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# Oxygen control and stressor treatments for complete and long-term suppression of nitrite-oxidizing bacteria in biofilm-based partial nitritation/anammox

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## Abstract

Mainstream nitrogen removal by partial nitritation/anammox (PN/A) can realize energy and cost savings for sewage treatment. Selective suppression of nitrite oxidizing bacteria (NOB) remains a key bottleneck for PN/A implementation. A rotating biological contactor was studied with an overhead cover and controlled air/$N_2$ inflow to regulate oxygen availability at 20°C. Biofilm exposure to dissolved oxygen concentrations <0.51±0.04 mg $O_2$ $L^{-1}$ when submerged in the water and <1.41±0.31 mg $O_2$ $L^{-1}$ when emerged in the headspace (estimated), resulted in complete and long-term NOB suppression with a low relative nitrate production ratio of 10±4%. Additionally, weekly biofilm stressor treatments with free ammonia (FA) (29±1 mg $NH_3$-N $L^{-1}$ for 3 h) could improve the NOB suppression while free nitrous acid treatments had insufficient effect. This study demonstrated the potential of managing NOB suppression in biofilm-based systems by oxygen control and recurrent FA exposure, opening opportunities for resource efficient nitrogen removal.


**Keywords:** Deammonification; Nitrification; *Nitrotoga*; *Nitrospira*; *Kuenenia*


## 1. Introduction

The development of partial nitritation/anammox (PN/A) in the main stream of a sewage treatment plant is important for the transition to energy-neutral sewage treatment (Verstraete and Vlaeminck, 2011). To reach this goal, most organic carbon (COD) is separated from sewage in a first stage and valorised as biogas by anaerobic digestion, while ammonium is removed as nitrogen gas via PN/A in a second stage. This PN/A pathway relies on the teamwork of two types of autotrophic bacteria:

aerobic and anoxic ammonium-oxidizing bacteria (AerAOB and AnAOB). Nitrite-oxidizing bacteria (NOB) are another type of autotrophic bacteria that can proliferate in the system and generate undesired competition for nitrite (and oxygen) to produce nitrate, lowering the nitrogen removal efficiency. These NOB can be classified into seven known genera (Daims et al., 2016), of which mainly three are frequently detected in nitrification studies: *Nitrobacter*, *Nitrospira*, and *Nitrotoga* (Duan et al., 2019a; Gustavsson et al., 2020; Ma et al., 2017; Poot et al., 2016). Although PN/A is already widely used in sidestream (reject water) and industrial applications (Lackner et al., 2014), its implementation in the main stream is challenged by lower temperatures, lower influent nitrogen concentrations and fast fluctuating loading rates (Lotti et al., 2015).  Selective NOB suppression remains one of the main challenges to achieve mainstream PN/A (Agrawal et al., 2018).

A variety of potentially successful strategies for PN/A implementation is proposed in literature. In general, they can be classified as either ON/OFF (promotion/suppression) and IN/OUT (retention&seeding/wash-out) strategies, and the combination of both is required to achieve stable mainstream PN/A (Agrawal et al., 2018). In this IN/OUT framework, densely aggregated growth of biomass in granules or biofilms is mostly used to improve the retention of the slowly growing AnAOB, next to creating aerobic and anoxic zones for simultaneous AerAOB and AnAOB activity. From the available technologies biofilm-on-support and granule systems, a rotating biological contactor (RBC) with discs is a convenient model for PN/A studies, given its potential to easily grow and retain relatively thick biofilms (e.g. Antileo et al. (2007) and Courtens et al. (2014)).

For the ON/OFF control, maintaining a residual ammonium concentration is essential as NOB proliferate at low ammonium concentrations (Liu et al., 2019; Poot et al., 2016). Additionally, it protects AnAOB in the deeper biofilm layers from oxygen penetration and inhibition by creating a continuous oxygen demand by AerAOB (Lotti et al., 2015). Oxygen availability is another key control parameter, but the optimal settings are still under discussion. Most studies apply continuous aeration at rather low DO setpoints (<1.0 mg $O_2$ $L^{-1}$) for biofilm reactors (Gilbert et al., 2014; Laureni et al., 2019; Wang et al., 2018; Yang et al., 2017). Lowering the DO setpoint to enhance NOB suppression was therefore often successfully applied in the past (De Clippeleir et al., 2011; Laureni et al., 2019). However, NOB are known to adapt to low DO conditions, particularly *Nitrospira*, and thus threatening the long-term NOB suppression (Liu and Wang, 2013; Wang et al., 2020b). Additionally, some studies reported no or even a negative effect after lowering the DO setpoint for NOB suppression (Gilbert et al., 2014; Malovanyy et al., 2015; Wang et al., 2018). Further research on the aeration settings is therefore needed, specifically towards long-term NOB suppression in the biofilms which is often overlooked.

Next to residual ammonium and DO control, recurrent exposure of the biomass to stressors such as free ammonia (FA) and free nitrous acid (FNA) is frequently used for NOB suppression as they are reported to be more vulnerable to this stress compared to AerAOB (Anthonisen et al., 1976; Vadivelu et al., 2006). Both stressors can be generated in-situ using either the ammonium-rich reject water as such or after full nitritation as nitrite, with tested concentrations and frequency in respect to the practical feasibility (Peng et al., 2020). Numerous studies showed the potential of (recurrent) FNA or FA treatments on flocculent sludge for selective NOB suppression over AerAOB (Duan et al., 2019a; Wang et al., 2016; Wang et al., 2017). However, research on these treatments is mostly limited to solely partial nitritation while the few existing studies on PN/A often excluded the AnAOB fraction (present in the biofilm) from the treatment to safeguard its activity (Wang et al., 2020a; Wang et al., 2018). Yet, excluding the AnAOB biofilm from treatments can result in a migration of NOB activity towards the biofilm as observed by Peng et al. (2020). Another potential risk is the adaptation of the NOB community towards these recurrent treatments (Duan et al., 2019a; Peng et al., 2020; Wang et al., 2016). More attention should therefore be given to the exposure of the biofilm, especially to AnAOB, by FNA or FA treatments to ensure long-term and robust NOB suppression in a PN/A system.

To advance the insights in controlling granules and biofilms for mainstream PN/A applications, this study focussed only on metabolic ON/OFF strategies based on (i) the oxygen regime, (ii) the use of chemical stressors FA and FNA, (iii) while continuously maintaining an ammonium residual (10±5 mg N $L^{-1}$). The oxygen regime focussed on the biofilm's alternated exposure to the reactor liquid (submerged DO), which was not controlled, and the headspace above the liquid (emerged DO), which could be controlled and was unique for this study. For the FA and FNA treatments, tested conditions were realistic with regards to typically available nitrogen from the side stream, with special attention to changes in the microbial community. Contrary to most other studies, the AnAOB-rich biofilm was included in these treatments. As a reactor configuration solely based on biofilm, an RBC with mature biofilm was operated under mainstream conditions for 822 days (20±2°C, 47±4 mg $NH_4^+$-N $L^{-1}$ influent). Synthetic sewage was chosen over real sewage to minimize variations over time as stability was needed to achieve generic insights. Additionally, as no or minimal

COD was present, it was more challenging to achieve low effluent nitrate levels as denitrification couldn't occur, but it allowed the calculation of AerAOB, NOB and AnAOB activity. Initially, a high emerged DO strategy was applied to the reactor. In later phases, this was combined with the application recurrent biofilm treatments with first FNA and second FA as a stressor. Afterwards, the emerged DO concentration was lowered in combination with the FA treatments. Finally, the FA treatments were halted and the isolated effect of the low emerged DO strategy was tested. Shifts in microbial community composition and potential activity were monitored over time. The overall goal was to achieve complete and long-term NOB suppression, while preserving AerAOB and AnAOB activity, as to obtain stable nitrogen removal via PN/A in the absence of COD.

