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Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam

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ABSTRACT

The first full-scale anammox reactor in the world was started in Rotterdam (NL). The reactor was scaled-up directly from laboratory-scale to full-scale and treats up to 750 kg-N/d. In the initial phase of the startup, anammox conversions could not be identified by traditional methods, but quantitative PCR proved to be a reliable indicator for growth of the anammox population, indicating an anammox doubling time of 10–12 days. The experience gained during this first startup in combination with the availability of seed sludge from this reactor, will lead to a faster startup of anammox reactors in the future. The anammox reactor type employed in Rotterdam was compared to other reactor types for the anammox process. Reactors with a high specific surface area like the granular sludge reactor employed in Rotterdam provide the highest volumetric loading rates. Mass transfer of nitrite into the biofilm is limiting the conversion of those reactor types that have a lower specific surface area. Now the first full-scale commercial anammox reactor is in operation, a consistent and descriptive nomenclature is suggested for reactors in which the anammox process is employed.

1. Introduction

The anammox process is the anoxic oxidation of ammonium with nitrite as electron acceptor (Van de Graaf et al., 1996). The process is performed by bacteria in the order of the Planctomycetales (Strous et al., 1999a) and is—with an estimated doubling time of 11 days (Strous et al., 1998)—characterized by an extremely slow growth rate. The autotrophic growth mode (in combination with the high maintenance requirement due to the slow growth rate) leads to an overall stoichiometry showing a relatively low biomass yield (Strous et al., 1998):

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

The substrate nitrite is toxic to anammox organisms at levels above 50–150 mg-N/L, and stops the process completely (Strous et al., 1999b).

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The anammox conversion was first observed in an autotrophic denitrification reactor with a sulfide-limiting loading for the denitrification process, in the presence of ammonium (Mulder et al., 1995). Since this first description, anammox-based processes for wastewater treatment have been developed (Jetten et al., 1997) and in recent years the first applications have occurred. In 2002, the first full-scale anammox reactor has been taken into operation at the sludge treatment plant Sluisjesdijk, Rotterdam, NL. The reactor was fed with partially nitritated sludge liquor from an adjusted nitritation process (Mulder et al., 2001; Van Dongen et al., 2001). The reactor is now fully operational (Abma et al., 2007).

In this paper we describe the course of the reactor startup followed by its stable operation. The operation is compared with anammox-related parameters previously reported in studies on laboratory scale. Furthermore, the various anammox-based processes now reaching full-scale are discussed and an evaluation of possible limiting factors in anammox reactor-configurations is discussed.

2. Experimental

2.1. The anammox reactor

The full-scale anammox reactor is a 70 m^3 reactor designed by Paques BV (Fig. 1). The reactor combines a high loading rate with efficient biomass retention, characteristics which the anammox process has in common with anaerobic wastewater treatment. The lower compartment (ca 40 m^3) is mixed by influent and downcomer flow as well as by gas recycled from the top of the reactor. On top of the lower compartment, gas is collected for the riser of the gas lift. The liquid moves from the lower compartment to the less mixed and thus stratified upper compartment, serving mainly for biomass retention and effluent polishing. The feed is introduced from the bottom of the reactor and is (during loads lower than ca 8 m^3/h or 150 kg-N/d) mixed with an additional recirculation flow from the effluent of the reactor to maintain adequate upflow velocity and shear stress to favor granule formation. The design load was 500 kg-N/d (7.1 kg-N/m^3/d) but the practical maximum loading is determined by the amount of nitrogen in the sludge digestate (on average ca 700 kg-N/d).

2.2. Description of the sludge treatment facility

The anammox reactor is located at the sludge treatment site Sluisjesdijk (Rotterdam, NL, 51°53′48.8″N, 4°27′13.8″E). This site treats the sludge of a municipal wastewater treatment plant Dokhaven (620,400 population equivalents (p.e.), defined as 136 g COD/per person). At the sludge treatment site, sludge is thickened and digested (residence time ca 30 days, temperature 32–33 °C). The centrifuged digestate (containing ca 1200 mg/l NH_4^+–N) can be heated or cooled and is fed to a SHARON-type nitritation reactor (Mulder et al., 2001). The temperature of the nitritation reactor is kept at 33 °C and the reactor can be operated with nitritation alone, or with nitritation–denitrification (see 2.3: startup strategy for details). Nitrite oxidation to nitrate is avoided by controlling aerobic residence time: by controlling the aeration period, the "aerated hydraulic retention time" is set long enough to enable growth of ammonium oxidizers, but short enough to lead to washout of (undesired) nitrite oxidizers (Hellinga et al., 1998; Mulder et al., 2001). The effluent of the nitritation reactor (largely) serves—after passing a tilted plate settler—as influent for the anammox reactor. The effluent of the anammox reactor is returned to the influent of the main WWTP.

2.3. Startup strategy

2.3.1. Scale up strategy

Based on 10 l lab scale experiments with the nitritation-anammox process on centrifuged sludge digestate from Sluisjesdijk (Van Dongen et al., 2001), the reactor was directly scaled up 70,000 fold to full-scale without building a pilot plant first. However, it was anticipated that some of the teething problems associated with new technologies—which normally would be identified and solved on pilot scale—would only show up during the startup of this first full-scale installation. To detect these expected problems, and to follow the startup well, several extra sample points and a flexible measurement loop were installed in addition to an online monitoring system.

2.3.2. Inoculation

The reactor was initially inoculated with nitrifying sludge from the Rotterdam-Dokhaven wastewater treatment plant. The inoculum had a sludge age of 7 days. After the startup, from day 622 to 1033, on 29 occasions with a total amount of 9.6 m^3, settled biomass from an anammox enrichment reactor was added in portions between 24 and 500 l. The enrichment reactor was a 5 m^3 well-mixed reactor connected to a settler located in Balk (NL), running at 35 °C with a conversion of 5 kg-N/m^3/d. The enrichment reactor had a hydraulic retention time of 0.7 days and was fed with synthetic medium containing—besides nutrients similar the medium of Van de Graaf et al. (1996)—1.8 kg-N/m^3 NH_4Cl and 1.6 kg-N/m^3 $NaNO_2$.

2.3.3. Control of nitrite concentration and loading

The concentration of nitrite during the startup is of crucial importance for growth: a too low amount will result in substrate limitation and thus slower growth, while concentrations above 50–150 mg-N/l can already lead to inhibition (Strous et al., 1999b; Egli et al., 2001; Dapena-Mora et al., 2007). These inhibition values are especially low compared with the nitrite concentration in a nitritation reactor running on sludge digestate (ca 600 mg-N/l).

