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SMOOTH TEMPERATURE DECREASING FOR NITROGEN REMOVAL IN COLD (9-15° C) ANAMMOX BIOFILM REACTOR TESTS

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Abstract

For N-rich wastewater treatment the anaerobic ammonium oxidation (anammox) and nitritation-anammox (deammonification) processes are widely used. In a deammonification moving bed biofilm reactor (MBBR) a maximum total nitrogen removal rate (TNRR) of 1.5 g N m^{-2} d^{-1} (0.6 kg N m^{-3} d^{-1}) was achieved. During biofilm cultivation, temperature was gradually lowered by 0.5° C per week, and a similar TNRR was sustained at 15° C. qPCR analysis showed an increase in *Candidatus Brocadia* quantities from 5×10^3 to 1×10^7 anammox gene copies g^{-1} TSS despite temperature lowered to 15° C. Fluctuations in TNRR were rather related to changes in influent NH_4^+ concentration. To study the short-term effect of temperature on the TNRR, a series of batch-scale experiments were performed which showed sufficient TNRRs even at 9-15° C (4.3-5.4 mg N L^{-1} h^{-1}, respectively) with anammox temperature constants ranging 1.3-1.6. After biomass was adapted to 15° C, the decrease in TNRR in batch tests at 9° C was lower (15-20%)

than for biomass adapted to 17-18° C. Our experiments show that a biofilm of a deammonification reactor adapted to 15° C successfully tolerates short-term cold shocks down to 9° C retaining a high TNRR.

Keywords: Deammonification, reject water, intermittent aeration, nitrite inhibition

Introduction

The anaerobic ammonium oxidation (anammox) is a efficient method for the treatment of NH_4^+-rich and low organic content reject water coming from anaerobic treatment occurring with the participation of anammox bacteria according to the following equation:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02\ N_2 + 0.256NO_3 + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \qquad \Delta G_0 = -360\ kJ\ mol^{-1} \qquad (1)\ [1]$$

Anammox process can be used in moving bed biofilm reactors (MBBRs)) as anammox bacteria are able to form a stable biofilm. According to [2] anammox process-based MBRRs have achieved a high total nitrogen removal rate (TNRR) ((>5 kg N m^{-3} d^{-1} (~1-2 g N $m^{-2}d^{-1}$)) at temperatures above 20°C. Also, an anammox MBBR can have up to 5 times higher TN removal efficacy than deammonifying systems [3].

Suitability of anammox biofilm system for autotrophic nitrogen removal at low temperatures has been shown through the TNRRs achieved at 10°, 15° and at 20°C (0.06, 0.1 and 0.18 kg N m^{-3} d^{-1}, respectively) [4].

Most studies on temperature effect on anammox process have been done with anammox-suspended systems and they have shown that it was most efficient to treat reject water in a temperature range of 25-40 °C ([5]). Regarding these facts, we define temperatures below 20° C as moderate temperatures for nitrogen removal similarly to other authors ([4]). The optimal temperature for most mesophilic anammox organisms was 40 ± 3 °C [6]. Also, [7] showed that at temperatures below 15°C the anammox process was stopped. Studies conducted with suspended anammox systems at 20-22 °C [8] and at 15-19 °C [9] have shown high TNRRs of 8.1 kg N m^{-3} d^{-1} at 20 °C and 0.5 kg N m^{-3} d^{-1} at 17 °C. [10] have accomplished the TNRR of 6.6 kg N m^{-3} d^{-1} at only 9.1 °C by adding 10 mL highly active anammox sludge to the reactor after every 2 days. Moreover, [11] performed anammox process in an upflow anaerobic sludge blanket reactor at 30 °C and 16 °C and achieved TNRRs of 5.72 kg N m^{-3} d^{-1} and 2.28 kg N m^{-3} d^{-1}, respectively yielding a value of temperature constant K=1.93. The growth rate of anammox bacteria is much lower (0.04 d^{-1}) at 20° C than above this temperature [4]. The latter studies with low-strength wastewaters showed an abrupt decrease in TNRR when temperature was decreased hastily.

A deammonification system- comprising partial nitrification (ammonium oxidation to nitrite) and anammox consortiums in a single process tank [12]) has typically lower building costs since two-stage systems require separate process tanks for both- nitritation and anammox processes [13]. Additional savings, comparing to a conventional nitrification-denitrification process, can be gained from less aeration (ca 60%), from the lack of demand for an organic carbon source (100%), and from decreased amount of excess sludge requiring handling. Also the emission of greenhouse gases (CO_2, NO_x etc.) is decreased [14].

Among deammonification systems, [13] started SBR at 25 °C and then gradually lowered the operation temperature down to 12 °C during 10 days remaining stable TN removal efficiency of 90% during 300 days. De Clippeleir et al. have achieved high TNRRs at low temperature of 15 °C [15]. Lotti et al. proved that low temperature effect increased when temperature was decreased [16].

On the whole, most temperature decreasing methods have been abrupt ([9]; [13]) not gradual. By means of gradual temperature lowering anammox biomass could be adapted to a lower temperature better, although there could be problems with the elevated propagation of nitrite oxidizing bacteria (NOB) as they have higher growth rate at low temperature compared to ammonium oxidizing bacteria (AOB) ([17]; [18]). Higher NH_4^+ concentrations would inhibit NOB activity through free ammonium [14] even at low temperatures, ensuring sufficient NO_2^- in the process and NO_2^- not being oxidized into non-usable form for anammox bacteria- NO_3^-.

The anammox process is affected by substrate limitation below 10 mg TN L^{-1} as studied earlier in biofilm systems [19]. Furthermore, the anammox process can lose half of its efficiency at nitrite concentration as high as 400 mg N L^{-1} [20] and/or by low temperatures.

