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Title

Aeration control strategies to stimulate simultaneous nitrification-denitrification via nitrite during the formation of aerobic granular sludge

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Abstract

In this study a sequencing batch reactor (SBR), treating synthetic wastewater (COD/N = 5), was operated in two stages. During stage I, an aeration control strategy based on oxygen uptake rate (OUR) was applied, to accomplish nitrogen removal via nitrite >80%. In stage II, the development of aerobic granular sludge (AGS) was examined while two aeration control strategies (OUR and pH-slope) maintained the nitrite-pathway and optimized the simultaneous nitrification-denitrification (SND) performance. Stimulation of slow growing organisms, (denitrifying) polyphosphate accumulating organisms (D)PAO and (denitrifying) glycogen accumulating organisms (D)GAO, lead to full granulation (at day 200, SVI_{10}= 47.0 mL/g and SVI_{30}= 43.1 mL/g). The average biological nutrient removal efficiencies, for nitrogen and phosphorus, were 94.6% and 83.7% respectively. Furthermore, the benefits of an increased dissolved oxygen concentration (1.0 – 2.0 mg O_2/L) were shown as biomass concentrations increased with approximately 2 g/L, specific ammonium removal rate and phosphorus uptake rate increased with 33% and 44% respectively. It was shown that the combination of both aeration phase length control strategies provided an innovative method to achieve SND via nitrite in AGS.

Keywords

Aeration control strategies; aerobic granular sludge, nitrite pathway; SBR; dissolved oxygen; biological nutrient removal

INTRODUCTION

Biological nutrient removal (BNR) from wastewater strongly relies on alternating operational conditions, i.e. aerobic, anoxic and anaerobic. Therefore, aerobic granular sludge (AGS) has been proposed as an very competitive alternative for the conventional activated sludge process (CAS) (Beun et al. 1999; De Kreuk et al. 2005; Adav et al. 2008). In AGS, aerobic and anoxic zones are integrated in the granule which makes it an ideal technology for simultaneous nitrification-denitrification (SND). In order to promote stable AGS formation, the SBR process offers the opportunity for a slow anaerobic feeding and thus selection of polyphosphate accumulating organisms (PAO) or glycogen accumulating organisms (GAO) (de Kreuk and van Loosdrecht 2004). During anaerobic conditions PAO and GAO take up volatile fatty acids (VFA) and store them internally as polyhydroxyalkanoates (PHA). Using anaerobic feeding, de Kreuk and van Loosdrecht, (2004) obtained stable granule formation at low DO concentrations. Furthermore, PAO will also contribute to the phosphorus removal from the wastewater (Mino et al. 1998). Additional carbon savings can be obtained, if denitrification is accomplished by denitrifying PAO or GAO (DPAO, DGAO) (Zeng et al. 2003; Oehmen et al. 2010). These organisms consume internal storage polymers, PHA, in order to reduce nitrite or nitrate, and in the case of DPAO contribute to phosphate uptake as well.

In last years, simultaneous nitrogen and phosphate removal by AGS has received much attention. However, most studies reported conventional nitrification via nitrate (Kishida et al. 2006; Lemaire et al. 2008b; Zhang et al. 2011; Pronk et al. 2015). Several authors, (Yilmaz et al. 2008; Gao et al. 2011; Coma et al. 2012; Guimarães et al. 2016) found that nitrogen removal was mainly occurring via nitrite in their AGS systems. However, the underlying reasons for this short-cut nitrification-denitrification in AGS remain largely unexplored. Nitrogen removal via the nitrite pathway reduces the oxygen demand in the nitrification stage by 25% and the COD demand in the denitrification stage by 40%, resulting in 20% less CO_2 emission and 30-50% less sludge production (Peng and Zhu 2006). Recently, Dobbeleers et al. (2017) showed the formation of aerobic nitrite granules (ANG) treating industrial wastewater, which has the great advantage to feature the benefits of (1) aerobic granular sludge and (2) nitrogen removal via nitrite.

Previous research (Andreottola et al. 2001; Marsili-Libelli et al. 2008; Dries et al. 2013) about online control of BNR - SBR processes, employing cheap and reliable online sensors as dissolved oxygen (DO), pH and ORP, mainly focused on the optimization of the efficiency and energy consumption of the nitrogen removal process. Aeration phase length control based on DO was used by Blackburne et al. (2008) and Lemaire et al. (2008a) to achieve short-cut nitrification denitrification via nitrite. However, little is known about the influence of aeration control strategies on the formation and performance of ANG.

This study investigates the formation and maintenance of ANG, in 2 stages, through the use of aeration phase length control strategies. During stage I, a strategy based on oxygen uptake rate (OUR) was used in order to achieve nitritation-denitritation in a conventional BNR system (Blackburne et al. 2008; Lemaire et al. 2008a). In stage II, the objective was to use of an additional aeration phase length control strategy based on pH-slope, to transform the conventional activated sludge into AGS, while SND was stimulated and abundant nitrite accumulation was avoided as much as possible. Moreover, the influence of the DO concentration on the SND and BNR performance was examined as well. In addition, through the combination of specific activity measurements, quantitative real-time polymerase chain reaction (qPCR) of the most important bacterial groups and analysis of the sludge parameters we aimed to elucidate the key-factors determining ANG.

MATERIALS AND METHODS

Reactor set-up

A lab-scale SBR with an internal diameter of 230 mm and maximum height of 400 mm was used in this study (H/D = 1.74). Feeding was performed by an electromagnetic IWAKI® ES pump (Tessenderlo, Belgium). Effluent withdrawal was accomplished by an electric ER-PLUS® valve (Eriks, Hoboken, Belgium) which was located on a height of 220 mm. A heidolph® RZR 2020 (Schwabach, Germany) mechanic mixer was used to maintain the sludge in suspension. An air pump (Rena, Annecy, France) connected to a ceramic air diffuser provided sufficient oxygen distribution through the mixed liquor.

The SBR sequence and performance of all hardware actions (mixer, air pump, feeding pump and discharge valve) were controlled by a Siemens® programmable logic controller (PLC) (Beersel, Belgium) which could be manipulated by a custom-build LabVIEW (National Instruments, Texas, USA) supervision program. Additionally, DO, pH and oxidation reduction potential (ORP) (Hach, Mechelen, Belgium) were continuously measured and visualized by the LabVIEW supervision program.

