Towards mainstream anammox

lessons learned from pilot-scale research at WWTP Dokhaven

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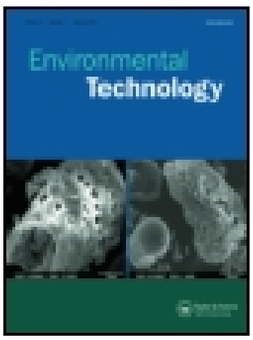

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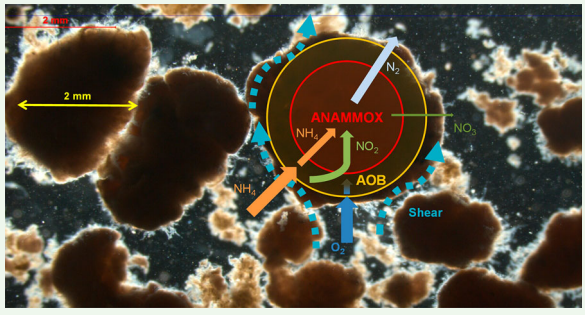
Towards mainstream anammox: lessons learned from pilot-scale research at WWTP Dokhaven

Maaike Hoekstra[a]*, Stefan P. Geilvoet[b], Tim L. G. Hendrickx[c], Charlotte S. van Erp Taalman Kip[b], Robbert Kleerebezem[a]* and Mark C. M. van Loosdrecht[a]*

[a]Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Delft, Netherlands; [b]Waterschap Hollandse Delta, Ridderkerk, Netherlands; [c]Paques BV, Balk, Netherlands

ABSTRACT

The aim of this research was to study the biological feasibility of the Partial Nitritation/Anammox (PN/A) technology to remove nitrogen from municipal mainstream wastewaters. During stable process operations at summer temperatures (23.2 ± 1.3°C), the total nitrogen removal rate was 0.223 ± 0.029 kg N $(m^3\ d)^{-1}$ while at winter temperatures (13.4 ± 1.1°C) the total nitrogen removal rate was 0.097 ± 0.016 kg N $(m^3\ d)^{-1}$. Nitrite-oxidizing bacteria (NOB) suppression was successfully achieved at the complete temperature range of municipal mainstream wastewater. Despite the presence of NOB as observed in activity tests, their activity could be successfully suppressed due to a relative low dissolved oxygen concentration. An overcapacity of ammonia-oxidizing bacteria and anammox activity was always present. Long-term stability is a focus point for future research, especially in relation to the stability of the biological oxygen demand removing step, preceding the PN/A reactor.



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Mainstream anammox; wastewater treatment; autotrophic nitrogen removal; low temperature

1. Introduction

One of the available technologies to treat municipal wastewater is the AB-system. Wastewater treatment plants (WWTPs) designed according to this technology are set up as a two-stage system with a higher loaded A-stage (Adsorption) and a lower loaded B-stage (Belebung) [1]. In the A-stage the biological oxygen demand (BOD) is removed and in the B-stage the ammonium is nitrified. To remove nitrate from the effluent of the nitrification process, wastewater is recycled to the A-stage where the BOD in the influent can serve as an electron donor for the denitrification process. The main advantage of this process is the relative low external energy requirement and compact construction, with full nitrification. An example of an AB-process upgraded to partial denitrification is the Dokhaven treatment plant in Rotterdam, the Netherlands [2].

In the two-sludge type AB-system, it was not simple to implement the pre-denitrification process for nitrogen elimination. Due to the large recycle flow required for returning nitrate-rich effluent from the B-stage to a partially anoxic A-stage, the hydraulic load on the settlers of both stages was strongly increased. The total nitrogen removal capacity of the treatment plant is still limited, since a further improvement would result in extremely large recycle flows. In AB-system-based treatment plants, it would therefore be favourable if complete nitrogen removal could be introduced in the B-stage of the

CONTACT Maaike Hoekstra m.hoekstra@hhnk.nl Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, vd Maasweg 9, Delft 2629 HZ, Netherlands
*Present address: Hoogheemraadschap Hollands Noorderkwartier, Stationsplein 137, Heerhugowaard 1703 WC, Netherlands.
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process. This can be achieved by implementing the Partial Nitritation/Anammox (PN/A) process in the current B-stage. In the PN/A process the first step of nitrification, the oxidation of ammonium to nitrite (nitritation), is combined with the anaerobic ammonium oxidizing (anammox) process. Both nitritation and anammox are autotrophic processes that do not require BOD, but the anammox bacteria do require a high sludge age for their implementation. Removal of BOD in the A-stage of the AB-process facilitates high sludge ages and high percentages of autotrophic biomass in the B-stage, potentially enabling the PN/A process.

The PN/A technology, can optimize the current municipal mainstream wastewater treatment technology [3]. There are multiple advantages of the process. Firstly, there is no longer a need for organic carbon in order to remove nitrogen through denitrification. The carbon in the wastewater can therefore be used for different purposes, for instance for the production of biogas [4]. Electricity produced from the biogas could make the treatment plant autarkic with respect to energy. This is especially of interest for countries with an unsecure electricity supply or to avoid the costs of connection to the electricity grid. The carbon in the wastewater could also be reused in different ways, for instance for producing biopolymers [5]. A second advantage of the PN/A technology is the use of biofilms for (part of) the biomass. Biofilms can lead to higher biomass concentrations in the reactor and therefore higher volumetric loading rates can be applied. Biofilms are easier to separate from water compared to sludge flocs, so a more compact sludge retention system could be built (compared to current secondary clarifiers). And lastly, due to the exclusion of heterotrophic denitrification and therefore the need for BOD, this technology could be implemented in streams with BOD:N ratios, too low for heterotrophic denitrification. Specifically for AB-systems that need upgrading for full denitrification, effluent recycle (pump energy) will no longer be required.

There are different ways of implementing the PN/A process in the B-stage of an AB-process [6]. Two types of organisms can be combined in a biofilm, in the form of granules or carrier material [7,8]. In this biofilm, the ammonia-oxidizing bacteria (AOB) are oxidizing part of the ammonium to nitrite and the anammox bacteria convert ammonium together with the produced nitrite, to dinitrogen gas. Since the organisms grow as a biofilm it is possible to combine the aerobic zone (for AOB) and the anoxic zone (for anammox bacteria) in a single reactor at the same time. Another possibility would be to cultivate the AOB as flocs in suspension and the anammox bacteria as a biofilm, the so-called hybrid system [9]. It is also possible to separate the AOB from the anammox bacteria in a two-stage system, with AOB in the first, aerated, reactor and anammox bacteria in the second reactor [10].

