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A novel technology with precise oxygen-input control: Application of the partial nitritation-anammox process

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ABSTRACT

Reliable and accurate oxygen-input control, which is critical to maintaining efficient nitrogen removal performance for partial nitritation-anammox (PN-A) process, remains one of the main operational difficulties. In this study, a novel, yet simple system (a simple process for autotrophic nitrogen-removal, SPAN) with precise oxygen-input control was developed to treat ammonium-rich wastewater via PN-A process. SPAN brings oxygen to biomass by circulating water and creating water spray (shower) at the water-air interface, and effectively balances the activities of core functional microorganisms through precise oxygen-input control. The oxygen-input rate is decided by the water circulation rate and shower rate and is measurable and predictable. Therefore, the required amount of oxygen for ammonium oxidation can be precisely delivered to the biomass by adjusting the circulation rate and shower rate. The results of two parallel SPAN reactors demonstrated that during long-term operation, the required oxygen input was precisely and reliably controlled. More than 99% of NH_4^+-N and 81% - 85% of total nitrogen were stably removed, with anammox bacteria contributing to more than 96% of total nitrogen removal. Anammox bacteria were efficiently enriched to the highest level among the key nitrogen-converting microbial groups, both in terms of abundance (8.17%) and nitrogen-conversion capacity, while ammonium oxidizing bacteria were well controlled to provide sufficient ammonium-oxidizing capacity. Nitrite oxidizing bacteria were maintained stable (relative abundance of 1.08%-1.88%) and their activity was effectively suppressed. This study provided a novel technology, SPAN, to precisely control oxygen input in PN-A system, and proved that SPAN was effective and reliable in achieving long-term high-efficiency nitrogen removal.

1. Introduction

Among the biological nitrogen removal alternatives, single-stage partial nitritation-anammox (PN-A) process has arisen as one of the most attractive ones during the last two decades (Sliekers et al., 2002; Third et al., 2001). Single-stage PN-A has advantages including lower aeration demand, no external organic carbon requirement, and lower excess sludge yield (Lackner et al., 2014; Li et al., 2017b), making it a promising part of energy-neutral or even energy-positive municipal/ wastewater treatment scheme. The single-stage PN-A process, as described by Eq. (1) (Third et al., 2001), consists of two autotrophic nitrogen conversion processes in a single reactor: ammonium-oxidizing microorganisms, mainly ammonium-oxidizing bacteria (AOB), oxidizing roughly half of the ammonium (NH_4^+) to nitrite (NO_2^-), and anammox bacteria converting the rest of NH_4^+ and NO_2^- to dinitrogen gas (Daverey et al., 2013). The successful application of PN-A relies on efficient retention of anammox bacteria and AOB, and stable interactions between them. Simultaneously, the undesired aerobic nitrite-oxidizing bacteria (NOB), which compete with anammox bacteria for nitrite and AOB for oxygen (Agrawal et al., 2018), must be well suppressed.

Oxygen is one of the most vital controlling parameters in PN-A process: it not only serves as a necessary electron acceptor for both

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AOB and undesirable NOB, but also acts as an inhibitor for anaerobic anammox bacteria (Agrawal et al., 2018; Kuai and Verstraete 1998). Thus, in practical application, the stability of PN-A systems relies on reliable and accurate control of oxygen input, while improper oxygen-input control leads to the imbalance of microbial communities (overgrowth of NOB and inhibition of anammox bacteria) and deterioration of nitrogen removal performance (accumulation of NH_4^+, NO_2^- or NO_3^-) (Lackner et al., 2014; Lotti et al., 2014).

$$0.85\boldsymbol{O}_2 + \boldsymbol{NH}_4^+ \rightarrow 0.435\boldsymbol{N}_2 + 1.3\boldsymbol{H}_2\boldsymbol{O} + 1.4\boldsymbol{H}^+ + 0.13\boldsymbol{NO}_3^- \quad (1)$$

However, reliable and accurate oxygen-input control via conventional oxygen-input control methods through diffused aerators, mechanical aerators, and combined aerators remains one of the main operational difficulties for the treatment of side-stream wastewater using PN-A process (Lackner et al., 2014), let alone for the even more challenging treatment of mainstream wastewater. Both diffused aeration and surface aeration are commonly used aeration techniques (Mueller et al., 2002). The innermost controller of the conventional oxygen-input control strategy is dissolved oxygen (DO) cascade control, which indirectly estimates the relationship between oxygen input and oxygen demand and controls oxygen input based on the DO concentration and the airflow rate (Åmand et al., 2013). This is not always reliable and largely results in a gap between the oxygen demand and the actual oxygen input for PN-A process, which is sensitive to the accuracy of oxygen-input control (Åmand et al., 2013; Lackner et al., 2014). In PN-A systems, the efforts to reduce this gap via conventional oxygen-input control methods mostly rely on adding other parameters such as oxidation–reduction potential (ORP), pH, and nitrogen concentrations into the control strategy (Lackner et al., 2014). This can be misleading as these parameters alone cannot directly reflect the actual oxygen input and do not always provide a good correlation with biomass activity or substrate depletion. For instance, DO concentration could remain within the target control range when oxygen input is higher than the demand of AOB because of the oxygen consumption by other microbes (Lackner et al., 2014; Li et al., 2016). Thus a gap between the oxygen demand and the oxygen input always remains. On the other hand, the incapability of precise oxygen-input control necessitates complex online aeration control strategies including pH-based aeration control, ammonia-based aeration control, and fixed-DO control, etc. (Klaus et al., 2017), and complex aeration control instruments such as online probes (probes measuring NH_4^+, NO_2^- and NO_3^- concentrations, DO concentration, pH, conductivity, etc.), distributed control systems (to receive signals and send commands), and modulating control valves (Christensson et al., 2013; Lackner et al., 2014; Qiu et al., 2020). According to a survey on full-scale PN-A installations treating side-stream wastewater, aeration control is one of the main operational difficulties: 30% of the surveyed plants experienced NH_4^+ accumulation (oxygen input < oxygen demand) lasting for several days to three weeks, and 50% of the surveyed plants experienced NO_2^- and NO_3^- build-up (oxygen input > oxygen demand) generally lasting up to several days for NO_2^- accumulation and several weeks for NO_3^- accumulation (Lackner et al., 2014). Thus, the establishment of a more reliable, easily accessible, and precise oxygen-input control method is required for the oxygen-sensitive PN-A process.

