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Overcoming Nitrite Oxidizing Bacteria Adaptation through Alternating Sludge Treatment with Free Nitrous Acid and Free Ammonia — Source link

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Overcoming nitrite oxidizing bacteria adaptation through alternating sludge treatment with free nitrous acid and free ammonia

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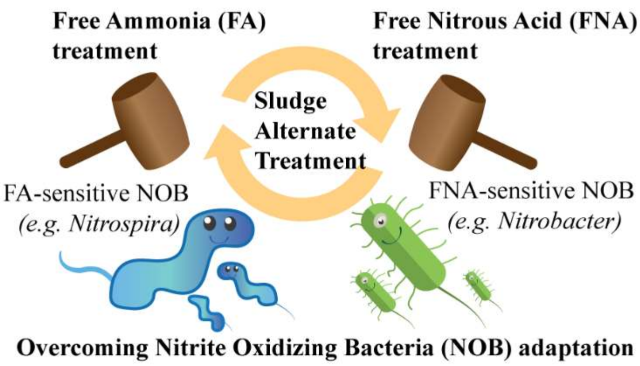
TOC Art


Free Ammonia (FA) treatment
Free Nitrous Acid (FNA) treatment
Sludge Alternate Treatment
FA-sensitive NOB
(e.g. Nitrospira)
FNA-sensitive NOB
(e.g. Nitrobacter)
Overcoming Nitrite Oxidizing Bacteria (NOB) adaptation

ABSTRACT

Stable suppression of nitrite oxidizing bacteria (NOB) is one of the major bottlenecks for achieving mainstream nitrite shunt or partial nitritation/anammox (PN/A). It is increasingly experienced that NOB could develop resistance to suppressions over an extended time, leading to failure of nitrite shunt or PN/A. This study reports and demonstrates the first effective strategy to overcome NOB adaptation through alternating sludge treatment with free nitrous acid (FNA) and free ammonia (FA). During over 650 days reactor operation, NOB adaptation to both FNA and FA was observed but the adaptation was successfully overcome by deploying the alternate treatment strategy. Microbial community analysis showed *Nitrospira* and *Nitrobacter*, the key NOB populations in the reactor, have the ability to adapt to FNA and FA, respectively, but do not adapt to the alternation. Stable nitrite shunt with nitrite accumulation ratio over 95% and excellent nitrogen removal was maintained for the last 8 months with only one alternation applied. N_2O emission increased initially as the attainment of nitrite shunt but exhibited a declining trend during the study. By using onsite-produced nitrite and ammonium, the proposed strategy is feasible and sustainable. This study brings the mainstream nitrite shunt and PN/A one step closer to wide applications.

Key words: *Nitrite shunt, Partial nitritation, adaptation, FNA, FA, Sludge treatment*

1. INTRODUCTION

Biological nitrogen removal via the nitrite shunt or the partial nitritation and anammox (PN/A, also called deammonification) process are two attractive carbon- and energy-efficient nitrogen removal technologies [1-3]. With nitrite rather than nitrate as the intermediate between nitrification-denitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow N_2$), the so-called nitrite shunt reduces the organic carbon demand (COD) for nitrogen removal and oxygen consumption for nitrification by 40% and 25%, respectively [4, 5]. The PN/A process, consisting of partial nitritation ($NH_4^+ \rightarrow NO_2^-$) followed by anaerobic ammonium oxidation (anammox, $NH_4^+ + NO_2^- \rightarrow N_2$), could reduce the COD and oxygen requirement even further, by nearly 100% and 60%, respectively [6, 7]. The saved organic carbon can be redirected to anaerobic digestion for bioenergy recovery [8].

The key to achieving nitrite shunt or partial nitritation is the suppression or elimination of nitrite oxidizing bacteria (NOB; $NO_2^- \rightarrow NO_3^-$) while maintaining the ammonium oxidizing bacteria (AOB; $NH_4^+ \rightarrow NO_2^-$). Many approaches have been demonstrated to selectively inhibit or eliminate NOB in mainstream wastewater treatment, relying on the different physiological characteristics of AOB and NOB. These approaches include the use of low dissolved oxygen (DO) [9-12], aeration control with aeration switched off when or even before complete ammonium oxidation is achieved [13-15] and short sludge retention time (SRT) [16-18]. More recently, strategies involving sidestream sludge treatment with ultrasonic [19], Free Nitrous Acid (FNA, the protonated form of nitrite) [20, 21], Free Ammonia (FA, the unionised form of ammonium) [22] and sulfide [23] have been proposed. Instead of suppressing NOB growth in the main reactor, these strategies feature recirculating sludge through a sidestream reactor, where NOB are selectively inactivated through the use of biocidal factors such as ultrasonic radiation, FNA, FA or sulfide. For example, Wang et al [22] achieved stable mainstream nitrite shunt with nitrite accumulation ratio (NAR: $NO_2^-{-}N/(NO_2^-{-}N+NO_3^-{-}N) \times 100\%$) over 90% in an SBR, by sidestream sludge treatment with FA. A total of 22% of the activated sludge from the SBR was transferred into an FA treatment unit, subject to 210 mgNH_3−N/L treatment condition for 1 day, where the NOB were selectively inactivated. The treated sludge was afterward recirculated back to the SBR.

Although many of the above approaches have been successfully demonstrated, the stable suppression of NOB is still one of the main bottlenecks for achieving the nitrite shunt or PN/A. Adaptation of NOB to the suppression factor(s) applied has been increasingly recognized as a crucial challenge to the stable suppression of NOB. For example, over long-term low DO conditions (0.16 and 0.37mgO_2/L), NOB gradually become more efficient in competition for DO with AOB by enriching the genus *Nitrospira*, eventually leading to failure of nitrite shunt [24]. The adaptation of NOB to low DO was recently confirmed at full-scale again caused by shifting dominant NOB population from *Nitrobacter* to *Nitrospira* [25]. Similarly, NOB adaptation was observed in systems with sidestream sludge treatment. Ma, et al. [26] observed a dominant NOB population shift from *Nitrospira* to *Nitrotoga* shortly after the establishment of the nitrite shunt by using FNA treatment (0.75 mgHNO_2-N/L), leading to collapse of the nitrite shunt. The nitrite shunt could not be restored even by further increasing the FNA concentration. NOB have previously also been shown capable of tolerating increasing levels of FA (up to 40-50 mgNH_3−N/L), causing the failure of several FA-established nitrite shunt systems [27-29].

