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ABSTRACT

The implementation of autotrophic nitrogen removal in the mainstream of a municipal wastewater treatment plant is currently pursued. Among the crucial unknown factors are the kinetic properties of anaerobic ammonium oxidising (anammox) bacteria at low temperatures. In this study we investigated the adaptation of a fast-growing anammox culture to a lower temperature. In a membrane bioreactor a highly enriched anammox community was obtained at 30°C, 25°C and 20°C. This culture was exposed to long- and short-term temperature changes. In short-term experiments the decrease in biomass-specific activity due to decrease in temperature can be described by an activation energy of 64 ± 28 kJ mol^{-1}. Prolonged cultivation (months) implies that cultivation at low temperatures resulted in deterioration of biomass-specific activity (Ea^{LT} 239 kJ mol^{-1}). The growth rate and specific anammox activity in the system decreased from 0.33 d^{-1} and 4.47 g NO$_2$-N g VSS^{-1} d^{-1} at 30°C to 0.0011 d^{-1} and 0.037 g NO$_2$-N g VSS^{-1} d^{-1} at 20°C. The reason for the deterioration of the system was related to the required long SRT in the system. The long SRT leads to an increase of non-active and non-anammox cells in the reactor, thereby decreasing the biomass-specific activity.

1. Introduction

Anammox bacteria are interesting organisms from both a microbiological and an engineering point of view. Anammox stands for ANaerobic AMMonium OXidation and refers to the anaerobic conversion of ammonium with nitrite to nitrogen gas (the conversion was first called anoxic ammonium removal by Mulder, 1989). The bacteria catalysing this conversion were discovered in a sulphide-dependent denitrifying stage of a wastewater treatment pilot plant in Delft, the Netherlands, and named after its conversion: the anammox bacteria [1]. These autotrophic organisms fix CO_2 by obtaining reduction equivalents from oxidation of nitrite to nitrate; the anammox bacteria therefore produce nitrate proportional to their growth rate [2].

From an engineering point of view the anammox bacteria are interesting for applications in wastewater treatment systems [3]. The anammox conversion makes a full autotrophic process for ammonium removal into nitrogen gas possible. This allows the conversion of the organic carbon into methane which can be used as energy supply for the wastewater treatment process. There is a whole set of partial nitritation/anammox

(PN/A) processes developed [4–6]. Implementation of the anammox process is mostly based on biofilm or granular sludge processes because through this method a high biomass concentration and therewith a high volumetric treatment capacity can be achieved.

The PN/A technology is currently applied in streams with high ammonium concentrations and mesophilic temperatures [4,7]. The application of PN/A in the mainstream of a wastewater treatment plant will be a major step forward in obtaining an energy-neutral wastewater treatment plant [8]. One of the biggest challenges for implementation of this technology in the waterline of a sewage treatment plant is the temperature of the wastewater; in moderate climates the wastewater temperature reaches 10°C during winter. Lab- and pilot-scale research on the mainstream application of anammox is currently pursued worldwide [9–12]. These studies usually focus on the full PN/A processes or actual wastewater, making it difficult to evaluate the exact contribution of the anammox conversion as well as the intrinsic impact of temperature on the anammox population.

Since anammox bacteria have a low growth rate, their kinetic characteristics are important in the process

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design. Verified kinetic information is however very sparsely available in the literature [13–15]. The cause of the limited availability of kinetic data can be found due to the method of cultivation of anammox bacteria. Anammox bacteria have a tendency to grow in biofilms and due to their low growth rate, the cultivation in biofilms is convenient. Biofilms can be developed in a granular sludge process or on carrier material, leading to high biomass concentrations in a reactor. Unfortunately identification of kinetic properties of bacteria in a biofilm process is complicated by the combined effect of microbial kinetics and mass transfer processes [16–18]. To solve this bottleneck, a membrane bioreactor (MBR) has been used to cultivate the anammox bacteria as planktonic cells or in small flocs [14,19,20]. The advantages of this system are subsequently the absence of mass transfer limitations. In biofilm systems not all cells will be exposed to the same conditions due to the substrate and product concentration gradients. The second advantage is the homogeneous distribution of cells and of the growth rate in the system. In a biofilm the actual growth rate of cells may be highly variable as a function of their location. And finally, an accurate establishment of a biomass dilution rate (i.e. growth rate) by controlled removal of biomass results in a well-defined growth rate in the steady state. In systems with biomass retention such as biofilms or flocs, a small fraction of the biomass washes out of the system with the effluent. This fraction is hard to identify and is often neglected, leading to an underestimation of the growth rate.

In previous research, using different biofilm-based reactor configurations, doubling times in the order of magnitude of 7–11 days were reported for cultures grown at 30°C [19,21,22]. In an MBR with a suspended cell culture, anammox doubling times down to 3 days were obtained at 30°C and accurate kinetic parameters could be measured [23]. The doubling times that are until now reported for anammox cultures at lower temperatures are much higher, 77 d at 10°C [24]; 17 d at 20°C [25]; 63 d at 10°C [26]; 80 d at 12.5°C [27]. For marine anammox species it is known that they can grow at temperatures below 10°C [28,29]; however kinetic characterisation of these anammox communities has not been reported. These data seem to indicate that the growth rate of anammox gets strongly reduced at a lower temperature. Therefore we aimed to study the potential growth rate under optimal conditions at lower temperatures.

