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Successful application of nitrification/anammox to wastewater with elevated organic carbon to ammonia ratios

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ABSTRACT

The nitrification/anammox process has been mainly applied to high-strength nitrogenous wastewaters with very low biodegradable organic carbon content ($< 0.5 \text{ g COD} \cdot \text{g N}^{-1}$). However, several wastewaters have biodegradable organic carbon to nitrogen (COD/N) ratios between 0.5 and $1.7 \text{ g COD} \cdot \text{g N}^{-1}$ and thus, contain elevated amounts of organic carbon but not enough for heterotrophic denitrification. In this study, the influence of elevated COD/N ratios was studied on a nitrification/anammox process with suspended sludge. In a step-wise manner, the influent COD/N ratio was increased to $1.4 \text{ g COD} \cdot \text{g N}^{-1}$ by supplementing digester supernatant with acetate. The increasing availability of COD led to an increase of the nitrogen removal efficiency from around 85% with pure digester supernatant to $>95\%$ with added acetate while the nitrogen elimination rate stayed constant ($275 \pm 40 \text{ mg N} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$). Anammox activity and abundance of anammox bacteria (AMX) were strongly correlated, and with increasing influent COD/N ratio both decreased steadily. At the same time, heterotrophic denitrification with nitrite and the activity of ammonia oxidising bacteria (AOB) gradually increased. Simultaneously, the sludge retention time (SRT) decreased significantly with increasing COD loading to about 15 days and reached critical values for the slowly growing AMX. When the SRT was increased by reducing biomass loss with the effluent, AMX activity and abundance started to rise again, while the AOB activity remained unaltered. Fluorescent *in-situ* hybridisation (FISH) showed that the initial AMX community shifted within only 40 days from a mixed AMX community to “*Candidatus Brocadia fulgida*” as the dominant AMX type with an influent COD/N ratio of $0.8 \text{ g COD} \cdot \text{g N}^{-1}$ and higher. “*Ca. Brocadia fulgida*” is known to oxidise acetate, and its ability to outcompete other types of AMX indicates that AMX participated in acetate oxidation. In a later phase, glucose was added to the influent instead of acetate. The new substrate composition did not significantly influence the nitrogen removal nor the AMX activity, and “*Ca. Brocadia fulgida*” remained the dominant type of AMX. Overall, this study showed that AMX can coexist with heterotrophic bacteria at elevated influent COD/N ratios if a sufficiently high SRT is maintained.

26 **KEYWORDS**

27 Biological nitrogen removal

28 Anammox

29 “*Ca. Brocadia fulgida*”

30 Denitrification

31 Nitrification

32 Urine separation

1. INTRODUCTION

The combination of nitrification and anammox is a cost saving alternative to conventional biological nitrogen removal via heterotrophic denitrification (Siegrist et al. 2008, Vlaeminck et al. 2012). Many studies have investigated various types of nitrification/anammox reactors with dedicated attention for the biomass growth mode as biofilm, flocs and/or granules (Vlaeminck et al. 2010), and the influence of process conditions such as the temperature (Vázquez-Padín et al. 2011), dissolved oxygen concentration (Strous et al. 1997), salinity (Liu et al. 2009) and inhibitory substances (Dapena-Mora et al. 2007).

The term "biodegradable organic carbon" refers to the difference between the dissolved COD in the influent and the dissolved COD in the effluent, i.e. the organic carbon, which is actually degraded in a reactor. So far, nitrification/anammox has mainly been applied for wastewaters with high ammonia concentrations and low concentrations of biodegradable organic substances such as digester supernatant, in which the ratio of biodegradable organic carbon to ammonia nitrogen (COD/N) is lower than 0.5 g COD·g N⁻¹ (Joss et al. 2009, van der Star et al. 2007, Wett 2007). In contrast to this, complete heterotrophic denitrification via nitrite requires a COD/N ratio of more than 1.71 g COD·g N⁻¹. However, several wastewaters have COD/N ratios between 0.5 and 1.71 g COD·g N⁻¹ and thus, contain elevated amounts of organic carbon but not enough for heterotrophic denitrification. If no organic substrate should be dosed, anammox bacteria (AMX) are needed for complete biological nitrogen removal. The application of the nitrification/anammox process has, for example, already been proposed as a cost efficient method for nitrogen removal from urine (Udert et al. 2008, Wilsenach and van Loosdrecht 2006). Stored urine has a theoretical COD/N ratio of approximately 1 g COD·g N⁻¹ (Udert et al. 2006) which can increase up to 1.5 g COD·g N⁻¹ (Bürgmann et al. 2011) due to ammonia volatilization during storage (i.e. a decrease of the ammonium concentration while the COD concentrations stays constant). AMX are also needed for a complete nitrogen removal in animal manure, especially piggery wastewater where the COD/N ratios can vary between 4 and 6.5 g COD·g N⁻¹, but this is sometimes also too low to achieve complete denitrification (Bernet and Béline 2009). COD/N

ratios of approximately $1 \text{ g COD} \cdot \text{g N}^{-1}$ are also expected in the recently discussed integration of anammox into mainstream wastewater treatment (De Clippeleir et al. 2013, Winkler et al. 2012a).

The possible effects of COD on the nitrification/anammox process depend on the type of COD. Firstly, biodegradable organic carbon fosters the growth of heterotrophic bacteria (HET) which compete with ammonia oxidising bacteria (AOB) for oxygen and with AMX for nitrite. Additionally, more heterotrophic growth will lead to a higher sludge production, which in turn often leads to a higher sludge loss, and thus to a decrease in the sludge retention time (SRT). Sludge loss is the total amount of sludge which leaves the system, i.e. the sludge in the effluent, the sludge in the samples and in case the SRT is regulated, also the sludge which is removed to maintain a certain SRT. A low SRT is particularly critical for the slowly growing AMX. Several studies reported decreasing AMX and AOB activities with high COD concentrations in the influent. Chamchoi et al. (2008) for instance, reported that AMX were outcompeted by HET when the COD/N ratio in the influent of a complete anoxic system was higher than $2.0 \text{ g COD} \cdot \text{g N}^{-1}$ (SRT not mentioned). Zhu and Chen (2001) observed a 70% reduction of AOB activity when the influent COD/N ratio of a nitrification reactor was between 1.8 and $3.5 \text{ g COD} \cdot \text{g N}^{-1}$ (SRT not mentioned). On the other hand, Desloover et al. (2011) successfully operated a partial nitrification reactor with an influent COD/N ratio of $3.70 \text{ g COD} \cdot \text{g N}^{-1}$ (SRT $1.7 \pm 0.5 \text{ d}$) and a subsequent anammox reactor with an influent COD/N ratio of $1.60 \text{ g COD} \cdot \text{g N}^{-1}$ (SRT 46 d). None of these studies discussed whether competition for nitrite, oxygen or space (low SRT) caused the observed problems and which were the crucial factors for a successful operation with elevated COD/N influent ratios. Often, important information to judge this, as e.g. the SRT and the type of COD, is missing.