The above experimental evidences suggest that it would be extremely challenging to suppress NOB growth using a single factor. It is likely that not all NOB populations are equally susceptible to the same factor, and consequently the less susceptible populations could outcompete others and emerge as the dominating NOB population, leading to suppression failure. It is also possible that certain strains may be able to develop physiological changes to better cope with a suppression factor [30].

While the crucial NOB adaptation challenge to stable mainstream nitrite shunt and PN/A has been realized, no successful solution has been demonstrated. In this study, we hypothesize that more sustainable NOB suppression may be achievable with the use of multiple factors sequentially. Specifically, we hypothesize that different populations of NOB may have different levels of intolerance to the biocidal effects of FNA and FA, and consequently, more sustainable NOB suppression may be attained when sidestream sludge treatment is alternated between using FNA and FA. A lab-scale sequencing batch reactors (SBR) receiving synthetic domestic wastewater were continuously operated for more than 650 days, during which NOB adaptation to FNA and FA was induced and then overcome through the use of the proposed alternate treatment strategy. The nitrogen removal performances including the nitrite accumulation ratio (NAR, NAR=NO_2^--N/(NO_2^--N+NO_3^--N)×100%) were closely monitored to reveal the effects of alternating FNA and FA sludge treatment on reactor performance. The activities and microbial communities of AOB and NOB were also monitored and analysed to provide insights into NOB adaptation and how it was overcome through alternate treatment. The nitrous oxide (N_2O) in the emitted gas was also monitored during the long-term study to evaluate impacts of the strategy on N_2O emissions.

2. MATERIAL AND METHODS

2.1 Reactor operation and monitoring

An SBR with a working volume of 8L was set up and operated in a temperature-controlled laboratory (22.0 – 23.0°C), seeded with activated sludge from a local municipal wastewater treatment plant (Luggage Point Sewage Treatment Plant, Brisbane, Australia). The SBR was operated for achieving mainstream nitrite shunt. Each cycle of the SBR operation is composed of anoxic feeding (60 min), anoxic reaction (60 min), aerobic reaction (180 min), sludge wasting (5 min), settling (50 min) and decanting (5 min) in sequence, lasting in total for 6 hours. The hydraulic retention time (HRT) was fixed at 24 hours by feeding 2L of synthetic wastewater (see below and Section 2.3 for composition) into the SBR in the feeding phase. The SRT was maintained at 15 days, with 133 mL mixed liquor wasted in each cycle in the wastage phase. To allow sufficient mixing during the feeding, anoxic, aerobic and wasting phases, a magnetic stirrer was applied and set at 250 rpm. The DO concentration was measured online using a set of dissolved oxygen meter and probe (TPS) connected to the programmable logic controller (PLC), and controlled between 1.5 -2.0 mgO_2/L through on/off control of the aeration. The airflow rate was kept at 1 LPM by a flowmeter (Cole-Parmer Valved Acrylic Flowmeter, 0.4-5 LPM) when aeration was switched on. The pH was measured with a set of pH meter and probe (miniCHEM, Labtek) but not controlled, varying between 7.1 and 7.4 over a typical cycle.

The reactor operation was divided into a baseline and an experimental period. In the baseline period, the SBR received a mixture of synthetic domestic wastewater and a synthetic anaerobic digestion liquor (see Section 2.3). The anaerobic digestion liquor contributed an additional 20% ammonium loading to the system. The experimental period was started when the SBR reactor reached steady-state as evidenced by the constant mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) concentrations, and constant effluent quality. In the experimental period, a sidestream sludge treatment unit was added (Figure S1). Additional to the sludge wastage flow (530 mL/day), 1790 mL of the mixed liquor was removed from the SBR daily (22% of the reactor solids), thickened to 100 mL and transferred into the sidestream sludge treatment unit. Instead of supplying the synthetic anaerobic digestion liquor into the SBR as in the baseline, the synthetic digestion liquor or partially nitrified digestion liquor, (equivalent amount of nitrogen in the form of ammonium or nitrite) was added into the treatment unit for FA or FNA treatment, conditional on experimental phases (see Section 2.2). After one day treatment, the treated sludge was recirculated to the SBR equally over four cycles during the feeding stage. The sludge treatment unit was equipped with a magnetic stirrer (250 rpm) to ensure sufficient mixing. Besides, a set of pH meter and probe (miniCHEM, Labtek) connected with PLC was installed for the unit to maintain the designed pH (specified in the following section) in the unit by adding HCl (1.0 M) or NaOH (1.0 M) via PLC.

The nitrogenous compounds concentrations, namely, NH_4^+-N, NO_2^--N, NO_3^--N and NO_x^--N (i.e. NO_2^--N+NO_3^--N) in the effluent of the SBR were analyzed 2-4 times a week. Cycle studies were performed weekly in the SBR by determining the nitrogenous compounds concentrations every half an hour throughout one cycle. In terms of activated sludge property, MLSS, MLVSS concentrations and sludge volume index (SVI_{30}) were measured weekly. AOB and NOB activities in the reactor were determined as the biomass-specific ammonium reduction and nitrate production rates, respectively, which were calculated by dividing the corresponding volumetric rates by the measured MLVSS concentration. Amplicon sequencing (16S ribosomal RNA sequencing) was performed in each phase of the reactor to assess the microbial community compositions of NOB. Quality control measures were taken for the sequencing analysis using MultiQC (version 1.3) (Table S1, Figure S2 in Supporting Information). Analytical details of the DNA extraction, PCR amplification and Amplicon sequencing are provided in Supporting Information (SI), Section 2.3 & 2.4. N_2O concentrations in the emission gas of the SBR was measured online during the steady state of each phase of the SBR. The methodology of N_2O measurement and the N_2O emission factor calculation are described in SI section 2.5. The steady state of the SBR was defined by the stable sludge concentration and also by the similar NO_3^--N, NO_2^--N and NH_4^+-N profiles in operational cycles. All the analytical procedures are presented in details in SI Section 2.

2.2 Overall experimental plan

The experimental period was divided into five phases.