Reactor operation

The SBR was operated at room temperature (18-22°C) in two separate stages. For stage I, seed sludge from the local WWTP (Antwerpen – zuid, Belgium) was used (3.5 gMLSS/L;SVI_{30} = 256.2 mL/g) . Each cycle 2L of wastewater was pumped into the reactor, resulting in a volume exchange ratio (VER) of 17%. The SBR cycle (Fig. S1) consisted of a pre-aeration phase (30 min) followed by 3 consecutive loops containing an anoxic feeding (20 min) followed by an extended anoxic phase (40 min) and an aerobic period (phase length control, minimum 20 min, maximum 45 min), thereafter one anoxic (40 min) - aerobic loop (phase length control, minimum 20 min, maximum 45 min) without feeding was executed, including a post-denitrification step (60 min), settling (120 min) and effluent withdrawal (10 min). During the aerobic steps, the DO level was controlled, using an on-off regulator, between 0.7 and 1.5 mg O_2/L. The aeration phase length was supervised by the aeration control strategy (see 2.4). pH was not regulated and varied between 7.1 and 8.0.

For stage II, sludge from the end of stage I was used to inoculate the SBR. During start-up, the operational strategy was manipulated in order to promote granule formation. The SBR cycle (Fig. S1) consisted of a pre-aeration phase (30 min), an anaerobic feeding (30 min) with prolonged anaerobic phase (60 min), to select slow growing organisms, followed by alternating aerobic (phase length control, 20 – 240 min) and anoxic (30 – 120 min) conditions. Based on the applied operational parameters, stage II was subdivided into three periods, period IIa, the start-up, period IIb, operated at a low DO (0.5 - 1.0 mg O_2/L), and period IIc, operated at a higher DO (1.0 - 2.0 mg O_2/L) (Table 1). The VER was progressively raised from 17% during period IIa to 29% in period IIc while the settling time was gradually shortened. A specific online control strategy, based on pH-slope and OUR, was developed to secure the alternation between aerobic and anoxic phases, to maximize SND, to retain the nitrite pathway (see 2.4) and to maintain an optimized reactor performance.

In both stages, a constant SRT of 20 days was maintained by periodically removing the excess sludge from the reactor.

The synthetic wastewater

Synthetic wastewater was used and had the following composition (mg/l): NaAc (1300), NH_4Cl (765), KH_2PO_4 (70) (stage I) ; KH_2PO_4 (162) (stage II), $MgSO_4$ (6); KCl (1), $NaHCO_3$ (550) and 1 mL/L of the trace elements solution described by Vishniac and Santer (1957). This resulted in following influent concentrations: 1000 mg COD/L, 200 mg NH_4-N/L and 16 mg P/L (stage I); 37 mg P/L (stage II).

Aeration control strategies

To achieve the nitrite-pathway, during stage I, an aeration control strategy based on the OUR was used. As demonstrated by Blackburne et al. (2008) and Lemaire et al. (2008a) a sharp drop in OUR will appear as soon as NH_4^+ oxidation is almost accomplished. By the use of an on/off DO control system, OUR values were calculated from the decreasing oxygen concentration. Through the use of a custom-build LabVIEW supervision program, the variable length of the aeration steps was determined by an adjustable OUR threshold value, minimum and maximum time. Every few days the OUR threshold was examined and adjusted if necessary.

For stage II, an additional control strategy, based on the pH profile was applied. Due to the on/off aeration control, the pH demonstrated a saw-tooth profile (Fig. 6), which was used by the pH-slope algorithm. First, the average of each decreasing pH flank was calculated. Subsequently, over a moving period of 3 pH-flank-averages, linear regression was conducted, with as a result, the pH-slope. Our hypothesis was that, once the pH-slope became negative, nitrification was the primary reaction and nitrite started to accumulate (Spagni et al. 2001). This pH-slope strategy was used to control the length of the first aeration step in order to maximise SND and to avoid elevated concentrations of nitrite, which acts as a precursor for NOB stimulation. The following aeration steps were controlled by the OUR control strategy to supervise the oxidation of NH_4^+. In order to avoid substantial nitrite accumulation, the maximum time of these subsequent aeration phases was limited to 40 min.

In-situ cycle measurements

During stage I, in-situ cycle measurements during the aerobic periods were carried out at least once a week to determine the "nitritation-degree" (ND).

$$ND\ (\%) = \frac{[NO_2^-]_{t_0}^{t_n}}{[NO_2^-]_{t_0}^{t_n} + [NO_3^-]_{t_0}^{t_n}}\ x\ 100 \qquad [1]$$

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

Through stage II, in-situ cycle measurements were performed during the total length of a SBR cycle, samples were taken every 10 - 30 min to obtain the profiles of nitrite, nitrate, ammonium and phosphate.

Ex-situ batch tests

Ex-situ batch tests were conducted in order to determine the specific phosphate uptake rates (PUR) with the different electron acceptors nitrite, nitrate and oxygen. The sludge was sampled from the SBR right before settling. Equal amounts (400 mL) of sludge were divided in separate beakers whereafter wastewater was added. In order to obtain maximum phosphate release and carbon uptake, an anaerobic step of 90 minutes was used. Subsequently, each electron acceptor was spiked to a different beaker. In case of the aerobic phosphate removal, an on/off DO control insured oxygen concentrations between 4 mg O_2 /L and 6 mg O_2/L. Furthermore,

allylthiourea (at 10 mg/L), was added in order to prevent nitrification (Ginestet et al. 1998). For the anoxic phosphate removal, a pulse of nitrate or nitrite was added to achieve an initial concentration of 40 mg NO_3-N/L and 10 mg NO_2-N/L. The nitrite dosage was kept low in order to avoid the potential inhibition caused by HNO_2 (Saito et al. 2004). Therefore, on regular times, the NO_2-N concentration was determined and when necessary an additional dosage of 10 mg NO_2-N/L was added. The pH was maintained between 7.4 and 8.0 throughout the entire experiment.

Microbial activity measurements and molecular quantification

The microbial activity measurements and qPCR procedure for molecular quantification were conducted as described by Dobbeleers et al. (2017).

Briefly, a custom-build respirometer was used to determine the specific nitrogen removal rates (SR) of both ammonium oxidizing bacteria (AOB), SR_{AOB}, and nitrite oxidizing bacteria (NOB), SR_{NOB}.

