The afterglow mystery of pulsed glow discharges and the role of dissociative electron-ion recombination

Reference:
Bogaerts Annemie.- The afterglow mystery of pulsed glow discharges and the role of dissociative electron-ion recombination
Journal of analytical atomic spectrometry - ISSN 0267-9477 - 22(2007), p. 502-512
Full text (Publisher's DOI): https://doi.org/10.1039/B618035C

Title

Biological nutrient removal from slaughterhouse wastewater via nitritation-denitritation using granular sludge: an onsite pilot demonstration

Short Title

Nitritation-denitritation granular sludge : an onsite pilot demonstration

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Abstract

BACKGROUND: In this study, an onsite pilot sequencing batch reactor (SBR) of 450L was operated at a local Flemish slaughterhouse in two consecutive periods. During period I, nitrogen removal via nitritation-denitritation (Nit/DNit) was achieved by the use of an innovative startup strategy without seed sludge and subsequently maintained by the use of aeration phase length control based on the oxygen uptake rate (OUR). In period II, the operational strategy was modified. In order to obtain Nit/DNit granular sludge (GS), an anaerobic (feast) feeding was introduced followed by a dynamic controlled (OUR based) aerobic (famine) reaction step

RESULTS: After 74 days the local (Flemish) legislation standards for COD (125 mg O_2/L) and T-N (15 mg N/L) were fulfilled. During period II, floccular sludge was partially transformed into granular sludge, which resulted in increased COD and NH_4-N conversion rates of 50% and 60% respectively. Average removal efficiencies for SCOD, N and P, during period II, were respectively 89.5 ± 4.2%, 88.8 ± 9.8% and 84.4 ± 9.3%. Despite the relatively low biomass concentrations (2.5 g/L), excellent sludge settling was reported as the SVI_{30} < 100 mL/g from day 167 onwards. Moreover, it was found that the operational strategy and temperature variability had a crucial impact on the microbial community dynamics and granule growth.

CONCLUSION: This study demonstrated the Nit/DNit BNR-GS system in a real operational environment, through the operation of an onsite pilot plant.

INTRODUCTION

With a share of 24% of the freshwater demand of the global food and beverage sector, meat processing industry is considered as a large water consumer[1]. The process steps with the highest water consumption are transport of animals, slaughtering activities and cleaning of the facilities. Slaughterhouse wastewater typically consists of a large fraction (40-60%) insoluble matter, such as fats, proteins and cellulose. Therefore pre-treatment of slaughterhouse wastewater is inevitable to degrade or remove the majority of the insoluble matter. Afterwards, a biological nutrient removal (BNR) step is recommended to remove the residual amount of soluble nitrogen (N), phosphorus (P) and biodegradable carbon (COD).

BNR by the use of granular sludge (GS) has been proposed as an energy efficient and sustainable technology for the treatment of synthetic[2–4], domestic[5–7] and industrial wastewater[8–12]. Stable BNR-GS processes rely on anaerobic (feast) feeding, to stimulate storage micro-organisms, followed by an aerobic famine reaction[4,13,14]. The organisms capable to store organic compounds, under anaerobic conditions, are known as slow growing organisms such as polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). In many (lab-scale) studies[2,7,14–16], the model substrate Volatile Fatty Acids (VFA) is known to stimulate the model PAO, *Candidatus* Accumulibacter or model GAO, *Candidatus* Competibacter.

However, for domestic wastewater, the GS technology was scaled up from lab- to pilot and finally full-scale applications during the last 20 years[7,17]. Under de commercial tradename Nereda®, there are currently more than 50, mainly for domestic wastewater, GS references worldwide (www.royalhaskoningdhv.com/en-gb/nereda). References regarding industrial wastewater mainly reported lab-scale applications[8,18–20]. Recently, Hasebe et al. (2017)[21] operated a nitrifying granular pilot plant treating real industrial wastewater originating from the electronic processing

industry. Although, onsite pilot research using GS for the treatment of diverse industrial wastewaters in a real environment still remains scarce.

Another energy efficient and sustainable technology, which has been studied extensively during the last decades is nitrogen removal via nitritation-denitritation (Nit/DNit)[22–24]. Whether the aim is full nitritation-denitritation, partial nitritation – anammox or one stage anammox, the common goal for all these processes is the repression and washout of nitrite oxidizing bacteria (NOB) and the promotion of ammonium oxidizing bacteria (AOB). Many techniques such as, low SRT, high temperature, free ammonia, low dissolved oxygen, the use of specific inhibitors, have been proposed to suppress NOB from an existing microbial population[25–28]. More recently, Dobbeleers et al. (2018)[29] demonstrated full nitritation-denitritation, treating slaughterhouse wastewater, by the use of an innovative start-up strategy without seed sludge in combination with aeration phase length control, based on the oxygen uptake rate (OUR), in a lab-scale SBR.

Combination of both technologies: (1) GS and (2) Nit/DNit leads to an energy efficient, single stage BNR process, Nit/DNit BNR-GS. Dobbeleers et al. (2017a)[9], already demonstrated the feasibility of the Nit/DNit BNR-GS process, at lab-scale, for the treatment of anaerobically pre-treated industrial wastewater from the potato processing industry. However, what is still missing from these previous studies is to fill in the gap between basic lab-scale research and real industrial application. In this study, the Nit/DNit BNR-GS system, was scaled-up to a pilot scale sequencing batch reactor (450L) to investigate the feasibility of the proposed technology in an real operational environment on-site at an industrial slaughterhouse. This allowed to investigate the effect of the wastewater variability and external factors such as temperature on the process performance. The objectives were twofold: (1) to achieve nitrogen removal via Nit/DNit through the use of an innovative startup strategy without inoculum as described by Dobbeleers et al.,

(2018)[29]; (2) the formation of granular sludge performing BNR via Nit/DNit as described by Dobbeleers et al. (2017a, 2017b)[9,30].

MATERIALS & METHODS

Situation sketch and Reactor setup

The mobile pilot SBR was operated on-site at a local slaughterhouse. Supplementary figure 1 pictures an on-site situation sketch. The pilot plant was provided by electricity and pressurized air, which was first reduced by a pressure regulator, from the slaughterhouse. In order to provide fresh wastewater, the pilot plant buffer tank was in direct connection with the full-scale buffer tank by the use of a submersible pump and driven by high and low level controls. Furthermore, a rectangular pilot-scale SBR reactor with a working volume of 450L, height of 1500 mm, length and width of 680 mm was used. Feeding was performed by a peristaltic pump, effluent withdrawal was accomplished by a submersible pump which was located at a height of 600 mm. Both feeding and effluent withdrawal were controlled by an ultrasonic level controller, which continuously monitored the level of the mixed liquor in the reactor. A mechanic mixer was used, to maintain the sludge in suspension. To provide sufficient oxygen distribution through the mixed liquor, an on/off controllable air valve connected to a membrane aeration unit provided sufficient oxygen distribution through the mixed liquor.

