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Combined Nitritation—Anammox: Advances in Understanding Process Stability

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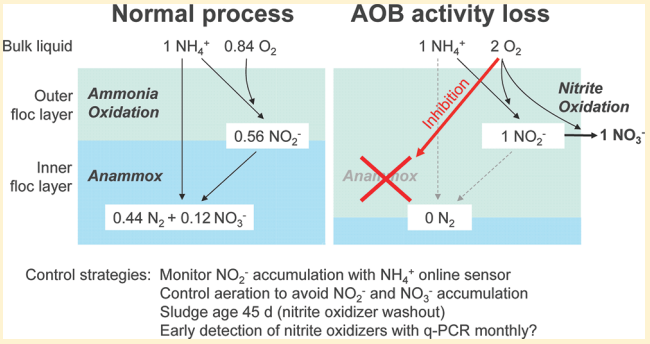
Supporting Information

ABSTRACT: Efficient nitrogen removal from wastewater containing high concentrations of ammonium but little organic substrate has recently been demonstrated by several full-scale applications of the combined nitritation—anammox process. While the process efficiency is in most cases very good, process instabilities have been observed to result in temporary process failures. In the current study, conditions resulting in instability and strategies to regain efficient operation were evaluated. First, data from full-scale operation is presented, showing a sudden partial loss of activity followed by recovery within less than 1 month. Results from laboratory-scale experiments indicate that these dynamics observed in full scale can be caused by partial inhibition of the ammonia oxidizing bacteria (AOB), while anammox inhibition is a secondary effect due to temporarily reduced O_2 depletion. Complete anammox inhibition is observed at 0.2 mg $O_2 \cdot L^{-1}$, resulting in NO_2^- accumulation. However, this inhibition of anammox is reversible within minutes after O_2 depletion. Thus, variable AOB activity was identified as the key to reactor stability. With appropriate interpretation of the online NH_4^+ signal, accumulation of NO_2^- can be detected indirectly and used to signal an imbalance of O_2 supply and AOB activity (no suitable online NO_2^- electrode is currently available). Second, increased abundance of nitrite-oxidizing bacteria (NOB; competing with anammox for NO_2^-) is known as another cause of instability. Based on a comparison of parallel full-scale reactors, it is suggested that an infrequent and short-term increased O_2 supply (e.g., for maintenance of aerators) that exceeds prompt depletion of oxygen by AOB may have caused increased NOB abundance. The volumetric air supply as a proxy for O_2 supply thus needs to be linked to AOB activity. Further, NOB can be washed out of the system during regular operation if the system is operated at a sludge age in the range of 45 days and by controlling the air supply according to the NO_3^- concentration in the treated effluent. Early detection of growing NOB abundance while the population is still low can help guide process operation and it is suggested that molecular methods of quantifying NOB abundance should be tested.



INTRODUCTION

Combined nitritation—anammox in a single sequencing batch reactor has been confirmed as an attractive option for nitrogen removal in high-strength wastewater due to the low specific costs of nitrogen removal[1,2] so that many full-scale projects are currently being realized. Now that anammox-based N-removal has been shown to reduce costs and energy consumption compared to denitrification-based processes, its process stability appears to be the major issue making operators reluctant to opt for this process.

The start-up times required at full scale have decreased dramatically in recent years, confirming the progress made in terms of better understanding of the process as well as by empirically testing new operation strategies: Abma et al.[3] used a granular reactor to show the feasibility of anammox start-up with an increase of observed performance (0.055 d^{-1}) close to the maximum growth rate observed in the lab (0.06 d^{-1}[4]). Additional full-scale examples confirm that start-up no longer requires years but can now be regarded as a well-controlled and reliable procedure lasting no more than several months.[5−8]

Further aspects currently under active discussion are a comparison of reactor configurations and process control options: single-stage versus two-stage reactors (i.e., with segregated nitritation and anammox stages), suspended growth versus attached biofilm or granular reactors, selection of sensors required for process control, and a detailed strategy for embedding these sensors in automated process control systems are among the topics most actively pursued both in the laboratories and in

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full-scale operation. According to the authors' knowledge, such direct comparisons of different options are still difficult, since the various treatment schemes are still being optimized.

The present work discusses full-scale data of suspended sludge nitritation−anammox reactors with the help of lab experiments as a contribution to understanding process stability issues. Several options for improved process control in treatments combining nitritation and anammox in a single reactor are presented. We aim to contribute to the applicability of the process as well as providing a sound basis for discussing the suitability of alternative processes.