This research had the following aims: cultivation of biomass for efficient running of a deammonifying biofilm reactor at lower temperatures (~15 ºC) by using gradual temperature lowering (0.5 ºC per week) in the treatment of high-strength undiluted wastewater. To determine the effect of adapting the biofilm to different cultivating temperatures and in order to mimicry short-term cold shocks, batch-scale experiments were conducted. This research aimed to find out the low temperature at which the TNRR was still sufficient for wastewater treatment in colder regions. Also, the temperature constants of Arrhenius equation were calculated in order to quantify the temperature dependency of the anammox reaction.

Materials and methods

Continuous reactor

A 20 L plexiglass MBBR filled ~50% with polyethylene carriers (specific surface 800 m^{-2} m^{-3}) (Aquamyc, Germany) was continuously fed with diluted reject water coming from the methane digester of the Tallinn municipal wastewater treatment plant (Estonia) having NH_4^+-N content of 680 (±76) mg N L^{-1} and COD of 600 (±200) mg L^{-1} [21]. Intermittent aeration cycles with 45 min. aeration (air flow rate 665 mL min^{-1})/ 45 min. non-aeration were applied. Within aerobic cycle DO concentration fluctuated between 0-1.5 mg L^{-1}. HRT of 0.5-2 d was applied. A detailed description of the start-up and operation of a single-step deammonification preceding experiments (before 400 days of operation) is given in [12, 22].

The reactor was started up and operated at a temperature of 26 ±0.5 °C for 2 years [14], then the temperature was set at 20° C for a year (see Fig. 1a, b). From 1420th day on, temperature was decreased by 0.5° C per ~week until reaching 15° C. Gradual temperature decreasing was done to adapt biomass smoothly to lower temperatures as abrupt temperature decrease ([13], [4]) causes more instabilities and lengthens biomass cultivation time.

Batch tests

60 batch tests were performed in 0.8-L volume three-necked glass bottles, which were filled with 200 biofilm carriers and placed on a magnetic stirrer located in a thermostated room. Firstly, during reactor operation period (before 600 days) 9 tests together with parallels were done at nitrite concentration of 30 mg NO_2^--N L^{-1}. Secondly, during reactor operation days 1500-1570, 33 tests together with parallels were done at nitrite concentration of 100 mg NO_2^--N L^{-1} using biomass adapted at 17-18º C. Thirdly, on reactor operation days 1570-1600, 12 tests together with parallels were done at nitrite concentration of 30 mg NO_2^--N L^{-1} using biomass adapted at 15º C.

NO_2^--N/NH_4^+-N ratio of 1.32/1 was maintained as a suitable anammox process stoichiometrical ratio [23]). The substrate concentrations used were 100 mg NO_2^--N L^{-1} and 80 mg NH_4^+ -N L^{-1} (or 30 mg NO_2^--N L^{-1} and 23 mg NH_4^+ -N L^{-1}), respectively. In addition, the reaction mixture contained micro- and macroelements as given in [21]. Anoxic conditions in the reaction cell were maintained by argon purging for 20 min after solutions preparation and reaction cell filling with biofilm carriers.

Batch tests were performed right after one another in a temperature range of 9–30 (±0.5) ºC after reactor adaption temperature was maintained for one month. Temperature was maintained in separate tests by a thermostated water bath. Sampling was done 0, 2, 4 and 6 h after the start of the test. TNRRs were calculated from the linear decrease of the summary

nitrogen concentrations during 6 h and calculated per hour and a gram of biofilm's wet weight.

pH in the range of 7.5-8 was maintained as optimum for the anammox proccess [5] by adding 0.4 g HCO_3^- L^{-1}.

Biofilm total suspended solids (TSS) of 2.62 g L^{-1} (6.55 g m^{-2}) was maintained in the test cell as concentration present on 200 carriers. Triplicate TSS measurements were done gravimetrically before the batch experiment using 3 × 20 carriers: the carriers were rinsed with distilled water and dried at 105 ºC for 24 h. After drying the carriers were weighed and the biomass was removed using chromic acid. The carriers were washed, dried and weighed again after 24 h at 105 ºC. TSS was calculated as the difference between the biomass of the dried and washed biofilm carriers.

Analytical methods

Nitrogen forms (NH_4^+-N, NO_2^--N, NO_3^--N) as well as COD were determined spectrophotometrically according to [24]. Hydroxylamine (NH_2OH) was measured spectrophotometrically according to [25]. Hydrazine (N_2H_4) was determined by the Hach Lange HydraVer 2 reagent (containing *p*-dimethylaminobenzaldehyde) and 0.5% solution of sulphamic acid was added to eliminate interference from nitrite and nitrate. pH and conductivity were measured by Jenway (Germany) and dissolved oxygen by Marvet Junior (Estonia) electrodes, respectively. FA was calculated according to equilibrium constant between NH_4^+/NH_3 ([26]).

Homogeneity of group variances and the difference between group means were checked using *f* -test and the two-way *t*-test, respectively. The level of significance was set at $\alpha < 0.05$. The MS Excel 2010 Analysis ToolPak software was used.

Temperature constant calculation

Temperature constants were calculated according to Van't Hoff's rule to evaluate the temperature constant at different temperatures [27]:

$$\frac{d(\ln K)}{dT} = \frac{\Delta H}{RT^2} \quad \text{or} \quad \frac{d(\ln K)}{d(1/T]} = -\frac{\Delta H}{R}, \qquad (2a, 2b)$$

where:

$\ln K$ - natural logarithm of temperature constant K;

T - absolute temperature °K;

ΔH - activation energy $Jmol^{-1}$;

$R = 8.314462(75)\ J.K^{-1}mol^{-1}$ - universal gas constant

Polymerase chain reaction (PCR) methodology, sequencing and

Biomass was mechanically removed using vortex mixer, followed by DNA extraction by MoBio Powersoil DNA isolation kit (USA) according to the manufacturer´s instructions. 5 biomass carriers were taken from the reactor. The PCR products were purified with the JETquick Spin Column Kit (GENOMED GmbH) and then sequenced. 25-50 mg of biomass was applied for DNA extraction.