Therefore, in this study, a novel, yet simple system with precise oxygen-input control (a Simple Process for Autotrophic Nitrogen-removal, SPAN) was developed to treat ammonium-containing wastewater via PN-A process. In SPAN system, the activities of core functional microorganisms are effectively maintained as desired to achieve stable and efficient nitrogen removal performance. This is realized in two ways: 1) the required amount of oxygen is precisely delivered to the microorganisms based on theoretical oxygen demand rather than parameters like DO concentrations; and 2) designing the reactor to accommodate biomass at a nearly zero-DO zone away from the oxygen-rich section in the reactor, which reduces the oxygen inhibition on anammox bacteria and maximizes the suppression of NOB by limiting the oxygen availability. In SPAN reactor, the oxygen transfer from atmospheric air to wastewater is achieved through creating a disturbance at the air-water interface by circulating/spraying wastewater. The SPAN system consists of a cylindrical reactor, three pumps for filling, drawing, and wastewater circulation, and timers for controlling the pumps. In SPAN reactor, biomass is located at the bottom of the reactor, and DO in the wastewater at the top of the reactor is delivered to the biomass via wastewater circulation. DO is depleted by AOB in the biomass to oxidize NH_4^+ to NO_2^-, thus creating a nearly zero-DO zone at the bottom of the reactor allowing the minimization of oxygen inhibition on anammox bacteria and maximization of suppression of NOB. The oxidized NO_2^- and the rest of NH_4^+ are converted to N_2 by anammox bacteria in the biomass. The DO-depleted wastewater is circulated back to the top of the reactor thus creating an oxygen gradient difference to allow the natural diffusion of oxygen from the air into water. If necessary, the oxygen transfer rate can be enhanced through creating disturbance at the air-water interface by creating water spray on the water interface (simulating a shower). Oxygen input is the function of the water circulation rate and the shower rate, and the oxygen input at various circulation rates/shower rates can be measured aforehand using clear water without biomass in an identical reactor. The theoretical oxygen demand for oxidizing about half of NH_4^+ to NO_2^- by AOB is calculated according to Eq. (1) based on the incoming NH_4^+-N. In this way, oxygen input is precisely controlled to meet the demand by controlling the water circulation rate and the shower rate. Therefore, the activities of the core functional microorganisms are effectively balanced through precise oxygen-input control and reactor design: AOB get just enough oxygen to oxidize ammonium, NOB overgrowth is suppressed because the delivered oxygen is advantageously depleted by AOB (Guisasola et al., 2005), and oxygen-inhibition on anammox is minimized. Therefore, stable and efficient nitrogen removal can be maintained in SPAN system.

In this study, two identical lab-scale SPAN reactors (R1 and R2) were set up and operated to verify the effectiveness of SPAN. The research aims were to investigate: 1) the long-term nitrogen removal performance at various oxygen inputs; 2) the capacities of the key nitrogen-conversion pathways to monitor the evolution of the key nitrogen-converting guilds, and 3) the dynamics of the microbial communities in one of the reactors (R1) through metagenomic analysis.

2. Materials and methods

2.1. Design and operation of SPAN

Two identical reactors, R1 and R2, were set up as SPAN reactors and run parallelly under the same conditions to confirm the effectiveness of the novel SPAN technology (Fig. 1). These two reactors were previously operated using conventional oxygen-input control methods. The SPAN reactor had an effective volume of 2.6 L and a height/diameter ratio of 11.5. Three peristaltic pumps were used, i.e., an influent pump, an effluent pump, and a pump for circulating water and creating shower via a by-pass line if necessary (Fig. 1); all pumps were controlled by programmable timers. The pump for water circulation drew water from the top of the reactor and delivered the water to the bottom of the reactor. With the water circulation rates in this study, the PN-A biomass, which was in a suspended growth form, resided only at the bottom part of the reactor (Fig. 1). The reactors were used to treat synthetic wastewater and were run as sequencing batch reactors (SBRs). One

Table 1
The operating conditions of R1 and R2.

Day	1–82	83–358	359–431
Stages	1	2	3
Influent NH_4^+-N (mg/L)	200	300	300
NLR (mg N/L/d) [a]	77	115	231
Circulation rate/shower rate (mL/min / mL/min)	70/0	70/0, 150/0, 300/0 [b]	200/88
Oxygen input (mg O_2/d)	314	314, 454, 858	1290

[a] nitrogen loading rate.
[b] day 83–158: 70/0; day 159–260: 150/0; day 261–358: 300/0.

SBR cycle included a 5-minutes fill phase, a 340-minutes reaction phase, a 10-minutes settling phase, and a 5-minutes draw phase. Wastewater circulation and the operation of the shower only happened during the reaction phase. The temperature was maintained at 30 °C by placing the reactors in a light-blocked thermostatic cabinet. The operation of the reactors was divided into three stages depending on the increasing nitrogen loading rate (NLR) (Table 1). In Stage 1, the nitrogen removal performance of SPAN reactors with precise oxygen-input control to meet the oxygen demand was investigated. In Stage 2, the reactors were operated under various water circulation rates to examine the nitrogen removal performance with lower or greater oxygen input than oxygen demand. In Stage 3, the reactors were operated to examine the effectiveness of SPAN technology with the addition of shower.

2.2. Determination of oxygen input

Oxygen input (mg O_2/d) was calculated according to Eq. (2) (Baquero-Rodríguez et al., 2018).

$$\text{Oxygen input} = \int_0^t k_L a \times (C_s - C_t) \times V \times dt \quad (2)$$

where k_La is the volumetric oxygen transfer coefficient (1/s), C_t is the DO concentration at time t, C_s is the oxygen saturation concentration, and V is the volume of the reactor. C_t was measured and recorded using a DO sensor (FDO925, WTW, Germany) and a portable meter (Multi3620, WTW, Germany). At the bottom of the reactor where the biomass resided, C_t was constant at 0 (Figure S1), so C_t was set as 0 during the calculations. C_s was measured using saline water (the same salinity of 0.22% as that in the reactor) at 30 °C. k_La was determined using the dynamic method according to Eq. (3) and (4) (Garcia-Ochoa and Gomez 2009; Rodgers et al., 2006; Zhan et al., 2006).

$$\frac{dC}{dt} = k_L a \times (C_s - C_t) \quad (3)$$

$$\ln\left(\frac{C_s - C_t}{C_s - C_0}\right) = -k_L a \times t \quad (4)$$

where C and C_0 are the DO concentration and DO concentration at time 0, respectively.

k_La values under various circulation rates and shower rates were determined in an identical reactor using saline water (0.22%, same with that in the reactors) with the absence of biomass. Firstly, DO in the saline water was removed by flushing dinitrogen gas, and then the saline water was circulated at the set circulation rate and shower rate. The DO concentrations during the tests were recorded and were used to calculate k_La. Because the temperature of the saline water was inevitably reduced to lower than 30 °C by the cold dinitrogen gas, k_La was determined at 20 °C and adjusted to at 30 °C as per Eq. (5) (Lee 2017; Vogelaar et al., 2000).

$$K_L a(T) = k_L a_{20} \theta^{T-20} \quad (5)$$

where $k_La(T)$ is k_La value at temperature T, k_La_{20} is k_La at temperature 20 °C, and θ is the theta factor (1.008 was used in this study (Lee, 2017)).