The SBR sequence and management of all hardware actuators (pumps, mixers and discharge) were controlled by a Siemens® programmable logic controller (PLC). In addition, a SC1000 module (Hach, Mechelen, Belgium) equipped with an dissolved oxygen (DO), pH, oxidation - reduction potential (ORP) and conductivity (EC) probe was connected to the PLC as well. A

custom-build LabVIEW (National Instruments, Texas, USA) supervision program allowed to setup the operational strategy and to monitor and visualize the online measured data.

Reactor operation

The pilot SBR reactor was operated in two operational periods. The aim of, period I (P-I), was to obtain nitrogen removal via Nit/DNit based on the innovative strategy described by Dobbeleers et al.(2018)[22]. No inoculum was used and the reactor was filled with slaughterhouse wastewater from the full-scale buffer tank. During the first 8 days of P-I, an operational strategy similar as described by Dobbeleers et al. (2018)[29] was used. Aerobic periods of 12h with on-off aeration control between 4 mg O_2/L and 6 mg O_2/L were ran and no additional feeding was added (Table 1). Later in P-I (from day 65), the operational strategy was carefully modified in order to stimulate AOB and prevent NOB growth by implementation of the aeration phase length control.

In the second period (P-II), the operational strategy was adapted in order to promote sludge granulation. During P-II, a single loop operational strategy consisting of an anaerobic feeding (20 min), with prolonged anaerobic phase (90 min) followed by an aeration step (dynamically controlled), a post anoxic step (90 min) and finally settling and effluent withdrawal (10 + 5 min) was operated. The volume exchange ratio (VER) varied between 15 and 23% depending on the wastewater composition variability. Furthermore, DO was controlled, using an on-off regulator, between 0.5 and 1.0 mg O_2/L, which also allowed to calculate the oxygen uptake rates (OUR) from the decreasing oxygen values. These OUR values were subsequently compared with the adjustable OUR threshold value by the OUR based aeration phase length control strategy, which led to a dynamic controlled SBR cycle.

Industrial slaughterhouse wastewater

In a first step, the raw wastewater of the slaughterhouse is treated by the use of a drum screen to remove larger particles. In a second step, this wastewater was pre-treated by an enhanced dissolved air flotation (DAF), through the addition of $FeCl_3$. This physio-chemical pre-treatment has an average COD removal efficiency between 50 and 80%. Thereafter, the pre-treated wastewater is stored in a buffer tank prior to the BNR step. As described previously, wastewater was directly drawn from this buffer tank by the use of a submersible pump. This allowed to operate the pilot plant with real wastewater variability. Table 2 summarizes the wastewater composition during the two operational periods.

Aeration phase length control

Due to an on-off aeration control during aerobic steps, OUR could be calculated from decreasing DO values. This online OUR calculation was performed automatically by the custom-build LabVIEW program. Furthermore, an adjustable OUR threshold value could be set and adjusted when necessary. It was shown that a sharp drop in OUR occurs as soon as the oxidation of NH_4-N is (almost) complete[22]. Consequently, if the custom-build LabVIEW program detects an OUR value which is below the threshold value, it will switch immediately to the next step in the SBR cycle (denitrification step). In order to build in some prudence in the control strategy, aeration steps always had a minimum (period I = 20 minutes; period II = 120 min) and maximum duration (period I = 45 minutes; period II = 480 min).

Analytical methods

COD samples were analyzed using a standard cuvette test (Hannah Instruments, Temse, Belgium). All other samples were first filtered over a glass microfiber filter (particle retention 1.2

µm), where after concentrations of phosphate (Hach Lange, Germany), ammonium (Hanna Instruments, Belgium), nitrite (Hach Lange, Germany), nitrate (Hanna Instruments, Belgium) and SCOD (Hanna Instruments, Belgium) were analyzed with standard cuvette tests (Hanna Instruments, Temse, Belgium). Dissolved organic carbon (DOC) was analyzed with a laboratory TOC Organic Carbon (TOC) analyzer (Sievers Innovox). The evolution of the sludge morphology was examined using an MOTIC (Xiamen, China) microscope. Biomass concentration and sludge volume index (SVI) measurements were conducted according to the standard methods (APHA, 1998).

In-situ cycle measurements

During P-I, in situ cycle measurements were carried out to determine the nitritation-degree (ND). Therefore, samples were taken every 10-30 min in order to obtain profiles of ammonium, nitrite and nitrate.

$$ND\ (\%) = \frac{[NO_2^- - N]_{t_0}^{t_n}}{[NO_2^- - N]_{t_0}^{t_n} + [NO_3^- - N]_{t_0}^{t_n}}\ x\ 100$$

With t_0 the start of the aeration phase and t_n the end of the aeration phase.

Ex-situ microbial activity & batch experiments

To examine the performance of the nitrogen removal via Nit/DNit, ex-situ microbial activity tests, according to Dobbeleers et al. (2017b)[30] were performed to determine the specific nitrogen removal rates (SR) of AOB (mg NH_4-N/gVSS.h) (SR_{AOB}) and NOB (mg NO_2-N/gVSS.h) (SR_{NOB}). These SRs were used to calculate the (%) NOB/AOB activity ratio which is an excellent precursor for the performance of Nit/DNit.

Moreover, also during P-II, ex-situ batch tests were conducted to analyze the maximum phosphate release rate (mg PO_4-P/gVSS.h) (PRR) and to determine the $P_{release}/C_{uptake}$ ratio. Biomass was sampled (400 mL) right before settling and washed with a solution of tap water enriched with 0.1 M $NaHCO_3$ and 0.05 M KCl. Then, an excess of industrial wastewater was fed to the biomass (typically 300 mL) wereafter anaerobic conditions were maintained for 120 min and samples were taken every 10 to 30 minutes.

Microbial community analysis by 16s RNA gene amplicon sequencing and quantitative PCR

Prior to the molecular analysis, DNA was extracted from triplicate biomass samples (500µL) as described by McIlroy et al. (2009)[31], with minor modifications.

At the end of P-I and during P-II, 16s rRNA gene amplicon sequencing targeting the V1-3 region was carried out in order to monitor the microbial population dynamics. Amplicons were generated with barcoded primers (IDT), and Phusion High-Fidelity DNA Polymerase (Thermo Scientific), as described by Kozich et al. (2013)[32]. PCR products were purified using the SequalPrep Normalization plate kit (Invitrogen), and pooled. The resulting library was further purified by gel extraction using NucleoSpin Gel and PCR Clean-up (Macherey Nagel), and diluted to obtain a 4 nM library. Amplicon sequencing was carried out on a Illumina Miseq system at the Centre for Medical Genetics (Edegem, Belgium) with the MiSeq Reagent Kit v2 (Illumina). The obtained paired-end reads were processed with the UPARSE pipeline[33]. Taxonomy prediction of the OTU sequences was carried out with MiDAS[34] as reference database. Further data analysis was done in R, using the ampvis package[35].