The PN/A technology is currently implemented in side stream flows of WWTPs and in industrial wastewater streams, characterized by high temperatures and high ammonium concentrations [11]. Laboratory and pilot-scale research on the mainstream application is currently pursued worldwide. But a limited amount of work is done on pilot-scale installations fed with real wastewater [9,12–14]. The laboratory and pilot-scale results indicate that the main challenge of implementing the technology is the low water temperatures during winter. At low temperatures, the total conversion rates decrease and suppression of nitrite-oxidizing bacteria (NOB) proved difficult [6,15,16].

Before converting treatment plants to this new technology, a proof of principle is needed, therefore a pilot-scale reactor was operated. The aim of this research was to evaluate the biological feasibility of the technology to remove nitrogen from municipal mainstream wastewaters. Therefore, different topics were studied. Firstly, the effect of decreasing temperatures on total nitrogen removal capacity. Secondly, the suppression of NOB needed for stability and finally the need for an overcapacity in activity of AOB and anammox bacteria. The set-up was designed to evaluate the potential volumetric conversion capacity on effluent of the A-stage at fluctuating temperatures, with an effluent demand of total soluble nitrogen below 10 mgN L^{-1} (current effluent requirement for most WWTPs in the Netherlands). A point of interest during this reactor run was the stability of the system. When applying a technology on a full scale in a WWTP the system needs to be resilient, not treating the wastewater will not be acceptable. Here, we describe the results and discuss the lessons learned.

2. Materials and methods

2.1. Dokhaven–Sluisjesdijk WWTP

The municipal WWTP at Dokhaven–Sluisjesdijk, Rotterdam, the Netherlands, has a treatment capacity of about 560,000 p.e. The treatment plant was designed as an AB-system and was built underground. In the A-stage BOD is removed in a high-loaded reactor by adsorption with the aim of maximizing biogas production by anaerobic digestion (hydraulic retention time (HRT) of 1 h in dry weather conditions, solid retention time (SRT) 0.3 d). In the B-stage, the remaining BOD is oxidized and ammonium is nitrified to nitrate (HRT 3 h; SRT 7 d). The treatment plant was not originally designed for denitrification. To remove

the nitrogen from the water, the effluent of the B-stage is recycled to the beginning of the A-stage, where the nitrate can be denitrified together with the incoming BOD. Phosphorus is chemically removed by $Fe^{3+}Cl_3$ dosage in the A-stage.

2.2. PN/A reactor set-up and operating conditions

The reactor has been operated from 2013 to 2016 and is comprised of a well-mixed reactor of four cubic metre and a separator for granular biomass retention. A schematic representation of the installation can be seen in Figure 1. The complete reactor run was divided into different periods, a description of the different periods can be found in Table 1. The influent of the reactor was the effluent of the intermediate sedimentation tank after the A-stage. Before the influent entered the reactor it was buffered in the buffer tank. In this tank NH_4Cl could be dosed and the temperature of the influent was controlled. In period 1–3 ammonium chloride was dosed to the reactor influent, to decrease the BOD/N ratio and to correct for the decrease in ammonium in the influent, due to the recycling of the effluent for denitrification, in period 4 the dosing was stopped. By changing the HRT, the volumetric nitrogen load was kept constant over all four periods. Oxygen transfer and mixing were done by fine-bubble aerators. The dissolved oxygen (DO) concentration was controlled by a gas recycling system with a constant gas flow to which fresh air was introduced. pH was controlled at 7.2 by the addition of 33 w/v% NaOH. The addition of NaOH was needed to compensate for the change in pH due the gas recycle and will not be needed in a full-scale application. The reactor was inoculated at the beginning of each phase with granular biomass from a full-scale one-stage PN/A side stream reactor operated at 30–35°C. In the biomass separator, the granular biomass was separated from the water by gravity settling and the settled granular biomass was returned to the reactor.

2.3. Calculating conversion rates

Based on the influent and effluent concentrations of the N-species, the conversion rates of the reactor were calculated. Based on the assumption that the only conversions taking place in the reactor were autotrophic nitrogen conversions, specific rates for anammox bacteria, AOB and NOB were derived. Due to the presence of oxygen in the reactor, it was assumed that the incoming BOD was converted aerobically and not through denitrification. The calculations are based on the stoichiometry in Equations (1)–(3), for the AOB, NOB and anammox conversions [17]. The ratio between nitrate production and ammonium consumption was used as an indicator of the significance of NOB activity. When there is only

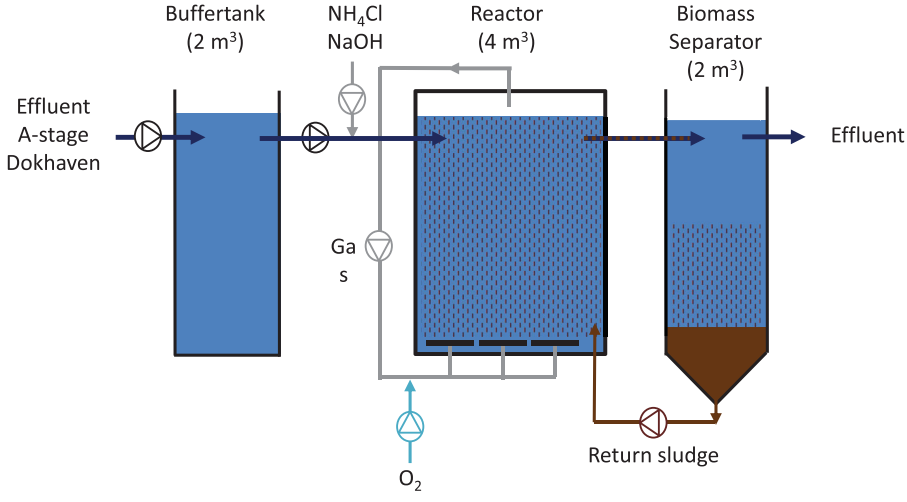

Figure 1. Schematic representation of the lay-out of the PN/A reactor.

Table 1. Overview of the process parameters in the different periods which are described in this publication.