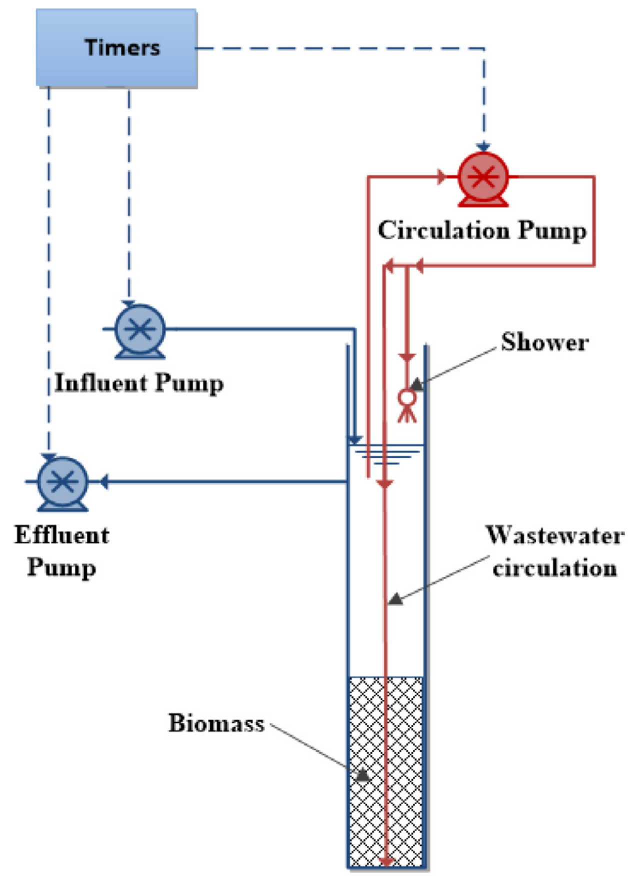

Fig. 1. Simplified scheme of the SPAN system (shower was used only in Stage 3).

2.3. Calculation of theoretical oxygen demand and actual oxygen consumption for nitrogen conversion

The theoretical oxygen demand (mg O_2/d) for nitrogen removal was calculated based on Eq. (1), i.e., 1.94 mg O_2 is needed to remove 1 mg NH_4^+-N. The oxygen consumption (mg O_2/d) by PN-A and by the production of NO_3^--N via full nitrification was considered. The actual oxygen consumption (mg O_2/d) by PN-A was calculated based on Eq. (1): the production of 1 mg N_2-N from NH_4^+-N requires 2.23 mg O_2. The calculation was made based on the total nitrogen (TN) removal instead of NH_4^+-N removal or NO_3^--N production because part of NH_4^+-N was oxidized to NO_3^--N by nitrification. The oxygen consumption (mg O_2/d) by full nitrification was calculated using Equation 6: 4.57 mg O_2 is needed to produce 1 mg NO_3^--N (NO_3^--N produced by nitrification = (measured NO_3^--N) – (NO_3^--N produced by PN-A), and NO_3^--N produced by PN-A was calculated based on Eq. (1)).

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (6)$$

2.4. Synthetic wastewater and seeding sludge

Synthetic wastewater mimicking ammonium-rich wastewater with high N/COD ratio, which was made from tap water according to Qiu et al. (Qiu et al., 2019), mainly contained 200 - 300 mg/L NH_4^+-N, 40 - 60 mg/L COD with N/ COD ratio of 4, 706 - 1059 mg/L $NaHCO_3$ (NH_4^+-N/HCO_3^- of 1.7), 170 mg/L $CaCl_2$ • $6H_2O$, 111 mg/L KH_2PO_4, 58 mg/L $MgSO_4$ • $7H_2O$, and trace elements. The two reactors were originally inoculated with one liter of suspended sludge taken from another PN-A reactor. The biomass maintained a form of suspended-growth flocs during the whole operation of the two reactors. On day 1, when the two reactors were operated as SPAN reactors, the sludge concentrations were 1340 ± 28 mg VSS /L in R1 and 1230 ± 14 mg VSS /L in R2 (the small difference was due to the previous operation using conventional oxygen-input control methods; VSS: volatile suspended solid).

2.5. Estimation of nitrogen removal by anammox bacteria in SPAN reactors

In this study, nitrogen removal by anammox bacteria was estimated using Eqs. 7–9:

$$\begin{aligned}&\text{Nitrogen removal by anammox bacteria}\\&\quad=\text{measured total nitrogen removal}\\&\qquad-\text{nitrogen removal by heterotrophic denitrification}\end{aligned}\tag{7}$$

$$\begin{aligned}&\text{Nitrogen removal by heterotrophic denitrification}\\&\quad=\text{COD consumption by heterotrophic denitrifiers}\\&\qquad\text{for denitrification}/(\mathrm{S_F/S_{NO_{3-}}}-\mathrm{N})\end{aligned}\tag{8}$$

$$\begin{aligned}&\text{COD consumption by heterotrophic denitrifiers for denitrification}\\&\quad=\text{total measured COD consumption}-\text{COD consumption by}\\&\qquad\text{heterotrophic denitrifiers for biomass growth}\\&\qquad-\text{COD consumption byaerobic heterotrophs}\end{aligned}\tag{9}$$

where, in Eq. 8, $S_F/S_{NO_{3-}-N}$ is the ratio of readily biodegradable organic matters (S_F) to nitrate plus nitrite nitrogen ($S_{NO_{3-}-N}$). According to the stoichiometry in ASM2 (Henze et al., 2000), the ratio of $S_F/S_{NO_{3-}-N}$ was calculated based on the yield coefficient of heterotrophic denitrifiers (Y_H, 0.5 g COD/g COD with nitrite or nitrate as the electron acceptor): $S_F/S_{NO_{3-}-N}$ = *2.86/(1-Y_H) = 2.86/(1–0.5)* = 5.72 g COD/g NO_3^--N, which means that 5.72 g COD is required for the complete denitrification of NO_3^--N (without considering COD consumption for biomass growth).

According to Eq. (7), an underestimation of nitrogen removal by heterotrophic denitrification would lead to an exaggerated contribution of anammox bacteria to nitrogen removal, though anammox bacteria were acutally the dominant nitrogen removal microorganisms in SPAN reactors. The underestimation of nitrogen removal by heterotrophic denitrification was avoided by assuming that COD consumption by heterotrophic denitrifiers for denitrification was equal to the total measured COD consumption, which means that the estimated COD consumption by heterotrophic denitrifiers for denitrification Eq. (9) and consequently, the calculated nitrogen removal by heterotrophic denitrification (Eq. (8), were higher than the actual levels. So in the estimation, COD consumption by aerobic heterotrophs and COD consumption by heterotrophic denitrifiers for biomass growth were both neglected Eq. (9).

2.6. Analytical procedures

Prior to analysis, effluent wastewater samples were pre-treated by filtration (pore size 0.45 µm). A Konelab analyzer (Thermo Scientific, USA) was used to measure the concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N. Standard methods were employed to determine VSS and COD concentrations (APHA, 1998). pH was determined using a pH meter (Multi3620, WTW, Germany). The salinity was determined using a salinity electrode and a portable meter (Extech DO700, FLIR Commercial Systems, USA). In order to investigate the maximum capacities of the main nitrogen-converting pathways, the nitrogen-conversion capacities of aerobic ammonium oxidation (AAO, mainly by AOB), anaerobic ammonium oxidation (anammox), aerobic nitrite oxidation (ANO, by NOB), and heterotrophic denitrification were tested in triplicate according to Qiu et al. (Qiu et al., 2019) (NOB activity tests were the same as those of AOB tests except that only NO_2^- instead of NH_4^+ was supplied).