In this study a highly enriched fast-growing anammox community at 30°C was used to study the effect of decreasing the temperature to 25°C and 20°C on the anammox bacteria. The short-term temperature dependency of the activity of the anammox-dominated community was measured in stable enrichments grown at 30°C, 25°C and 20°C, using dynamic temperature experiments. Short-term temperature effects were compared to data of long-term-cultivated enrichments.

2. Material and methods

2.1. Reactor operations

The experiments described in this paper were conducted in an MBR with a working volume of 10 L. The reactor set-up and general operational properties have been described elsewhere [14,19,23]. The biomass in the reactor originates from the full-scale anammox internal circulation reactor of Dokhaven WWTP in Rotterdam [5]. Prior to this experiment the biomass was used in other studies [14,23] and the reactor had been in operation for more than 6 years. When the temperature of the reactor was shifted, 15 mL of sludge from the low temperature mainstream anammox pilot reactor in Dokhaven WWTP in Rotterdam was added [30] in order to increase the microbial diversity in the system.

The synthetic medium utilised was composed of 30 mM ammonium sulphate (840 mg NH$_4$-N L^{-1}); 60 mM sodium nitrite (840 mg NO$_2^-$-N L^{-1}); 0.51 mM of Ca^{2+} (added as Ca$_2$Cl$_2$·2H$_2$O); 0.41 mM of Mg^{2+} (added as MgSO$_4$ 7H$_2$O); 5 mM of phosphate (added as pH-buffer by dissolving KH$_2$PO$_4$ and K$_2$HPO$_4$ salts); 0.08 mM of Fe^{2+} (added as FeSO$_4$·7H$_2$O) and 1.25 mL L^{-1} of trace element solution [19]. During the 20°C run the EDTA concentration was elevated from 0.08 to 0.11 mM, because precipitations were observed in the medium.

The system was first operated at 30°C for 12 months, followed by a 10-month period of 25°C and finally a 12-month period of 20°C. The changes in temperature were made in steps of 1°C per day. The hydraulic and solids retention time (HRT and SRT) are depicted in Table 1. The HRT was set with the aim to operate under nitrite-limiting conditions (below 0.5 mg NO$_2$-N L^{-1}). The feeding flow and sludge bleed were monitored by weighting the sludge collection vessel and medium vessel, calculating the flow by measuring the weight difference over a period of time. The SRT was controlled by purging mixed liquor (5 min every 30 min) at different flow rates and sampling. The dilution rate was controlled

Table 1. Reactor configurations of the enrichment reactor used in this study.

	30°C	25°C	20°C
Hydraulic retention time (d)	1.65	2.5	2.45–11.83
Solids retention time (d)	3–4.20	10	50–950
Loading rate (mg NO$_2$-N L^{-1} d^{-1})	510	336	71–350

close to the maximal growth rate of the bacteria, based on measurements of the residual nitrite concentrations. Before feeding to the reactor, the medium was flushed with nitrogen gas in order to remove traces of oxygen. To keep anoxic conditions the reactor was continuously sparged at 80 mL min^{-1} with 95%N$_2$–5%CO$_2$ gas. The pH was controlled at a value of 7 using a 0.1 M bicarbonate buffer using an applikon ADI 1030 Bio controller.

2.2. Analytical methods

Daily assessments of nitrite and nitrate concentrations in the reactor were executed with Merckoquant® test strips. Weekly measurements of ammonium, nitrite and nitrate were conducted using Hach Lange cuvette tests and analysed with a LASA 20 spectrophotometer.

    The concentrations of solids, determined as total suspended solids and the fraction corresponding as volatile suspended solids (VSS), were determined according to Standard Methods [31]. The yield was based on measurements of the biomass production and nitrite consumption.

2.3. Specific anammox activity

The maximum specific anammox activity (SAA^{max}) ($q_{NO_2^-}^{max}$, mg NO$_2$–N gVSS^{-1} d^{-1}) was obtained via batch testing procedure. The medium was fed to the reactor at an elevated rate for a period of 15–20 min to obtain a nitrite concentration between 20 and 30 mg NO$_2$-N L^{-1}. At this point the substrate feed was stopped and the nitrite uptake rate was determined by measuring nitrite depletion. Ammonium was not limited during stable reactor operation; therefore a surplus of ammonium (around 150 mg NH$_4$-N L^{-1}) was present in the solution during these tests. The sludge and effluent control were switched off during this period of time. The biomass concentration (g VSS L^{-1}) was used to convert the volumetric conversion to biomass-specific anammox activity.