- In Phase I (Day -110 – 61), sidestream sludge treatment by FNA was commissioned. An FNA concentration of 4.23 mgN/L (NO_2^-=550 mgN/L, pH = 5.5, T=22 ºC) was applied. The study starts from day 0, as a continuation of a previous work[31] (shown in negative days for background information).
- In Phase II (Day 62 – 145), the FNA treatment was suspended on Day 62, mimicking possible interruption that could happen in real-life applications. FNA treatment was resumed 7 days later, until the end of Phase II. The objective of Phase II was to verify if treatment interruptions could disrupt the nitrite shunt established in Phase I, and if such interruptions would induce NOB adaptation.
- In Phase III (Day 146 – 240), sludge treatment with FA was applied, replacing FNA treatment in Phase II, in order to verify if NOB adaptation to FNA identified in Phase II could be overcome. The FA treatment condition was chosen according to Wang et al [22] (210 mg NH_3-N/L, NH_4^+-N+NH_3-N=800 mg N/L; pH=8.9).
- In Phase IV (Day 241 – 338), the FA treatment was interrupted for a week (Day 241-247) to verify if treatment interruptions could disrupt the nitrite shunt established in Phase III by FA treatment, and if such interruptions would induce NOB adaptation.
- In Phase V (Day 339 – 650), sludge treatment with FNA was firstly applied, replacing FA treatment in Phase IV, in order to verify if NOB adaptation to FA identified in Phase IV could be overcome with FNA-based treatment. The FNA treatment concentration applied

in Phase V (FNA=3.07 mgN/L, NO_2^-:400 mgN/L, pH=5.5) was slightly relaxed from the conditions applied in Phase I&II. Phase V further aimed to demonstrate long-term stability of NOB suppression by the alternate treatment when an early sign of adaptation (NAR < 90%) is observed. The switch from FNA to FA on day 447 divided the Phase into Phase Va and Vb.

2.3 Wastewater composition

In this study, synthetic rather than real wastewater was used, in order to maintain consistent compositions of wastewater. Synthetic wastewater enables more conclusive comparison of results in different phases of this long-term study. It also maintains designed FNA and FA concentrations stably for sludge treatment and ensures FNA or FA was the only significant inhibitor.

The synthetic domestic wastewater has a total Kjeldahl nitrogen (TKN) and chemical oxygen demand (COD) concentration of 50 mgN/L and 300 mg/L, respectively. It comprised per litre: 153 mg NH_4Cl (40 mg NH_4^+-N), 83 mg milk powder, 61 mg starch, 60 mg sucrose, 29 mg yeast extract, 12 mg peptone, 45 mg CH_3COONa, 14 mg KH_2PO4, 13 mg K_2HPO4, 600 mg $NaHCO_3$, 2.5 mg $FeSO_4{\cdot}7H_2O$, 0.44 mg $CaCl_2$, 0.19 mg $NaMoO_4{\cdot}2H_2O$, 0.19 mg $MgCl_2$, 0.13 mg $CoCl_2{\cdot}6H_2O$, 0.06 mg $ZnCl_2$, 0.06 mg $MnCl_2{\cdot}4H_2O$, 0.06 mg $CuSO_4$, 0.06 mg H_3BO_3 and 0.04 mg $NiCl_2{\cdot}6H_2O$.

The synthetic anaerobic digestion liquor was simulated with NH_4HCO_3. In the baseline period, it was directly added into the feed to the SBR, increasing the ammonium loading by 20% [32]. In the FA-based treatment periods, it was fed to the sludge treatment reactor to support sludge treatment, and then recirculated to the SBR with FA-treated sludge (Figure S1). During the implementation of FNA treatment, the ammonium in the digestion liquor was assumed to be converted into nitrite by sidestream partial nitritation (Figure S1). The converted digestion liquor was simulated with $NaNO_2$ and fed to the treatment reactor for sludge treatment by FNA, and then returned to the SBR with FNA-treated sludge (Figure S1).

2.4 Batch tests determining the suppression of NOB after treatment

Batch tests were performed in all phases to determine the activities of NOB after sidestream treatment, to assess the effectiveness of sidestream FNA or FA treatment on NOB suppression. The mixed liquor withdrawn from the reactor was firstly thickened and then treated with FNA or FA as applied in the corresponding phase (refers to the experimental plan). To measure the activities of NOB following the sludge treatment, the treated sludge was washed using the synthetic SBR influent to remove the inhibition of FA or FNA and finally diluted to the volume prior to treatment. Sodium nitrite stock solution was afterwards dosed into the mixed liquor, resulting in an initial nitrite concentration of 25 mgN/L. Continuous aeration and mixing was then provided to test the activity of NOB. pH was maintained between 7.1 to 7.4 by PLC via the dosing of 0.5M HCl and 0.5M $NaHCO_3$. Each test lasted for 8 hours and mixed liquor samples were taken at 2 hours intervals, for ammonium, nitrite and nitrate analysis. MLSS and MLVSS were determined at the end of each test. The biomass specific nitrate production rates (NOB activities) were then obtained by dividing the volumetric nitrate production rates (linear regression) by the MLVSS concentration.

3. RESULTS AND DISCUSSION

3.1 NOB adaptation and the proposed sludge alternate treatment strategy

As shown in Figure 1, nitrite shunt was maintained in Phase I stably with a high NAR of 87.5 ± 0.9% by sludge treatment using FNA. The observation of low NOB in-reactor activities of 0.5 ± 0.2 mg (NO_3^-–N)/g(MLVSS)/h and hardly detectable NOB activities after FNA treatment of 0.05 ± 0.003 mg (NO_3^-–N)/g(MLVSS)/h suggested effective NOB suppression by FNA treatment (Figure 2). However, as soon as the FNA treatment was intentionally interrupted at the start of Phase II (Day 62), the NOB activity recovered rapidly, resulting in the plunge of NAR from 81.4% to 5.3% in 7 days before FNA sludge treatment was restored. The reintroduction of FNA treatment immediately reversed the decline of NAR. However, the same FNA treatment approach could no longer suppress the NOB to the previous level, leading to a varying NAR in the range of 13% to 50% (29.5 ± 2.5%) in Phase II. The NOB in-reactor activities increased from 0.5 ± 0.2 mg(NO_3^-–N)/g(MLVSS)/h in Phase I to 2.4 ± 0.5 mg(NO_3^-–N)/g(MLVSS)/h (obtained between Day 102-144) (Figure 2). On Day 144, the end of Phase II, the NOB activities have reached above 3.5 mg(NO_3^-–N)/g(MLVSS)/h in the reactor and 1.74 mg(NO_3^-–N)/g(MLVSS)/h after FNA treatment; while NAR has dropped to less than 5%. NOB have clearly developed resistance to the FNA treatment, which led to failure of the nitrite shunt (Figure 1).

