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Abstract

This study compared two anammox sequencing batch reactors (SBR) for one year. $SBR_{constantT}$ was kept at 30°C while temperature in $SBR_{loweringT}$ was decreased step-wise from 30°C to 20°C and 15°C followed by over 140 days at 12.5°C and 10°C. High retention of anammox bacteria (AnAOB) and minimization of competition with AnAOB were key. 5-L anoxic reactors with the same inoculum were fed synthetic influent containing 25.9 mg NH_4^+-N/L and 34.1 mg NO_2^--N/L (no COD). Specific ammonium removal rates continuously increased in $SBR_{constantT}$, reaching 785 mg NH_4^+-N/gVSS/d, and were maintained in $SBR_{loweringT}$, reaching 82.2 and 91.8 mg NH_4^+-N/gVSS/d at 12.5 and 10°C respectively. AnAOB enrichment (increasing hzsA and 16S rDNA gene concentrations) and adaptation (shift from *Ca. Brocadia* to *Ca. Kuenenia* in $SBR_{loweringT}$) contributed to these high rates. Rapidly settling granules developed, with average diameters of 1.2 ($SBR_{constantT}$) and 1.6 mm ($SBR_{loweringT}$). Results reinforce the potential of anammox for mainstream applications.

Key words: biological nitrogen removal; shortcut nitrogen removal; cold anammox; microbial community structure

1. Introduction

Partial nitritation/anammox (PN/A) is an autotrophic shortcut nitrogen removal process in which, after partial nitritation, Anammox bacteria (AnAOB) perform anoxic oxidation of ammonium with the produced nitrite as electron acceptor. This process requires less oxygen, abandons the need for organic carbon, and yields a lower sludge production. It can thus present a more cost-efficient treatment compared to the conventional nitrification/denitrification process (Vlaeminck et al. 2012). Many advancements have been made and by the end of 2013, close to one hundred full scale side-stream anammox installations, usually treating digestate, landfill leachate or reject water, have seen the day (Lackner et al. 2014, Mulder et al. 1995, Siegrist et al. 2008, Strous et al. 1999). The next development goal is to introduce anammox in the mainstream or waterline of sewage treatment plants in order to further improve their efficiency in terms of energy consumption (and hence economics) and greenhouse gas emissions.

The main challenges for PN/A implementation on pretreated sewage, so-called mainstream PN/A, are associated with the development of robust methods to suppress nitrite oxidizing bacteria (NOB) and promote the growth and activity of AnAOB under relatively low influent nitrogen concentrations (40-80 mg NH_4^+-N/L) and non-negligible amounts of biodegradable organic carbon (which allow ordinary heterotrophic organisms, OHO, to develop and compete for nitrite through denitrification). Furthermore, and of particular interest for regions with a temperate (or cold) climate, relatively low sewage temperatures (below 15°C

down to 10°C, or even below) drastically decrease AnAOB growth rates and activity (Cao et al. 2017, Vlaeminck et al. 2012). Their optimal operating temperature has been reported around 35°C - 40°C (Strous et al., 1999, Isaka et al. 2008).

Several attempts have been made to understand and model the short term impact of decreasing temperature on the specific ammonia removal rate (SARR) by calculating Arrhenius temperature coefficient (also called θ-value) or Activation Energy (E_a). These studies reported a sharp decrease in anammox activity from 15°C and down (up to 10-fold between 30 and 10°C). Maximum reported SARR values (after conversion from N_2 production to NH_4^+ consumption based on the theoretical stoichiometry) at 10°C did not go beyond 14 mg NH_4^+-N/gVSS/d (Lotti et al. 2014c, Dosta et al. 2008). However, short-term batch tests do not include the impact of adaptation and selection on the temperature sensitivity of the biomass, and are therefore not sufficient to assess the full potential of anammox at lower temperatures.

Several reactor strategies have been tested for anammox at lower temperature, with different types of biomass (flocs, granules or carrier-supported biofilms), and types of wastewater. Adaptation could be demonstrated through a shift in optimal temperature upon quickly or gradually decreasing temperature in Moving Bed Biofilm Reactor (MBBR) (Gilbert et al. 2014, Gustavsson et al. 2014) and Sequencing Batch Reactor (SBR) systems (Gilbert et al. 2015, Hu et al. 2013). The formation, enrichment and maintenance of anammox granules at

lower temperatures has been reported on both synthetic wastewater at 10°C (Hendrickx et al. 2014, Lotti et al. 2014a) and real wastewater at 16°C (Ma et al. 2013) and 10°C (Lotti et al. 2014b). In another study where hybrid sludge (flocs + wall attached biofilm) was formed, decreasing temperature caused the SARR to drop tenfold, but the system's performance remained stable at 12°C (Laureni et al. 2015). Stable P/NA performance was also observed when decreasing the temperature to 15°C in 2 MBBRs, however a sudden drop in temperature caused a significant yet reversible drop in anammox activity (Laureni et al. 2016). Interestingly, *Brocadia* was the dominant anammox genus in all of the abovementioned studies throughout the adaptation to and operation at low temperature. It should be mentioned that all those studies at low temperature were perfomed in the presence of oxygen and/or organic carbon and the temperature decrease was often abrupt (big steps) and/or little time (days to a couple of months) was given to renew the slow growing AnAOB. To our knowledge, no study has been done under pure anoxic autotrophic conditions with granular sludge at low temperature.

The objective of this study was to reveal the maximum potential of so-called 'cold anammox' (for temperatures below 15°C), also monitoring potential change in the microbiological structure and the physical properties of granular sludge. In order to achieve an adapted and enriched anammox community, a long-term reactor experiment was designed in which the microbial competition was limited through using anoxic reactors, avoiding AOB and NOB activity, in the absence of organic carbon, avoiding OHO. To do so, an anammox

sequencing batch reactor (SBR) operated at high temperature (30°C) was compared to one subjected to subsequent temperature drops for almost 1 year. The impact of lowering the operational temperature from 30 over 20, 15 and 12.5 to 10°C on nitrogen removal performance and factors linked to biomass enrichment/adaptation and morphology was examined.

2 Material and Methods

2.1 Set-up and operation of the reactors

Two anammox SBR were operated in parallel, composed of identical, airtight, mixed, and jacketed vessels (5L), equipped with pH (H 8481 HD, SI Analytics), temperature and dissolved oxygen (DO) probes (VisifermTM, Hamilton). The SBR mode is comprised of four characteristic phases: (1) filling phase (30 minutes, inflow of 2.5L influent, hence a volumetric exchange ratio, VER, of 50%), during which oxygen was removed from the liquid through stripping with N_2 gas. For the next 30 minutes, the reactors were flushed with a mixture of N_2 and CO_2 gas to ensure anoxic conditions and set the pH back to 7.5 before starting the actual (2) reaction phase with a variable duration (between 4h and 11.3h). The length of the reaction phase was shortened (lengthened) to increase (decrease) the ammonium loading rate (ALR) when needed. After the reaction phase, mixing was stopped for 30 minutes during the (3) settling phase before starting the (4) discharge phase where 2.5L of supernatant was removed before starting the next cycle. Those operating conditions led to maintain in the system particles that have a settling velocity higher than 0.29 m/h.