The startup involved two phases. During the first 900 days, the startup regime was characterized by a relatively high influent flow rate (on average 3.6 m^3/h, HRT = 19.4 h) with a low concentration of nitrite (on average 120 mg-N/l). The low nitrite level was achieved by operating the nitritation reactor as a nitritation–denitrification reactor by alternating periods of aeration (for nitritation) with non-aerated periods with methanol dosing (for denitrification). This mode of operation was similar to that of the nitritation reactor before the anammox reactor was installed. During the startup of the anammox reactor, the aim was to produce an effluent

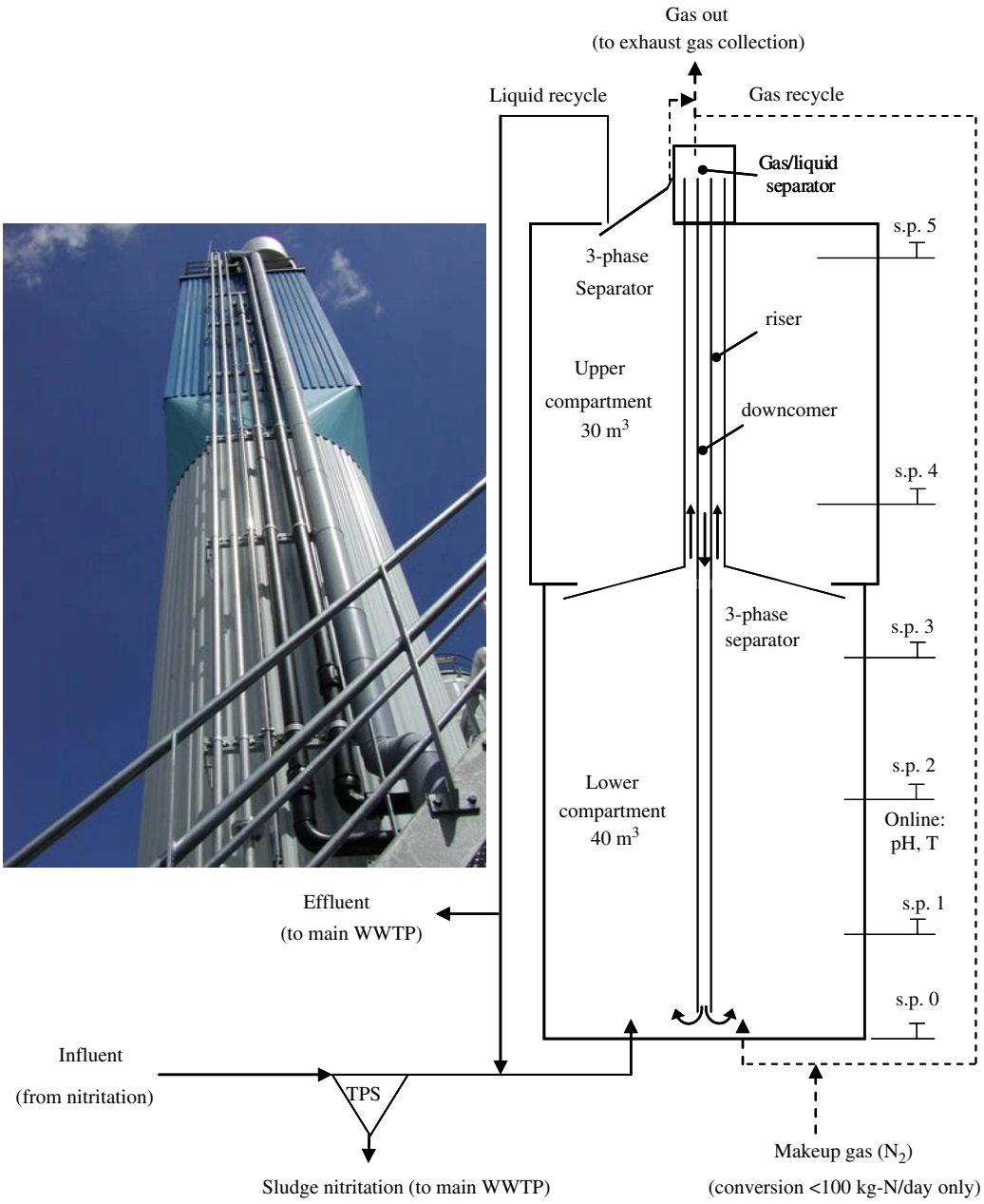

Fig. 1 – The full (70 m^3) scale anammox reactor in Rotterdam designed to treat 500 kg-N/d. The photograph shows the upper compartment (dark grey), as well as the lower compartment (light grey). The schematic picture shows the internal gaslift, gas recycle, six sample points (s.p.), as well as the tilted plate settler (TPS) *before* the anammox reactor.

containing nitrite at non-toxic levels. An additional economical advantage of this mode of operation was that the nitrogen removal of the sludge treatment as a whole remained high during this phase in the startup of the anammox reactor.

In the second part of the startup, after about 900 days, methanol dosing to the nitritation reactor was completely stopped and the reactor was running as a nitritation reactor with nitrite effluent concentrations close to 600 mg-N/l. In this phase, the loading rate was carefully balanced with available conversion capacity in the anammox reactor by varying the influent flow rate. The nitrite:ammonium ratio of circa 1:1—which is required for the anammox process—was obtained automatically because the nitrification is limited by the amount of alkalinity. Therefore, the acidifying effect of nitrification could be balanced for circa 50% by the carbonate present in the sludge digestate (Van Dongen et al., 2001).

2.4. *Ammonium, nitrite and nitrate measurement*

Daily measurements of ammonium, nitrite and nitrate served as basis for calculation of nitrogen load and conversion rate. Influent concentrations in the anammox reactor were based on the daily averaged effluent concentration of the nitritation

reactor. An automated sampling device was employed for daily flow-rate proportional sampling. The sample was stored at 4 °C. For anammox effluent samples, a daily grab-sample from the effluent of the anammox reactor was taken and analyzed immediately. Ammonium (47–130 mg-N/l), nitrite (0.6–6 mg-N/l) and nitrate (0.23–13.5 mg-N/l) were detected using commercial test kits (brand: Dr. Lange test kits, Hach Lange GmbH, Düsseldorf, DE, kits LCK304, LCK 341 and LCK339, respectively). The analysis was performed on a designated spectrophotometer (LASA 20).

2.5. *Quantitative polymerase chain reaction (Q-PCR)*

Q-PCR of partial 16S rDNA genes was performed on samples taken every 2–5 days during the first 1000 days of the startup. Samples were taken from sample point two of the reactor (Fig. 1).

2.5.1. *DNA extraction*

DNA extraction was performed as described by Zhou et al. (1996). However, after initial centrifugation and before the final centrifugation a bead beating step was introduced using 1 ml of Zirconia/Silica 100 μm beads and 1 ml of extraction buffer (pH 8.0, containing 100 mM Tris-HCl, 100 mM EDTA, 100 mM phosphate, 1.5 M NaCl).

2.5.2. *Q-PCR*

Extracted DNA was diluted 10 times with Tris-EDTA buffer. 1 μl of the diluted DNA extract was mixed with 19 μL of hybridization essay (LightCycler DNA master hybridization probe, from Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, CH) for Q-PCR. Q-PCR was performed using a LightCycler 2.0 (Roche Diagnostics). Pla46F (Neef et al., 1998) was used as forward primer. A reverse primer (AMX667R) was designed to amplify part of 16S rDNA genes of the anammox cluster containing 621 base pairs. Three types of hybridization probes were designed and mixed in a 1:2:1 molar ratio: (i) AMX361 (hybridizing with all known anammox bacteria), (ii) AMX381 (hybridizing with all known anammox bacteria) and (iii) AMX382 (hybridizing with Kuenenia and Brocadia). Primer and probe details and sequences can be found in Table 1. The Q-PCR method was validated and calibrated by using serial dilution of a lab-scale anammox enrichment from a toilet-water treatment. Detection in the enrichment was linear with the concentration in the range from 10^1 to 10^7 cells/ml. In view of the large amount of non-anammox DNA in the sample 10^4 cells/ml was taken as the detection limit.

The program for the Q-PCR assay consisted of 2 min denaturation at 95 °C, followed by 50 cycles of denaturation (heating to 95 °C) immediately followed by annealing combined with real time-detection (15 s at 55 °C) and elongation (27 s at 72 °C). The procedure was concluded with a melt-curve analysis between 40 and 85 °C.