Representatives of NOB *Nitrospira spp.* and *Nitrobacter spp.* were analysed according to [28]. To identify *Nitrospira* strains the primer sets NSR1113f / NSR1264r were used.

Pla46f /Amx368r primers were used for targeting anammox bacteria. Nested PCR was carried out according to the thermocycling parameters described by by manufacturer. PCR-DGGE for detecting diversity of the most abundant microorganisms was conducted using the eubacterial primer set GC-BacV3f / 907r as described previously by manufacturer.

DGGE was performed using INGENY the PhorU System (INGENY, the Netherlands). PCR products were loaded on a 30–65% denaturing gel and run for 17 h at 90V at a constant temperature of 60°C. The gels were stained with an ethidium bromide solution in an 1× Tris- acetate- acetic acid-ethylenediaminetetraacetic acid (TAE) buffer to observe the bands by UV transillumination, subsequently the bands were excised for further reamplification and sequencing.

Sequencing

The gene sequences were amplified in a Mastercycler Personal thermocycler (Eppendorf, Germany). The PCR reaction products were applied to agarose gel electrophoresis of a 1% agarose (SeaKem® GTG® Agarose, FMC Bioproducts, Rockland, Maine, USA) gel, which was stained with ethidium bromide and visualized under UV transillumination.

PCR for sequencing was performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit **(**Life Technologies Corporation, USA) [21]. The acquired sequences were compared to the available database sequences via a BLAST (Basic Local Alignment Search Tool) search and the related sequences were obtained from the GenBank.

For pyrosequencing universal 8F and 357R sequences were used for the PCR amplification of V2–V3 hypervariable regions of 16S rRNA genes.

Phylogenetic analysis

In order to determine the phylogenetic position of the anammox 16S rRNA gene sequence acquired, it was compared with the available database sequences via a BLAST search, obtaining the related sequences

from the GenBank. Further analysis was carried out with the MEGA software version 5.0 with the neighbour-joining method.

Quantitative polymerase chain reaction (qPCR)

QPCR was conducted with primer sets Amx694F(GGGGAGAGTGGAACTTCTG) and Amx960R(GCTCCACCGCTTGTGCGAGC), which amplify about 285 bp fragments from most anammox bacteria 16S rDNA [29].

Cloning for the standard was performed using the Thermo Scientific InsTAclone PCR cloning kit according to the manufacturers' instructions. JM109 cell line was used. Plasmid was purified from selected colonies using the GeneJET Plasmid minipreparation kit (Thermo Scientific). Dilutions of purified plasmid were used as standard in the qPCR reaction.

PCR amplification and detection were performed in optical 96-well reaction plates. The PCR temperature programme was initiated during 12 min at 95°C, followed by 45 cycles of 10 sec at 94°C, 20 sec at 58°C, and 20 sec at 72°C. Each PCR mixture (10 μL) was composed of 2 μL of 5x HOT FIREPol Eva Green qPCR Supermix (Solis BioDyne, Estonia), 0.25 μL of forward and reverse primers (100 μM) and 1 μL of template DNA.

Results and discussion

The biomass in the MBBR was adapted to 17°–18° and 15° C after which batch tests were performed at various temperatures to determine the accelerating effect of low temperature adaption on short-term low-temperature tests and rate losses at higher temperature adaption in short-term tests. Long-term temperature effect on continuous MBBR operation (Fig. 1) and a short-term temperature effect on batch tests were studied. Also, the effect of different NO_2^- concentrations (100 mg N L^{-1} and 30 mg N L^{-1}) on batch tests' TNRRs was assigned to determine substrate concentration effect [20].

Periods of operation of the continuous reactor

Deammonifying biofilm cultivation was started up at 26° C using nitrifying biofilm carriers [12, 14] and the reactor was operated long-termly (1600 days). The operation of the reactor can be divided into three main periods based on operation temperature: >20° C, 20° C and below 20° C. The behaviour of the MBBR was clearly different at 26° C and 20° C (days 1-1100 and 1100-1419, respectively) compared to period when temperature was maintained below 20° C.

On days 400-616, the increase of the TNRR was from 0.1 g N m^{-2} d^{-1} (0.04 kg N m^{-3} d^{-1}) to 0.6 g N m^{-2} d^{-1} (0.24 kg N m^{-3} d^{-1}). Before the start of

the temperature decreasing the reactor had achieved a relatively high average TNRR of 1.7 g N m^{-2} d^{-1} (0.68 kg N m^{-3} d^{-1}) at 26 °C and 1.5 g N m^{-2} d^{-1} (0.60 kg N m^{-3} d^{-1}) at 15-20 °C (from the 1420th day on), respectively. After the temperature was decreased for 0.5° C per week, there were small decreases in the TNRR, but the rate stabilized shortly after biomass adaptation (Fig. 1). When the TNRR was 1.65 g N m^{-2} d^{-1} (0.60 kg N m^{-3} d^{-1}), on day 1500 it decreased within a week to 1.3 g N m^{-2} d^{-1} (0.52 kg N m^{-3} d^{-1}) when the temperature was decreased from 17° C to 16.5° C. It can be noted that when the temperature was decreased more during a short time interval, a larger decrease in the TNRR caused greater instabilities in the system and higher effluent NH_4^+ concentrations, latter could be removed through post-treatment by nitrification-denitrification process as there was residual COD present in the effluent (>300mg L^{-1}). It would confirm that gradual temperature decreasing could be beneficial to sustain a sufficient TNRR by cultivated biofilm, and as in practice temperature normally does not decrease that abruptly lower than 15º C, cultivated biomass can be used for treatment of wastewater having temperatures 15-30º C. Moreover, the general trend of the TNRR during the third operation period when temperature was lowered from 20° C to 15° C was rather increasing (Fig. 1) as also proven by statistical analysis.