DNA extraction from sludge samples was carried out with the NaTCA method (McIlroy et al. 2009). Quantitative PCR analysis of the most important bacterial groups, AOB, NOB, PAO, GAO and the total 16S rRNA bacterial abundance, Eubacteria, was performed with primers, amoA-1F/amoA-1R targeting *Nitrosomonas* (Rotthauwe and Witzel 1997), NxrBF169/NxrBR638 targeting *Nitrospira* (Pester et al. 2014), PAO541f/PAO846r targeting 16S rRNA of *Candidatus Accumulibacter* (Fukushima et al. 2007), GAOQ989f/GAM1278r targeting 16S rRNA of *Candidatus Competibacter* (Nielsen et al. 1999; Crocetti R. et al. 2002) and Universal1055f/Universal1392r (Ferris et al. 1996). For every sample, the MLVSS and DNA concentration were used to calculate the amount of target cells per g biomass and the NOB/AOB absolute quantification ratio.

Analytical methods

All samples were filtered over a glass microfiber filter (particle retention 1.2 µm) in order to measure the concentrations of phosphate (Hach, Mechelen, Belgium), COD (Hanna Instruments, Temse, Belgium), ammonium (Hanna Instruments, Temse, Belgium), nitrite (Hach, Mechelen, Belgium) and nitrate (Hanna Instruments, Temse, Belgium) with standard cuvette tests. Biomass concentrations and Sludge Volume Index (SVI) were determined according to Standard Methods (APHA 1998). The evolution of the sludge morphology was observed using a MOTIC microscope (Xiamen, China).

SND efficiency

According to Dobbeleers et al. (2017), calculation of the SND was based on the total amount of NO_x-N formed divided by the amount of NH_4-N oxidized. A distinction was made between SND_1, during the first aeration step, and the cumulative SND_c throughout the complete reaction cycle.

$$SND_1\ (\%) = 1 - \left(\frac{[NO_x^-]_{t_0}^{t_n}}{[NH_4^+]_{t_n}^{t_0}}\right) x\ 100 \qquad [2]$$

With t_0 start of aeration 1 and t_n the end of aeration n;

$$SND_c\ (\%) = 1 - \left(\frac{[NO_x^-]_{t1_0}^{t1_n}}{[NH_4^+]_{t1_n}^{t1_0}} + \frac{[NO_x^-]_{t2_0}^{t2_n}}{[NH_4^+]_{t2_n}^{t2_0}} + \frac{[NO_x^-]_{t3_0}^{t3_n}}{[NH_4^+]_{t3_n}^{t3_0}} + \frac{[NO_x^-]_{t4_0}^{t4_n}}{[NH_4^+]_{t4_n}^{t4_0}}\right) x\ 100 \qquad [3]$$

With $t1_0$ start of aeration 1 and $t1_n$ the end of aeration ; $t2_0$ start of aeration 2 and $t2_n$ the end of aeration 2; ….

RESULTS

Stage I: obtaining the nitrite-pathway

During stage I, a step-feed SBR with aeration phase length control was operated during 165 days to achieve nitrogen removal via nitrite for > 80%. Therefore, the ND-degree and NOB/AOB activity ratio were periodically analysed (Fig. 1A). Based on the results of these ND-degree and NOB/AOB activity, three consecutive periods were defined; start-up, Ia (day 1-27), transition, Ib (day 28 – 105) and maintenance, Ic (day 106 -165). During the start-up period Ia, some minor improvement could be observed concerning both parameters (See Table S1 for more details). More significant is the enhancement in the transition period Ib, where the ND-degree increased from 12.8% towards 100.0%. Meanwhile, the NOB/AOB activity ratio decreased from 63.6% to 21.1%. During period Ic; the nitrite-pathway was maintained for 60 days with an average ND of 99% and an average NOB/AOB activity ratio of 12.1%. In the same time, the specific nitrogen removal SR_{NOB} decreased from 6.5 mg N/gVSS.h in period Ia to 1.3 mg N/gVSS.h in period Ic. In addition, it has to be mentioned that during the entire operational period, N-removal was above 90% with an average of 95.3%.
Fig. 1B presents a specific in-situ measurement on day 81 (stage I). As nitrification proceeds, declining NH_4^+-N concentrations and rising NO_x^--N concentrations can be observed. Moreover, it is shown that, the sharp drop of OUR already occurs before NH_4^+-N is fully depleted.

Stage II: transforming the floccular activated sludge into aerobic granular sludge while maintaining the nitrite pathway

Performance of the nitrite-pathway and nutrient removal
Sludge from stage I, period Ic, was used to inoculate the SBR in stage II. A slow anaerobic feeding was chosen to stimulate PAO and/or GAO in order to promote aerobic granulation.
Fig. 2 plots the NOB/AOB activity ratio, N and P removal during stage II. Throughout the entire stage II operation, N-removal was above 90% (average 94.6 ± 5.6) with just a few exceptions due to some aeration pump failures. Furthermore, the average P-removal was 83.7 ± 8.6% and the average COD-removal was 93.8 ± 3.2% (data not shown). From the start of stage II, the NOB/AOB activity ratio was below 10% and remained this low for approximately 300 days. The average SR_{AOB} was 9.6 ± 3.2 mg N/gVSS.h, while the average SR_{NOB} was only 0.4 ± 0.3 mg N/gVSS.h. These findings illustrate that, during stage II, BNR was mainly performed via the nitrite-pathway. However, minor activity of NOB was observed at all time.

The seed sludge, which was used to start-up stage I, from the WWTP (Antwerpen – zuid), and sludge at the end of stage II (period IIc), were extracted and qPCR analysis was conducted for specific targets of AOB, NOB, PAO, GAO and the total 16S rRNA of Eubacteria (Fig. 3). Important to note that the investigated target organisms , except for the PAO which remained more or less constant, were relatively more abundant at the end of the reactor operation. This illustrates the positive effect of a well-defined reactor strategy on the microbial community. Specific for the nitrifiers (Fig. 3), the relative increase of the AOB population was 2.5 times higher than the relative increase of the NOB population. Furthermore, the major increase of the GAO population, from 0.03% to 9.18%, at the end of stage II did not affect the EBPR performance. As the PAO/GAO ratio decreased from 37.80 ± 5.75 to 0.11 ± 0.01, the P-removal increased above 90% at the end of stage II.

Progress of granulation

During stage II, sludge characteristics were monitored intensively. Fig. 4 shows the progress of MLSS and SVI throughout the entire SBR operation. As the seeding sludge from stage II originated from stage Ic, poor settling could be observed (SVI_{10} = 300.9 mL/g ; SVI_{30} = 220.2 mL/g). In period IIa, a sharp drop in SVI values could be noticed, reaching, SVI_{10} = 110.1 mL/g and SVI_{30} = 67.1 mL/g at day 35. The MLSS concentration increased from 2.69 g/L on average in period IIa to 3.62 g/L in period IIb. In addition, low SVI values (on average, SVI_{10} = 67.4 mL/g and SVI_{30} = 48.3 mL/g), could be maintained during this period, corresponding to well settling sludge. Increasing the DO concentration in period IIc resulted in higher MLSS concentrations, from 3.95 g/L on average during the first 42 days to 5.31 g/L on average during the last 73 days. On day 284, SVI values of 26.0 mL/g for SVI_{10}, 25.2 mL/g for SVI_{30} were obtained. The corresponding SVI_{10}/SVI_{30} ratio was 1.03, which has been stated as an excellent indication of granule formation (de Kreuk et al. 2007).