2.6. *FISH*

Fluorescence in situ hybridization (FISH) analysis of the anammox population was performed regularly in the first 400 days of the startup to detect anammox growth. The planctomycetales probe Pla46, as well as the AMX820 (Brocadia and Kuenenia) probe were employed. On day 1330, FISH was performed to determine the genus of the anammox population of the reactor in Rotterdam and the inoculation reactor in Balk. The probes used were AMX368 (hybridizing with all known anammox bacteria), AMX820 (Kuenenia and Brocadia) and KST-157 (Kuenenia). Probe and hybridization details (Schmid et al., 2000, 2003, 2005) are available at Probebase (Loy et al., 2003).

2.7. *Online measurements*

Temperature, conductivity and pH sensors (Endress+Hauser, Reinach Ch) were located in a measurement loop in the lower compartment of the reactor. The pH sensor was calibrated once every two months. Values were averaged over 24 h for further evaluation. Nitrite concentrations at sample point 2 and in the effluent were determined spectrophotometrically every 20 min using a fully automated analyzer (ADI 2019, Applikon, Schiedam NL).

2.8. *Granule size*

1000 ml of reactor volume was sieved on sieves with a 0.25 and 0.45 mm mesh. The sieved granules were collected, resuspended in water, and the volume was estimated using an Imhoff funnel after settling.

Table 1 – Probes and primers used in Q-PCR

Name	Probe sequence[a] 5′–3′	Hybridization	Reference
Pla46F	GGATTAGGCATGCAAGTC	Planctomyctales	Neef et al. (1998)
AMX667R	ACCAGAAGTTCCACTCTC	Anammox	This work
AMX361	AGAATCTTTCGCAATGCCCG-F	Anammox[b]	This work
AMX381	L-AAGGGTGACGAAGCGACGCC	Most Anammox	This work
AMX382	L-AAGGTGACGAAGCGACGCC	Kuenenia and Brocadia	This work

[a] Fluorophores mentioned: L = LightCycler Red 640, F = fluorescein isothiocyanate.
[b] This probe partly overlaps with FISH probe AMX368 (GCTTT**CGGGCATTGCGAA**).

2.9. Control of granulation

In view of the low growth rate of anammox bacteria, and the required high loading rate, efficient biomass retention is required. A high upflow velocity (Beun et al., 2000) is a crucial factor in the formation of well settling biomass that can remain in the reactor. The hydraulic retention time varies with varying N-load during the startup. Therefore, the hydraulic retention time cannot be used efficiently to maintain a high upflow velocity. Thus, from day 1130 on, a flow-adjustable recycle stream from the top of the reactor was mixed with the influent to maintain a sufficiently high upflow rate (2–3 m/h) during the phases in the startup when the influent rate was not high enough to ensure sufficient granulation.

2.10. Sludge withdrawal

After the reactor was converting at its design capacity of 500 kg-N/d, sludge was removed periodically from the bottom of the reactor. A total amount of 36 m^3 of sludge was removed on 26 occasions from day 1341 to day 1432 in amounts varying from 0.5 to 2 m^3. The sludge was used to inoculate an anammox reactor in Lichtenvoorde (NL) and a one reactor nitritation–anammox process in Olburgen (NL).

3. Results

3.1. Description of the startup

By analyzing the behavior or biomass growth and anammox activity (Figs. 2 and 3), the startup period of the anammox reactor can be divided into two main phases.

First 800 days: periods of growth, but no detectable anammox activity: The reactor was inoculated with activated sludge from the main treatment plant, since the effect of inoculation with excess anammox sludge from available enrichments (on 1–25 l scale) would not be significant on a 70 m^3 scale. The presence of (low amounts) of anammox bacteria was shown already on lab scale by the successful anammox enrichment from activated sludge of the Dokhaven wastewater treatment plant (Van Dongen et al., 2001). The predominant activity in the first period was a rather low nitrite conversion (ca 0.025 kg-N/m^3/d, Fig. 2), probably due to denitrification consuming nitrite with some excess COD in the anammox reactor. The nitrite removal was not coupled to ammonium removal, nor could nitrate production be detected as would be expected if the anammox process were the predominant process. Since the anoxic oxidation of ammonium is (by definition) a clear indicator of anammox activity, the *absence* of ammonium removal indicated that anammox activity was not visible from the conversion rate. In order to smoothen the rather fluctuating measurements and have a more robust indication of when ammonium removal occurred during a sufficiently long time interval, a 30-day moving average was applied to the daily measured conversion rates. The time-averaged ammonium conversion reached a value higher than 0.02 kg NH_4^+/m^3/d only from day 530. Significant nitrate production was not detected at all during this period. Q-PCR, however, provided an indication that anammox bacteria grew in the reactor (Fig. 2). From day 73 on, a first period of growth could be detected by molecular methods.

Day 800–1250: periods of increase in conversion: From day 800 on, the 30-day moving average of the ammonium conversion was always significant and thus anammox activity could be detected by traditional measurements (Fig. 3). Nitrate production, also a clear indicator of anammox activity, was only

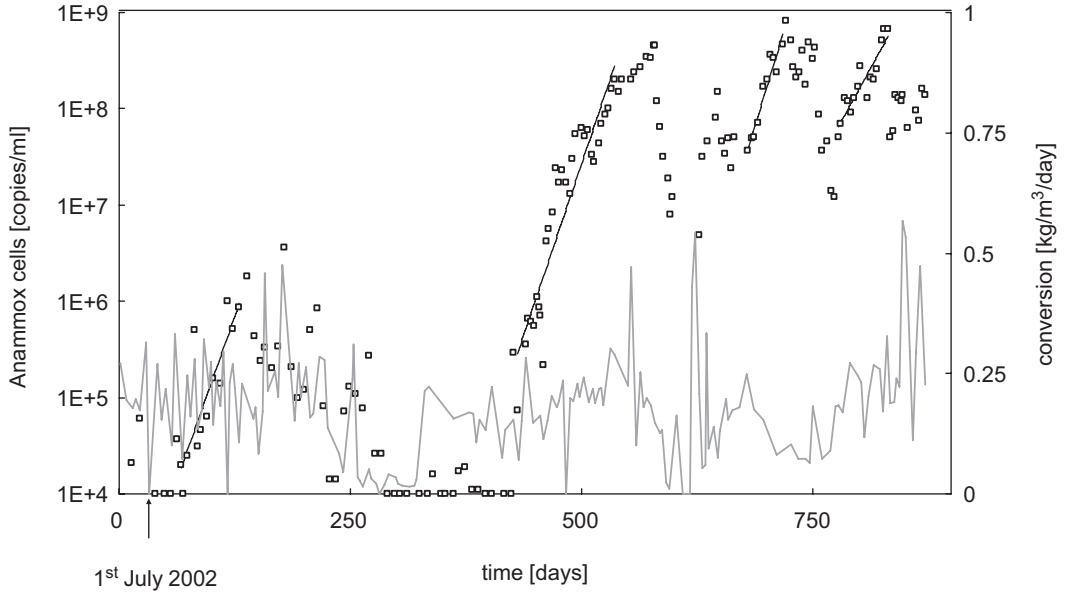

Fig. 2 – Startup of the anammox reactor. The anammox cell concentration as measured by Q-PCR (open squares); the lines fitting the concentrations during periods of exponential growth for estimation of growth rates μ (thick solid lines); 30 days moving average of the nitrogen conversion (grey line).