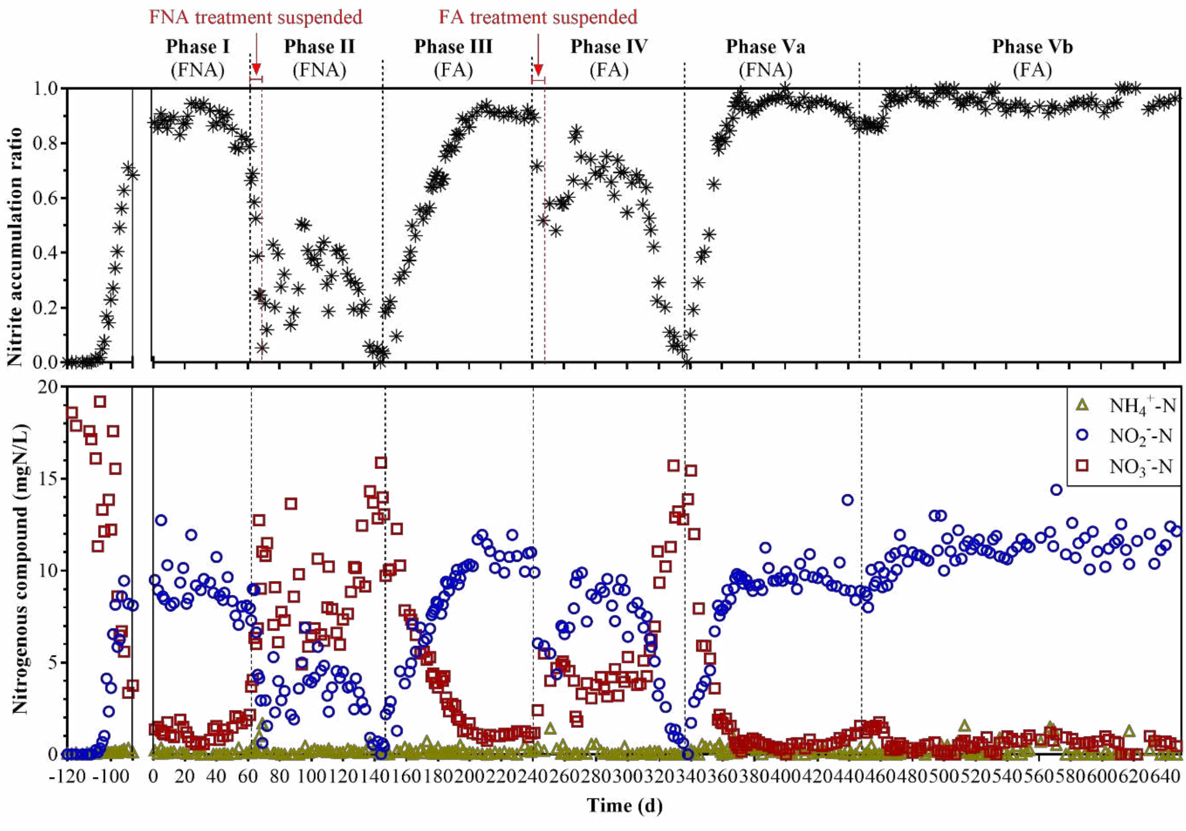


Figure 1 Nitrite accumulation ratio (NO_2^-−N/(NO_2^-−N + NO_3^-−N) × 100%) in the effluent of the reactor during the study period (Upper), and the effluent NO_2^-−N, NO_3^-−N and NH_4^+−N concentrations during the study period (Lower). Details of data between day -90-0 can be found in Duan, et al. [31].

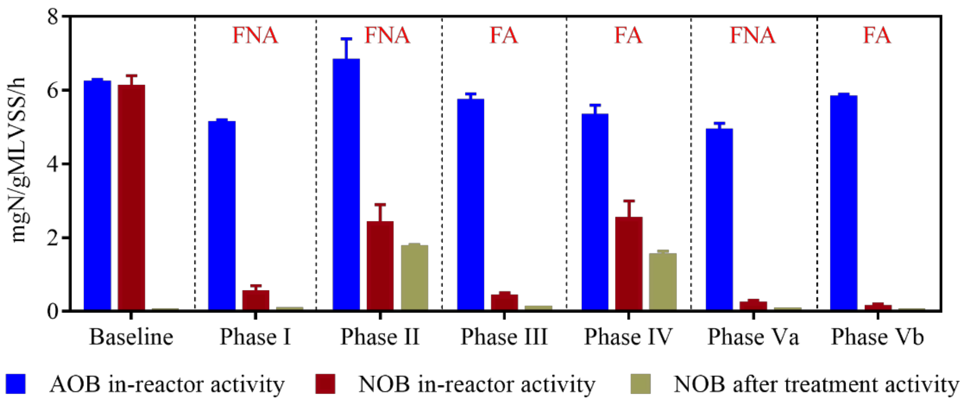


Figure 2 AOB, NOB in-reactor activities and NOB activities after sidestream sludge treatment during steady state of each phase. For Phase II & IV, NOB in-reactor and after treatment activities shown were determined towards the end of Phase II & IV (specified in text).

To address the identified NOB adaptation, FA treatment was implemented in Phase III. While the NOB had been resistant to FNA treatment, batch tests (on Day 139) showed much-reduced NOB activities after FA treatment, at 0.095 ± 0.002 mg(NO_3^-–N)/g(MLVSS)/h (Figure 2). The gradual increase of NOB activities and decrease of NAR during Phase II reversed instantly following the introduction of the FA-based sludge treatment (Figure 1). The NOB in-reactor activities were progressively suppressed to 0.4 ± 0.1 mg(NO_3^-–N)/g(MLVSS)/h and NAR reached over 90% in 60 days. The nitrite shunt was re-established with an even higher NAR (90.5 ± 0.5%) than Phase I. This was due to the lower NOB in-reactor activities detected in Phase II than Phase I (Figure 2), suggesting switching to FA treatment was effective in suppressing FNA resistant NOB.