2.7. Metagenomic high throughput sequencing

Biomass samples (5 mL) were collected on day 1, day 57, day 121, day 247, day 351, and day 388 for metagenomic sequencing (Sangon Biological Engineering, China). The DNA extraction methods were provided in supplementary information. Illumina HiSeq platform was used for high-throughput sequencing to produce 150 bp paired-end reads. The raw reads were quality filtered by trimming the adaptor sequences and ambiguous nucleotides, removing short reads (< 35 bp), and excluding low-quality sequences (quality score < 20) using Trimmomatic (version 0.36). The cleaned sequences (Table S1) were assembled into contigs using IDBA_UD (version 1.1.2) with default parameters. Open reading frames (ORFs) were predicted from contigs using Prodigal (version 2.60). ORFs longer than 100 bp were translated into protein sequences. CD-HIT (version 4.6), SAMtools (version 0.1.18), and Bowtie2 (version 2.1.0) were used to remove the redundant sequences and determine gene abundance. Taxonomic annotation was conducted by searching the reads against the NCBI Nr database with the E-value cut-off of 10^{-5}. The relative abundance was calculated based on total detected sequences. The reported sequences were deposited in the GenBank under the accession number PRJNA606508.

3. Results and discussion

3.1. Determination of the oxygen input

k_La values at various water circulation rates and shower rates were determined (Figure S2 and Table S2) and based on k_La values the oxygen input was calculated. Then, a non-linear surface regression analysis was conducted to investigate the empirical, quantitative correlation of the oxygen input to the water circulation rate and shower rate. This was described by Eq. (10) (R^2=0.96) and Fig. 2. Based on the equation, the oxygen input was predicted at various water circulation rates and shower rates.

$$\text{Oxygen input, mg } O_2/d = -214.6 + 351.1e^A + 2.8e^B + 1555e^{A+B}\tag{10}$$

where $A = -e^{\frac{25.8-C}{42.8}}$, $B = -e^{\frac{-15.5-S}{123.3}}$, C is the water circulation rate (mL/min), and S is the shower rate (mL/min).

Good agreement was observed between the predicted oxygen input and the measured oxygen input (Figure S3). Eq. (10) and Fig. 2 indicate that the increased water circulation rate will deliver more oxygen into the SPAN system, and the contribution of shower with water circulation can introduce much more oxygen

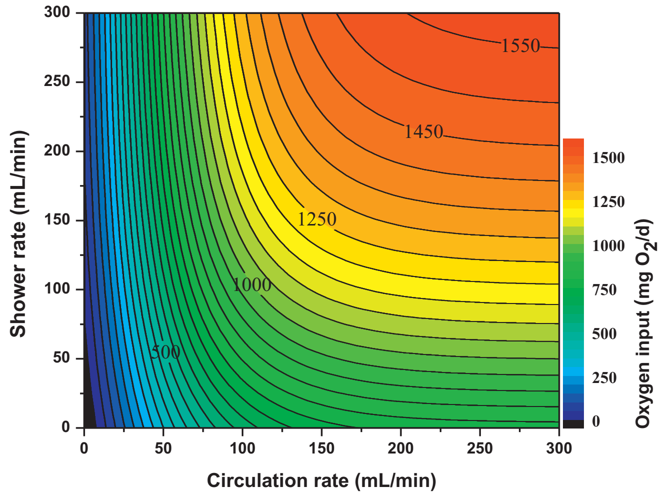

Fig. 2. Heatmap showing the dependence of the oxygen input on the water circulation rate and shower rate.

than simply employing water circulation. For instance, at the water circulation rate of 300 mL/min, oxygen input of 858 mg O_2/d was achieved, while 1290 mg O_2/d was achieved at the water circulation rate of 200 mL/min and the shower rate of 88 mL/min (Figure S3). The introduction of the shower improved the k_La value by creating more disturbance at the water-air interface and thus improved the oxygen transfer. Therefore, from the perspective of minimizing the amount of circulated wastewater, the circulation-shower mode is a better choice than the circulation-only mode.

3.2. Performance of SPAN

3.2.1. Nitrogen-removal performance

The performance of the two SPAN reactors (R1 and R2) ran in parallel is shown in Fig. 3. Because similar performances were achieved in the two reactors, so only the results of R1 were discussed in detail. In Stage 1 (day 1- day 82), the two reactors were operated at a water circulation rate of 70 mL/min (oxygen input of 314 mg O_2/d) and fed with 200 mg NH_4^+-N/L at an NLR of 77 mg N/L/d. At this NLR, the theoretical oxygen demand for PN-A to convert all the NH_4^+-N was 389 mg O_2/d (Figure S3). In R1, around 86% of NH_4^+-N was removed (Figure S4), while the average NO_2^--N and NO_3^--N concentrations were 0.2 mg N/L and 55 mg N/L, respectively, leading to a final TN removal efficiency of 68%.

The ratio of NO_3^--N production to NH_4^+-N consumption can indicate how well the NOB suppression is controlled: larger than the theoretical value of 0.13 (Eq. (1)) indicates certain NO_3^--N production by NOB; the larger the ratio is, the more NO_3^--N is produced by NOB. The ratios of the two reactors showed a gradual decline to 0.23 (Fig. 4), indicating NOB-suppression. A small amount of COD in the influent (40 mg/L) was removed, leaving effluent COD of around 23 mg/L in R1 (Fig. 4). The biomass in the two reactors was gradually enriched to roughly 2000 mg VSS/L from the original level, suggesting the efficient biomass retention of SPAN system (Fig. 4). No intentional sludge discharge was conducted and the effluent VSS concentration was about 20 mg/L, which means sludge retention time (SRT) was 67 - 107 days. During this stage, the initial anammox capacities (55 mg N/L/d) were far less than those of AAO (424 mg N/L/d) and ANO (292 mg N/L/d) (Fig. 5). The differences in nitrogen conversion capacities between R1 and R2 were caused by previous operation before they were run as SPAN reactors. But still, stable nitrogen removal efficiency was achieved without NO_3^--N build-up. These results proved that regardless of the low anammox capacity and high NOB capacity, stable nitrogen removal was reliably achieved in the SPAN reactors by controlling the oxygen input to meet the oxygen demand.