Secondly, specific organic compounds can also be directly toxic for AOB and AMX. Complete inhibition of AOB was for instance observed in the presence of $1000 \text{ mg} \cdot \text{L}^{-1}$ propionate, $1000 \text{ mg} \cdot \text{L}^{-1}$ butyrate, $2000 \text{ mg} \cdot \text{L}^{-1}$ acetate (Gomez et al. 2000), $3.7 \text{ mg} \cdot \text{L}^{-1}$ phenol or $1.3 \text{ mg} \cdot \text{L}^{-1}$ cresol (Dyreborg and Arvin 1995). For AMX, Güven et al. (2005) observed a complete loss of activity in the presence of $23 \text{ mg} \cdot \text{L}^{-1}$ methanol.

Thirdly, due to the ability of some anammox species to oxidise fatty acids while reducing nitrate (Güven et al. 2005, Kartal et al. 2007), fatty acids could foster AMX activity and increase their competitiveness. Apparently, the fatty acids are not integrated into biomass but are instead oxidised to carbon dioxide, while the carbon for growth is fixed from CO₂ via acetyl-CoA. The oxidation of fatty acids might only occur for the conservation of energy, which could in turn lead to a higher biomass yield for AMX (Kartal et al. 2008), but this hypothesis has not yet been proven.

So far, one-stage nitrification/anammox systems have mainly been operated with influent COD/N ratios below 0.5 g COD·g N⁻¹. In one of two studies which were performed with elevated COD/N influent ratios in biofilm systems, Chen et al. (2009) observed a significant reduction of the nitrogen removal efficiency (NRE) from 79% to below 52% when the influent COD/N ratio was increased from 0.5 to 0.75 g COD·g N⁻¹ (synthetic influent, type of COD and SRT not mentioned). In contrast to this, Jia et al. (2012) achieved almost 95% total NRE with an influent COD/N ratio of 1.28 g COD·g N⁻¹ (synthetic solution with glucose, SRT not mentioned). In case of suspended sludge systems, Lackner and Horn (2013) reached 80-85% total nitrogen removal with an influent COD/N ratio of 1.00 ± 0.27 g COD·g N⁻¹ (wastewater with high COD concentration, but type of COD and SRT are not mentioned) in a sequencing batch reactor. In our own previous studies with a suspended sludge system and five-times diluted urine (1.4 -1.5 g COD·g N⁻¹) we observed process instabilities (Udert et al. 2008) and a population shift to a new stable state characterised with a low nitrogen elimination rate and limited AMX activity (Bürgmann et al. 2011). In most of these studies the type of COD is not known and especially in case of urine, it is not possible to separate the influence of the elevated COD/N influent ratio from the influence of other wastewater compounds on the process.

Higher SRTs and having several zones with different redox conditions (oxic and anoxic zones) are advantages of biofilm systems for the slowly growing AMX. However, the handling and especially the start-up of suspended sludge systems is simpler, and most of the full-scale anammox applications in Switzerland are operated with suspended sludge (e.g. Joss et al. (2009)). We therefore considered

suspended sludge systems to be more relevant for practical applications and decided to use a suspended sludge system for our study.

The goal of this study was to test the feasibility of the nitrification/anammox process for wastewaters with COD/N ratios of about $1.4 \text{ g COD} \cdot \text{g N}^{-1}$, which is relevant for stored urine. Since acetate accounts for about 50% of the total COD in stored urine (Udert et al. 2013), we also wanted to test whether the oxidability of acetate provides a specific advantage for AMX. In order to minimise other influences from potentially toxic influent compounds, a sequencing batch reactor was operated with digester supernatant, which is a typical substrate for nitrification/anammox processes, and the influent COD/N ratio was increased stepwise up to $1.4 \text{ g COD} \cdot \text{g N}^{-1}$ by adding increasing amounts of acetate. In the end, acetate was replaced with glucose, which supposedly cannot be degraded by AMX directly.

2. MATERIAL AND METHODS

2.1 Reactor operation

The lab-scale reactor was started with suspended biomass from a nitrification/anammox reactor treating sludge digester supernatant (WWTP Limmattal, Dietikon, Switzerland). This reactor is operated with hydrocyclones which enriches the granules (Wett et al. 2010). We collected a combined sample of the hydrocyclone under- and overflow that yielded an overall average volumetric particle size of $300 \pm 6 \mu\text{m}$. This mixture of granules and flocs is subsequently termed 'suspended biomass'. The initial concentration of volatile suspended solids (VSS) in the lab-scale reactor was $\sim 4 \text{ g} \cdot \text{L}^{-1}$. In the first phase of the experiment, the sludge retention time (SRT) was not controlled, i.e. sludge loss only occurred through washout in the effluent (the effluent withdrawal occurred at a constant depth) and sampling (for VSS concentration in the reactor and FISH samples). Subsequently, from day 129 onwards, the sludge from the effluent was returned to the reactor to increase the SRT. The SRT was calculated based on the VSS concentration in the reactor and the VSS loss per day. The reactor was operated with a liquid volume of 6.7 L and was equipped with sensors for online measurement of conductivity (Tetracon 325,

WTW, Weilheim, Germany), dissolved oxygen (TriOxmatic 700, WTW, Weilheim, Germany), water level (Waterpilot FMX 167, Endress und Hauser, Reinach, Switzerland), pH, temperature, ammonia, nitrate and potassium (CAS40d, Endress und Hauser, Reinach, Switzerland). The reactor was fed with digester supernatant from the WWTP Werdhölzli in Zürich, Switzerland (composition see Table 1). The temperature was regulated at 30°C with a thermostat. pH was not controlled and fluctuated between 7.8 to 8.3 at the beginning of each cycle and 7.0 to 7.3 at the end.

The reactor was operated in sequencing batch mode. After the feeding period (~ 3 min), a 20 min anoxic phase was followed by alternating aerobic (40 min) and anoxic phases (20 min) until the ammonia concentration decreased below 10 mg N·L⁻¹. Afterwards, the sludge was allowed to settle for 20 min prior to effluent withdrawal (~ 9 min). The effluent was withdrawn at a constant depth of 10 cm below the initial water level. Together with the sedimentation time of 20 min, this results in a minimum settling velocity of 0.3 m·h⁻¹. The exchanged volume per cycle was 1.3 L, i.e. about 20% of the liquid volume present and the cycle time is varied between 8.5 and 18 h (supporting information S1). During aerobic phases, the reactor was aerated with 81 mL·min⁻¹ air, which resulted in average oxygen concentrations of 0.07 mg·L⁻¹. On day 160, the aeration rate was accidentally increased from 81 to 228 mL·min⁻¹. However, the average oxygen concentration did not change.

The influent COD/N ratio was increased in a stepwise manner every 2 weeks by adding the required volume of a sodium acetate solution (Figure 1). At first, sodium acetate was added to the influent tank (peak on day 14). However, with this method acetate was degraded in the influent tank, and the desired influent COD/N ratio could not be reached. In order to solve this problem, a separate pump (diaphragm metering pump, beta 4, ProMinent, Regensdorf, Switzerland) and tank were added for the sodium acetate solution. This pump was coupled to the influent pump (peristaltic pump, Vario Pumpsystem, Ismatec, Glattbrugg, Switzerland) which itself was coupled to a water level sensor. From day 159 onwards, the acetate was replaced by glucose maintaining a COD/N ratio of 1.4 g COD·g N⁻¹.

