Anammox and partial nitritation in the mainstream of a wastewater treatment plant in a temperate region (Denmark)

Anja Kamp, Lars Ditlev Mørck Ottosen, Nikolaj Bang Thøgersen, Niels Peter Revsbech, Bo Thamdrup and Mikkel Holmen Andersen

ABSTRACT

The Marselisborg WWTP (Aarhus, Denmark) fed the mainstream nitrification/denitrification tanks with excess sludge from a sidestream DEMON tank for more than three years to investigate if anammox can supplement conventional nitrification/denitrification in a mainstream of a temperate region. To evaluate this long-term attempt, anammox and also denitrification rates were measured in activated sludge from the main- and sidestream at 10, 20 and 30 °C using ^15N-labelling (stable isotope) experiments. The results show that anammox contributes by approximately 1% of the total nitrogen removal in the mainstream tanks and that anammox conversion rates there are approximately 800–900 times lower than in the DEMON. A distinct temperature dependence of both anammox and denitrification rates was also confirmed, however, results from different temperatures did not significantly alter relative shares, e.g. anammox rates in activated sludge from the nitrification/denitrification tanks are also negligible at 30 °C. This indicates that the anammox bacteria abundance in the nitrification/denitrification tanks is too low to play an important role and that an adaptation to lower temperatures had not occurred. Additional in situ measurements in the nitrification/denitrification tanks further revealed that full nitrification dominates over partial nitritation. Dominant nitritation-anammox is therefore excluded per se and also nitrite shunt activities are not particularly supported.

Key words | deammonification, DEMON, denitrification, nitrification, nitrite shunt, nitrogen removal

INTRODUCTION

The dominant source of fixed nitrogen in most municipal wastewater treatment plants (WWTPs) is NH$_4^+$ which can reach concentrations of up to a few mmol L$^{-1}$ and would lead to strong eutrophication if released untreated into aquatic environments. Conventionally, NH$_4^+$ is removed in municipal WWTPs via biological nitrogen removal (BNR) by alternating nitrification and denitrification in nitrification/denitrification tanks of the mainstream (van Hulle et al. 2010). During nitrification, NH$_4^+$ becomes oxidized to NO$_2^-$ and NO$_3^-$, which in turn are reduced to N$_2$ that is emitted to the atmosphere. However, NH$_4^+$ oxidation during nitrification requires O$_2$ associated with high aeration costs. Also, denitrification might be an additional cost factor, because it is an anoxic but heterotrophic N-removal process and therefore sometimes requires additional organic carbon which has to be purchased to keep microbial conversions by denitrification efficient (Lackner et al. 2014).

Only two decades ago it was discovered that NH$_4^+$ can also be removed by anaerobic ammonium oxidation in WWTPs (anammox; Mulder et al. 1995). By the anammox process, the bacteria oxidize NH$_4^+$ with NO$_2^-$ to N$_2$, which is an autotrophic N-removal pathway without the need of

Anja Kamp (corresponding author)
Lars Ditlev Mørck Ottosen
Biological and Chemical Engineering, Department of Engineering, Aarhus University, Aarhus, Denmark
E-mail: anjakamp@bios.au.dk; tara@gmx.de

Anja Kamp
Nikolaj Bang Thøgersen
Niels Peter Revsbech
Aarhus University Centre for Water Technology, Department of Bioscience, Aarhus University, Aarhus, Denmark

Bo Thamdrup
Department of Biology, University of Southern Denmark, Odense, Denmark