One reactor, $SBR_{constantT}$, was kept at the reference temperature of 30°C throughout the entire experiment while in the second reactor, $SBR_{loweringT}$, a stepwise decrease from 30°C to 20°C, 15°C, 12.5°C and finally 10°C was imposed corresponding to phases I, II, III, IV and V respectively. The decrease of temperature was deliberately slow to let the time for the microbial community to adapt to new conditions. More specifically the temperature pattern was chosen according to the following philosophy:

- Step by step, each step being maintained for at least 2 months, until the Ammonium Removal Efficiency (ARE) recovered a value of 100%, and the Volumetric Ammonium Removal Rate (VARR), i.e. the anammox conversion rate, reached steady state value;
- The temperature delta was not conservative, but progressively smaller (10°C, 5°C, 2.5°C, 2.5°C), assuming that the last temperature steps would be more detrimental to the anammox bacteria considering previous litterature indicating a higher activation constant at low temperature;
- The rate of temperature decrease was linear with a slope of 2°C/day, 2°C/day, 1°C/day, 0.5°C/day for the last, again expecting that the last temperature step would be more critical for anammox bacteria.

Finally the decrease from 30 to 10°C took 7 months. Calculations were made in order to evaluate if this time was sufficiently long to allow the renewal of most of the biomass initially contained in the reactor considering the AnAOB biomass yield and imposed nitrogen load. These calculations are given in the discussion.

The reactors were fed with the same synthetic influent which was stored at 4°C in a 150L tank. The influent contained per liter 168.0 mg of $NaNO_2$, 98.8 mg of NH_4Cl, 165.0 mg of $MgSO_4 \cdot 7H_2O$, 143.8 mg of $CaCl_2 \cdot 2H_2O$, 6.3 mg of EDTA-$Na_2 \cdot 2H_2O$, 775.4 mg of $NaHCO_3$, 30.0 mg of KH_2PO_4, and 9.1 mg of $FeSO_4 \cdot 7H_2O$. Hence, the total nitrogen content was 60 mg N/L, a typical value for municipal wastewater (Metcalf 2003), made up of ammonium and nitrite in a 1/1.32 ratio correspondig to the stochiometry found by (Strous et al. 1998): 25.9 mg N-NH_4^+/L and 34.1 mg N-NO_2^-/L. Per liter of influent, 2 mL of a trace element solution was added, which contained per liter 430 mg of $ZnSO_4 \cdot 7H_2O$, 240 mg of $CoCl_2 \cdot 6H_2O$, 1.21g of $MnCl_2 \cdot 4H_2O$, 250 mg of $CuSO_4 \cdot 5H_2O$, 183 mg of $Na_2MoO_4 \cdot 2H_2O$, 274.6 mg of $NiSO_4 \cdot 7H_2O$, 50.1 mg of $Na_2SeO_3 \cdot 5H_2O$, 60.9 mg of $Na_2WO4 \cdot 2H_2O$, 14 mg of H_3BO_3 and 15g of EDTA-$Na_2 \cdot 2H_2O$.

2.2 Biomass inoculum mix

To provide a high microbial AnAOB diversity, four different non cold-adapted biomasses from stable, long running systems containing AnAOB and operated at rather high temperature (26-30°C) were mixed. Half of the biomass types originated from smaller lab-scale set-ups treating synthetic influent, the others originated from full or pilot scale systems treating centrate from a sewage sludge digester. Three of the systems were PN/A (aerobically operated), the fourth system was anammox (anoxic). The resulting inoculated biomass concentration was 2.3 g VSS/L.

2.3 Anammox activity and chemical analyses

The actual ARE were determined by measuring outlet concentrations of ammonium roughly every two days. Comparable information was obtained from nitrite measurements.

The systems being operated in sequencing batch mode kinetics could be followed directly during the reaction phase of the cycles. The *in situ* VARR was measured two to three times per week. Samples of mixed liquor (8mL) were taken throughout the reaction phase of one cycle. The ammonium uptake rate was calculated by linear regression (no substrate limitation for ammonium concentration higher than 5 mgN/L for which a linear decrease was observed). Roughly every one or two months, mixed liquor samples were taken from the reactors to determine total (TSS) and volatile (VSS) suspended solids concentrations. For biomass sampling a protocol was carefully set up to collect representative samples. During sampling, the agitation speed was increased (from 50 to 100 rpm) for maximal homogenization of the reactor liquid (and at least one minute before sampling). Sampling and VSS measurements were done in duplicate, and the sample volume (2x50 mL) was chosen to obtain good reproducibility. For each phase, an average biomass concentration (g VSS/L) was calculated (two to three measurements) together with the average *in situ* VARR during the last two weeks (three to four measurements). From this, the *in situ* SARR at the end of each phase was estimated. Standard deviations were calculated for average VARR and VSS and propagated in the error bars, allowing for an objective comparison. Student tests (t-tests) were performed to

compare mean SARR values between reactors and between consecutive phases within the same reactor.

All water samples were centrifuged (4°C, 2 minutes at 2,591x*g*) and supernatant was filtered through 0.2 µm membrane filters prior to nitrogen compounds analysis. Nitrite and nitrate were quantified using spectrophotometric methods, analyses were done automatically using the SMARTCHEM200 (AMS, Italy). Ammonium concentration was measured according to the spectrophotometric Nessler method (APHA 1992). Suspended solids (TSS and VSS) were measured in duplicate according to Standard Methods 2540D and E (APHA 1992).

2.4 Particle size distribution of the biomass aggregates

The particle size distribution (PSD) was determined after 354 days. Triplicate samples of mixed liquor were taken, and particles were visualized with a WILD M420x1.25 (optical zoom set at x6.4) stereomicroscope, operated using a NIKON DS-U2 control unit combined with NIS-elements F software (v.3.2). Photos were taken using a NIKON DS-Fi1 digital microscope camera. For each triplicate, 50 photos were taken. The three series of photos were combined to give one set of 150 photos per reactor for image analysis.

The first step of the image treatment consisted of a conversion to greyscale using IrfanView software (v.4.10). Next, using Visilog software (v.6.7), the pixel-scale values were converted by a scaling factor (which was calibrated for the magnification). Each grey-scale image underwent a binarization step after which

the surface of each particle was quantified and used to estimate the sphere-equivalent diameter. The used script was made so that particles touching the edge of the photo or with a diameter smaller than 15 µm would not be detected (the detection limit corresponded to a minimal number of 10 pixels in the image analysis script). The obtained data was then treated in Excel with a visual basic macro to obtain the number, surface and volume weighted PSD along with additional information such as mean diameters.

2.5 Microbial community analyses

Triplicate samples of 1.5 mL mixed liquor were taken from each reactor, one was used for microbial community analyses, and the other two were stored as back-up. This was done on average once every two to three weeks with higher sampling density around each transition between phases. After centrifugation (20 min at 19,000x*g*), supernatant was removed and the remaining biomass was immediately frozen in liquid nitrogen. DNA extraction on one of these samples was performed using the FastDNA spinkit™ from MP biomedical following the provided Fastprep® protocol. The extracted DNA was stored at -20°C untill further use.