The improved sludge settling characteristics could also be related to the sludge morphology which shows small aggregation between day 1 and day 51 (Fig. 5). Later, in period IIb, on day 76, the first granule-shaped biomass could be observed. Thereafter, on day 118, dark spots appeared which suggests the formation of anaerobic/anoxic zones (Fig. 5). The size of the sludge increased to a maximum of 0.5 mm. During the final period IIc, mature, strong and regular shaped granules with an average size between 0.5 and 1 mm were observed, which supports the excellent settleability and high biomass retention which was discussed previously.

Performance of ANG under the control of aeration phase length control strategies

The objective of present study was to exploit BNR using SND via nitrite as much as possible through the use of aeration phase length control strategies. Fig. 6 shows a typical in-situ cycle measurement (stage IIc, day 257). During the anaerobic feeding phase, release of phosphate was observed while acetate was taken up, according to the storage of PHA by PAO. These effects can also be observed from the pH-profile. In the first aerobic phase, after the anaerobic feeding, a strong decline in phosphate and ammonium can be observed, whereas only a small amount of nitrite (3 mg NO_2-N/L) accumulated and no nitrate was measured (Fig. 6A). Comparing this minor nitrite production to the significant amount of ammonium being oxidized (27.2 mg NH_4-N/L), clearly confirms the occurrence of SND. The initial pH increase during the first aerobic phase results from the oxidation, by (D)PAO, of the stored organic matter. Subsequently, as nitritation becomes the primary reaction, a pH decrease can be observed. As presented in Fig. 6B, the first aerobic phase ended as soon as the pH-slope exceeded -0.25 pH units/h. Using this threshold value, an enhanced SND performance could be achieved while abundant nitrite accumulation was avoided.

Thereafter, in the first anoxic phase, a complete denitritation could be observed. Moreover, a decline in the phosphate concentration was detected as well, suggesting the presence of DPAO. During subsequent aerobic conditions, most of the stored organic matter is degraded, and thus no longer available for denitritation, which causes elevated nitrite accumulation. (Fig. 6A). As result of these increased nitrite accumulation, the SND obtained, during the consecutive aerobic phases decreased (SND_1= 70.2%, SND_2 = 32.0%, SND_3 = 23.0% and SND_4 = 10.0%).

While the pH-slope strategy was used to control the first aerobic phase, the subsequent aerobic phases were controlled by the OUR control strategy in order to regulate the ammonium oxidization and avoid abundant nitrite accumulation, as discussed above (see 3.1). This OUR control strategy is illustrated in Fig. 6B, where the aeration periods finishes as the OUR drops below a threshold value of 0.89 mg O_2/L.min (= 53 mg O_2/L.h) during loop 4.

Influence of the DO on the SND-BNR performance of ANG

In stage II of present study, distinction was made between, period IIb (DO 0.5 - 1.0 mg O_2/L) and period IIc (DO 1.0 – 2.0 mg O_2/L) to examine the influence of DO on the SND and BNR performance (Table 2). It was shown that, for both periods, the average SND_c, over the whole SBR cycle, was less compared to the average SND_1, during the first aeration loop. This findings correspond to the previous results, discussed in section 3.2.3, where the SND performance showed a decrease during consecutive aerobic phases. Due to the higher DO in stage IIc, 2.3% less SND_1 and 13.2% less SND_c performance was achieved (Table 2). However, due the higher DO concentrations, the N-loading rate increased with approximately 25% and the COD-loading rate or F/M ratio increased with almost 30% (table 2).

In addition, in-situ cycle measurements and ex-situ activity measurements were conducted throughout the different periods of stage II (IIb and IIc). These data were used to calculate the overall ammonium uptake rate (AUR), SR_{AOB}, SR_{NOB} and phosphorus uptake rate (PUR), (table 3).

As stated before the increased DO, in period IIc, led to a slightly lower SND (table 2). However, the AUR and the SR_{AOB} on the other hand, increased with 33% and 24% respectively. This increased ammonium uptake rates confirm the enhanced nitrogen loading rate of 25% during stage IIc. A negligible activity was obtained for SR_{NOB} (table 3), indicating the excellent performance of the nitrite-pathway. Finally, the PUR obtained during normal reactor conditions, increased with 44%.

During stage IIc several ex-situ batch experiments were performed to determine the PAO and DPAO activities. In order to investigate DPAO activity, batch tests were conducted with either nitrite or nitrate as electron acceptor. Table 4 summarises the different PUR values together with the PUR_{DPAO} / PUR_{PAO} ratio. These results show that the aerobic PUR is approximately twofold compared to the anoxic PUR (Meinhold et al. 1999) and that, both, nitrite and nitrate could be used as electron acceptor, but PUR obtained with nitrite, was 17% higher.

DISCUSSION

Achieving nitrogen removal via nitrite

The achievement of the nitrite route using an OUR-based aerobic phase length control strategy, in stage I, is in agreement with earlier reports by Blackburne et al. (2008) and Lemaire et al. (2008a). The use of this OUR drop in order to finish the aeration step allows to switch immediately to the next anoxic feeding step (denitrification). As a consequence, NOB will receive less energy for growth during aerobic phases, which ultimately leads to a repressed population (Lemaire et al. 2008a). Furthermore, Lemaire et al. (2008a) reported 80-100% nitrite accumulation after 100 days through the use of aerobic phase length control, which corresponds to the time needed in our study. Moreover, a strong negative correlation (Pearson correlation = -0.91) between the ND and NOB/AOB activity ratio could be determined (Fig. 1A), which evidences the use of the NOB/AOB activity ratio in stage II, to keep track of the nitrite pathway.

Molecular quantification by the use of qPCR, confirms the increasing SR_{AOB} throughout stage I and II, but does not explain the decreasing SR_{NOB}. Nevertheless, a certain parallel between the NOB/AOB activity ratio and NOB/AOB quantification ratio can be observed, for the inoculum 94.2% versus 110.5% and at day 319, 10.5% versus 43.2%. These results suggest that NOB are clearly inhibited in their activity (0.4 ± 0.3 mg N/gVSS.h), however, their presence in the biomass remains remarkable.

