Sinks and sources of anammox bacteria in a wastewater treatment plant – screening with qPCR

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ABSTRACT

The deammonification process, which includes nitritation and anammox bacteria, is an energy-efficient nitrogen removal process. Starting up an anammox process in a wastewater treatment plant (WWTP) is still widely believed to require external seeding of anammox bacteria. To demonstrate the principle of a non-seeded anammox start-up, anammox bacteria in potential sources must be quantified. In this study, seven digesters, their substrates and reject water were sampled and quantitative polymerase chain reaction (qPCR) was used to quantify both total and viable anammox bacteria. The results show that mesophilic digesters fed with nitrifying sludge (with high sludge ages) can be classified as a reliable source of anammox bacteria. Sludge hygienization and dewatering of digestate reduce the amount of anammox bacteria by one to two orders of magnitude and can be considered as a sink. The sampled reject waters contained on average \( > 4.0 \times 10^4 \) copies mL\(^{-1}\) and the majority of these cells (\( > 87\% \)) were viable cells. Furthermore, plants with side-stream anammox treatment appear to have higher overall quantities of anammox bacteria than those without such treatment. The present study contributes to the development of sustainable strategies for both start-up of anammox reactors and the possibility of improving microbial management in WWTPs.

Key words | anaerobic ammonium oxidizing bacteria (anammox), digestion, full-scale, qPCR, reject water treatment, start-up strategy

INTRODUCTION

Anaerobic ammonium oxidation, known as anammox, is an effective way of converting ammonia into nitrogen gas in wastewater streams, using nitrite as an electron acceptor. This process occurs in several natural environments such as marine and freshwater sediments and water columns (Schmid et al. 2007) as well as in engineered wastewater systems (Mulder et al. 1995; Beier et al. 1998). In wastewater treatment plants (WWTP), the use of autotrophic anammox bacteria is increasing in popularity because it reduces aeration demand and does not require an additional carbon source (Strous et al. 1998). However, since the bacteria are slow growers in comparison to other bacteria used in nitrogen wastewater treatment, the right growth conditions must be implemented in the WWTP.

One of several necessary conditions for successful growth and maintenance of the bacteria in the system is sufficient sludge age. Common techniques to achieve this are biofilm applications, as well as granular sludge or sludge with gravity selection. The critical sludge age depends on the temperature of the process. The optimum temperature for the anammox process was initially found to be mesophilic, around 35–40 °C, (Strous et al. 1999). In side-stream reject water treatment (illustrated in Figure 1 in Methods), dewatering of mesophilic (or thermophilic) digestate allows the process to be performed in the mesophilic temperature range. No successful selection of anammox bacteria was done at thermophilic conditions in a study performed by Toh et al. (2002).

When starting up a new anammox reject water treatment plant, optimum temperatures (30–35 °C) are beneficial to enable high activity and high growth rates in order to reduce start-up time. Another important factor is availability of a seeding source (inoculum) for the anammox bacteria. The source of inoculum may vary depending on the technique selected (biofilm, granular or sludge). For sludge and granular systems, it has become common to seed new plants with a small amount of anammox sludge from an existing plant. By contrast, for biofilm systems, our previous...
work and a number of other studies have shown that external seeding is not necessary to start up the process (Schneider et al. 2009; Zekker et al. 2012; Kanders et al. 2016, 2014). These start-up strategies assume that there are sufficient quantities of anammox bacteria in the influent reject water. In this paper, we aim to verify this assumption and also investigate if this is independent of the sludge treatment, such as hygienization.

Hygienization of sludge at elevated temperatures prior to digestion is a common strategy for reducing pathogens and/or breaking up cells to increase the biomethane potential. Since the microbiological composition in the digester is likely to influence the composition of the reject water, these aspects must also be taken into account when selecting start-up strategies for side-stream treatment.