In addition, the most important species of the microbial community were quantified by the use of qPCR as described by Dobbeleers et al. 2017a[9]. These groups involve AOB (targeting the amoA genes of *Nitrosomonas*[36]), NOB (targeting the NxrB genes of *Nitrospira*[37]), PAO (targeting the 16S

rRNA of *Candidatus* Accumulibacter[38]) and GAO (targeting the 16S rRNA of *Candidatus* Competibacter[39]).

Results

Startup: nitrogen removal via Nit/DNit (Operational period I)

To start up the pilot SBR reactor, no inoculum was used in order to prevent NOB activity and growth from the beginning. Therefore, the operational strategy was carefully modified during the first 64 days (Table 1). Wastewater was added at day 1 and, to stimulate biomass growth, oxygen was provided continuously (between fixed DO set points, see Table 1) during the following 8 days. It can be observed from Figure 1, that mostly (S)COD degradation took place during this period. As a result of the ammonification process the NH_4-N concentration in the reactor increased during this start-up. After 20 days of reactor operation, the SCOD effluent values fulfilled the local (Flemish) discharge limits (125 mg COD/L) and during the residual course of P-I an average SCOD removal efficiency of 92.8 ± 5.4% could be maintained. The effluent NH_4-N concentration remained almost unchanged (excluding the initial increase due to ammonification) during the first 50 days. Starting from day 36, NO_2^- started to appear at the end of an aeration step (> 0.5 mg N/L), suggesting the initiation of AOB activity. Contrary, it did not immediately lead to decreasing NH_4-N concentrations, which was probably caused by elevated influent NH_4-N. Afterwards, starting from day 50, in a period of 25 days the effluent NH_4-N, in combination with the effluent NO_2^- and NO_3^-, reached the local (Flemish) legislation discharge standard (15 mg T-N/L). This allowed to apply from day 65, the aeration phase length strategy, based on OUR. This dynamic strategy was the key to maintain Nit/DNit in combination with a high NH_4-N removal efficiency during the residual period of period I with an average of 98.4 ± 5.4%.

From day 40 onwards, in-situ cycle measurements were performed at least biweekly to determine the ND. It can be observed from Figure 1, that the ND varied between 80% and 100% during the entire course of P-I, confirming nitrogen was mainly removed via Nit/DNit. In addition, from day 72, ex-situ activity measurements were carried out on a weekly base in order to analyze SR_{AOB}, SR_{NOB} and to calculate the NOB/AOB activity ratio. Figure 1 shows the increasing SR_{AOB}, the low SR_{NOB} (< 0.5 mgN/gVSS.h) and the low NOB/AOB activity ratio, (< 10%). To verify these activity-based measurements, qPCR analysis was performed on biomass sampled at day 98 to determine the abundance of AOB (target amoA *Nitrosomonas*) and NOB (target nxrB *Nitrospira,*). For AOB, 7.11 ± 1.01x10^{10} cells/gVSS were found whereas for NOB, 1.41 ± 0.09x10^{9} cells/gVSS were detected, resulting in a molecular NOB/AOB quantification ratio of 1.8 ± 0.3% for those specific target genes. However, 16s rRNA amplicon sequencing showed that *Candidatus* Nitrotoga (representing 0.05% of the reads) and not *Nitrospira* (representing 0.02% of the reads) was the most abundant NOB. Because of this the previously mentioned molecular NOB/AOB quantification ratio based on qPCR is inaccurate. Nevertheless, as the AOB genus *Nitrosomonas* represented 3.11% of the reads, the molecular techniques confirmed NOB repression and AOB enrichment and thus Nit/DNit as the main pathway for nitrogen removal.

On-site pilot research involves some external influences, such as wastewater variability and temperature fluctuations, causing additional challenges for biomass growth and the prevention of NOB growth. Figure 2 shows the influence of varying temperature on the biomass concentration expressed as ML(V)SS, and the ammonia utilization rate (AUR). Since the reactor start up (P-I) took place from early March until mid-July, it explains the overall increase in temperature. Both parameters, ML(V)SS and AUR, are significantly correlated with temperature as the Pearson coefficients are respectively 0.81 ($n = 73$; $P < 0.0001$) and 0.91 ($n = 57$; $P = 0.0002$). From day 65 onwards, an SRT of 25 days was applied, whereafter the biomass concentration

stabilized regardless of the rising temperature. In contrast, the AUR was not affected by the SRT regulation and increased further during the course of period I.

Formation of Nit/DNit BNR-GS (Operational period II)

During period II, the process parameters were modified in order to stimulate sludge granulation. This implied the introduction of an anaerobic feeding step to promote slow growing organisms, such as PAO and GAO, followed by an aerobic/anoxic reaction step. However, nitrogen removal via Nit/DNit also remained an important aim of the study. To compromise both objectives (granulation and Nit/DNit), the DO concentration was controlled by an on-off regulation between 0.5 and 1.0 mg O_2/L and the OUR control strategy secured the end of aerobic step as soon as the OUR dropped below the adjustable threshold value. In order to examine biomass aggregation, sludge characteristics such as SVI, ML(V)SS and morphology were monitored rigorously. Supplementary Figure 2 shows the evolution of the sludge morphology during P-II. On day 1, loose – irregular small particles can be observed, which gradually evolve to somewhat denser aggregates between day 21 and 142. Well-shaped granules can be distinguished between day 175 and 225, with dimensions up to 500 µm.

It has to be mentioned that loose – irregular floccular sludge was part of the mixed liquor at all times. In addition, sludge growth above 3 gMLSS/L could not be achieved (Figure 3). Although, as observed from Figure 3A, SVI values gradually decreased over almost the entire course of P-II (to SVI_{30} < 100 mL/g from day 167 onwards), indicating excellent sludge settling. A disturbance can be noticed between day 65 and 90, which was caused by feeding pump issues resulting in 2 episodes without feeding for 3 days and one overload event.

Performance of the Nit/DNit BNR-GS

During period II, BNR performance via Nit/DNit was examined intensively. Average removal efficiencies of (S)COD, N and P were (Figure 3) respectively 89.5 ± 4.2%, 88.8 ± 9.8% and 84.4 ± 9.3%. The feeding disturbance between day 65 and 90, as mentioned in the section above, also affected the N-removal efficiency. Disregarded this incident, effluent concentrations of SCOD, N and P fulfilled the local (Flemish) legislation discharge standards (125 mg SCOD/L, 15 mg T-N/L and 2.5 mg PO_4-P/L) at all time.