Mikkel Holmen Andersen
DHI, Aarhus, Denmark

This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC BY 4.0), which permits copying, adaptation and redistribution, provided the original work is properly cited (http://creativecommons.org/licenses/by/4.0/).
organic carbon input (Strous et al. 1998) and therefore more cost efficient. Efficient BNR by anammox in municipal WWTPs is well-established in NH$_4^+$-rich sidestreams of reject water from anaerobic digesters (Lackner et al. 2014; Li et al. 2018). Here, high temperatures favor the microbial anammox activity and growth (van Hulle et al. 2010), and the low organic carbon content after the digestion suppresses heterotrophic denitrifying bacteria that would otherwise outcompete the slow growing anammox bacteria (Jenni et al. 2014). Another cost-efficient advantage of anammox is the coupling to partial nitritation (nitrification-anammox process), which requires less aeration energy due to oxidation of NH$_4^+$ only to NO$_2^-$ by ammonia-oxidizing bacteria (AOB) and not further to NO$_3^-$ by nitrite-oxidizing bacteria (NOB). In fact, not all of the NH$_4^+$ needs to be oxidized to NO$_2^-$ because NH$_4^+$ itself serves as one of the substrates for anammox.

Since the discovery of anammox, more than 114 WWTPs around the world had implemented full-scale anammox in 2015 (Ali & Okabe 2015) to improve BNR at lower costs; including the Marselisborg WWTP in Aarhus, Denmark. At Marselisborg WWTP, anammox was fully implemented in March 2015 in a sidestream single-stage aerobic/anoxic deammonification process (DEMON tank) with suspended granular sludge and cyclone operation (Hippen et al. 1997; Wett et al. 2007; Li et al. 2018). From the beginning, the Marselisborg WWTP also enriched the mainstream with excess sludge from the DEMON in the hope to achieve a reasonable relative proportion of anammox in BNR and with this an energy-saving anammox effect in the mainstream (Li et al. 2018). This was a pioneering long-term experiment as it was not known if (a) the anammox bacteria could remain in the mainstream without controlled cyclone operation and (b) anammox activity would adapt from 30–35 °C in the DEMON to mainstream temperatures that vary between 7 and 20 °C throughout the seasons in Denmark.

To evaluate if this long-term experiment was successful, a highly specific $^{15}$N-labelling approach (stable isotope) was used to measure anammox and also denitrification rates in activated sludge from the DEMON and mainstream. The $^{15}$N-labelling experiments were conducted in the laboratory under in situ conditions with untreated activated sludge, but temperatures were adjusted to 10, 20 and 30 °C to (a) calculate the temperature dependence of anammox and denitrification, (b) estimate on a potential cold adaption of anammox in the mainstream, and (c) make statements on the relative importance of anammox and denitrification in the respective tanks.

In addition to anammox and denitrification rate measurements, this study also aimed to discover if anammox or denitrification in the mainstream might be coupled to shortcut biological nitrogen removal (SBNR), like the nitrification-anammox process in the DEMON. Another potential energy-saving SBNR process in the mainstream is a nitrite shunt. Via a nitrite shunt, NO$_2^-$ produced by AOB is immediately used by denitrifiers, and full nitrification to NO$_3^-$ by NOB is largely suppressed. To test for potential SBNR mechanisms, in situ time series measurements of O$_2$, NH$_4^+$, NO$_3^-$ and NO$_2^-$ were performed in the mainstream of Marselisborg WWTP and the actual time-related N-conversions were analyzed.

**MATERIALS AND METHODS**

**Temperature-dependent anammox and denitrification rate measurements in the side- and mainstream**

**Sampling and experimental set up for $^{15}$N-labelling experiments**

For anammox and denitrification rate measurements, activated sludge samples were collected from approximately 0.5–1 m water depth in the DEMON and nitrification/denitrification tanks at Marselisborg WWTP in May 2018.

Rate measurements were done at 10 °C, 20 °C and 30 °C, each. Rate measurements at 20 °C were started immediately after the activated sludge arrived to the home laboratory. Activated sludge samples for rate measurements at 30 °C were kept at 20 °C and measured within 5 h after sampling, and activated sludge samples for rate measurements at 10 °C were stored at 4 °C and measured within 24 h after sampling.