Period	Inoculation date	Number of days	HRT (d)	NH_4Cl dosing	Temperature range (°C)	Influent NH_4 ($mgNH_4$-N L^{-1})	Influent BOD/N
1	Feb 2014	91	0.12 ± 0.02	+	16.1–21.1	33.0 ± 7.5	0.6 ± 0.2
2	May 2014	153	0.11 ± 0.01	+	18.8–25.5	32.6 ± 5.4	0.4 ± 0.1
3	Dec 2014	83	0.10 ± 0.00	+	10.4–17.5	30.3 ± 4.9	1.2 ± 0.4
4	July 2015	180	0.05 ± 0.01	–	10.5–24.7	14.2 ± 3.8	2.5 ± 1.8

AOB and anammox activity, 7.5% of the consumed NH_4-N will be converted to NO_3-N.

$$NH_4 + 0.09HCO_3 + 1.38O_2 \rightarrow 0.09CH_{1.4}O_{0.4}N_{0.2} + 1.04H_2O + 0.98NO_2 + H^+, \quad (1)$$

$$NO_2 + 0.0025NH_4 + HCO_3 + 0.49O_2 \rightarrow 0.0125CH_{1.4}O_{0.4}N_{0.2} + 0.0075H_2O + NO_3 + 0.0025H^+, \quad (2)$$

$$1.146NO_2 + 1NH_4 + 0.071HCO_3 + 0.057H^+ \rightarrow 0.986N_2 + CH_{1.74}O_{0.31}N_{0.2} + 2.002H_2O + 0.161NO_3. \quad (3)$$

2.4. Ex situ batch test

Ex situ batch tests were conducted regularly to identify the maximum activity of AOB, NOB and anammox bacteria. To measure the maximum anammox specific activity (SA^{max}_{AMX} ($mgNO_2$-N (gVSS d)$^{-1}$)), anoxic monomeric batch tests were used, these tests are described elsewhere [18]. In short, the experiments were performed in bottles with OxiTop heads. The granular biomass was washed (0.2 mm sieve) and suspended in filtered supernatant (filtered through filter paper). The pH of the medium was maintained at 7 using 25 mM HEPES (N-2-hydroxylehyl-piperazine-N0-2-ethane sulphonic acid) buffer, the pH was set using NaOH. Nitrate was added to the solution to avoid low redox values. The bottles were made anoxic by sparging with N_2 gas and incubated in a temperature-controlled shaker. After stabilization of the headspace pressure a substrate solution was injected, leading to concentrations of 25 $mgNH_4$-N L^{-1}, 25 $mgNO_2$-N L^{-1} and 21.8 $mgHCO_3$ L^{-1}. By measuring the increase of the headspace pressure (due to N_2 production), the anammox activity could be calculated. The substrate solution was injected three times and reported values are averages of the last two injections in four different bottles. Experiments were executed at 20°C and at the reactor temperature.

To measure the maximum AOB and NOB activity (SA^{max}_{AOB} ($mgNO_2$-N (gVSS d)$^{-1}$); SA^{max}_{NOB} ($mgNO_3$-N (gVSS d)$^{-1}$)), aerobic batch tests were used. In these tests mixed liquid of the reactor was used, the pH was stabilized at 7 using 25 mM HEPES buffer, the pH was set by using NaOH and temperature was controlled by placing the bottles in a temperature-controlled water bath (16–18°C). A flow of compressed air was used to mix and aerate the sample through fine-bubble aeration. At the start of the experiment, concentrated ammonium and nitrite solutions were fed to the bottles to obtain a concentration of 40 mg NH_4-N L^{-1} and 20 mg NO_2-N L^{-1}. Conversion rates were measured by measuring N-species concentrations at different time points. Ammonium, nitrite and oxygen were not limited during these experiments. Experiments were done in duplicates.

2.5. Analytical methods

Analytical measurements for inorganic N-compounds were conducted with an online grab sample of the influent and effluent, filtered samples were measured online every half an hour with a spectrophotometric method (Applikon Biotechnology, Delft). In addition to the online measurements, 24 h collected samples (time and flow proportional) were measured with Dr. Lange test kits for N-species. Measurements for BOD and biomass concentration (gTSS L^{-1} and gVSS L^{-1}) were carried out according to standard methods [19].

The morphology and size distribution of the granules were monitored by image analysis, with a Lexmark Optra image analysis system.

FISH was used to analyse the distribution of the different microorganisms in the granule. The collected granules were washed in phosphate buffer and fixed using paraformaldehyde. Slicing was accomplished by embedding the granules in a tissue freezing medium (Leica Microsystems) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 20 µm slices. Dried slices were stored on a microscopic glass slide, and FISH was performed. Hybridization with fluorescent-labelled oligonucleotide probes and analysis of the samples were performed as described in Lotti et al. [17], a formamide concentration of 35% was used for hybridization with the probes specific for Kuenenia-like and Brocadia-like anammox bacteria (AMX-820) [20], AOB (mix of NEU-653, NSO-190 and NSO-1225) [21,22], NOB (mix of NTSPA-0712 and NIT-1035) [23,24] and eubacteria (mix EUB-338) [25].

Genomic DNA was extracted using the Ultraclean Microbial DNA extraction kit supplied by MOBIO laboratories Inc. (CA, USA) according to the manufacturer's protocol, combined with an additional heating step of 5 min heating at 65°C and 5 min beat-beating, to ensure maximum yields. The extracted genomic DNA was subsequently used for a two-step PCR reaction targeting the 16Sr-RNA gene of most bacteria and archaea. For this we used the primers, U515F (5′ – GTGYCAGCMGCCGCGGTA – 3′) and U1071R (5′ – GARCTGRCGRCRRCCATGCA – 3′) as used by Wang et al. [26]. The first amplification was performed to enrich the 16s-rRNA genes. The following chemicals were used: 2× iQ™ SYBR® Green Supermix (Bio-rad, CA, USA), 500 nM primers each and finally 1–50 ng

genomic DNA added per well to a final volume of 20 µL. The protocol was denaturation at 95°C for 5 min and 20 cycles at 95°C for 30 s, 50°C for 40 s, 72°C for 40 s and a final extension at 72°C for 7 min. During the second step, 454-adapters (Roche) and MID tags at the U515F primer were added to the products of step one. The second step was similar to step one, except that Taq PCR Master Mix (Qiagen Inc, CA, USA) was used, the programme was run for 15 cycles and the template was diluted 10 times. After the second amplification, 24 PCR products were pooled equimolar and purified over an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Netherlands). The resulting library was sent for 454 sequencing and run in 1/8 lane with titanium chemistry by Macrogen Inc. (Seoul, Korea).