# 2. Materials and Methods

## 2.1. Rotating biological contactor (RBC)

An RBC with mature biofilm was operated at 20±2°C for 900 days, subdivided in 10 operational phases, of which 822 days with mainstream influent. The RBC consisted of 2 sets of 20 discs with a disc radius, thickness, and interspace of 15, 0.5, and 1 cm, respectively. The biofilm thickness was estimated at 3.2±0.5 mm using a calliper. Manual harvest of the biofilm was occasionally performed to prevent biofilm on neighbouring disks to merge, hereby maintaining sufficient biofilm-liquid contact area in the disc interspace. The reactor volume varied with the applied submersion level, ranging from 51, 65, and 77 L for an immersion level of 50, 70, and 87%, respectively.

The disc rotation speed remained unchanged at 1.8 rpm, yielding a consecutive exposure of 17 s to emerged and submerged conditions (at 50% immersion). Synthetic feed under mainstream operation consisted of tap water spiked with $(NH_4)_2SO_4$ (47±4 mg N $L^{-1}$), 5 mg $NaHCO_3$ (mg N)$^{-1}$, 7.5 mg $KH_2PO_4$-P $L^{-1}$ and 0.01 ml $L^{-1}$ of trace elements solutions A and B (van de Graaf et al., 1996). Previous to this study, the RBC was operated under sidestream conditions applying high-strength influent ammonium concentrations of 1144±104 mg $NH_4^+$-N $L^{-1}$ (Courtens et al., 2014). During the transition to mainstream conditions (Phase I), the temperature was lowered on day 0 from 28-30°C to 20±2°C while the influent ammonium concentration was lowered in a stepwise manner from 399±42 to 47±4 mg $NH_4^+$-N $L^{-1}$ over the first 78 days. The influent flow rate was increased from 18 to 246 L $d^{-1}$ to maintain a similar volumetric N loading rate according to De Clippeleir et al. (2011). After this transition period, the flow was manually changed to maintain a sufficient effluent residual ammonium concentration (≥ 5 mg N $L^{-1}$) and ranged from 88 to 246 L $d^{-1}$, resulting in a hydraulic residence time of 6.3-13.9 hours. No COD was added to the influent, except for Phase IV in which acetic acid was sometimes dosed at a COD/N ratio of 2.0 (day 371-382) and 0.25-0.5 (day 422-463), to lower the submerged DO level and study the influence of COD. These concentrations are in line with the expected ratio of pre-treated sewage, e.g. after high-rate activated sludge and primary settling, as well as with other studies (Laureni et al., 2019; Ma et al., 2015; Malovanyy et al., 2015; Wang et al., 2020a). The acetic acid was dosed from a concentrated stock solution to avoid biological degradation.

## 2.2. Treatments with free nitrous acid (FNA) and free ammonia (FA)

Starting from phase II, FNA and FA were successively used as stressors for the treatments in the RBC. Details on the treatment conditions are given in Figure 1 and Figure 4. In phase II, only one biofilm segment of all discs (A) was treated with FNA by stopping the rotation of RBC when the pre-marked segment A was submerged. This was done as a precaution to avoid complete loss of AnAOB activity if the treatment would have been toxic to AnAOB. For the later phases with FNA or FA treatment, both segments A and B were treated simultaneously using the disc rotation speed of 1.8 rpm. The formerly mentioned precaution was no longer applied as ex-situ batch tests showed limited effect on AnAOB activity by FA. Before the treatments were applied in the reactor, two sets of exploratory batch tests were executed to evaluate the impact of varying FNA/FA shock conditions, such as stressor concentration, contact time, pH, etc. on AerAOB, NOB, and AnAOB in the biofilm. The methodology of the batch tests is described in Section 2.4. Based on the results of batch tests, FNA treatment strength increased from 0.4 to 3.7±0.2 mg of $HNO_2$-N $L^{-1}$ (pH 6.0, 20°C) and contact time from 1 h to 24 h. The FA treatment remained unchanged at 29±1 mg of $NH_3$-N $L^{-1}$ (pH 8.0, 20°C) for a 3 h contact time. The pH target was based on previous research by Peng et al. (2020) and was in line with literature values (Wang et al., 2020a; Wang et al., 2016; Wang et al., 2018).

For each FNA respectively FA treatment, the reactor liquid in the RBC was replaced with $NO_2^-$- (275 to 1427±79 mg N $L^{-1}$, pH 6.0, 20°C) respectively $NH_4^+$-containing (759±28 mg N $L^{-1}$, pH 8.0, 20°C) solutions, with the required stressor concentration. The treatment was terminated by discharging all the stressor solution and replacing it with the previously collected reactor effluent. Samples were regularly taken during the treatment to monitor nitrite and ammonium concentration dynamics during each treatment period. The results showed that their concentrations remained relatively stable. Hence, only the pH was controlled during the treatments using a pH sensor (Consort SP12x) and Consort 3600 controller, dosing 1M HCl or NaOH to maintain the intended FNA and FA concentration.

## 2.3. Oxygen availability control through overhead cover

Starting from Phase VI, an overhead cover was added to regulate oxygen availability. The cover was made from PVC with a transparent, acrylic window. The trapped headspace had a total volume of 145 L at an immersion level of 50%. From day 535 onwards, an artificially air flow consisting out of compressed air (0.3-7.5 L $min^{-1}$) and nitrogen gas (0-10 L $min^{-1}$) was added to control the emerged (headspace-exposed) and submerged (water-exposed) DO concentration. Both gases were mixed before entering the RBC.  The gas flow followed the same direction as the liquid flow. The emerged DO concentration after 17 s of total emerged time was assumed to be equal to the maximum saturation corresponding to the gas mixture applied (0.8-21% $O_2$), as 85% and 100% saturation at the surface of the biofilm were reported to be reached already after respectively 1.5 and 4 s of air exposure (Wang et al., 2018). This was calculated using Henry's law and the Van 't Hoff equation with the following constants: $H_{cp} = 1.2 \times 10^{-5}$ mol/kg/bar; temperature dependency ($d \ln(H_{cp})/d(1/T)$) =  1700 K (Warneck and Williams, 2012). The submerged DO was measured via a HQ30D portable dissolved oxygen meter. The DO level control was purposely lifted twice in Phase VII and VIII by adjusting the gas mixture (Figure 4 a). During the FA treatment period (3 h), no DO control was present as the overhead cover was temporarily removed.

## 2.4. Potential activity batch tests

The potential or maximum activities of AerAOB, NOB, and AnAOB in the biofilm were determined in ex-situ batch tests at 20°C. For each test, biofilm was collected from multiple discs and segments, blended, and transferred to a nitrogen-free medium (250 mg $NaHCO_3$ $L^{-1}$ demineralized water) to achieve a homogenous sample. For the aerobic AerAOB/NOB tests, the biomass was added into open glass flasks and constantly mixed by a magnetic stirrer at 150 rpm (DO >7.0 mg $O_2$ $L^{-1}$ and pH around 7.0). For the anoxic AnAOB tests, the sludge was flushed with nitrogen gas ($N_2$) in penicillin bottles for 30 mins, sealed, and constantly mixed by a magnetic stirrer at 150 rpm. Then, 50 mg of $NH_4Cl$-N $L^{-1}$ and 50 mg of $NaNO_2$-N $L^{-1}$ were spiked at the beginning of all batch tests. Regular samples were taken every hour to monitor the changes in ammonium, nitrite, and nitrate concentrations, for a total period of 4-5 h. The biomass concentration was determined at the end of the test. Each test was performed in triplicate. The maximum potential activities of AnAOB, AerAOB, and NOB in biofilm were estimated by extrapolating the maximum volumetric activities measured in the batch tests by means of the biomass concentration.