The performance of Nit/DNit was investigated by ex-situ activity measurements and molecular analysis, quantification by qPCR and 16s rRNA amplicon sequencing. Figure 3B and Figure 5 show these results for P-II. As it could be expected after P-I, the AOB rates (SR_{AOB}) were substantially higher than NOB rates (SR_{NOB}). Besides that, the AOB (qPCR target *Nitrosomonas*) abundancies were significantly higher than the NOB (qPCR target *Nitrospira)* abundancies. However, at the start (first 50 days) of P-II there was some inconsistency between the NOB/AOB activity and molecular quantification ratio based on qPCR, 47.8% versus 15.8% respectively. 16s rRNA sequencing revealed that during this start of P-II, again *Candidatus* Nitrotoga was found to be more abundant than *Nitrospira* (Figure 5). Later in P-II, *Nitrospira* became the major NOB and hereby also the consistence between NOB/AOB activity and qPCR quantification ratio increased.

Besides the detailed investigation regarding the N removal species, ex-situ batch tests and molecular analysis were also conducted for bacterial groups involved in P-removal and C-uptake (Figure 3). As demonstrated by qPCR quantification, for the specific targets *Candidatus* Accumulibacter, *Candidatus* Competibacter, and the $P_{release}/C_{uptake}$ ratio, PAO rather than GAO predominated the system (PAO/GAO qPCR ratio > 20 and $P_{release}/C_{uptake}$ ratio > 0.25) during the first 125 days. In addition a relatively high phosphorus removal efficiency (generally > 80%) from

the start of P-II was achieved. According to 16s rRNA sequencing (Figure 5), *Candidatus* Accumulibacter was found to be the most abundant PAO during the start of P-II. Although other PAO genera such as *Dechloromonas*, *Candidatus* Accumulimonas, *Candidatus* Obscuribacter and *Tetrasphaera* could be detected as well. At the same time (start of P-II), only a limited percentage of the overall reads could be related to GAO Genera such as *Candidatus* Competibacter, *CCM19a* and *CPB_S60*. In the further course of P-II, the $P_{release}/C_{uptake}$ ratio decreased (< 0.25) while the GAO abundancies and especially the target Candidatus *Competibacter* gradually increased, leading to PAO (qPCR target Candidatus *Accumilbacter)*/GAO (qPCR target *Candidatus* Competibacte*r)* ratio < 1 and thus a GAO dominated system (Figure 3 and 5). Despite this GAO dominance, the average P removal of 84.4 ± 9.3% and PRR of 7.4 ± 2.4 mg P/gVSS.h could be maintained. 16s rRNA sequencing confirmed the presence of PAO in the biomass as a diverse group of PAO genera such as *Candidatus* Accumilibacter*, Dechlormonas* and *Candidatus* Obscuribacter could be detected (Figure 5).

A typical in-situ cycle measurement performed on day 196 (P-II) is shown in Figure 4. During anaerobic conditions, 78% of the influent DOC is taken up from the liquid phase and presumably stored as PHA. Concurrently phosphates are released to the mixed liquor. During aerobic conditions, a PUR of 12.2 mg P/gVSS.h led to full uptake of phosphorus after 120 min. Regarding the N-conversions, complete ammonium removal was observed, with only a minor amount of nitrite accumulation after 60 min of aeration. Moreover, simultaneous nitritation-denitritation (SND) during the aerobic step can be clearly observed from Figure 4. The calculated SND was 69.3% indicating that most of the nitrogen was denitrified during the aerobic phase. Furthermore, only a negligible amount of nitrate was formed after 180 min aeration, confirming nitrogen removal occurred mainly via Nit/DNit.

Effect of granulation strategies on the nutrient loading rates

Due to the variable influent wastewater and the application of the phase length control strategy, the average cycle length turned out to be variable as well. As a consequence, this was also reflected in the nutrient loading rates which are shown in Table 3. For P-I, the loading rates are given after the fulfillment of the Flemish discharge limits at day 75. Taken in account that the average temperature during P-I (day 75 – 125) was higher (22.4°C) than during P-II (20.4°C), Table 3 clearly shows the increased loading rates during P-II, which might be related to the positive effect of sludge granulation.

Microbial population dynamics during operational period II

In P-II the operational parameters where selected to stimulate slow growing organisms, PAO and GAO, with the ability to store organic matter during anaerobic conditions, ultimately leading to sludge granulation[13]. The evolution of the microbial community was examined by 16s rRNA amplicon sequencing to provide population dynamics during the transformation from floccular sludge to granular sludge. Figure 5 shows a heatmap, representing the 30 most abundant genera, of the shifting microbial community. *Thauera* and *Zoogloea* were detected during the entire P-II as most abundant genera. Both genera are known as denitrifiers, with the ability to store organics substrates as PHA, and producers of amyloid adhesins in the extracellular polymeric substances (EPS)[40]. Another genus, *Ferruginibacter*, which is involved in the biosynthesis and exportation of EPS[41], also increased during P-II. As already mentioned, PAO genera and more specifically *Candidatus* Accumulibacter where found to be more abundant in the first half of P-II, while GAO genera and more specifically *Candidatus* Competibacter dominated the second half of P-II. Other important genera with a noteworthy function are hydrolysers such as *MK04, Terrimonas* and

Aquabacterium, and filamentous bacteria such as Candidatus *Villogracilis* and *Haliscomenobacter*[34].

Discussion

Influences on the startup of an onsite pilot reactor achieving nitrogen removal via Nit/DNit

In this study, an onsite pilot SBR was operated in P-I to obtain nitrogen removal via Nit/DNit, using an innovative start-up strategy without inoculum and with aeration phase length control during the treatment of slaughterhouse wastewater. It was demonstrated that sufficient (S)COD removal, according to the Flemish discharge limits (125 mgCOD/L), was achieved after 20 days. However, for nitrogen, the Flemish discharge limits (15 mgN/L) were only achieved after 75 days of reactor operation. Through the use of the innovative start-up strategy, an average ND of 96.7% and NOB/AOB activity ratio of 4.4% were achieved over the entire course of P-I. These results confirm that NOB could be prevented from the start and nitrogen removal was mainly performed via Nit/DNit. Similarly, Dobbeleers et al. (2018)[29] achieved full nitrogen removal after 59 days, using a similar start-up strategy for the treatment of slaughterhouse wastewater in a lab-scale SBR. Moreover, several other authors[22,30,42,43] reported varying start-up times, from less than 40 days to more than 300 days, using seed sludge and aeration control strategies as single parameter to achieve nitrogen via Nit/DNit. It is tempting to assume that the start-up time of 75 days in this study was highly influenced by the operational temperature as significant Pearson correlations were found regarding biomass growth and AUR to the increasing temperature. Temperature as such is one of the most, if not the most important operational parameter influencing growth and activity of microbial communities. As temperature rises, chemical and enzymatic conversions rates increase, until a certain maximum temperature at which cell denaturation starts to occur. It is generally accepted that most bacterial groups present in wastewater treatment plants, such