Effect of aeration phase length control strategies on the performance of ANG

Comeau et al. (1986) proposed an anaerobic biochemical model for PAO, in which proton transfer from the cell is described during the anaerobic acetate uptake. Spagni et al. (2001) translated this mechanism towards a decreasing bulk media pH which is related to anaerobic PO_4^+-P release. In contrast, in the aerobic/anoxic biochemical model, the presence of an electron acceptor is used, to create proton transfer towards the cell which allows phosphate uptake (Comeau et al. 1986). According to this aerobic model, the bulk media pH will increase as long as phosphate uptake proceeds. These knowledge was used as a starting point to develop the pH-slope aeration control strategy (see 2.4). During the first aerobic phase, SND occurred as only minor amounts of nitrite accumulated (Fig. 6). Moreover, it was shown that this SND was associated with significant phosphate uptake as well, suggesting anoxic phosphate uptake, by DPAO, was the main BNR pathway. DPAO can use stored PHA for growth and phosphate uptake, which results in a proton uptake from the mixed liquor (Fig. 6). However, as aerobic conditions continue, the stored PHA gets strongly reduced, which results in an unbalanced nitritation-denitritation process. Less protons will be removed by (D)PAO while nitritation retains its proton production leading to an overall pH decrease (Ahn 2006). The importance of the pH-slope aeration control strategy is, that it can simultaneously monitor (anoxic) phosphate uptake as well as nitrifying activity, which makes it a perfect control strategy to maximize DPAO based SND as much as possible and prevent the accumulation of elevated nitrite concentrations. Guisasola et al. (2007) also showed that the use of a negative proton production rate could be used for the online monitoring of the EBPR process. Our study proved that, the average SND_1, was at least 88.3 ± 5.2% (Table 2) at a COD/N ratio of 5, indicating the usefulness of the pH-slope strategy. Moreover, the combination of the OUR strategy which controls the NH_4^+-N oxidation, and the pH-slope strategy to maximize the DPAO based SND as much as possible, provides a dynamic energy efficient ANG process, which makes this technology perfectly suitable for small industries without the necessity of high investment or operational costs. Moreover, for an even better time management and reactor efficiency, control strategies considering anaerobic and anoxic periods could be very advantageous to implement. Several authors (Kishida et al. 2003; Won and Ra 2011; Dries 2016) already described the opportunities of online pH, ORP and conductivity measurements to determine the end of the denitrification phase. Ultimately, it would be valuable to develop a dynamic system which regulates the entire SBR cycle for an efficient nutrient removal, formation and maintenance of granulated biomass.

Influence of the DO concentration on the SND

As mentioned in section 3.2.4, the higher DO in stage IIc resulted in 2.3% less SND_1 and 13.2% less SND_c (Table 2). These results are in line with previous research, stating low DO as an important factor to achieve enhanced SND performance (Zeng et al. 2003; Third et al. 2003; de Kreuk and van Loosdrecht 2004; Kishida et al. 2006).

However, the enhanced loading rates for COD and N, emphasize the importance of finding the right balance between DO level, SND performance and process efficiency. Similarly, Coma et al. (2012) reported more than 50% SND, at a constant DO of 2.0 mg O_2/L, when nitrite was present at low concentrations in the effluent. Yilmaz et al., (2008) achieved 85.7% and 62.5% SND during respectively, the first 3h and the entire aerobic phase at a DO (2.0 - 2.5 mg O_2/L), which clearly supports our findings related to SND_1 and SND_c .

(D)PAO and (D)GAO pathways for nitrogen removal

The ex-situe anoxic batch tests with nitrite and nitrate showed that both could be used by DPAO, however, the reduction of nitrite turned out to be more favorable for phosphate uptake. These findings are in agreement with Flowers et al. (2009), who suggested the existence of two DPAO clades: DPAO I, with the ability to reduce nitrate, and DPAO II, that can only use nitrite to accomplish anoxic phosphate uptake. In contrast, Saad et al. (2016) found that neither DPAO I nor DPAO II can reduce nitrate. They suggest the presence of flanking bacteria which are in symbiosis with DPAO for the reduction of nitrate to nitrite in a primary step. Bassin et al. (2012) also proved a symbiotic relationship between DGAO that convert nitrate to nitrite, and DPAO II that reduce nitrite to nitrogen gas. Similar as in our study, Yilmaz et al. (2008) operated an anaerobic-aerobic granular sludge system and found that nitrogen was mainly removed via nitrite. However, they observed almost no DPAO activity with nitrate as electron acceptor.

In our study, the development of slow growing organisms (PAO and GAO) was the main factor to stimulate granulation in stage II (de Kreuk and van Loosdrecht 2004; Pronk et al. 2015). Phosphorus removal efficiencies and PUR's suggest the presence of an enriched PAO population. However, according to the qPCR analysis it was the GAO population which increased the most. These findings illustrate the presence of both of PAO and GAO in the granules without undesirable effects on the P-removal. Bassin et al. (2012) also experienced full phosphorus removal using a mixed culture of PAO and GAO. In future work, it would be advantageous for the ANG process to analyse the distribution of the PAO clades I and II.

This study describes an innovative technology to treat wastewaters with low COD/N values regulated by a dynamic aeration control strategy. The combination of the 2 aeration phase length control strategies proved that, (1) AGS could be formed, (2) SND could be maximized and (3) that nitrogen was removed via nitrite. Unlike in other studies which reported AGS in combination with N-removal via nitrite (Yilmaz et al. 2008; Coma et al. 2012; Bassin et al. 2012; Guimarães et al. 2016), this study provides a great opportunity to achieve AGS performing SND via nitrite, in a controlled manner.