MATERIALS AND METHODS

Pilot and Full-Scale Sequencing Batch Reactor (SBR) Equipment. The pilot and full-scale sequencing batch reactor equipment used was compatible and consists of a fully stirred reactor, a stirrer, a feed pump, a decanter, and an aeration unit. The process is controlled by a programmable logical controller equipped with online sensors for the water level, ammonium, nitrate, volumetric airflow control to the aeration unit, soluble oxygen, temperature, pH, and conductivity. The SBR cycle always comprises a feeding phase, an aeration phase, a mixing phase, a sedimentation phase, and a discharge phase; at full scale, a pause of up to several days was included between the discharge phase and the subsequent feeding phases to adapt to the incoming load, while the pilot-scale reactor was operated without pausing. A complete cycle typically lasts between 6 and 10 h under normal conditions, depending primarily on the amount of supernatant to be treated, parameter settings like the aeration rate, and the sludge conditions. During start-up or a washout phase of nitrogen oxidizing bacteria (NOB), a cycle can also last up to several days. Full-scale data are taken from the nitritation anammox reactor at the wastewater treatment facility (WWTP) of Zurich-Werdholzli, which is equipped with two parallel reactors of 1400 m^3 each. The operational parameters and influent of the two reactors were identical except for one detail: the reactor with growing NOBs was equipped with old aerator membranes, while the other one was fitted with new aerators prior to being put into operation. An aerator-cleaning procedure was consequently performed every 2 weeks only on reactor South (Figure 4): each cleaning event resulting in 2 h of increased aeration (2600−2800 $m^3 \cdot h^{-1}$ after dosing with formic acid for scale removal). The pilot experiments were performed in a 400 L reactor fed with supernatant originating from the sludge dewatering plant at the WWTP Zurich-Werdholzli. The sludge originated from the full scale installation at the same WWTP (see ref 6 for more details). For the NOB washout experiment, sludge was taken from the full-scale reactor at WWTP Niederglatt (Switzerland), a 180 m^3 reactor equipped identically with the one in Zurich, at a time (December 2009) when the sludge featured high NOB activity.

Data Processing. Data processing was performed with Matlab (MathWorks Inc., Natick, USA) by directly accessing the raw data stored by the reactor's supervisory control and data acquisition (SCADA) system, with a time resolution of 10 s for the pilot-scale unit and 1 min for the full-scale installation.

The ammonium depletion rate was obtained by linear regression of the ammonium online signal (Endress+Hauser, ion-selective ammonium electrode Minical NAM 760) during the aeration phase, when ammonia oxidation and anammox occur simultaneously directly in the reactor.

The biomass growth or net activity (N removal) increase rate μ is calculated as follows:

$$\mu = \frac{\ln\left(\frac{r_t}{r_0}\right)}{t} \quad (1)$$

where r_0 and r_t are the rates of ammonium depletion at the beginning of the cycle and at a time t, respectively.

Fluorescent in Situ Hybridization (FISH) and Confocal Laser Scanning Microscopy. Sludge samples, taken regularly over 40 days, were fixed by adding 100 μL of 37% (v/v) stabilized formaldehyde (Sigma Aldrich, Germany) to 1 mL of sludge and incubated on ice for 1 h. The samples were subsequently washed three times by centrifugation for 3 min at 4 °C and 10 000g and resuspended in 1 mL of phosphate-buffered saline solution (PBS pH 7.2). Fixed samples were added to a 1:1 (v/v) mixture of ethanol/PBS and stored at −20 °C. A quantity of 10 μL was then applied to a well of epoxy-masked slides (Marienfeld, Lauda-Königshofen, Germany), dried for 15−30 min at 46 °C and sequentially dehydrated for 3 min in 50%, 80%, and 100% (v/v) ethanol/PBS solutions on ice. Next, 10 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8, 0.01% SDS, 35% formamide) and 1 μL of each fluorescent-labeled probe mix were added for staining all bacteria with 30 ng μL^{-1} of Cy5-labeled EUB-mix probe,[9,10] 30 ng μL^{-1} of Cy3 labeled AOB-mix probe,[11−14] and 50 ng μL^{-1} of FLUO-labeled NOB-mix[11,15] were added to the sample and incubated for 90 min at 46 °C in a humidified chamber (50 mL Greiner tubes). Following hybridization, a washing step was performed for 10 min at 48 °C in a buffer (0.07 M NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA) and the slides were dipped for 2 s into ice-cold deionized water. The slides were immediately dried under compressed air and mounted with Citi-fluor AF1 (Citifluor Ltd., London, UK). Oligo-nucleotide probes were obtained from Thermo-Fisher Scientific (Ulm, Germany). For visualization, at least 20 randomly acquired fields of view were recorded by confocal microscopy (Leica, SP5, Germany). Three images per field of view were acquired sequentially by using the 488, 520, and 633 nm laser lines to detect Cy3, Cy5, and FLUO signals, respectively. For one channel the same setting of the detector sensitivity was maintained for every field of view. Around 5% of the 600 images were not considered for image analysis due to overexposure or low biomass.