as nitrifiers can be considered as mesophilic organisms with optimum growth rates between 28 and 36°C[44]. Marais and Ekama (1976)[45] already showed that an increase of 6°C resulted in a 50% rise of the heterotrophic growth rate. Similar as for chemical reactions, the temperature effect on the growth rate and activity of micro-organisms can be described by the Arrhenius equation ($\mu = \mu_{20°C}\ \theta^{(T-20)}$). In this study the startup of the pilot SBR occurred at a reactor temperature of 12°C, resulting in a theoretical AOB growth rate of μ_{AOB} of 0.402 day^{-1}, which is only half of the theoretical growth rate at 20°C (μ_{AOB} = 0.801 day^{-1}). In contrast, the theoretical NOB growth rate at 12°C is significantly higher ($\mu_{NOB\text{-}12°C}$ = 0.566 day^{-1}), suggesting NOB to be unavoidable. However, due to the startup without biomass, ammonia and not nitrite was the only available substrate. As a consequence AOB and more specifically the genus *Nitrosomonas* was the first to appear. Through the use of a step-feed operation in combination with aeration phase length control, any nitrite accumulation is denitrified before NOB are able to oxidize it to nitrate. This was also confirmed by molecular analysis of the NOB genera. Only a very low read abundance of *Nitrospira*, *Candidatus* Nitrotoga and no *Nitrobacter* was found at day 98. As already mentioned, a significant linear correlation was observed between the temperature and the AUR during the start-up with as a consequence, also impacted the discrepancy in growth rate between AOB and NOB, favouring AOB and repressing NOB even more. For future research, it would be advantageous, to investigate the dynamically controlled innovative start-up strategy continuously at low temperature (for example 12°C).

Determining factors for the formation of GS at an onsite pilot reactor

During P-II, the operational strategy was modified in order to promote sludge aggregation. Therefore, an anaerobic feeding with extended anaerobic phase was introduced, followed by an aerobic step and a post-anoxic step. Alternating anaerobic, aerobic/anoxic conditions favoring

PAO and/or GAO, have been proposed as one of the most important factors for GS formation combined with a stable BNR[4,13]. In this study molecular analysis by qPCR and 16s rRNA amplicon sequencing showed that *Candidatus* Accumulibacter predominated over *Candidatus* Competibacter at the start, but that the dominance turned around from day 170 onwards. Although, PAO genera such as Candidatus *Accumulibacter, Dechlormonas* and Candidatus *Obscuribacter* were still present in the mixed liquor during the second half of P-II and sufficient P-removal could be maintained. These findings indicate that PAO competition by GAO for organic substrates are not necessarily causing problematic P-removal.

Primary granulation was observed from day 20 and ultimately leaded to aggregates of 0.5 mm and SVI_{30} values below 100 mL/g after 175 days. These observations seem to indicate that the predominance of GAO genera was the key to form larger granules. However, biomass concentrations remained rather low, with an average MLSS of 2.5 g/L during the last 50 operational days, and some fraction of floccular sludge remained present at all times. Weissbrodt et al. (2013)[4] stated that sludge granulation depends on (1) operational conditions and (2) predominant organisms. Moreover, Weissbrodt et al. (2013)[4] found that spontaneous granulation occurred in PAO and/or GAO dominated microbial communities under steady state conditions. From molecular DNA analysis we could observe that neither PAO or GAO genera dominated the overall microbial community but the denitrifiers and EPS producers *Thauera* and *Zoogloea* were found to be the genera with the highest read abundance. These findings are in agreement with researchers such as Meunier et al. (2016)[46] and Zhang et al. (2016)[47], who studied microbial dynamics during sludge granulation and also found high read abundances of *Zoogloea* and *Thaurea.* EPS have been generally recognized to be key elements in the matrix structure of GS[48,49]. Thus, both genera (*Zoogloea* and *Thaurea)* will contribute to the structural EPS matrix which is needed to imbed other slower growing micro-organisms such as PAO and

nitrifiers[50]. In addition to the operational strategy and predominant organisms, Pronk et al., (2015a)[51] stated that full granulation is only possible if the biomass particle is fully saturated with organic substrate under anaerobic conditions. However, many studies regarding biomass saturation[2,14–16], only use the model VFA substrates such as acetate and propionate. In this study real industrial wastewater was used, containing a complex mixture of diverse organic substrates (VFA, sugars, amino acids,…) resulted in an anaerobic carbon uptake (ACU) of 78% (at day 196) and 73% (at day 216). According to Pronk et al., (2015a)[51], this incomplete ACU will lead to formation of hybrid (combination of granular and floccular) sludge in the subsequent aerobic phase. Moreover, due to the variable wastewater characteristics, steady state conditions are impossible to achieve. Although, longer operation of the onsite pilot reactor might have led to improved granulation with higher biomass concentrations as well. As an example, Weissbrodt et al., (2013)[4] achieved granulation in a GAO dominated lab-scale SBR after 450 days. Therefore it would be advantageous if future research would focus on long-term onsite pilot-scale experiments in order to verify the industrial applicability of GS in terms of varying loading rates and external process circumstances such as temperature.

Factors affecting BNR performance via Nit/DNit of the onsite pilot SBR during period II

Sufficient BNR removal could be maintained during the entire course of period II (feeding issue not taken into account). Moreover, the Flemish discharge limits for (S)COD, TN and PO_4-P were fulfilled as well. These results can be considered as highly relevant regarding the industrial applicability of the Nit/DNit BNR-GS process. In addition to this excellent BNR performance and despite the relatively low MLSS of 2.5 g/L, the average loading rates in period II for COD, and NH_4-N were significantly higher, 50, and 60% respectively than during P-I. Liu and Tay (2004)[48] already proposed that higher specific conversion rates are one of the major benefits of the GS process.

A possible reason for the low biomass concentration in combination with higher sludge conversion rates might be that the applied SRT of 20 days was insufficient to obtain high biomass concentrations. Future research should focus on how to achieve higher biomass concentrations for this type of industrial wastewater without compromising the conversion rates and thus the activity of the microbial-community.

As already mentioned, contradictory results regarding NOB/AOB ratios were obtained, at the start from P-II (first 50 days), when comparing the qPCR analysis (NOB target *Nitrospira)* and the activity measurements. It was shown that *Candidatus* Nitrotoga was found to be the most abundant NOB during this startup period, which caused an inaccurate molecular NOB quantification as it was not targeted by qPCR analysis (qPCR NOB target *Nitrospira*) and elaborates, the relatively high NOB activity (SR_{NOB}) at the start of P-II.. Moreover, as the NOB growth rate exceeds the AOB growth rate at temperatures below 20°C[45], this might have led to an increased NOB activity. Moreover *Candidatus* Nitrotoga was found to be a nitrite oxidizer under cold circumstances and can outcompete *Nitrospira* under elevated nitrite (1.0 mg NO_2-N/L) concentrations[52]. During the start-up of P-II, the reactor temperature was < 18°C (prior to the startup of P-II the reactor temperature was even below 15°C). As a consequence of these initial lower temperatures and with Nit/DNit as major nitrogen removal pathway, this might have led to increased NOB abundancies, of *Candidatus* Nitrotoga, until day 70. Later between day 70 and 125, due to the slightly higher temperature (18-20°C) and the fact that more and more nitrite was removed simultaneously, *Nitrospira* became the more abundant NOB. Starting from day 150, the reactor temperature was generally above 23°C resulting in an increased SR_{AOB} (6 mg N/gVSS.h). Hellinga et al. (1998)[25] and Mulder et al. (1998)[53] already showed the influence of high process temperature on the nitrifier activity. Moreover, these authors proposed the SHARON process, operated between 30-40°C as sole parameter to achieve nitrogen removal via Nit/DNit.