2.2 Sampling

Aliquots from the influent tank, the effluent tank, and the reactor were taken three times a week. Samples for the analysis of dissolved compounds were immediately filtered (MN GF-5, pore size 0.45 μm , Macherey-Nagel, Düren, Germany) and stored in the fridge for 1-2 days prior to analysis. Total suspended solids (TSS) and VSS samples were processed immediately after sampling. Aliquots for the determination of the particle size distribution were taken on day 0, 115 and 194 and stored overnight prior to analysis. For the batch activity measurement, the necessary amount of sludge (based on the VSS concentration) was removed from the reactor immediately before the start of the experiment. Sludge samples for FISH analysis were taken once a week in triplicate.

2.3 Batch activity measurements

For the batch activity measurements, biomass was taken from the reactor, washed once, and diluted with sodium phosphate buffer ($0.01 \text{ mol} \cdot \text{L}^{-1}$, pH 8.35) to a total volume of 0.6 L in order to obtain a VSS concentration of approximately $1 \text{ g} \cdot \text{L}^{-1}$. 1.2 mL of a trace element stock solution (Kuai and Verstraete 1998) was added. The temperature was kept at 30°C . For AMX activity tests, the reactor was closed and anaerobic conditions were established by sparging the solution with $600 \text{ mL} \cdot \text{min}^{-1} \text{ N}_2$ gas containing 2% CO_2 for 20 min prior to the experiment. The pH was increased to 7.2 by adding NaOH solution and kept constant during the experiment (7.2 ± 0.1) by regulating the CO_2 flow ($0\text{--}60 \text{ mL} \cdot \text{min}^{-1}$). $60 \text{ mg N} \cdot \text{L}^{-1}$ of both NH_4^+ (NH_4Cl) and NO_2^- (NaNO_2) were added, and liquid samples for the analysis of the nitrogen compounds were taken every 30 min for a total duration of 2 h. During the experiment, only the headspace was flushed with N_2 ($540 \text{ mL} \cdot \text{min}^{-1}$) and 10 to $60 \text{ mL} \cdot \text{min}^{-1}$ of 20% CO_2 in N_2 gas to keep the pH constant. After the 2 h, the anoxic phase continued until the NO_2^- concentration reached values below $20 \text{ mg N} \cdot \text{L}^{-1}$. Then, the reactor was opened, the airflow was switched to synthetic air (80% N_2 , 20% O_2), and the sludge was aerated until reaching at least $4 \text{ mg O}_2 \cdot \text{L}^{-1}$ in order to determine the activity of AOB and nitrite oxidising bacteria (NOB). NH_4^+ (NH_4Cl) was added to reach a minimum ammonia concentration of $80 \text{ mg N} \cdot \text{L}^{-1}$. Afterwards, aliquots were taken every 30 min for a

total duration of 2 h for the analysis of the nitrogen compounds. Samples for the measurement of TSS and VSS were taken at the beginning (before AMX activity tests) and at the end (after AOB and NOB activity test) of each experiment. At the end of each batch experiment, the filtered sludge was returned to the reactor.

AMX (NH_4^+ consumption), AOB (NH_4^+ consumption), and NOB (NO_3^- production) activities were calculated as the slopes from 5 data points using the least squares method. The measurement uncertainties of the resulting rate are given as the standard error of the calculated slopes. In order to determine the maximum possible activities in the sequencing batch reactor (SBR; $\text{mg N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$), the calculated values in $\text{mg N}\cdot\text{gVSS}^{-1}\cdot\text{d}^{-1}$ were multiplied with the VSS concentration measured in the SBR on the same day.

In order to compare the AMX activity with the FISH data, we assumed that the conditions in the batch activity measurements allowed a maximum growth, that the assimilation of ammonium into biomass can be ignored due to the low yield of AMX, and thus that AMX quantities can be calculated with the measured rates as follows:

$$X_{AMX} = r_{NH4} \times \frac{Y_{AMX}}{\mu_{max,AMX}} \quad (1)$$

where X_{AMX} is the AMX concentration ($\text{mg COD}\cdot\text{L}^{-1}$), r_{NH4} is the measured maximum ammonia degradation rate ($\text{mg NH}_4\text{-N}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$), Y_{AMX} is the yield of AMX ($\text{mg COD}\cdot\text{mg}^{-1}\text{NH}_4\text{-N}$), and $\mu_{max,AMX}$ is the maximum growth rate of AMX (d^{-1}). Yields and growth rates rather than substrate uptake rates (q_{max}) were used for the calculations, because values for these two parameters are directly available in modeling studies. We used a maximum growth rate of 0.08 d^{-1} and a yield of $0.159\text{ g COD}\cdot\text{g}^{-1}\text{ N}$ according to Lackner et al. (2008). Some of the previous studies reported much faster growth rates for AMX with doubling times in the range of 2 to 4 d. However, it is not yet clear under which conditions AMX are able to grow that fast and doubling times in the range of 8 to 10 days have been reported far more often. The authors therefore think that the use of a growth rate with a doubling time of 8.7 d is

adequate in this case. For the conversion from $\text{mg COD}\cdot\text{L}^{-1}$ to $\text{mg VSS}\cdot\text{L}^{-1}$, we assumed a biomass composition of $\text{CH}_2\text{O}_{0.5}\text{N}_{0.15}$, which results in a conversion factor of $1.51 \text{ g COD}\cdot\text{g}^{-1} \text{ VSS}$. As such, $X_{\text{AMX}} (\text{mg VSS}\cdot\text{L}^{-1}) = 1.316 \cdot r_{\text{NH}_4}$.

2.4 Fluorescence *in situ* hybridisation (FISH)

Biomass samples were fixed in a 4% paraformaldehyde solution, washed with phosphate-buffered saline, and stored in phosphate-buffered saline–ethanol (1:1) at -20°C until further processing (Amann et al. 1990). Prior to hybridisation, the samples were diluted five times with PBS and homogenised by repeated drawings with a needle on a syringe (inner diameter $210 \mu\text{m}$). Three $10 \mu\text{L}$ aliquots of each sample were spotted on a Poly L-lysine coated slide. The oligonucleotide probes used are listed in the supporting information S2. For DAPI staining, $15 \mu\text{L}$ of DAPI solution ($0.33 \mu\text{g}\cdot\text{mL}^{-1}$ in ultra-pure water) was added to each spot, and the slides were incubated for 10 min at room temperature in the dark. Subsequently, the slides were washed with cold (4°C) ultra-pure water and dried at room temperature.

For microscopy, the slides were embedded with the embedding medium (Citifluor, Citifluor Ltd, Leicester, United Kingdom or Vectashield, Vector Laboratories Ltd, Peterborough, United Kingdom), covered with a cover slide, and analysed with an epifluorescence microscope (Axioskop 2 plus, Zeiss, Feldbach, Switzerland resp. DMI 6000B, Leica, Heerbrugg, Switzerland).