2.4. Dynamic temperature response

The short-term temperature response was analysed via dynamic temperature experiments. The temperature profile was adjusted from the initial temperature to 15–30°C and back to the initial temperature in the course of 8 h. The feeding flow was adjusted and monitored, such that nitrite would not become limiting or exceed 25 mg NO$_2$-N L^{-1}. The dynamic temperature response was fitted with the Arrhenius equation (Equation (1)) using nonlinear regression for determination of the apparent activation energy (Ea, kJ mol^{-1}). The data were fitted with a singular

Arrhenius coefficient for the entire temperature range (15–30°C), using the measured SAA (mg NO$_2$-N g VSS^{-1} d^{-1}) to fit SAA^{max} (mg NO$_2$-N g VSS^{-1} d^{-1}), and optimal temperature in T_{ref} (C) and error (r).

$$SAA(T) = SAA^{max} * \left(\frac{\exp(-Ea/r*T)}{\exp(-Ea/r*T_{ref})} \right). \quad (1)$$

2.5. Microbial community analysis

An extensive analysis of the microbial community was performed using denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridisation (FISH). Samples were taken every 1–2 months to evaluate any community changes during different temperature periods. To estimate the average floc size, the size of 50 flocs was measured, with an image analyser.

2.5.1. Fluorescence in situ hybridisation
The collected samples were washed in phosphate buffer and fixed using paraformaldehyde. Hybridisation with fluorescent-labelled oligonucleotide probes and analysis of the samples were performed as described in Lotti et al. [24]; a formamide concentration of 30% was used for hybridisation of the probes used in this study (Table 2). The rough estimation of the size of the side population was based on the analysis of 20 microscopic fields.

2.5.2. Denaturing gradient gel electrophoresis
The collected samples were washed in a phosphate buffer and centrifuged. The pellet was stored at −80°C. DNA extraction and DGGE were carried out as described by Lotti et al. [23]. Extracted DNA was used to amplify the 16S rRNA gene using a primer with near perfect matches to most anammox species: AN314 and a bacterial specific primer: Bac907rM (Table 2). After purification of the PCR products out of the agarose gel and sequencing, the obtained sequences were compared to sequences stored in GenBank using Blastn.

Table 2. Oligonucleotides probes used in this study for FISH analysis and primers used for DGGE.

Oligonucleotide	Target organism	Sequence (5′–3′)	Reference
Bfu-613 FISH	C. Brocadia fulgida	GGA TGC CGT TCT TCC GTT AAG CGG	[44]
Amx1015 FISH	C. Brocadia anammoxidans	GAT ACC GTT CGT CGC CCT	[43]
Kst1273 FISH	C. Kuenenia stuttgartiensis	TCG GCT TTA TAG GTT TCG CA	[43]
EUB 338 FISH	Bacteria	GCT GCC TCC CGT AGG AGT	[45]
AN314 DGGE	Anammox bacteria	CCT ACG GGA GGC AGC AG	[46]
Bac907rM DGGE	Bacteria	CCG TCA ATT CMT TTG AGT TT	[47]

3. Results

3.1. Long-term operation at three different temperatures

The MBR was operated for 12 months at 30°C, for 10 months at 25°C and for 12 months at 20°C. Stable conversions and biomass concentrations were achieved at each temperature. Several studies have found that ammonium and nitrate do not inhibit the anammox process up to concentrations of 1 g N L^{-1}, but nitrite has been reported as an inhibitive compound. Nitrite levels above 350–400 mg NO$_2$-N L^{-1} completely inhibit the process [32,33]. Therefore, nitrite concentration was kept below 30 mg NO$_2$-N L^{-1}, during batch tests. Since cells were grown almost suspended, oxygen inhibition can occur at very low values. Special care was taken to prevent trace amounts of oxygen entering the system. To avoid accumulation of nitrite during reactor operations the volumetric loading rate was decreased stepwise at the different temperatures by increasing the HRT. At each temperature the SRT was adjusted to the maximal achievable growth rate of the biomass, based on the residual nitrite concentration in the reactor. A small but measureable nitrite concentration ensures the anammox cells operate close to their maximal growth rate.

During the stabilisation at the different temperatures, the biomass concentration and the maximum specific anammox activity (SAA^{max}) were measured. The results of the operation at 20°C are depicted in Figure 1. At day 100 the sludge removal was stopped and the only biomass removed from the system was during sampling. Therefore the SRT was increased from 50 to 950 days,

Table 3. Kinetic parameters for the anammox-enriched community obtained at three different temperatures during long-term cultivation.

Parameters	30°C	25°C	20°C
Y_{X/NO_2-N} (g VSS gNO$_2$-N^{-1})	0.098 ± 0.01	0.111 ± 0.017	0.028 ± 0.001
SAA$^{max\ LT}$ (g NO$_2$-N g VSS^{-1} d^{-1})	4.19 ± 0.001	0.904 ± 0.174	0.0368 ± 0.0058
NO$_2$/NH$_4$	1.219 ± 0.08	1.00 ± 0.01	1.16 ± 0.03
NO$_3$/NH$_4$	0.211 ± 0.07	0.14 ± 0.11	0.10 ± 0.01

leading to an increase in the biomass concentration. This change was introduced, because the SAA decreased and nitrite started to accumulate in the system. From day 70 to 190 the activity decreased from 250 to 30 mg NO$_2$-N g VSS^{-1} d^{-1}.