Following the 7-day interruption to FA-based sludge treatment at the beginning of Phase IV, the NAR decreased to 57.8% at the end of the 7-day period. The NOB activity recovery was slower than that in Phase II. Following the resumption of FA treatment, NAR recovered to around 70%. The nitrite shunt was maintained relatively stable by FA treatment for around 45 days (Day 266-310, Figure 1), before a gradual decline of NAR initiated on Day 310, which eventually resulted in no nitrite accumulation on Day 338. The average NOB in-reactor activity of 2.5 ± 0.5 mg(NO_3^-–N)/g(MLVSS)/h towards the end of Phase IV (Day 312-335) and NOB activities of 1.51 ± 0.12 mg(NO_3^-–N)/g(MLVSS)/h after FA treatment on Day 338 both suggested developed NOB resistance to FA treatment (Figure 2).

Following the alternation to FNA treatment in Phase V (Day 339), the nitrite shunt was rapidly restored in three weeks. In Phase V, the in-reactor NOB activities progressively decreased to 0.2 ± 0.1 mg(NO_3^-–N)/g(MLVSS)/h in 33 days (Figure 2), which resulted in the steady increase of NAR from 0 to 98.5%. NAR stabilized at 94.2 ± 0.5% for the following three months. The much-depressed NOB activities (Figure 2) indicated that the FA resistant NOB communities could be suppressed by FNA treatment. A decrease of NAR detected from 93.0% to 85.2% (Day 441-447, Figure 1), indicated an early signal of NOB adaptation to FNA treatment. This triggered an alternation from FNA-based sludge treatment to FA-based sludge treatment, according to our experimental design. The decline of NAR was stopped immediately while the NAR did not rise as expected (day 447-461, Figure 1). It was found out on Day 461 that the biofilm attached to the DO probe caused over-aeration in the reactor, as confirmed by a substantially higher fraction of 'Aeration on' periods (~70%), in comparison to other periods (~50%). Following the cleaning of the DO probe, NAR increased instantly, from 86.3% to 97.8% in 6 days (Day 461- 466, Figure 1). The slight decrease of NAR prior to the switch was also likely a result of over-aeration, suggesting that over-aeration favours NOB growth. NAR in the remaining period of Phase V (Vb) was maintained at the highest level in this study, 95.9 ± 0.4%, as a result of the effective suppression on the NOB activities, at 0.1 ± 0.1 mg(NO_3^-–N)/g(MLVSS)/h (the lowest in-reactor activity in this study). The early development of NOB adaptation to FNA observed in Phase V was shown to be restrained and stable suppression of NOB was ensured by the alternate treatment.

The alternate treatment approach not only tackled the NOB adaptation challenge, it also suppressed the NOB more effectively than FNA or FA treatment alone. This is reflected by the reduced NOB in-reactor activities and the elevated NAR. The NOB in-reactor activities decreased with the alternate treatment effectively applied (0.5 ± 0.2, 0.4 ± 0.1, 0.2 ± 0.1 and 0.1 ± 0.1 mg(NO_3^-–N)/g(MLVSS)/h, in Phase I, III, Va and Vb, respectively). The NAR increased accordingly, 87.5 ± 0.9, 90.5 ± 0.5, 94.2 ± 0.5 and 95.9 ± 0.4% in Phase I, III, Va and Vb. With same treatment conditions, the NAR achieved in this study is higher than sole FNA treatment approach (90.0 ± 0.5%) or FA treatment approach (91.3 ± 0.5%) reported previously [22, 31].

This study also revealed that NOB could unlikely develop resistance to the alternate treatment strategy, which promised the long-term stable NOB suppression. While the NOB have developed resistance to FNA in Phase II, after receiving sludge treatment by FA in Phase III & IV, the resistance to FNA was not observed in Phase V (FNA treatment) anymore. Similarly, the FA resistance of NOB identified in Phase IV did not withstand FA treatment in Phase Vb. The loss of resistance suggested that proposed alternate treatment approach might always be effectual controlling NOB adaptation due to the alternation nature of this approach.

The impact of the sludge alternate treatment approach on the nitrogen removal performance was assessed with the analysis of nitrogenous compounds in the effluent of the SBR (Figure 1, Table S2) and the SBR cycle studies (Figure S3). Compared with the baseline, the alternate treatment did not considerably affect the AOB activities in the reactor, therefore ensured satisfactory ammonium removal in all experimental phases with above 99.5% ammonium converted (Figure 1 & 2, Table S1). The approach has positive impacts on the TN removal performance due to the nitrite shunt attainment. Effects of nitrite shunt on enhancing nitrogen removal have been reported in many previous studies [2]. Detailed results and analysis on nitrogen removal of this study can be found in Supporting Information Section 3. It should be pointed out that while successful NOB suppression and TN removal enhancement have been achieved in this study, the nitrite level in the effluent was relatively high (⩾10 mgN/L), which is toxic to the aquatic life[33]. The effluent nitrite level could be reduced by applying multi-step feeding regime[31]. Furthermore, aeration control, e.g., low DO control, could be applied in the system to attain simultaneous nitritation and denitritation, whereby the nitrite in effluent could be significantly reduced[34, 35].