In order to map AnAOB enrichment, qPCR was used to quantify the AnAOB based on phylogenetic primers (16S rRNA) and functional primers (hzsA). Also, to follow up the composition of the overall community, MiSeq Illumina sequencing was performed by the GenoToul Genomics and Transcriptomics facility (GeT-PlaGe, Auzeville, France). Sequencing data was processed using the pipeline FROGS, one of the tools proposed on Galaxy, an open web-based platform for genomic research.

3. Results

3.1. Reactor performance

3.1.1. Start-up of the reactors

Both reactors were started with the same seeding sludge and operated identically at 30°C for 62 days to allow for adaptation to the new incubations conditions. During this period, both reactors showed a very comparable evolution both in ARE (Figure 1.b) and VARR, starting out around 48 mg NH_4^+-N/L/d on day 19 and increasing to around 74.4 mg NH_4^+-N/L/d on day 54 (Figure 1.a). Because of this increase in volumetric activity, the duration of the reaction time was decreased twice resulting in an increase ALR: from 40.8 mg NH_4^+-N/L/d to 47.0 mg NH_4^+-N/L/d (on day 24) and later to 55.4 mg NH_4^+-N/L/d (on day 31). While a biomass concentration of 2.3 g VSS/L was initially added, levels of 0.70 and 0.76 g VSS/L, for $SBR_{constantT}$ and $SBR_{loweringT}$ respectively, were retrieved on day 67. This was caused by washout of the smallest aggregates during the first two months of operation, this is illustrated by the increased loss of VSS in the effluent during phase I.

Figure 2 shows the average *in situ* specific ammonium removal rates (SARR) at the end of each operating phase. By the end of the phase I, in which both reactors had been operated identically, they had reached very similar *in situ* SARR (103 and 99 mg NH_4^+-N/gVSS/d for $SBR_{constantT}$ and $SBR_{loweringT}$ respectively). On day 62, temperature in $SBR_{loweringT}$ was decreased to 20°C, whereas from this point on, operating conditions in $SBR_{constantT}$ were left unchanged for the rest of the experiment (T=30°C, ALR = 55.4 mg NH_4^+-N/L/d).

3.1.2. Activity evolution at constant temperature (30°C)

For the control reactor $SBR_{constantT}$, as shown in Figure 1.a, maximal volumetric activity has continuously increased upon inoculation: at the beginning of period II, it increased slightly from 79.2 mg NH_4^+-N/L/d on day 69 to 87.6 mg NH_4^+-N/L/d on day 103. On this day, a leak causing a slight entrance of oxygen (0.05 mg O_2/L) in the headspace was detected. After this leak was repaired, the activity increased even more rapidly to 129.8 mg NH_4^+-N/L/d in only three days. This increase in activity with time in $SBR_{constantT}$ continued almost linearly during the overall study. Finally maximal VARR reached about six times its initial value up to 336 mg NH_4^+/L/d at the end of the experiment. At this high VARR, all of the ammonia and nitrite were consumed during the first 45 minutes of the reaction phase. The VARR was higher than the ALR, translating in a part of the reaction phase in which conditions were endogeneous. The ARE remained close to 100% during the entire running period (Figure 1.b). The average effluent concentrations were 0.6 mg NH_4^+-N/L and 0.1 mgNO_2^-N/L over the entire operating period.

As VSS decreased (roughly by a factor 1.7) during the experiment, the evolution of specific activity is of particular interest: the *in situ* SARR increased by a factor 7.5 between the end of phase I (103 mg NH_4^+-N/gVSS/d) and the end of phase V (785 mg NH_4^+-N/gVSS/d). This suggests enrichment and/or an adaptation of the anammox biomass; this point will be addressed in the discussion section.

3.1.3. Activity evolution at decreasing temperature (30°C to 10°C)

As shown in Figure 1.a, each temperature decrease step in $SBR_{loweringT}$ resulted in a decrease of anammox activity which temporarily affected the removal

efficiency. This effect was most pronounced when switching from 30 to 20°C (beginning of phase II) and from 15°C to 12.5°C (beginning of phase IV), resulting in a 64.8% and 76.7% drop in activity respectively. However, partial recovery after the temperature drop from 30°C to 20°C, activity took 6 weeks, significantly more than the 3 days required for recovery after the drop from 15° to 10°C, which was probably the result of an adaptation and/or enrichment of the biomass during the 146 days between these events (see chapter 3.2). The effects of decreasing temperature from 20°C to 15°C and 12.5°C to 10°C were less significant, as activity loss was lower (36.3% and 28.4% respectively) and activity recovered within the month. Considering the activity measured at the end of each phase, maximal VARR decreased from 76.2 to 41.0 mg NH_4^+-N/L/d for temperature decrease from 30°C to 20°C (phases I and II), but was relatively similar at the end of the next three phases (III, IV, V), reaching 24.0 mg NH_4^+-N/L/d at the end the study. For this reason, it was possible to maintain the nitrogen load constant during the phases II, III, IV and V without impacting that much the ARE, which remained also relatively high during the study, ranging between 57-100% for $SBR_{lowering}$ (Figure 1.b). The average effluent concentrations were 2.7 mg NH_4^+-N/L and 3.3 mgNO_2^--N/L over the entire operating period.

In $SBR_{loweringT}$ the VSS concentration also decreased during the experiment, from 0.76 g VSS/L to 0.24 g VSS/L. When temperature was decreased to 20°C, *in situ* SARR dropped by 38% between the ends of phase I and II. During this time, the biomass concentration decreased from 0.76 g VSS/L to 0.56 g VSS/L.

316 For the next 11 weeks, SARR remained rather stable, reaching 65.6 mg NH_4^+-
317 N/gVSS/d at the end of phase III while biomass concentration decreased from
318 0.56 g VSS/L to 0.39 g VSS/L. Further decreases in temperature affected
319 transiently the SARR but did not negatively impact the final SARR obtained
320 after several weeks at the end of the temperature step. On the contrary SARR
321 increased slightly to 82.2 mg NH_4^+-N/gVSS/d at the end of phase IV and
322 reached 91.8 mg NH_4^+-N/gVSS/d at the end of phase V. This is the highest
323 reported SARR at such low temperature. These results demonstrate a very
324 successful adaptation and operation of the granular sludge anammox process
325 at 10°C.

326 **Figure 1**

327 **Figure 2**

328 Student tests (data not shown) clearly showed that the SARR in phase II, III, IV
329 and V were significantly different between both reactors. The tests showed no
330 significant difference between two consecutive phases in the same reactor.

331 **3.2 Biomass aggregate size and microbial community analysis**

332 *3.2.1 Biomass particle size distribution*

333 On day 1, both reactors contained a dark brownish and very heterogeneous
334 hybrid sludge, made up mainly of small flocs and suspended biomass and a
335 small amount of granules, features linked to the individual constituents of the
336 inoculum (Table 1). From size fractionation of each biomass type (through
337 sieving) and their respective contribution to the inoculum mix, it was estimated
338 that that 59% of the aggregates were smaller than 315 µm (data not shown).
339 During the first 5 months of reactor operation, the biomass evolved to a bright
340 red, predominantly granular sludge. During the last period of operation (phase

V), even at 10°C, the granules demonstrated very good settling properties due to their size and density: To illustrate this, SVI was determined to be 34 mL/g and 26 mL/g for $SBR_{constantT}$ and $SBR_{loweringT}$ respectively on day 323. Futhermore, most of the solids were settled in less than 10 minutes during the settling phase. This corresponds to settling velocities higher than 0.88 m/h. Analysis of the particle size distribution (PSD) was performed on day 354.