After a standard error correction by Macrogen Inc. the library was imported into CLC genomics workbench v7.5.1 (CLC Bio, Aarhus, DK) and (quality, limit = 0.05) trimmed to a minimum of 200 bp and de-multiplexed resulting in five samples with an average of 5908 reads per sample with an average length of 497 bp. A build-it SILVA 123.1 SSURef Nr99 taxonomic database was used for BLASTn analysis on the reads under default conditions. The top 20 most abundant genera are displayed in the supplemented material.

3. Results

The PN/A pilot-scale reactor was operated for 3.5 years, under different influent conditions and temperatures. The operational period of the reactor is described in four periods, in which a stable process was obtained (Table 1). During stable process operations, at summer temperatures (23.2 ± 1.3°C), a total nitrogen removal rate of 0.223 ± 0.029 kg N $(m^3\ d)^{-1}$ was achieved and at winter temperatures (13.4 ± 1.1°C) the total nitrogen removal rate was 0.097 ± 0.016 kg N $(m^3\ d)^{-1}$. The average granular size over the complete period of operation was 1.2 ± 0.4 mm. The average nitrite concentration in the reactor was 1.1 ± 1.0 mgNO_2-N L^{-1}. Graphs of loading rates, removal rates and temperature can be found in the online supplemental material.

3.1. Effect of temperature on conversions

During periods 3 and 4 the temperature decreased to temperatures below 15°C, either due to the natural decent of the wastewater temperature (period 3) or due to forced cooling of the influent (period 4). The temperature effect on the conversion rates observed is presented in Figure 2. In period 3 (winter 2014–2015) the decrease in temperature had no negative effect on the conversions (Figure 2(A)). In period 4 (winter 2015–2016) there was a small negative trend in conversions (due to a lower ammonium removal rate) and an increase in nitrate production at decreasing temperatures (Figure 2(B)) (for NO_3 concentrations, see the online supplemented material). It is important to note that the reactor under the influence of a protozoa bloom at this time. In period 4 the BOD/N ratio in the influent of the pilot reactor was higher compared to period 3. The fluctuations in BOD/N (Table 1) could not directly be related to the changes in temperature.

3.2. Maximal biomass capacity vs actual activity

The reactor was started up several times with sludge originating from full-scale side stream PN/A reactors containing granular biomass. The sludge was immediately active under municipal mainstream conditions and no adaptation period was needed. If the inoculation sludge did contain a significant fraction of floccular biomass, this fraction was washed out of the reactor in the first week of operation. The overcapacities of the AOB, NOB and anammox activity (Table 2) were estimated based on the ratio of the SA^{max} (maximal specific activity in tests outside of the reactor) and the SA (actual specific activity in the reactor). Rates can be found in the overview table in the online supplemented materials. During the entire run there was an overcapacity of the anammox activity. A smaller AOB overcapacity was observed and NOB were present in the biomass, but barely active during continuous operation of the reactor. This also led to large NOB overcapacity values. A visual representation of the granules can be seen in Figures 3 and 4.

For municipal mainstream application of an anammox-based technology, it is important that anammox bacteria are retained and remain active within the system. If the actual activity (SA_{amx} in the reactor during normal operation) was compared to the maximal activity in batch tests in the laboratory (SA^{max}_{AMX}), the anammox overcapacity was fluctuating, but always present. Directly after inoculation the anammox overcapacity was the highest, after prolonged cultivation overcapacity decreased, due to a decrease in SA^{max}_{AMX} in time. The nitrogen removal rates of the biomass in the reactor were not limited by the anammox capacity, but limited by nitrite production since only low concentrations of nitrite were detected. In nitrification batch tests, at high oxygen concentrations (>5 mg L^{-1}), the nitrogen mass-balance closed based on ammonium, nitrite and nitrate, so there was no anammox activity at these oxygen concentrations. Based on the ex situ batch tests at different temperatures, an anammox temperature dependency was estimated: an increase of