3.2 NOB community shifts

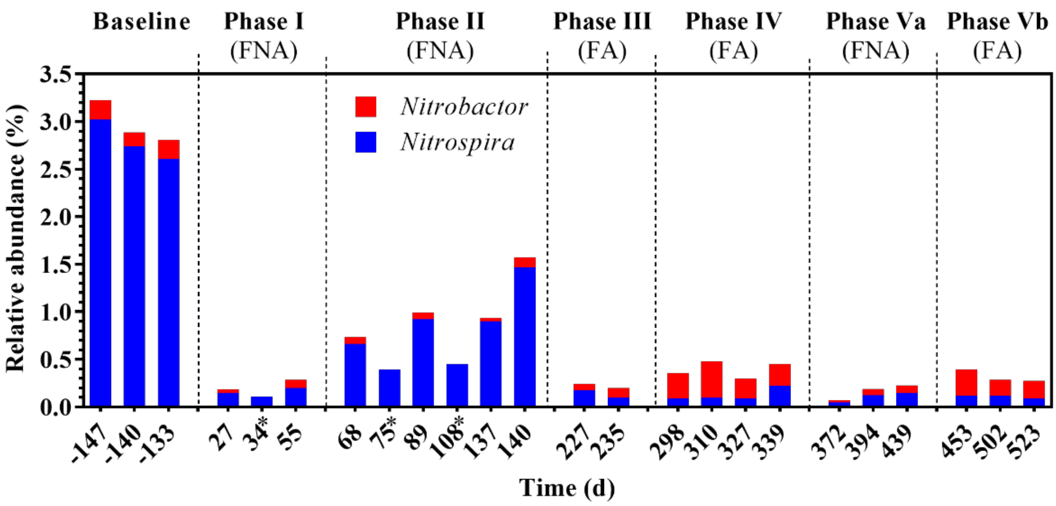


Figure 3 Relative NOB genus abundances (%) during the study, determined by 16S rRNA Amplicon sequencing. Quality control was conducted for the sequencing analysis and the data are provided in SI. *for days 34, 75 and 108, *Nitrobactor* was not detected.

NOB communities were monitored to understand the mechanisms underlying the NOB adaptation and mitigation. Opposite NOB population shifts were observed in acclimation to FNA and FA (Figure 3), supporting the process data. *Nitrospira* was already at a relatively higher abundance than *Nitrobacter* in Phase I, and increased by five times in Phase II from 0.13 ± 0.06% to 0.78 ± 0.15% (s.e.m.), dominating NOB community by the end of Phase II. Higher abundance of *Nitrospira* than *Nitrobacter* in Phase I&II implied that *Nitrospira* was more tolerant to FNA treatment. It is apparent that the resistance of NOB to FNA treatment observed in Phase II was due to the enhanced growth of *Nitrospira*. The growth of *Nitrospira* was likely accelerated by the suspension of FNA treatment at the beginning of Phase II. The growth of *Nitrospira* was successfully restrained by FA treatment and its abundance decreased by nearly 10-folds to 0.08 – 0.16% ($p<0.01$). In comparison, the abundance of *Nitrobacter* remained unchanged until interruption of FA treatment. The interruption to FA treatment at the beginning of Phase IV stimulated the growth of *Nitrobacter*, with the *Nitrobacter* abundance tripled from 0.10% at the end of Phase III to 0.27 ±0.04% at the end of Phase IV. The microbial data in Phase IV clearly showed that *Nitrobacter* has a higher level of tolerance to FA compared to *Nitrospira*. The reduction of *Nitrobacter* abundance in Phase Va supported the effectiveness of the FNA treatment on suppressing *Nitrobacter* growth. In Phase Vb, low abundance levels of both *Nitrobacter* and *Nitrospira* were sustained (Figure 2). Stable nitrite shunt was maintained for more than 10 months with NAR still being above 95% at the end of this experimental study. The results showed the effectiveness and reliability of the proposed alternate treatment strategy for NOB control.

Microorganisms could adapt to inhibiting environment by reversibly adjusting their physiology to maximize resource utilization while maintaining structural and genetic integrity by repairing and minimizing damage to cellular infrastructure [30]. FNA and FA are inhibitory or even biocidal[36, 37] to microorganisms while NOB are more sensitive to these than AOB[22]. The biocidal effects of FNA are likely due to the oxidative damages by various reactive nitrogen and oxygen species dissociated from FNA. These reactive species can lead to oxidative damage to cellular proteins, cell membrane and nucleic acids [37, 38]. The *Nitrosomonas* genus of AOB have been reported to adapt to FNA by upregulating a number of known oxidative stress enzymes and energy generating enzymes[37]. In comparison, the inhibitory effects of FA are attributed to the passive diffusion of FA molecule into cells, causing proton imbalance or potassium deficiency[39]. The cells must then consume energy in proton balancing, potassium pumping[40]. The FNA and FA treatments likely drove selections of NOB populations that are better suited to readjust physiology to manage and mitigate the consequences of distinct stresses resulting from FNA and FA, namely *Nitrospira* to FNA treatment and *Nitrobacter* to FA treatment. However, the mechanism of NOB adaptation to FNA or FA is still unclear. A comprehensive understanding of the adaptations of NOB to different kinds of stresses requires in-depth research on proteins, genetics and cell membranes, which is recommended for future research. A better understanding of the controlling mechanisms of NOB adaptation would facilitate further optimization of the proposed approach.

It should be noted that *Nitrospira* and *Nitrobacter* were the two key NOB populations in our reactor and no other known NOB was found in this study, which agrees with most real scenarios. It is generally accepted that *Nitrospira* (dominant) and *Nitrobacter* are the prevailing NOB in most WWTPs [41-44]. Therefore, the strategy demonstrated in this study could be applicable to most WWTPs. However, it was recently found that the genus *Nitrotoga* could as well be an important nitrite oxidizer in some WWTPs [44, 45]. More importantly, *Nitrotoga* reportedly developed resistances to FNA treatment [26]. The effectiveness of the proposed strategy thus remains to be investigated in *Nitrotoga*-abundant activated sludge systems.

3.3 Promoted but declining N_2O emissions

N_2O is a potent greenhouse gas and an ozone-depleting matter that can be produced in biological nitrogen removal, leading to serious environmental problems[46]. The N_2O concentration in the reactor off-gas was monitored to assess consequence of applying the alternate treatment strategy on N_2O emission. The N_2O emission from the treatment tank is also measured and shown to be negligible (Figure S4).

