Title
One-stage nitritation - anaerobic ammonium oxidation at low temperatures in a moving bed biofilm reactor.

Authors
Frank Persson*, Razia Sultana*, Britt-Marie Wilén, Malte Hermansson, Fred Sörensson, Ann Matsson, Elzbieta Plaza

*These authors have equally contributed to the study.

Addresses and affiliations
Frank Persson (corresponding author), Britt-Marie Wilén:
Division of Water Environment Technology, Department of Civil and Environmental Engineering, Chalmers University of Technology, SE 412 96 Göteborg, Sweden.
frank.persson@chalmers.se

Razia Sultana, Elzbieta Plaza:
Department of Land and Water Resources Engineering, Royal Institute of Technology (KTH), SE 100 44 Stockholm, Sweden.

Malte Hermansson, Fred Sörensson:
Department of Chemistry and Molecular Biology, Microbiology, University of Gothenburg, Box 462, SE 405 30 Göteborg, Sweden.

Ann Matsson:
Gryaab AB, Box 8984, SE 402 74 Göteborg, Sweden.

Abstract
Bacteria capable of anaerobic ammonium oxidation (anammox) enable autotrophic nitrogen removal. Organic carbon in wastewater can instead be utilized for energy production. However, anammox-based processes are not yet used at any extent for treatment of the main stream at wastewater treatment plants. One of the reasons for this is the challenge for the slow growing autotrophic bacteria to work at low temperatures. Here we investigate one-stage nitritation-anammox at temperatures of 13-16°C in a pilot moving bed biofilm reactor (MBBR) receiving reject water from anaerobic sludge digestion. At a target nitrogen loading rate of 1 g NH_4^+-N m^{-2} d^{-1} the average nitrogen removal rate was 0.81 g NH_4^+-N m^{-2} d^{-1} and 0.55 g NH_4^+-N m^{-2} d^{-1} at 16°C and 13°C respectively. At low temperatures oxygen control is important to avoid oxygen penetration to the deeper parts of the biofilm, which causes inhibition of the anammox bacteria and as a result nitrite accumulation. Hence, the process was operated at conditions to limit the activity of the aerobic ammonium oxidizing bacteria (AOB) by oxygen availability. The biofilm biomass was dominated by anammox bacteria, with 1.0×10^{14} copies m^{-2} (16S rRNA), with considerably fewer AOB of 2.1×10^{12} copies m^{-2} (amoA), as measured by quantitative PCR. Cell specific conversion rates of anammox bacteria and AOB were estimated at 0.3-0.5 fmol N $cell^{-1}$ d^{-1} and 7-9 fmol N $cell^{-1}$ d^{-1}, respectively. The study shows the applicability of one-stage nitritation-anammox in MBBRs at low temperatures and highlights the importance of quantification of AOB and anammox bacteria for understanding process performance.

Keywords
Ammonium oxidizing bacteria (AOB), anammox bacteria, deammonification, low temperature, moving bed biofilm reactor (MBBR)

Introduction

Nitrogen removal using bacteria capable of anaerobic ammonium oxidation (anammox) is today an established technology for treatment of concentrated high temperature streams e.g. reject water from sludge digestion. Anammox-based processes have the benefits of being autotrophic and hence no organic carbon is needed. The nitrogen removal process could be performed in two stages, with a separate aerated nitritation reactor where aerobic ammonium oxidizing bacteria (AOB) oxidize half of the influent ammonium to nitrite and a subsequent anoxic anammox reactor where anammox bacteria oxidize the remaining ammonium using nitrite as electron acceptor (e.g. Jetten et al., 1997). The nitrogen removal process could also be performed in one stage, with AOB and anammox bacteria in the same reactor, which requires tightly controlled aeration to enable aerobic AOB activity while preventing oxygen inhibition of the anoxic anammox bacteria (Sliekers et al., 2002).