For each temperature experiment, 400 mL activated sludge was transferred into a 500 mL incubation bottle (BlueCap Bottle; DURAN®) which was placed in a temperature-controlled water bath (Supplementary Figure 1, available with the online version of this paper). The sludge was gently mixed during the entire incubation using a magnetic stirrer below the water bath. The incubation bottle was sealed with a gas tight butyl rubber stopper containing inflow- and outflow ports to introduce anoxia with He, and to connect a 50 mL glass syringe for pressure adjustments (Fortuna® Optima®; wet glass piston prevents gas loss); the total headspace volume was 240 mL at $t_0$ (bottle plus pressure adjustment syringe). Anoxia was confirmed via O$_2$ measurements with an oxygen sensor spot (small planar optode, diameter <1 cm) inside the closed bottle.
using an outside bare optical fiber detector (SPFIB, Bare Optical Fiber) connected to a FireSting GO2 (PyroScience GmbH, Aachen, Germany).

**Start of \( ^{15}\text{N} \)-labelling experiments and sub-sampling in the laboratory**

To start the \( ^{15}\text{N} \)-labelling experiments for the anammox rate measurements, 1 mM \( ^{15}\text{NH}_4 \) (99 atom %; Cambridge Isotope Laboratories, Tewksbury, MA, USA) and 200 \( \mu \text{M} \) \( ^{14}\text{NO}_2 \) (to prevent substrate limitation) were added to the activated sludge from the DEMON and nitrification/denitrification tanks, respectively. To start the \( ^{15}\text{N} \)-labelling experiments for the denitrification rate measurements, 200 \( \mu \text{M} \) \( ^{15}\text{NO}_2 \) (98 atom %; Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to the activated sludge from DEMON and nitrification/denitrification tanks, respectively.

For \( ^{15}\text{N} \)-labeled \( \text{N}_2 \) determination, 5 mL headspace from each incubation bottle was taken with a gas tight syringe (SGE Analytical Science, TRAJAN) through the rubber stopper directly after adding \( ^{15}\text{N} \)-label (\( t_0 \)) and thereafter every 10 min for a total of 1 h (\( t_1 \text{ to } t_6 \)). One mL of the headspace in the sampling syringe was discarded and the remaining 4 mL were transferred to 6 mL exetainers (Labco EXETAINER® Vials), preflushed with He-flushed 0.5% \( \text{ZnCl}_2 \) solution, while the excess solution was expelled. Gas samples were stored upside down until analysis. Prior to each sampling, the headspace of the pressure adjustment syringe was gently mixed with the headspace of the incubation bottle by moving the piston of the pressure adjustment syringe several times a few millimeters up and down (for proof of concept see Supplementary Figure 2, available online). After each sampling, the piston of the pressure adjustment syringe lowered automatically and pressures below atmospheric were thus prevented.

To determine the mole fractions of \( ^{15}\text{NH}_4^+ /^{14}\text{NH}_4^+ \) and \( ^{15}\text{NO}_2^- /^{14}\text{NO}_2^- \) label, respectively, 2 mL subsamples for \( \text{NH}_4^+ \) and \( \text{NO}_2^- \) determinations were taken with a syringe through the rubber stopper of the incubation bottle directly before and after adding of \( ^{15}\text{N} \)-label. Samples were sterile filtered (0.22 µm) and frozen (–20 °C) until further analyses.

For measurements of suspended solids (SS), i.e. total suspended solids (TSS) and volatile suspended solids (VSS), per volume of activated sludge, the sludge was vigorously mixed after the experiment, and 40 mL sludge from each incubation bottle was transferred into pre-weighed porcelain vials which were heated at 100 °C for 3 days (TSS) and thereafter at 500 °C for 24 h (VSS = TSS – inorganic compounds).