The overall aim of this study was therefore to gain an overview of anammox sources in a WWTP, and specifically, to investigate and evaluate the possibilities for internal seeding of new side-stream treatment steps. In our study, we:

- evaluated whether anammox bacteria were present in different types of digesters and their reject waters, and if so
- whether the pre-hygienization of the substrate (wastewater sludge/co-substrate) entering the digester influences the quantity and viability of anammox bacteria.

Further, if an anammox treatment is already present at the WWTP as a side-stream process, we investigated whether it influenced the presence and the quantity of anammox bacteria in the main stream of the plant, downstream of the side-stream.

We used quantitative polymerase chain reaction (qPCR) to detect, quantify and measure viability of anammox bacteria.
**METHOD**

**Plant characterization and selection**

Operational data were collected from several plants prior to the study to enable selection of plants that digest different substrate compositions (wastewater sludge, household food waste, manure, industrial food waste, slaughterhouse waste and septic wastewater), utilize different hygienization methods (none, in-digester at 55 °C, 1 h at 70 °C, and 20 min at 135–160 °C), and operate at different digestion temperatures (mesophilic, 37–40 °C, or thermophilic, 55 °C). The existence of side-stream anammox treatment was also an important factor.

Three WWTPs and four biogas production plants were selected and numbered (1–7) in the first sampling round. The plants are presented in Table 1 and have been selected for their relevance to the objectives of this study.

- Plants 1, 2, 4, 5 and 7: five plants; mesophilic digestion.
- Plants 3 and 6: two plants; thermophilic digestion.
- Plants 2, 3, 4, 6 and 7: five digesters; mainly with wastewater sludge as substrate, two of which have no addition of external substrate.
- Plants 1 and 5: two digesters that do not use wastewater sludge as substrate.
- Plants 1, 3, 5 and 7: four plants; apply pre-hygienization to the substrate before digestion two of which apply hygienization in the normal range (70 °C), and two at high temperature range (135–160 °C).
- Plants 2, 3, 4, 6 and 7: five plants; digestate is dewatered and the liquid fraction (reject water) is either treated separately for nitrogen removal with a deammonification method or directly recirculated back to the main biological treatment.
- Plants 2 and 6: two out of three selected WWTPs have nitrogen side-stream treatment with anammox (biofilm processes).

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant type</th>
<th>Substrate to digester</th>
<th>Sludge age (d)</th>
<th>Hygienization</th>
<th>Digestion mode</th>
<th>HRT (d)</th>
<th>Anammox at plant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biogas plant</td>
<td>Food waste from households</td>
<td>20 n/a</td>
<td>1 h, 71 °C</td>
<td>Mesophilic</td>
<td>35 No</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Manure</td>
<td>30 n/a</td>
<td>37–38 °C</td>
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<tr>
<td></td>
<td></td>
<td>Food waste from industry</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Slaughterhouse waste</td>
<td>10 n/a</td>
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<tr>
<td>2</td>
<td>WWTP w digesters</td>
<td>WW sludge with biological nitrogen removal</td>
<td>70 16</td>
<td>No</td>
<td>Mesophilic</td>
<td>17 Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Food waste from households</td>
<td>30 n/a</td>
<td>1 h, 70 °C</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>Biogas plant</td>
<td>WW sludge with no biological nitrogen removal</td>
<td>100 3–4 and biofilm</td>
<td>1 h, 70 °C</td>
<td>Thermophilic</td>
<td>14 No</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Food waste from industry (screenings, starch, fat)</td>
<td>65 6</td>
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<tr>
<td>4</td>
<td>WWTP w digesters</td>
<td>WW sludge with biological nitrogen removal</td>
<td>35 n/a</td>
<td>THPb, 20 min at 135 °C</td>
<td>Mesophilic</td>
<td>27 No</td>
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<td></td>
<td></td>
<td>Food waste from industry</td>
<td>100 n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Biogas plant</td>
<td>WW sludge with biological nitrogen removal</td>
<td>100 22</td>
<td>In digester for 2 h</td>
<td>Thermophilic</td>
<td>12 Yes</td>
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<td></td>
<td></td>
<td>Food waste from households</td>
<td>100 3</td>
<td>biofilm</td>
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<tr>
<td></td>
<td></td>
<td>Septic wastewater</td>
<td>12 n/a</td>
<td>THPb, 20 min at 160 °C</td>
<td>Mesophilic</td>
<td>16 No</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 | Selected plants with relevant information for evaluation: their substrate composition to digester, hygienization method, digestion temperature, digestion hydraulic retention time (HRT) and presence/absence of side-stream anammox treatment