Four samples (day 4, 73, 157 and 194) were screened for the most common AMX types (probe sequences are listed in the supporting information S2). Clear signals in relevant quantities were only detected with Bfu613 and Amx820. Therefore, a combination of these two probes was used as an approximation for total AMX. The real amount of total AMX might be higher, as not all AMX types have yet been identified. However, based on the good agreement between the measured AMX quantities with FISH and the calculated AMX quantities from the batch activity measurements (Figure 3), the authors are confident that the main part of the present AMX were detected. Potential autofluorescence of “*Ca. Brocadia fulgida*” was tested and only registered in the FLUO channel. Since all AMX probes were

used with Cy3 dye, the autofluorescence did not pose a problem in this study. The failure of AMX368, which is specific for all anammox bacteria including Bfu613, has also been observed by Liu et al. (2013).

For the quantification of “*Ca. Brocadia fulgida*” and ‘total AMX’, 15 pictures were taken at random locations and processed with the with ImageJ freeware (ImageJ 1.46r, Wayne Rasband, National Institutes of Health, USA). For the conversion of the measured quantities of AMX in % of total bacteria (DAPI) in $\text{g VSS} \cdot \text{L}^{-1}$, we assumed that the whole VSS mass in the reactor was active biomass. Thus we multiplied the measured percentages with the measured VSS concentrations in the reactor.

2.5 Calculation of the sludge production

The sludge production of AMX, AOB and HET was each calculated using the following formula, which can be derived from the biomass and the substrate mass balance (supporting information S3):

$$P_{Xi} = \frac{Q \cdot Y_i \cdot \Delta S_i}{1 + b_i \cdot SRT} \quad (2)$$

where P_{Xi} is the sludge production of bacteria i ($\text{mg VSS} \cdot \text{d}^{-1}$), Q is the flow rate ($\text{L} \cdot \text{d}^{-1}$), Y_i is the yield of bacteria i ($\text{mg COD} \cdot \text{mg}^{-1} \text{ COD}$ resp. $\text{mg COD} \cdot \text{mg}^{-1} \text{ N}$), ΔS_i is the degraded substrate by bacteria i ($\text{mg COD} \cdot \text{L}^{-1}$ resp. $\text{mg N} \cdot \text{L}^{-1}$), and b_i is the endogenous respiration rate of bacteria i (d^{-1}). Equation 2 is based on the assumption that the growth rate μ is constant, which is not true in a SBR. However, the assumption is a good approximation if we consider average values over the whole cycle. Furthermore, as the exchange volume was rather low (about one-fifth of the reactor volume), concentration changes and thus also deviations from the average μ over the cycle were also rather small.

The yields and the endogenous respiration rates used are listed in the supporting information S3. The total sludge production $P_{X,\text{tot}}$ was then calculated as the sum of the sludge production by AMX, AOB, and HET.

2.6 Analytical methods

NH_4^+ in the effluent and in the samples from the batch activity measurements was analysed on a flow injection analyser (Foss FIA star 5000, Rellingen, Germany). NH_4^+ in influent samples and COD were measured photometrically with vial tests (Hach Lange, Düsseldorf, Germany). NO_2^- and NO_3^- were analysed on an ion chromatograph (Compact IC 761, Metrohm, Herisau, Switzerland). TSS and VSS were determined according to standard methods (American Public Health Association 2005).

Particle size distribution analysis was performed on a mastersizer (Mastersizer 2000, Malvern Instruments Ltd, Malvern, United Kingdom). Samples were dispersed in water in the Hydro 2000s module until a laser obscuration of 10-15% was established prior to the measurement. The measurement range was 0.5 μm to 2 mm. Each sample was analysed in triplicate.

3. RESULTS

3.1 Nitrogen removal

During the operation with digester supernatant and acetate addition, the NER remained stable at a level of $270 \pm 30 \text{ mg N} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$ (Figure 1). Following the switch from acetate to glucose on day 159, a significant change of the NER occurred when the NER reached values in the range of $370 \pm 25 \text{ mg N} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$. This increase of the NER was most likely due to the (accidental) increase of the aeration rate on day 160. The increased nitrite effluent concentrations on day 161 and 164 (unbalance between AMX and AOB activity due to the increased availability of oxygen) support this hypothesis. Since it was not the goal of this study to maximise the NER, the aeration rate was kept constant during the operation with acetate, and this most likely did not provide enough oxygen for the maximum nitrogen removal capacity of the reactor.

During the whole operation phase, the nitrogen removal efficiency (NRE) gradually increased from $87.9 \pm 3.9\%$ with pure digester supernatant to $94.9 \pm 0.6\%$ with acetate addition and finally to $98.0 \pm$

1.0% with glucose addition (Figure 1). This coincided with a gradual decrease of the nitrate effluent concentration from above 100 mg N·L⁻¹ with pure digester supernatant to 29.2 ± 3.2 mg N·L with acetate addition and finally to 9.4 ± 4.2 mg N·L during the last operation phase with glucose addition.

3.2 Activity measurements of nitrifiers and anammox bacteria

The maximum activities of AMX, AOB, and NOB are given in Figure 2. The AMX activity gradually decreased from an initial value of 840 mg NH₄⁺-N·L⁻¹·d⁻¹ with digester supernatant down to 160 mg NH₄⁺-N·L⁻¹·d⁻¹ after 6 weeks at an influent COD/N ratio of 1.4 g COD·g N⁻¹ with acetate. This decreasing AMX trend was countered by the increase of the SRT on day 129, which resulted in activities of 225 and 220 mg NH₄⁺-N·L⁻¹·d⁻¹ 3 and 4 weeks after the SRT increase, respectively. After the change to glucose on day 159, the maximum AMX activity further increased to 275 and 353 mg NH₄⁺-N·L⁻¹·d⁻¹.

The AOB activity increased from 320 mg NH₄⁺-N·L⁻¹·d⁻¹ with digester supernatant to 1695 mg NH₄⁺-N·L⁻¹·d⁻¹ after 2 weeks with an influent COD/N ratio of 1.4 g COD·g N⁻¹ with acetate. Afterwards, the AOB activity reached a stable level of around 1830 mg NH₄⁺-N·L⁻¹·d⁻¹ and was not influenced by the increase of the SRT on day 129 or the switch from acetate to glucose on day 159. The lower value on day 115 was probably an outlier.

The NOB activity developed very similarly to the AMX activity. After an initial increase from 410 mg NO₃-N·L⁻¹·d⁻¹ with digester supernatant to 860 mg NO₃-N·L⁻¹·d⁻¹ at an influent COD/N ratio of 0.6 g COD·g N⁻¹, the maximum NOB activity continuously decreased to 350 mg NO₃-N·L⁻¹·d⁻¹ after 6 weeks at an influent COD/N ratio of 1.4 g COD·g N⁻¹. However, after the increase of the SRT on day 129 NOB activity decreased to an even lower level of around 185 mg NO₃-N·L⁻¹·d⁻¹. Similar to the AMX activity, NOB activity was not influenced by the switch from acetate to glucose on day 159.

During the whole operation, the maximum NOB activity was relatively high and in the same range as the anammox activity. However, compared to the batch activity measurements where conditions for NOB were ideal with oxygen concentrations of at least 4 mg·L⁻¹, very little oxygen was available in the

reactor. Regarding an average oxygen concentration around $0.066 \text{ mg}\cdot\text{L}^{-1}$ and a half saturation constant of $2.2 \text{ mg}\cdot\text{L}^{-1}$ (Lackner et al. 2008), the actual NOB activity in the reactor was on average only ~3% of the maximum NOB activity. The actual NOB activity in the reactor was thus considered to be negligible for the total N turnover.