**Analyses and calculations for \( ^{15}\text{N} \)-labelling experiments**

The isotopic composition of \( \text{N}_2 \) was determined via gas chromatography – isotope ratio mass spectrometry (GC-IRMS; SerCom) for calculation of the accumulation of \( ^{15}\text{N} \) labeled \( \text{N}_2 \) as \( ^{29}\text{N}_2 \left( ^{14}\text{N}^{15}\text{N} \right) \) and \( ^{30}\text{N}_2 \left( ^{15}\text{N}^{15}\text{N} \right) \) in excess of the natural abundance. Ammonium was analyzed with the salicylate method (Bower & Holm-Hansen 1980), and \( \text{NO}_2^- \) was analyzed with the Griess reaction using the protocol by Garcia-Robledo et al. (2014). The mole fractions of the \( ^{15}\text{N} \)-label in the respective \( \text{NH}_4^+ \) or \( \text{NO}_2^- \) pools (\( F_{\text{N-substrate}} \)) were calculated from the concentration of \( ^{15}\text{N} \)-labeled substrate added, \( ^{15}\text{N-substrate} \), and the total substrate concentration, \( ^{15}\text{N-substrate} + ^{14}\text{N-substrate} \), according to the review on \( ^{15}\text{N} \)-incubation experiments by Holtappels et al. (2011):

\[
F_{\text{N-substrate}} = \frac{^{15}\text{N-substrate}}{^{15}\text{N-substrate} + ^{14}\text{N-substrate}}
\]

\( \text{N}_2 \) produced by anammox (\( A \)) was quantified from the rate of accumulation of excess \( ^{29}\text{N}_2 \left( ^{14}\text{N}^{15}\text{N} \right), P^{29}\text{N}_2 \), over time in incubations with \( ^{15}\text{NH}_4 \), using the following expression:

\[
A = \frac{P^{29}\text{N}_2}{F_{\text{NH}_4}}
\]

and \( \text{N}_2 \) produced by denitrification (\( D \)) was quantified from the rate of accumulation of excess \( ^{30}\text{N}_2 \left( ^{15}\text{N}^{15}\text{N} \right), P^{30}\text{N}_2 \), over time in incubations with \( ^{15}\text{NO}_2^- \), using the following expression (for details see Thamdrup & Dalsgaard 2000, 2002; Thamdrup et al. 2006):

\[
D = \frac{P^{30}\text{N}_2}{(F_{\text{NO}_2})^2}
\]

Accumulation rates of \( ^{15}\text{N} \)-labeled \( \text{N}_2 \) (± standard errors) were calculated from the linear regression of the measured excess labeled \( \text{N}_2 \) per time (\( n \geq 4 \) timepoints) and were related to TSS and VSS, respectively. The decreasing headspace volume (because of pressure adjustment syringe; see above) and the \( \text{N}_2 \) dissolved in the sludge (Bunsen coefficient: \( \text{N}_{\text{water}}/\text{N}_{\text{gas}} = 0.015 \)) were considered for all calculations.

The \( Q_{10} \) value (temperature coefficient) for a changing N-conversion rates (\( R_1 \) and \( R_2 \)) as a consequence of an increasing temperatures by 10 °C (\( T_1 \) and \( T_2 \)) was calculated using the following equation (e.g. Reyes et al. 2008):

\[
Q_{10} = (R_2/R_1)^{10/(T2-T1)}
\]
Shortcut biological nitrogen removal in the mainstream

Measurements of oxygen dependent nitrogen conversions

To estimate the potential of partial nitritation vs. full nitrification, time series measurements of $O_2$, $NO_2$, $NO_3$, $NH_4$ and pH were performed in the nitrification/denitrification tank of Marselisborg WWTP close to (approximately 2 m distance), within (directly in) and outside (more than 10 m distance) an aeration zone at 1.5 and 3–4 m water depth in August 2018 (several large bottom aeration zones are placed in some but not all areas of the tank; for details see Supplementary Figure 3, available online). Date, time, temperature and pH during sampling are shown in Table 1.

O$_2$ and pH were measured in situ with a HQ40d Multi Meter (Hach Lange ApS, Bronshøj, Denmark). Samples for NO$_2$, NO$_3$ and NH$_4$ were taken from the respective water depths with a homemade pump, sterile filtered (0.22 μm), immediately frozen and kept at $-20^\circ$C until further processing. NO$_2$ and NO$_3$ were analyzed with an NOx analyzer connected to a reaction chamber (CLD 66 s plus a Liquid NO Setup; ECO PHYSICS AG, Duernten, Switzerland) using the VCl$_3$ reduction method (Braman & Hendrix 1989), and NH$_4$ was measured with the salicylate method (Bower & Holm-Hansen 1989).