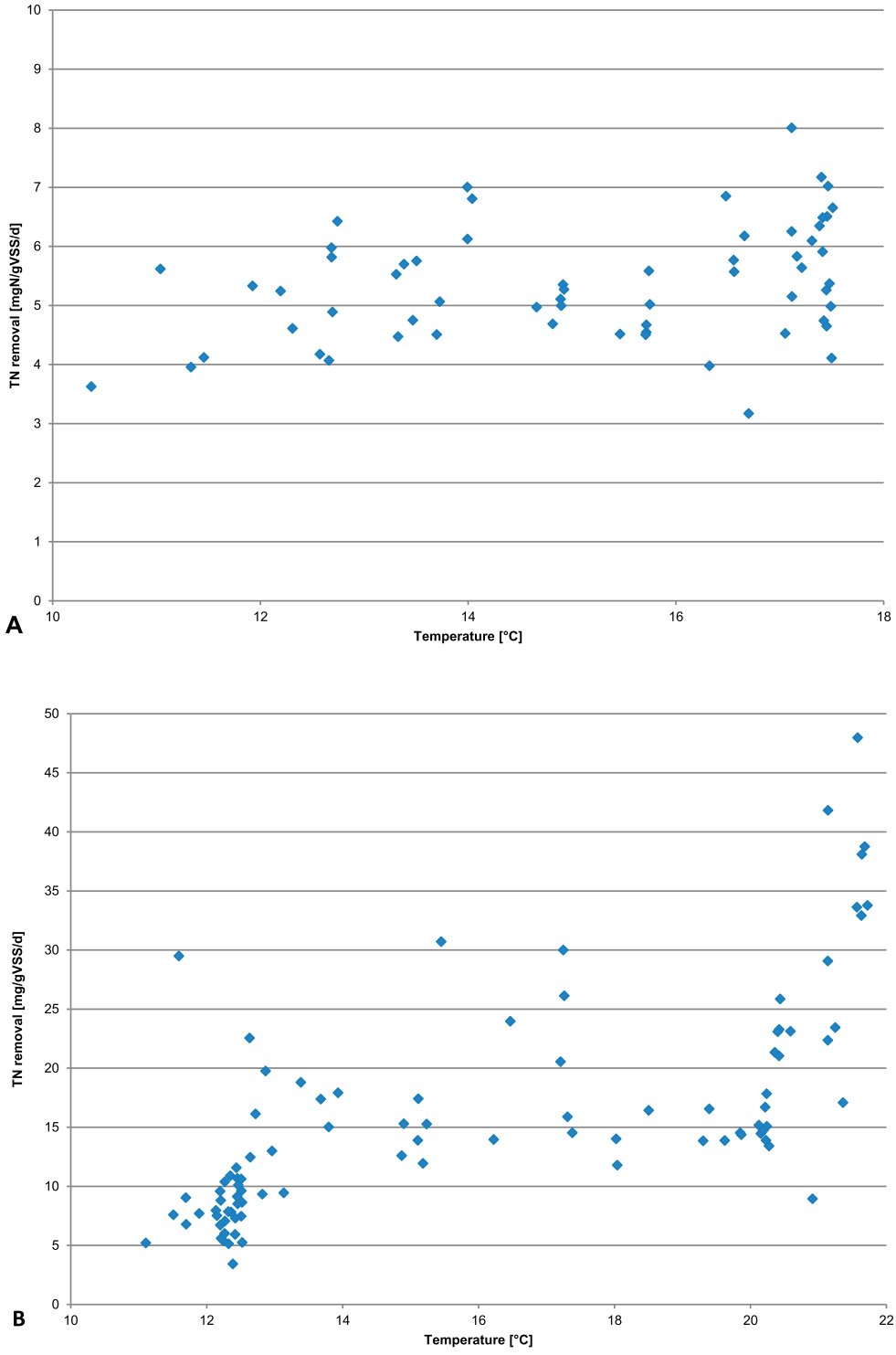

Figure 2. Effect of temperature on the total nitrogen removal, in A: period 3 and B: period 4. Average BOD/N ratio of period 3 was 1.2 ± 0.4 and 2.5 ± 1.8 for period 4.

5°C (15–20°C) increased the SA_{AMX}^{max} with a factor 2 and an activation energy of 96 kJ mol^{-1} (12–24.5°C).

There was a large difference between the SA_{NOB} and the SA_{NOB}^{max}, this indicates that NOB was successfully suppressed in the reactor (Table 3). In batch tests, with a surplus of oxygen, ammonium and nitrite present, the sludge could be fully nitrifying. But in the pilot reactor, with low DO concentrations and anammox bacteria competing for nitrite, aerobic nitrite oxidation was minimal. The effect of DO on the suppression NOB activity in

Table 2. Ratio of maximum activity (in ex situ tests) and activity in reactor, values are based on biomass-specific rates.

Period	Number of days after inoculation	Anammox	AOB	NOB
1	1	4.06	6.06	89.30
2	22	2.76	1.64	21.99
3	6	16.47	3.43	$SA_{NOB}^{max} = 0$
4	3	12.52	2.39	8.49

the pilot reactor is illustrated in Figure 5. High DO concentrations were present during a short period of time. At these high oxygen concentrations, the reactor was fully nitrifying, but after lowering the DO the anammox pathway became active again.

As in the case of the anammox bacteria activity, there was always an overcapacity of AOB, although this overcapacity was fluctuating. The AOB activity was the limiting process step in the reactor, not due to maximum AOB capacity, but due to oxygen limitation.

3.3. Long-term stability

During different reactor runs, the long-term stability was a point of concern. The longest period of stable performance that was achieved was six months. Some perturbations were caused by clear technical problems in the pilot-plant or the full-scale A-stage reactor, but there have also been periods without evident technical problems in which the nitrogen removal rate would eventually decrease. Decrease in nitrogen removal rates was in some cases due to an increase of NOB or a decrease in AOB activity. As it was difficult to get clear what caused the observed losses of process stability, the process was reseeded with biomass several times so that the focus could be on nitrogen removal via the anammox route and suppression of NOB activity. Once more knowledge and experience are gained in this area, research/demonstration focus can switch to long-term stability, but this could not be done in this pilot research yet.

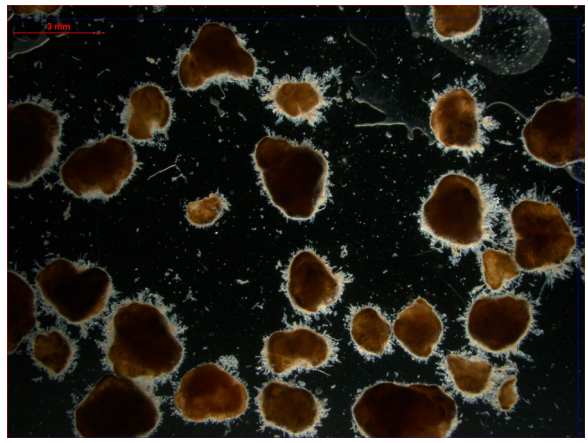

Figure 4. Granules after 120 days in the reactor in period 4.

It was observed that during periods with instable A-stage performance the conversions decreased, probably due to high BOD in the incoming water leading to growth of heterotrophic bacteria and protozoa. An incidental peak load of BOD and particulates from the A-stage caused no problem for the conversions in the reactor, but long-term exposure to these conditions led to a decrease in nitrogen conversions. In the online supplemented materials, a figure can be found showing the correlation between the increase of the nitrogen load to the B-stage (an indication for an instable A-stage) and the decrease in nitrogen removal in the mainstream anammox reactor.

3.4. Biomass composition

The microbial community within the biomass was analysed by sequencing samples at the beginning and end of period 2, the end of period 3 and at the beginning and end of period 4. The observed species related to autotrophic nitrogen removal are shown in Table 4. The dominant anammox species identified in the reactor was always *Candidatus Brocadia*, in period 3 an

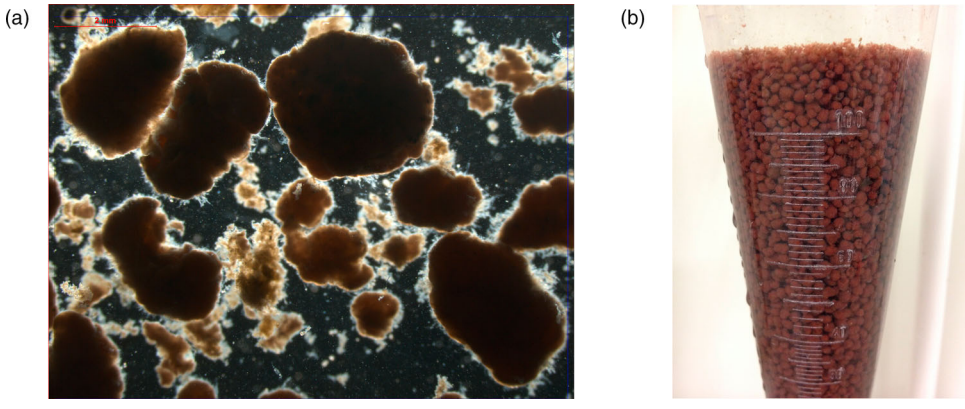

Figure 3. Granules in the inoculum of the PN/A reactor at the beginning of period 2.

