻¹), it is not surprising that a biomass retention system, such as an FBR with biofilm attached to solid support, is required. Indeed, enrichment cultures in suspended continuous cultures, even at a dilution rate as low as 0.01 h⁻¹ failed, and complete conversion of ammonium only occurred in this system when sufficient biomass had accumulated on the fermenter walls.

It has been shown that nitrite, an intermediate in nitrate reduction, can accumulate in waste-water-treatment systems during changes in the reactor loading (Gommers *et al.*, 1988). It is therefore logical to suggest that the Anammox process with nitrate as electron acceptor could be the overall reaction of at least two types of bacteria, one of which reduces nitrate to nitrite using sulfide or degradable organic material as an electron donor. The second species would be the one that oxidizes ammonium with nitrite as the electron acceptor. Due to the direct supply of nitrite, the nitrite-providing nitrate-reducing organism would no longer be necessary in the Anammox enrichment culture using synthetic medium. The observed difference between the inhibitory effect of chloramphenicol on the original sludge (van de Graaf *et al.*, 1995) and the non-inhibitory effect of chloramphenicol on the enrichment culture could be explained by assuming that the nitrite-producing reaction was the one sensitive to chloramphenicol. Given the extremely low growth rate of the new enrichment, a short-term (24 h) effect of chloramphenicol (i.e. inhibition of protein synthesis) is unlikely.

The role of nitrate formation is not yet clear. The most likely explanation is that reducing power is required for CO₂-fixation. When the general formula 'CH₂O' for biomass is used, the production of 0.22 mol NO₃⁻ from NO₂⁻ would allow the fixation of 0.0425 CO₂ according to the reaction:



The anaerobic conversion of 20 mM ammonium (with 0.22 mol NO₃⁻ as a by-product) would thus allow the production of 26 mg dry weight. This is close to the

observed 20 mg biomass produced. This is of the same order of magnitude as aerobic ammonium-oxidizing bacteria such as *Nitrosomonas europaea*, which produce 25 mg dry weight from the same amount of ammonium (de Bruijn *et al.*, 1995). The possibility that nitrate would be formed by oxidation of NO_2^- to NO_3^- by *Nitrobacter* species is very unlikely in view of the very low quantity of O_2 penetrating into the reactor.

The original sludge had an anaerobic ammonium removal rate of $0.4 \text{ kg N m}^{-3} \text{ d}^{-1}$ (Mulder *et al.*, 1995). After enrichment with synthetic medium, the ammonium removal rate was $3 \text{ kg NH}_4^+ \text{ m}^{-3} \text{ d}^{-1}$ (equivalent to $2.4 \text{ kg N m}^{-3} \text{ d}^{-1}$). The total nitrogen removal of the Anammox reactor, including the conversion of nitrite, was $4.8 \text{ kg N m}^{-3} \text{ d}^{-1}$. In aerobic, nitrifying FBRs, ammonium conversion rates of $1.8\text{--}2.9 \text{ kg N m}^{-3} \text{ d}^{-1}$ have been obtained, depending on the pre-treatment system (Mulder *et al.*, 1986). Higher nitrification rates of $5 \text{ kg N m}^{-3} \text{ d}^{-1}$ were recently reached with a biofilm airlift suspension reactor (Tijhuis *et al.*, 1994). Compared with these aerobic systems, the Anammox process can contribute significantly to the nitrogen removal from waste water with low carbon content.

The consistent presence of the nitrifiers in the Anammox sludge suggests that they can survive long periods of anaerobiosis, as shown by Abeliovich (1987). Poth (1986) showed that a new isolate, identified as a *Nitrosomonas* species, was able to produce N_2 under anaerobic conditions. More recently, it has been shown that *Nitrosomonas europaea*, under strictly anaerobic conditions, utilized nitrite as electron acceptor, and pyruvate as energy source (Abeliovich & Vonshak, 1992), and pure and mixed cultures of *Nitrosomonas eutropha* were able to denitrify with hydrogen and ammonium as electron donor (Bock *et al.*, 1995). If the consistent but low numbers of nitrifiers [$10^3\text{--}10^4 \text{ (mg VS)}^{-1}$] present are compared with the value of $9 \times 10^9 \text{ cells (mg dry wt)}^{-1}$ (biomass of $35 \text{ mg dry wt l}^{-1}$ and $3 \times 10^8 \text{ cells ml}^{-1}$) of a pure culture of *Nitrosomonas europaea*, their contribution to the Anammox is highly unlikely. Even if the nitrification of the aerobic nitrifier was the same under anaerobic conditions as under aerobic conditions, their activity would be three orders of magnitude too low to explain the observed ammonium oxidation rate of $800 \text{ nmol NH}_4^+ \text{ h}^{-1} \text{ (mg VS)}^{-1}$.

The presence of ether lipids seems to be confined to the most ancient micro-organisms (e.g. the Archaea or the deepest phylogenetic branches within the Bacteria), thus suggesting that the ether linkage in the lipids could have appeared during the evolution of life before the ester types. Membrane lipids of the members of the order *Thermogotales* are based on ester and ether linkage (Gambacorta *et al.*, 1994). Also members of the genus *Aquifex* were found to possess diethers, but not archaeal diethers. Knowledge of the lipids can be used for taxonomic purposes, once catalogues of signature lipids have been developed (Gambacorta *et al.*, 1994).

The findings reported here show that the observed increase in Anammox capacity of the enrichment is

directly related to an increase in morphologically conspicuous micro-organisms, an increase in ether lipids and an increase in cytochromes. These properties might all be due to one and the same organism, although the possibility remains that two or more micro-organisms are responsible for the Anammox reaction. In the future, molecular techniques, such as 16S RNA analysis, will be used to unravel the consortium structure.

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