Strategy for suppression of the NOBs

As selecting ammonia oxidizing bacteria (AOB) instead of nitrite oxidizing bacteria (NOB) is challenging at temperatures below 20° C [17], elevated free ammonium (FA) was used. FA spiking (influent FA concentration periodically increased to 7.5 mg N L^{-1} through feeding) in combination with short HRT (0.5 d) applied in reactor between days 0-700 enabled low NOB activity and maximum sufficient TNRR of 0.7 g N m^{-2} d^{-1} (0.28 kg N m^{-3} d^{-1}).

Nevertheless, representatives of NOB *Nitrospira spp*. present in the reactor system are known for their fairly high tolerance for low dissolved oxygen concentrations [30] and are responsible for higher NO_3^- production than stoichiometric ratio. Lower NO_3^- production after 700 days was achieved when the maintained FA concentration in the reactor was increased to 23 mg NH_3-N L^{-1}. Higher FA spikes can be achieved by feeding system with elevated pH (>8) and elevated NH_4^+-N concentration (600-1000 mg N L^{-1}) wastewater having temperatures above 20º C. Apparently, high effluent NH_4^+-N concentrations were present in the effluent, which could be solved through increasing air flow rate shortly after NOBs permanent outcompetition or decreasing TN loading rate.

Similarly to batch tests, no obvious increase in the ratio of effluent NO_3^--$N_{produced}$/NH_4^+-$N_{consumed}$ (that in reactor was on average 0.27/1) during

temperature lowering was present in reactor operation. It showed that gradual temperature lowering still enabled NOB out-competition even at lower temperatures and, on the other hand, non-inhibitory FA concentrations (according to [31] 13-80 mg N L^{-1}) for mature anammox biofilm were present.

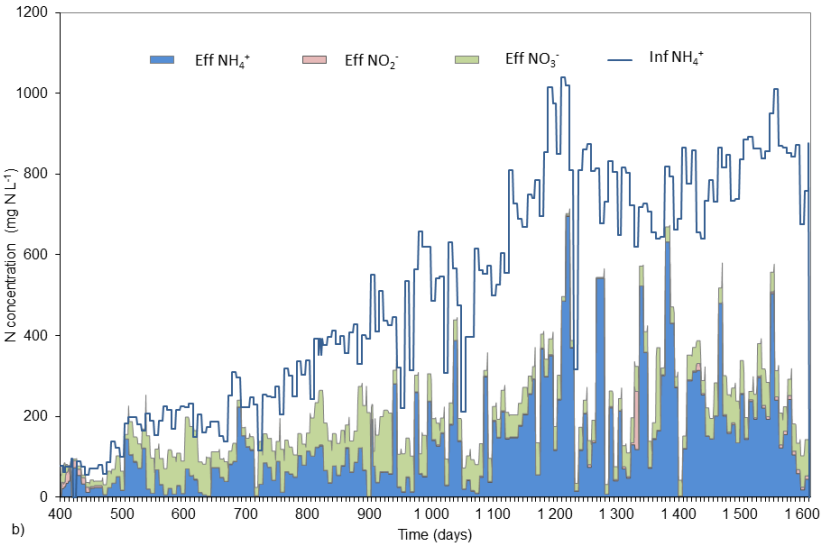

Figure 1. N species concentrations in biofilm reactor within 400 – 1600 days.

Anammox stoichiometric ratio

NO_2^- and NH_4^+ decrease and NO_3^- formation of the cultivated biofilm typical of the anammox process are shown on Fig. 2. NO_2^- concentration decreased averagely 1.35 times faster than NH_4^+ concentration in a temperature range of 15-30° C (Table) being similar to earlier propositions ([23]; [7]).

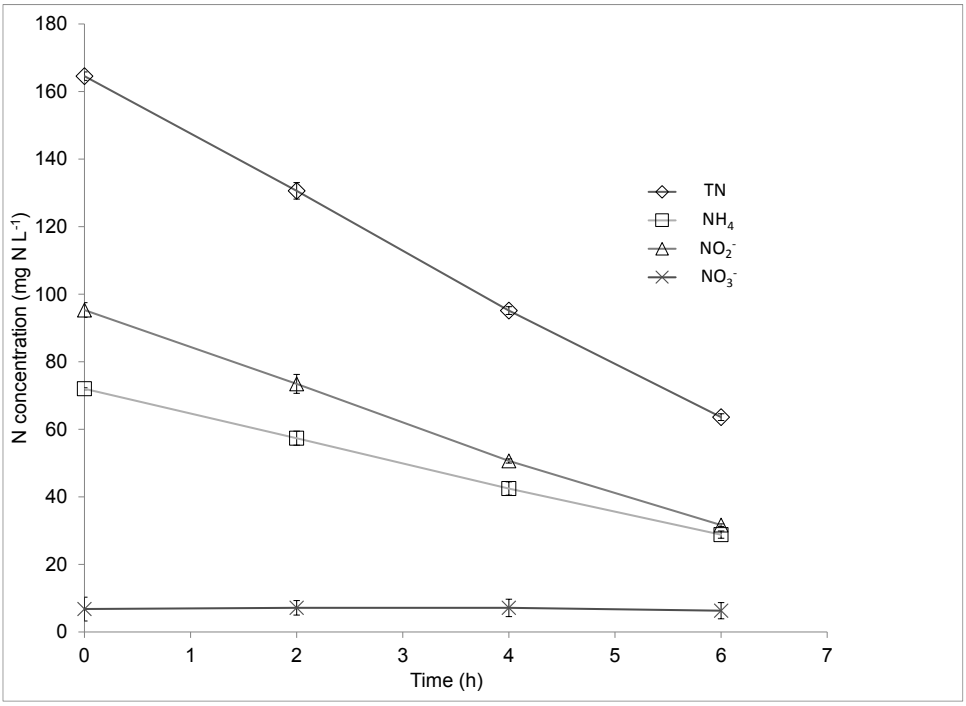

Figure 2. TN and nitrogen species concentration decrease in biofilm carriers with different applied temperatures at 100 mg NO_2^--N L^{-1} at adaption temperature of 17-18 °C. Error bars represent standard deviation of 3 parallels of independent tests.