Quantification of FISH Images. Quantification of FISH images consisted in measuring the biosurfaces of each population. The images were treated using ImageJ (http://rsb.info.nih.gov/ij/) and a self-written macro. The threshold value was manually estimated for around 40 images (average = 30). It was then applied to all images to convert them from 8-bit to binary form. The suitability of the threshold was visually checked for each set of images. Biosurfaces of AOB to EUB, NOB to EUB, and AOB to NOB were then calculated.

Analytical Methods for Liquid Samples. Commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany, Test LCK303, LCK339, LCK340, LCK342; spectrophotometer type LASA 26) were used to test the accuracy of the online ammonium sensors as well as for the offline measurement of ammonium, nitrite, nitrate, and COD. In the NOB washout experiment, colorimetric nitrite tests with test strips (Nitrite-test, 0−10 mg $NO_2^- - N \cdot L^{-1}$, Merck KGaA, Darmstadt, Germany) were used.

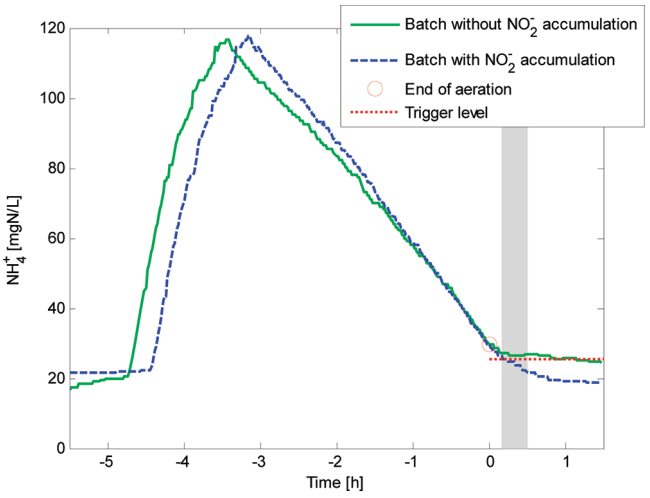

Figure 1. Overlay of a typical online NH_4^+ signal of two complete batches without (December 5, 2008, solid line) and with (December 9, dashed line) NO_2^- accumulation, respectively. For better comparison, the time scale has been shifted to fit zero to the end of the aeration phase. The gray area indicates the proposed observation time to check for NO_2^- accumulated during the preceding aeration. The dotted line shows the trigger level for switching to manual operation.

Monitoring NO_2^- Accumulation with the NH_4^+ Probe. Since an NO_2^- online sensor is currently not available, the NO_2^- concentration at the end of each batch was obtained by interpreting the online NH_4^+ signal (Figure 1): hereto the NH_4^+ depletion between 5 and 20 min after switching off the aeration was used, and the stoichiometric factor of 1.32 NO_2^- per NH_4^+ consumed by anammox[16] was considered.

NH_4^+ depletion requires either soluble O_2 (by AOB) or NO_2^- (by anammox). After the blower is switched off, oxygen is still available for about 5 min: it takes several minutes to shut down the blowers and to relax the membrane aerator; it takes half a minute for the bubbles to rise to the top of the reactor and less than a minute for the biomass to deplete the typically <0.5 mg $O_2 \cdot L^{-1}$ of soluble oxygen. During the following 20 min, the NH_4^+ depletion is monitored and assumed to depend principally on depletion of the residual NO_2^- accumulated in the reactor during the preceding aeration phase. NO_2^- formation due to heterotrophic denitrification is substrate-limited due to the low organic content of the influent and is thus low under normal operating conditions (as confirmed by the constant NO_3^- values; data not shown). On the basis of screening the online data, a threshold value of 2 mg NH_4^+—$N \cdot L^{-1}$ depletion is proposed (corresponding to 2.6 mg NO_2^-—$N \cdot L^{-1}$ according to the stoichiometry): if it is exceeded at the end of the aeration cycle, this indicates imbalanced O_2 supply and NO_2^- depletion. If this occurs, the control algorithm should trigger an automatic feeding stop and switch to manual operation with reduced aeration.