COMPLIANCE WITH ETHICAL STANDARDS

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1 Summary of the different periods of stage II and the applied parameters

Day	Period	VER (%)	DO (mg/L)	Settling time (min)
1-35	IIa	17-23	0.5-1.0	30-15
36-202	IIb	23	0.5-1.0	15-10
203-319	IIc	23-29	1.0-2.0	10-4

Table 2*:* Influence of the DO on the SND (SND_1 and SND_c) and the loading rates (mg COD, N/gVSS.day) in stage II (Average ± SD)

	SND_1 (%)	**SND_c (%)**	**COD - loading (mg COD/gVSS.day)**	**N - loading (mg N/gVSS.day)**
Stage IIb (DO: 0.5 – 1.0 mg/L)	90.4 ± 2.6	76.5 ± 4.6	159.5 ± 33.7	35.6 ± 6.1
Stage IIc (DO: 1.0 – 2.0 mg/L)	88.3 ± 5.2	66.4 ± 7.7	224.1 ± 36.9	46.8 ± 8.9

Table 3 Specific nitrogen uptake rate (AUR) and phosphorus uptake rate (PUR) during stage II, obtained during normal reactor operation (in-situ measurement), and maximum specific ammonium uptake rate (SR_{AOB}) and nitrite uptake rate (SR_{NOB}) during stage II, obtained by ex-situ activity measurements. Distinction is made for the different DO conditions during the SBR operation (Average ± SD)

	In-situe		*Ex-situe*	
	AUR (mg NH_4-N/gVSS.h)	**PUR** (mg PO_4-P/gVSS.h)	**SR_{AOB}** (mg NH_4-N/gVSS.h)	**SR_{NOB}** (mg NO_2-N/gVSS.h)
Stage IIb (DO: 0.5 - 1.0 mg/L)	5.4 ± 0.7	11.6 ± 1.7	8.7 ± 2.9	0.3 ± 0.1
Stage IIc (DO: 1.0 – 2.0 mg/L)	7.2 ± 2.0	16.7 ± 2.9	10.8 ± 3.3	0.5 ± 0.4

Table 4*:* Maximum specific phosphate uptake rates (PUR) during stage II, obtained by ex-situ batch experiments for different electron acceptors; nitrite, nitrate and oxygen. All experiments were conducted during stage IIe (Average ± SD)

	PUR **(mg PO_4-P/gVSS.h)**		
electron acceptor	*NO_2-N*	*NO_3-N*	*O_2*
	9.7 ± 0.9	8.4 ± 0.8	18.4 ± 0.2
PUR_{DPAO}/PUR_{PAO}	0.53	0.46	

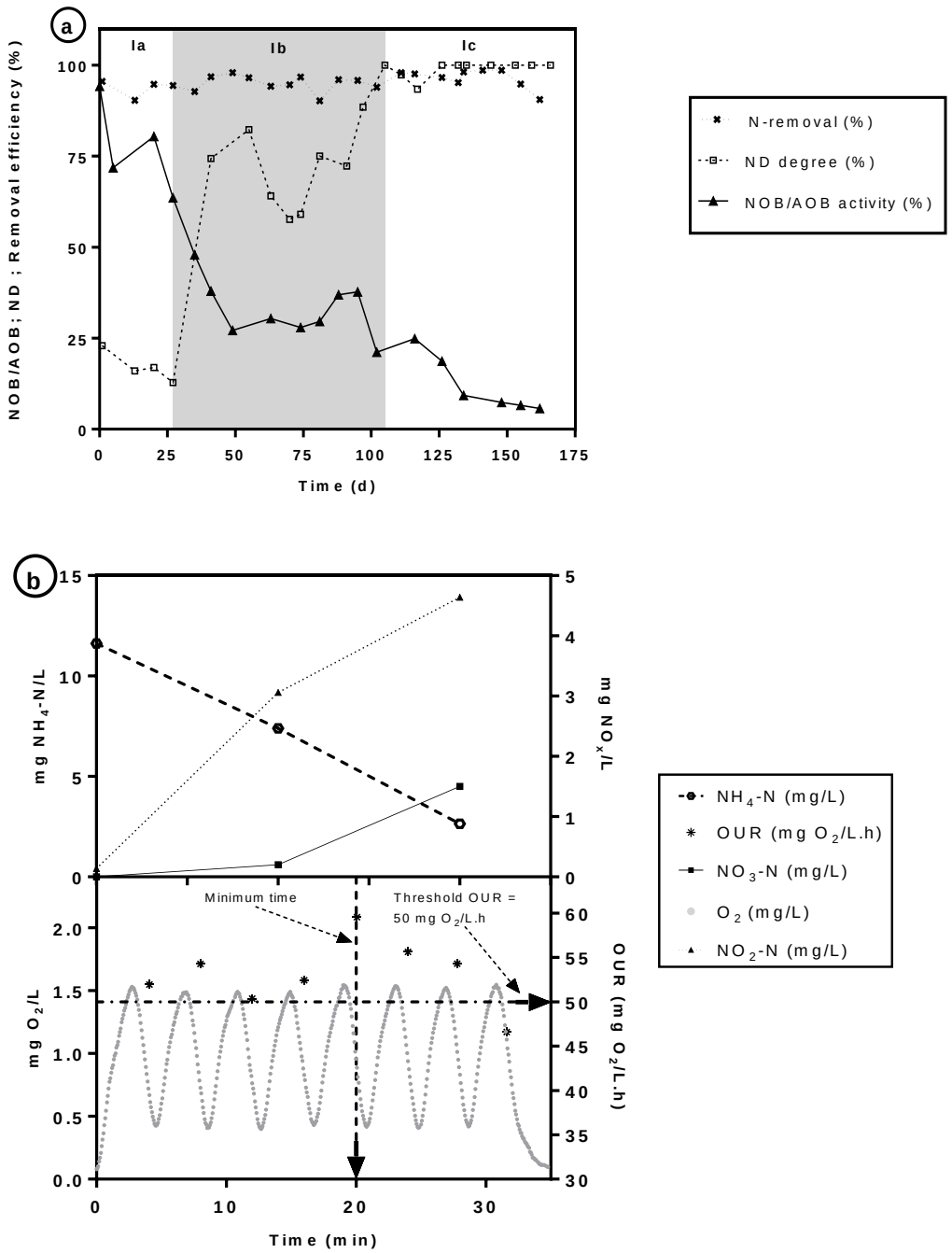

Fig. 1 Development of the nitrite-pathway (NOB/AOB activity ratio; ND) and performance of the N-removal during stage I. (Ia: start-up period; Ib: transition period; Ic: maintenance period) (a) In-situ measurement performed on day 81 (stage I – aeration loop 3): profiles of NH_4^+-N, NO_3^--N and NO_2^--N and progress of the OUR control strategy (b)