3.3 Anammox bacteria abundance

The results from the FISH measurements are displayed in Figure 2. “*Ca. Brocadia fulgida*” increased from $\sim 0.2 \text{ g VSS}\cdot\text{L}^{-1}$ during the operation with digester supernatant to $\sim 0.7 \text{ g VSS}\cdot\text{L}^{-1}$ with an influent COD/N ratio of $0.8 - 1.07 \text{ g COD}\cdot\text{g N}^{-1}$. The increase of “*Ca. Brocadia fulgida*” together with the decrease of ‘total’ AMX (estimated with Bfu613+Amx820) indicates that “*Ca. Brocadia fulgida*” was able to out-compete other AMX types relatively quickly (40 days), and this is probably due to its ability to oxidise acetate. Afterwards, with an influent COD/N ratio of 1.16 and $1.4 \text{ g COD}\cdot\text{g N}^{-1}$, “*Ca. Brocadia fulgida*” continuously decreased to a minimum of $0.04 \text{ g VSS}\cdot\text{L}^{-1}$ on day 129 but remained the dominant type of AMX, even after the switch from acetate to glucose on day 159.

In Figure 3, we plotted the measured AMX quantities with FISH against the calculated AMX quantities from the batch activity measurements. Since the measured maximum AMX activity on day 31 was much higher than the other activities (Figure 2) and also much too high for the corresponding quantity that was measured with FISH (most right point in Figure 3), this point is considered to be an outlier. The slope of a straight line that best fits the remaining ten points in Figure 3 according to the least square method was calculated to be 1.05 ± 0.09 . In our study, the measured AMX quantities with FISH thus correlated surprisingly well (correlation coefficient 0.973) with the results from the batch activity measurements. However, it should be kept in mind that the uncertainty of FISH and activity based biomass estimations is high. Several assumptions contribute to the considerable uncertainty, e.g. the values of the growth rate and the yield of AMX was assumed based on literature values and the exact amount of active VSS is not known.

3.4 Organic substrate removal

Based on the assumption that the yield of HET with acetate is much higher than the yield of AMX with acetate, we expected that an involvement of AMX in acetate oxidation could be demonstrated based on the sludge production in different scenarios. All three scenarios assume a complete autotrophic nitrogen removal and negligible NOB activity. Scenario 1 assumes a complete heterotrophic COD removal, while scenarios 2 and 3 assume a maximum possible COD oxidation by AMX (supporting information S4) and heterotrophic degradation of the remaining COD.

For heterotrophic COD removal, the yield was assumed to be the average value for COD removal with oxygen, nitrite and nitrate (supporting information S5). Due to the very low oxygen concentration, AMX activity and HET denitrification cannot be excluded during aerobic phases. It is therefore not possible to gain any information on the degradation pathways based on oxygen mass transfer data and cycle measurements. Furthermore, in single-stage nitrification/anammox processes with elevated COD/N influent ratios, it is generally not possible to assess the activities of the different bacterial groups based on mass balances.

To our knowledge, the effect of acetate oxidation on the yield of AMX has not yet been determined. Apparently, acetate is not integrated into biomass, but the oxidation probably occurs for the conservation of energy, which could lead to a higher biomass yield for AMX. For this study, we assumed that the true yield of AMX with acetate oxidation is between zero and the AMX yield with ammonia ($0.159 \text{ g COD} \cdot \text{g N}^{-1}$; Strous et al. (1998)). These values are based on the assumption that, as ammonia is their main substrate, the AMX yield with ammonia is maximised and it is therefore unlikely, that the AMX yield with acetate is higher. Since ammonia and acetate oxidation produce the same amount of energy per electrons released, we converted the normal AMX yield with ammonia to $0.093 \text{ g COD} \cdot \text{g COD}^{-1}$ based on the electrons released during the two reactions (supporting information S3). We then calculated scenario 2 with no biomass growth and scenario 3 with the normal AMX yield of $0.093 \text{ g COD} \cdot \text{g COD}^{-1}$ as the two extreme scenarios.

The measured and the calculated sludge productions for the three scenarios are displayed in Figure 4. However, the sludge production in all three scenarios was relatively similar, and there was no clear difference between complete heterotrophic acetate degradation (scenario 1) and an involvement by AMX (scenario 2 and 3). Furthermore, the measured sludge production was much higher than the calculated ones for the three scenarios, which could have been due to higher actual yields and/or lower actual endogenous respiration rates than the ones used for the calculation (supporting information S3). Therefore, this approach did not allow us to judge the involvement of AMX in acetate oxidation, but it did show that the sludge production exceeded stoichiometric expectations.

3.5 Sludge retention time

During the start-up phase with digester supernatant, the SRT increased from 130 d to ~ 350 d (Figure 1). Subsequently, when the influent COD/N ratio was increased, the SRT rapidly dropped to values below 50 d. At the same time, sludge washout in the effluent increased (supporting information S6). Based on visual observations, this was due to an increase of the sludge level at the end of the sedimentation phase and, thus cannot be attributed only to the turbidity of the supernatant.

Because the SRT was not controlled, and sludge loss only occurred through washout in the effluent and sampling, and because the sedimentation time and the sludge withdrawal regime was not changed, a decrease of the SRT was expected due to increasing heterotrophic growth with increasing availability of COD. However, a simultaneous decrease of the VSS concentration in the reactor (Figure 1) showed that the decrease of the SRT must have been, at least partly, caused by worse settleability of the sludge. The worse settleability could be due to a decrease of the particle size (Figure 5).

Between the beginning of the operation (day 1) and day 115, the average particle size decreased from $300 \pm 6 \mu\text{m}$ to $104 \pm 3 \mu\text{m}$. Since smaller particles settle more slowly than bigger ones with the same density, the decreased floc size probably caused a general lower settleability. An influence of inorganic solids can be excluded because the VSS/TSS ratio remained constant during the whole operation period ($0.843 \pm 0.033 \text{ g VSS} \cdot \text{g TSS}^{-1}$).

From day 129 onwards, the sludge from the effluent was returned to the reactor in order to increase the SRT. This resulted in an average SRT of 702 ± 48 d for the remaining operation period.

4. DISCUSSION

4.1 Influence of the COD/N ratio on nitrogen removal

Despite the increasing influent COD/N ratio, the decreasing SRT, and the changing bacterial activities, the NER remained stable and was thus not influenced by the increase of the influent COD/N ratio from pure digester supernatant up to $1.4 \text{ g COD} \cdot \text{g N}^{-1}$ with acetate. In contrast to this, the NRE clearly increased with increasing influent COD/N ratio. This was expected because the additional COD allows more denitrification of the nitrate produced by AMX.