RESULTS AND DISCUSSION

Sudden Activity Loss. An event of uncontrolled activity loss and recovery during full-scale operation is shown in Figure 2: normal NH_4^+ depletion rates are seen until 6th August. On the 7th, the operator noticed a significant reduction of the ammonium depletion rate and consequently reduced the air supply by 40% to maintain typical dissolved oxygen levels during aeration

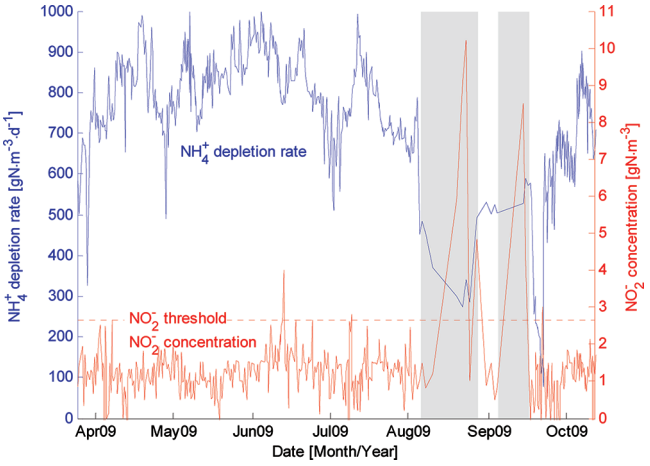

Figure 2. Ammonium depletion rate during aeration (blue, left axis) and nitrite concentration at the end of each aeration cycle (red, right axis; derived from the NH_4^+ signal) in the full-scale reactor (North lane) of Zürich-Werdhölzli. One data point for each SBR cycle is plotted. The gray area indicates operation at reduced aeration. The horizontal line at 2.6 mg NO_2^-—$N \cdot L^{-1}$ indicates the proposed threshold value for triggering the end of the automatic reactor feeding.

(0.4—0.6 mg$O_2 \cdot L^{-1}$, data not shown; the air supply was reduced from 0.86 to 0.5 $m^3_{air} \cdot m^{-3}_{reactor} \cdot h^{-1}$; combined nitritation—anammox is normally operated under oxygen-limiting conditions, so that the activity is proportional to the air supply). Nevertheless, a significant increase of the accumulated NO_2^- is observed at the end of the aeration phase, indicating significant inhibition of the NO_2^- depletion by anammox. For about 1.5 months the reactor was operated at reduced aeration and with one batch loading every 2.5 days on average (the rest being stirred idle time; under normal operating conditions, three batches are processed each day). Nevertheless, Figure 2 shows that the actual activity probably recovered faster: the 1.5 months taken to return to the previous operational settings represent a cautious choice of the operator having to handle an unexpected performance problem at full scale. On 17th September, regular operation was resumed and NH_4^+ depletion rates quickly returned to normal levels. No other interventions were carried out during this period. The direct cause of the activity loss has not yet been identified, but it is speculated that a toxic compound was contained in the influent supernatant.

Different effects have been discussed to explain how anammox inhibition can occur during combined nitritation—anammox. Kindaichi et al.[17] describe inhibition of anammox within sections of a biofilm reactor and speculate that organic compounds originating from the biomass may be the cause. Methanol has been shown to irreversibly inhibit anammox[18] but is very unlikely to occur in digester supernatant. In presence of degradable organic carbon, denitrifiers outcompete anammox for nitrite,[19,20] but since this is leading to NO_2^- depletion rather than accumulation, this case is not deemed relevant for the performance loss discussed above.

Nitrite toxicity has been discussed.[21] Recently Abma et al.[3] describe that up to 42 mg NO_2^--$N \cdot L^{-1}$ did not affect process performance of granular sludge. Suspended sludge systems have been successfully started up at nitrite concentrations in the range of 100—200 mg NO_2^--$N \cdot L^{-1}$,[22] thus proving that nitrite toxicity is not relevant for long-term operation under typical conditions.

Sulfide has been shown to affect anammox activity <20 mg $S^{2-} \cdot L^{-1}$ [23]; sulfide or compounds containing S^{2-} can be contained in

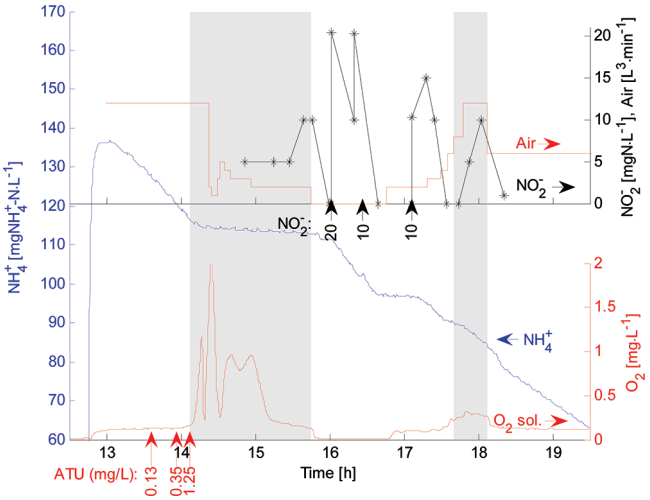

Figure 3. Experiment showing reversible inhibition of the anammox activity at bulk liquid oxygen concentrations <0.2 mg $O_2 \cdot L^{-1}$. The gray areas indicate times of complete (left) or partial (right) anammox inhibition. The vertical red arrows show the addition of allylthiourea (ATU) with indication of the totalized soluble concentration in mg·L^{-1}; the vertical black arrows show nitrite addition with numerical indication of the mg NO_2^--N·L^{-1} added at each event. The horizontal arrows in the legend indicate the respective *Y* scale.

digester supernatant (e.g., in bio-P plants where Fe^{3+} is not used to precipitate phosphorus).