## 2.5. Physicochemical analyses

Samples were taken periodically from the influent-inlet and effluent-outlet spots to monitor the performance, as well as during the potential activity batch tests. Samples were filtered (0.2 µm) and stored at 4°C until $NH_4^+$, $NO_2^-$ and $NO_3^-$ concentrations were determined using a San++ Automated Wet Chemistry Analyzer (Skalar, The Netherlands). Additionally, total and volatile suspended solids (TSS and VSS) concentrations were measured for all the batch tests according to the standard methods (APHA, 1998). The oxygen concentration in the artificial air flow was occasionally verified with a gas chromatograph (Shimadzu, Japan) and matched the calculated value.

The nitrate production ratio was calculated by dividing the nitrate production rate by the ammonium conversion rate. The relative nitrite consumption by AnAOB and NOB was determined using a mass balance applying the in literature described stoichiometric values for AerAOB, NOB (Barnes and Bliss, 1983) and AnAOB (Lotti et al., 2014b; Strous et al., 1998). To explore the correlation of effective controlling parameters (i.e., emerged DO and submerged DO) with $NO_3^-$-N production ratio, Spearman's correlation was analysed (IBM® SPSS® Statistics 26) for phases VII and VIII.

## 2.6. Microbiome analysis

The bacterial community in the biofilm of the RBC was frequently analysed. Biofilm samples, compiled from multiple discs and segments, were stored at -20°C prior to analysis. Total DNA content was extracted using the Powerfecal kit (Qiagen), according to the manufacturers protocol (excluding incubation steps) and eluted in 100 µL. The V4 region of the 16S rRNA gene was amplified using dedicated dual-index paired-end sequencing primers (Kozich et al., 2013) and sequenced on the MiSeq Desktop sequencer (M00984, Illumina) at the Medical Genetics research group (University of Antwerp, Belgium). Analysis was performed as described in (Peng et al., 2020). In short, raw reads were denoised using DADA2 (Callahan et al., 2016) and downstream processed in R using an in-house developed package (https://github.com/Swittouck/tidyamplicons). Sequencing data is available on the European Nucleotide Archive (ENA) with accession number PRJEB45279.

# 3. Results and Discussion

## 3.1. FNA treatments insufficiently suppressed NOB activity

The transition to mainstream conditions (Phase I) resulted in a high contribution of unwanted NOB activity, with an average nitrate production ratio and TN removal efficiency of 44±4 and 44±4%, respectively, by the end of Phase I (Figure 1). Weekly FNA biofilm treatments were initiated to selectively suppress this high NOB activity. Initially (Phase II), only one segment of the biofilm (A) was treated and the strength was gradually increased over the first 80 days (0.4 to 2.0 mg $HNO_2$-N $L^{-1}$ for 1 to 12 h). Throughout Phase II, no clear changes in performance could be observed (Figure 1), apart from the shift on day 259 when the rotation was halted for 41 h. This technical error resulted in a long-lasting decrease in ammonium conversion and TN removal rate at similar nitrate production. Simultaneously and unexpectedly, the potential AerAOB/NOB activity increased from 0.9-1.4 to 2.3-2.5 (Figure 2, day 262 vs. 322) in contrast to the reactor performance. The reason for this remains unclear. Prior to that disturbance, some effect of the FNA treatments was however shown as the drop in potential AerAOB/NOB activity ratio was smaller for segment A (treated), from 1.8 to 1.4, compared to segment B (untreated) which dropped to 0.9 (Figure 2, day 161 vs. 262). This implies that the FNA treatments did result in some selective NOB suppression, but insufficient to improve the overall performance. Subsequently, segment B was included in the treatments from Phase III onwards, while the strength was further increased up to 3.7±0.2 mg $HNO_2$-N $L^{-1}$ for 24 h. By the end of Phase III, the potential AerAOB/NOB activity ratio of segment A and B were similar again and increased to 1.8-1.9 (Figure 2, day 357). Despite this increase, the reactor performance itself remained unchanged and maintained a high nitrate production ratio of 51±8%. A possible explanation could be the unchanged potential AnAOB/NOB activity ratio of 0.8-1.1 during Phase III, as the presence of sufficient AnAOB activity to act as a nitrite sink is important to achieve NOB suppression (Seuntjens et al., 2020). **Overall, the FNA treatments failed to completely suppress NOB activity and establish full PN/A**, as the nitrate production ratio remained elevated at 44-61% with a TN removal efficiency of 27-39% in Phases III-IV (Figure 2). This was rather unexpected, as multiple studies showed good NOB suppression in both flocs and biofilm using FNA treatments (Peng et al., 2020; Wang et al., 2014; Wang et al., 2018), in contrast to this study. A possible explanation for this inconsistency could be the presence of *Nitrospira* and *Nitrotoga* as dominant NOB genera rather than *Nitrobacter* (Figure 3), as the former ones are known to be less vulnerable towards FNA stress (Duan et al., 2019a; Ma et al., 2017). Nevertheless, successful suppression of *Nitrospira* by FNA was demonstrated in flocs by Wang et al. (2016) and Wang et al. (2020a). The thickness of the biofilm could also have protected the NOB from the FNA stress as thicker biofilm were shown to be more resilient to FNA stress (Jiang et al., 2011), although the extended contact time of 24 h should be sufficient to penetrate the whole biofilm. **The inhibiting effect of FNA on AnAOB activity was limited.** Exploratory batch tests showed a 58-73% preservation of AnAOB activity within the first 6 h after a single FNA treatment (2-4 mg $HNO_2$-N $L^{-1}$ at pH=5.5-6.0 for 8 h). Treatments at a lower pH of 5.0 for 12 h however resulted in a preservation of only 1.4-18% and were therefore not tested in the reactor. The limited inhibitions observed in the batch tests (pH ≥ 5.5) were confirmed by the reactor test, as both the AnAOB relative abundance and potential activity as well as the observed TN removal could be preserved after multiple treatments (Figure 1, 2 and 3). An initial decline in potential AnAOB activity could however be observed during the first 200 days, from 34±7 to 10±1 mg $NH_4^+$-N $L^{-1}$ $d^{-1}$, but stabilised afterwards (Figure 2).

**The effect of the COD addition was inconsistent but did not result in lasting NOB suppression.** Acetic acid was periodically added to the influent in Phase IV at a COD/N ratio of 2.0 (day 371-382) and 0.25-0.5 (day 422-463). At high dosage, a sudden drop in ammonium conversion and nitrate production rate to 38±12% and ~0%, respectively, was observed which only slowly recovered once the addition was stopped. As the submerged DO concentration remained unchanged and even temporarily increased after the COD addition was halted, it was assumed that the COD was mainly aerobically consumed by heterotrophs, competing with NOB (and some AerAOB) for oxygen and lowering their activity. This is in line with the studies of Laureni et al. (2016) and Seuntjens et al. (2020) where almost all COD (>80%) was consumed aerobically, even at low DO setpoints of 0.05-0.3 mg $O_2$ $L^{-1}$ while no harmful effect on AnAOB was observed. Conversely, the lower COD addition did not result in any sudden changes in conversion rates but induced a continuous increase in nitrate production ratio. Moreover, stopping the COD addition revealed the loss of almost all AnAOB activity while no shift in submerged DO level could be observed. The cause of these inconsistent results remains unclear. Stopping the COD addition at day 463 and additionally the weekly FNA treatments at day 484 both failed to restore the AnAOB activity, with a nitrate production ratio up to 100% and TN removal efficiency < 10%.