In comparison to conventional biological nitrogen removal using nitrification – denitrification, anammox-based processes save energy due to the decreased needs for aeration and organic carbon and less sludge is produced, which saves labor and costs at the wastewater treatment plants (WWTPs) (Siegrist et al., 2008). Furthermore, greenhouse gas emissions are expected to decrease by using anammox processes, since N_20 is not produced by anammox bacteria and CO_2 is taken up and utilized as carbon source (Kampschreur et al., 2008). The organic carbon content in the wastewater can instead be used for methane production, which together with the decreased need for aeration can make WWTPs net-energy producers, instead of consumer as with the conventional nitrification – denitrification system (Siegrist et al., 2008). Despite these major potential advantages with the anammox technology, it is not yet an established technology for nitrogen removal in the main stream of wastewater, comprising about 85% of the total nitrogen flow at a conventional WWTP (but see Wett et al. 2012). Among the reasons for this is the challenge to obtain enough active biomass of the slow growing anammox bacteria and AOB, which is especially important at the low wastewater temperatures during winter when growth rates are very low. Biomass retention is also crucial for obtaining reasonable removal rates at the low concentrations of nitrogen (< 80 mg N L^{-1}) in the wastewater main stream (De Clippeleir et al., 2011). Besides being important for potential nitrogen removal in the main wastewater stream, anammox-based processes at low temperatures are also interesting for treatment of e.g. landfill leachates (Cema et al., 2007) and reject water from psychrophilic anaerobic digesters (Vazquez-Padin et al., 2009).

In marine sediments, anammox bacteria have been found to be highly active at low temperatures with rate optima as low as 12°C (Rysgaard et al., 2004). In engineered systems, a limited number of studies have investigate nitrogen conversions at low temperatures by anammox bacteria separately (Cema et al., 2007; Dosta et al., 2008; Isaka et al., 2008) or by AOB and anammox bacteria in a one-stage configuration (Vazquez-Padin et al., 2011). Even though sub-optimal removal rates were observed at low temperatures, these studies have indicated that anammox-based processes may be feasible at temperatures below 20°C. Detailed knowledge is however yet lacking on the dynamics and interactions of AOB and anammox bacteria and how to control the process at these conditions.

The aim of this study was to (i) investigate the process performance at temperatures of 16°C and 13°C of one-stage partial nitritation – anammox in a MBBR for autotrophic nitrogen removal

from an anaerobic sludge digestion supernatant and (ii) to quantify the microorganisms responsible for the major nitrogen removals in the MBBR using quantitative PCR.

Methods

The MBBR pilot

The MBBR pilot plant had a volume of 0.2 m^3 and was filled to 40% with Kaldnes K1 biofilm carriers with a specific surface area of 500 m^2 m^{-3}. Stirring with mixers and continuous air supply from the bottom of reactor kept the carriers in motion. Temperature was controlled by a cooler and a heater connected to a thermostat. The pilot MBBR was situated at Hammarby Sjöstadsverk (Centre for municipal wastewater purification), Stockholm Sweden and received reject water from anaerobic sludge digestion from the Bromma WWTP, Stockholm Sweden. The pilot MBBR was equipped for on-line measurements (Cerlic Controls AB, Sweden) of conductivity, pH, redox and dissolved oxygen, which was used as a control parameter. Samples of influent and effluent were taken and filtered for analysis of inorganic nitrogen species and chemical oxygen demand (COD) using dr. Lange test tubes. The pilot reactor was operated at a target nitrogen loading rate (NLR) of 1 g NH_4^+-N m^{-2} d^{-1}.

PCR and quantitative PCR

DNA was separately extracted from 30 mg of biofilm originated from three carriers using the FastDNA spin kit for soil (MP Biomedicals, France). Concentrations of the extracted DNA were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). In order to screen for presence of nitrogen converting organisms, end-point PCR was performed with primers for 16S rRNA genes of anammox bacteria (Tsushima et al., 2007) and amoA genes of AOB (Rotthauwe et al., 1997) and ammonia oxidizing archaea (AOA; Francis et al., 2005). Presence or absence of PCR products was checked by agarose gel electrophoresis. Organisms giving positive results in the screening were further quantified with quantitative PCR (qPCR) using the SYBR green chemistry on an IQ5 Bio-Rad thermal cycler (Bio-Rad Laboratories). The abundance of total bacteria was quantified using universal primers for 16S rRNA genes (Park et al., 2010).