Time series data of NH$_4$, NO$_2$ and NO$_3$ during aeration were further used to calculate the net consumption and net production rates of NH$_4$, NO$_2$ and NO$_3$ during the time of aeration (linear regressions and standard errors of the respective slopes).

RESULTS

Distinct temperature dependence of anammox and denitrification

Anammox and denitrification rates in the DEMON and nitrification/denitrification tanks showed a clear temperature dependence with highest rates at 30 $^\circ$C, medium rates at 20 $^\circ$C, and lowest rates at 10 $^\circ$C (Figure 1). The TSS/VSS ratio was $2.1 \pm 0.1$ and $1.7 \pm <0.1$ (mean ± standard derivation) in the DEMON and nitrification/denitrification tanks, respectively, which is reflected in the higher anammox and denitrification rates per VSS, i.e. per biomass and other organic material.

The temperature dependence of the N-conversion rates affected anammox in particular. In the DEMON, anammox rates were $7.7 \pm 0.6$ and $72.9 \pm 7.2\ \mu$mol gVSS$^{-1}$ h$^{-1}$ at 10 $^\circ$C and 30 $^\circ$C, respectively (mean ± standard error), and thus increased nearly 10 times at higher temperature (Figure 1), which corresponds to an anammox Q$_{10}$ value of 5.0. Anammox rates also showed a strong temperature dependence in the nitrification/denitrification tanks. Here, the rates were $0.011 \pm 0.002$ and $0.082 \pm 0.004\ \mu$mol gVSS$^{-1}$ h$^{-1}$ at 10 $^\circ$C and 30 $^\circ$C, respectively (Q$_{10}$ value: 2.7). Denitrification also exhibited a distinct temperature dependence, but was less sensitive than anammox. Thus, rates increased from $1.3 \pm 0.1$ to $2.7 \pm 0.2\ \mu$mol gVSS$^{-1}$ h$^{-1}$ at 10 $^\circ$C and 30 $^\circ$C, respectively, in the DEMON (Q$_{10}$ value: 1.4; Figure 1), and from $1.17 \pm 0.07$ to $6.33 \pm 0.41\ \mu$mol gVSS$^{-1}$ h$^{-1}$ at 10 $^\circ$C and 30 $^\circ$C, respectively, in the nitrification/denitrification tanks (Q$_{10}$ value: 2.3).

Anammox in the mainstream is negligible

Anammox was the dominant dissimilatory N-removal pathway in the DEMON and denitrification was the dominant dissimilatory N-removal pathway in the nitrification/denitrification tanks, at which denitrification rates in the DEMON are almost half as high as in the nitrification/denitrification tanks (Figure 1). At 30 $^\circ$C, which is in the range of the in situ temperature of the DEMON (30–35 $^\circ$C), anammox rates were 30 times higher than denitrification rates. At 10 $^\circ$C, which covers the in situ temperature of the nitrification/denitrification tanks during the seasons in Denmark except for the summer, denitrification rates were 115 times higher than anammox rates, and at 20 $^\circ$C, which covers the in situ temperature during summer, denitrification rates were 173 times higher than anammox rates in the nitrification/denitrification tanks. The anammox process is therefore negligible for BNR in the mainstream of Marselisborg WWTP.