$SBR_{loweringT}$ displays a broader PSD, indicating more variation in granule size compared to the narrower PSD of $SBR_{constantT}$, showing a rather uniform granule size. Also, the maximum observed diameter is higher at low temperature, this is also reflected in a bigger volume median diameter: D(0.5) = 1551µm for $SBR_{loweringT}$ vs. D(0.5) 1215µm for $SBR_{constantT}$. Some granules around 3.3-4.4mm were detected at 10°C, but not observed at 30°C. These results showed that lowering the temperature was not detrimental to the granulation process, and can even generate bigger granules. This is likely due to the lower AnAOB growth rates at low temperatures, which resulted in stronger granules with a higher density (as illustrated by their lower SVI of 26 mL/g compared to 34 mL/g at 30°C). Consequently, granules were less susceptible to breakage, explaining why larger diameters were observed at lower temperatures.

3.2.2 Evolutions in the microbial community

16S rRNA sequencing showed that, right after reactor start-up (day 10), AnAOB made up 64.2% and 57.7% of the total community in SBR_{ref} and SBR_{lowT} respectively. By the end of phase I (day 60) the AnAOB abundances had increased to 79.2% and 80.5% respectively, enrichment was taking place. At

365 the end phase IV abundances in $SBR_{constantT}$ and $SBR_{loweringT}$ were only slightly
366 higher, 81.2% and 87.2% respectively, indicating an AnAOB enrichment that
367 mainly occured in phase I. The evolution of the microbial population at genus
368 level was monitored throughout the experiment for both reactors.
369
370 Figure 3 shows the evolution at genus level within the AnAOB community from
371 16S rRNA sequencing data. Both reactors started with the same inoculum,
372 which is reflected in a very similar anammox composition at day 10 with a
373 majority of *Ca. Brocadia* (81%), complemented with minor fractions of *Ca.*
374 *Kuenenia* (8%) and *Ca. Jettenia* (11%).
375
376 As long as both reactors were operated identically (the first 60 days), they
377 showed a comparable evolution in their AnAOB profile: an increase in *Ca.*
378 *Kuenenia* abundance together with a slightly lower decrease in *Ca. Brocadia*
379 abundance. *Ca. Jettenia* abundance dropped dramatically to 1% in both
380 reactors, and, regardless of operational temperature, could not re-establish
381 itself dominantly within the community. The initial changes in the AnAOB
382 community during the first 60 days are likely the result of acclimatization to the
383 imposed reactor operating conditions. Regarding the significant loss of
384 suspended solids observed during the first period, a selection by selective
385 retention during settling was likely to occur. Indeed it was observed that the loss
386 of *Ca. Brocadia* (-15%) and *Ca. Jettenia* (-10%), caused by washout of smaller
387 aggregates, made the relative AnAOB abundance of *Ca. Kuenenia* increase
388 (+25%).

In $SBR_{constantT}$ at 30°C, there was no further major shift. *Ca. Brocadia* remained dominant, and even increased its fraction during Phase II to V, at the expense of a drop in *Ca. Kuenenia* abundance. The population repartition remained roughly unchanged and *Ca. Brocadia* made up 90% of the AnAOB community on day 293 while *Ca. Kuenenia* represented less than 10%.

In contrast, when reducing the operating temperature to 20°C in $SBR_{lowering}$ a remarkable evolution in the AnAOB community occurred: the *Ca. Brocadia* abundance dropped quickly (from 73 to 29%) whilst the *Ca. Kuenenia* abundance increased just as quickly (from 26 to 68%). Further operation at lower temperatures (15, 12.5 and finally 10°C) did not significantly alter the dominance with the AnAOB community, which on day 293 was comprised of 74% of *Ca. Kuenenia* and 26% *Ca. Brocadia*.

Figure 3

In parallel, the AnAOB enrichment in the biomass was assessed by qPCR at three moments in time. Results in Figure 4 indicate a considerable AnAOB enrichment occurring in both reactors, as reflected from the increase in both hzsA and 16S rDNA copy levels. Interestingly, in both systems most of the abundance increase occured in the first half of the reactor operation time, by the end of Phase II, with little further enrichment over the second half. The observed trend was similar for hzsA and 16S rDNA copy levels expressed ‘per g of biomass’ or ‘per ng of DNA’.

Figure 4

4. Discussion

4.1 Enrichment and adaptation favoring high specific activities

Our goal was to investigate the maximum potential of anammox at low temperatures (<15°C). After gradually decreasing from the initial temperature of 30°C in $SBR_{loweringT}$, the activity stabilized at 82.2 and 91.8 mg NH_4^+-N/gVSS/d at 12.5 and 10°C, respectively. The *in situ* SARR in the reference reactor at 30°C ($SBR_{constantT}$) increased continuously, reaching up to 785 mg NH_4^+-N/gVSS/d. Compared to literature, these specific removal rates at 10°C are considerably higher than previously reported values from reactor studies at low temperature, as illustrated in Table 1. Explanations for these differences in SARR are likely linked to differences in enrichment and/or adaptation degree, which is the consequence of a set of factors fixed in the experimental strategy. obtained a slightly higher SARR at after 131 days of operation 12°C compared to this study after 63 days at the same temperature. The final SARR obtained in this study after adaptation to 10°C was the same as the SARR obtained by Hu *et al.* at 12°C and significantly higher than the SARR obtained at 10°C without adaptation in that same study (Hu et al. 2013).

Table 1

Maximizing space for AnAOB in the biomass is a key factor in both enrichment and adaptation, and yielded from low competition with AnAOB and high retention of AnAOB. Feeding with an autotrophic influent in anoxic conditions indeed minimized competition with AOB, NOB and OHO, the average ratio of nitrite to ammonium removal was 1.27±0.19 and 1.20±0.11 for $SBR_{constantT}$ and $SBR_{loweringT}$ respectively, which was close to the stoichiometry reported by Strous (Strous et al. 1998). Note that, the systems in other studies contained

COD or oxygen (Table 1). Maximizing biomass retention enables the slow(est) growing AnAOB to stay or develop in the system. In our study, this was implemented by imposing a low minimum settling velocity, keeping solids settling at > 0.3 m/h into the system. In both reactors, regardless of operational temperature, the bacteria aggregated in rapidly settling granules, which facilitated biomass retention. Nonetheless, biomass retention was not perfect, and sampling and washout renewed an estimated 70 and 100% of the produced AnAOB biomass in $SBR_{constantT}$ and $SBR_{lowering}$ respectively, based on the nitrogen loading and theoretical growth yield. Anoxic SRT values of 162 and 164 days, for $SBR_{constantT}$ and $SBR_{loweringT}$ respectively, were obtained, from the estimated solids balance. In other words, operating the systems for more than two times the SRT should in theory replace more than 85% of the original biomass.