## 3.2. Oxygen availability control achieved selective NOB suppression

**Strict oxygen control achieved complete suppression of NOB activity and resulted in a good reactor performance.** Both the submerged and emerged DO levels were lowered by covering the reactor's headspace (Phase VI onwards) and adding an artificial air flow consisting of compressed air and nitrogen gas (day 535). Controlling the oxygen availability in combination with weekly FA treatments in Phase VI succeeded in selectively suppressing NOB and restoring nitrogen removal via PN/A, after almost all AnAOB activity was lost in the earlier Phase V (Figure 4). Even after the recurrent FA treatments were stopped in Phase VII, the contribution of NOB in nitrite consumption kept declining, showing the supremacy of the oxygen availability control, and eventually reached 0% (Figure 4 D). During the final Phase X, a balanced microbial community was achieved consisting of on average 85±4% nitrite consumption by AnAOB, 13±4% by NOB and 2±2% residual nitrite for almost 100 days. The effect of the FA treatments will be discussed in detail in Section 3.3.

The lowest NOB activity was measured in Phases VIIa (day 595-644) and VIIc (day 661-693) in which virtually no NOB activity was present for 46 and 33 days, respectively, with an average nitrate production ratio of 10±4 and 11±4% (Table 1). This was in line with the theoretical nitrate production by PN/A of 8-11%, indicating full suppression of NOB (Lotti et al., 2014b; Strous et al., 1998). This obtained nitrate production ratio is low compared to similar studies without influent COD, ranging from 21-40% (De Clippeleir et al., 2013; De Clippeleir et al., 2011; Gilbert et al., 2014; Peng et al., 2020), showing the added value of this research. Kwak et al. (2012) reported a lower ratio of 2%, which is however unrealistically low in the absence of COD, while the other low values were all observed in combination with COD addition (COD/N of 1-3), potentially removing nitrate via denitrification.

**The importance of the oxygen availability control and its reversibility was confirmed after purposely imposing distortions to the oxygen control in absence of FA treatments.** In Phase VIIb (day 645-660), the emerged DO concentration was increased to initially 5.06±1.10 mg $O_2$ $L^{-1}$ for 8 days and afterwards to 8.57±0.48 mg $O_2$ $L^{-1}$ for another 9 days, resulting in an elevated submerged DO concentration of 1.01±0.13 and 1.95±0.08 mg $O_2$ $L^{-1}$, respectively. This caused an immediate increase of the nitrate production ratio from 8±2% to initially 64±7% and afterwards to 87±7%. Once the original DO settings were restored (Phase VIIc, day 661-693), the nitrate production ratio immediately returned to 11±2%. This fast response induced by the oxygen control was also observed by Gilbert et al. (2014), for which a reduction in DO levels resulted in short-term NOB suppression but once the original DO was restored, NOB prevailed again. Full NOB suppression was however not achieved in contrast to this study. In Phase VIIIa (day 694-729), the emerged DO concentration was stepwise increased to determine the tipping points in nitrate production ratio and thus selective NOB suppression. A first increase (day 694-704) was found after increasing the emerged (0.95±0.18 to 1.41±0.31 mg $O_2$ $L^{-1}$) and submerged (0.41±0.04 to 0.51±0.04 mg $O_2$ $L^{-1}$) DO concentration, resulting in an elevated nitrate production ratio from 11±2% to 21±2%. A second increase (day 707-714) occurred when the emerged and submerged DO concentrations reached 2.29±0.29 and 0.59±0.05 mg $O_2$ $L^{-1}$, respectively, which further boosted the nitrate production ratio to 35±9% and continued to increase afterwards. Another interesting observation was that the higher DO levels in this phase did not seem to increase the ammonium conversion rate but solely boosted the nitrate production by NOB. The absence of an increased AerAOB relative abundance, in contrast to the sharp increase in NOB relative abundance, strengthens the observation that NOB are proliferating more than AerAOB at these slightly higher DO concentrations (Figure 3, day 689 vs. 729). A Spearman's correlation analysis showed that the nitrate production ratio was positively correlated with the emerged DO concentration during Phases VII-VIIIa (rho = 0.90, $p < 0.0001$).

Surprisingly, reverting the DO setpoints, initially to the second tipping point (day 719-729), secondly to the first tipping point (day 730-731), and finally to the original values (day 732-900), did not restore the performance in contrast to Phase XIIb (day 645-660). A similar observation was made in Phase VI (day 553), where a 3-day disturbance in the artificial airflow caused an immediate increase in nitrate production ratio from 37±6% to 100%, which was also not fully reversible.  A possible explanation for these inconsistent observations could be a difference in NOB genera present: for the reversible experiment in Phase VIIb, *Nitrotoga* was the sole NOB genus present while in the experiment in Phase VIIIa, both *Nitrotoga* and *Nitrospira* were present, the latter increasing in relative abundance (Figure 3). This difference in enrichment is most likely due to the lower imposed submerged DO level of 0.51-0.59 mg $O_2$ $L^{-1}$ in Phase VIIIa versus 1-2 mg $O_2$ $L^{-1}$ in the reversible Phase VIIb, as *Nitrospira* can dominate the NOB community under oxygen-limited conditions (Wang et al., 2020b; Yu et al., 2020). Since *Nitrospira* are known to have a higher oxygen affinity compared to AerAOB (Liu and Wang, 2013), their presence could explain the limited reversibility of the DO control. Other studies like Wang et al. (2018) reported similar failure of system recovery using low DO levels once *Nitrospira* were enriched. The weekly FA treatments were therefore restarted in Phase IX and successfully suppressed most NOB activity, including *Nitrospira*, in combination with the DO control.

The effectiveness of limiting the oxygen availability by reducing the DO setpoint has previously been reported (De Clippeleir et al., 2011; Laureni et al., 2019), while the complete suppression of NOB and especially the reversibility of the DO control were seldomly observed unlike for this study.  Despite multiple reports that process control strategies solely based on DO level are not effective (Agrawal et al., 2018; Courtens et al., 2014) our study proved that it can nevertheless be achieved when nitrate formation by NOB was already fully suppressed. If the NOB are still active in the system, additional strategies such as recurrent FA treatments may be needed until full suppression has been established.

**The operational window to enable mainstream PN/A with little to no NOB activity consisted of a submerged DO setpoint <0.51±0.04 mg $O_2$ $L^{-1}$ and emerged DO setpoint <1.41±0.31 mg $O_2$ $L^{-1}$**, derived from the previously discussed observations. These DO setpoints are rather high compared to most other studies, reporting optimal submerged setpoints of <0.1 to 0.4 mg $O_2$ $L^{-1}$ (Gilbert et al., 2015; Kwak et al., 2012; Laureni et al., 2019; Wang et al., 2018; Yang et al., 2017). However, these studies used rather thin biofilms (~300 µm vs. 3200±500 µm) or combined biofilm and flocculent sludge, allowing lower DO concentrations because of lower diffusion limitations in the flocs. Sole biofilm systems with a thick biofilm, such as some RBC setups, report in general higher values of 1.2-3.1 mg $O_2$ $L^{-1}$ (Antileo et al., 2007; De Clippeleir et al., 2013; De Clippeleir et al., 2011).

From the perspective of the biofilm itself, the DO concentration it is exposed to rapidly changes over time, switching from submerged to emerged DO concentration every 17 s at 50% submersion level. This rapid switch could also be interpreted as some form of intermittent aeration: due to the frequent exposure to a lower DO concentration in the submerged stage, transient anoxic zones will occur in the biofilm. Although intermittent aeration is effective in selectively suppressing NOB (Bekele et al., 2020; Gilbert et al., 2014; Kornaros et al., 2010), it remains unclear if it also had a considerable effect on the performance. In this case, controlling the oxygen availability was more likely key to achieve complete NOB suppression. The emerged DO concentration could therefore be argued to be the most critical parameter, as this mainly determines the submerged DO concentration (Courtens et al., 2014). Moreover, a stronger positive correlation was found between the nitrate production ratio and the emerged rather than submerged DO concentration during Phases VII-VIIa of 0.90 and 0.82 ($p < 0.0001$), respectively. However, NOB suppression was also observed in RBC setups without an overhead cover, with consequently an emerged DO concentration of up to 9 mg $O_2$ $L^{-1}$ (Antileo et al., 2007; De Clippeleir et al., 2013). Additional research is therefore needed to clarify the importance of both the submerged and emerged DO concentration.