During stable reactor performance the different process parameters were measured. Based on these values different kinetic parameters were calculated, which are reported in Table 3. The biomass yield and the nitrite to ammonium ratio remained constant at 30°C and 25°C. Since the biomass yield remained constant, the SAA$^{max\ LT}$ and the maximum growth rate, decreased with a similar percentage from 30°C to 25°C. In this case the changes in decay and maintenance rate are not incorporated, since both values will be small and no significant changes were expected. The trend of the affinity constant for nitrite during long-term operations cannot be accurately defined because too strongly fluctuating residual nitrite concentrations were measured (between 0.015 and 0.8 mg NO$_2$-N L^{-1}), compared to the fluctuations in SAA$^{max\ LT}$.

The values for growth at 20°C are different from the values at higher temperatures. The amount of nitrate
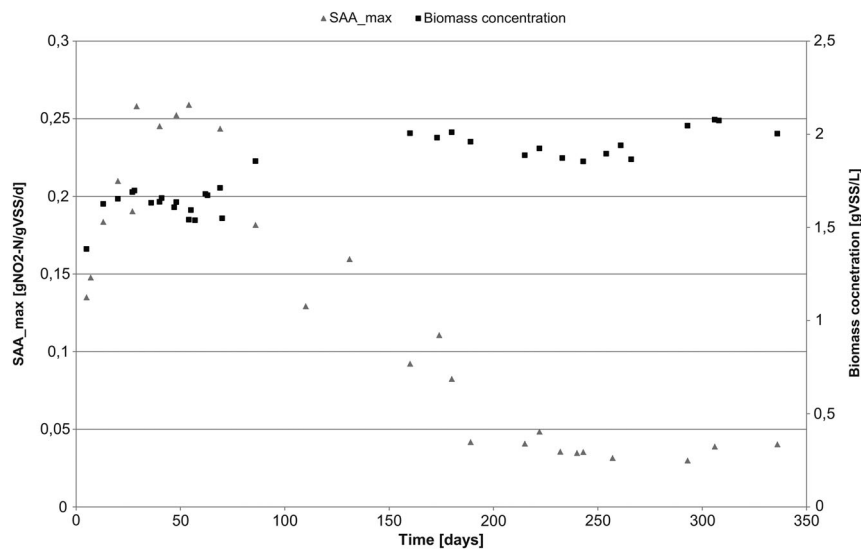

Figure 1. Maximum specific anammox activity (SAA$^{max\ LT}$) during long-term cultivation of anammox bacteria in an MBR at 20°C. Biomass concentration is shown in black squares and SAA$^{max\ LT}$ in grey triangles.
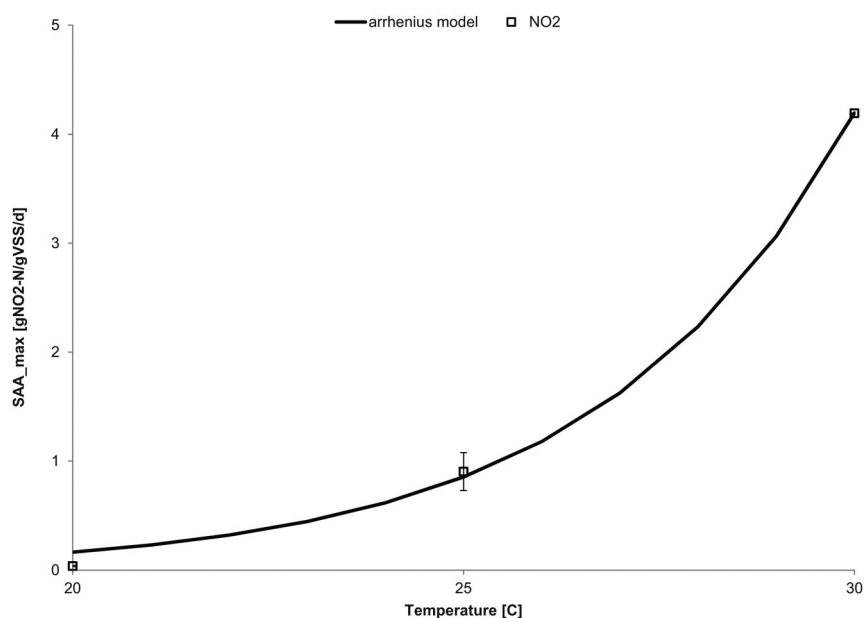
Figure 2. Maximum specific anammox activity as a function of temperature after long-term cultivation at 30℃, 25℃ and 20℃. Arrhenius equation is fitted (solid line) through SAA$^{max\ LT}$ data points (open squares); the data points are averages of long-term stable reactor performance and corresponding standard deviations. The error bars of the data points at 20° and 30° are too small to be seen.

produced per ammonium consumed decreased significantly with decreasing temperatures. The long-term SAA$^{max\ LT}$ trend with temperature was fitted with an Arrhenius equation (see Figure 2). A significant decrease in SAA$^{max\ LT}$ is seen with a high apparent activation energy of 239 kJ mol^{-1}.