Enrichment in both systems was witnessed by the increase in relative AnAOB abundance in the biomass (qPCR data; Figure 4) in both reactors. According to the qPCR data, most of the enrichment occurred in the first half of the experiment. In $SBR_{loweringT}$, adaptation was observed at the level of the AnAOB community, with a major shift in dominant AnAOB genus from *Ca. Brocadia* to *Ca. Kuenenia* (16S rRNA gene sequence amplicons; Figure 3). Even though it was not studied here, physiological adaptations within a given AnAOB species could have occurred. There is for instance evidence that AnAOB are able to alter their lipid membrane composition through ‘addition synthesis’ as a form of homeoviscous adaptation to changes in *in situ* temperature (Rattray et al.

462 2010). Such type of adaptation might also explain the shift in temperature
463 optimum for AnAOB without significant changes in the dominant genus reported
464 by other authors (Gilbert et al. 2014, Hu et al. 2013).
465
466 In order to assess the impact of long-term enrichment and adaptation on the
467 AnAOB performance, a parallel study was performed to test the immediate
468 effect of exposure to low temperatures on the anammox activity, on the four
469 types of biomass used in the inoculum mix. Based on recalculated values from
470 this batch test, the SARR roughly doubled after 67 days of operation at 30°C
471 (end of Phase I; Table 2). After long term operation at 30°C in $SBR_{constantT}$, the
472 SARR was 16 times higher than the activity of the inoculum at 30°C.
473 Comparably, the final SARR at 12.5-10°C in $SBR_{loweringT}$ was 8-15 times higher
474 than the recalculated inoculum rates under these respective temperatures. As a
475 consequence the SARR ratios (low/high temperature) were similar in the short
476 and long term tests: on short term, exposure to 12.5 and 10°C yielded
477 respectively 20 and 13% of the SARR at 30°C, while the respective values were
478 17 and 12% for the long-term reactor test (see Table 2) .
479 **Table 2**
480 **4.2 Potential AnAOB genus niche differentiation**
481 The results point towards a *Ca. Kuenenia* preference to colder temperatures
482 (20°C and below) and a *Ca. Brocadia* preference to 30°C whereas *Ca. Jettenia*
483 was outcompeted in both reactors. It is interesting to note that each system
484 nonetheless maintained both genera, so the niche differentiation or
485 competitional advantage was insufficiently strong to out-compete the one or the
486 other.

In literature *Ca. Kuenenia* has been only observed as predominant in studies at relatively high temperature. A complete replacement of *Ca. Brocadia* by *Ca. Kuenenia* was observed in an anoxic anammox MBBR at 38°C (van der Star et al. 2008). The authors suggested that nitrite limitation was the main driver for this transition, based on the hypothesis that *Ca. Kuenenia* would be an affinity (K) strategist and *Ca. Brocadia* a growth rate (r) strategist. This hypothesis was challenged recently when the opposite switch was observed: *Ca. Brocadia* replaced *Ca. Kuenenia* as the dominant genus in the biofilm of two IFAS reactors operated between 24 and 29°C. The authors claimed that in this case, the tolerance towards higher and toxic nitrite levels affected the selection more than their affinity for low substrate levels (Zheng et al. 2016). *Ca. Kuenenia* has a higher reported tolerance towards nitrite than *Ca. Brocadia*: inhibition occurs for concentration of 180 mg/L and 70 mg/L respectively (Schmid et al. 2003). Neither of these hypotheses seems applicable to our study, since nitrite concentrations were too low to cause inhibition (maximum 17 mg N/L after feeding) yet were never limiting in any of the reactors (operated with rapid feeding in SBR mode). However, from Phase II onwards, the VARR in $SBR_{constantT}$ was higher than the VALR, meaning that the AnAOB were subjected to endogenous conditions during the final part of the cycle whereas the AnAOB in $SBR_{lowering}$ knew no endogenous period since the VARR was stabilized around the VALR throughout the entire experiment. This presence or absence of an endogenous period may have also played a role in the niche differentiation between *Ca. Brocadia* to *Ca. Kuenenia.*

This is the first time a switch from *Ca. Brocadia* to *Ca. Kuenenia* is observed at low temperature. Almost every other low temperature study, reported that *Ca. Brocadia* was and remained the dominant genus throughout the experiment, suggesting that this genus had a competitive advantage over other anammox genera at low temperature (Gilbert et al. 2015, Hendrickx et al. 2014, Hendrickx et al. 2012, Hu et al. 2013, Laureni et al. 2016, Laureni et al. 2015, Lotti et al. 2014a, Sánchez Guillén et al., 2016). Note that during these studies there was always a presence of oxygen and/or organic carbon. In the study by Sánchez Guillén and colleagues the maximum DO was 0.2%, but even at such low concentration AnAOB activity can be impacted (observed in this study when a leak was detected on day 69). It is interesting to note that in the before mentioned study, average granules size decreased throughout the reactor operation. The fact that granule size increased in this study was probably due to the higher NLR and lower mixing intensity (the SBR reactor in the other study was fitted with baffles which most likely increased the shear force on the granules).Only one study reported on *Ca. Jettenia* as most dominant AnAOB genus in a PN/A Expanded Granular Sludge Bed (EGSB) reactor operated under low DO conditions (0.8 mg/L) to treating COD containing effluent of an upflow anaerobic fixed bed (UAFB) executing anaerobic digestion (AD) (Gao et al. 2015). It is possible that the absence of inhibition (in the case of oxygen) and of competition/partnerships with other bacteria (AOB, NOB and OHO) played a role in the niche differentiation between *Ca. Brocadia* and *Ca. Kuenenia* observed in this study at low temperature. Available data on activation energies

535 (E_a) do not allow to differentiate in temperature sensitivity between the two
536 discussed genera. Two studies working with *Ca. Kuenenia* reported E_a-values
537 of 63 kJ/mol between 10°C-40°C (Dosta et al. 2008) and 93 kJ/mol between
538 6°C and 22°C (Isaka et al. 2008), these values fall within the same range as the
539 ones obtained for *Ca. Brocadia* (Hendrickx et al. 2014, Lotti et al. 2014c).
540
541 Further research should focus on understanding the influence key drivers such
542 as high/low affinity for substrate, sensitivity to low temperature and/or to
543 inhibitors etc. have on the niche differentiation between different AnAOB genera
544 and if/how this affects process kinetics/stoechiometry.