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*aOnly at food waste from households.

bTHP, Thermal hydrolysis process.

WW, Waste water; n/a, not applicable.
All digesters and their mixed substrates were sampled; if hygienization was used, samples were taken before and after this step. Only one digester was sampled at each plant, even though some plants had two or even three digesters. Where applicable, reject water from centrifuges and anammox-treated reject water were sampled. In addition, samples were taken from the return sludge as well as from the influent in all of the WWTPs. Influent samples were taken from raw influent wastewater or after the sand-and-grit trap, and were used as an influent reference for the main biology. Plant 1 did not have a full-scale dewatering unit, but reject water was produced in the laboratory by centrifugation (2,000 g for 10 min; Sorvall Lynx 4000, Thermo Scientific).

Sample points were numbered and are shown in a conceptual block scheme in Figure 1. The first digit indicates the plant (1–7) (see Table 1) and the second digit indicates the sample point (0–6):

- 0 – Untreated influent to wastewater plant
- 1 – Substrate to digester before hygienization step
- 2 – Substrate to digester
- 3 – Digester
- 4 – Reject water
- 5 – Anammox-treated reject water
- 6 – Return sludge in activated sludge system.

All samples were collected during April and May 2017 and analysed for physical and chemical parameters and anammox bacteria content. After evaluation of data from the first qPCR round, three of the most interesting plants (plants 1, 4 and 7) were selected for a second round of sampling three months later. In this second round both qPCR (for total quantification) and viability PCR (vPCR) were performed.

Operating data for the digesters at the time of sampling was provided by the process engineers at each plant. Temperature at each sampling point was given as the normal operating temperature. The pH was measured either at the time of sampling (with a portable HQD40/Intellical pHC 101 from Hach Lange, Germany) or in the laboratory within 24 h of sampling (Radiometer, Copenhagen). Samples for chemical and physical parameters were kept cold and analysed within 24 h, unless otherwise stated. The microbiological samples were stored at −18 °C and analysed 4–8 weeks later.

### Chemical and physical parameters

Before filtering, sludge samples were first centrifuged (Sigma 2–15 for 15 min at 13,500 rpm or Thermo, Heraeus Labofuge 200 for 10 min at 5,000 rpm). Water samples and supernatant from the centrifuged sludge samples were filtered using a 0.45 μm syringe filter and analysed for inorganic nitrogen compounds (NH₄-N, NO₂-N and NO₃-N) with Hach cuvett tests (LCK 302/303, 342, 340; Hach Lange, Germany) using a spectrophotometer (Dr Lange Lasa 100). The soluble organic fraction (sCOD) was analysed with a similar procedure (LCK 114/400/014; Hach Lange, Germany) using filtered samples. Analyses of total and volatile solids (TS and VS, respectively) were conducted on unfiltered samples according to 2540B and E (APHA 2005) respectively, however total solids was measured only once after 24 h of drying.