<table>
<thead>
<tr>
<th>Water depth (m)</th>
<th>Date</th>
<th>Time (CET)</th>
<th>Temp. (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Side (A)</td>
<td>Side (B)</td>
<td>Side (C)</td>
</tr>
<tr>
<td>1.5</td>
<td>14.08.18</td>
<td>10:38–12:02</td>
<td>13:09–14:49</td>
<td>15:11–16:33</td>
</tr>
<tr>
<td>3–4</td>
<td>30.08.18</td>
<td>10:52–12:45</td>
<td>13:46–15:40</td>
<td>16:28–18:04</td>
</tr>
</tbody>
</table>
Full nitrification dominates over partial nitritation in the mainstream

The investigation of a possible SBNR process, like nitritation-anammox or a nitrite shunt in which nitritation is coupled to denitrification, largely excluded its presence in the nitrification/denitrification tanks of Marselisborg WWTP during the time of our sampling in August 2018. During aeration, NH₄⁺ oxidation was largely mirrored by the production of NO₃⁻/CO₃, indicating full nitrification, while NO₂⁻ concentrations varied little in general (Figure 2). In case of partial nitritation, NH₄⁺ would only become oxidized to NO₂⁻, and NO₂⁻ might either accumulate for later denitrification in anoxic conditions, or immediately be taken up by e.g. simultaneous nitritation/denitrification. However, NO₂⁻ wouldn’t become further oxidized to NO₃⁻ as observed in this study. Also, the net consumption rates of NH₄⁺ and net productions rates of NO₃⁻ are in the same range (Figure 3), which again stresses that partial nitritation in the nitrification/denitrification tanks of Marselisborg WWTP can be largely excluded. The NO₂⁻ turnover to NO₃⁻ is so immediate that it cannot be captured, or might be partially intracellular (comammox; Daims et al. 2015). The sampling site at 3–4 m water depth close to an aeration zone (bottom water) is, however, an exception. Here, the
consumption of NH$_4^+$ is not completely mirrored by the production of NO$_3^-$ (Figure 2) and also the net consumption and net production rates are not in the same range (Figure 3).

DISCUSSION

As part of an energy-efficiency plan, Marselisborg WWTP successfully implemented sidestream anammox with the DEMON process. To explore whether anammox might also contribute with a reasonable relative proportion to BNR in the nitrification/denitrification tanks of Marselisborg WWTP, excess sludge from the DEMON was continuously redirected into the nitrification/denitrification tanks for more than three years. The continuous feeding approach was selected since it would be directly applicable in conventional activated sludge WWTPs with anaerobic sludge digestion and anammox deammonification of reject water from the digester, without significant redesign of processes and infrastructure. The results of our $^{15}$N-labelling rate measurements show, however, that despite the innovative long-term initiative of Marselisborg WWTP, anammox rates in the mainstream only reach approximately 1% of the measured denitrification rates, and are thus negligible in the mainstream (Figure 1). To our knowledge, these are so far the only reported results of a long-term trial to introduce anammox in a mainstream during ongoing operation of the WWTP.

A well-known challenge of implementing anammox in a mainstream of temperate regions, is the adaption of anammox activities to lower temperatures (Hu et al. 2013; Hoekstra et al. 2018). The temperature optimum of anammox bacteria in wastewater systems is around 30–40 °C (reviewed by van Hulle et al. 2013), which corresponds to the conditions in the DEMON, but mainstream temperatures in temperate regions vary between 7 and 20 °C. Our $^{15}$N-labelling rate measurements at 10, 20 and 30 °C confirm a pronounced temperature dependence of anammox activities (Figure 1) and, moreover, a significant cold adaption of DEMON reactor grown annamox bacteria in the mainstream nitrification/denitrification tanks cannot be observed. The calculated $Q_{10}$ values from anammox rates in the DEMON and nitrification/denitrification tanks are in the same range. However, the lower temperatures in the mainstream and the lack of cold adaption, respectively, cannot be the only factor for negligible anammox rates in the nitrification/denitrification tanks of the mainstream. Also, in our 30 °C experiments with activated sludge from the nitrification/denitrification tanks, anammox rates were so low that anammox could not contribute significantly to BNR, even if the mainstream temperature at Marselisborg WWTP would be 30 °C.