Calculations

The removal rates of anammox bacteria and AOB were estimated as g N m^{-2} d^{-1}, based on the nitrogen balances and stoichiometry of the anammox reactions (Strous et al. 1998). HRT = hydraulic retention time, RSS = Reactor specific surface.

$$\Delta N = \left[NH_4^+\text{-}N_{influent}\right] - \left(\left[NH_4^+\text{-}N_{effluent}\right] + \left[NO_2^-\text{-}N_{effluent}\right] + \left[NO_3^-\text{-}N_{effluent}\right]\right)$$

$$\text{Nitrogen removal rate, NRR (anammox)} = \frac{\Delta N}{HRT \times RSS}$$

$$\text{Aerobic ammonium oxidation rate, AOR (AOB)} = \frac{\left[NH_4^+\text{-}N_{influent}\right] - \left[NH_4^+\text{-}N_{effluent}\right] - \frac{\Delta N}{2.04}}{HRT \times RSS}$$

The removal rates together with qPCR data for anammox bacteria and AOB were utilized for assessments of cell specific conversion rates, given a fmol N $cell^{-1}$ h^{-1}, with the assumption of 2 gene copies of amoA per cell of AOB of eutrophic *Nitrosomonas* taxa (Norton et al., 2002) and 1 gene copy of 16S rRNA per anammox bacterium (Strous et al., 2006).

Free ammonia (FA) and free nitrous acid (FNA) were calculated as described in Yamamoto et al. (2008).

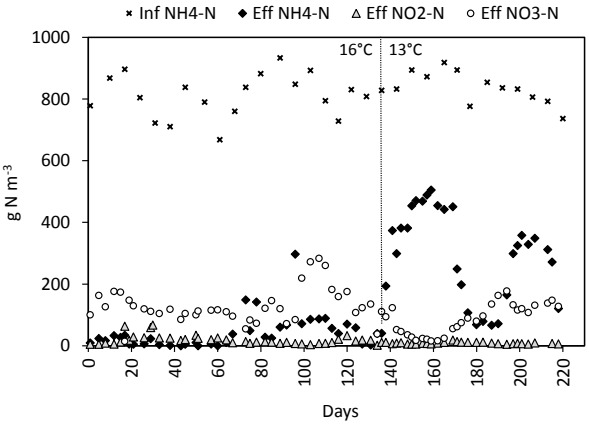
Results and Discussion

Process performance

The target loading rate was 1 g N m^{-2} d^{-1} during the entire experimental period with the intention of obtaining comparable data for the different temperatures, rather than to estimate the maximum removal rate of the MBBR at any given temperature. Aeration was set with the primary aim of providing AOB with oxygen while simultaneously avoiding inhibition of the anammox bacteria, which was successful since nitrite, which is inhibitory for anammox bacteria, did not accumulate in the reactor (Figure 1a).

At 16°C in the pilot MBBR percentage removal was 77% ± 9% (average ± st. dev.) for total nitrogen and 95% ± 7% for ammonium. At this period, the air supply was fluctuating around 1 mg L^{-1}, but due to operational problems with the on-line DO meter no clear registration of data for this period was obtained. However, air supply increased temporarily at the end of the period concomitantly with an increase in nitrate concentrations and decreased nitrogen removal rate, presumably due to an increased NOB activity. Levels of FA and free nitrous acid (FNA) were low (Figure 1b), except at day 96 when the aerobic ammonium oxidation rate was particularly low, which resulted in an elevated concentration of FA.

Decreasing the temperature to 13°C decreased the removal rate (Figure 1c) with a percentage removal of 54% ± 13% for total nitrogen and 65% ± 16% for ammonium. During this period, the impact of aeration was tested. Aeration was set aiming at bulk DO concentrations of 1.0 mg O_2 L^{-1} during days 135-161 and 193-205. At these conditions, nitrogen removal rates were gradually decreasing. At DO concentration of 1.2-1.3 mg O_2 L^{-1} during days 162-192 and days 206-224, increasing removal rates were observed, clearly showing the importance of adequate aeration for the AOB and thereby on the entire nitrogen removal in the biofilm. The pronounced effect of aeration may have been amplified by the increased pH during the periods of low aeration (1.0 mg O_2 L^{-1}) via the lowered AOB activity. This increased pH resulted in elevated concentrations of FA at these periods (Figure 1b) which may have inhibited the activity of AOB and anammox bacteria (Li et al., 2012). Interestingly, nitrate concentrations in the effluent were elevated at the end of the 13°C period, indicative of NOB activity, despite the rather high FA concentrations. NOB are generally assumed to be more sensitive to FA than AOB and anammox bacteria, but an acquired tolerance of NOB for FA to concentrations that were inhibitory for AOB and anammox bacteria has been described in one-stage anammox microbial consortia (Li et al., 2012).