### Quantification of anammox bacteria by PCR

Anammox bacteria were quantified with a TaqMan fluorogenic qPCR method using DNA extracted from the samples as previously described. DNA was extracted from the samples (200 μL) using the Qiagen QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). This kit contains reagents and consumables needed for cell lysis and DNA purification by membrane filtration. The extracted DNA was stored at −20 °C prior to PCR amplification. The primer set (AMX-808-F, AMX-1040-R) and the probe (AMX-931), designed by Hamersley et al. (2007) to target all known anammox or anammox-like bacteria, was used. The target region is within the 16S rRNA gene. The PCR amplification conditions were as follows: 2 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 5 s. DNA extracted from an anammox enrichment culture obtained from an MBR system (99% related to Brocadia sp. 40) was used as positive control and standard. All samples and standards were analysed in duplicate. The qPCR amplification curves were processed and concentrations were determined using the Rotor-Gene Q-series system software, version 2.3.1 (Qiagen). The assay can detect 500 copies in 1 mL sample (limit of detection of the method including the DNA extraction).

Viable anammox bacteria in selected samples were quantified by vPCR. To distinguish between dead and viable anammox bacteria, the samples were treated with propidium monoazide bromide (PMA), which penetrates cells with a damaged cell wall, intercalates the DNA and prevents DNA amplification. In the present study, we used the method described by Delgado-Viscogliosi et al. (2009) and Mansi et al. (2014) to determine the concentration of viable anammox bacteria. No reliable results were obtained regarding the concentration of viable anammox bacteria from either the hygienized substrates or the digester because these samples contain a high amount of organic matter and
microbial biomass, respectively, that retains propidium monoazide and consequently reduces the available concentration of this DNA intercalating agent to mask dead anammox cells. PMA limitations when applied to raw wastewater were also reported by Varma et al. (2009). Only samples taken before or without hygienization (sample point 1 or 2) and reject water samples (sample point 4) gave reliable results.

A multivariate linear regression including Pearson coefficients was made between the quantification of anammox bacteria (log copies g⁻¹ VS) and the chemical data (pH, T, NH₄-N, NO₂-N, NO₃-N, PO₄-P, sCOD, TS, VS, NH₃, C/N and sludge retention time) in the samples by using Excel Analysis Toolpak (Microsoft Office).

RESULTS AND DISCUSSION

Parameters for pH, temperature, inorganic nitrogen compounds, soluble organics and total and volatile solids for each sample point in the first round of analysis are shown in Supplementary information (S1), available with the online version of this paper. In the second sampling round, only samples from sample points 11, 14, 42, 44, 71 and 74 were taken, and only TS and VS were analysed, these results are also presented in S1.

The concentrations of anammox bacteria in each sample were determined by qPCR and are expressed in copies mL⁻¹ sample including standard deviation (Figure 2(a)). To facilitate the comparison between samples, the concentration was normalized against the organic content of the sample, measured as volatile solids. The normalized concentrations (in copies gVS⁻¹) are presented in Figure 2(b), in plant order with standard deviation. Substantial amounts of anammox bacteria were found in all samples except sample 40, in which the quantities were below the detection limit (500 copies mL⁻¹) of the qPCR method. The amount of bacteria ranged from 8.9×10⁴ to 1.2×10⁹ copies gVS⁻¹, and it can be concluded that anammox bacteria are present in all plants independent of process configuration or substrate used for digestion.

The statistical analysis showed that the quantity of anammox (log copies gVS⁻¹) was positively correlated with pH, NO₂-N, NH₃-N and sludge retention time but only pH was significant (p < 0.05). The correlations were weak (had a low coefficient) but the positive correlation with these parameters could be expected from an anammox habitat. The parameters with the strongest negative correlation were temperature, PO₄-P and sCOD, and all of them were significant (p < 0.05), see Supplementary information (S2), available with the online version of this paper. Wang et al. (2015) showed a significant positive coefficient for temperature but their samples did not include any samples above digestion temperature.

It should be noted that this is a limited set of data and caution is advised when extrapolating from these results. Nevertheless, the sections below include some interpretation to open a broader discussion. The interpretations are mostly based on relative numbers within the study, but some careful comparisons are made to other studies using absolute anammox quantities.