One at-first-glance convincing explanation for the low anammox rates in the mainstream is simply the low abundance of anammox bacteria. In the DEMON, anammox granules become continuously enriched during cyclone operation and are thus numerous and also very well visible with the naked eye. In fact, approximately 22% of the DEMON sludge consists of anammox granules (TSS/dry weight [DW]; Supplementary Table 1, available with the online version of this paper). In contrast, no additional...
measures were taken by Marselisborg WWTP to keep and further enrich anammox granules in the mainstream nitrification/denitrification tanks. The entrainment of anammox granules from the DEMON to the nitrification/denitrification tanks is not controlled, and the solids retention time (SRT) at Marselisborg WWTP might, with six and 12 days between summer and winter month, be too short, such that anammox granules become washed out too fast. Actually, only 29 granules were counted during a systematized screening of 250 L water from the nitrification/denitrification tanks, which accounts to approximately 0.045% of the sludge in the nitrification/denitrification tanks (TSS/DW; Supplementary Table 1). The DEMON sludge therefore contains a 500 times higher fraction of anammox bacteria, which nearly explains the approximately 800–900 times higher anammox process rates measured in the DEMON (Figure 1). The short SRT also largely prevents a sufficient growth of anammox bacteria. Even at optimal growth conditions with temperatures between 50 and 40 °C, anammox doubling times are 7–14 days (reviewed by Ali & Okabe 2015) and are thus in the same range as the SRT. At lower temperatures, doubling times can yet increase by 10-fold (Lotti et al. 2014), though actual doubling times will depend on respective anammox species and growth conditions. A promising action for enhancing anammox bacteria abundance would be to increase their residence via a prolonged SRT. A significantly increased SRT, and thus a further decoupling of SRT to hydraulic retention time (HRT) would, however, require a complete process redesign and probably a much more efficient recovery of C-sources via primary sludge extraction, before partial oxidation of NH₄⁺ to NO₂⁻ as the initial step in anammox. The configuration would thus be moving towards a situation similar to conditions in the DEMON tank, but at lower temperatures and higher C-content. Other possibilities for increasing the anammox bacteria abundance would be the installation of permanent bacterial carriers or cyclone-like operations, and with this allowing anammox bacteria to grow and adapt to lower temperatures, respectively. Indeed, several experimental laboratory studies demonstrate that anammox-specific nitrogen removal activities, including growth, adapt to lower temperatures, if required time is given (Isaka et al. 2006; Cema et al. 2007; Hu et al. 2013; Hendrickx et al. 2014), also in simulated mainstream conditions (Lotti et al. 2014).