3.2. Microbial community analysis

In the interest of accurate estimations of the kinetic parameters it is preferred to have a free suspended (planktonic) cell culture or small flocs without significant mass transfer limitations. In previous experiments conducted at 30℃ cells could be grown in single cells. When the operational temperature of the system was decreased to 25℃, the biomass flocculated with floc sizes of approximately 50–150 μm. Microscopic images showed that the small flocs were composed of filamentous bacteria and anammox bacteria (Figure 3). In the 20℃ period of operations the amount of non-anammox bacteria increased significantly; there was a fraction of biomass present in the reactor which did not hybridise with the anammox and Eubacterial FISH probe (Figure 4). These organisms are probably gram-positive bacteria, since no hybridisation took place in the FISH experiment for gram-negative bacteria. Higher organisms, protozoa, were occasionally observed in the sludge as well (picture in supplemented material Figure S7). Due to the growth in the form of flocs, it was impossible to obtain free cells in a fish protocol and counting

individual cells was therefore impossible. A rough estimation of the side population could be made; at 30℃ there were hardly any non-anammox cells present; at 20℃ there were 20–40% of non-anammox cells present. The increase in side population was related to the increased SRT. The increased SRT will lead to the accumulation of more heterotrophs, grown on the minimal organic carbon present in the influent as well on exudates or decay products of anammox bacteria.

Throughout this study the anammox bacteria population was shown to be a member of the genus *Candidatus Brocadia*, based on FISH. Hybridisation took place with the anammox probe for *C. Brocadia fulgida* (BFU-613). No hybridisation took place with the *C. Brocadia anammoxidans* – specific probe (AMX-1015) or the *C. Kuenenia stuttgartiensis* – specific probe (KST-1273) (data not shown).

Microbial community analysis by sequencing of the dominating DGGE bands confirmed 99% identity with *C. Brocadia TKU 1 & 2*, at all temperatures (supplemented material Figure S8). FISH analysis revealed that anammox was the dominating species in bio volume in the reactor. However, a side population can clearly be seen in microscopic images. The bacterial DGGE results show three groups of bands with the highest intensity present throughout all temperature operations. Those sequences were blasted, corresponding with denitrifying bacterium enrichments and a chloroflexi bacterium. Due to the simple composition of the culture, no metagenomics sequencing was executed.
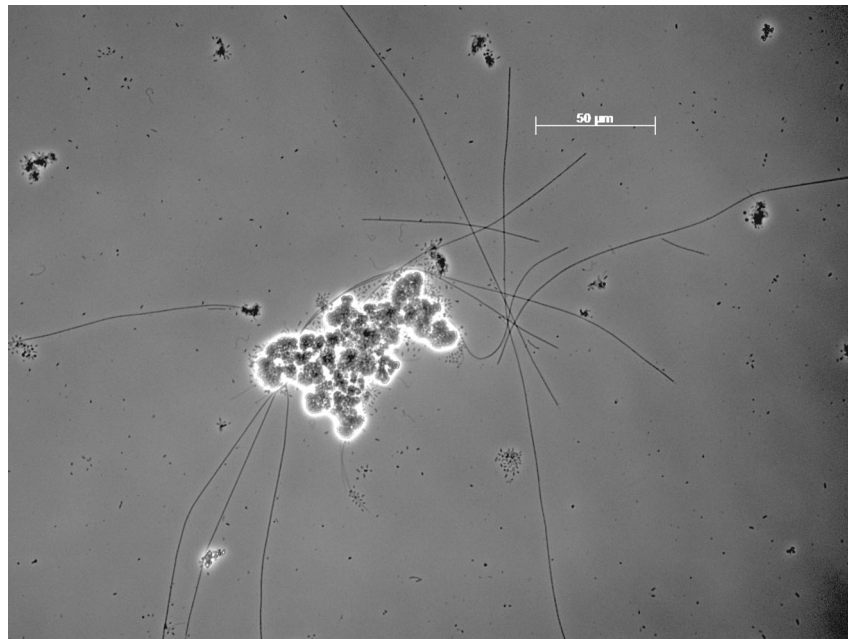
Figure 3. Microscopic phase contrast picture of floc and filamentous bacteria, during 25°C cultivation.