Assuming no significant NOB activity and that all nitrogen is converted via nitrification/anammox, an influent COD/N ratio of $0.31 \text{ g COD} \cdot \text{g N}^{-1}$ would already be sufficient to denitrify all nitrate produced by AMX (11% nitrate production, $2.86 \text{ g COD} \cdot \text{g}^{-1} \text{ NO}_3\text{-N}$, assuming complete COD degradation). Based on the influent COD/N ratio, complete denitrification should thus be possible from day 21 on (Figure 1). However, because this was a mixed aerobic/anoxic system, part of the COD would also have been degraded with oxygen. Furthermore, while the nitrate is produced continuously during the whole cycle, COD is only added once in the beginning, and it was rapidly degraded during the first 1-2 hours of each cycle, part of it also with oxygen. These two factors can explain why denitrification was still incomplete at an influent COD/N ratio of $1.4 \text{ g COD} \cdot \text{g N}^{-1}$.

4.2 Nitrite removal by AMX, HET, and NOB

Batch experiments and FISH data indicated that the maximum AMX activity decreased in the SBR. According to Monod kinetics, this decrease of the maximally possible activity could have been balanced out by higher substrate concentrations, but ammonia and nitrite concentrations were similar in all cycles. Therefore, the actual activity of the AMX in the SBR must have decreased as well.

One possible explanation for decreasing AMX activity is increasing competition for nitrite by HET with increasing availability of COD. Because the NER remained constant up to the maximum influent COD/N ratio of 1.4 g COD·g N⁻¹ with acetate (day 1 - day 159; Figure 1) but the actual AMX activity in the SBR decreased, heterotrophic denitrification must have increased in order to still achieve the same nitrogen removal. This is supported by the fact that the actual AOB activity in the reactor, and thus the nitrite production, increased as well (because ammonia, nitrite and oxygen concentrations were similar in all cycles it can be assumed that the actual AOB activity developed proportionally to the maximum activity in Figure 2).

However, simultaneous to the decrease of the AMX activity and the increase of denitrification by HET, the SRT strongly decreased (Figure 1). Thus, the decrease of the AMX activity and abundance might also have been due to wash out, which could occur as soon as the SRT is lower than the inverse of the actual growth rate of AMX. The fact that AMX activity started to increase again as soon as a high SRT was established by sludge recycling from day 129 onwards suggests, that the decrease of AMX activity was at least partly due to the decrease of the SRT. Furthermore, this increase clearly shows that AMX are able to stay in the system and compete with HET up to influent COD/N ratios of 1.4 g COD·g N⁻¹ if the SRT is high enough. However, the remaining experiment was too short to reach the initial high activity of AMX. Therefore, it remains unclear to what extent the AMX activity decreased due to competition by HET.

NOB activity developed very similarly to the AMX activity and also decreased with increasing COD/N ratio respectively decreasing SRT (Figure 2). This indicated that NOB also suffered from the increased competition by HET for nitrite and, because their actual growth rate was limited by nitrite, NOB were also affected by the decreasing SRT. However, in contrast to AMX activity, NOB activity did further decrease after the SRT was increased by sludge recycling from day 129 onwards. This could mean that AMX were mainly affected by the SRT decrease while the competition for nitrite by HET was more significant for NOB.

The initial presence of NOB can be explained with the fact that the inoculum was taken from a reactor which was operated with digester supernatant. Due to the lower availability of biodegradable COD, HET denitrification was low and therefore more nitrite was available for NOB. Therefore, NOB were able to grow in the original reactor and thus present in the inoculum. The fact that they sustained over longer periods in our reactor can be explained with the high initial SRT. Even with the lowest average SRT of 17 d and zero net growth (i.e. endogenous respiration equals growth), it would take 40 d for a 90% reduction of the NOB population.

4.3 Acetate oxidation by anammox bacteria

Previous studies have shown that “*Ca. Brocadia fulgida*” is able to oxidise acetate and out-competes other AMX types in the presence of acetate (Kartal et al. 2008, Winkler et al. 2012a, Winkler et al. 2012b). Our study confirmed that “*Ca. Brocadia fulgida*” was able to outcompete other AMX types in the presence of acetate (Figure 2). However, the dominance of “*Ca. Brocadia fulgida*” alone does not prove that AMX participated in acetate oxidation.

Because the sludge production in all three tested scenarios was very similar (Figure 4), it was not possible to prove or exclude an involvement of AMX in acetate oxidation based on the sludge production. The fact that “*Ca. Brocadia fulgida*” remained the dominant type of AMX after the switch from acetate to glucose does not support an involvement of AMX in acetate degradation. Güven et al. (2005) tested the influence of glucose on AMX and did not find any positive or adverse effect. Thus, it is not likely that AMX are able to oxidise glucose. If AMX and specifically “*Ca. Brocadia fulgida*” would have had an advantage with acetate, this should have resulted in a lower activity with glucose, probably also coupled with a switch of the dominant type of AMX. The increasing trend of AMX activity can be explained with the increased SRT. Whether glucose would support another AMX type cannot be judged with our experiment, because the experiment was too short for another type of AMX to reach significant concentrations.

4.4 Causes for the decrease of the sludge retention time

A high SRT is essential for the slowly growing AMX. A decrease of the SRT due to higher sludge production of HET with an increasing influent COD/N ratio could thus be critical for AMX. Indeed, the SRT rapidly decreased in our study and reached values in the range of the minimally required SRT for AMX. The lower SRT was caused by worse settleability of the sludge. However, the worse settleability of the sludge was not due to increased HET sludge production but was caused by a decrease of the floc size.

There are two possible reasons for the observed decrease of the floc size. First of all, the reactor from where the inoculum was taken was equipped with hydrocyclones and thus selecting for dense and coarse particles. The different operating conditions in the reactor used for this study, e.g. the low shear forces, might have induced a mechanical breakup of the bigger particles. Secondly, HET mainly grow in small and light flocs due to their fast growth. Therefore, the decrease of the SRT might still have been caused by HET, but rather due to the quality of the flocs than due to the amount of the produced biomass.

Based on our own observation, an elevated influent COD/N ratio does not necessarily cause a decrease of the SRT. During the operation of a one-stage nitrification/anammox process with five times diluted urine in the same sequencing batch reactor (Bürgmann et al. 2011), the average SRT was 170 days even though the biomass concentration was with an average of $8.7 \text{ g TSS} \cdot \text{L}^{-1}$ much higher than in this study (average influent COD/N ratio of $1.5 \pm 0.3 \text{ g COD} \cdot \text{g N}^{-1}$; constant minimum settling velocity). The sodium and the chloride concentrations of five times diluted urine are around 2.5 respectively 8.5 times higher than in digester supernatant and Fernández et al. (2008) showed that sodium chloride can increase the settleability (anammox process with activated sludge). A high salinity might thus be beneficial for the treatment of wastewaters with elevated influent COD/N ratios. However, in case of Schaubroeck et al. (2012), higher sodium chloride concentrations led to a higher proportion of small aggregates coupled to nitrite accumulation, and thus had a negative effect on the process.

In cases where the SRT reaches critical values for AMX, there are a number of measures to increase the SRT. Apart from the option chosen in this study (returning all sludge from the effluent back into the reactor), an increase of the SRT might also be achieved with a longer sedimentation time or by increasing the settleability of the sludge. The latter could be achieved by using another reactor setup, which allows the formation of larger or denser flocs or of granules. While the mechanisms for aerobic (Adav et al. 2008) and anaerobic (Hulshoff Pol et al. 2004) granulation are well known, the exact physical or chemical triggers for granulation in one-stage nitrification/anammox systems are not clear yet (Vlaeminck et al. (2010)).