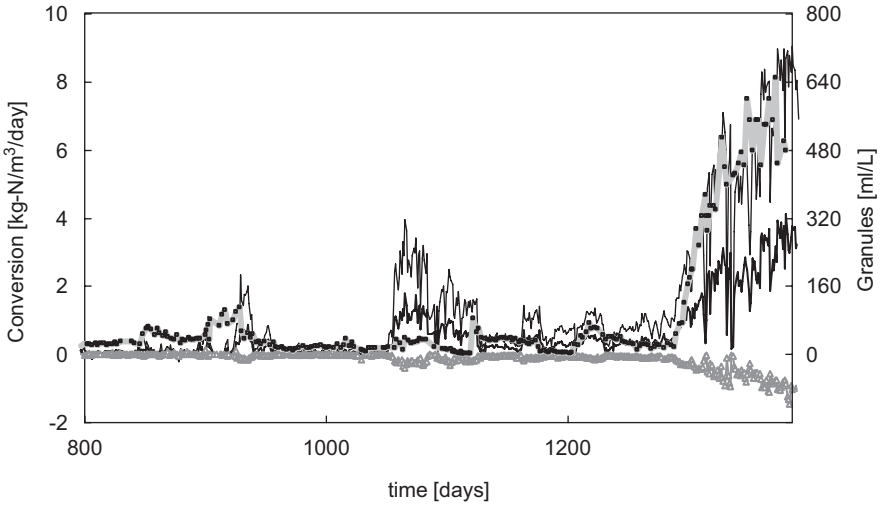

Fig. 3 – N-conversion rate in the anammox reactor between days 800 and 1400 until the design conversion of 7.1 kg-N/m^3/d was achieved. Conversion of total nitrogen (thin black line), ammonium (thick black line), and nitrate (open triangles, negative, because it is production) are shown on the left axis. The relative volume of granules (thick grey line with black squares, Imhoff value) is shown on the right axis.

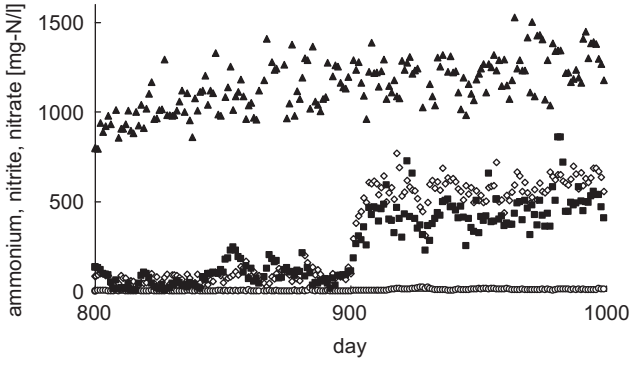

Fig. 4 – Evolution of concentrations of N-compounds during the transition from operation of SHARON reactor as nitritation–denitrification reactor with methanol addition (until day 900) to operation with nitritation only. Influent ammonium (closed triangles), effluent ammonium (closed squares), nitrite (open diamonds) and nitrate (open circles).

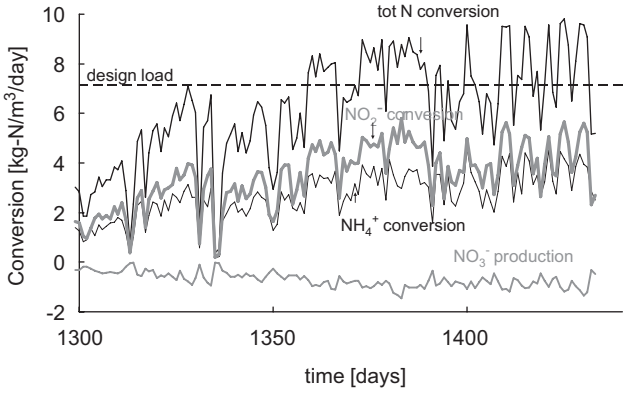

Fig. 5 – Conversion during the days that the final the design load was achieved. Total nitogen conversion (thick black), nitrite conversion (thick grey), ammonium conversion (thin black), nitrate production (indicated as negative conversion; thin grey).

significant from day 925 on, while granule formation was significant from day 848 (Fig. 3). On day 900 the operation of the nitritation reactor was changed from nitritation–denitritation to nitritation only, which led to a rise in nitrite level of the anammox influent (Fig. 4). The nitrite level rose from 130 ± 268 to 575 ± 175 mg-N/L.

Several periods of growth could be distinguished, but for various reasons (see further section "Problems during start-up"), the enhanced conversion levels could not be maintained. In all those periods along with the conversion, the amount of granules increased.

3.2. *Stable operation*

From day 1235 on a stable conversion took place which could be gradually increased until the design load of 500 kg-N /d (7.1 kg-N/m^3/d) was reached on day 1359. Sludge was removed to serve as an inoculum for new reactors (Lichtenvoorde, Olburgen) and if possible, the load was increased. The maximum attained value of 9.5 kg-N/m^3/d was limited by the available influent load and is not a maximum volumetric conversion of the anammox reactor (Fig. 5).

3.3. *Problems during start-up*

Often during the startup (day 0–1250) the increasing anammox biomass concentration or activity could not be maintained due to various accidents. A list of problems is given below:

- *Fall in conversion due to incidental nitrite toxicity.* This was caused by a too high loading rate and occurred

mainly when nitrite analyzers were defect, or not well calibrated.

- *Washout of biomass due to sudden changes in the hydraulic regime*. When during once-through operation (before day 1134, when no liquid recycle flow existed), the loading rate was increased, the upstream velocity was increased accordingly. This incidentally led to biomass washout because of too sudden increase in upstream velocity.
- *Breakthrough of toxic methanol from nitritification*. Methanol is extremely toxic to anammox (Güven et al., 2005), and in the period that methanol was dosed on the nitritation reactor (until day 900) this issue was of continuous concern. Although mixing-time calculations and incidental measurements indicated that methanol was converted before reaching the anammox reactor and although breakthrough of methanol into the anammox reactor was never detected, it was incidentally suspected to have caused problems.
- *Freezing of pipes leading to a stop in the influent*. During the winter of 2002 (around day 150), due to periods of low influent flow, freezing of the anammox influent lead to a stop in the feed.
- *Incidental failing of pumps and compressor; hampering mixing or loading*. Mechanical problems occurred once in a while, mainly in the first 500 days of the startup.
- *Discharge of wastewater from mobile chemical toilets directly in the sludge line*. This wastewater contains amongst others nitrification inhibitors. These chemicals (or their degradation products during the sludge digestion or nitritation) had a strong adverse effect on the anammox process. The conversions in the nitritation process were, however, not visibly influenced. If this wastewater had been supplied to the main treatment plant (as is normal routine), then this would not have been a problem in view of the large dilution in the main plant.

3.4. *Exponential increase in biomass and conversion*

Under laboratory conditions the maximum reported attainable doubling time was 11 days (Strous et al., 1998). In view of this low growth rate it is interesting to see if this value was also attained in the full-scale reactor. For this purpose, the growth rate and doubling time of the organisms were determined from four periods of exponential increase in anammox cell copies during the preliminary phase of the startup (Fig. 2 and Table 2), and for six periods of exponential increase in conversion (Fig. 6 and Table 3). Only those periods of exponential increase were taken into account, where no inocuation had taken place during the weeks before the calculation. Growth rates from the Q-PCR data were determined with the least-squares method from the logarithm of the number of gene copies/ml with respect to time:

$$\ln C = \ln C_0 + \mu \cdot t$$

where C_0 and C are the gene copy concentrations (gene copies/ml) initial and at time, t (days), respectively, and μ is the specific biomass growth rate (day^{-1}).