545 **4.3 Towards implementation of partial nitritation/anammox**

546 The high activities obtained at low temperatures in this study reinforces the
547 potential of anammox for mainstream application. A key strategy to obtain
548 enrichment and adaptation was to eliminate competition and maximize retention
549 while applying a step-wise decrease in temperature. Process design and
550 operation will define what activity level can be reached and should therefore be
551 aimed at maximizing the abundance of adapted AnAOB in the biomass.
552
553 In the end, the high maximal potential AnAOB activities under low temperatures
554 provide a basis for to conceive new PN/A design solutions and associated
555 operational strategies. If 50% of the activity at 10°C could be maintained in a
556 full-scale process with a solids content of 3 g VSS/L, it would work at 135 g
557 NH_4^+-N/m^3/d which corresponds to a hydraulic retention time of about 8 hours,
558 this is a conventional value for activated sludge treating systems domestic

559 sewage. Given the nitrite feeding and anoxic conditions in this study, a two-
560 stage PN/A approach seems to be suitable to go as close as possible to the full
561 potential of an enriched/adapted community, as it avoids the competition with
562 nitrifiers, aerobic heterotrophs and with part of the anoxic heterotrophs.
563 Selective AnAOB retention and enrichment can also be implemented in a one-
564 stage approach, by selectively increasing the SRT of AnAOB compared to the
565 SRT of the competitors, like in IFAS PN/A systems (Veuillet et al. 2014) or
566 through the use of a selective screen (Han et al. 2016). Future work should
567 verify whether equally high potential rates can be obtained when making a
568 faster and seasonally realistic transition from higher to lower temperatures. Five
569 year temperature gradient data from a WWTP in Germany showed a
570 temperature decrease from 20°C (summer) to 10°C (winter) at a rate of 2°C per
571 month. During the winter temperature could acutely drop down to 7°C for a
572 short period of time (weeks) (Gilbert et al. 2014). In addition, more research
573 should be dedicated to determine any influence of low temperatures on the
574 growth yield. One study in a PN/A SBR estimated that the growth yield lowered
575 with decreasing temperature (Hu et al. 2013) while in another anammox
576 granular MBBR/SBR study no such impact was observed (Hendrickx et al.
577 2014).

578 **5. Conclusions**

579 Minimal competition and high AnAOB retention resulted in unprecedented
580 removal rates of 82 and 92 mg NH_4^+-N/g VSS/d at 12.5 and 10°C respectively.
581 AnAOB enrichment (indicated by increasing hzsA and 16S rRNA gene
582 concentrations) and adaptation at genus level (indicated by a shift from *Ca.*

583 *Brocadia* to *Ca. Kuenenia* in $SBR_{loweringT}$) contributed to this performance. Well
584 settling granules were formed and maintained at both 30°C and 10°C, indicating
585 that lowering temperature is not detrimental to granulation and can even
586 increase granule size. These results provide new insights that reinforce the
587 potential of cold anammox applications for mainstream N-removal.
588
589 E-supplementary data of this work can be found in online version of the paper

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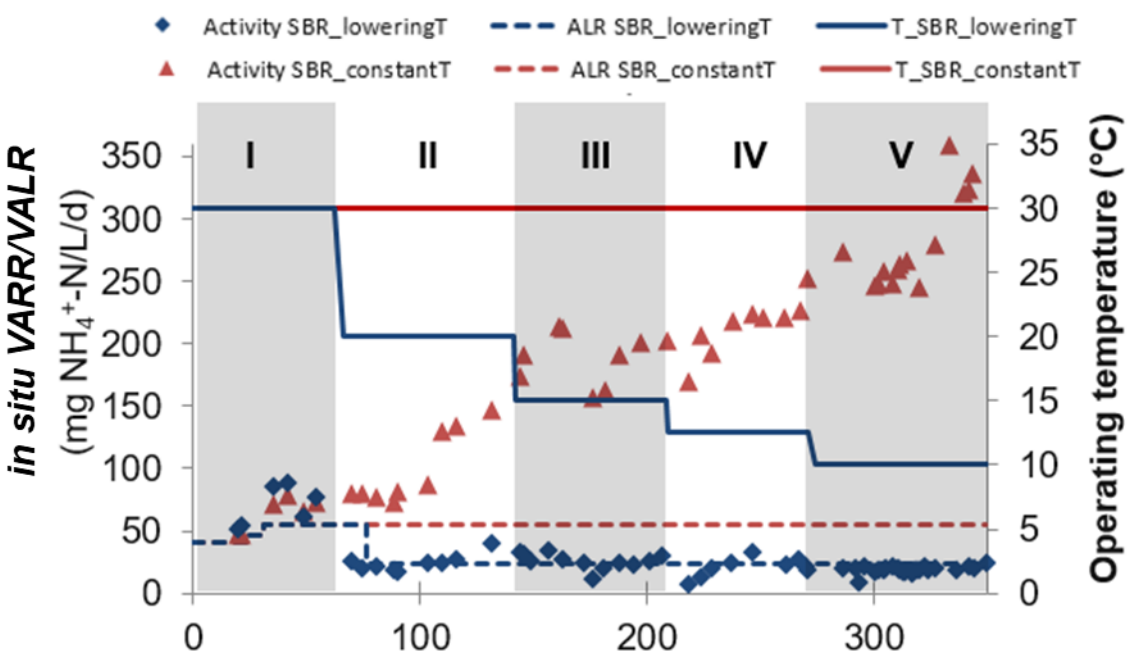
(a)



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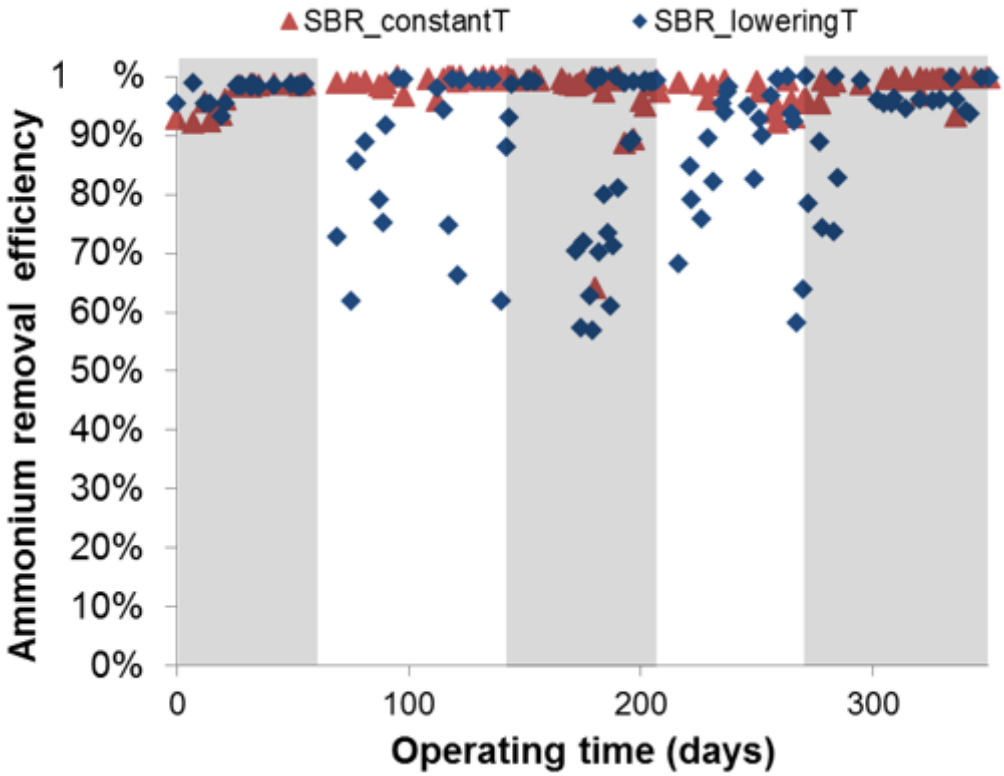
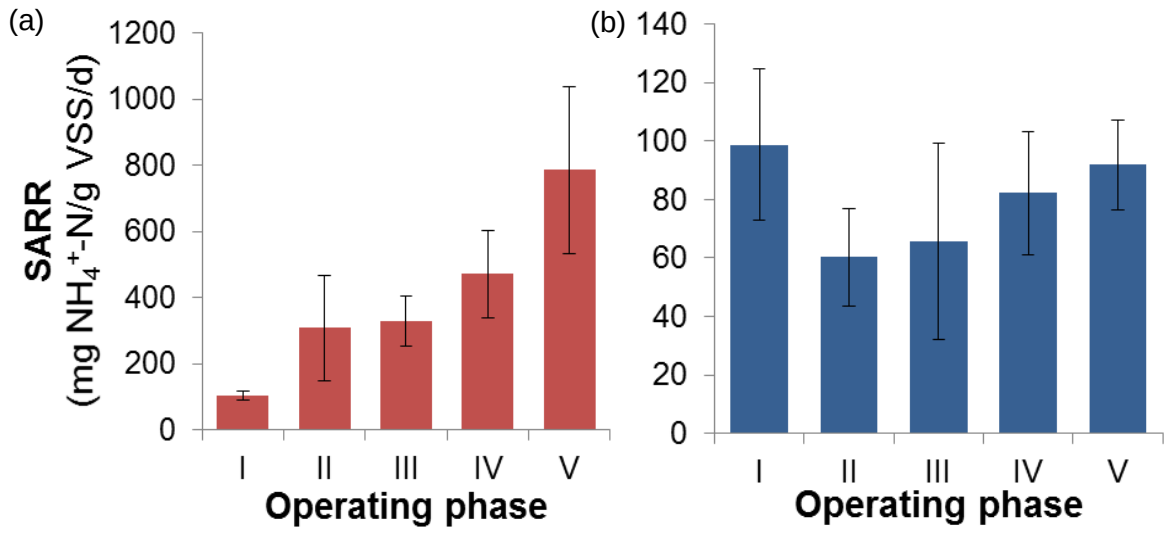