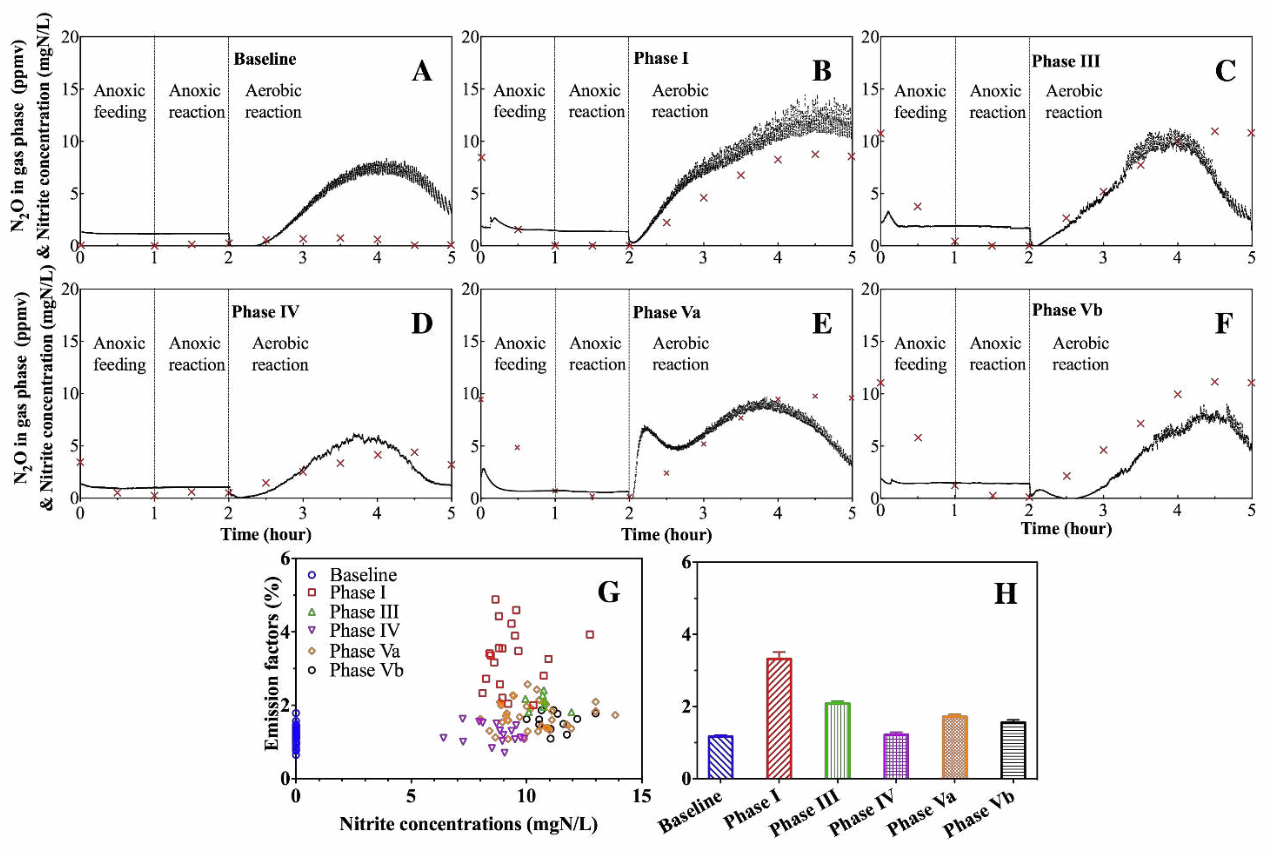


Figure 4 Gas phase N_2O concentrations over a typical SBR cycle at steady states of Baseline (A), Phase I (B), III (C), IV (D), Va (E) and Vb (F) in the SBR (red crosses indicate the according nitrite concentrations during the cycle; N_2O data for Phase IV was obtained when NAR was relatively constant at ~70%); (G) Emission factors measured during steady states of each phase plotted against the effluent nitrite concentrations on the same measurement cycle, and (H) the average emission factors during the steady states of each phase, error bars are shown as standard errors in replicate cycle measurements (n>=10).

The gas phase N_2O monitoring in the SBR (Figure 4) suggested that although the N_2O emission was stimulated following the initial attainment of nitrite accumulation, a gradual decline of N_2O emissions occurred during the long-term study. Before the implementation of the alternate treatment, sidestream sludge treatment by FNA applied to the SBR in Phase I promoted the N_2O emission substantially: the N_2O emission factor nearly tripled, from 1.17 ± 0.04 % to 3.32 ± 0.19%. The observed increase of N_2O emission was likely caused by the nitrite accumulation. An increase in nitrite concentration is generally recognised to promote N_2O emission in mainstream wastewater treatment [47, 48]. Partial nitrification systems are expected to produce more N_2O than the conventional full nitrification systems [49], which is consistent with our observations. However, when the alternate treatment (by FA) was applied in Phase III, significantly less N_2O was emitted (emission factor: 2.08 ± 0.07%) than that in Phase I. The N_2O emission was reduced further when the sludge treatment was alternated in Phase Va and Phase Vb, with emission factors down to 1.72 ± 0.06 and 1.56 ± 0.07%, respectively (Figure 4). It should be noted that the nitrite accumulation levels increased over the mentioned phases, but the nitrite increase did not stimulate more N_2O emission, contrary to the current understanding that higher nitrite levels induces more N_2O production. Although the N_2O

emission factor is still higher than that in the baseline, the gap narrowed from an absolute 2.15% difference to 0.4%.

The mechanism underlying the N_2O emission reduction is speculative and remains to be further investigated. Nevertheless, we hypothesise that this could be related to adaptation of AOB to the changing environment, i.e., nitrite accumulation environment. A stronger correlation of N_2O emission and nitrite concentrations in the reactor is observed in Phase I than in other phases. During typical SBR cycles (Figure 4A-F), gas phase N_2O concentrations only agreed well with nitrite concentrations in Phase I, while N_2O concentrations decreased substantially towards the end of aeration in other phases. Similarly, plotting emission factors against the effluent nitrite concentrations (Figure 4G), stimulation effects can also be observed in Phase I results, when higher nitrite concentration caused higher N_2O emission. However, nitrite concentrations did not exhibit clear correlations with N_2O emission in other phases. The observation could imply that the changing environment (increase of nitrite) initially stimulated AOB for N_2O production in Phase I. However, AOB afterwards adapted to the nitrite accumulation environment. Therefore, the nitrite concentration could no longer stimulate the N_2O emissions in the later phases. A similar observation was reported in the long-term study of Ahn, et al. [50], where a lab-scale bioreactor operated sequentially from full nitrification to partial nitritation. It was observed that the N_2O emissions peaked during the transition from full nitrification to partial nitritation period and stabilized gradually, suggesting the adaptive capability of the involved microbial communities (likely AOB). Yu et al [51] recently also reported decreasing N_2O emission from AOB during the development of AOB adaptation to oxic-to-anoxic transitions. The AOB adaptation was regulated at protein levels, which reduced the accumulation of the intermediates (nitric oxide and hydroxylamine) for N_2O production, leading to the decrease of N_2O emission.