However, this high temperature based nitritation process might not be applicable in regions with milder or cold climate conditions. In the present study, the combination of (1) temperature variation, (2) low DO[27] and (3) aeration phase length control[9,22] as most important factor in this study, influenced the performance of Nit/DNit.

Temperature not only affected the NOB/AOB dominance, it also might have its influence on the PAO/GAO competition as well. GAO are known as PAO competitors for the anaerobic uptake of organic substrates, without contributing to P removal[54]. The PAO (Candidatus *Accumulibacter*) dominance, at the start of the P-II clearly corresponds to the lower average temperatures (< 20°C). In contrast, GAO dominance (Candidatus *Competibacter*) was observed from day 150, corresponding to higher average temperatures (> 23°C). These results are in agreement with other studies reporting GAO dominance at temperatures > 20°C[14,54]. Similar as in the present study, Bassin et al. (2012)[14] and (Dobbeleers et al., 2017a, 2017b)[9,30] also reported excellent P removal in mixed PAO/GAO cultures. Recently Nielsen et al. (2019)[55] stated that in full-scale enhanced biological phosphorus removal (EBPR) plants it turned out that GAO are not causing problems to the P-removal efficiencies but rather are a sign of good-performing EBPR systems with an excess of carbon available.

Conclusion

In this study we demonstrated the Nit/DNit BNR-GS system using an onsite pilot SBR in a real operational environment. During this pilot-test the system was challenged by real wastewater and temperature variability, at a local Flemish slaughterhouse. We have shown the ability to achieve (1) Nit/DNit (> 80%) and (2) formation of GS outside a laboratory environment. It was found that complete nitrogen removal via Nit/DNit could be achieved at 75 days by the use of an innovative startup strategy without inoculum and at initial temperature of 12°C. Interestingly, for

NOB *Candidatus* Nitrotoga was found to be almost equally abundant as *Nitrospira*. Furthermore, formation of GS was reported, although a mixture of granular and floccular sludge and thus hybrid sludge was present at all-time. *Thauera* and *Zoogloea* were found to be the important organisms during the transition to GS. Despite the low biomass concentration (2.5 g/L), increased nutrient removal rates were found due to sludge granulation. Moreover, the BNR effluent values fulfilled the Flemish legislation standard at all times. We believe this study is highly relevant for the further scale-up of the Nit/DNit BNR-GS system. Therefore future research should focus on strategies to obtain higher biomass concentrations. In addition, this study also illustrates the impact of industrial wastewater containing different carbon sources on granulation, which gives opportunities for fundamental research related to the impact of variable carbon sources on sludge aggregation.

Acknowledgements

This work was funded by the Flanders Innovation and Entrepreneurship Agency (grant number 131325)

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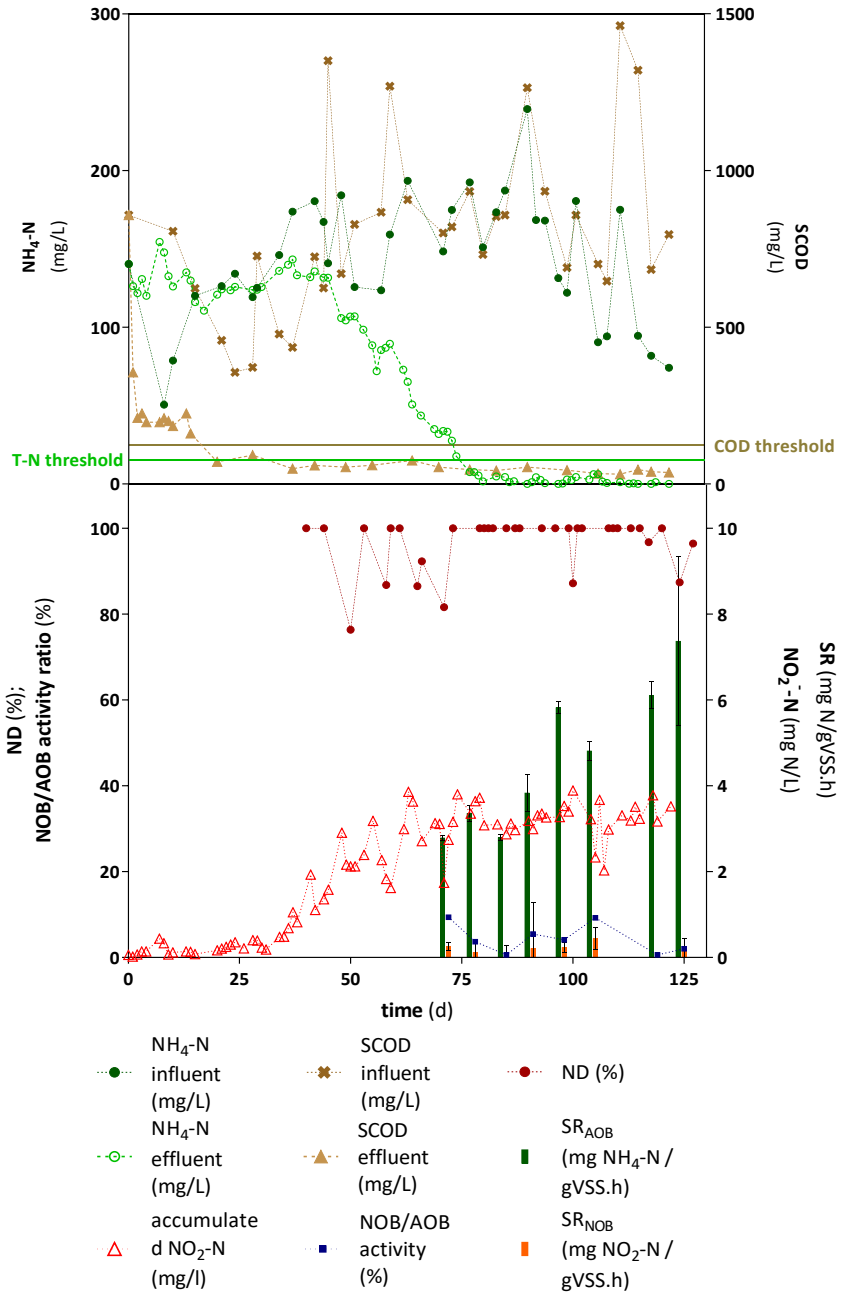

Figure 1. Start-up of the pilot reactor aiming nitrogen removal via Nit/DNit, without seed sludge (operational period I). Evolution of in- and effluent SCOD (mg/L) and NH_4-N (mg/L) (above). Progression of the nitrite accumulation (measured at the end of an aeration phase) (mg/L), ND (%), SR_{AOB} (mg NH_4-N/L), SR_{NOB} (mg NO_2-N/L), NOB/AOB activity ratio (%) (below) (error bars = SD).