NO_3^- concentration decreased by temperature lowering (NO_3^--$N_{produced}$/ NH_4^+-$N_{consumed}$<0.1/1) being smaller than characteristic of anammox process (NO_3^--$N_{produced}$/ NH_4^+-$N_{consumed}$<0.26/1). In earlier studies, [4] has found that, lower temperature has brought along higher NO_3^--$N_{produced}$/NH_4^+-$N_{consumed}$ ratio, indicating efficient NOB outcompetition at a lower temperature in our study.

The nitrogen conversion rates and temperature constant calculations in batch tests

Batch tests were done with MBBR carriers before 600 days and during 1500–1600 days (Fig. 2, 3) within 3 periods: At adaption temperature of 26° C (before 600 days), at 17–18° C (from 1500th day on) and on the third period (from day 1570 on) at 15° C. TNRRs achieved for the performed batch tests at the highest temperature (30° C) were not significantly higher (p- value >0.05 in all cases) than at other temperatures as shown in Table.

Table. TNRR of the reactor and batch tests biomass and significant differences (*p-values*) compared with the highest test TNRR. Other authors' temperature constants and respective parameters were added.

Reactor temp. (°C)	**Reactor TNRR (g N m^{-2} d^{-1})**	**Batch temp. (°C)**	**Batch [NO_2^-] (mg N L^{-1})**	**Batch TNRR (mg N L^{-1} h^{-1})**	***p-values* of batch TNRR**	**Batch temp. coefficient**	**NO_2^--$N_{consumed}$/ NH_4^+-$N_{consumed}$**
26	0.5	25	30	4.1	0.2	1.3	1.29
26	0.5	21	30	2.9	0.3	1.2	1.26
26	0.5	15	30	2.7	0.2	1.4	1.23
17-18	1-1.3	30	100	8.6	Reference	1.5	1.39
17-18	1-1.3	25	100	7.1	0.3	-	1.49
17-18	1-1.3	22.5	100	6.5	0.3	1.4	1.35
17-18	1-1.3	20	100	6.0	0.2	1.4	1.36
17-18	1-1.3	15	100	5.1	0.2	1.4	1.34
17-18	1-1.3	12	100	4.0	0.4	1.6	1.27
17-18	1-1.3	9	100	3.6	0.2	1.5	1.24
15	1.2-1.6	30	100	7.3	0.2	-	1.29
15	1.2-1.6	22.5	100	6.3	0.3	1.5	1.22
15	1.2-1.6	15	100	5.4	0.4	1.4	1.35
15	1.2-1.6	9	100	4.3	0.2	1.4	1.24
15	1.2-1.6	30	30	7.3	0.2	1.0	1.35
15	1.2-1.6	22.5	30	6.3	0.2	1.6	1.05
15	1.2-1.6	15	30	5.4	0.4	1.3	1.48
15	1.2-1.6	9	30	4.2	0.2	1.4	1.39
16-30	5.7 **	-	<5	-	-	1.93 [15]	1.24
10-19	1.7	-	9-19	-	0.001*	12.7 [31]	1.11-1.32
20	0.26**	25	<5	2		3.24 [15]	1.11-1.54

Temperature effect on biomass adapted to temperature of 26° and 17–18°C

During the first period reactor temperature was 26 °C the TNRRs were the lowest and quite highly dependent on temperatures maintained in the batch tests. After biomass adaption to lower temperatures its tolerance to lower temperatures increased as well as TNRR.

During the second period (cultivation at 17–18° C), the TNRR was the highest at 30 °C, decreasing with lowered temperature (Fig. 3). At 22.5 °C the TNRR had decreased approximately 25%, when comparing it to the value measured at 30 °C. At the applied temperature of 9 °C, the TNRR measured in a batch test was still 3.58 mg N g^{-1} TSS h^{-1} or approximately 40% of the value measured at 30 °C, showing low temperature tolerance of the biomass applied in tests.

