Improved nitrogen removal in upflow anaerobic sludge blanket (UASB) reactors by incorporation of Anammox bacteria into the granular sludge

J.E. Schmidt*, D.J. Batstone and I. Angelidaki
Environment and Resources DTU, Building 115, The Technical University of Denmark, DK-2800 Lyngby, Denmark (*E-mail: jes@er.dtu.dk)

Abstract
Upflow anaerobic sludge blanket reactors may offer a number of advantages over conventional mixed-tank, SBR, and biofilm reactors, including high space-loading, low footprint, and resistance to shocks and toxins. In this study, we assessed the use of upflow anaerobic sludge blanket (UASB) reactor technology as applied to anaerobic ammonia removal, or Anammox. Four 200 ml UASB reactors were inoculated with 50% (by volume) anaerobic granular sludge and 50% flocular sludge from different sources (all with the potential for containing Anammox organisms). Tools used to assess the reactors included basic analyses, fluorescent in-situ hybridisation, and mathematical modelling, with statistical non-linear parameter estimation. Two of the reactors showed statistically identical Anammox activity (i.e., identical kinetic parameters), with good ammonia and nitrite removal (0.14 kgNH₃ m⁻³ reactor day⁻¹, with 99% ammonia removal). The third reactor also demonstrated significant Anammox activity, but with poor identifiability of parameters. The fourth reactor had no statistical Anammox activity. Modelling indicated that poor identifiability and performance in the third and fourth reactors were related to an excess of reduced carbon, probably originating in the inoculum. Accumulation of Anammox organisms was confirmed both by a volume loading much lower than the growth rate, and response to a probe specific for organisms previously reported to mediate Anammox processes. Overall, the UASB reactors were effective as Anammox systems, and identifiability of the systems was good, and repeatable (even compared to a previous study in a rotating biological contactor). This indicates that operation, design, and analysis of Anammox UASB reactors specifically, and Anammox systems in general, are reliable and portable, and that UASB systems are an appropriate technology for this process.

Keywords Anaerobic; Anammox; FISH; granule; model; parameter identification; UASB

Introduction
The processes for traditional nitrogen removal consist principally of two sub-processes; nitrification and denitrification. Objectively, these can not be considered as a sustainable process, as they do not satisfy the requirements for low energy costs, and low sludge production. A very promising new, and more sustainable process for nitrogen removal is the anaerobic ammonium oxidation (Anammox) process (Strous et al., 1997). In this process ammonium is oxidized to nitrogen gas with nitrite as electron acceptor.

A major obstacle for utilization of the Anammox process is the slow growth rate of the Anammox bacteria (doubling time approximately 11 days), which requires a special reactor configuration in order to maintain high biomass concentration in the reactor. Increased biomass retention has been achieved in several configurations including fluidized bed, fixed bed reactors, sequencing batch reactors and rotating biological contactors (Strous et al., 1997; Siegrist et al., 1998; Strous et al., 2002). In 2002, the first full scale Anammox reactor was started at Dokhaven wastewater treatment plant, Rotterdam (The Netherlands).

Another problem for utilization of the Anammox process is competition with heterotrophic denitrification. When enough organic matter is available, heterotrophic denitrification will dominate (Jetten et al., 1998).
Upflow anaerobic sludge blankets (UASB) reactors may offer the same advantages to Anammox that they currently provide in anaerobic digestion (Lettinga and Hulshoff-Pol, 1991), including integral sludge retention, high space-loading, low footprint and good resistance to shocks and toxins. In the present paper we studied the introduction of a de novo biodegradation ability; anaerobic ammonia oxidation (Anammox), into anaerobic granular sludge by inoculation of sludge with the Anammox bacteria. To our knowledge, similar experiments have never been performed for investigation of the Anammox process in high rate reactors like the UASB reactors.

Material and methods

Experimental method for continuous feed experiments

Four 200 ml UASB reactors were inoculated with 40 ml granules from a full scale UASB reactor treating wastewater from a paper mill factory. Additional 40 ml inoculums were taken from the following sources and used in reactors 1–4 consecutively.

- Reactor 1: Sludge from Lundtofte wastewater treatment plant receiving municipal wastewater, Denmark.
- Reactor 2: Sludge from Novozymes wastewater treatment plant in Kalundborg, Denmark.
- Reactor 3: Sludge from a trickling filter with biomass that could perform the Anammox process in Stuttgart, Germany.
- Reactor 4: Sludge from a pilot plant at CP Kelco, Denmark, where the Anammox process was investigated.