**Higher submersion levels did not result in better NOB suppression.** The submersion level was sometimes changed throughout the experiment (Phase I, III, IV and VI) to lower the submerged DO concentration when the emerged DO control was not yet installed. Although increased submersion levels resulted in reduced submerged DO concentrations, no long-lasting improvement in NOB suppression could be observed (Figures 1, Phase I day 65 and Phase III day 340). This is contradicting the results of Courtens et al. (2014) and Antileo et al. (2007) for whom long-term NOB suppression was more effective at higher submersion levels. However, these experiments were conducted under sidestream conditions, which in general favour AerAOB activity over NOB due to their higher relative growth rate at higher temperatures, and the presence of higher FA concentration to which NOB are more sensible (Agrawal et al., 2018). Raising the submersion level to 87% in Phases III-IV resulted in a submerged DO concentration of 0.95±0.29 mg $O_2$ $L^{-1}$, with a minimum of 0.66±0.08 mg $O_2$ $L^{-1}$ in combination with COD addition (Phase IV). However, long-lasting decrease in nitrate production ratio was only observed once the emerged and submerged DO levels were below 1.41±0.31 and 0.51±0.04 mg $O_2$ $L^{-1}$, respectively (Phase VI onwards). This could explain the limited effect of the submersion level, as the reactor conditions did not allow to reach a sufficiently low submerged DO level by solely increasing the submersion level.

**Good preservation of potential NOB activity despite little observed activity.** Both provocation experiments revealed high potential NOB activity although almost no activity was observed prior to increasing the DO levels. The longest period with neglectable NOB activity (nitrate production ratio ≤15%) was prior to the reversible Phase VIIb (46 days), followed by Phase VIIIa (33 days). NOB are known to persist for a long period (several months) in granular sludge in absence of observable nitrate production (Bartroli et al., 2010; Lotti et al., 2014a; Poot et al., 2016). A possible explanation for this unexpected high presence of NOB is their ability to reverse its main oxidative reaction in the presence of COD (Koch et al., 2015). As no COD was dosed in the corresponding phases, they could have utilised the in-situ assimilated and endogenous COD. The addition of sludge retention time control could help to physically remove the NOB from the reactor and thus improve the overall stability (Agrawal et al., 2018).

## 3.3. FA treatments improved the NOB suppression

**Weekly FA treatments (29±1 mg $NH_3$-N $L^{-1}$ for 3 h) were conducted in Phases VI and IX and improved the selective NOB suppression.** In the first half of Phase VI, prior to the DO control, a small decline in nitrate production rate at equal ammonium conversion rate could be observed, resulting in a slightly improved performance. This trend started however already a few days before the first treatment, complicating the discussion. Moreover, the subsequent implementation of the oxygen availability control (day 536) seemed to have a larger impact on the performance since complete NOB suppression could be maintained in Phase VIIa in the absence of the recurrent FA treatments, as discussed in Section 3.2.

The beneficial effect of the FA treatments became clearer in Phase IX, when solely the DO control did not manage to immediately restore the performance after *Nitrospira* were enriched in the biofilm (Figure 3 and 4). In combination with the DO control, the weekly biofilm FA treatment did manage to restore the performance and suppress and partially wash-out both *Nitrospira* and *Nitrotoga*. This was in accordance with Duan et al. (2019b) who effectively restrained the growth of *Nitrospira* using recurrent FA treatments. However, since the recurrent FA treatments were mostly applied in combination with the oxygen availability control, it remains difficult to pinpoint its additional effect. More attention to the isolated effect of the FA treatments should be given in follow-up research.

**AnAOB activity in the biofilm seemed to be protected from the potentially inhibiting effects of the FA treatments.** The exploratory batch tests showed only a 1.6% reduction in potential AnAOB activity while no loss of AnAOB activity could be observed in the reactor itself (Figure 4). This is in sharp contrast with Peng et al. (2020), encountering severe losses in AnAOB activity after exposing the biofilm to a similar weekly FA treatment (30 mg $NH_3$-N $L^{-1}$ for 1 h), forcing them to stop the treatment of the biofilm. Differences in biofilm morphology, a thin biofilm on a K1 carrier versus the thick biofilm in this study, might explain this discrepancy. Additionally, differentiation in AnAOB genera could also possibly explain this dissimilarity as Ca. *Brocadia* was the dominating AnAOB genus in the study of Peng et al. (2020) while the biofilm in our study was dominated by Ca. *Kuenenia*.

**The recurrent FA treatments can be used as an extra tool to deal with unbalanced situations**, as occurred in Phase IX, since they were proven not to be crucial to maintain complete NOB suppression (e.g. Phase VII). This is in sharp contrast with Duan et al. (2019b) who observed an immediate collapse in performance once the weekly FA treatment was stopped in a flocculent system. The presence of AnAOB activity as a nitrite sink could explain this difference, as it is a key factor in NOB suppression (Seuntjens et al., 2020). However, the combination with the strict DO control remains important, as illustrated by Wang et al. (2018) who observed the reoccurrence of NOB in the biofilm after the DO was increased from 0.20 to 0.65 mg $O_2$ $L^{-1}$.

## 3.4. Microbial community

The nitrifying community, consisting of AerAOB, NOB and AnAOB, had an overall relative abundance ranging from 13-28% throughout the whole experiment (Figure 3). The AerAOB community was dominated by the sole genus *Nitrosomonas*, consisting of many different ASV (1-22). For NOB, both *Nitrospira* and *Nitrotoga* were present in high numbers, regularly switching in most abundant genus and ASV. *Nitrobacter* was solely detected on day 422 at a low abundance of 0.08%. Ca. *Kuenenia* was the only detected AnAOB genus until some Ca. *Brocadia* appeared from day 749 onwards. Despite a noticeable increase in relative abundance of Ca. *Brocadia* at the end of the experiment, Ca. *Kuenenia* remained by far the dominating genus with a relative abundance of 11% compared to 1% on day 779.

## 3.5. Application potential

The application of the oxygen availability control in combination with recurrent FA treatments was proven to successfully suppress all NOB activity and resulted in good TN removal via PN/A. The best reactor performance was achieved in Phase VIIc, with an average TN removal efficiency of 67±4% and low nitrate production ratio of 11±2%. However, this TN removal efficiency was restricted by the imposed residual ammonium concentration (≥ 5 mg N $L^{-1}$) and the absence of influent COD to remove the nitrate produced by PN/A (8-11%), limiting the efficiency to a maximum of 80%. Extra residual ammonium (10-25%) and sometimes some residual nitrite (≤10%) were the main limitations to achieve a higher TN removal efficiency. In a full-scale application, the process conditions would be further optimised, and the residual ammonium control would be lowered towards the final basins, thus improving the removal efficiency and effluent quality. Additionally, some COD would be present in the influent that can be used to remove the produced nitrate with smart process design. The TN loading rates in the best performing Phase VIIc was on average 132±7 mg N $L^{-1}$ $d^{-1}$, comparable with the nitrogen stage of full-scale installations.

The findings in this manuscript are generic and can be applied to several types of biofilm-based systems. As for RBC, although their application is less common, they are often covered in practice or can easily be covered, which with some further adjustments with headspace restriction of oxygen levels could be feasible as a strategy for mainstream PN/A. For alternative biofilm-based technologies such as moving bed biofilm reactor (MBBR) or integrated fixed-film activated sludge (IFAS), these insights would also apply although the exact settings should be translated to this new configuration.