Partial Inhibition of AOB May Explain the Activity Loss. Pilot-scale experiments in the 400 L SBR reactor as shown in Figure 3 confirm that partial inhibition of the AOB can result in NO_2^- accumulation and thus explain the temporary activity loss shown in Figure 2. The chronology of the experiment of Figure 3 is given below. Prior to the experiment, the pilot SBR was operated continuously for several months with a normal process rate.

- Time 12.45: start of a normal SBR cycle by feeding with untreated supernatant leading to the rise of the NH_4^+ concentration to ca. 137 mg NH_4^+-N·L^{-1}
- 12.45 to 13.30: regular operation with continuous aeration (at 12 L·min^{-1}; the higher specific aeration compared to the full-scale reactor is due to a reactor depth of only 1 m at pilot scale compared to 5.5 m at full scale) leading to normal NH_4^+ depletion via the nitritation-anammox process at a typical rate of ca. 20 mg N·L^{-1}·h^{-1}.
- 13.30 to 14.05: stepwise addition of allylthiourea in three aliquots to reach a concentration of 1.25 mg ATU·L^{-1}. The oxygen increase confirms the inhibition of the AOB leading to reduced depletion of the supplied O_2. The very slow NH_4^+ depletion slope confirms that the activity of the anammox biomass is also reduced.
- 14.25 to 14.55: the air supply is reduced in steps from 10 to 2 L·min^{-1} to avoid soluble oxygen concentrations >0.5 mg $O_2 \cdot L^{-1}$. NO_2^- starts accumulating, since its depletion (anammox) is completely inhibited, while its formation (AOB) is only partly inhibited (note that NO_2^- concentrations were measured using colorimetric test strips with an assumed relative error of approximately 50%).
- 15.45 to 16.45: the air supply is completely switched off. Within less than 20 min, the NH_4^+ depletion is back to its initial rate, thus confirming that the anammox inhibition disappears as soon as (a) the soluble O_2 concentration is depleted, and (b) NO_2^- is available.[24,25] So the continued presence of ATU does not interfere with the anammox activity. To confirm this, NO_2^- is added twice (20 mg NO_2^--N·L^{-1} at 16.05 and another 10 mg NO_2^--N·L^{-1} at 16.25).
- 16.45 to 17.40: the air supply is gradually increased without the soluble oxygen exceeding 0.2 mg $O_2 \cdot L^{-1}$. The fact that the rate of increase of soluble O_2 at an aeration rate of 6 L·min^{-1} is now much slower than immediately after addition of the inhibiting ATU (i.e., between 14.05 and 15.00) shows that ATU has been partly degraded within 2.5 h so that the AOB inhibition has diminished significantly. The last addition of 10 mgNO_2^--N·L^{-1} at 17.05 confirmed that the anammox activity is limited by the availability of NO_2^-.
- 17.40 to 18.05: further increase of aeration leads to complete inhibition of the anammox and thus to NO_2^- accumulation.
- 18.05 onward overnight: reduced aeration (6 L·min^{-1}) without any NO_2^- accumulation.
- Next day: the reactor returned to its usual aeration rate (12 L·min^{-1}) with a NH_4^+ depletion rate of ca. 20 mg N·L^{-1}·h^{-1} as before the experiment, thus confirming complete degradation of ATU and the reversibility of the inhibition of the anammox biomass (growing too slowly for significant recovery overnight).

The rationale of the interpretation of the experiment in Figure 3 is as follows: decreasing AOB activity resulted in lower O_2 depletion, leading indirectly to the inhibition of anammox by increased oxygen concentrations inside the flocs. Since anammox activity has been found in different activated sludge samples,[5,26] it can be expected that these anaerobic bacteria are also well adapted to survive prolonged exposure to oxygen. Thus, partial toxic inhibition of AOB is seen as a plausible explanation of the sudden performance loss and NO_2^- accumulation described previously at full scale. During start-up of the full scale installation at Zurich-Werdholzli, a comparable activity loss has been observed with the decrease in AOB activity preceding the drop in anammox activity by weeks.[6] According to the modeling of Hao et al.,[27] alternatively a significant decrease in typical sludge particle diameter might also have caused the observed N removal performance loss and NO_2^- accumulation: to achieve optimal anammox activity inside flocs of decreasing size, aeration must occur at a lower soluble oxygen set point; since typical floc diameter is currently not routinely monitored, such a change would go unnoticed (except in case of resulting in significant impaired sludge settling which did not occur).