In Stage 2, NH_4^+-N concentration in the influent was increased to 300 mg N/L (NLR was 115 mg N/L/d), and the theoretical oxygen demand for PN-A to convert all NH_4^+-N was 583 mg O_2/d. To examine the nitrogen removal performance of SPAN with less or more oxygen input than the oxygen demand, the reactors were operated under various water circulation rates: 70 mL/min during day 83–158 (oxygen input 314 mg O_2/d); 150 mL/min during day 159–260 (oxygen input 454 mg O_2/d), and 300 mL/min during day 261–358 (oxygen input 858 mg O_2/d). With a less oxygen input than the oxygen demand (day 83–158), the effluent NO_3^--N concentrations and NO_2^--N concentrations remained at the same level as those in Stage 1, while the NH_4^+-N concentrations rose to 97 mg N/L in R1, which led to the deterioration of NH_4^+-N removal efficiency to 68% (Figure S4), and TN removal efficiency to 50% (Fig. 3). During day 159–260, the water circulation rate was increased from 70 mL/min to 150 mL/min which enhanced the oxygen input to 454 mg O_2/d. The effluent NO_3^--N concentrations and NO_2^--N concentrations roughly remained unchanged, but the effluent NH_4^+-N concentrations decreased to 60 mg N/L, which improved the TN removal efficiency to 64% (Fig. 3). These results indicate that with a smaller oxygen input than the theoretical oxygen demand, stable nitrogen removal performance was maintained using SPAN, but the improvement of TN removal efficiency was restricted by the shortage of oxygen to oxidize NH_4^+. The water circulation rate was further increased from 150 mL/min to 300 mL/min during day 260 - 358 so the oxygen input of 858 mg O_2/d exceeded the oxygen demand of 583 mg O_2/d. With an excessive amount of oxygen, the effluent NH_4^+-N concentrations immediately decreased to mostly less than 10 mg N/L in the two reactors. NH_4^+-N removal efficiencies of more than 96% were achieved in R1 except during day 280 - 303 when the circulation pump malfunctioned (Figure S4). NO_3^--N concentrations in R1 increased and then leveled off at about 74 mg N/L, while NO_2^--N concentrations mostly remained less than 0.5 mg/L (Fig. 3). NO_3^--N concentrations resulted from the increased nitrogen removal by anammox and the enhanced NOB activity due to the excessive oxygen input. Overall, stable TN removal efficiency of about 75% in R1 was achieved with excessive oxygen input. Around 65% of the influent COD was consumed leading to an average effluent COD of 21 mg/L in Stage 2 (Fig. 4). Taken together, in SPAN reactors, stable nitrogen removal was achieved with less or more oxygen input than the theoretical oxygen demand. When oxygen input was less than the theoretical oxygen demand, the TN removal efficiency was low due to the shortage of oxygen to oxidize NH_4^+-N. When the oxygen input was more than the theoretical oxygen demand, TN removal efficiency was immediately improved, though NO_3^--N concentration increased to a higher but controllable level.

In Stage 3, the NLR was increased to 231 mg N/L/d (Table 1), which meant theoretically 1166 mg O_2/d of oxygen was required for PN-A process to remove all NH_4^+-N. Together with the wastewater circulation rate of 200 mL/min, a by-pass creating shower at the rate of 88 mL/min was introduced to provide an oxygen input of 1290 mg O_2/d. With higher NLR, the biomass concentrations increased to 2350 mg VSS/L in R1 (Fig. 4). The effluent NH_4^+-N gradually decreased to less than 1 mg N/L achieving removal efficiencies of more than 99% (Figure S4). The effluent NO_2^--N concentrations first slightly rose to around 2 mg N/L and then decreased to 0 until the end of this stage. Impressively, effluent NO_3^--N concentrations significantly decreased in both reactors, with only 55 mg N/L in R1, which meant only 16 mg NO_3^--N /L was produced by NOB (PN-A produced 39 mg NO_3^--N /L according to Eq. (1)) and the NOB activity was almost completely suppressed. This caused the decrease of NO_3^--N-production/ NH_4^+-N-consumption ratio to

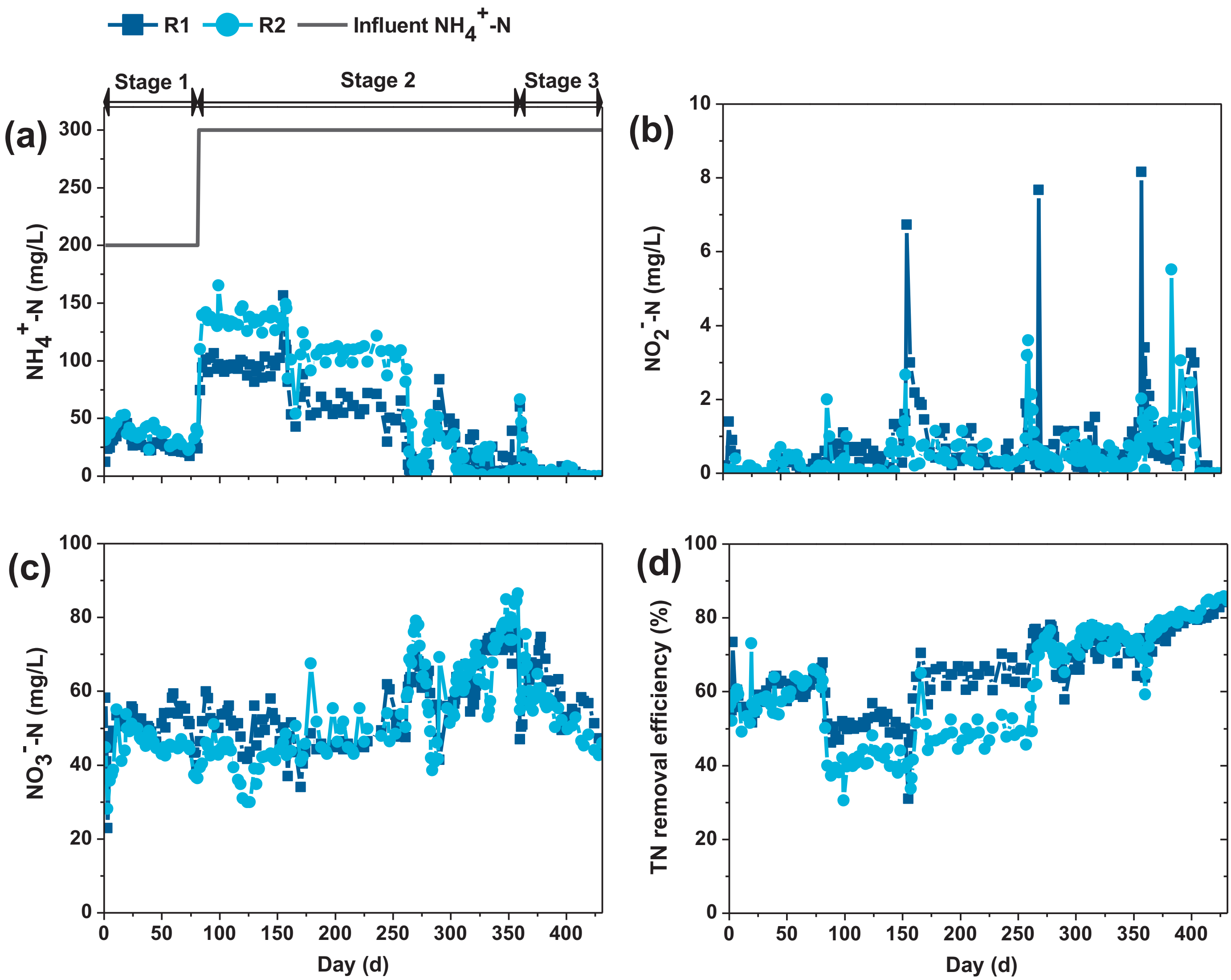

Fig. 3. Nitrogen removal performances of R1 and R2. (a): influent and effluent NH_4^+-N concentrations of the two reactors; (b) effluent NO_2^--N concentrations; (c) effluent NO_3^--N concentrations; and (d) total nitrogen (TN) removal efficiencies.