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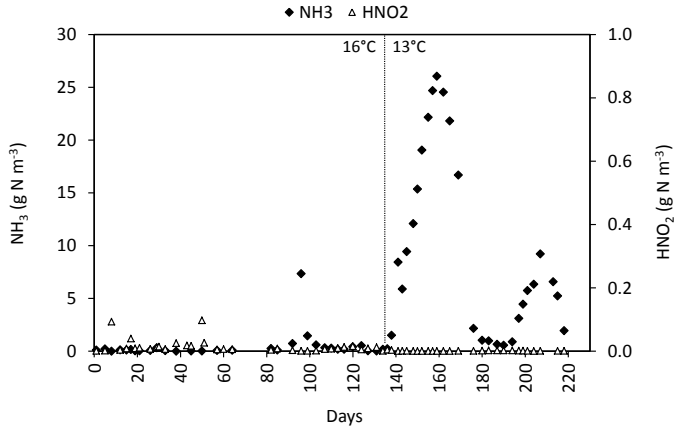

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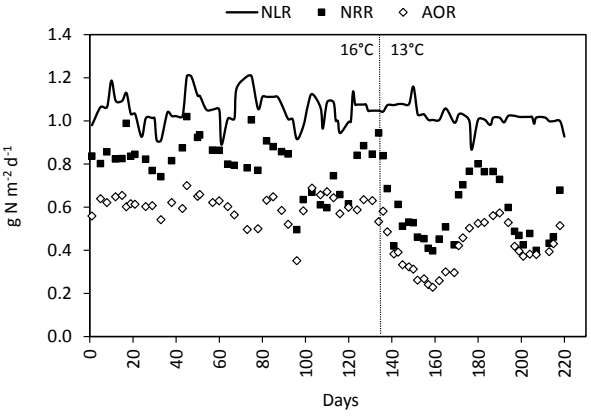

C

Figure 1. Operational data of the pilot MBBR operated at 16°C and 13°C. Concentrations of nitrogen (A). Concentrations of free ammonia and free nitric acid in the reactor (B). Removal

rates showing nitrogen loading rate (NLR), nitrogen removal rate (NRR) and aerobic ammonium oxidation rate (AOR) (C).

The average COD concentrations in the influent water of 471 mg L^{-1} corresponded to an average COD/N ratio in the influent of 0.50 g g^{-1}. During the study period average COD removal was 0.064 g COD m^{-2} d^{-1}. This would correspond to a removal of nitrogen via denitrification of 0.015 g N m^{-2} d^{-1}, assuming a ratio of COD/N of 4.2 g g^{-1} for denitrification (Carrera et al., 2004), corresponding to in average 2.5% of the total nitrogen removal in the MBBR pilot. Hence, denitrification was considered to be of limited importance for the nitrogen removal, presumably due to a low biodegradability of the organic carbon remaining in the influent water, the supernatant after anaerobic sludge digestion.

Table 1. Overview of studies with anammox processes at low temperatures (below 20°C).