Natural occurrence of anammox

For a reference, water mainline anammox value samples were taken from the influent of three WWTPs (sample points 20, 40 and 60). Two of the three influent samples were sampled from the influent to the mainstream wastewater treatment (samples 20 and 60) and showed relatively high amounts of anammox (1.4–7.8×10⁴ copies mL⁻¹), while in the third sample (40), which was collected directly at the influent pipe line, anammox was below the detection limit of the method (500 copies mL⁻¹). These results clearly show that anammox bacteria are already present in the influent to the mainstream biology. Water samples collected in the influent mainline can be expected to exhibit high variability due to both variations in flow and uneven mixtures of different waters. However, in one sample point (20), it was observed that a minor sludge stream was recirculated back before this sample point, which might explain the presence of anammox bacteria. The results for naturally occurring anammox in the influent can be compared with a study from Wang et al. (2015), who found in the order of 10⁵ anammox abundance in aerated grit chambers in five different WWTP (copies g⁻¹ MLVSS). It should be noted that water streams with low dry solids content will contain less biomass.

Influence of hygienization on anammox quantities and viability

At some of the investigated biogas plants, the substrate is hygienized prior to digestion. The qPCR results presented in Figure 2 show evidence of sterilization in plants 5 and 7, where the amount of the anammox DNA decreases between sample points 1 and 2. These plants employ sludge hygienization using the thermal hydrolysis process (THP), which also applies pressure in order to achieve high temperatures (135–165 °C for 20 min). However, anammox bacteria
counts in the range of $10^4$–$10^5$ copies gVS$^{-1}$ still remain after hygienization, as some of the anammox genome is detectable and can be amplified. Plants 1 and 3 employ hygienization in the normal range (1 h at 70 °C). These plants did not show any decrease of anammox quantities as detected by qPCR, but it should be emphasized that the qPCR detects all anammox DNA and does not distinguish between viable and non-viable bacteria before or after the hygienization step. The detection of anammox bacteria after hygienisation is probably due to the resistance of the phosphodiester bonds of the DNA molecule to high temperatures. Temperature gradients in the thermal hydrolysis reactor due to the
The inhomogeneous nature of the sludge and food waste samples might also prevent fragmentation of the DNA molecule. Plant 6 uses a different hygienization method (2 h at 55°C). The absolute amounts of anammox in copies mL⁻¹ (Figure 2(a)) decrease from substrate to digester in this plant, but this could also be due to competition from other bacteria in the digester.

The second sampling round three months later had two purposes: first, to investigate the viability of the anammox bacteria and how this was influenced by hygienization and digestion; and second, to compare the qPCR results of the second sampling round to those from the first round. Three plants were selected (plants 1, 4 and 7) and the focus was on analysis of differences in viability of anammox during hygienization and digestion. Only samples taken before or without hygienization (sample point 1 or 2) and reject water samples (sample point 4) gave reliable results, as described in Methods. These six samples were analysed for amounts of total and viable anammox bacteria. Figure 3 shows that all samples exhibited a very high fraction of viable anammox bacteria (42–128%). Values indicating more than 100% viable bacteria were within one standard deviation. This indicates that the anammox bacteria entering the digester via the substrate and those leaving the digester via the reject water were viable, which is an important result from this study. The data from Figure 2(b) (first round) and Figure 3 (second round) show that two out of three plants exhibited very similar values, within the same order of magnitude, which indicates that values generated in this study were relatively stable over time. However, the reject water sample (74) from the first round is a suspected outlier due to two reasons. First, it was two orders of magnitude higher than in the second round and second, it is not reasonable that the anammox quantity would increase by one order of magnitude from the digester to the centrifuge. Still, the data are kept but marked with arrows in the diagrams. In all three plants selected for the second sample round, the total numbers of anammox increased from substrate to reject water (Figure 3). This indicates an enrichment of viable anammox bacteria during digestion.