The loading rate was always adjusted to fit the possible conversion rate, and thus to avoid limitation. This results in a balance between loading rate and conversion

$$R = \left[\frac{\phi_V}{V}(C_{in} - C)\right] = \frac{\mu}{Y_{sx}} C_x$$

where R is the conversion rate (kg-N/m^3/d), ϕ_V is the flow rate (m^3/d), V is the reactor volume (m^3), Y_{sx} is the yield of biomass on nitrite (kg-C/kg-N) and C_x (kg-C/m^3) is the biomass

Table 2 – Exponential increase in anammox cells during the initial phase of the startup, as determined by Q-PCR

Start day	Duration (days)	μ (day^{-1})	Doubling time[a] (days)	RR^2
67	63	0.063	11.1	0.70
431	105	0.065	10.6	0.84
680	38	0.073	9.5	0.92
778	54	0.040	17.3	0.83

[a] The biomass doubling time is $\ln 2/\mu$.

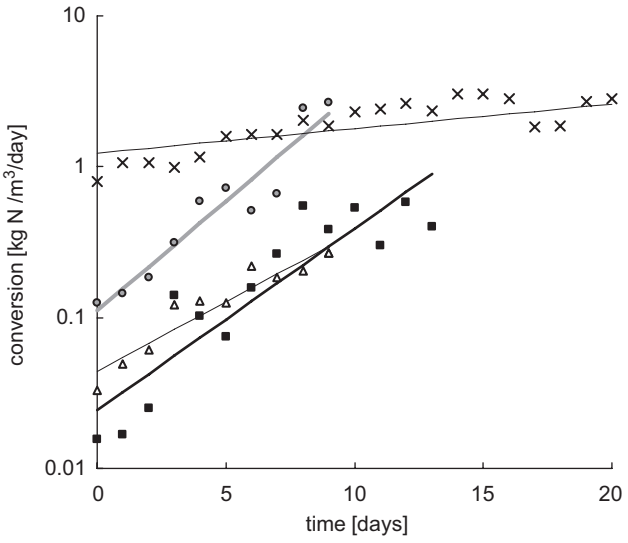

Fig. 6 – Determination of growth rates from periods of exponential increase in conversion from days 887 (closed squares), 912 (open triangles), 967 (grey circles) and 1284 (signs) (see Table 2). Solid lines represent fits for exponential growth.

Table 3 – Exponential increase in total nitrogen conversion, as determined from the mass balance

Start day	Duration (days)	μ (day^{-1})	Doubling time[a] (days)	RR^2
887	13	0.28	2.8	0.81
912	9	0.21	3.3	0.91
967	9	0.34	2.1	0.91
1284	44	0.037	18.5	0.67

[a] The doubling time calculated from the conversion is $\ln 2/\mu$.

concentration. During exponential growth this becomes:

$$\ln R = \ln\left(\frac{\mu}{Y_{sx}} C_{x,0} e^{\mu t}\right) = \ln\left(\frac{\mu}{Y_{sx}} C_{x,0}\right) + \mu t = \ln R_0 + \mu t,$$

where R_0 is the initial conversion (kg-N/m^3/d). This is similar to the equation for increase in the biomass concentration. For the periods of exponential increase in anammox cell copies, doubling times of 9–17 days were estimated. The doubling times based on conversion ranged from 2–18 days.

3.5. *FISH*

From the weekly FISH analysis in the first 400 days, only an *estimate* of the relative population could be given. At the low levels of anammox organisms present, this resulted in low but fluctuating amounts. Anammox organisms, however, could be detected nearly always.

The population (as determined on day 1330) consisted of "*Brocadia anammoxidans*" with a relative abundance of 50–60%. The inoculation reactor in Balk contained "*Kuenenia stuttgartiensis*" with a similar relative abundance. This means that in the inoculation reactor and the full-scale reactor different anammox bacteria were present.

3.6. *Stoichiometry*

The ratios between nitrate production and ammonium consumption ($Y_{NO_3^-/NH_4^+}$), as well as the ratio between nitrite conversion and ammonium conversion ($Y_{NO_2^-/NH_4^+}$) are given as a function of the total nitrogen conversion rate in Fig. 7. Both ratios vary considerably on days where the total N conversion is below 1–1.5 kg-N/m^3/d, but become stable at conversions above 1.5 kg-N/m^3/d. The average value for $Y_{NO_2^-/NH_4^+}$ at a conversion above 1.5 kg-N/m^3/d is 1.31 (average over 193 days, standard deviation (st.dev.) of the mean 0.032, st. dev. 0.46) and the $Y_{NO_3^-/NH_4^+}$ is 0.25 (193 days, st. dev. of the mean 0.006, st. dev 0.09). These values are well in line with values found on lab scale reactors with standard stoichiometry (Strous et al., 1998). The pH and temperature in the reactor at different levels of conversion are presented in Fig. 8. It is apparent from these data that the anammox process in this

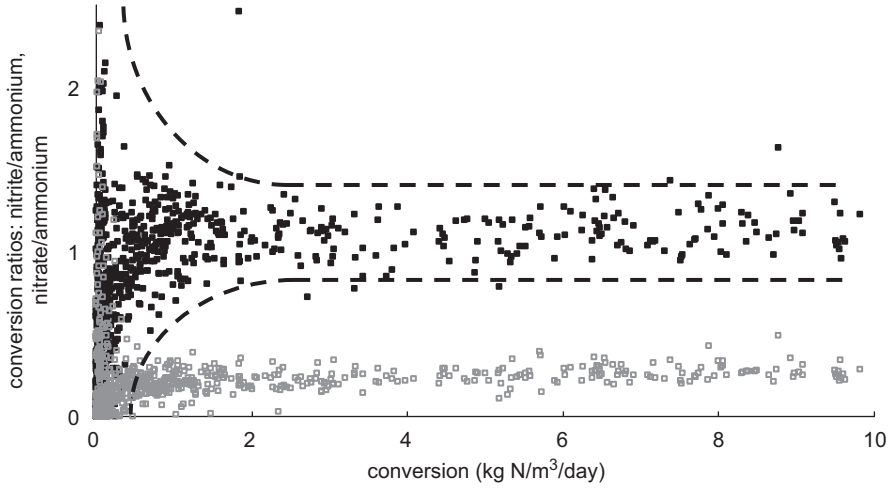

Fig. 7 – Conversion ratio of nitrite and ammonium ($Y_{NO_2^-/NH_4^+}$ closed squares); ratio between nitrate production and ammonium conversion ($Y_{NO_3^-/NH_4^+}$ open squares).

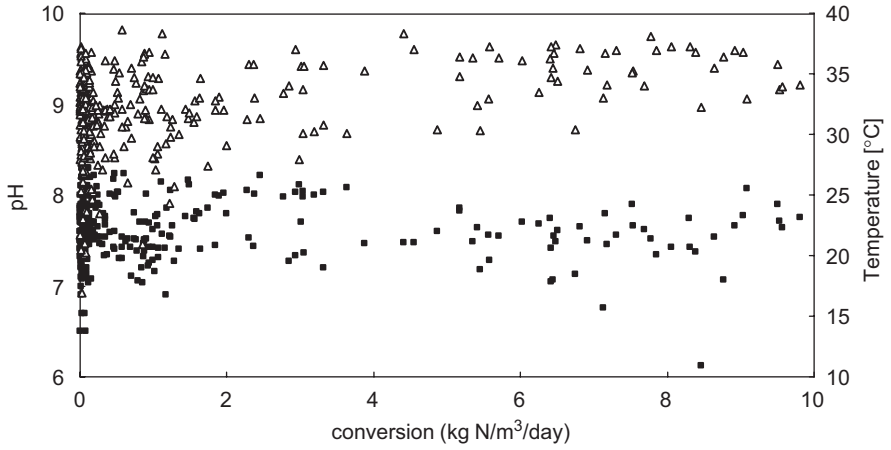

Fig. 8 – pH (closed squares) and temperature (open triangles) at different conversion ratios.

reactor configuration can run stably at a 6–10 kg-N/m^3/d level in a pH range between 7 and 8 and a temperature range of 30–40 °C.