3.4 Implications to wastewater treatment

This study proposed a promising approach that successfully overcome the NOB adaptation challenge and maintained a stable nitrite shunt in mainstream wastewater treatment. While the strategy is demonstrated in a mainstream nitrite shunt system, it can theoretically be applied to mainstream PN/A system, too. Also, the strategy is independent of using SBR and can be applied to a continuous flow system. This approach can effectively address the critical NOB adaptation challenge and thus ensuring a more stable NOB suppression. Stable mainstream NOB suppression is required for mainstream nitrite shunt or PN/A process, which provide opportunities for carbon-efficient nitrogen removal thus enabling higher energy recovery and lower operational costs. While the concepts of mainstream nitrite shunt or PN/A have been proposed for decades [6, 52] and a large number of lab-scale studies have been reported, its full-scale application is scarce. The stable suppression of NOB is one of the major barriers for the scale-up of such process [53]. The approach proposed in this study showed very promising results in overcoming the NOB adaptation issue, making a significant step forward in achieving reliable mainstream nitrite shunt or PN/A process.

The observed reverse community shifts and successful demonstration of the proposed strategy suggested that alternating among appropriate NOB controlling strategies could potentially be a general approach coping with NOB adaptation challenges in a nitrite shunt and PN/A system. For example, the adaptation of NOB to low DO was reportedly due to NOB community shift from *Nitrobacter* to *Nitrospira* [24, 25]. This problem could potentially be dealt with by using FA treatment, which was shown selecting *Nitrobacter* against *Nitrospira*. Generally, if a specific shift of NOB community is observed due to the adaptation to one NOB controlling strategy, the adaptation could likely be controlled by applying another NOB controlling strategy that selects against these NOB species. Alternating between suitable NOB controlling strategies could potentially address most NOB adaptation challenges in a nitrite shunt or PN/A system. The sludge alternate treatment with FNA and FA is a successful (and the first) demonstration of this principle.

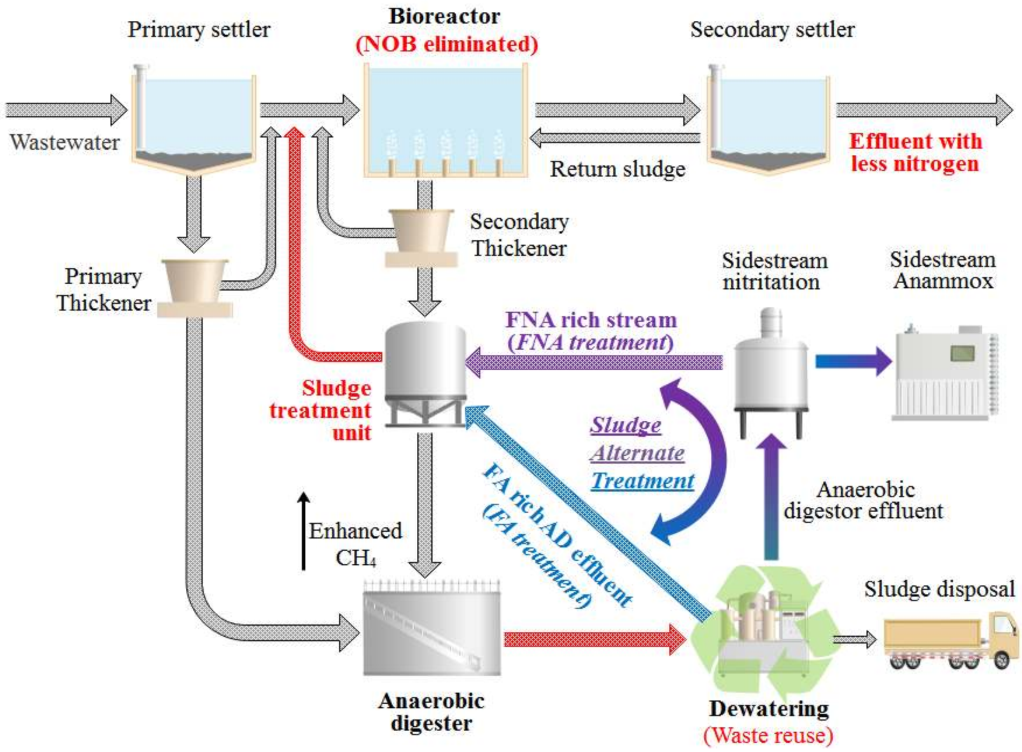


Figure 5. The closed-loop system implementing the proposed sludge alternate treatment strategy in a WWTP.

The proposed alternate treatment approach is an economically attractive and pragmatic approach for water utilities. While the proposed approach could incur extra operational costs due to chemical addition (HCl or NaOH) and capital costs due to the need for a sidestream nitritation unit (for WWTPs without sidestream treatment) and sludge treatment unit, the approach is overall economically favourable. Previous economic analyses performed for FNA-based and FA-based approaches revealed that both approaches are economically favourable due to significant savings in aeration energy, methanol addition and sludge handling, despite the additional costs of FNA or FA treatment[22, 31]. The proposed approach makes use of the anaerobic sludge digestion liquor, as the source of FNA and FA, respectively. Anaerobic digestion liquor is a waste stream with high ammonium available, which can also be transformed into high levels of nitrite stream by sidestream nitritation. It sets an excellent example for the paradigm shift of the WWTPs from 'linear economy' to 'circular economy' (Figure 5). The implementation of the proposed alternate strategy is practical. The strategy requires regular alternations between these two approaches. Unlike alternating with other approaches, the shared similarities of the FNA and FA approaches will unlikely over-complicate the alternate treatment operations in WWTPs. The switch from FNA-based to FA-based approach can be simply implemented by feeding the ammonium-rich anaerobic sludge digestion liquor directly to the sludge treatment unit, by-passing the FNA production reactor, vice versa, as illustrated in Figure 5.

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Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website. Additional figures (Figures S1−S4), tables (Tables S1− S2), and texts referenced in the main text showing information on schematic diagram of experimental setup, analytical procedures, quality control for sequencing analysis, nitrogen removal performance analysis and N_2O in the sludge treatment unit.

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