**Arguments for anammox bacteria thriving in digester**

The quantities of anammox in the digester were in general higher than the quantities in the corresponding substrate. The quantities in the substrate range from $8.9 \times 10^4$ to $2.9 \times 10^8$ copies gVS⁻¹ while the quantities in the digesters ranged from $1.1 \times 10^7$ to $3.6 \times 10^8$ GU gVS⁻¹ (presented in Figure 2(b)). When evaluating normalized samples (copies gVS⁻¹), the increase in anammox bacteria is expected since the organic content in the digester is decreased due to digestion. However, when we examine the relative amounts (presented in Figure 2(a) in copies mL⁻¹) we observe the following trends:

- anammox bacteria increased in number between sample points 2 and 3 in the digesters run in mesophilic mode, while
- anammox bacteria were stable (plant 3) or decreased in number (plant 6) in the digesters run in thermophilic mode.

This implies that anammox bacteria are enriched in digesters in mesophilic conditions. This is unexpected and novel, but seems reasonable since everything necessary for anammox growth is available in a digester, such as high sludge age, nitrite, ammonia and bicarbonate. Sludge age in the digesters, which in this case is equal to the hydraulic...
retention time, ranges from 12–35 days (Table 1). This is long enough to retain anammox bacteria in the system. Nitrite was either undetectable or present at negligible levels in the digester (0–0.21 mg NO₂-N L⁻¹ from S1). However, nitrate can be detected in the substrate to the digesters in the range of 2–22 mg NO₃-N L⁻¹ (see S1). Since the digesters are fed continuously with nitrate, the nitrate may be reduced to nitrite when used as an electron acceptor, and nitrite together with available ammonia and bicarbonate can support growth of anammox. Contrastingly, ammonia is available in excess, or even inhibiting concentrations, with levels of 24–395 mg NH₃-N L⁻¹. Bicarbonate is assumed to be supplied continuously by the heterotrophic and the acidogenic bacteria in the digester. Other environmental parameters such as temperature (37–38 °C), pH (7.3–7.9), micronutrients and a reducing environment are also in the range for growth of anammox bacteria. Co-existence between anammox bacteria and methanogens has likewise been shown by Gonzalez-Martinez et al. (2015), in which Archaeal methanogens were found in three full-scale anammox plants.

Our results from the present study indicate the presence of anammox bacteria, and the possibility that they may even thrive in the digester. Assuming that anammox bacteria enrichment in a digester is limited by nitrite and that the digester retention time (15 d) is longer than the actual doubling time, then an example can be calculated. An average mesophilic digester (V = 3,000 m³, 2% VS and no detectable NO₃), is fed with 200 m³ d⁻¹ substrate containing 10 mg NO₃-N L⁻¹, which in the digester is reduced to NO₂ and used as substrate. By using the yield (0.12 g DW g⁻¹ NH₄-N⁻¹) and the conversion rate of anammox (0.66 g NH₄-N g⁻¹ DW d⁻¹) calculated from Strous et al. (1999) and the stoichiometry 1:1.32:1.02 (Strous et al. 1998), we can calculate that the influent can sustain an anammox biomass of 2.65 kg dry weight per digester, and the conversion rate would be 0.57 gN g⁻¹ anammox d⁻¹. According to Strous et al. (1999), this is 87% of the maximum conversion rate. Further, this would produce approximately 1.5 kg N d⁻¹ as nitrogen gas, which could result in an error source in the nitrogen mass balance of the digester. Whether the nitrogen gas produced in the digester comes from anammox conversion or from ordinary heterotrophic conversion cannot be evaluated without performing N-labelling experiments.

**Sludge management and internal bioaugmentation**

A WWTP is a complex system with several, often biological, processes linked together. In such a system, downstream processes are influenced by the system upstream. Examples of such effects include how microbes in wastewater sludge may influence the microbial community in the digester, how digesters influence the community in the reject water treatment, and similarly how reject water treatment potentially influences microbial flora in the main biology. The following sections discuss the interaction between the different biological treatment steps (main biology, digestion and side-stream treatment, as described in Figure 1).