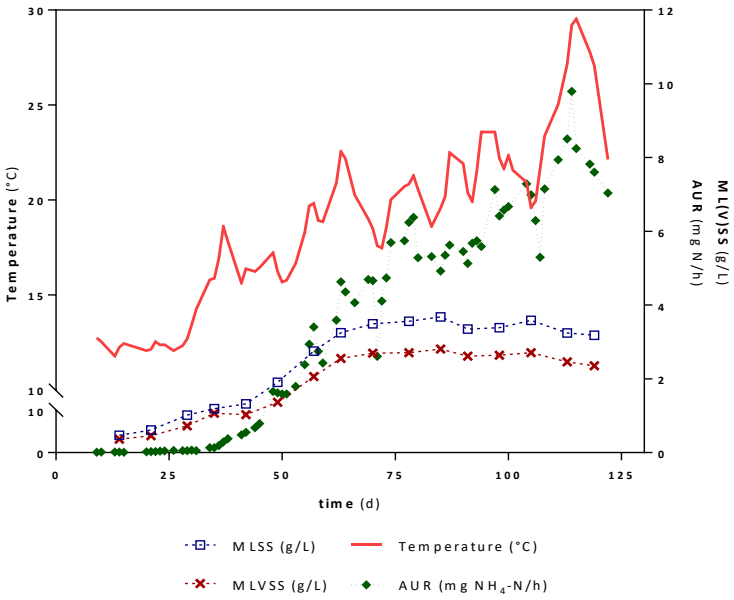

Figure 2. Influence of the reactor temperature (°C) on the biomass growth, (ML(V)SS (g/L)) and ammonia uptake rate (AUR) (mg NH_4-N/h) during operational period I.

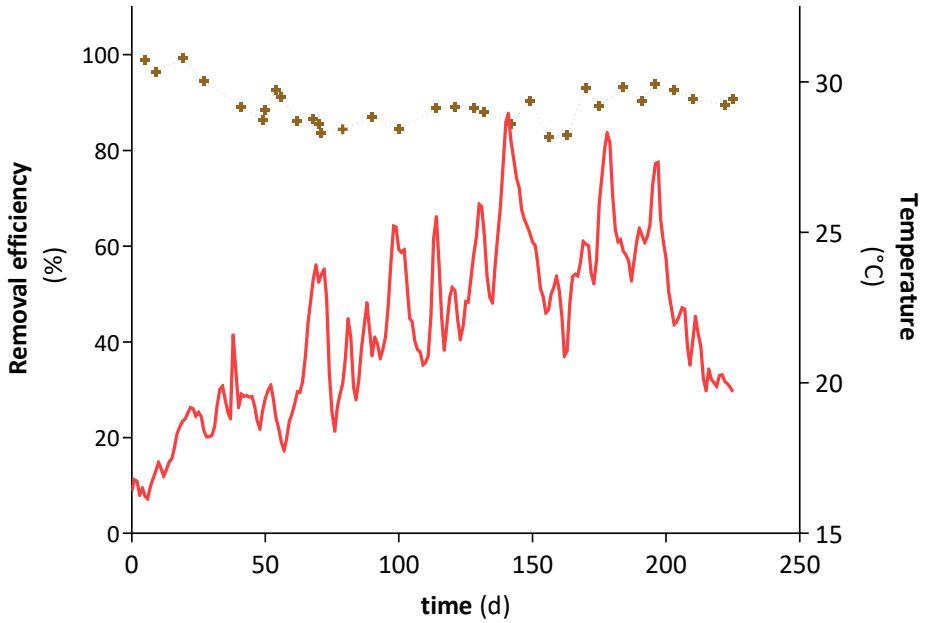

Figure 3. Evaluation of the Nit/DNit BNR-GS operational strategy during period II of the pilot plant SBR operation. Reactor temperature variance, sludge parameters (SVI and MLSS) and SCOD removal efficiency (%) **(A)**. Nitrogen removal efficiency (%), SR_{AOB} (mg NH_4-N/gVSS.h), SR_{NOB} (mg NO_2-N/gVSS.h), AOB and NOB target cells per g biomass (cells/gVSS), NOB/AOB activity and quantification ratio (%) **(B)**. Phosphorus removal efficiency (%), PRR (mg P/gVSS.h), $P_{release}/C_{uptake}$ (mol/mol), PAO and GAO target cells per g biomass (cells/gVSS) and PAO/GAO quantification ratio (%) **(C)**.

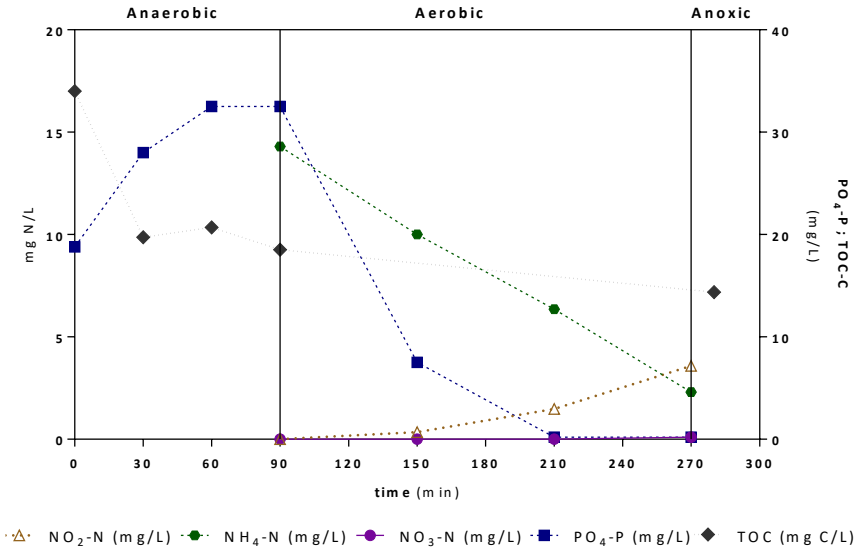

Figure 4. Typical in situ cycle measurement on day 196 showing profiles of NH_4-N, NO_2-N, NO_3-N, PO_4-P and TOC-C (mg/L). An anaerobic feeding step of 20 min, without mixing, took place right before time = 0