Since the recurrent FA treatments were shown to improve the selective suppression of NOB activity but became obsolete once the NOB were suppressed, they would rather be used occasionally to overcome unbalanced periods such as start-up, and to counter the continuous bio-augmentation of NOB via the influent (Duan et al., 2019b) or other process upsets. The ammonia-rich on-site reject water, present on most larger scale sewage treatment plants, is sufficient to generate the required FA concentration using the tested weekly frequency (Peng et al., 2020).

One of the potential risks is the capacity of NOB to adapt to stressful conditions such as low DO and FA/FNA treatments, as described in multiple studies (Duan et al., 2019a; Liu and Wang, 2013; Peng et al., 2020; Wang et al., 2020a). In this study however, no signs of adaptation were observed for neither the FA treatments, illustrated by the good results after its reintroduction in Phase IX, nor for the oxygen availability control. Additional tests are however required to fully verify the feasibility of both control strategies on full-scale, especially towards the application at lower temperatures (<20°C) and validation with real pre-treated sewage.

# 4. Conclusions

The complete and long-term suppression of NOB in a biofilm-based RBC was achieved by controlling the oxygen availability at DO concentrations <1.41±0.31 and <0.51±0.04 mg $O_2$ $L^{-1}$ for the biofilm respectively emerged above and submerged in the water. Additionally, weekly FA treatments (29±1 mg $NH_3$-N $L^{-1}$ for 3 h) of the biofilm could improve the NOB suppression while FNA treatments (up to 3.7±0.2 mg $HNO_2$-N $L^{-1}$ for 24 h) had only limited effects. Interestingly, AnAOB activity could withstand both treatments, thus expanding their application potential. These FA treatments could be used during start-up or process upsets.

E-supplementary data of this work can be found in online version of the paper.


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# Figure Captions

**Figure 1** Reactor performance in Phase I-IV (day 0-480). Main variables per phase are shown on top, additional details in Section 2.2 ("N/A" = no treatment applied). (a) Submerged and emerged DO concentration, (b) TN removal efficiency, $NH_4^+$-N conversion efficiency and $NO_3^-$-N production ratio, (c) volumetric TN loading, $NH_4^+$-N conversion and $NO_3^-$-N production rate, and (d) relative $NO_2^-$-N consumption by AnAOB and NOB, and residual $NO_2^-$-N. Yellow zones in panel (d) correspond to periods with lacking data or COD addition.

**Figure 2** Potential activity batch tests results, including AerAOB, NOB and AnAOB activity of both segments A and B. Error bars indicate standard deviation of the triplicate measurements.

**Table 1** Reactor performance summary of operational Phases V-X, chosen at stable operational conditions during the "calculation period (day)".

**Figure 3** Evolution of the relative abundance of all identified NOB (red), AerAOB (green) and AnAOB (blue) amplicon sequence variants (ASV) in the biofilm, expressed relatively over the total community. Segment A, B and A & B refer respectively to the treated and untreated segment in Phase II, and the simultaneous treatment of both segments from Phase III onwards.

**Figure 4** Reactor performance in Phase V-X (day 481-900). Main variables per phase are shown on top. (a) Submerged and emerged DO concentration, (b) TN removal efficiency, $NH_4^+$-N conversion efficiency and $NO_3^-$-N production ratio, (c) volumetric TN loading, $NH_4^+$-N conversion and $NO_3^-$-N production rate, and (d) relative $NO_2^-$-N consumption by AnAOB and NOB, and residual $NO_2^-$-N. Yellow zones in panel (d) correspond to periods with lacking data or COD addition.

# Tables and Figures

**Figure 1**

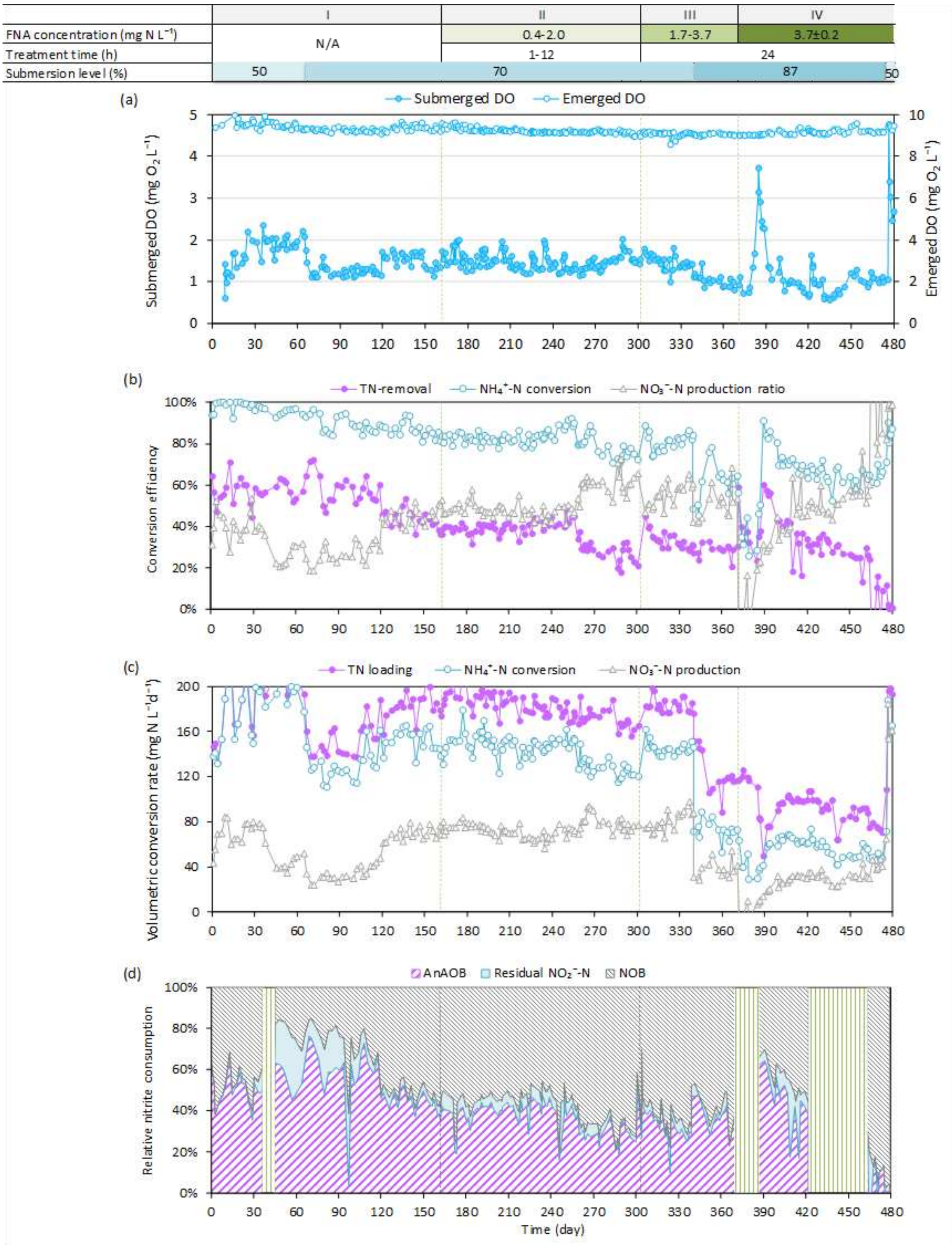

I
II
III
IV
FNA concentration (mg N L⁻¹)
N/A
0.4-2.0
1.7-3.7
3.7±0.2
Treatment time (h)
1-12
24
Submersion level (%)
50
70
87
50
(a)
Submerged DO
Emerged DO
Submerged DO (mg O₂ L⁻¹)
Emerged DO (mg O₂ L⁻¹)
(b)
TN-removal
NH₄⁺-N conversion
NO₃⁻-N production ratio
Conversion efficiency
(c)
TN loading
NH₄⁺-N conversion
NO₃⁻-N production
Volumetric conversion rate (mg N L⁻¹d⁻¹)
(d)
AnAOB
Residual NO₂⁻-N
NOB
Relative nitrite consumption
Time (day)