5. CONCLUSIONS

Based on the results of our study we conclude the following:

- Nitritation/anammox with influent COD/N ratios of $1.4 \text{ g COD} \cdot \text{g N}^{-1}$, which is similar to the typical ratio for stored urine ($1.5 \text{ g COD} \cdot \text{g N}^{-1}$), is feasible provided a sufficiently high SRT is maintained.
- The observed decrease of the SRT was the most critical factor for the slowly growing AMX. Since the VSS concentration in the reactor decreased and the minimum settling velocity remained constant, the SRT decrease was not caused by a higher sludge production. Instead, the sludge settleability decreased due to a decrease of the average floc size. Potential reasons for this were increased HET growth in small flocs and the breakup of bigger particles, presumably due to different operational conditions compared to the inoculum reactor.
- The availability of acetate quickly and strongly influenced the anammox community. “*Ca. Brocadia fulgida*” became the dominant AMX type, which is most probably due to its ability to oxidise acetate. It was, however, not possible to prove nor exclude an involvement of AMX in acetate oxidation based on the sludge production.

- The switch from acetate to glucose addition did not negatively influence the activity of “*Ca. Brocadia fulgida*”. This suggests that “*Ca. Brocadia fulgida*” can also successfully compete with HET in the presence of other types of COD as long as the organic compounds are not toxic.

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TABLES

Table 1 Composition of the digester supernatant

Compound	Concentration (average $\pm$ standard deviation)	Number of measurements
pH [-]	8.3 $\pm$ 0.2	83
Cond _{30°C} [mS·cm ⁻¹]	7.3 $\pm$ 0.3	83
TIC [mg·L ⁻¹]	710 $\pm$ 100	83
NH ₄ ⁺ [mg N·L ⁻¹]	740 $\pm$ 60	83
NO ₂ ⁻ [mg N·L ⁻¹]	< 0.50	22
NO ₃ ⁻ [mg N·L ⁻¹]	< 0.50	22
COD [mg·L ⁻¹]	140 $\pm$ 50	83
COD _{inert} [mg·L ⁻¹]	180 $\pm$ 22	83
PO ₄ ³⁻ [mg P·L ⁻¹]	13 $\pm$ 3	22
SO ₄ ²⁻ [mg·L ⁻¹]	4.3 $\pm$ 0.1	2
Cl ⁻ [mg·L ⁻¹]	96 $\pm$ 2	2
K ⁺ [mg·L ⁻¹]	130 $\pm$ 9	12
Na ⁺ [mg·L ⁻¹]	230 $\pm$ 29	7
Ca ²⁺ [mg·L ⁻¹]	110 $\pm$ 40	5
Mg ²⁺ [mg·L ⁻¹]	20 $\pm$ 7	6

FIGURE CAPTIONS

Figure 1 SBR performance, including the nitrogen elimination rate (NER) and the nitrogen removal efficiency (NRE), during operation with pure digester supernatant and increasing influent COD/N ratios due to acetate and glucose addition.

Figure 2 Maximum AMX, AOB and NOB activities in the SBR measured in batch experiments, and quantification of AMX with FISH. Both were recalculated based on the VSS concentration at the moment of sampling. Outliers are marked with a star.

Figure 3 Comparison of AMX abundance as calculated from the batch activity measurements and FISH with a linear regression (continuous line, $y = 1.048 \cdot x - 0.184$, $R^2 = 0.947$). For a perfect correlation, points would have to be on the dotted line. The data point marked with a star is considered to be an outlier. The error bars represent the standard deviations.

Figure 4 Measured and calculated total sludge production for three different scenarios. Scenario 1 assumed that all COD was degraded by HET. Scenarios 2 and 3 assumed a maximum possible COD oxidation by AMX, with a zero and normal AMX yield, respectively.

Figure 5 Particle size distribution at different time points during the SBR operation

HIGHLIGHTS

- Nitritation/anammox with high influent COD/N ratio (acetate and glucose addition)
- SRT is the key for successful AMX competition with HET at high influent COD/N ratio
- SRT decrease due to a change of the floc morphology caused washout of AMX
- Dominance of “*Candidatus Brocadia fulgida*” indicated acetate utilisation by AMX

Supporting Information for**Successful application of nitrification/anammox to wastewater with elevated organic carbon to ammonia ratios**

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This Supporting Information contains 9-page document, four tables and two figures and additional cited literatures, including this cover page.

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Figure S1. Cycle time during the whole experiment

S2 Oligonucleotide probes

Table S1. Oligonucleotide probes used for the screening of AMX, sequences according to Loy et al. (2003)

Probe	Target organism(s)
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Amx1900	Ca. Brocadia anammoxidans and Ca. Kuenenia stuttgartiensis
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Ban162	Ca. Brocadia anammoxidans
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Kst157	Ca. Kuenenia stuttgartiensis
Kst1275	Ca. Kuenenia stuttgartiensis
Sca1309	Ca. Scalindua
Scabr1114	Ca. Scalindua brodae

S3 Sludge production

The rate of substrate concentration change due to utilization by bacteria can be calculated as:

$$r_{su} = -\frac{\mu_{max}}{Y} \times \frac{S}{K_S + S} \times X$$

where r_{su} is the rate of substrate concentration change ($\text{mg S} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$), μ_{max} is the maximum growth rate of the bacteria (d^{-1}), Y is the yield of the bacteria ($\text{mg COD} \cdot \text{mg S}^{-1}$), X is the bacteria concentration ($\text{mg COD} \cdot \text{L}^{-1}$), S is the substrate concentration ($\text{mg} \cdot \text{L}^{-1}$) and K_S is the half saturation constant for substrate S ($\text{mg} \cdot \text{L}^{-1}$).

The growth rate of the bacteria can be formulated as:

$$r_g = \mu_{max} \times \frac{S}{K_S + S} \times X - b \times X$$

where r_g is the growth rate of the bacteria ($\text{mg COD} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$) and b is the endogenous respiration rate of the bacteria (d^{-1}).

The mass balance for substrate utilisation is:

$$\frac{dS}{dt} V = Q \Delta S + \frac{\mu_{max}}{Y} \times \frac{S}{K_S + S} \times X V$$

where V is the reactor volume (L), Q is flowrate ($\text{L} \cdot \text{d}^{-1}$) and ΔS is the degraded amount of substrate S ($\text{mg} \cdot \text{L}^{-1}$). Assuming steady state, this equation can be solved for the biomass concentration X as:

$$X = \frac{1}{HRT} \times \frac{\Delta S \times Y}{\mu_{max} \times \frac{S}{K_S + S}}$$

where HRT is the hydraulic retention time.