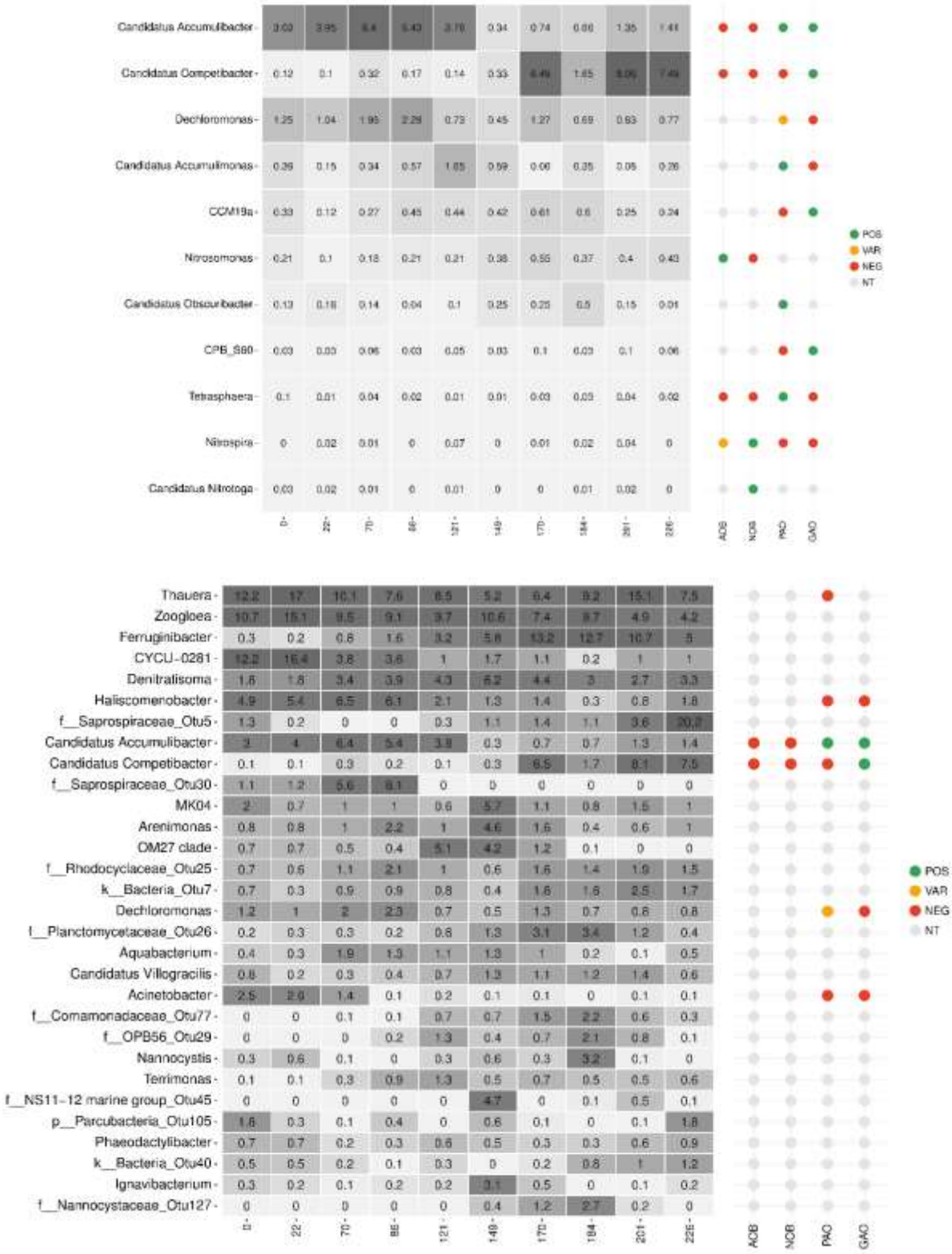

Figure 5. Heatmaps of the microbial population dynamics (up to the genus level) during P-II, the most abundant, PAO, GAO, AOB & NOB organisms (top) and overall top 30 organisms present (bottom). Each number specifies the % of the reads which can be contributed to a specific organism.

Table 1. Overview of the operational strategy during the startup, period I (P-I), of the pilot SBR reactor.

Days	Feeding	anoxic (min)	aerobic (min)	on/off aeration control	Settling + effluent discharge (min)
1-8			720	4.0 – 6.0 mg O_2/L	
9-24	2 loops VER = 5%	120	510	1.0 – 2.0 mg O_2/L	75 + 10
25-47	3 loops VER = 8%	65	120	1.0 - 2.5 mg O_2/L	75 + 10
48-55	3 loops VER = 13%	45	80	1.0 - 2.5 mg O_2/L	50 + 10
56-64	4 loops VER = 13%	45	50	1.0 – 2.0 mg O_2/L	50 + 10
65-125	5 loops VER = 11%	45	max. 45	1.0 – 2.0 mg O_2/L	50 + 10

Table 2. Summary of the wastewater characteristics, (S)COD, NH_4-N, PO_4-P (mg/L) in the two operational periods.

	Period I				**Period II**			
	Min	Max	Average ± SD	CV (%)	Min	Max	Average ± SD	CV (%)
COD (mg/L)	750	1932	1223 ± 336	28	884	2028	1361 ± 275	20
SCOD (mg/L)	356	1462	809 ± 274	34	418	1310	813 ± 172	21
NH_4-N (mg/L)	50.6	239.2	142.7 ± 40.9	29	78.8	247.0	160.9 ± 31.5	20
COD/N ratio	2.51	14.0	5.95 ± 2.55	43	2.52	9.49	5.20 ± 1.47	28
PO_4-P (mg/L)					3.80	34.50	16.13 ± 6.88	43
COD/P ratio					21.3	221.6	68.30 ± 47.50	69

Table 3. Nutrient loading rates, (S)COD (mg (S)COD/gVSS.day), NH_4-N (mg N/gVSS.day) and PO_4-P (mg P/gVSS.day) during both reactor periods. For P-I these loading rates were considered from day 75, after the fulfilment of the Flemish discharge limits.

	Period I (Average T = 22.4°C)				**Period II** (Average T = 20.4°C)			
	Min	Max	Average ± SD	CV (%)	Min	Max	Average ± SD	CV (%)
COD loading rate (g COD/gVSS.day)	0.001	0.237	0.146 ± 0.055	38.0	0.049	0.598	0.272 ± 0.085	31.2
SCOD loading rate (g SCOD/gVSS.day)	0.001	0.237	0.127 ± 0.052	40.6	0.031	0.376	0.164 ± 0.054	32.7
NH_4-N loading rate (mg N/gVSS.day)	2.61	26.95	16.88 ± 6.03	35.8	6.42	82.67	32.80 ± 11.1	34.5
PO_4-P loading rate (mg P/gVSS.day)					0.534	8.526	3.133 ± 1.609	51.4