4. Discussion

4.1. Direct startup

To save time and resources, the anammox technology was scaled up directly from lab scale to full-scale. This approach has proven to be effective in the introduction of other new nitrogen removing technologies (e.g. BABE (Salem et al., 2004) and SHARON (van Kempen et al., 2001)), mostly because the operational costs (mainly personnel) of a pilot plant are approximately the same as when running a full-scale plant. At low conversion rates, the slow growth rate of the anammox bacteria made the assessment of different process changes relatively time-consuming, which would not have been different at pilot-scale. The determination of growth of anammox bacteria by amplification with Q-PCR was one of the solutions to overcome this problem. However, to be fully effective, the Q-PCR should be used on daily rather than on weekly basis. Because of the time-delay between sample-taking and the communication of the results of the Q-PCR during this startup, the inoculation with seed sludge from an enrichment reactor in Balk proved to be very helpful to see the effects of changes in wastewater composition or process conditions. The reactor in Balk was running on synthetic feed under well-known lab-conditions, and thus the startup was relatively easy. The size of the reactor (4 m^3) was large enough to produce enough sludge to actually see the effects after inoculation.

In the same period as the anammox reactor startup took place, a one reactor anammox process was scaled up in Strass (Austria) in a step-wise way, from a 5 l inoculum (from a pilot scale anammox reactor in Zürich (Fux et al., 2002)) to 300 l, to 2.4 m^3, and finally to 500 m^3 (Wett, 2006). The whole scale-up took also more than 3 years. This is another indication that, at least for the anammox process, directly building a full-scale plant did not cost extra time and saved the cost of running a pilot plant on-site.

4.2. Growth rate

The detection of anammox bacteria by Q-PCR has been the only parameter from which the success of the enrichment during the first 900 days could be determined. Only recently the use of Q-PCR for quantification of anammox bacteria has been reported (Tsushima et al., 2007), but detection was normally mainly based on conversions or population estimation from FISH. Q-PCR has proven to be very valuable during the initial phase of the startup, when anammox conversions were too low to reflect the biomass growth. To our knowledge, this is the first successful industrial application of quantitative PCR as a biomass growth monitor. During the four identified phases of exponential biomass growth (Fig. 2, Table 2), doubling times of 9–17 days could be estimated, which is close to the reported doubling time of 10.5 days (Strous et al., 1998). It is possible however that the actual growth rate was slightly higher and that part of the produced biomass was removed with the effluent. Suspended solid concentrations in influent and effluent were only monitored incidentally, and a possibly higher growth rate due this washout could not be calculated. If one tries to use the increase in N-conversions to indirectly characterize the biomass growth, much quicker "doubling times" (2–3 days) are obtained. This unusually high increase in activity seemed to occur only for short periods (ca one week), suggesting that the boost of N-removal here does not correspond to growth, but rather to biomass reactivation after certain process disturbances. However, a reliable biomass estimate could not be given because of the large differences in conditions within the reactor volume during this phase of the startup. The results of FISH-analysis during the first 400 days could not be quantified, and also a reliable trend could not be distinguished. This shows once again that (contrary to Q-PCR) when the population is not strongly enriched, FISH cannot be used to follow the growth.

4.3. Anammox species

The enriched species ("Brocadia anammoxidans") is different from the inoculation reactor ("Kuenenia stuttgartiensis"). Interestingly, it is also different from the dominant species in two other enrichments from Rotterdam B-stage sludge, where the enriched organism was "Kuenenia stuttgartiensis" (Van Dongen et al., 2001) or "Brocadia fulgida" (Kartal et al., 2004). Apparently the anammox-diversity of the nitrifying sludge is (at least in Rotterdam) large, and which organism is enriched is determined not by inoculation but by (largely unknown) niche differentiation or other minor aspects. The kinetics and stoichiometry of all these anammox bacteria are similar, so for conversion it gives no differences. The anammox bacteria in the inoculum from Balk were clearly active when introduced in the full-scale reactor. However, finally they seemed to be replaced by "Brocadia anammoxidans".

4.4. Granule formation

Increases in volumetric N-conversion were always coupled to an increase in the volume of the biomass granules in the reactor (Fig. 3). During the final phase of the startup (after ~1300 days), the N-conversions seem linearly dependent on the total volume of biomass granules. This strongly indicates that in the period of the sharpest improvement in conversion most of the active biomass was present in the granules. In other periods of growth the total volume of granules also increased but to lesser extent than in the final period. Probably in those periods a significant part of the activity was performed by the suspended biomass.

4.5. Volumetric conversion

The maximum attained volumetric conversion rates in the reactor (more than 9 kg-N/m^3/d) were limited by the availability of nitritated sludge digestate and thus it is not clear if this is a maximum capacity related to the reactor-type. The full-scale N-conversions are high compared to the usual

conversion reported at lab scale, but the gas production is comparable to that of anaerobic methanogenic reactors (ca 10 m^3 gas/$m^3_{reactor}$/d). For 0.5–10 l lab reactors, 0.2–2 kg-N/m^3/d are normally obtained (Strous et al., 1998; Furukawa et al., 2003; Wyffels et al., 2004), although a maximum of 8.9 kg-N/m^3/d has also been reported (Sliekers et al., 2003). The footprint of the reactor (only 10 m^2) gives rise to a high surface specific conversion (75 kg-N/$m^2_{reactor}$/d). Because all substrates (ammonium, nitrite and (bi)carbonate) are available in the liquid phase, and because the liquid concentration of the final product (N_2) is not disturbing the process, gas–liquid transfer is not a process rate limiting step in granular sludge anammox reactors. The lab scale maximum conversions are therefore generally limited by the biomass retention capacity. In the full-scale reactor, however, biomass retention has proven to be more effective. The low biomass concentrations in the top part of the reactor, where the anammox biomass is actually separated from the effluent, show that biomass washout is very small (SRT is 45–160 days based on effluent measurements). The maximum attainable conversion is therefore probably only limited by the amount of biomass present in the lower compartment.

4.6. *Choice of reactor type*

4.6.1. *Anammox reactors*

Several types of reactors under various names (Box 1, Table 6) have been employed for pilot scale and full-scale installations (Table 4) where anammox is the dominant process. The

Box 1–Terminology

In the seventies, the common knowledge that ammonium could be oxidized under oxic conditions only, and even so not directly to dinitrogen gas, was challenged by Broda's (1977) prediction of anoxic ammonium oxidation with nitrite or nitrate instead of oxygen as electron acceptor. Despite his postulation of these "lithotrophs missing from nature", it took more than 10 years before they were found.