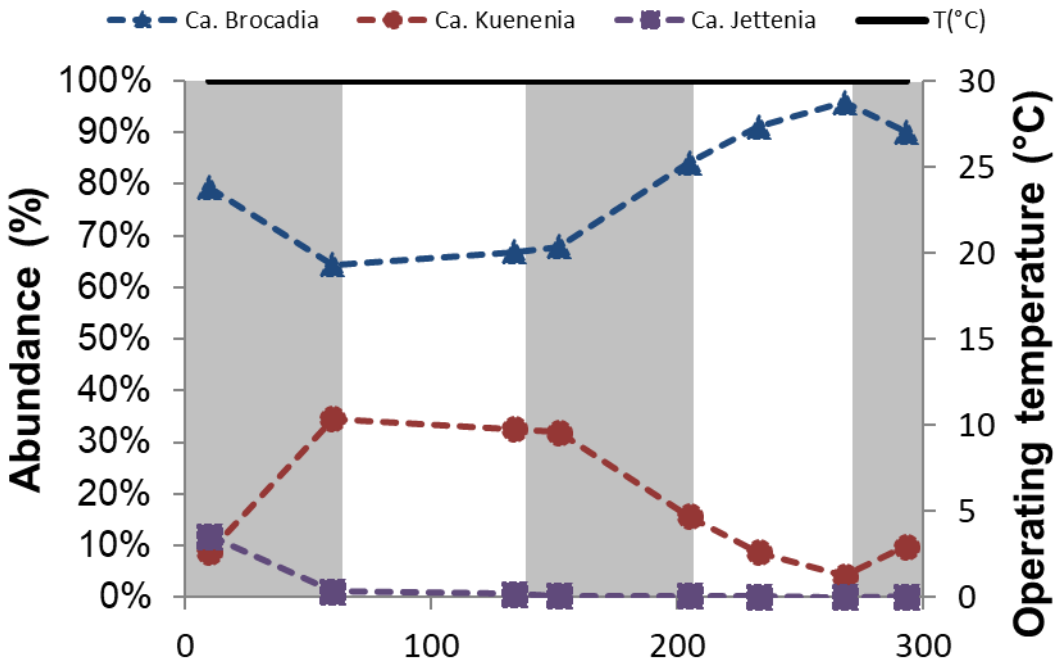
710 **Figure 1** – Evolution of the operating temperature, *in situ* volumetric ammonium removal rate and
711 volumetric ammonium loading rate (a) and ammonium removal efficiency (b) in $SBR_{constantT}$ (red) and
712 $SBR_{loweringT}$ (blue).
713



714 **Figure 2** – Average *in situ* specific ammonium removal rates (SARR) at the end of each operating phase
715 for $SBR_{constantT}$ (a) and $SBR_{loweringT}$ (b). The corresponding days at the end of each phase are 62 (I), 141
716 (II), 208 (III), 271 (IV) and 349 (V). Error bars represent standard deviations (n≥3)

717

(a)