All four reactors were fed with treated wastewater from Lundtofte wastewater treatment plant and ammonia and nitrite were added at 40 mg L\(^{-1}\) and 50 mg L\(^{-1}\), respectively. After 34 days of operation nitrate was added in the final concentration of 50 mg L\(^{-1}\). During the last period of the experiment, the concentrations of the different nitrogen compounds were further changed as indicated below. The hydraulic retention time (HRT) for the whole experiment was approximately 2.5 days except for Reactor 2 where the HRT was decreased for the last period of the experiment. The flow rate in the reactors was set to 2 m h\(^{-1}\) through recirculation of the treated wastewater. Liquid feed flow was approximately 100 ml d\(^{-1}\) total feed (excluding recycle).

Photometric measurements with the autoanalyzer

The AutoAnalyzer is an automated system to carry out diverse photometric measurements. It performs all the necessary steps including mixing, heating and measuring automatically. The samples are brought into a continuous stream of reagents that goes through all the necessary steps until the measurement (Technicon Corporation 1969).

Nitrite was measured by reaction with sulfanilamide giving a diazo compound that couples with N-1-naphthylene-ethyldiamine to produce a red coloured component. The red colour is measured at 520 nm.

Nitrate was measured by reduction to nitrite with hydrazine sulfate in alkaline solution using copper as catalyst and nitrite was measured as above. Interfering cations were eliminated on line by an ion exchanger.

The ammonium was determined spectrophotometrically by reaction with indophenol. Ammonium reacts in alkaline solution with hypochlorite to monochloramine. This was converted with phenol and hypochlorite (in excess) to indophenol, which is intensely blue in alkaline media. The blue colour was measured at 660 nm. Sodium nitroferricyanide was added to catalyze the reaction. EDTA was used to prevent the precipitation of metal hydroxides.
Fluorescent in-situ hybridisation

Granules were embedded in OCT (Sakura Finetek), after treatment in a 15% sucrose:OCT series, and sectioned at 20 µm on a cryomicrotome. Fluorescence in-situ hybridisation (FISH) was used to investigate abundance and presence of Anammox microbes using the general Anammox probe AMX820 (Egli et al., 2001), together with EUB338(+) supplemented (Daims et al., 1999) to identify all bacteria. Hybridization conditions were 20% formamide/46°C using the method of Hugenholtz et al. (2001).

Modelling and parameter estimation

The model of Koch et al. (2000) was implemented in Aquasim 2.1d (Reichert, 1994), with physicochemical extensions to estimate pH, and a simple biological group to describe heterotrophic denitrification and denitrification. Aerobic organisms and associated components were omitted. The ionic components (for pH calculation) were implemented as differential variables as used in the ADM1 (Batstone et al., 2002), with cations, anions, nitrate and nitrite as saline ions, and ammonia/ammonium and carbon-dioxide/bicarbonate as acid/base pairs. Gas stripping was also included, as the reactors were closed units. Temperature correction of Anammox uptake was included according to Koch et al. (2000). Competitive substrate uptake for nitrate and nitrite was included as for butyrate/valerate in Batstone et al. (2002). The reactors were implemented as fully mixed, fixed volume reactors, with biomass retention simulated by a biomass recycle stream. Inputs were recorded concentrations (variable) at the given flowrate. Initial conditions were steady state at the average concentrations. Influent pH was fitted to the measured influent pH by addition of sodium hydroxide (in the model). Initial and default parameters were as according to Koch et al. (2000). Model source files including inputs and initial conditions in Aquasim 2.1 format are available from the corresponding author.

The main parameter estimation technique used was evaluation of the multi-parameter surface by calculation of a critical objective function value (Batstone et al., 2003). This gives a “true” region or volume of parameter confidence for a given number of parameters, taking into account parameter correlation and non-linearity, given normal residuals (which were so in this case). F distribution values were variable, because of the variable number of points (normally 40), and a 95% confidence was applied ($F_{2,40,0.05} = 3.2317$). The iterative surface gradient-type search method was written in C++, and embedded within the Aquasim “fit” function. The objective function used was residual sum of squared errors (RSS) in nitrite ($S_{NO2}$), and ammonia/ammonium ($S_{NH}$).

Results and discussion

The four lab-scale UASB reactors were inoculated with granular sludge in addition to sludge from different sources as described in “Material and methods”. The concentrations of ammonia, nitrate and nitrite were followed over time. In Figure 1, the concentrations of the nitrogen compounds in the influent and the removal efficiency for reactor 1 are shown during startup. Results from reactors 2 and 3 are shown in Figure 2.

The concentration of nitrate and nitrite in the effluent was low from the beginning of the experiment, indicating that organic matter was available for heterotrophic denitrification. After 35 days of operation the concentration of the nitrate in the influent was increased to approximately 120 mg L$^{-1}$, so only nitrate was used for heterotrophic denitrification.