Table 3. Average specific NOB activity for all four periods SA_{NOB} in the reactor and SA_{NOB}^{max} in the ex situ batch test.

Period	SA_{NOB} mgN (gVSS d)$^{-1}$	SA_{NOB}^{max} mgN (gVSS d)$^{-1}$
1	3.6 ± 3.0	35.8 ± 12.1
2	3.2 ± 2.8	41.5 ± 6.1
3	0.2 ± 01	10.4 ± 6.0
4	0.4 ± 0.4	33.6 ± 27.6

Table 4. Sequencing results with the relative abundance on the genus level of the organisms involved in nitrogen removal.

Period	Number of days after inoculation	AMX Broccadia (%)	AMX Jettenia (%)	AOB (%)	NOB nitrospira (%)
2	22	3.91	0.70	1.46	0.84
	146	1.22	1.13	0.49	0.35
3	201	8.90	#N/A	2.10	1.36
4	2	3.99	0.70	4.02	#N/A
	202	1.27	#N/A	1.98	2.70

uncultured *Brocadia* species and in the other periods *Candidatus Brocadia sinica*. In periods 2 and 4 a small fraction of uncultured *Candidatus Jettenia* was present in the reactor. At the beginning of each phase, the reactor was inoculated with fresh sludge. The inoculation sludge of periods 2 and 4 originated from the same full-scale PN/A reactor, this might explain the presence of *Jettenia* and the different types of *Brocadia*. In general no change in types of anammox species was observed after inoculation. In period 3, the relative abundance of anammox bacteria was higher compared to the other two sequenced periods, but the SAA_{amx}^{max} of this sludge was relatively low, which could be due to the presence of a large fraction of inactive (anammox) biomass. The AOB and NOB species present in the sludge were uncultured *Nitrosomonas* and *Nitrospira* species. An overview of the other species identified in the biofilm is presented in the online supplemented material. There was no indication of the presence of a complete nitrifier (comammox bacterium) based on QPCR results [27].

FISH analysis was performed on sliced granules. These results, for the sludge at the end of period 4, can be found in Figure 6. In Figure 6(A) it can be seen that the core of the granule (left bottom) consisted of anammox bacteria, further away from the core was a layer of AOB and on the outside (right top) was a layer of bacteria that did not hybridize with the probe for AOB or anammox bacteria, these are probably heterotrophs. In a second FISH experiment (B) it became clear that the NOB were present in a deeper layer of the biofilm, compared to the AOB (core is in the left top).

An important question on biomass composition is what happens to the heterotrophic biomass, did the autotrophic and heterotrophic biomass experience the same SRT? Or is there an uncoupling of the two SRTs, for instance, due to the growth of heterotrophs in suspension and autotrophs in a biofilm. Based on the removal of BOD and ammonium in the reactor, an

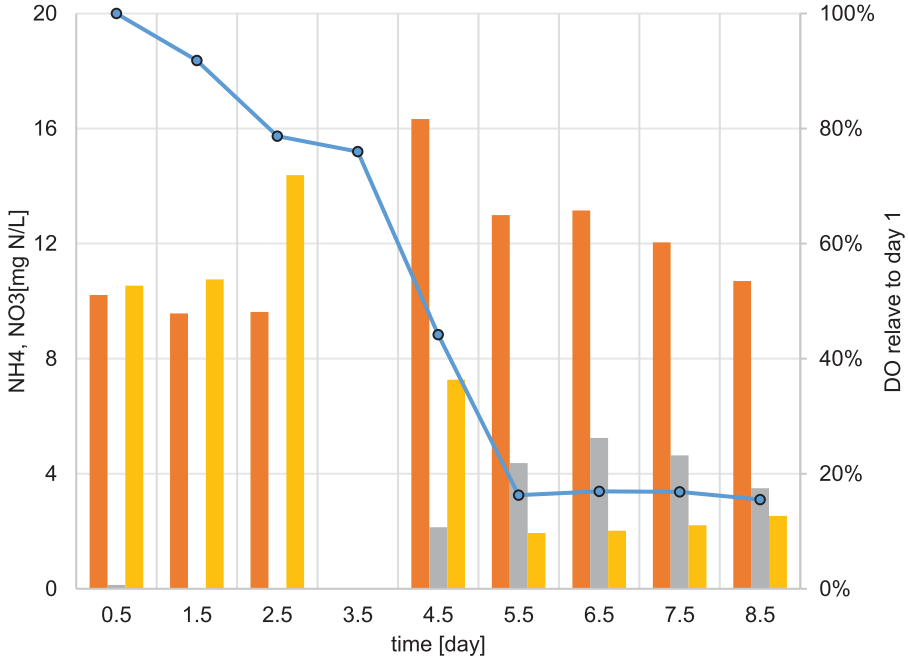

Figure 5. Influent and effluent of the reactor with different oxygen concentrations (data are 24 h averaged values), temperature is constant at 24°C. Influent ammonium in orange (first) bars, effluent ammonium in grey (second) bars, effluent nitrate in yellow (third) bar and relative DO in blue line (colour online only).

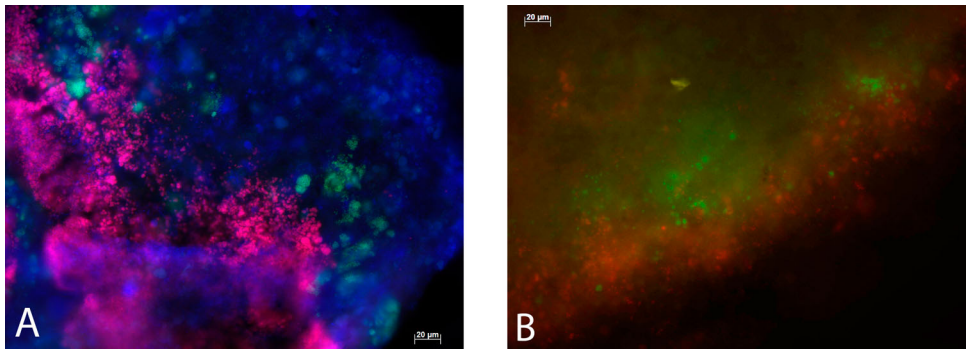

Figure 6. FISH analysis performed on sliced granules at the end of period 4, hybridization was accomplished with A: CY3-red (anammox), Cy5-blue (Eubacteria) and Fluos-green (AOB) labelled probes. The abruption on the right side of the picture is the outside of the granule. B: CY3-red (AOB) and Fluos-green (NOB) labelled probes. Core of the granule is in the left top. Size of the scale bars is 20 µm (colour online only).