The low abundance and corresponding low process rates of anammox in the nitrification/denitrification tanks of Marselisborg WWTP might, however, have several explanations and may not be tracked back to the short residence only. Factors such as an unfortunate pH or limited nutrient availability, or a potential combination of these factors, also have a strong influence on anammox activities (review by Li et al. 2018; Hoekstra et al. 2018). One essential factor to keep the anammox process active, is a continuous and sufficient supply of NO₂ for NH₄⁺ oxidation. In the DEMON, this is achieved by nitrification-anammox. In the nitrification/denitrification tanks of Marselisborg WWTP, NH₄⁺ largely becomes oxidized to NO₃⁻ during aeration essentially without net production of NO₂, i.e. full nitrification dominates over partial nitritation (Figures 2 and 3). A crucial question is therefore if the low abundance and activity of anammox bacteria in the nitrification/denitrification tanks might also be a consequence of a limited NO₂ accumulation and a herewith connected potential diffusion limitation into anammox granules. And if so, whether enhanced partial nitritation would enhance anammox activities, including growth, in the mainstream. Enhanced partial nitritation would not only be beneficial for strong nitritation-anammox, but also for a nitrite shunt, in which nitritation is coupled to NO₂ driven denitrification (Soliman & Eldyasti 2016), saving both aeration costs and costs for additional organic carbon supply. For a successful implementation of partial nitritation for either nitritation-anammox or a nitrite shunt, AOBs have to outgrow NOBs, i.e. full nitrification needs to be suppressed during aeration (Soliman & Eldyasti 2018). In the nitrification/denitrification tanks of Marselisborg WWTP, this might happen to some extent in the bottom sludge, in particular at the sampling site close to an aeration zone, where low NO₂ production was measured during aeration (Figures 2 and 3). Interestingly, in this area close to an aeration zone, the O₂ concentration was so low even during aeration (O₂ turnover faster than O₂ supply) that also simultaneous nitrification/denitrification could take place, which hints to partial nitritation. Other (micro)habitats of the obviously heterogeneous nitrification/denitrification tank might show comparable characteristics. Patterns can also change with daily and seasonal variations of the wastewater nutrient composition, microbial composition of the activated sludge and other parameters like O₂ concentration and pH, which all influence microbial N-­conversion rates. The exact interplay of parameters that favor AOB growth over NOB growth, and thus partial nitritation, is complex (reviewed by Soliman & Eldyasti 2018). For example, a temperature >24–25 °C favors AOB growth, whereas a temperature between 10 and 20 °C favors NOB growth. The temperatures during our in situ
measurements on SBNR were 19.0 and 21.5 °C, respectively, which should neither favor AOB nor NOB strongly. Lower temperatures outside of summer in Denmark would, however, be less conducive for partial nitritation in a mainstream. Also, the pH in the nitrification/denitrification tanks of Marselisborg WWTP was relatively low, approximately 7 (Table 1). This in particular has a negative effect on AOB, which have a pH growth optimum between 7.9 and 8.2. In contrast, NOB have a pH growth optimum between 7.2 and 7.6, and thus have an advantage at a pH around 7. The anammox process itself, would, however, not be affected by the low pH. The optimum pH for the growth and activity of anammox bacteria used in wastewater treatment is in the range of 6.7–8.3 (Strous et al. 1999).

CONCLUSION

During ongoing daily operations of WWTPs, biotic and abiotic parameters are subject to large fluctuations. Increasing the anammox bacteria abundance in the nitrification/denitrification tanks by e.g. extending the SRT while keeping a good substrate-to-microorganisms ratio, and adjusting favorable conditions for partial nitritation for either nitritation-anammox or a nitrite shunt in a mainstream are therefore challenging tasks (Kouba et al. 2017). Good partial nitritation might be reached by continuous monitoring of parameters that influence AOB versus NOB growth and a subsequent immediate response to alter potentially unfavorable conditions. Tackling this might be essential to establish the necessary continuous and sufficient NO₂⁻ supply for anammox growth and activity in the mainstream and could be, therefore, the decisive foundation for the establishment of nitritation-anammox to a reasonable relative proportion of BNR. An inoculation of AOB and anammox biofilms on, for example, permanent carriers might be of additional help to keep and enrich the desired bacteria in the nitrification/denitrification tanks, and with this to hopefully enhance BNR by nitritation-anammox, even in a mainstream of a temperate region.

ACKNOWLEDGEMENTS

Per Overgaard Pedersen from Aarhus Vand (the WWTP operating organization in Aarhus, Denmark) is recognized for inviting us to contribute with this study to the Vandsektorens Udviklings- og Demonstrationsprogram, which was financed by Dansk Vand og Spidlevandsforening, Denmark (project ID 88.2016). The Poul Due Jensen Foundation is acknowledged for additional financial support. Flemming Husum from Aarhus Vand is thanked for help and advice during sampling at the WWTP and for fruitful discussions. Nils Risaard-Petersen from Aarhus University is acknowledged for the initial idea to contribute with 15N-labelling experiments to this study. Karina Bomholt Oest is thanked for running the isotope ratio mass spectrometer, and Lars Borregaard Pedersen for various help in the laboratory.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


Hoekstra, M., de Weerd, F. A., Kleerebezem, R. & van Loosdrecht, M. C. M. 2018 Deterioration of the anammox process at
decreasing temperatures and long SRTs. *Environmental Technology* 39 (5), 658–668.


First received 18 January 2019; accepted in revised form 5 April 2019. Available online 17 April 2019