**Figure 2**

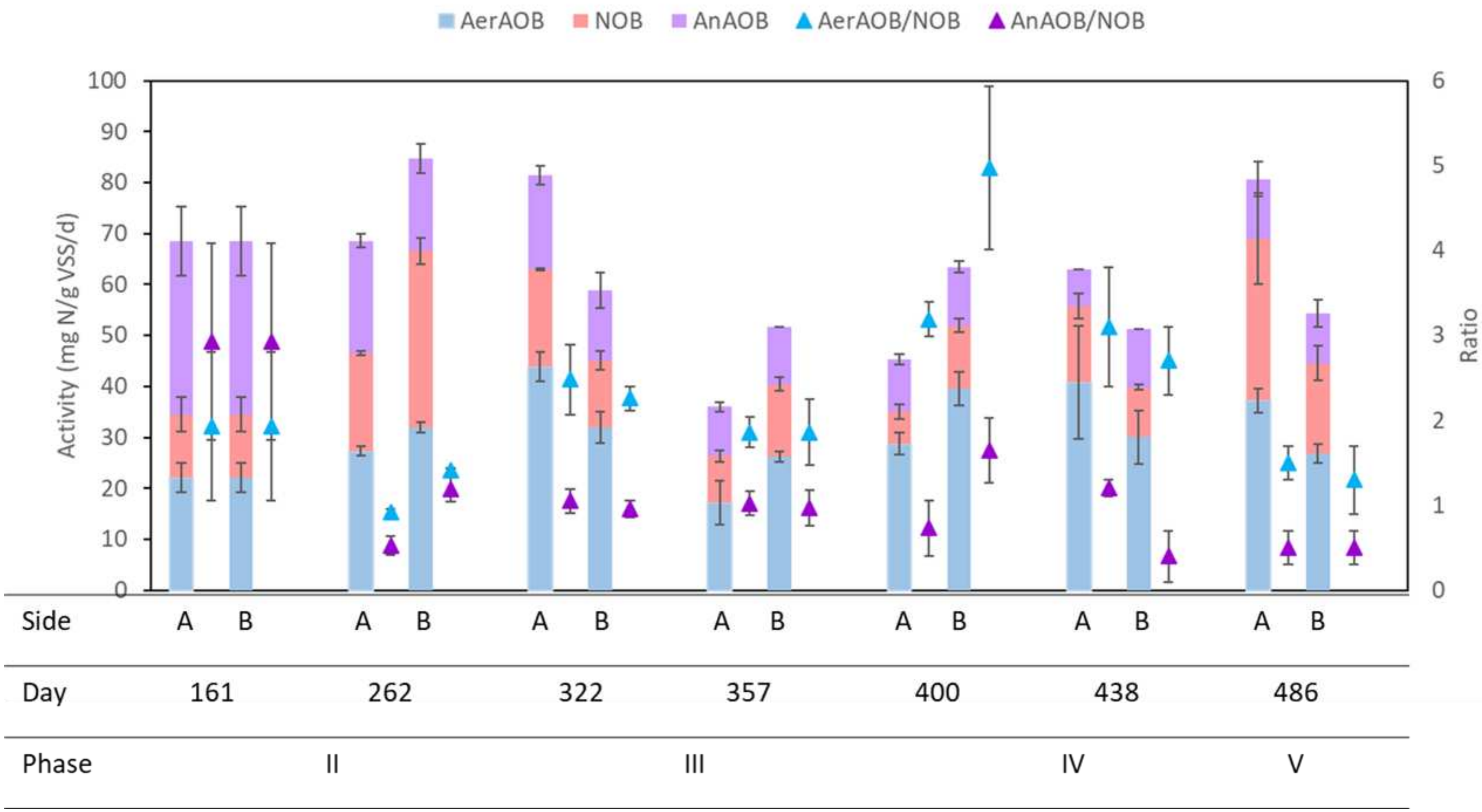
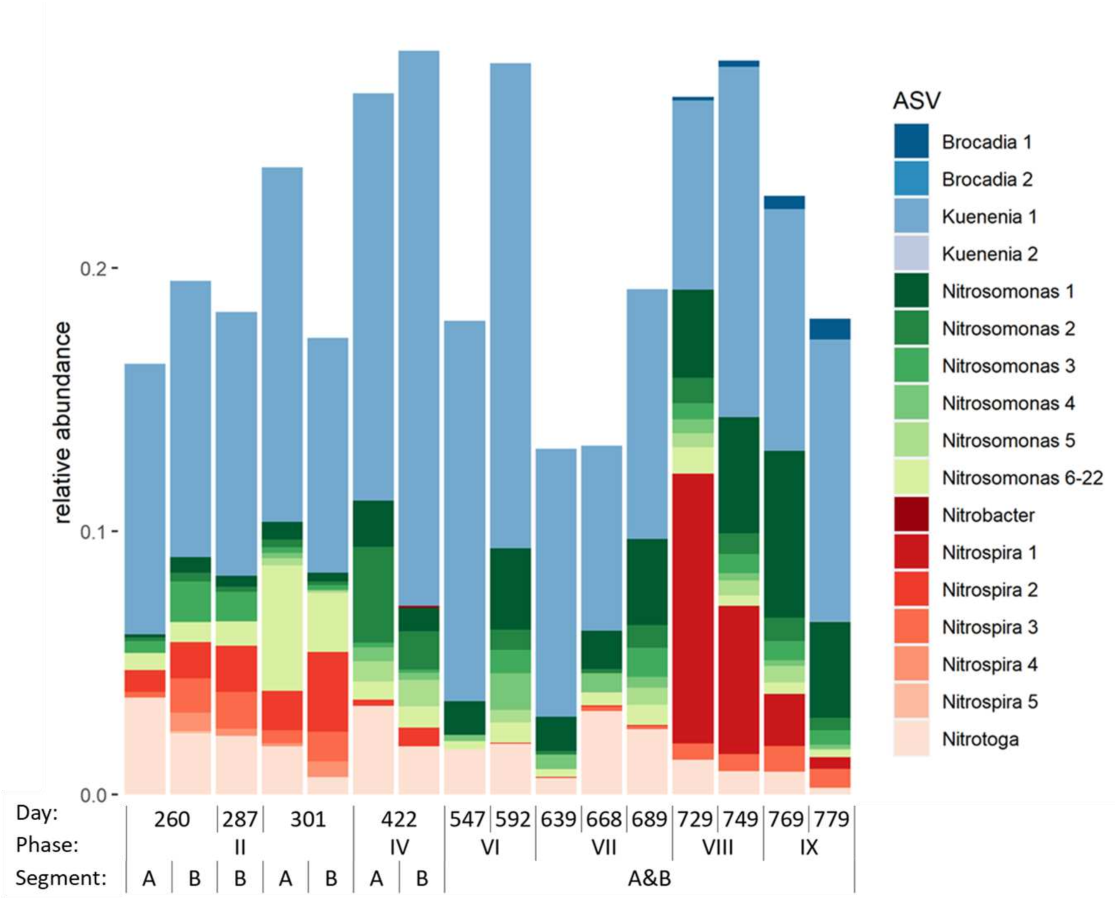