3.3. Short-term temperature response

The short-term temperature response was analysed via dynamic temperature experiments; the feed flow was adjusted and monitored, such that nitrite would not become limiting (0.5 mg NO_2 L^{-1}) or exceeding 30 mg NO_2-N L^{-1}. An example of an experiment is depicted in Figure 5. The temperature profile was adjusted from 25°C to 15°C, to 30°C and back to the initial temperature in the course of 8 h. Every half hour nitrite concentrations were measured. The dynamic temperature response in activity was fitted with the Arrhenius equation using nonlinear regression for determination of the apparent activation energy (Ea, kJ mol^{-1}).

Dynamic temperature experiments were performed with the stable biomass for 30°C, 25°C and 20°C. The results of the experiments are shown in Figure 6
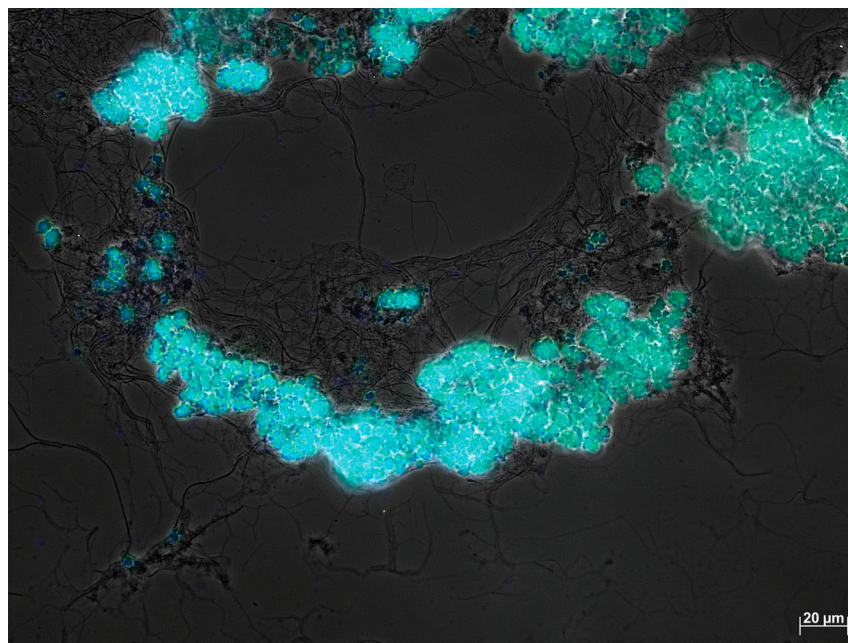

Figure 4. FISH picture of the biomass enriched at 20°C, hybridised with Eubacteria (blue) and AMX (BFU 613, green). Unstained side population can be clearly seen. There are a limited number of cells that are stained with the eubacteria probe and not with the anammox bacteria probe (readers are referred to the online version for colour figures).
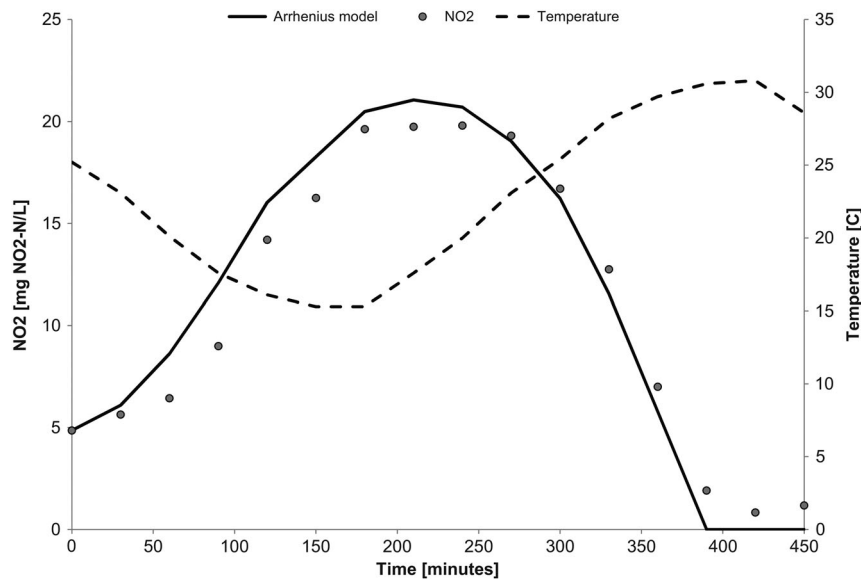
Figure 5. Dynamic temperature experiment; biomass was cultivated at 25°C. Temperature (dashed line), nitrite samples (filled circles) and fitted nitrite concentrations based on the best estimate for the activation energy in the reactor model (solid line).

and Table 4. In Figure 6 it can be seen that the SAA decreased with decreasing temperatures in a short-term test and the SAA$^{max\ ST}$ was lower for the biomass cultivated at lower temperatures.

Table 4 presents the data of the eight different dynamic temperature experiments, the differences in the activation energies fall within the standard deviations of the different measurements. The r^2 of the fit is rather low, indicating a suboptimal fit when using one activation energy for the entire temperature range.