Short-Term Aeration Pulses May Lead to Healthy NOB Population. At Zürich-Werdhölzli, two full-scale reactors are operated in parallel. After 2 years of successful operation, one of the two reactors (South) was found to have developed quite a strong NOB population, as evidenced by the effluent nitrate values increasing from typically below 15 mg NO_3^--N·L^{-1} (up to the end of June 2009) to over 200 mg NO_3^--N·L^{-1} in mid-October 2009 (>30% of the influent NH_4^+), while the effluent of reactor North was 10 mg NO_3^--N·L^{-1} on average, and never increasing above 35 mg NO_3^--N·L^{-1} (data not shown). The operational parameters and influents of the two reactors were identical except for one detail: the reactor with growing NOBs was equipped with old aerator membranes requiring a cleaning procedure resulting in a significant increase of the aeration during 2 h every 2 weeks (Figure 4); during this time of increased aeration, the bulk O_2 concentration did not increase significantly above 1 mg$O_2 \cdot L^{-1}$, and so did not reach alarming levels of

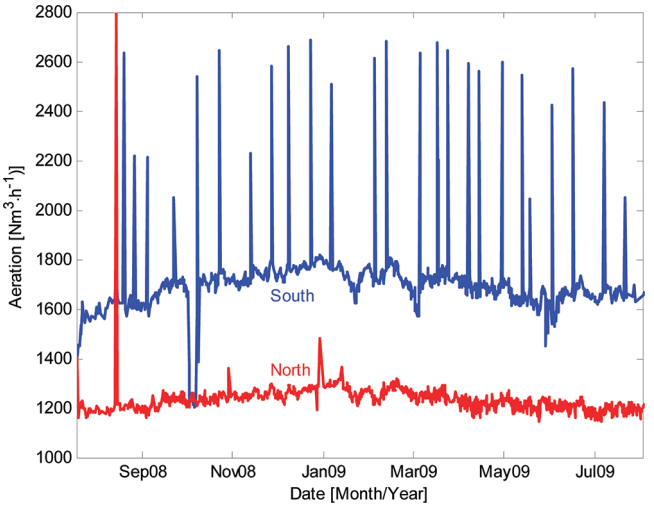

Figure 4. Average air supply rate during aeration in the two reactors operated in parallel. The aeration spikes in reactor South show the aerator membrane cleaning procedure performed only in this reactor, on average every 2 weeks.

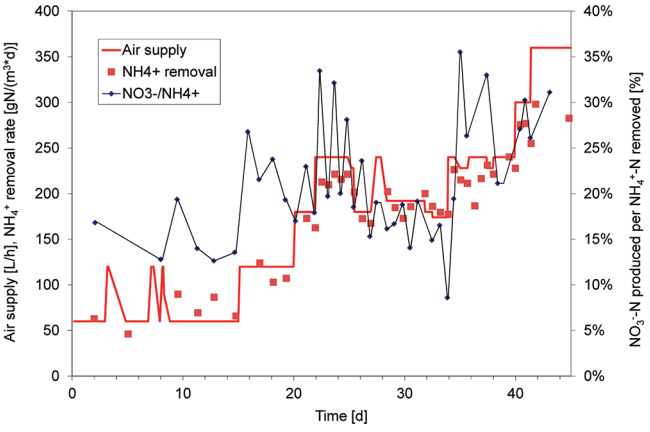

Figure 5. Experimental washout of nitrite oxidizers under normal operating conditions. Over 45 days the air supply and the NH_4^+ removal rate (left axis) could be increased in steps without the ratio of NO_3^- formed per NH_4^+ removed (right axis) permanently exceeding 30%.

short-term exposure. It is assumed that during these cleaning events the oxygen supply exceeded the oxygen depletion rate of the AOB, thus leading to reversible inhibition of the anammox, and transiently to optimal growing conditions for NOB, with NO_2^- accumulating and O_2 not completely depleted by AOBs. This growth is assumed to have eventually led to a healthy NOB population successfully competing with AOB for O_2 and with anammox for NO_2^-. The activity in reactor North also decreased gradually in July and August 2009 as previously described (Figure 2). During the same period in reactor South, the NO_3^- values started increasing at a rate of about 0.05 d^{-1} without showing any NO_2^- accumulation. It is thus concluded that the postulated toxic influent led in both reactors to an inhibition NO_2^- depletion by anammox, but also provided further support to NOB growth in reactor South, where an increased abundance of NOB had previously developed, while NO_2^- accumulation was observed in reactor North.

After the event, reactor South was emptied and its sludge discarded. By splitting the uncontaminated sludge of reactor North between both reactors, reactors South and North were back to full capacity within a few weeks.