**Nitrogen sludge influences digester anammox quantities**

Whether the digester provides favorable conditions for anammox bacteria may need extensive research, but there is firm evidence that nitrifying sludge contains anammox bacteria, as shown by several previous studies (Chamchoi & Nitisoravut 2007; Toh et al. 2002; Tang et al. 2013; Tao et al. 2013). The quantities of anammox bacteria in the substrate to the digester did not seem to influence the absolute quantities in the digester. However, the digesters that were linked to a WWTP, and hence received activated sludge with nitrogen removal, had among the highest quantities of anammox. Further, digesters fed mainly with wastewater sludge having high sludge ages tended to have more anammox than digesters fed with lower sludge ages. For example, three plants with similar HRT (15 ± 2 d): plant 4, 6-day sludge, 1.3 × 10⁸; plant 2, 16 days, 1.7 × 10⁸; and plant 6, 22 days, 3.6 × 10⁸ copies gVS⁻¹. This seems reasonable since the sludge age would be expected to be limiting on the amount of anammox in the wastewater mainstream at corresponding temperatures.

Smaller quantities were found in the digesters at plants 3 and 5. The digester at plant 3 treats WWTP sludge with very low sludge age (3–4 d), and therefore the substrate would not be expected to contain anammox. The digester at plant 5 only receives food waste, which would not be expected to contribute anammox bacteria to the digester either. This, in combination with the thermal hygienization process, may explain the smaller amounts of anammox. However, the digester at plant 1 exhibits high anammox, despite the substrate not containing nitrogen sludge and the digester being fed with mainly food waste. This plant also receives manure, which could contribute to increased anammox levels (Wang et al. 2017), or maintain favourable conditions in the digester for anammox microbial growth, as discussed earlier in this section. To summarize, the quantities of anammox bacteria in the digesters are influenced by type of substrate, and substrates containing nitrifying sludge
(higher sludge ages) tend to result in higher anammox quantities in the digester.

Digester or reject water quantities influence seeding of side-stream processes at anammox start-up

No clear correlation could be observed between the amount of anammox in the digester and in the reject water (sample points 3 and 4, in Figure 2). The reason for this is thought to be linked to the dewatering stage. Despite the fact that all plants in this study used centrifugation as the dewatering technique, the performance and reject water quality (gTS L⁻¹) usually depends on centrifugation settings, choice of polymer, concentrations, dosage, etc. Plants 1, 4 and 6 show a strong decrease in quantities of anammox bacteria across the dewatering unit, which indicates that centrifugal forces in the range of 2,000 g (as in the full-scale centrifuges in this study) are more effective for separating anammox granules than other organic material. By comparison, centrifugation above 3,000 g is usually used when separating cells in the laboratory, but anammox bacteria, with their dense anammoxosome, might separate with lower forces. This means that an effective dewatering technique (high g forces and low TSS concentration in reject water) may reduce the feeding of anammox bacteria from the digester to the reject water by separating the anammox cells to the sludge fraction. This is not crucial once an anammox plant is already in operation, but may be of interest during start-ups when the quantities of anammox bacteria in the reject water are critical for seeding the new process.

To further discuss the suitability of using reject water as the sole seeding source for anammox start-up, we can perform an example calculation, assuming that the influent contains 1.0 × 10⁵ copies mL⁻¹ anammox (data from Figure 2(a)) and needs to be increased by a factor of 10,000 to provide a suitable capacity (1.0 × 10⁹ copies mL⁻¹) (Hu et al. 2010; Tao et al. 2013; Rikmann et al. 2017) for nitrogen removal via anammox. Given that the process performance is limited by a doubling time of 7.7 days (at 30 °C, calculated from previous start-up studies), a start-up based on only these indigenous bacteria would take 13.3 doubling times (2^13.3 = 10,086) or 102 days. This is in agreement with earlier findings from start-up studies where full capacity was reached within 100 days in a full-scale plant (Kanders et al. 2016).