4. Discussion

4.1. Combination of long- and short-term effect of temperature

If the results of the long-term cultivation and the short-term dynamic temperature experiments are combined, it is evident that the long-term temperature impact is much stronger. The maximum biomass-specific activity, after prolonged cultivation at decreasing temperatures, decreases dramatically (Ea^{LT}, 239 kJ mol^{-1}) compared to the short-
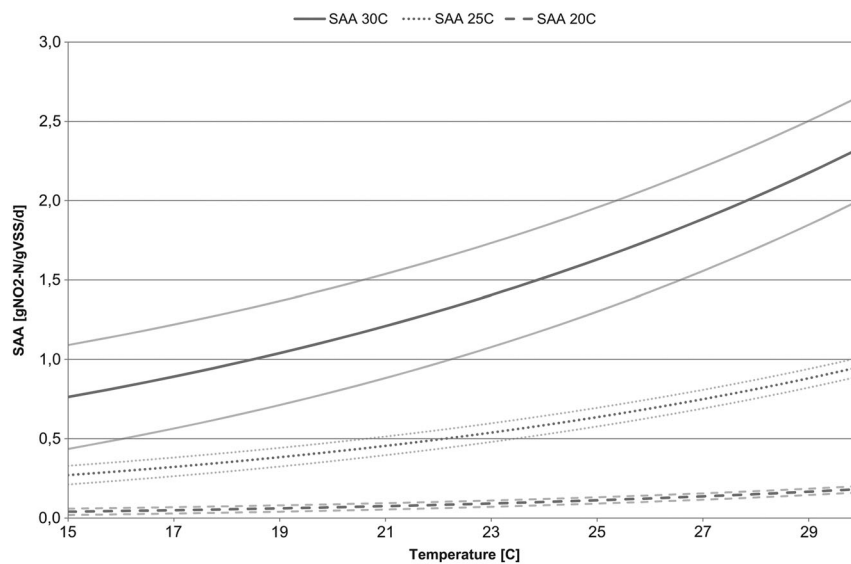
Figure 6. Temperature dependency of the specific anammox activity (SAA) as estimated from dynamic temperature experiments with biomass cultivated at 30°C (solid line), 25°C (dotted line) and 20°C (dashed line). Results are averages of multiple experiments (2 or 3) at 30°, 25° and 20°, with the combined standard deviations of these experiments as slightly transparent lines.

Table 4. Arrhenius model parameters obtained from short-term temperature experiments for anammox biomass cultivated at different temperatures.

Parameters	Unit	30°C	25°C	20°C
Ea	kJ mol^{-1}	54.1 ± 20.1	61.0 ± 12.8	73.6 ± 46.6
SAA$^{max\ ST}$	g NO$_2$-N g VSS^{-1} d^{-1}	2.39 ± 0.82	0.95 ± 0.47	0.19 ± 0.18
r^2	–	0.73 ± 0.20	0.94 ± 0.03	0.84 ± 0.087

Notes: Data are averages for two dynamic temperature experiments performed during 30°C cultivation period and three dynamic temperature experiments from the 25°C and 20°C cultivation periods.

term temperature responses (EaST, 64 ± 28 kJ mol^{-1}). As shown in Figure 1, the biomass-specific activity (SAA$^{max\ LT}$) slowly decreases during long-term cultivation at low temperatures. In Figure 6 it can be seen that the maximum activity at 30°C is lower for the biomass grown at 20°C, compared to the biomass grown at 30°C. But the short-term temperature effect is comparable for all cultivation temperatures; a short-term temperature dependency independent of the cultivation temperature is observed. The deterioration of the long-term biomass-specific activity is not reversible in a short-term experiment.

The short-term temperature dependency obtained for anammox bacteria in this study is similar with the results obtained by other authors with similar cultivation temperatures Ea: 70 kJ mol^{-1} for 20–43°C [15] 63 kJ mol^{-1} for 10–40°C [32] and 83.1 kJ mol^{-1} for 15–30°C [34]. Activation energies of 61 kJ mol^{-1} for 6.5–37°C and 51 kJ mol^{-1} for 2–13°C have been reported for anammox cultures from marine sediments that were adapted to colder climates (≤10°C) [28,29].

During all the short-term dynamic temperature experiments, the SAA always increased with temperature. This indicates that the optimum temperature, for anammox biomass cultivated at 30°C, 25°C and 20°C, is equal to or higher than the maximum temperature (=30°C) tested in these experiments. A change in temperature optimum for anammox bacteria, cultivated at different temperatures, has been reported in literature for a C. *Brocadia fulgida*-like strain and a marine anammox strain [29,35]. Nonetheless, this observation was not confirmed in other studies [34]. Also in this study no strong adaptation of the optimum temperature was observed.