Assuming that the influent does not contain any biomass, the biomass mass balance is:

$$\frac{dX}{dt} V = (Q_e \times X_e - Q_s \times X_s) + \left(\mu_{max} \times \frac{S}{K_S + S} - b \right) \times X V$$

where Q_e is the effluent flowrate ($L \cdot d^{-1}$), X_e is the concentration of biomass in the effluent ($mg \text{ VSS} \cdot L^{-1}$), Q_s is the sampling volume ($L \cdot d^{-1}$) and X_s is the concentration of biomass in the sampling volume ($mg \text{ VSS} \cdot L^{-1}$). Assuming steady state, this equation can be transferred to

$$\mu_{max} \times \frac{S}{K_S + S} = \frac{1}{SRT} + b$$

where SRT is the sludge retention time (d).

If we substitute this equation into the above equation for the biomass concentration X , we get:

$$X = \frac{SRT}{HRT} \times \frac{\Delta S \times Y}{1 + b \times SRT}$$

In steady state, the total biomass production of a reactor can be expressed as:

$$P_{X,tot} = \frac{X_{tot} \times V}{SRT}$$

where $P_{X,tot}$ is the total sludge production ($mg \text{ COD} \cdot d^{-1}$) and X_{tot} is the total sludge concentration in the reactor ($mg \text{ COD} \cdot L^{-1}$). Assuming that the total sludge in a reactor is a homogenous mixture of different types of bacteria, this equation can also be used to calculate the sludge production of any type of bacteria present in the reactor:

$$P_{X,i} = \frac{X_i \times V}{SRT}$$

where the concentration of bacteria X_i can be substituted with the above equation for X_i :

$$P_{X,i} = \frac{Q \times Y_i \times \Delta S_i}{1 + b_i \times SRT}$$

For the conversion of $mg \text{ COD} \cdot d^{-1}$ to $mg \text{ VSS} \cdot d^{-1}$, we assumed a biomass composition of $C_5H_7O_2N$ for HET and AOB respectively $CH_2O_{0.5}N_{0.15}$ for AX, which results in conversion factors of $1.42 \text{ g COD} \cdot g \text{ VSS}^{-1}$ and $1.51 \text{ g COD} \cdot g \text{ VSS}^{-1}$, respectively.

The yields and the endogenous respiration rates that were used for the calculation of the sludge productions are composed in the following table. The yield of AMX during acetate oxidation was calculated from the normal AMX yield during ammonium oxidation based on the produced electrons during ammonium and acetate oxidation, respectively. The half reactions according to Metcalf and Eddy (2003) are:

During ammonium oxidation, 3/14 mole e^- are released per g N. The normal AMX yield of 0.159 g COD·g N⁻¹ can thus be converted to 0.742 g COD·mole e^- ⁻¹. Together with an electron release of 1/8 mole e^- per g COD (1 mole acetate equals 64 g COD) during acetate oxidation, this results in a maximum yield of 0.093 g COD·g COD⁻¹ for AMX during acetate oxidation.

Table S2. Yields and endogenous respiration rates for the calculation of the sludge production of HET, AOB and AMX

	Y	Unit	Reference	b	Unit	Reference
HET with acetate	0.591	g COD·g COD ⁻¹	SI S5	0.62	d ⁻¹	Lackner et al. (2008)
HET with glucose	0.726	g COD·g COD ⁻¹	SI S5	0.62	d ⁻¹	Lackner et al. (2008)
AOB	0.15	g COD·g N ⁻¹	Lackner et al. (2008)	0.13	d ⁻¹	Lackner et al. (2008)
AMX	0.159	g COD·g N ⁻¹	Lackner et al. (2008)	0.003	d ⁻¹	Lackner et al. (2008)
AMX	0.093	g COD·g COD ⁻¹	calculated			

S4 Maximum possible COD oxidation by AMX

According to the stoichiometry of the AMX reaction (Strous et al. 1998), AMX produce 0.26 mole nitrate per mole ammonium degraded. Assuming a complete autotrophic nitrogen removal and neglecting NOB activity, the maximum possible nitrate production can be calculated.

Since the exact stoichiometry of acetate oxidation by AMX is not known, we decided to calculate the required amount of nitrate reduced per amount of acetate oxidised based on the COD difference between nitrate and ammonium, which is 4.57 g COD·g N⁻¹. Together with the maximum possible nitrate production, this allows to estimate the maximum possible acetate oxidation by AMX.

S5 Yield of heterotrophic bacteria

The yield of heterotrophic bacteria with acetate and with glucose was calculated according to the procedure in Metcalf and Eddy (2003). The calculation based on the following oxidation and reduction reactions:

The energy released from the oxidation reduction reactions ΔG_R can be calculated by combining the oxidation reaction with the respective reduction reactions and are composed in the following table for heterotrophic growth with acetate or glucose as substrate as well as oxygen, nitrite and nitrate as electron acceptors.

Table S3. Energy released from the oxidation (acetate and glucose) reduction (oxygen, nitrite and nitrate) reactions ΔG_R in kJ/mole e^-

	oxygen	nitrite	nitrate
acetate	105.82	120.91	99.35
glucose	120.10	135.19	113.63

The free energy to convert one electron equivalent of the carbon source to cell material ΔG_S (kJ/mole e^-) can be calculated with the following equation:

$$\Delta G_S = \frac{\Delta G_P}{K^m} + \Delta G_C$$

where ΔG_P is the free energy to convert one electron equivalent (e^- eq) of the carbon source to the pyruvate intermediate (kJ/ e^- eq), K is the fraction of energy transfer captured (-), m is +1 if ΔG_P is positive and -1 if energy is produced (-) and ΔG_C is the free energy to convert one electron equivalent of pyruvate intermediate to one electron equivalent of cells (kJ/ e^- eq).

The value of ΔG_C is +31.41 kJ/ e^- eq (McCarty 1975), K is 0.6 and ΔG_P can be calculated from combining the oxidation of acetate resp. glucose to carbon dioxide with the reduction of carbon dioxide to pyruvate as 8.10 kJ/mole e^- and -6.18 kJ/mole e^- , respectively.

The fraction of e^- mole of substrate used for cell synthesis per e^- mole of substrate used f_s (-) resp. the yield can then be calculated by combining the following two equations:

$$K \times \Delta G_R \times \frac{f_e}{f_s} = -\Delta G_S$$

$$f_e + f_s = 1$$

where f_e is e⁻ mole of substrate oxidised per e⁻ mole of substrate used (-) and solving them for f_s :

$$f_s = \frac{1}{1 + \frac{-\Delta G_S}{K \times \Delta G_R}}$$

The so calculated yields of HET growth for the substrates acetate and glucose and the different electron acceptors oxygen, nitrite and nitrate are composed in the following table.

Table S4. Yields of HET growth for the substrates acetate and glucose and the three different electron acceptors oxygen, nitrite and nitrate as well as the average yield for all three electron acceptors in g COD·g COD⁻¹

	oxygen	nitrite	nitrate	average
acetate	0.585	0.618	0.570	0.591
glucose	0.723	0.745	0.711	0.726

For the calculations in the paper we assumed that HET consumed all three electron acceptors with the same ratio and thus average yields of 0.591 g COD·g COD⁻¹ for acetate and 0.726 g COD·g COD⁻¹ for glucose.

S6 VSS loss with the effluent

Figure S2. VSS loss per effluent. From day 129 on, the sludge in the effluent was returned to the reactor in order to increase the SRT

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