Another alternative for successful start-up of a deammonification plant (nitritation and anammox) is a mixture of nitrogen sludge and anaerobic sludge, as suggested by Tang et al. (2015) and supported by the present study.

Thus, the availability of anammox-rich water streams in WWTPs, together with new findings reporting lower anammox doubling times (Lotti et al. 2015) and improved knowledge regarding ‘optimal conditions’ for growing anammox, means that there will soon be few arguments left for seeding new plants with external inoculum, provided that the plant fulfills the conditions presented here.

Side-stream anammox treatment influences main biology in WWTP

Two plants in the study had a separate side-stream treatment containing anammox (biofilm, DeAmmon technique). The return sludge in the main biology at these plants (plants 2 and 6) has higher quantities of anammox bacteria than plant 4, which has no side-stream anammox treatment, and can be seen as a reference plant. The higher presence of anammox in the main stream could be due to actual seeding or augmentation from the side-stream treatment to the main biology (see the conceptual scheme in Figure 1) or any of the two following reasons: the two plants both have high sludge ages (15 and 22 days), and they do not have a separate hygienization process. Both factors may have a positive effect on the amount of anammox, as discussed in the previous sections. Our measurements show that there is a relatively small number of anammox cells in the effluent from the side-streams and hence its contribution is rather limited. Nevertheless, it should be observed that the total quantities increase from the effluent of reject water treatment to the return sludge stream. This indicates that the anammox from the reject water treatment enrich in the main biology. In early anammox research, Hippen et al. (1997) showed that a rotating biological contactor (biofilm technique) could be successfully operated at temperatures around 15 °C, with an established anammox process. The mainstream anammox would not be limited by the seeding source or the temperature, but rather the ability to maintain high sludge age to keep a critical amount of anammox bacteria in the system. With the knowledge that the mainstream biology already contains anammox bacteria, future research could optimize the conditions further, in order to increase autotrophic growth in the mainstream treatment.

CONCLUSION

In conclusion, all samples tested (from mainline biology, hygienized and non-hygienized substrate, digested sludge and reject water) contained significant amounts of anammox
bacteria. Reject water contained $4 \times 10^4$ copies mL$^{-1}$ and the majority of these were viable cells. This provides conclusive evidence of the suitability of indigenous bacteria for starting up a side-stream anammox treatment. Sludge from mesophilic digesters fed with nitrifying wastewater sludge (high sludge age) can be considered as a safe source for anammox bacteria. Hygienization with THP was the only (high sludge age) can be considered as a safe source for ana

tox bacteria. Hygienization with THP was the only treatment that caused a clear reduction of anammox quantities, but hygienization in general should be seen as a sink for anammox bacteria as well as the dewatering process, due to the separation of biomass and the liquid phase. Further, plants with side-stream anammox treatment exhibited higher overall quantities of anammox bacteria than the reference plant without anammox side-stream treatment.

ACKNOWLEDGEMENTS

The authors would like to acknowledge all the biogas and WWTP plants for participating in the study and sharing operational data with us, and Karen Kock (ISAH) for her work with the PCR analyses. We would also like to thank the students Linnea Andersson and Viktor Ljungkvist for help in the laboratory as well as Olle Holst and Lene Nordrum at Lund University for contributing with valuable inputs to the manuscript.

This research is partly financed by a Piia scholarship from Vinnova (Swedish Governmental Agency for Innovation Systems), with co-funding from Purac AB and ABB AB.

REFERENCES


Schneider, Y., Beier, M. & Rosenwinkel, K.-H. 2009 Impact of seeding on the start-up of the deammonification process with different sludge systems. In: 2nd IWA Specialized Conference ‘Nutrient Management in Wastewater Treatment Processes’, Krakow, Poland.