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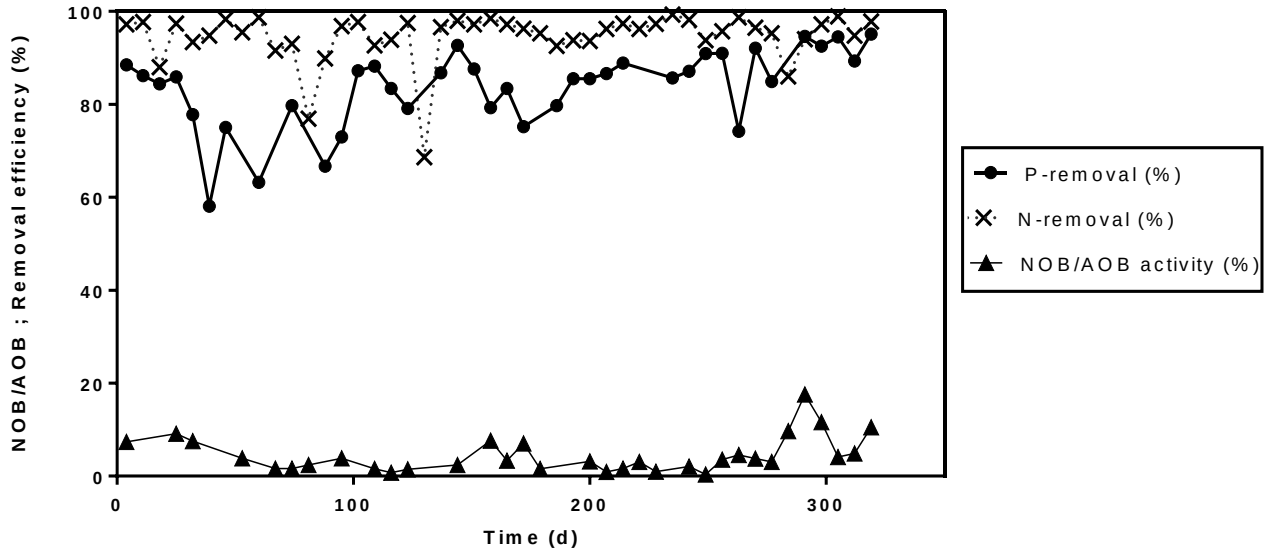

Fig. 2 Performance of the nitrogen and phosphorus removal; maintenance of the nitrite-pathway in stage II

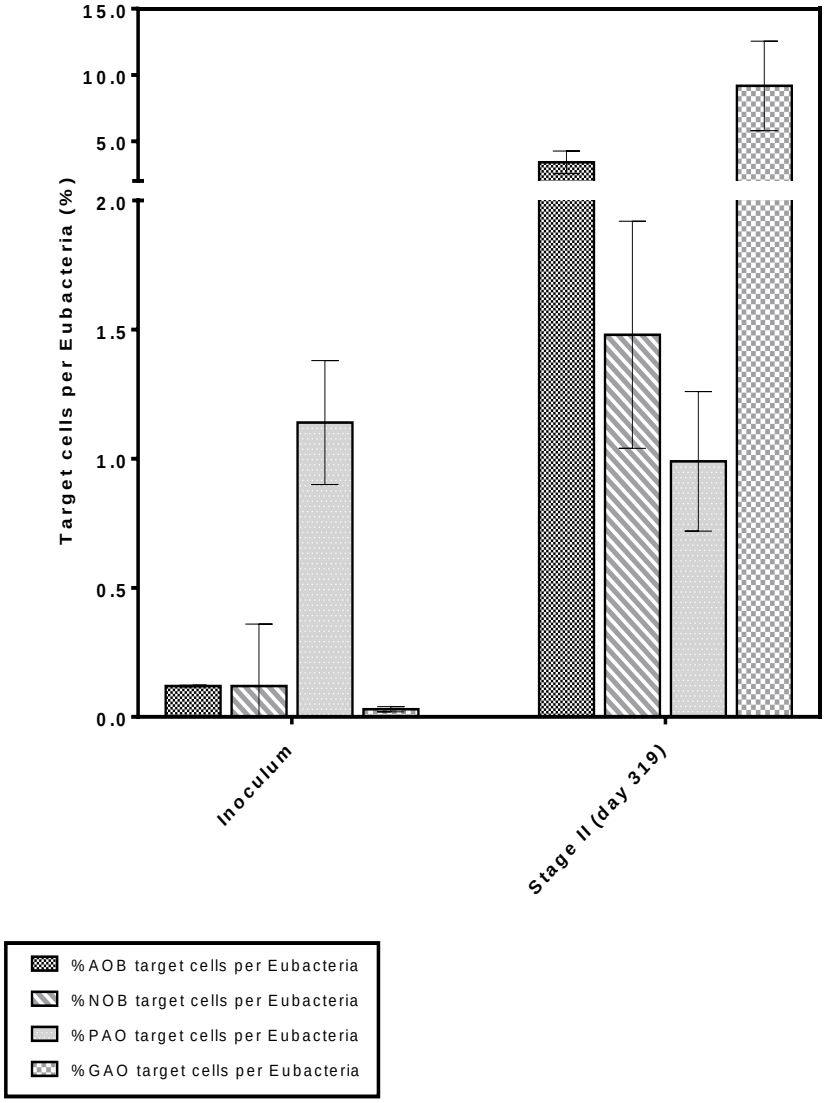

Fig. 3 Relative abundance of the specific AOB, NOB, PAO, GAO target cells per Eubacteria; biomass sampled from the inoculum (stage I) and at day 319 (stage II) (error bars = SD)

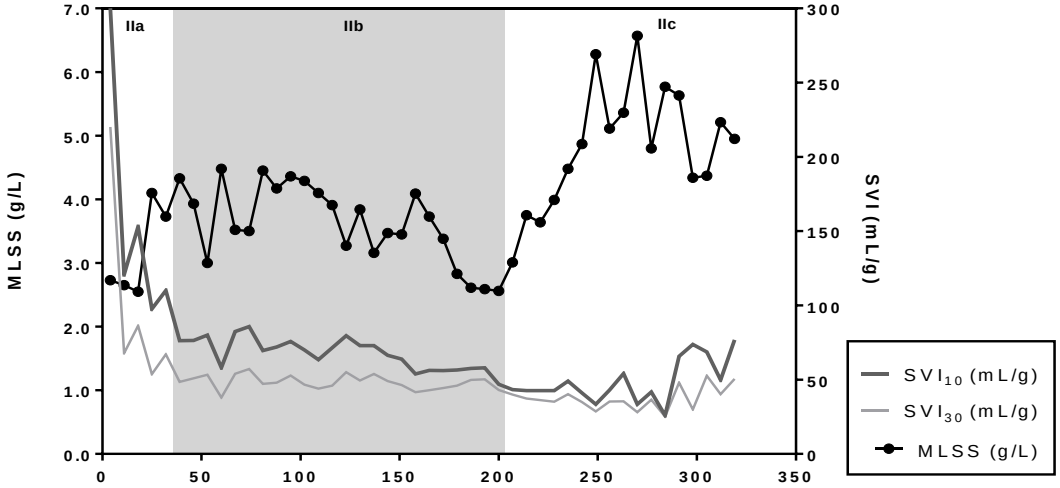

Fig. 4 Evolution of the sludge parameters during stage II: MLSS, SVI_{10}, SVI_{30} (See table 1 for the description of the different periods IIa, IIb and IIc)