Table 5. Biomass yields for anammox bacteria, AOB and heterotrophic bacteria and the corresponding biomass production rate, according to average substrate consumption rates (349 mg BOD $(m^3\ d)^{-1}$ and 138 mgNH_4-N $(m^3\ d)^{-1}$), the conversion factor from BOD to VSS of was 1.4 used. Fifty-five per cent of the converted ammonium was consumed by AOB.

	$Y_{XH/BOD}$ gBOD gBOD^{-1}	$Y_{XA/NH4}$ gBOD gNH_4-N^{-1}	$Rate_X$ mgVSS (L d)$^{-1}$	Community composition %	Reference
AOB		0.15	8.1	4.7	[48]
Anammox bacteria		0.16	7.1	4.1	[49]
Heterotrophs (O_2)	0.63		157.1	91.2	[50]

estimation of the community composition of the grown biomass was made. The average BOD load over the periods described in this paper was 0.349 ± 0.338 kg BOD $(m^3\ d)^{-1}$ and the average ammonium removal rate was 0.151 ± 0.070 kg NH_4-N $(m^3\ d)^{-1}$. If all species in the biomass experienced the same SRT, the system would be strongly dominated by heterotrophs (>90%), as shown in Table 5. From the qualitative evaluation of the FISH analysis (Figure 6), it was estimated that the fraction of autotropic biomass was around 80%, significantly higher than the theoretical 10% if the SRT of heterotrophs and autotrophs would be the same. Another indication of the uncoupling of the SRTs was the amount of biomass in the system. The calculated amount of grown heterotrophic bacteria would be 157 mgVSS $(L\ d)^{-1}$, if this was retained in the system the amount of biomass would have grown with 4.7 gVSS L^{-1} in a month, this is higher than the measured increase in the system. Therefore, it can be concluded that the SRTs of the heterotrophic and autotrophic biomass was uncoupled.

4. Discussion

In this study, a reactor design for a PN/A system with a purely granular biomass was used. During stable process operations, at summer temperatures (23.2 ± 1.3°C) the total nitrogen removal rate was 0.223 ± 0.029 kg N $(m^3\ d)^{-1}$ and at winter temperatures (13.4 ± 1.1°C) the total nitrogen removal rate was 0.097 ± 0.016 kg N $(m^3\ d)^{-1}$. Effluent polishing was not a goal of this research, therefore small amounts of ammonium (5 mgNH_4-N L^{-1}) were present in the effluent. The removal capacities obtained in this study are high compared to conversions reported in literature (at 20°C) for a B-stage (0.210 kg N $(m^3\ d)^{-1}$, [12]), an aerobic granular sludge system (0.17 kg N $(m^3\ d)^{-1}$, [28]) and a conventional activated sludge system (0.1 kg N $(m^3\ d)^{-1}$, [29]). Indicating that a full granular PN/A system could achieve at least comparable volumetric loads to conventional systems.

The different periods with decreasing temperatures showed different effects on the nitrogen removal. In period 3 the decrease in temperature, and therefore decrease in biomass-specific activity, was compensated by an overcapacity of the sludge and no change in nitrate production per ammonium consumed was observed. In period 4 an increase in nitrate production was observed at decreasing temperatures.

4.1. NOB suppression

Different operational strategies to suppress NOB are suggested in the literature for instance the use of: Intermitted aeration, [30–32], sequential batch operation [16] and NH_4:DO ratio [33,34]. In the completely mixed tank reactor used in this study, the NH_4:DO was successfully used to suppress the NOB activity, even if the NOB

were present in the granular sludge. Although full suppression was not achieved, the NOB activity could be limited to a fraction of 0.1–0.5 of ammonium conversion. In the different periods, it was shown that nitrate production was observed, but did not show a continuously increasing trend, demonstrating that the NOB activity could be suppressed, thus preventing full nitrification from eventually taking place. In addition, the reversibility of nitrate production by NOB in ex situ tests showed that NOB was present and that full nitrification could take place, but by proper DO control full nitrification was prevented by suppression of the NOB activity.

Biofilm simulations in general suggest that AOB tend to grow more on the outside and NOB deeper in the biofilm [35,36]. This was also confirmed by experimental observations and by FISH images of granules from the pilot reactor [37–39]. This means that NOB will indeed suffer more from oxygen limitation than AOB and can therefore be effectively suppressed by oxygen limitation. Suppression of NOB activity is essential for proper process control, strategies relying solely on population changes will have a delay in control actions of several days/weeks making them less practical.

4.2. Ammonium conversion

The nitrogen removal rates presented in this study are limited by the AOB activity, due to the limits of oxygen mass transfer. Under stable conditions, the AOB conversions in the reactor are lower than the maximal AOB capacity, due to oxygen limitation. In biofilm systems, the concentration of the different substrates needs to be balanced [34]. Increasing the DO could lead to less anoxic volume in the granules and consequently partial inhibition of anammox bacteria. For a good conversion, the nitrite produced in the outer layers of biofilms/granules needs to be converted in the anammox core. The oxygen concentration determines the depth of the aerobic AOB activity layer and the remainder of the biofilm/granule volume is available for anammox conversion. This ensures that DO concentration, biofilm/granule thickness and ammonium loading rates are strongly coupled. An increase in biomass (more granules or a thicker layer of AOB) could increase the AOB conversions.

The average SA_{AMX}^{max} (20°C) during the described reactor run was 70 mgN $(gVSS\ d)^{-1}$. Other values reported in literature are diverse: SA_{AMX}^{max} 68.8 mgN $(gVSS\ d)^{-1}$ (20°C enriched anammox reactor) [40], SA_{amx} 600 mgN $(gVSS\ d)^{-1}$ (20°C enriched anammox reactor) [41], SA_{AMX}^{max} 50 mgN $(gVSS\ d)^{-1}$ (15°C PN/A reactor) [16] and SA_{AMX}^{max} 13.5 mgN $(gTSS\ d)^{-1}$ (20°C PN/A reactor) [42]. The general conclusion is that the order of magnitude of the anammox activity that can be maintained in this system is in a similar range as the reported values in the literature. The contribution of denitrification to the pressure build-up can be excluded since no gas production could be measured after all ammonium was consumed in the first injection.