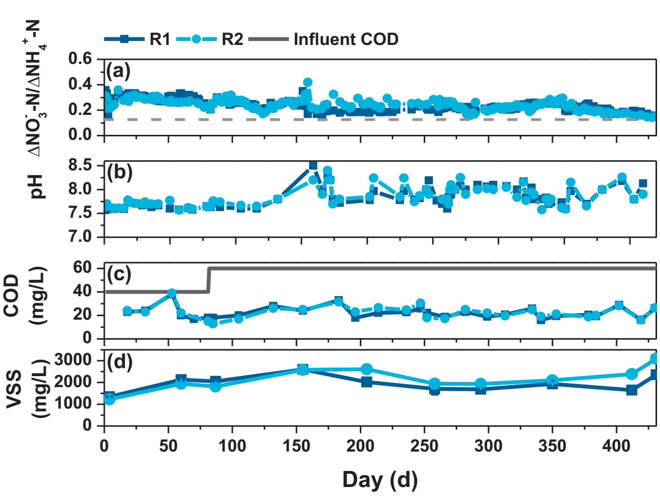

Fig. 4. Performance of R1 and R2 other than nitrogen removal. (a) ratio of NO_3^--N production/NH_4^+-N consumption (ΔNO_3^--N/ΔNH_4^+-N); (b) effluent pH; (c) effluent COD concentration, and (d) biomass concentrations.

0.18 in R1 (Fig. 4). As a result, a high average TN removal efficiency of 81% was achieved (Fig. 3). At the end of this stage, TN removal efficiencies of 82% and 85%, which were close to the maximum theoretical level of 87%, were maintained in R1 and R2. Many PN-A systems, where conventional oxygen-input control methods were used under similar conditions with this study, have been reported. In a PN-A reactor, an average TN removal efficiency of 65.6% was achieved with an average DO level of 0.33 mg/L and aeration rates of 0.5–0.6 L/min (Li et al., 2017a). In a continuous stirred tank reactor PN-A system, TN removal efficiencies ranged 70% - 81% depending on the effectiveness of aeration control based on DO concentrations (Liu et al., 2017). Results of other PN-A systems where oxygen input was controlled based on DO concentrations had TN removal efficiencies of 61% (DO = 0.5 - 0.6 mg/L) (Zhang et al., 2010), 43% (DO = 0.3 mg/L) (Xiao et al., 2015), and 60% - 90% (DO = 0.5 - 1.5 mg/L) (Zheng et al., 2016). In PN-A systems where pH-based aeration control method was used to control oxygen input, TN removal efficiencies were 75% (average value), 78% (average value), and 67% –85% in an integrated fixed film activated sludge reactor, an SBR, and a moving bed biofilm reactor, respectively (Graham and Jolis, 2017; Klaus et al., 2017). The nitrogen removal performances of the two SPAN reactors were stabler and

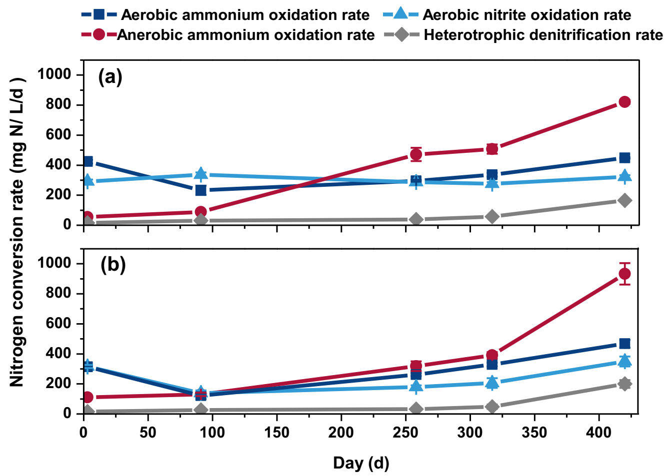

Fig. 5. Capacities of the key nitrogen-conversion pathways in R1 (a) and R2 (b) based on unit reactor volume (mg N/ L/d).

higher than the reported PN-A systems using conventional oxygen-input control methods.

COD removal, which remained at approximately the same level as that in Stage 2 (Fig. 4), was used to estimate the contribution of anammox bacteria to nitrogen removal (Section 2.5). Anammox bacteria were estimated to contributed more than 98%, 96%, and 97% to TN removed in Stage 1, Stage 2, and Stage 3 of both R1 and R2, respectively (Figure S4). Overall, with precise oxygen-input control, high TN removal efficiency and NH_4^+-N removal efficiency, which were close to the maximum theoretical values, were stably achieved in the SPAN reactors. These results proved the extremely high efficiency and reliability of SPAN technology in removing nitrogen from ammonium-rich wastewater.

3.2.2. Nitrogen-conversion capacities of the key pathways

Capacities of four key nitrogen-converting pathways were measured by ex-situ activity tests, i.e., AAO (mainly by AOB), ANO (mainly by NOB), anammox, and heterotrophic denitrification. The capacities based on the unit volume of the reactor (mg N/ L/d), which indicate the dynamics of the key nitrogen-converting guilds and suggests the nitrogen-conversion potential, are shown in Fig. 5. The results of capacities based on unit biomass weight are shown in Figure S5. Similar nitrogen-conversion capacities of the four pathways were observed in R1 and R2, so only the results of R1 are discussed in detail. ANO capacities showed an initial rapid decrease in both reactors, followed by a slight decrease to 277 mg N / L/d in R1, while in some of the reported studies, a significant increase of NOB activities was observed leading to the accumulation of nitrate (Joss et al., 2011; Liu et al., 2017; Wang et al., 2015). The capacities of AAO first decreased and then gradually increased to higher levels of 448 mg N/ L/d in R1. The AAO capacities gradually exceeded those of ANO in both of the two reactors, suggesting that in SPAN reactors, AOB were not restricted by the oxygen-input control and were able to effectively over-compete NOB. Another factor that contributed to the suppression of NOB was the temperature of 30 °C, which led to a higher oxygen affinity of AOB than NOB and encouraged the growth of anammox bacteria (Qiu 2019; Tomaszewski et al., 2017; Vannecke and Volcke, 2015). This helped AOB and anammox bacteria compete with NOB for oxygen and nitrite, respectively, and assisted in the suppression of NOB (Agrawal et al., 2018). These results prove that with precise oxygen-input control in SPAN reactors, efficient and stable nitrogen-removal was still achieved, even with high NOB activity (comparable with AOB activity). In Stage 1 and Stage 2, heterotrophic denitrification capacities were much lower than those of the other three pathways, and increased to 166 N/ L/d in Stage 3 due to a higher NLR. In Stage 1, due to the low NLR, the anammox capacities remained stable at 55 mg N/ L/d in R1. Along with the stepwise increase of NLR to higher levels in Stage 2 and Stage 3, anammox capacities rapidly exceeded those of all other three pathways and increased to the final levels of 821 mg N/ L/d in R1, which was higher than the reported value of 680 mg N/ L/d (Wang et al., 2015). The enhanced anammox activities demonstrated that SPAN was effective in retaining and enriching anammox bacteria, especially compared with the reported studies where a rapid decrease of anammox activity was observed (Liu et al., 2017; Wang et al., 2015). The high anammox capacity together with the oxygen-input control accounted for the extremely high nitrogen-removal performance in Stage 3. These results prove that in SPAN reactors, anammox activity was efficiently and rapidly enhanced to the highest one among the key nitrogen-conversion pathways, especially with a higher NLR; AOB activity was also improved and over-competed the activity of NOB which was effectively suppressed.