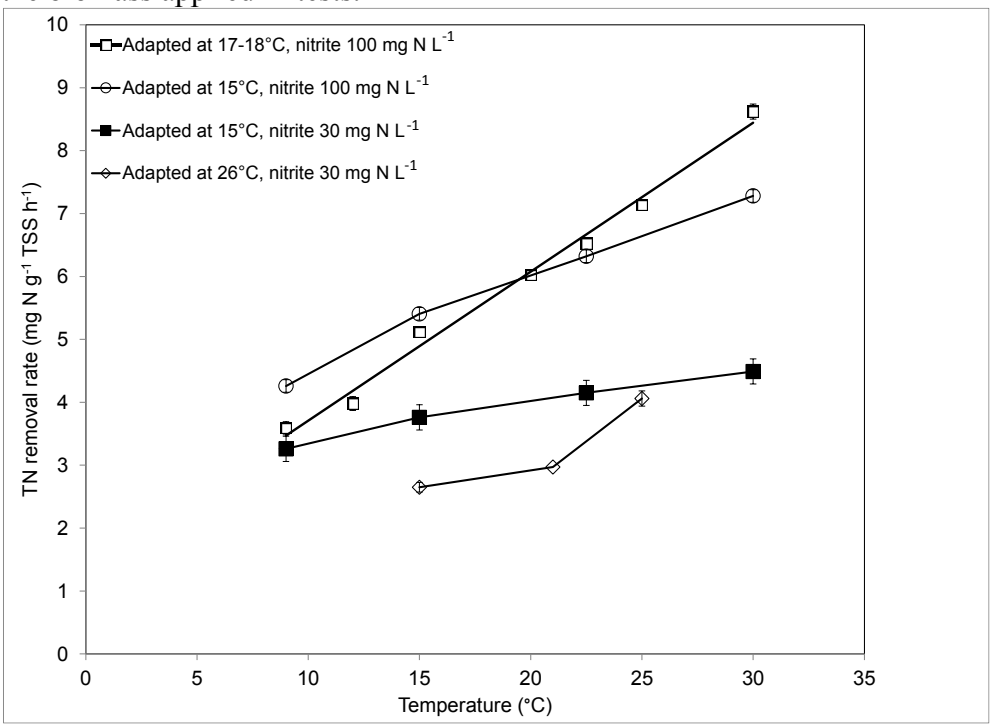

Figure 3. TN removal rates of biofilm carriers with different applied temperatures at NO_2^- concentrations of 100 mg NO_2^--N L^{-1} and 30 mg NO_2^--N L^{-1} with different applied temperatures in the reactor. Error bars represent standard deviation of 3 parallels of independent tests.

In a temperature range of 9-30 °C, a decrease in the TNRR was linear without an abrupt rate drop - a fact that is in disagreement with the results reported by [7] who observed decrease in the TNRR almost to zero below 15 °C. We can conclude that a slow, gradual lowering of temperature down to 17 °C is beneficial from the point of view of practical applications since anammox biomass adapted to lower-temperature has an ability to perform autotrophic nitrogen removal in a short term at a temperature as low as 9 °C. This has been considered unfeasible in the earlier studies ([9]) as long as cryoprotectants have not been applied [13]). By comparison, [32] operated a nitritation–anammox MBBR at 19-10 °C, when lowering of the temperature was done relatively abruptly by 3 °C in each period, TNRR remained stable

in a temperature range of 19–13 °C (1.67- 0.55 g N m^{-2} d^{-1}) using low NO_2^- concentrations (Table). However, at 10 °C TNRR has been low (average TNRR was 0.17 g N m^{-2} d^{-1}), which eventually have led to a failure of reactor work when the NO_2^- concentrations reached 200 mg N L^{-1} [32].

Temperature constants calculated by us for deammonification biofilm in the temperature range of 9-30 °C were 1-1.6 being close to the temperature constant of 1.93 in the temperature range of 16-30° C achieved by [11]. Surprisingly, compared to our results, the data from [32] leads to a higher value of the temperature constant within the temperature range of 10-19°C. Differences on constants could be explained by using different biomass (biofilm/granules) as well as different applied conditions during the tests.

Temperature effect on reactor adapted to lower temperature of 15 °C

On the third test period the MBBR was adapted to 15 °C. In batch experiments at 9 °C the TNRR of the adapted biomass was 4.3 mg N g^{-1} TSS h^{-1}, whereas at the same temperature it was 15% lower for the biomass adapted to a temperature of 17-18° C.

The obtained results indicated that the biomass adapted to 15 °C in reactor showed higher TNRRs on a relative scale at a lower temperature (9 °C) rather than at a higher temperature (30 °C). TNRRs for the biomass adapted to lower temperatures (15 °C) in the MBBR were higher in batch tests at 9–15 °C as compared with the rates achieved with the biomass cultivated at 17-18 °C. Respective lower-temperature-adapted biomass showed lower TNRRs in batch tests at a higher temperature range of 20–30 °C. Short-term back-adaption to a higher temperature was not observed, proving permanent biomass adaption at a low temperature by the applied strategy.

These experimental results indicate that slow, gradual temperature lowering is substantial for efficient operation of the reactor at a lower temperature [7].

Due to adaption of biomass to low temperature, increased TNRRs in the batch tests occurred using biomass adapted to a lower temperature (15 °C) in the MBBR even at 9 °C when TNRRs decreased less compared to the biomass adapted to a higher temperature (17-18 °C) in comparison with [7].

In cold regions ocean floor sediment anammox bacteria strains different from which are present in wastewater treatment have been shown to be able to adapt to temperatures as low as to 9 °C. Mostly, anammox process has been observed, *in-situ* in estuary deposits in a temperature range of 15-19 °C [33], which was one of the reasons for adaption temperature choice in our case. After adaptation to temperatures ~15° C, with the temperature exceeding 20 °C TNRRs, however, had decreased [33]. It showed that

anammox bacteria *Planctomycetales Scalindua* strains do not adapt back to high temperatures and there could be assumed that *Candidatus Brocadia* strains have same mechanism.

Nitrite concentration effect

A series of batch-scale experiments were performed in a period when the MBBR biomass was adapted to lower temperature (15 °C) to determine the effects of nitrite concentration on the TNRR. The biomass in the MBBR system was able to tolerate high concentrations of nitrite (100 mg N L^{-1}) in the batch assays, whereas in the tests with lower nitrite concentrations (30 mg N L^{-1}) TNRR was lower. High NO_2^- tolerance could be achieved for thick biofilm cultivation by a long-term operation whose findings are contradictory to previous studies [7]. Among TNRRs for both concentrations at different temperatures significant differences between tests were not observed (*p-values* >0.05) (Table).