4.3. Reactor design

There are different methods for implementing PN/A technology in the mainstream of a municipal wastewater treatment plant [6,43]. The reactor described in this paper was operated as a completely mixed tank under continuous gas mixing. This design was selected for its simplicity, but there is a limitation for optimization of the effluent quality. In a full-scale implementation a plug flow set-up or sequential batch reactor would be chosen, in which optimization for effluent quality is possible while achieving adequate repression of NOB activity by inclusion of a gradient in DO in the plug flow bioreactor.

Due to the low substrate concentration in the influent of the mainstream PN/A reactor, and potential high conversion rates, the HRT of this (kind of) system will always be short (few hours). The low temperatures that mainstream WWTPs operate under during winter, the SRT needed to retain the autotrophic biomass in the reactor will be long. For a reactor experiencing yearly temperature fluctuations like the Dokhaven plant, an SRT of 100 days is needed to obtain an average of 80% nitrogen removal. Combining these two restrictions will lead to a small HRT:SRT ratio, which will result in the need for an efficient autotrophic biomass retention, while obtaining preferential wash out of the inevitably formed heterotrophic biomass.

One of the main challenges will always be separating granules from the effluent. Several options have been proposed in the literature, like cyclones or tilted plate clarifiers. Their practical operation aspects at a large scale are still to be evaluated. This system for biomass (as biofilm) retention will be integrated into the PN/A reactor. The existing/secondary clarifiers will be used for the removal of heterotrophic floccular biomass.

One of the risks using granular sludge is trapping of the granules in sludge flocs that leave these high-rate separation systems together with the effluent. Especially when a preceding A-stage gets disturbed, a significant amount of sludge can wash out of the intermediate clarifier in the PN/A-stage, leading to floc/granule separation problems. Inadequate retention of granular biomass results in a decrease of biofilm surface area, limiting the conversions required. This problem can be avoided when mobile or fixed carriers are used instead of granular biomass, since they are easier to retain in the reactor. The disadvantage

of carrier material is the limitation of the surface, therefore higher DO concentrations (energy use) or bigger reactors, compared to the granular system, will be needed.

4.4. Impact of BOD

Independent of the reactor design chosen and the optimized process control, the PN/A system will always be dependent on the treatment performance of the BOD removing step. In combination with a good BOD removing step before the PN/A process, this technology can significantly improve the wastewater treatment plant [3,4,44]. By decreasing the size of the reactors (due to higher volumetric loading rates) and energy consumption of the entire plant.

During the operation of the reactor at the Dokhaven treatment plant, it was observed that the conversion rates of the PN/A reactor decreased when the A-stage was instable. The incoming BOD was most likely consumed by heterotrophs. This layer of heterotrophs could have multiple negative effects. Firstly, the heterotrophic layer could act as an additional diffusion layer that increases the external mass transfer [45]. Secondly, the heterotrophic growth could consume so much oxygen that the AOB becomes oxygen limited. And finally, it is possible that the heterotrophic layer changes the stratification in the biofilm and will therefore make the suppression of NOB more difficult. If the AOB and NOB grow in a mixed biofilm and not in a stratified biofilm (in which NOB grow deeper in the biofilm compared to AOB) it is hard to suppress NOB due to substrate limitation. Particulates in the influent of the PN/A reactor caused protozoa blooms in the sludge. The heterotrophic biomass and protozoa were observed as a fluffy growth on the outside of the granules.

A-stage instability was often correlated with rain weather conditions leading to large hydraulic loading of the A-stage. Uncoupling rainwater from sewage water within the sewage system, will therefore simplify the implementation of the PN/A technology for domestic wastewater treatment. In a plug flow reactor design, the negative impact of BOD in the influent on the PN/A process can be minimized. When the BOD to ammonium ratio is elevated, high aeration levels in the first part of the reactor could be introduced, oxidizing all the BOD quickly, to avoid oxygen competition between heterotrophs and AOB.

Based on the possible energy gain as the main driver for implementing PN/A technology, we strongly suggest that implementation of the PN/A technology to a wastewater treatment plant is combined with the optimization of the BOD removing step, prior to the PN/A reactor. The vulnerable floc structure in the A-stage might lead to limitations in A-stage optimization [2]. The decrease in temperature of the sewage might lead to a decrease in activity of the A-stage, which will lead to an increase of incoming BOD for the PN/A system during winter [46]. Possibilities are the use of membranes, sieves or sand filters. The use of a membrane for biomass separation in the A-stage might lead to problems with clogging and fouling at municipal mainstream wastewater temperatures [47]. A combination of two techniques might be a solution, for instance, an A-stage with a settler and subsequently a sand filter.

The FISH and sequencing data show different results in the abundance of autotrophic nitrogen converting organisms. Estimations based on FISH results indicate that 80% of the biomass was composed of AOB, NOB or anammox bacteria. In the sequencing results less than 15% of the counts are related to nitrogen conversions. This difference is probably caused by limitations in DNA extraction from the biofilm.

4.5. Outlook

We propose that the next step in the development of a municipal mainstream PN/A process focusses on the long-term stability. In the research described in this publication, the stability in the long-term is insufficient. The cause of the decrease of the nitrogen removal rate cannot always be identified. It is possible that it is related to the competition between AOB, NOB and anammox bacteria, but also the bloom of heterotrophic bacteria and protozoa may have played an important role.

The PN/A technology will not be implemented in the wastewater treatment plant of Dokhaven immediately, the current B-stage with the recycling for denitrification is sufficient to achieve effluent standards of 20 mg N L^{-1}. Future research will focus on the resilience of the system and a proof of principle regarding the effluent quality, based on a different reactor design. If the Dokhaven treatment plant is required to generate a better effluent quality in the future, the (partial) adaptation of the B-stage to PN/A reactor is a viable and good option.

5. Conclusion

During stable process operations, high nitrogen removal rates were obtained. Suppression of NOB activity was successfully achieved at the complete temperature range of municipal mainstream wastewater based on DO concentration. Overcapacity of AMX was always present. The AOB activity was the limiting process step. Long-term stability will be a point for future focus, especially the stability of the BOD removing step

preceding the PN/A reactor. The incoming BOD in the reactor will lead to growth of heterotrophic bacteria and protozoa.

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