AerAOB
NOB
AnAOB
AerAOB/NOB
AnAOB/NOB
Activity (mg N/g VSS/d)
Ratio
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
Side
A
B
Day
161
262
322
357
400
438
486
Phase
II
III
IV
V

**Table 1**

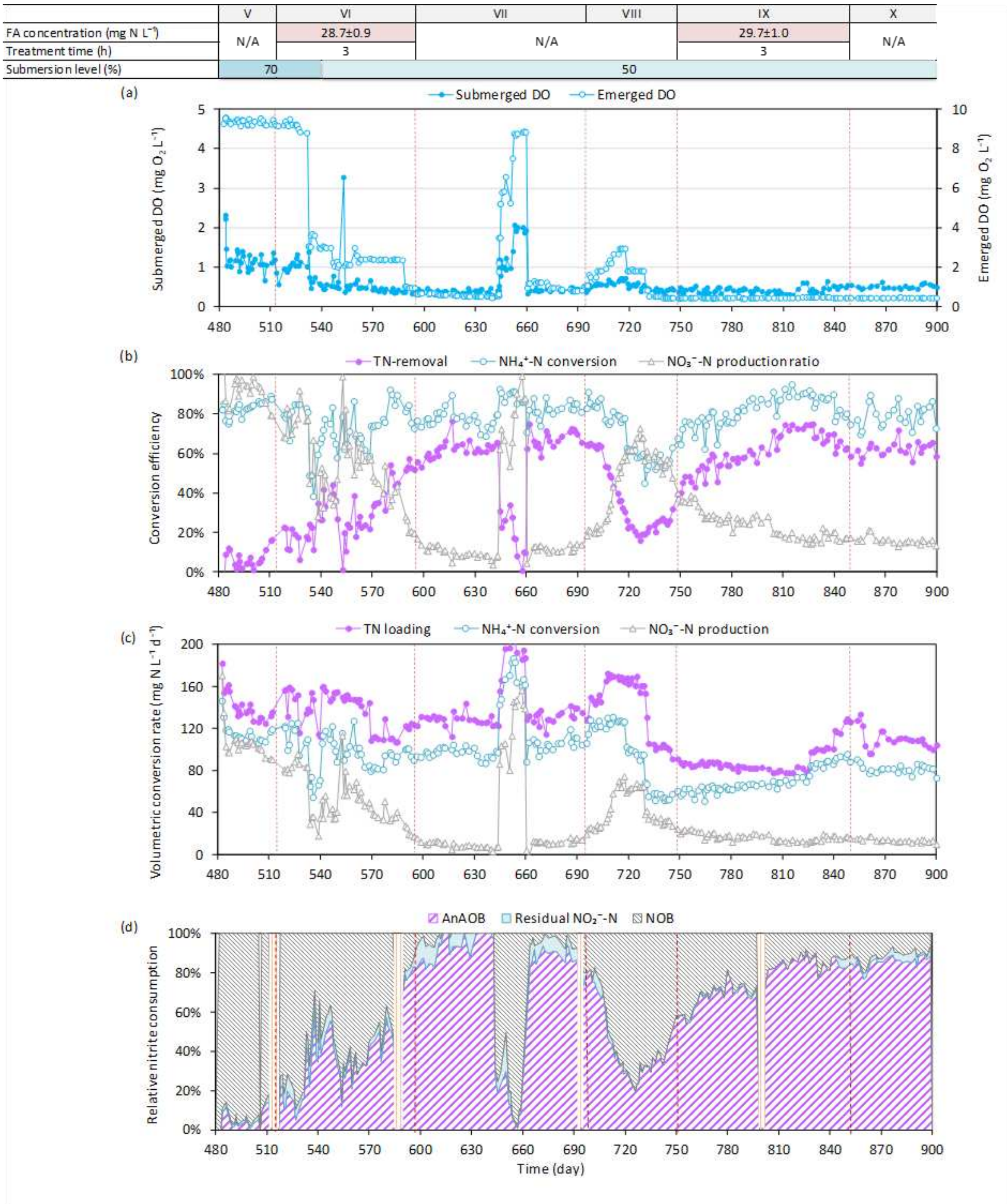
| Phase | V | VI | | VII | | | VIII | | IX | | X |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subphase (day) | **V** (484-513) | **VIa** (514-532) | **VIb** (533-594) | **VIIa** (595-644) | **VIIb** (645-660) | **VIIc** (661-693) | **VIIIa** (694-729) | **VIIIb** (730-748) | **IXa** (749-805) | **IXb** (806-849) | **X** (850-900) |
| Emerged DO (mg $L^{-1}$) | 9.31 ± 0.11 | 9.12 ± 0.18 | 2.52 ± 0.45 | 0.54 ± 0.08 | 7.20 ± 1.62 | 0.95 ± 0.18 | 1.03 to 2.91 | 0.48 ± 0.14 | 0.39 ± 0.02 | 0.42 ± 0.02 | 0.41 ± 0.01 |
| Submerged DO (mg $L^{-1}$) | 1.13 ± 0.19 | 0.99 ± 0.18 | 0.48 ± 0.11 | 0.38 ± 0.10 | 1.42 ± 0.47 | 0.41 ± 0.04 | 0.54 ± 0.08 | 0.41 ± 0.05 | 0.40 ± 0.07 | 0.42 ± 0.10 | 0.50 ± 0.05 |
| FA concentration (mg N $L^{-1}$) | N/A | 28.0 ± 1.2 | 29.0 ± 0.7 | | | N/A | | | 29.8 ± 1.0 | 29.7 ± 1.1 | N/A |
| TN loading (mg N $L^{-1}$ $d^{-1}$) | 138 ± 10 | 146 ± 15 | 137 ± 19 | 128 ± 6 | 191 ± 17 | 132 ± 7 | 159 ± 12 | 102 ± 4 | 85 ± 3 | 96 ± 16 | 111 ± 9 |
| TN removal (%) | 7 ± 5 | 17 ± 6 | 30 ± 11 | 62 ± 5 | 18 ± 11 | 67 ± 4 | 16 to 65 | 25 ± 3 | 54 ± 7 | 69 ± 5 | 62 ± 4 |
| Nitrate production ratio (%) | 91 ± 6 | 77 ± 8 | 52 ± 15 | 10 ± 4 | 77 ± 14 | 11 ± 2 | 18 to 73 | 55 ± 4 | 29 ± 5 | 17 ± 2 | 16 ± 2 |

**Figure 3**



relative abundance
0.2
0.1
0.0
ASV
Brocadia 1
Brocadia 2
Kuenenia 1
Kuenenia 2
Nitrosomonas 1
Nitrosomonas 2
Nitrosomonas 3
Nitrosomonas 4
Nitrosomonas 5
Nitrosomonas 6-22
Nitrobacter
Nitrospira 1
Nitrospira 2
Nitrospira 3
Nitrospira 4
Nitrospira 5
Nitrotoga
Day: 260 287 301 422 547 592 639 668 689 729 749 769 779
Phase: II IV VI VII VIII IX
Segment: A B B A B A B A&B

**Figure 4**



V
VI
VII
VIII
IX
X
FA concentration (mg N L⁻¹)
N/A
28.7±0.9
N/A
29.7±1.0
N/A
Treatment time (h)
3
3
Submersion level (%)
70
50
(a)
Submerged DO
Emerged DO
Submerged DO (mg O2 L-1)
Emerged DO (mg O2 L-1)
(b)
TN-removal
NH4+-N conversion
NO3−-N production ratio
Conversion efficiency
(c)
TN loading
NH4+-N conversion
NO3−-N production
Volumetric conversion rate (mg N L-1 d-1)
(d)
AnAOB
Residual NO2−-N
NOB
Relative nitrite consumption
Time (day)