- In an anoxic reactor *in Delft (NL)* which was fed with nitrate and ammonium, unexpectedly ammonium was removed (Mulder, 1989; Van de Graaf et al., 1990; Mulder et al., 1995). The responsible organism for the oxidation of ammonium was cultivated with nitrite as electron acceptor (Van de Graaf et al., 1996) and the process was dubbed the *anammox process*. In 1999 it was found that the anammox process was performed by dedicated organisms in the order of the Planctomycetales (Strous et al., 1999a).
- The high removal of ammonium from a nitrifying Rotating Disk Contactor *in Mechernich (DE)* in the absence of COD was noticed and thought to be linked to the aerobic oxidation of ammonium to dinitrogen gas (Hippen et al., 1997). The process was dubbed *aerobic deammonification*.
- Also in a rotating biological contactor *in Köllikon (CH)*, high N-losses occurred (Siegrist et al., 1998). They were attributed to either a combination between nitritation and anammox, or to the activity of nitrifyers alone.
- In lab-scale nitrifying rotating disk contactors *in Gent (BE)* nitrogen losses occurred, which were attributed to the activity of nitrifyers in the oxic and anoxic layers of the biofilm (Kuai and Verstraete, 1998). The process was named oxygen limited autotrophic nitrification–denitrification (OLAND).

During the following years, it was discovered that the N-losses in the nitrifying systems in Switzerland (Egli et al., 2001), Germany (Helmer et al., 2001) and Belgium (Pynaert et al., 2003) were all caused by partial nitritation by aerobic ammonium oxidizers, followed by anoxic ammonium oxidation by anammox organisms in the deeper layers of the biofilm. The finding of unexplainable N-losses at different locations—in combination with lacking consensus on both the responsible mechanism as well as the type of organisms performing the reaction—has led to several names for processes where anammox organisms play a major role. These names, combined with others (often based on the names mentioned above) for certain configurations of these processes have lead to an unclear terminology in the (scientific) literature (Table 6).

Because the first full-scale anammox installations are now running, we suggest clarifying this situation by the following *descriptive* terms, which are unambiguous for the scientific community:

- The *anammox process* for the anoxic combination of ammonium and nitrite to form dinitrogen gas.
- *One reactor nitritation–anammox* process as the occurrence of nitrite production and the anammox process in one (aerated) reactor.
- *Two reactor nitritation–anammox* process for the partial oxidation of ammonium to nitrite, followed by an anoxic reactor, where only the anammox process takes place.
- *One reactor denitrification–anammox* for the anoxic processes denitrification of nitrate to nitrite combined with the anammox process. This was the original process configuration in which anammox was discovered (Mulder et al., 1995).
- The *anammox reactor* for the reactor in which only the anammox process takes place.
- *Anammox organisms*: the dedicated organisms (until now always found in the order Planctomycetales) capable of performing the anammox process.

Table 4 – Conversions in full-scale and pilot scale (>1 m³) reactors for the anammox process and the one reactor nitritation–anammox process

Process	Location	Reactor type	Volume (m^3)	Area conversion[a] (g-N/m^2/d)	sp. Max. conversion (Kg-N/m^3/d)	Limitation	Organism	Reference
Anammox process	Rotterdam (NL)	Granular sludge reactor	70	n.d.	10 (20)[b]	Feed (nitrite)	Brocadia	This work
	Lichtenvoorde (NL)	Granular sludge reactor	100	n.d.	1	Feed (nitrite)	Kuenenia	Abma and Haarhuis, pers. comm.
	Hattingen[c] (DE)	Moving Bed	67	5	1	Na[d]	n.d.	Thöle et al. (2005)
	Mie prefecture (JP)	Granular sludge reactor	58	n.d.	3	Feed (ammonium)	n.d.	Abma and Tokutomi, pers.comm.
	Balk (NL)	Granular sludge reactor	5	n.d.	4	Feed (nitrite)	Kuenenia	This work
	Stockholm[e] (SE)	Moving Bed	2	0.5	0.1	Feed (nitrite)	Brocadia[f]	Gut et al. (2006)
	Zürich (CH)	SBR	2.5	n.d.	2	Feed (nitrite)	n.d.	Fux et al. (2002)
One-reactor nitritation–anammox process	Olburgen (NL)	Bubble Column	600	n.d.	1.2	Feed	Brocadia	Abma and Haarhuis, pers. comm.
	Strass (AT)	SBR	500	n.d.	0.6	Feed	n.d.	Wett (2006)
	Glarnerland (CH)	SBR	400	n.d.	0.4	Feed	n.d.	Nyhuis et al. (2006)
	Pitsea (GB)	RDC[g]	240	7	1.7	Feed	Scalindua	Schmid et al. (2003)
	Hattingen[h] (DE)	Moving Bed	102	6	1	Not reported	n.d.	Thöle et al. (2005)
	Mechernich (DE)	RDC	80[i]	2	0.6[d]	Aeration	n.d.	Hippen et al. (1997)
	Köllikon (CH)	RDC	33	2	0.4	Feed	Kuenenia[j]	Siegrist et al. (1998)
	Stockholm[e] (SE)	Moving Bed	4	2	0.5	Aeration	Brocadia	Szatkowska et al. (2007)

[a] Conversion per area of biofilm.
[b] Estimated conversion in the lower compartment.
[c] Reactor 2, when no aeration was employed there, and Reactor 1 was operated as a nitritation reactor.
[d] Setup changed before maximum conversion was reached.
[e] Reactor was first operated as a two reactor nitritation–anammox process, followed by one reactor nitritation–anammox operation.
[f] Reported by Gut (2006).
[g] Plant consists of 4 lines of 3 RDC's in series. The values for the first cylinders are presented.
[h] Reactor 1.
[i] Not reported, value estimated based on a biofilm surface of 250 m^2/m^3.
[j] Organism was enriched from this source (Egli et al., 2001), but if this is the dominant organism, is unknown.

volumetric conversion in these reactors is markedly lower than that in the reactor in Rotterdam, and it is generally limited by availability of substrate. All substrate-limited reactors are—by definition—over-designed, and it is not possible to compare reactor types based on these conversions. Therefore maximum conversions for different anammox reactors were estimated by calculating the rates of possible process steps which are limiting the conversion (Table 5). Apart from the hydraulic limitation in the granular sludge reactor from Rotterdam, nitrite flux to the biofilm is another potential limitation. The maximum nitrite flux to an anammox-containing biofilm was calculated using the half-order substrate flux derived from penetration theory assuming zero-th order reaction kinetics (Harremoës, 1977). By assuming a concentration of nitrite of 15 mg-N/l in the bulk, a conversion flux of 17 g-NO_2^-–N/m^2/d (equivalent to 30 g-N/m^2/d) can be estimated. Due to lower biofilm specific areas, rotating disk reactors and moving bed reactors attain therefore maximum conversions of 7 kg N/m^3/d respectively, based on this limitation. The small diameter of the granular sludge (ca 1 mm) yields—based on nitrite limitation—a conversion capacity which is so high (90 kg N/m^3/d), that nitrite limitation cannot be the main limitation in this reactor. The efficiency of biomass retention or eventually the gas–liquid separation unit determines in this case the maximum conversion.

Table 5 – Estimated volumetric conversion limitations and fluxes (shown in brackets) in different types of reactors for the anammox process and the one reactor nitritation–anammox process

	Reactor type	Particle diameter (m)	Surface area[a] (m^2/m^3)	Maximum volumetric conversion rate (kg N/m^3/d)[b] (conversion flux (g N/m^2/d))			
				Limiting process			
				Nitrite penetration[c,f,g]	Hydrodynamic limitation	Oxygen	
Anammox process	Granular sludge	0.001	3000	90 (30)	—	—	***12***[i]
	Biofilm moving bed	0.01	250	***7 (30)***	—	—	n.d.
	Biofilm packed bed[j]		250	***7 (30)***	—	—	n.d.
	Biofilm sheets reactor		250	***7 (30)***	—	—	n.d.
One reactor nitritation–anammox process	Airlift/ Bubble column	0.001	3000	89 (30)	15 (5)	***8***	n.d.
	Rotating disk contactor		250	7 (30)	***2.5 (10)***[k]	n.d.	n.d.
	Moving Bed	0.01	250	7 (30)	**1.2 (5)**	8	n.d.
	Sequencing Batch Reactor	0.001	3000	89 (30)	15 (5)	***8***	n.d.