Low activation energies of 17.7 and 29.7 kJ mol^{-1} for NO_2^- concentrations of 30 and 100 mg N L^{-1}, respectively, were determined. Earlier, activation energies of the anammox process have reached much higher values-in a range of 67 to 86 kJ mol^{-1} [4] than our results, probably because of different systems applied: they used suspended biomass instead of biofilm system used by us. Literature data agreed with the values of 70 kJ mol^{-1} obtained by [34] and [4] , which were close to 63 kJ mol^{-1} obtained by [7]. It could be assumed that in our case less energy (water treatment at lower temperature) was needed for efficient and more economical water treatment process making it applicable to low temperature mainstream wastewater treatment.

Based on experimental results, we conclude that during reactor operation, the biomass in the MBBR system had developed a high degree of NO_2^- tolerance and, in disagreement with most literature sources [20], had shown the highest TNRR at a high NO_2^- content (100 mg N L^{-1}) and a lower TNRR at a low NO_2^- (30 mg N L^{-1}) content.

Quantitative anammox 16S rRNA analysis

In the reject water (TSS 0.75 g L^{-1}) from which anammox organisms were enriched, anammox quantities of 1×10^4 copies g^{-1} TSS were present. Quantitative 16S rRNA analysis showed an increase in anammox organisms' quantities from 5×10^3 anammox gene copies g^{-1} TSS from the start of the operation to 1×10^7 anammox gene copies g^{-1} TSS at the end of reactor operation (day 1660) (Fig. 4). Abrupt increase in the anammox gene copy number at the end of operation despite temperature lowering could show a good adaption of anammox organisms to low temperature after a certain gene copy numbers have been exceeded (10^5 copies g^{-1} TSS) in the system.

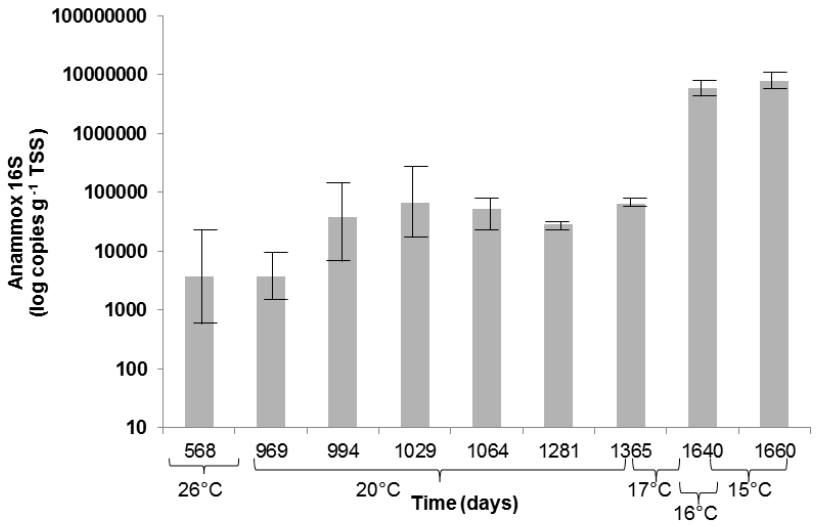

Figure 4. Logarithmic values of *Planctomycetes* gene copies in the biomass of MBBR dependent on reactor temperature. Error bars represent standard deviation of 3 parallels of independent tests.

Conclusion

The effects of low temperature on the deammonification the maximum total nitrogen removal rate (TNRR) of the deammonification reactor of 1.5 g N m^{-2} d^{-1} (0.60 kg N m^{-3} d^{-1}) at a low temperature (15 °C) was shown.

TNRR at 9 °C with the biomass cultivated at 17-18 °C was only 40% as compared with that of for highest temperature of 30 °C applied. The adaptability of biomass to lower temperatures by gradually lowering the reactor's temperature from 18 °C to 15 °C was studied in batch tests. The biomass adapted to 15 °C showed a quite sufficient TNRR (3.6 g N g^{-1} TSS h^{-1}) in the batch tests even at 9 °C. The results showed that if the MBBR was adapted to 17 °C, the batch experiment at 30 °C had a high TNRR (8.6 mg N g^{-1} TSS h^{-1}) whereas at a low temperature of 9 °C the batch experiment showed a loss in the TNRR (3.6 mg N g^{-1} TSS h^{-1}) as compared with the biomass adapted to 15 °C.

In order to quantify the temperature and substrate dependency of the anammox reaction, the calculated activation energies were 17.7 and 29.7 kJ mol^{-1} for NO_2^- concentrations of 30 and 100 mg N L^{-1}, respectively, showing that a high NO_2^- concentration increased TNRR. Nitrite concentration of (30

mg N L^{-1}) limited the TNRR of the anammox process in comparison with the highest one (100 mg N L^{-1}).

Anammox bacteria *Planctomycetales clone P4* and *uncultured planctomycetes clone Amx_P055-8* sequences, which were the closest (98 and 99% similarity, respectively) relative to *Candidatus Brocadia fulgida* increased till day 1600 of reactor operation to 1×10^7 anammox gene copies g^{-1} TSS despite the temperature decreasing to 15 °C.

Regarding to practical applications, biofilm deammonification system could be set up at colder regions for treatment of mainstream wastewater when developing biomass with gradual temperature adaption methods.