Process	Reactor	Reference	Influent type	Influent conc. (kg-N m^{-3})	Temp. (°C)	Loading rate (kg-N $m^{-3}d^{-1}$)	Removal rate (kg-N $m^{-3}d^{-1}$)
Anammox	SBR, granules	Dosta et al. (2008)	Synthetic medium	300	18	0.3	0.29
	RBC	Cema et al. (2007)	Landfill leachate + NH_4^+ and NO_2^-	500-1400	17	1.1	0.5
	Gel carrier	Isaka et al. (2008)	Synthetic medium	340	18	3.2	1.2
					12	1.2	0.6
					6.3	0.6	0.3
One-stage Nitritation-Anammox	SBR, granules	Vazquez-Padin et al. (2011)	Anaerobic sludge digestion supernatant	200-350	15	0.7	0.2
	MBBR	This study	Anaerobic sludge digestion supernatant	670-930	16	0.21	0.16
					13	0.2	0.11

A handful of studies have investigated separate anammox reactors and reactors with one-stage partial nitritation – anammox at low temperatures (Table 1). Despite the different aims of the studies, fairly similar removal rates have been observed, with the exception of the study by Isaka et al. (2008) where the anammox processes was investigated separately using synthetic wastewater and gel entrapped anammox bacteria, with extraordinary high biomass concentrations, which may explain the high removal rates measured in this particular study.

Microbial community analysis

Anammox bacteria were making up a large and stable fraction of the total biomass (38% ± 6%, average ± st. dev.), as measured by the ratio of 16S rRNA gene copy numbers of anammox bacteria to "all" bacteria targeted by the universal primers (Figure 2). Even though quantitative comparisons of 16S rRNA copy numbers are uncertain, due to the different cell specific copy numbers of 16S rRNA genes of different bacteria (Acinas et al., 2004), this high ratio demonstrate that anammox bacteria were a dominating guild in the biofilm. The numbers of AOB were considerably lower. With the assumption of two amoA copy numbers per cell of AOB and

one copy number of 16S rRNA per anammox cell, the ratio of AOB/Anammox was 2% ± 0.7% (average ± st. dev.), in the biofilm. In addition no ammonium oxidizing archaea (AOA) were observed in any of the samples, as measured by PCR with primers for the archaeal amoA gene. AOA are ubiquitous in nature and are important for global nitrogen transformations and AOA have been detected also at WWTPs, but their role for nitrogen removal in wastewater treatment is yet unclear (You et al., 2009).

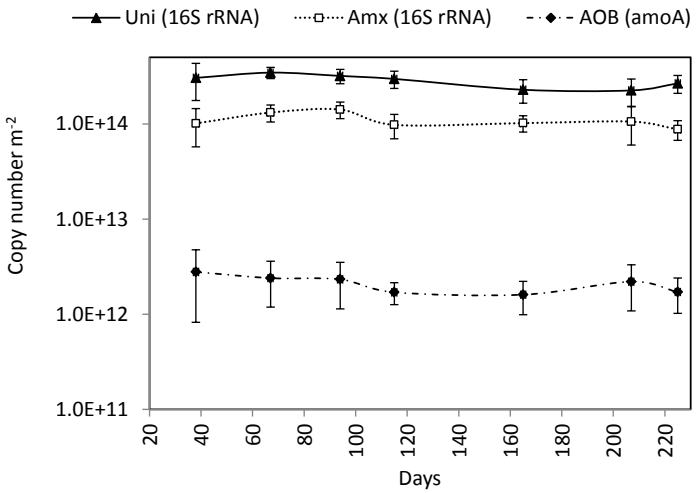

Figure 2. Gene copy numbers in the pilot MBBR pilot of the general bacterial community (Uni), anammox bacteria (Amx) and AOB, as measured by qPCR. Average ± standard deviation (n = 3).

The relative numbers of AOB and anammox bacteria to total bacteria in functional one-stage partial nitritation – anammox microbial communities vary a lot between the few rather different studies where such information has been provided. Park et al. (2010) showed a high abundance of AOB, dominating the biofilm biomass, with fewer anammox bacteria in a CANON MBBR, while De Clippeleir et al. (2011) showed 22-30% AOB and 5-8% anammox bacteria in an Oland RBC at 25°C. In a comparison between five CANON up-flow reactors rather equal abundances of AOB (26-60%) and anammox bacteria (22-46%) was observed at 20-30°C (Liu et al., 2012), while a higher abundance of anammox bacteria (70%) than AOB (10%) was detected in highly aerated biofilms in a RBC at 35°C (Liu et al., 2008).