To sum up, these results demonstrated that SPAN technology was outstanding in several ways. Firstly, the oxygen input can be precisely and reliably controlled to meet the oxygen demand, which can not be guaranteed in PN-A systems using conventional oxygen-input control methods as mentioned above (Lackner et al., 2014). Secondly, the reliable and precise oxygen-input control can be realized simply by adjusting the wastewater circulation/shower rate without using complex online control systems such as fixed-DO aeration control, pH-based aeration control, and ammonia-based aeration control (Klaus et al., 2017). Similar with surface aeration, SPAN system does not have diffuser clogging problem which happens to diffused aeration systems (Mueller et al., 2002). Thirdly, in SPAN reactors, the activities of the key microbial guilds can be easily and effectively controlled in the desired way, i.e., to enhance the anammox activity, maintain the AOB activity, and suppress the NOB activity. PN-A systems using conventional oxygen-input control methods are vulnerable to the loss of anammox biomass, DO-caused inhibition on anammox bacteria (Lackner et al., 2014), nitrite accumulation and nitrate accumulation due to reduced anammox activity and enhanced NOB activity (Daverey et al., 2013; Li et al., 2017a). Thus, strategies including the above-mentioned aeration control, maintaining a high FA concentration, SRT control, and even re-inoculation (replacing the sludge), have to be used to suppress NOB overgrowth (Agrawal et al., 2018; Joss et al., 2011; Wang and Gao 2016). Fourthly, high nitrogen removal efficiencies close to the maximum theoretical levels can be maintained during long-term operation, while unstable nitrogen removal efficiencies of 60% −81% were achieved in reported PN-A systems using conventional oxygen-input control methods (Graham and Jolis, 2017; Klaus et al., 2017; Li et al., 2017a; Liu et al., 2017; Zhang et al., 2010).

3.3. Microbial community structure in the SPAN

Biomass samples from R1 were taken on day 1, 57, 121, 247, 351, and 388 for metagenomic analysis and to gain microbial insights into the SPAN reactor.

3.3.1. Community structure in the SPAN reactor

Fourteen phyla with relative abundance higher than 1% were detected (Figure S6). The relative abundance of the most abundant phylum *Proteobacteria,* which the phylum AOB belong to (Li et al., 2017b), gradually increased from the initial 25.3% to 38.8%. *Planctomycetes* was the second most abundant phylum, and its relative abundance also showed a gradual increase from 6.0% to 16.7%, except for a decrease during day 57 - day 121. The six reported anammox genera including *Candidatus Scalindua, Candidatus Jettenia, Candidatus Brocadia, Candidatus Anammoxoglobus, Candidatus Kuenenia*, and *Candidatus Anammoximicrobium*, are all affiliated with the phylum *Planctomycetes* (Ali et al., 2015). The relative abundances of three phyla, i.e., *Chloroflexi, Firmicutes*, and *Verrucomicrobia*, decreased to a lower level. The phylum *Nitrospirae* which includes the NOB genera *Nitrospira*, slightly varied between 1.0% and 1.4%. This agrees with the ANO capacities (Fig. 5) and proves the well-controlled NOB-suppression with precise oxygen-input control in SPAN. The relative abundances of other phyla generally remained stable.

At the species level, 17 species with relative abundances of higher than 1% were detected in R1 (Figure S7). *Candidatus Jettenia* caeni, one of the identified anammox species, gradually increased from 0.06% to the highest abundance of 7.1%. The relative abundance of *Nitrosomonas europaea*, an AOB species, remained at 0.16% - 0.74% except on day 57 when the relative abundance temporarily increased to 2.70%. Six species, *Paracoccus versutus, Sulfuritalea hydrogenivorans, Leptolyngbya valderiana, Planctomycetaceae bacterium* SCGC AG-212-F19, *Caldilinea aerophile*, and *Zavarzinella formosa* also displayed a rising trend to 1.0%−2.2%. Among them, *Paracoccus versutus* have the ability to convert NO_3^- into molecular nitrogen (Baker et al., 1998). The relative abundance of *Pedosphaera parvula, Ignavibacterium album, Chlorobi bacterium* OLB4, and *Chloroflexi bacterium* OLB14 all displayed a peak value around day 57, then decreased and leveled off at the level of less than 1% on day 351 and day 388. The other species generally had the highest relative abundances in Stage 1 and gradually reduced to the lowest level on day 388.

3.3.2. Dynamics of nitrogen-converting species in the SPAN reactor

Five key nitrogen-converting microbial microorganisms were detected including anammox, AOB, NOB, comammox (complete nitrification by a single microorganism (van Kessel et al., 2015)), and AOA (Fig. 6). Among these guilds, the total relative abundance of anammox bacteria had the most outstanding increase from 0.61% to the highest value of 8.17% and the increase happened mainly after day 121 (Fig. 6(a)), which agrees with the results of the capacity tests (Fig. 5). This proves that with precise oxygen-input control and nearly zero DO in the sludge (Figure S1), the slow-growing anammox bacteria were effectively retained and enriched in SPAN reactor, which is a considerable challenge for the reactors equipped with conventional oxygen-input control methods, partly due to inhibition caused by DO (Jin et al., 2012; Ma et al., 2016). Four anammox species were detected (Fig. 6(b)), among which *Candidatus Jettenia* caeni was the dominant anammox species achieving the highest relative abundance of 7.13%, while *Candidatus Kuenenia* stuttgartiensis, *Candidatus Brocadia* sinica, and *Candidatus Brocadia* fulgida had relative abundances of less than 0.5%. The maximum relative abundance of anammox bacteria in this study was higher than the reported values of 3.4% (at phylum level), 0.5%, and 1.5%, in suspended growth PN-A sludge flocs (Li et al., 2017a; Liu et al., 2017; Xiao et al., 2015), and was even higher than 4.8% in the mixture of biofilms and flocs and 5.0% in biofilms (Liu et al., 2017; Nhu Hien et al., 2017). The relative abundance of NOB, which ranked the second-highest guild except in Stage 1 when AOB showed a higher relative abundance, generally remained stable and slightly increased from around 1.08% to 1.88% during day 1 - 247, and then gradually decreased to 1.43% on day 388. This agrees with the results of the ANO capacities. Five NOB species that are phylogenetically affiliated to two genera *Nitrospira* and *Nitrobacter* were detected (Fig. 6(c)). The relative abundance of the most abundant species, *Nitrospira* sp. OLB3, remained between 0.29% and 0.72%. These results prove that without using complexed control strategies, NOB were effectively suppressed from proliferating and were restricted to produce NO_3^--N (only 16 mg N/L was produced by NOB). AOB first experienced an increase from 1.14% on day 1 to 3.65% on day 57 and then dramatically decreased to 0.42%, which probably resulted from the inhibition of high NH_4^+-N concentrations in Stage 2. Thereafter, the AOB abundance gradually recovered to 0.79% on day 388. The low relative abundance of AOB was the result of precise oxygen-input control, which meant that only the required amount of oxygen was delivered to and mainly consumed by AOB. AOB consumption and precise oxygen-input control led to the nearly zero DO levels at the bottom of the reactor in this study (Figure S1). This was the main reason that AOB were able to grow at low DO levels. Though AOB had low relative abundance, they had high AAO capacities (Fig. 5) to oxidize NH_4^+-N (indicated by NLR), which means AOB were also well controlled in SPAN reactors. The common *Nitrosomonas* europaea was the most dominant AOB species showing a similar trend with that of the total AOB and achieved a final relative abundance of 0.39% (Fig. 6(d)). The relative abundance of the other two AOB species, *Nitrosomonas* eutropha, and *Nitrosomonas* communis, mostly remained below 0.1%.