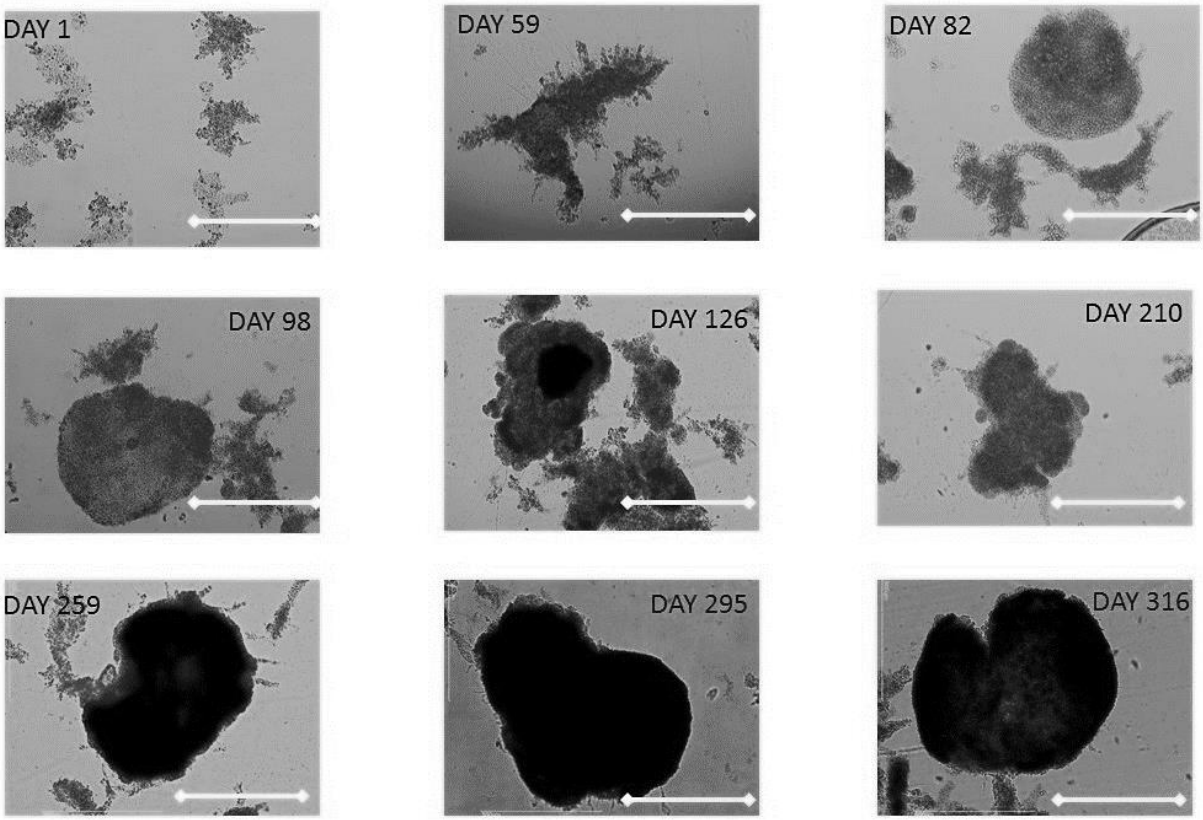

Fig. 5 Evolution of the sludge morphology during stage II (bar = 500 µm)

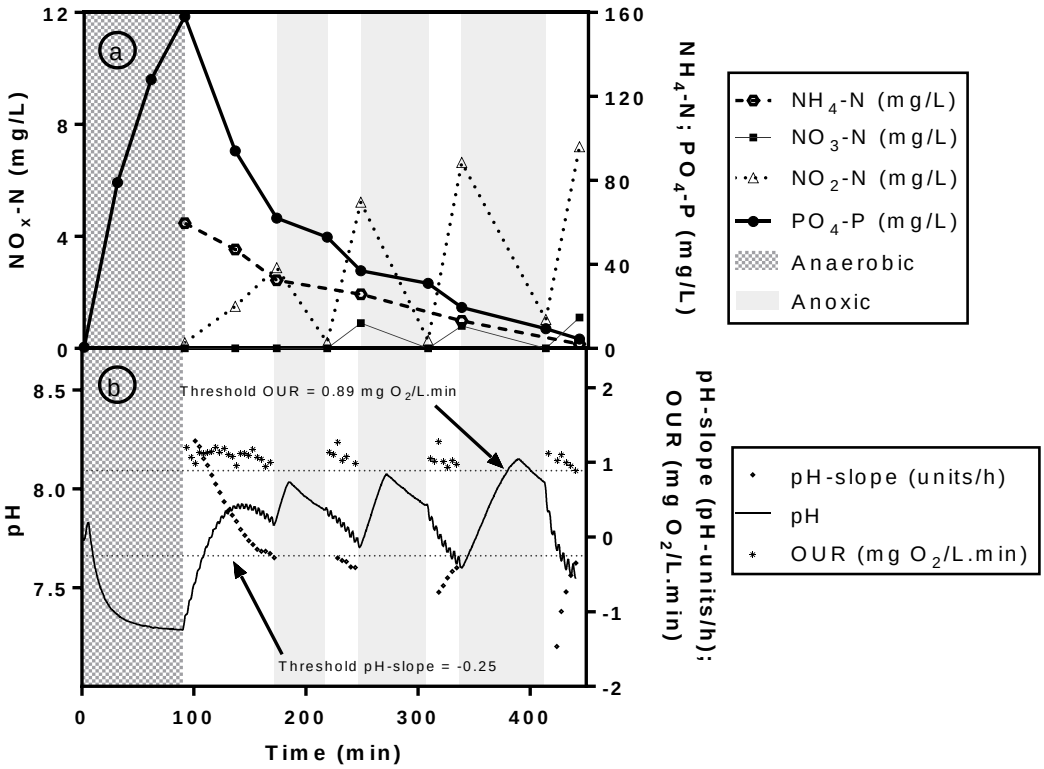

Fig. 6 Typical in-situ cycle measurement on day 257: Evolution of NH_4-N, NO_3-N, NO_2-N and PO_4-P (mg/L) (a) pH, pH-slope, OUR (mg O_2/L.min) profiles and threshold values of the aeration control strategies (b)