The strongest limitation for each reactor is shown in bold italic.

[a] Specific surface areas are estimated based on (Nicolella et al., 2000) and the references cited in Table 4.

[b] In one reactor nitritation–anammox reactors the ammonium conversion is the amount of ammonium that is removed (not only the amount of ammonium oxidized by aerobic ammonium oxidizing bacteria to nitrite).

[c] Based on penetration into the biofilm (assuming zero-th order conversion kinetics) $\Phi_i = \sqrt{2q_{ix}C_xD_iC_{b,i}}$ (Harremoës, 1977) where subscript i is nitrite or oxygen, biofilm concentration $C_x = 80$ kg DW/m^3.

[d] Assumed nitrite bulk concentration = 15 mg-N/L; diffusion coefficient $D_{NO_2^-} = 1.9 \times 10^{-9}$ m^2/s, specific oxygen conversion rate (anammox) $q_{nitriteX} = 8.3 \times 10^{-6}$ kg NO_2^-–N/kg DW/s (Strous et al., 1998).

[e] Nitrite was assumed to be the limiting substrate in the anammox reaction for the anammox reactor (to avoid nitrite toxicity, Strous et al., 1998) as well as for the two reactor nitritation-anammox process (to prevent in-growth of nitrite oxidizers Third et al., 2001).

[f] Assumed oxygen bulk concentration = 1 mg/l; Diffusion coefficient $D_{O_2} = 2.4 \times 10^{-9}$ m^2/s, specific oxygen conversion rate (nitrifyers) $q_{nitriteX} = 3.2 \times 10^{-5}$ kg O_2/kg DW/s (Wiesmann, 1994).

[g] Oxygen consumption is supposed to be only caused by aerobic ammonium oxidizers; nitrite oxidizers are supposed to be not significantly present in the one-reactor nitritation-anammox process.

[h] Oxygen transfer of 0.55% per meter height was assumed (Heijnen and Van 't Riet, 1984) and the superficial gas velocity taken at 0.025 m/s (flooding limit).

[i] Gas production of more than 10 $m^3_{gas}/m^3_{reactor}$/d will lead to failing biomass retention in this type of reactors (Nicolella et al., 2000).

4.6.2. *One reactor nitritation-anammox processes*

The combined nitritation-anammox process is also under several names (Box 1, Table 6) applied on full-scale, and also found incidentally in existing nitrifying rotating disk contactors (Table 5). For nitritation–anammox reactors, besides the nitrite limitation mentioned above, oxygen consumption by aerobic nitrifyers plays a role in process design. Oxygen transfer was indeed indicated as the limiting process for a lab-scale air-lift (Sliekers et al., 2003) and for a pilot scale moving bed reactor (Szatkowska et al., 2007). The oxygen limitation can stem from the slow diffusion into the biofilm or from the gas–liquid transfer. For the oxygen gas–liquid transfer rate a simplified correlation was used assuming a transfer of 0.55 v% air per meter of reactor height (Heijnen and Van 't Riet, 1984), whereas the oxygen penetration in the biofilm was calculated with the same approach as for nitrite. Our estimations show that oxygen penetration is limiting the rotating disk contactor and the moving bed reactor with conversions of 2.5 and 1.2 kg N/m^3/d, respectively. For the other reactors, gas–liquid oxygen transfer is potentially limiting as well. With a superficial gas velocity of 0.025 m/s the oxygen transfer is ca 15 kg O_2/m^3/d (equivalent to a conversion of 8 kg-N/m^3/d).

4.7. *Recommendations for future startups*

The low maximum growth rate of anammox bacteria will lead to a longer startup period for any anammox process compared to other nitrogen removal technologies. However, the startup period can be considerably shortened if the following two factors are taken into account:

- The biomass produced by the existing anammox reactor can be used for the inoculation of other reactors, requiring only 3–5 successful anammox doubling times to achieve full operation.
- Inoculation with large amounts of active biomass enables a fast check if the installation (including safety and automation systems) is functioning well. For example, the effect of unexpected toxicities can be seen immediately.

Table 6 – Process options and names for nitrogen removal systems involving the anammox process

Process name proposed in here	Number of reactors		Source of nitrite	Alternative process names	First reference
Two-reactor nitritation–anammox process (Fux et al., 2001)	2	NH_4^+	nitritation	SHARON[a,b]-anammox	Van Dongen et al. (2001)
				Two stage OLAND[c]	Wyffels et al. (2004)
				Two stage deammonification	Trela et al. (2004)
One-reactor nitritation–anammox	1	NH_4^+	nitritation	aerobic deammonification	Hippen et al. (1997)
				OLAND[c]	Kuai and Verstraete (1998)
				CANON[d]	Third et al. (2001)
				aerobic/anoxic deammonification	Hippen et al. (2001)
				deammonification	Seyfried et al. (2001)
				SNAP[e]	Lieu et al. (2005)
				DEMON[f]	Wett (2006)
				DIB[f,g]	Ladiges et al. (2006)
One-reactor denitrification–anammox process		NO_3^-	denitrification	anammox[h]	Mulder et al. (1995)
				DEAMOX[i]	Kalyuzhnyi et al. (2006)
				denammox[j]	Pathak and Kazama (2007)

Notes:
[a] Acronym of Sustainable High rate Ammonium Removal Over Nitrite; the name only refers to nitritation where nitrite oxidation is avoided by choice of residence time and operation at elevated temperature.
[b] Sometimes the nitrification–denitrification over nitrite is addressed by this term.
[c] Acronym of Oxygen-Limited Autotrophic Nitrification Denitrification.
[d] Acronym of Completely Autotrophic Nitrogen removal Over Nitrite.
[e] Acronym of Single-stage Nitrogen removal using the Anammox and Partial nitritation; name only refers to the process on a biofilm surface layer.
[f] Name only refers to the process in an SBR under pH-control.
[g] Acronym of Deammonification in Interval-aerated Biofilm systems.
[h] System where anammox was found originally. The whole process was originally designated as "anammox".
[i] Acronym of DEnitrifying AMmonium OXidation; this name only refers to denitrification with sulfide as electron donor.
[j] Acronym of DEnitrification–anAMMOX process; this name only refers to denitrication with organic matter as electron donor.

As an example, initial results of the startup of a full-scale one-stage nitritation-anammox reactor treating a digested potato-wastewater in Olburgen (NL) (startup to design capacity in 6 months) indeed confirm that the faster startup of an anammox-based N-removal process is possible with the larger biomass amounts now available and applying the lessons learned during the startup of the Rotterdam process.

5. Conclusion

The anammox process is the first wastewater treatment process based on notoriously slow-growing microorganisms that has reached the commercial and full-scale level.

For the first time, the startup could be successfully monitored with Q-PCR during the periods when conversion did not give a clear proof of anammox activity yet. The biomass growth rate during periods of exponential growth could be estimated from Q-PCR to be 9–17 days, which is well in correspondence with literature values. However, periods of faster (2–3 days) exponential increase in N-conversion seemed to be correlated more to an increase in the biomass activity, rather than to biomass production.

Future startup of anammox reactors can take advantage of the experiences gained in Rotterdam, and can use sludge from this reactor as inoculum. The large inoculum will enable the fast check of the reactor performance, and decrease the number of multiplications of anammox organisms required before the design conversion is reached; thus decreasing the startup time of other anammox reactors.

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