After the addition of extra nitrate the digestion of the organic inoculum proceeded faster. This can be seen in the fact that the ammonium concentration in the reactor was higher than in the feeding itself (between days 37 and 70, negative removal rates). The increased ammonium concentration in the effluent could also be due to the degradation of hydrazine ($N_2H_4$ is degraded to $NH_3$ and $N_2$ under $NO_2$ limiting conditions). At the end of the experiment the
ammonium concentration in the effluent was nearly zero while the nitrite concentration stayed close to zero, indicating that significant anaerobic ammonia oxidation was occurring in the reactors 1–3. Similar results were obtained for reactors 1, 2 and 3. However, in reactor 4, NO₂ and NO₃ were removed only by denitrification, while no ammonia was removed, indicating that anaerobic ammonia oxidation was not occurring. As indicated by modelling, this was mainly due to a lack of NO₂, probably caused by heterotrophic denitrification, with the inoculum as carbon source (see below).

The reported doubling time for the Anammox is approximately 12 days (Jetten et al., 1998) with a hydraulic retention time in our reactors of approximately 2 days, indicating that the Anammox bacteria were immobilized in the granular sludge.

The loading to reactor 1 was, at the end of the experiment, approximately 0.1 kgNₑtotm⁻³ reactor day⁻¹. This is lower than reported for other Anammox systems (up to 2.6 kgNₑtotm⁻³ reactor day⁻¹), but in the same range as activated sludge systems (0.1 kgNₑtotm⁻³ reactor...
day\(^{-1}\)). To optimize the treatment the HRT in reactor 2 was decreased while in reactor 3 the concentration of N-compounds was increased (see Figure 2).

In reactor 2 the loading could be increased to 0.52 kgN\(_{\text{tot}}\) m\(^{-3}\) reactor day\(^{-1}\) and for ammonia up to 0.14 kgN-NH\(_3\) m\(^{-3}\) reactor day\(^{-1}\), still with approximately 99% removal of ammonia and nitrite. This indicates that the loading could be increased even more. The removal of nitrate decreased, indicating a lack of organic carbon for denitrification.

In reactor 3, inhibition of the Anammox reaction was observed when the concentration of nitrite in the influent increased to over 150 mg L\(^{-1}\). Only 36% of the ammonia was removed at the end of the experiment.

Organisms responding to the AMX820 probe were present, in relatively low amounts, mainly located in the inner portion of the granule. Immobilisation patterns were also different in the three reactors, indicating different origins. In reactors 1 and 2, organisms responding to the AMX820 probe were scattered throughout the granule, with greater abundance further in the granule. In reactor 3, they were largely in relatively dense colonies (Figure 3).

The model results (with simulation from 30–150 days) indicated significant (with 95% confidence) Anammox activity in reactors 1–3. In reactor 4 and possibly 3, evaluation indicated there was significant carbon supply additional to that in the feed. This was probably due to decay of residual biomass from inoculation. The level of additional carbon could be approximated as 0.3 g L\(^{-1}\) with respect to feed, or 0.03 g d\(^{-1}\) total in reactor 4. The simulations with an optimal parameter set are shown for nitrite and ammonia in reactor 2 in Figure 4. The results for reactor 1 are very similar, while reactors 3 and 4 predict more poorly (unless additional carbon is added in the simulation).

Because ammonia and nitrite were pulsed together (at day 100), it was difficult to separate the half saturation coefficients \((K_S)\) for ammonia and nitrite. In this study, parameter

**Figure 3** FISH images of slides of granules from reactor 1 (left) and 3 (right)

**Figure 4** Model results showing nitrite (○), and ammonia (×), and simulations of the same (continuous lines fitting the data points) in reactor 2 from days 70–130
spaces were estimated for the maximum uptake rate at 20°C ($k_{m20} = \mu_{max}/Y$; kmoleN kgCOD$^{-1}$ d$^{-1}$) and $K_{S,NO2}$ (M) in reactors 1, 2, and 3. The confidence space for reactor 4 could not be estimated. The figure shows R3 (left), and detail (inset right), including the confidence spaces for reactor 1 (R1) and reactor 2 (R2) with $K_{S,NH} = 8 \times 10^{-5}$M (left), and $K_{S,NH} = 0.0015$M (right). Also shown is the parameter set used by Koch et al. (2000), without confidence estimates. The error bars indicate 95% linear, uncorrelated estimates of parameter confidence as estimated by secant parameter estimation. These are just for comparison, and the iterated spaces should be used as “true” parameter confidence space. Note that the confidence spaces for R1 and R2 with high $K_{S,NH}$ do not cross the zero $K_{S,NO2}$ vector (but come very close).