Stable operation of the very similar CANON system has been shown at lab scale with synthetic wastewater to require oxygen limiting conditions,[25,28] i.e., to avoid inhibition of anammox as well as for allowing AOBs to outcompete NOBs. According to Wiesmann et al.,[29] it is expected that the significantly higher affinity for oxygen of AOB compared to NOBs is responsible for this competition (half-saturation constant of 0.6 compared to 2.2 mg $O_2 \cdot L^{-1}$). Similarly, Hawkins et al.[30] showed in a nitrification reactor under saturated DO conditions that NOB activity was suppressed as long as AOBs were not substrate limited; the observed rise in NOB activity after depletion of ammonium indicates that diffusive mass transfer limitation combined with higher affinity of AOB to oxygen is responsible for suppressing NOB activity. According to present experience from full-scale operation, the treatment of digester supernatant results in a different competition situation for NOBs: numerous full-scale combined nitritation−anammox system face at times effluent NO_3^- concentrations >100 mg NO_3^--N·L^{-1} (thus achieving reduced N removal), in spite of keeping O_2 availability rate limiting and avoiding NH_4^+ limitation (personal communication from various operators). In several circumstances, the problem of NOB activity could be solved only by discarding the NOB containing sludge and reinoculation from another reactor devoid of NOBs. Thus, better understanding and strategies for reducing NOB activity are still a relevant issue for process stability. Hao et al.[27] confirm in their modeling effort that the ratio of different affinity constants is crucial to NOB versus AOB/anammox population dynamics and currently still uncertain, thus explaining the discrepancy between the mentioned lab- and full-scale operation.

NOB Washout during Regular Operation. Previously published experiments[6] had shown that the decay rate of NOB is too slow to decrease the abundance of NOB without active sludge withdrawal. Unpublished results in our lab showed that NH_3 concentration in a typical range (i.e., up to pH 8 and 200 mg NH_4^+-N/L equivalent to 10 mg NH_3-N/L) do not to impact on NOB growth sufficiently to be suitable for controlling their growth.

The experiment illustrated in Figures 5 and 6 demonstrates that under regular operating conditions a strong population of NOB can be washed out by sludge removal at a sludge age of around 45 days. In addition, the availability of O_2 and NO_2^- was limited to restrict NOB growth by controlling the aeration. During the experiment, the aeration was slowly increased in steps in order to stop the effluent NO_3^- concentration from permanently exceeding 30% of the removed NH_4^+-N: a step increase was performed only after the NO_3^- production had dropped below 20% of the NH_4^+ removal. At each step increase of the aeration rate, the NO_3^- production increased transiently, but was reduced to below 20% after a week or two of operation at an unchanged aeration rate. Prompt depletion kept the concentrations of O_2 and NO_2^- below <0.5 mg$O_2 \cdot L^{-1}$ and <1 mgNO_2^--N·L^{-1}, respectively, for the entire duration of the experiment. Since several groups had shown a higher affinity of AOB toward O_2 compared to NOBs, it is expected that competition for molecular oxygen rather than for NO_2^- is crucial for limiting NOB growth.[5,30]

Control of the Aeration Rate. The aeration must be controlled in order to match the O_2 supply to the AOB activity, since providing more oxygen than the AOB are able to deplete promptly leads to NOB growth and may reversibly inhibit anammox

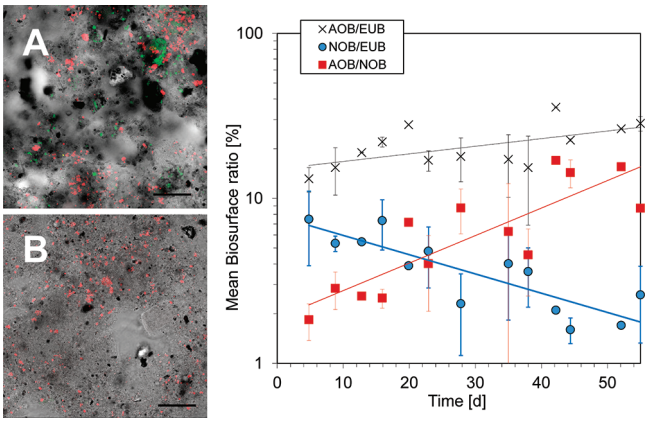

Figure 6. Confocal laser scanning microscopy images taken from nitritation anammox sludge on day 5 (A) and day 55 (B) of the NOB washout experiment. FISH probes used: AOB in red, NOB in green, transmitted light grayscale. The black bar indicates 50 μm. The graph on the right shows the image analysis confirming the trend of decreased NOB to AOB activity ratio shown in Figure 5. Error bars indicate the standard deviation where multiple images have been taken ($2 \leq n \leq 4$, n indicating the mean of 20−40 images analyzed per well).