Being located at the biofilm surface layer, AOB and other aerobic bacteria are considerably more subjected to detachment than are anammox bacteria, which reside in the deeper anoxic parts of the biofilm (see e.g. Winkler et al., 2012). A low temperature means low growth rate, while detachment rate is virtually independent of temperature. Hence, the AOB population in a one-stage nitritation-anammox biofilm with limited oxygen supply, and thereby limited possibility to establish at deeper layers in the biofilm, may get difficulties of maintaining a high density in mature biofilms. This situation would be exaggerated at low temperatures due to the slow growth rates, which may help explain the low relative abundance of AOB compared to anammox bacteria in this study.

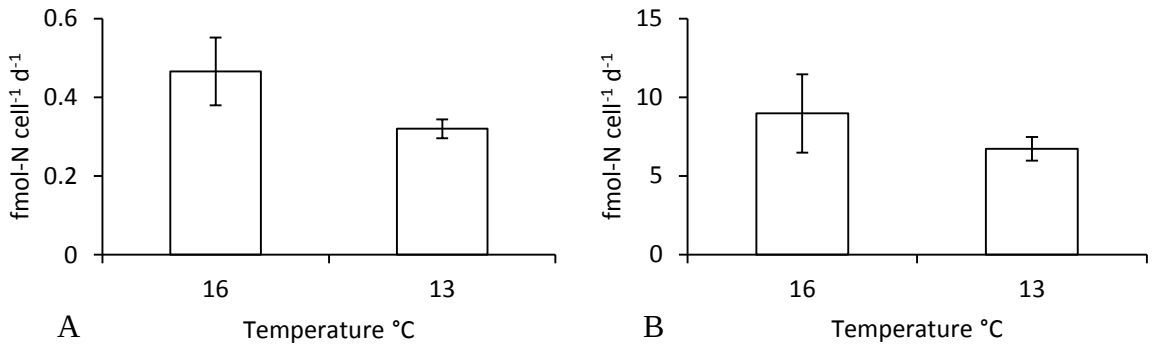

Figure 3. Specific nitrogen conversion rates for anammox bacteria (A) and specific ammonium oxidation rates for AOB (B), at different operational temperatures of the MBBR.

For estimations of specific nitrogen conversion rates, we made the assumption that denitrification was negligible for the process, given the low measured removal of COD (see above). The specific nitrogen conversion rates of anammox bacteria (Figure 3a), given as femtomoles (fmol) per cell per day, were within the range of reported values of 0.09-0.12 fmol-N $cell^{-1}$ day^{-1} (Tsushima et al., 2007) and 2-20 fmol-N $cell^{-1}$ day^{-1} (Kuypers et al., 2003). Likewise, the estimated specific ammonium oxidation rates of AOB (Figure 3b) were within the large range of values measured from AOB in mature wastewater biofilms; 48 fmol-N $cell^{-1}$ day^{-1} (Daims et al., 2001), 2.9 fmol-N $cell^{-1}$ day^{-1} (Kindaichi et al., 2006), 7-91 fmol-N $cell^{-1}$ day^{-1} (Lydmark et al., 2007). For both anammox bacteria and AOB, specific conversion rates were higher at 16°C than at 13°C. Specific conversion rates of biofilm bacteria represent average values and it is reasonable to assume that the bacteria at different depths of the biofilm are not equally active, due to differences in the availability of substrates, carbon and electron acceptors (Gieseke et al., 2005). In the present study, this is especially relevant for the anammox bacteria, which have an extensive depth distribution in the biofilm, as revealed by fluorescence in situ hybridization in conjunction with confocal microscopy (data not shown), while the AOB were located at a thin layer at the water interface of the biofilm, and hence would be considerably less affected by mass transport limitations.

Conclusions

One-stage nitritation – anammox processes can be operated at temperatures of 13-16°C by carefully adjusting the DO concentrations to control the ammonium oxidation rate by AOB. This is relevant for treatment of the main stream of wastewater where temperatures are low, and also for treatment of concentrated nitrogen streams at low temperatures.

Despite temperatures of 13-16°C, the anammox bacterial community was maintained at a high density in the MBBR, while the AOB community was considerably smaller. As a consequence the cell specific conversion rates were much higher for AOB than for the anammox bacteria.

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