The long-term temperature effect on the SAA of this system was bigger compared to data reported in literature. After decreasing the temperature, the maximum biomass-specific activity slowly decreased, in terms of weeks. This observation is inconsistent with results obtained in other studies where prolonged cultivation of anammox did not result in a slow decrease in time of the SAA as observed in this work [32,34,35]. The point of discussion in these studies is the question

whether the cultivation times are long enough to properly study the long-term temperature effects, because the long-term temperature effects occurred over a period of moths in this study. In literature higher growth rates are reported for anammox cultures at lower temperatures, 0.009 d^{-1} at 10°C [24]; 0.04 d^{-1} at 20°C [25]; 0.011 d^{-1} at 10°C [26]; 0.0087 d^{-1} at 12.5°C [27], the main difference between these studies and the work described in this paper is the way of cultivation, the absence of a biofilm in our study.

4.2. Side population

Anammox bacteria have to date never been isolated in pure culture. A side population is always present in anammox cultivations; therefore all the anammox strains are still referred to as *candidatus*. The increasing amount of side population biomass in this study can be linked to the increasing SRT. At the high dilution rates imposed at 30°C, prokaryotic and eukaryotic grazers may have been washed out from the system. At the increasing SRT values required to avoid wash-out of anammox at lower temperatures, the undesired side population maintains itself in the system. The side population can grow directly on anammox bacteria through grazing, grow on decaying biomass or grow on microbial products excreted by anammox. Fourthly, it is possible that organisms grow on the Chemical Oxygen Demand present in the demineralised water, used to prepare the medium (personal communication, E.I. Prest, 2015;[36]). Some of the organisms of the side population are denitrifiers and can consume part of the nitrate produced by anammox bacteria. This is the likely cause of the changed ammonium to nitrate ratio at different cultivation temperatures; the consumption of nitrite by denitrifying bacteria or the change in stoichiometry of the anammox bacteria cannot be ruled out. The type of organisms in the side population is observed in similar systems [37,38].

In a biofilm system anammox cells potentially are better protected against these direct or indirect grazing communities [39]. Unfortunately there are no inhibitors known that will only inhibit the side population and not the anammox bacteria; therefore it was not possible to repress selectively the non-anammox biomass.

4.3. Possible explanation for the observed strong long-term temperature effect

The results of this study would imply that long-term cultivation at low temperatures results in deterioration in terms of biomass-specific conversion rates; the negative effect on the growth yield is less severe. This kinetic

deterioration does not occur during short-term temperature fluctuations.

The decrease in activity could be explained by the fact that a part of the biomass dies, becomes inactive or is not anammox biomass. At both downward steps in the temperature the SRT is increased substantially; therefore non-anammox cells can accumulate in the system and thereby give a decreased value of the measured SAA [36]. In our reactor we observed a side population, which could lead to a decrease in specific activity. At the long SRT applied at 20℃ protozoa were observed in the sludge; these protozoa might graze on the anammox biomass. The fact that the SAA is decreasing much more compared to the yield could be explained by the presence of dormant or dead anammox cells. The cells are formed, but do not retain their activity. These cells might be identified by FISH, but do not contribute to conversions in the reactor [40–42]. Unfortunately the standard life/dead staining procedure cannot be used on anammox cells due their different membrane composition with ladderane lipids. All these factors will have a small impact on the relative change in activity during short-term temperature fluctuations as compared to long-term temperature cultivation.

The above explanations can explain the strong long-term temperature effects on primarily the SAA and secondary the growth yield observed in this study, which have not been reported in literature before. This is most likely an indirect effect: The increased SRT required for maintaining anammox biomass at lower temperatures facilitated a side population that grows on anammox biomass. Kinetically this suggests an increase in the decay coefficient, resulting in a decrease of the net-growth rate of the anammox bacteria, requiring an increase in SRT. This can be a self-amplifying effect, where an increase in SRT imposes an increase in decay/grazing, and thereby with a decrease in the net-growth rate of anammox, requiring an increase in SRT again.

The major difference between this study and the studies described in literature is the cultivation in biofilms in all studies in literature. There were good reasons to try to omit a biofilm in this study; in a free cell and fast-growing culture, the kinetic parameters of anammox bacteria could have been accurately measured. But due to the formation of flocs from 25℃, the possible dormancy of a part of the anammox population and the increased side population, the measurements of the kinetic parameters might be concealed. The next step in this topic of research will be finding out whether the decrease in growth rate described in this study and in literature is intrinsic for anammox bacteria or whether it is a flaw of the cultivation technologies. The answer to this question will also be important for the application of the PN/A technology in the mainstream of the wastewater treatment plant.

5. Conclusion

In a MBR a fast-growing highly enriched anammox culture was obtained at 30℃. This culture was exposed to long- and short-term temperature fluctuations. Long-term cultivation at low temperatures resulted in a very strong decrease in growth rate, yield and biomass-specific activity. While short-term experiments showed a regular temperature response (activation energy (Ea) of 64 ± 28 kJ mol^{-1}) the long-term temperature effect was very strong (Ea was 239 kJ mol^{-1}). To maintain an anammox culture in the membrane reactor, the SRT was increased at decreasing temperatures. This increase in SRT induced an increase in non-active anammox biomass and an increase in the side population, suggesting an indirect temperature effect of long-term cultivation at temperatures lower than 30℃.

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