activity.[27] Figure 3 shows that the oxygen concentration as measured in the bulk may not be taken as a measure for maximum sustainable operation without considering further indications of activity: while a good N removal rate was achieved at 0.2 mg$O_2 \cdot L^{-1}$ under normal operating conditions, at the same bulk oxygen concentration but with decreased activity of the AOB the anammox biomass was completely inhibited, resulting in optimal growth conditions for NOBs (i.e., sufficient O_2 and NO_2^- were available simultaneously). The same conclusion is drawn from the oxygen concentrations monitored at full scale in the event illustrated in Figure 2: controlling the aeration according to the dissolved oxygen did not allow avoiding NO_2^- accumulation.

It is thus suggested that the aeration rate be controlled according to the soluble NO_2^- concentration: as long as the NO_2^- formed by the AOB is promptly depleted by the anammox, the aeration rate is sustainable, but as soon as NO_2^- starts accumulating to >2 mg NO_2^--N·L^{-1}, this indicates that conditions propitious for NOB growth are present, so the aeration rate is too high.

Since reliable online NO_2^- measurement in activated sludge with an accuracy of <1 mg NO_2^--N·L^{-1} and a background nitrate concentration somewhere between 0 and 200 mg NO_3^--N·L^{-1} remain a challenge, the nitrite concentration can currently be assessed either by sampling and offline measurement or indirectly by monitoring the NH_4^+ depletion in the absence of soluble oxygen (as discussed previously). Since anammox biomass exhibits rather slow growth of 0.06 d^{-1},[4] the aeration is normally also increased at a rather slow rate (i.e., increase of the aeration rate once weekly during the start-up phase), while downward corrections must be carried out significantly more quickly (e.g., in case a toxicant is present in the influent). According to current experience with supernatant originating from digestion of municipal sludge (supplemented with up to 20% industrial waste), automatic monitoring of the NO_2^- at the end of each batch (i.e., every 8 h) suffices under normal operating conditions for SBR operation at an exchange volume of up to 25%.

Finally, the experiment shown in Figures 5 and 6 suggests that it is appropriate to limit the aeration rate for transforming up to 20% of the oxidized ammonium to nitrate, thus limiting the growth of the NOBs to an extent resulting in washout at a feasible sludge age of 45 days.

Compliance with the above rules results in limiting the net activity of nitritation−anammox by the air supply, so that the N removal rate is proportional to the volume of air supplied under normal operating conditions. The aeration of the system is consequently controlled more effectively by the volumetric air flow rate than by the soluble O_2 concentration as measured in the bulk liquid (as is usual for activated sludge processes).

Detecting NOB Growth. Process stability requires the NOB population to be kept constantly at a very low level. As discussed previously (Figure 4), NOB growth can be slow and go unnoticed for long time, since effluent NO_3^- may also result from anammox directly (transforming ca. 10% of the removed nitrogen to nitrate) and may be depleted by denitrification to an extent depending on the availability of degradable COD. In view of the process stability issues discussed in this article, it is assumed that operators are interested in methods with sufficiently low quantification limits, so that the NOB levels may be controlled on a monthly basis and remain well below those apparent from high NO_3^- production.

Because of its better quantification limit, the quantitative polymerase chain reaction (qPCR; detecting down to a few copies per sample[31]) is expected to be more suitable than FISH, since the latter has a typical quantification limit of 2−5% of the biomass.[32] Several authors have applied qPCR to anammox sludge, but for other purposes and mostly focusing on quantifying the anammox rather than the NOB.[7,26,30,33,34,35] Thus, the applicability of qPCR for this purpose has not yet been confirmed.

Floc Structure. The reversible inhibition of the anammox biomass in the presence of molecular oxygen means that the biofilm may be clearly structured in terms of activity (i.e., AOB activity in the outer layer and anammox activity in the inner anaerobic core), but this need not translate to a corresponding stratification of the biomass itself: according to the reversibility of the inhibition of anammox, a homogeneous distribution of this biomass cannot be excluded, possibly resulting from flocs growing and falling apart randomly, thus not featuring clear structures in biomass stratification, as observed also by ref 5. The direct analysis of granular sludge by Vlaeminck et al.[36] did not show a clear stratification (i.e., with anammox embedded as a core while AOB and NOB were located in the outer layers) in some reactors while it did in others (e.g., ref 37). This issue of relevance to understanding the conditions needed to avoid NOB growth has consequently not yet been resolved, at least to the author's knowledge.

■ ASSOCIATED CONTENT

Supporting Information. Table of the oligonucleotide probes used for FISH analysis of AOB, NOB and all bacteria. This material is available free of charge via the Internet at http://pubs.acs.org.

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