Three newly discovered comammox species were detected, i.e., *Candidatus Nitrospira* inopinata, *Candidatus Nitrospira* nitrosa, and *Candidatus Nitrospira* nitrificans (Daims et al., 2015; van Kessel et al., 2015). But they were less abundant (<0.1%) and the relative abundance of the total comammox gradually decreased from 0.24% to 0.13% suggesting that they were also well managed along with the NOB. AOA were the least abundant ones among the five key nitrogen-converting guilds with their total relative abundance of around 0.02%. In conclusion, with the precise oxygen-input control in the SPAN reactor, anammox bacteria were efficiently enriched with higher abundances than other microorganisms, AOB were well controlled to provided sufficient AAO capacity, and NOB were maintained stable and effectively suppressed.

3.4. Outlook

The presented results prove the reliability and high efficiency of SPAN reactor and the effectiveness of precise oxygen-input control in removing nitrogen from ammonium-rich wastewater. More than 99% of NH_4^+-N and more than 81% of TN were stably removed during the long-term operation. Energy consumption of SPAN technology is one of the concerns. Besides the energy-input for feeding and withdrawing, the water circulation is the only source of energy consumption for the SPAN system. The water circulation rates of 1.2 m/h - 5.3 m/h in SPAN system (corresponding to 70 mL/min - 300 mL/min) were comparable with the internal water recirculation rates of 2.3 m/h - 4.5 m/h in reported PN-A systems (Li et al., 2017b; Zhang et al., 2010), and less than that of 16 m/h in another PN-A expanded granular sludge bed reactor (Wang and Gao, 2016). Considering internal water recirculation is only used to create uniformity and extra energy is needed to supply oxygen for the reported PN-A systems (but is needless for SPAN system), energy-consumption of SPAN system will be comparable with, if not less than, the reported reactor configurations. This study aimed to prove the effectiveness of SPAN concept, so the optimization of parameters for large-scale applications was not conducted. For example, the high height/diameter ratio of the laboratory-scale SPAN reactor is a major factor that impacts the scalability potential of this technology, and thus deserves further optimization study prior to larger-scale applications. Future research work aiming at the reduction of energy consumption and field application, such as the investigation of the effectiveness of alternative water circulation methods (mechanical stirrer inside the reactor vs. pump above the reactor), the use of more efficient shower system, and the examination of the effectiveness of SPAN in the mainstream conditions, are recommended.

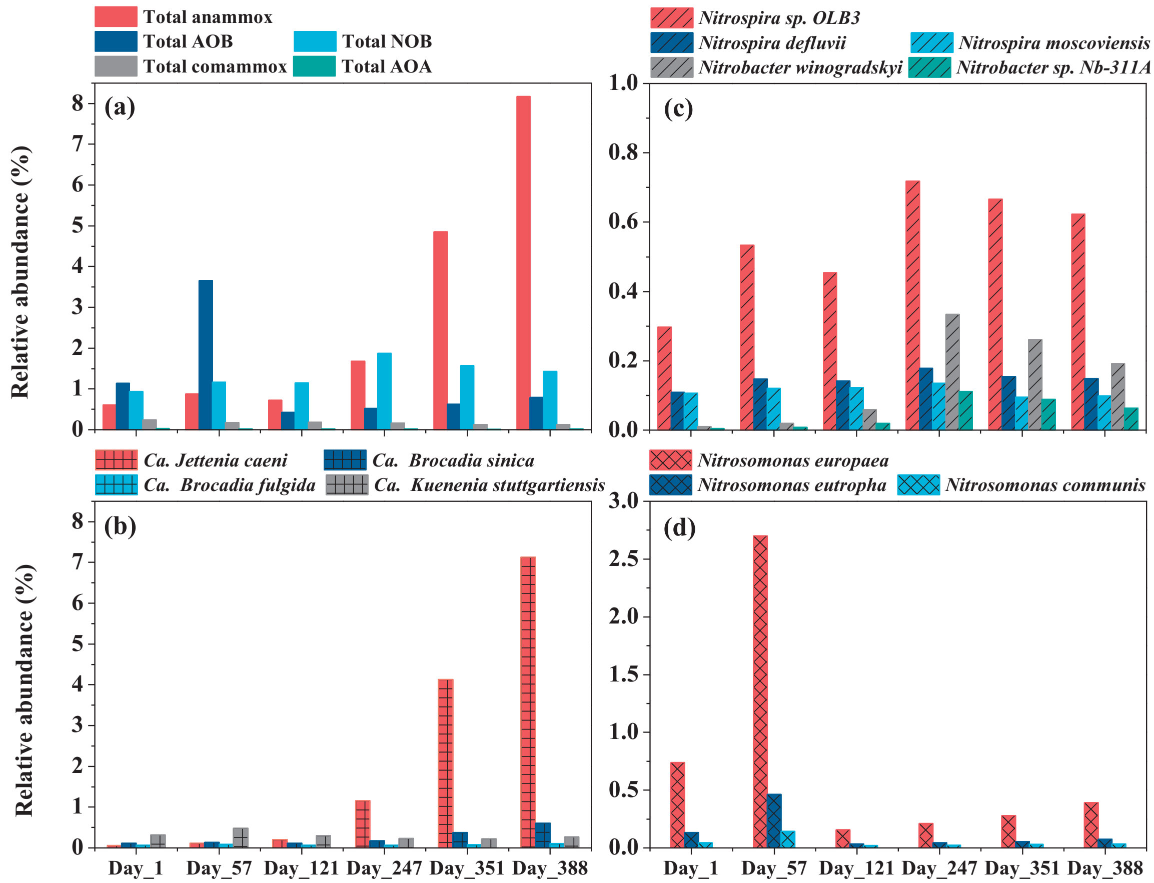

Fig. 6. Relative abundance of the nitrogen-converting microbial guilds. (a) total relative abundances of AOB, NOB, comammox, and AOA, where "Total" represents the sum relative abundances of the same functional group; (b) relative abundance of anammox bacteria; (c) relative abundance of NOB, and (d) relative abundance of AOB. .

4. Conclusions

This study demonstrated the robustness and high efficiency of SPAN and the effectiveness of precise oxygen-input control in removing nitrogen from ammonium-rich wastewater. The quantitative correlation among the oxygen input, the water circulation rate, and the shower rate was established. The circulation-shower mode was proven to be more efficient than the circulation mode in delivering oxygen. More than 99% of NH_4^+-N removal and 81% of TN removal (up to 85%) were removed. Nitrogen was mainly removed by anammox bacteria, contributing to more than 98%, 96%, and 97% of the TN removal in Stage 1, Stage 2, and Stage 3 of both R1 and R2, respectively. The key nitrogen-converting microbial guilds were effectively controlled as required both in terms of relative abundance and nitrogen-converting capacities. Anammox bacteria were efficiently enriched to be the most abundant microorganisms with a relative abundance of 8.17%, AOB were well controlled to provide sufficient AAO capacity, and NOB were effectively suppressed from proliferating and producing NO_3^--N. This study provides a new perspective for the precise oxygen-input control and successful operation of PN-A systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116213.

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