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References:
Puyol, D., et al., *Kinetic characterization of Brocadia spp.-dominated anammox cultures.* Bioresource Technology, 2013. **139**: p. 94-100.
van Loosdrecht, M.C.M., *Innovative Nitrogen Removal*. Biological wastewater treatment: Principles, modelling and design, ed. M. Henze, et al. 2008, London: IWA Publishing.
Lackner, S. and H. Horn, *Comparing the performance and operation stability of an SBR and MBBR for single-stage nitritation-anammox treating wastewater with high organic load.* Environmental Technology, 2013. **34**(10): p. 1319-1328.
Hendrickx, T.L.G., et al., *Autotrophic nitrogen removal from low strength waste water at low temperature.* Water Research, 2012. **46**(7): p. 2187-2193.
Strous, M., et al., *Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge.* Applied and Environmental Microbiology, 1997. **63**(6): p. 2446-2448.
Wang, Z., et al., *Start up of a gravity flow CANON-like MBR treating surface water under low temperature.* Chemical Engineering Journal, 2013. **217**: p. 466-474.
Dosta, J., et al., *Short- and long-term effects of temperature on the Anammox process.* Journal of Hazardous Materials, 2008. **154**(1-3): p. 688-693.
Isaka, K., et al., *Ammonium removal performance of anaerobic ammonium-oxidizing bacteria immobilized in polyethylene glycol gel carrier.* Applied Microbiology and Biotechnology, 2007.

Cema, G., et al., *Nitrogen removal rates at a technical-scale pilot plant with the one-stage partial nitritation/Anammox process.* Water Science and Technology, 2006. **54**(8): p. 209-217.
Jin, R.C., et al., *The effect of sulfide inhibition on the ANAMMOX process.* Water Research, 2013. **47**(3): p. 1459-1469.
Ma, B., et al., *Performance of anammox UASB reactor treating low strength wastewater under moderate and low temperatures.* Bioresource Technology, 2013. **129**: p. 606-611.
Zekker, I., et al., *Achieving nitritation and anammox enrichment in a single moving-bed biofilm reactor treating reject water.* Environmental Technology, 2012. **33**(6): p. 703-710.
Hu, Z.Y., et al., *Nitrogen Removal by a Nitritation-Anammox Bioreactor at Low Temperature.* Applied and Environmental Microbiology, 2013. **79**(8): p. 2807-2812.
Zekker, I., et al., *Deammonification process start-up after enrichment of anammox microorganisms from reject water in a moving-bed biofilm reactor.* Environmental Technology, 2013. **34**(23): p. 3095-3101.
De Clippeleir, H., et al., *One-stage partial nitritation/anammox at 15 A degrees C on pretreated sewage: feasibility demonstration at lab-scale.* Applied Microbiology and Biotechnology, 2013. **97**(23): p. 10199-10210.
Lotti, T., R. Kleerebezem, and M.C.M. van Loosdrecht, *Effect of Temperature Change on Anammox Activity.* Biotechnology and Bioengineering, 2015. **112**(1): p. 98-103.
Hellinga, C., et al., *The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water.* Water Science and Technology, 1998. **37**(9): p. 135-142.
Gilbert, E.M., et al., *Low Temperature Partial Nitritation/Anammox in a Moving Bed Biofilm Reactor Treating Low Strength Wastewater.* Environmental Science & Technology, 2014. **48**(15): p. 8784-8792.
Wett, B., et al. *Development of Enhanced Deammonification Selector*. in *WEFTEC 2010*. 2010. New Orleans, US.
Lotti, T., et al., *The effect of nitrite inhibition on the anammox process.* Water Research, 2012. **46**(8): p. 2559-2569.
Zekker, I., et al., *Anammox enrichment from reject water on blank biofilm carriers and carriers containing nitrifying biomass: operation of two moving bed biofilm reactors (MBBR).* Biodegradation, 2012. **23**(4): p. 547-560.
Zekker, I., et al., *Modification of nitrifying biofilm into nitritating one by combination of increased free ammonia concentrations, lowered HRT and dissolved oxygen concentration.* Journal of Environmental Sciences-China, 2011. **23**(7): p. 1113-1121.

van de Graaf, A.A., et al., *Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor.* Microbiology-Uk, 1996. **142**: p. 2187-2196.
Greenberg, A.E., L.S. Clesceri, and A.D. Eaton, *Standard Methods for the Examination of Water and Wastewater*. 1992, Washington DC: American Public Health Association.
Frear, D.S.a.B., R.C. , *Spectrophotometric method for determining hydroxylamine reductase activity in higher plants.* Anal. Chem. , 1955. **27**: p. 1664.
Anthonisen, A.C., et al., *Inhibition of nitrification by ammonia and nitrous acid.* Journal Water Pollution Control Federation, 1976. **48**(5): p. 835-852.
Atkins, P.W., *Physical Chemistry, 5th edition.* 1994.
Dionisi, H.M., et al., *Quantification of Nitrosomonas oligotropha-like ammonia-oxidizing bacteria and Nitrospira spp. from full-scale wastewater treatment plants by competitive PCR.* Applied and Environmental Microbiology, 2002. **68**(1): p. 245-253.
Ni, B.J., et al., *Microbial and Physicochemical Characteristics of Compact Anaerobic Ammonium-Oxidizing Granules in an Upflow Anaerobic Sludge Blanket Reactor.* Applied and Environmental Microbiology, 2010. **76**(8): p. 2652-2656.
Mota, C., et al., *High levels of nitrifying bacteria in intermittently aerated reactors treating high ammonia wastewater.* FEMS Microbiology Ecology, 2005. **54**(3): p. 391-400.
Waki, M., et al., *Nitrogen removal from animal waste treatment water by anammox enrichment.* Bioresource Technology, 2007. **98**(14): p. 2775-2780.
Persson, F., et al., *Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation-anammox at low temperatures.* Bioresource Technology, 2014. **154**: p. 267-273.
Teixeira, C., et al., *Potential rates and environmental controls of anaerobic ammonium oxidation in estuarine sediments.* Aquatic Microbial Ecology, 2012. **66**(1): p. 23-32.
Strous, M., J.G. Kuenen, and M.S.M. Jetten, *Key physiology of anaerobic ammonium oxidation.* Applied and Environmental Microbiology, 1999. **65**(7): p. 3248-3250.