Figure 5 Parameter confidence spaces (with 95% confidence) for $k_{m20} (= \mu_{max}/Y$; kmoleN kgCOD$^{-1}$ d$^{-1}$) and $K_{S,NO2}$ (M) in reactors 1, 2, and 3. The confidence space for reactor 4 could not be estimated. The figure shows R3 (left), and detail (inset right), including the confidence spaces for reactor 1 (R1) and reactor 2 (R2) with $K_{S,NH} = 8 \times 10^{-5}$M (left), and $K_{S,NH} = 0.0015$M (right). Also shown is the parameter set used by Koch et al. (2000), without confidence estimates. The error bars indicate 95% linear, uncorrelated estimates of parameter confidence as estimated by secant parameter estimation. These are just for comparison, and the iterated spaces should be used as “true” parameter confidence space. Note that the confidence spaces for R1 and R2 with high $K_{S,NH}$ do not cross the zero $K_{S,NO2}$ vector (but come very close).

spaces were estimated for the maximum uptake rate at 20°C ($k_{m20} = \mu_{max}/Y$) and the nitrite half saturation coefficient ($K_{S,NO2}$). The objective function was residual sum of squares (RSS) for nitrite and ammonia. Residuals were normally distributed.

Parameter spaces were estimated for a $K_{S,NH}$ of $8 \times 10^{-5}$M (1.1 mgN L$^{-1}$), and $K_{S,NH}$ of 0.0015 M (21 mgN L$^{-1}$). The low $K_{S,NH}$ allowed estimation of the lower limit for $K_{S,NO2}$ (particularly in reactor 3), while the higher $K_{S,NH}$ was the same as that used by Koch et al. (2000), and therefore allows comparison with this study. The parameter spaces are shown in Figure 5, and indicate that the parameters for reactors 1 and 2 are statistically the same (and also reactor 3, but with a very different shape). The confidence space for reactor 4 could not be estimated, as it was very large, and included the vector with $k_{m0} = 0$ (i.e., no removal). The results also indicate that the three reactors were significantly (but not greatly) slower than that studied by Koch ($k_{m20}$ of 0.038 kmoleN kgCOD$^{-1}$ d$^{-1}$, $K_{S,NO2} = 0.000143$ M), even when the same $K_{S,NH}$ of 0.0015 M was used (see spaces in Figure 5). It is important to note that the uncorrelated, linear error bars are completely different from the “true” confidence space, especially when error is high (i.e., R3), and $K_{S}$ is low. Therefore iterated space should generally be used (or at least evaluated). This is heavy computationally, but computer time is cheap (and getting cheaper), and the tools are relatively easy to use.

The similarity between R1 and R2 indicates that parameters describing Anammox activity are very repeatable across similar reactors (with different seed), and because the parameters are quite close to Koch et al. (2000), across different reactors, with different origin of biomass. Koch estimated parameters based on a batch test using biomass from a landfill-leachate treating rotating biological contactor in Switzerland. Because the space did not change heavily with a large change in $K_{S,NH}$, it also appears that even with this non-optimal experimental data set, it is quite possible to estimate all three parameters describing Anammox activity effectively, given slightly different experiments.

Conclusions

Significant Anammox activity was found in three of the four UASB reactors operated using statistical methods as applied to a dynamic model. Retention of Anammox microbes in the
granular sludge was demonstrated both by fluorescence in-situ hybridization using an appropriate probe, and by hydraulic retention times lower than reported Anammox doubling times by a factor of 6. High loading rates could be achieved; up to 0.14 kg N-NH$_3$ m$^{-3}$ reactor day$^{-1}$, at 99% ammonia removal. Statistical analysis of the model kinetic parameters also indicated that the key Monod kinetic parameters were highly repeatable between two of the reactors, and in the other reactors, differences were likely caused by heterotrophic denitrification with inoculum as carbon source. This was also probably the reason for poor performance in the fourth reactor (without significant Anammox activity).

We also found reasonable agreement between our parameters, and those published in a previous study from a rotating biological contactor. While not statistically the same, the parameter sets were close, and differences may be explained by variance in the previous study, or variant independent reactor parameters (such as sludge retention time, or residual and input COD).

**Acknowledgements**

We thank the following people for supplying us with seed sludge: Birgit Neumüller, Stuttgart University, Germany, Pernille Lungsi, CP-Kelco, Denmark and Peter Frode Pind, Novozymes, Denmark. Peter Reichert generously allowed us access to the Aquasim 2.1d source code. The students Stefan Heffner, Jacob Kragh Andersen, Kristian West Barnes and Tore Linde helped with experiments and reactor operation.

**References**