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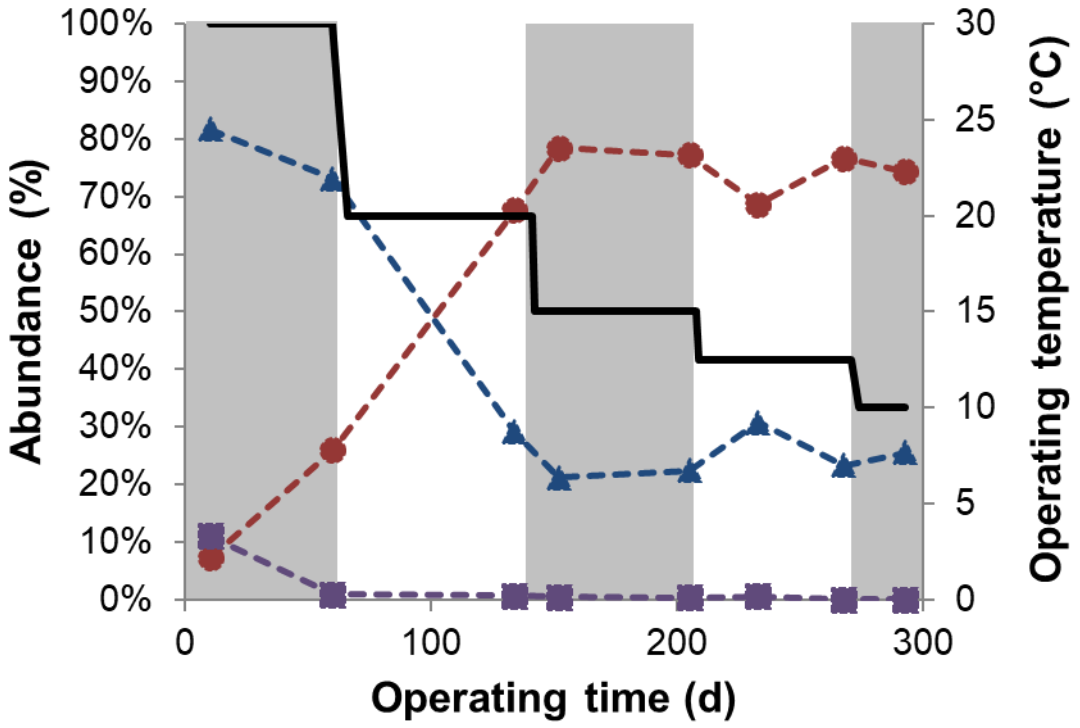
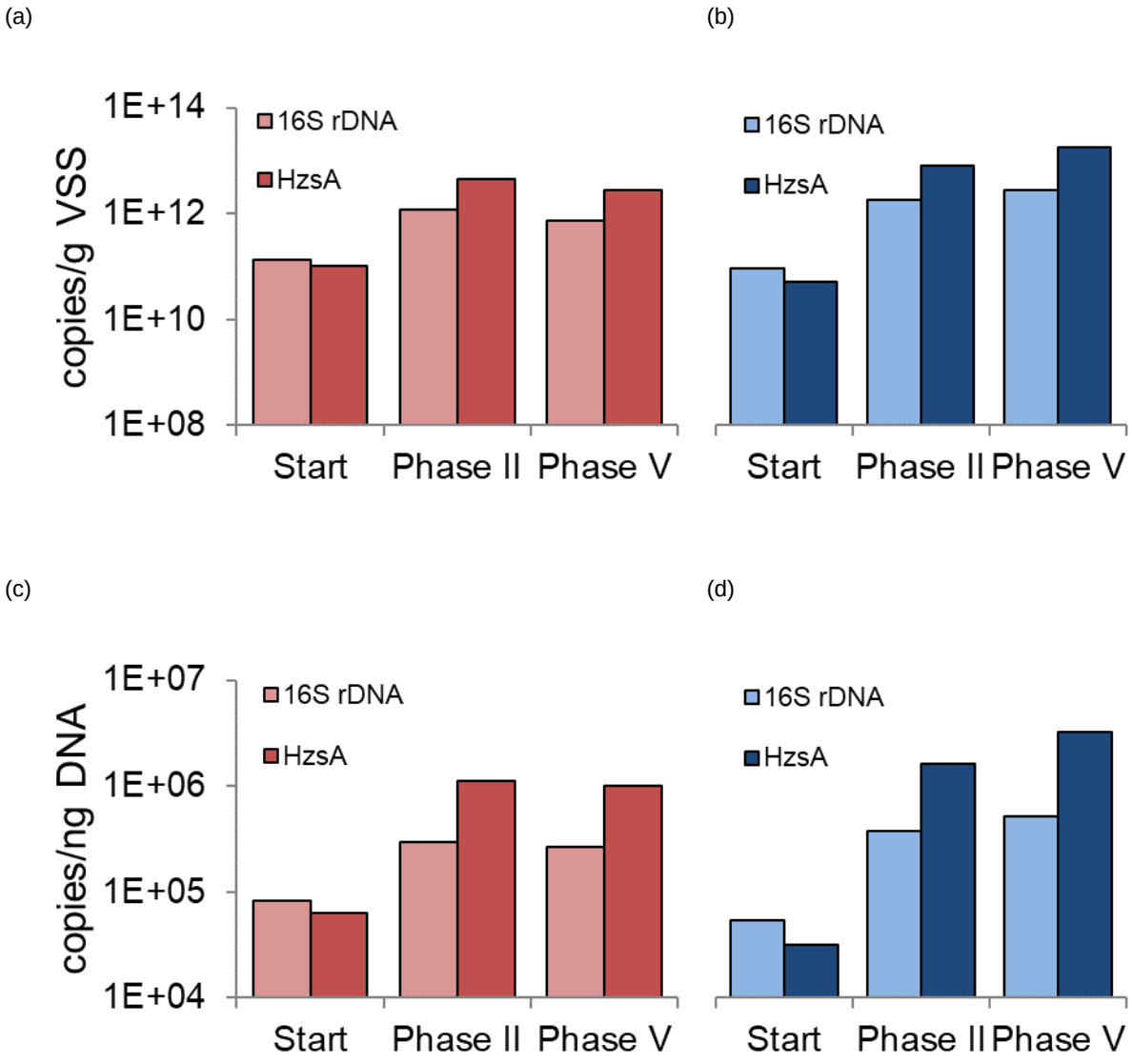


718 **Figure 3 –** Evolutions within the AnAOB community for $SBR_{constantT}$ (a) and $SBR_{loweringT}$ (b): relative
719 abundances of *Ca. Brocadia*, *Ca. Kuenenia* and *Ca. Jettenia* genera, along with the operating
720 temperature.



721 **Figure 4** - Evolution of overall AnAOB abundance as determined by qPCR analyses based on AnAOB
722 16S rDNA (lighter bars) and the HzsA functional gene (darker bars). Top row shows the data expressed
723 per g of VSS for SBRconstantT (a) and SBRloweringT (b). Bottom rows show the same data expressed
724 per ng of DNA for SBRconstantT (c) and SBRloweringT (d). The corresponding sampling times for Start,
725 Phase II and Phase V are 10, 134 and 293 days, respectively.

Table 1 - Specific ammonium removal rates (SARR, expressed in mg NH_4^+-N/g VSS/d) and selected influencing factors for anammox based processes at low temperatures (<15°C) and in different reactor configurations. Moving Bed Biofilm Reactor (MBRR), Sequecing Batch Reactor (SBR), Partial Nitritation/Anammox (PN/A), Chemical oxygen demand (COD)

Process configuration; Process type	**Substrates for competitors**	**T_{low} (°C)**	**Period lowering T (d)**	**Period at T_{low} (d)**	**SARR**	**Reference**
Granular MBBR/SBR; Anammox	COD	10	-	722	13-19	Hendrickx *et al*. 2014
SBR; PN/A	O_2	12	10 (25-12°C)	131	93	Hu *et al*. 2013
Granular upflow fluidized bed; Anammox	COD	10	127 (20-10°C)	151	35	Lotti *et al.* 2014b
Granular SBR; Anammox	O_2	13.2	523 (30.5-13.2°C)	75	15.1	Sánchez Guillén *et al.*, 2016
Granular SBR; Anammox	-	12.5	208 (30-12.5°C)	63	82	This study
		10	257 (30-10°C)	78	92	

731 **Table 2** – Comparison of the short vs. long term effect of temperature decrease on SARR. Between
732 brackets the Phase number from the long-term reactor experiment is given. For the short term test,
733 biomass was directly exposed to lower temperatures and the data from individual biomass tests were used
734 to calculate a weighted average, based on the different biomass contributions in the inoculum of the
735 reactor test.

	Short term experiment	**Long term experiment**	
	Inoculum mix	$SBR_{constantT}$	$SBR_{loweringT}$
SARR 30°C (mg NH_4^+-N/gVSS/d)	49.2	104 (I); 471 (IV); 785 (V)	98.7 (I)
SARR 12.5°C (mg NH_4^+-N/gVSS/d)	9.99	-	82.2 (IV)
SARR 10°C (mg NH_4^+-N/gVSS/d)	6.30	-	91.8 (V)
SARR ratio 12.5°C/30°C (%)	20	-	17*
SARR ratio 10°C/30°C (%)	13	-	12**

736 * ratio $SBR_{loweringT}/SBR_{constantT}$ for Phase IV

737 ** ratio $SBR_{loweringT}/SBR_{constantT}$ for Phase V

Electronic Annex

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