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Improve nitrogen removal of the biofilm single-stage PN/A process by optimizing the intermittent aeration strategy

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ABSTRACT

This study demonstrated a novel technique to achieve partial nitritation/anammox (PN/A) to treat inorganic low-strength wastewater by using a 10 L continuous reactor equipped with fiber carriers. Aeration control was the key factor in single-state PN/A performance stability under oxygen-limiting conditions. Intermittent aeration was successfully adapted in this study to achieve over 80% nitrogen removal at the nitrogen loading rate of 0.12–0.16 kg N m^{-3} d^{-1}. The nitrite-oxidizing bacteria activities were inhibited by intermittent aeration due to the alternate between aerobic to anoxic conditions. Ammonium-oxidizing bacteria (AOB) and anammox bacteria could be symbiotically supported within the biofilm with a specific activity of anammox bacteria that was 1.5 times higher than that of AOB. The biomass was efficiently retained by using fiber carriers. The results obtained from this study could bring the possibility of applying an energy-saving and efficient biofilm single-stage PN/A process in tropical regions.

1. Introduction

Nitrogen removal from wastewater prior to environmental discharge is essential to avoid eutrophication (Vu et al., 2020; Aditya et al., 2022). In conventional wastewater treatment, nitrogen is removed by sequential nitrification and denitrification. The traditional nitrification/denitrification process is energy intensive as aeration is required to create an aerobic condition for nitrification, then an anoxic condition and sometime an additional carbon source is required to reduce nitrate to nitrogen gas in the denitrification step (Wollmann et al., 2019). In recent years, direct oxidation of ammonium under anaerobic conditions, known as the anammox process, has been introduced as an alternative to the nitrification/denitrification process. Anammox process is considered more environmentally friendly due to reducing the aeration energy of wastewater treatment, no need to use an external carbon source, and less waste sludge production (Le et al., 2022a,b; Zhang et al., 2022).

Single-stage partial nitritation/anammox (PN/A) process is a popular technique of the anammox-based deammonification process in WWTPs. In the single-stage PN/A process, both nitritation and Anammox are simultaneously achieved

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in one reactor. According to Eq. (1), part of the incoming NH_4^+–N is first oxidized to NO_2^-–N (partial nitritation) by ammonium-oxidizing bacteria (AOB). Then anammox bacteria convert the remained NH_4^+–N and produce NO_2^-–N to nitrogen gas (N_2) (Eq. (2)). The overall PN/A reaction can be described by Eq. (3).

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{1}$$

$$NH_4^+ + 1.32\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O \tag{2}$$

$$NH_4^+ + 0.85\ O_2 \rightarrow 0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O \tag{3}$$

So far, PN/A process has been successfully applied to the treatment of high-strength wastewater (Eskicioglu et al., 2018) [e.g., digested sludge liquor (Han et al., 2020; Valenzuela-Heredia et al., 2022), livestock manure digester liquor (Kwon et al., 2019; Sheng et al., 2020), and landfill leachate (Phan et al., 2017; Magrí et al., 2021)]. However, its application to the treatment of low strength MWW (referred as the mainstream process) remains challenging. Firstly, anammox bacteria has been known as autotrophic bacteria with the shortest doubling time of approximately two days (Zhang et al., 2017). Therefore, the challenge of applying a single-stage PN/A is to maintain the AOB and anammox biomass, while the NOB will be washed out. This difficulty could be solved by retaining AOB and anammox in granular biomass or forming biofilm on the surface of carriers, whereas washing out NOB under low DO conditions (< 1.0 mg/L) (Malovanyy et al., 2015; Liu and Yang, 2017; Wang et al., 2018). In the granular or biofilm structure, AOB placed in the aerobic outer layer, while anammox bacteria were retained in the anaerobic inner space, that were protected from the changes of the cultured environment (Laureni et al., 2016). Various published studies indicated that biofilm was simpler and more effective than other anammox immobilizing methods because of the improved ability to retain biomass within the reactor and contributed to increase in the activity of the whole system (Adams et al., 2020; Wang et al., 2022). Kosgey et al. (2020) and Rong et al. (2022a) also indicated that the biofilm configuration could retain anammox biomass and be convenient in controlling the population of NOB, and thus the high activity and develop the relative abundance of anammox bacteria in other types of reactors. Azari et al. (2020) showed that an integrated fixed film activated sludge (IFAS) reactor with K3 Kaldnes carriers could improve the ability to retain the anammox population in the deep layer of biofilm, while PN reaction occurred in aerobic outer space.

Another challenge in stabilizing the long-term performance of the PN/A process is to ensure symbiotic growth between anammox and ammonium-oxidizing bacteria. It is also essential to inhibit nitrite-oxidizing bacteria (NOB) to ensure nitrite availability for the anammox process. Various researchers reported that free ammonia (FA), free nitrous acid (FNA), DO control, high ammonium and high temperature are effective methods which use to inhibit NOB (Tian et al., 2013; Han et al., 2020). A single-stage PN/A process were applied to treat a high-strength wastewater at temperatures above 25 °C and achieved a high nitrogen removal efficiency (Han et al., 2020; Qian et al., 2022). Qian et al. (2022) applied a very high FA concentration of above 40 mg L^{-1} to inhibit NOB growth when treating high-strength wastewater (NLR of 1.2 gN L^{-1} d^{-1}) in a biofilm gas-lift single-stage PN/A reactor. A Sharon/Aanammox process was also developed by Milia et al. (2015) to suppress NOB at a NRL of 1.0–1.5 g N L^{-1} d^{-1} and high temperature of 35 ±0.5 °C. Ammonia conversion of the Sharon was approximately 64%, and the effluent NO_2–N to NH_4^+–N ratio of 1.58, which was suitable for the following anammox process. The nitrogen removal efficiency after the anammox process was 89 ± 4%, the stoichiometric ratio NH_3–N:NO_2^-–N:NO_3^-–N of 1:1.24:0.19. Tian et al. (2013) fed a hybrid sequencing batch reactor with raw leachate (TN of 2459 ± 138 mg N L^{-1}) at DO in a range from 0.5–1.0 mg L^{-1}. The NOB population was inhibited by both FA of 10–130 mg N L^{-1} and FNA of 0.01–0.03 mg N L^{-1}, while AOB had no inhibition under these conditions. They achieved the desired NO_2^-–N to NH_4^+–N ratio of 1.1–1.3 with the initial NH_4^+–N of 1200 mg NL^{-1} at 31 °C. These results indicated that the high concentration of influent ammonium nitrogen and high temperature, or another word high FA concentrations were effective strategies to inhibit NOB in PN/A processes. However, in the case of mainstream wastewater, which had a low concentration of ammonium nitrogen (<100 mg N L^{-1}) and low temperature (< 30 °C), the inhibitor FA and high temperature, like the operating conditions of Sharon for suppressing NOB growth was difficult to achieve. By contrast, intermittent aeration is considered a promising strategy to suppress NOB communities in a single-stage mainstream PN/A process in the near future. Until now, different studies have applied the intermittent aeration strategy with a wide range of DO (0.1–2.0 mg L^{-1}) (Zhang et al., 2014; Liu and Yang, 2017; Azari et al., 2020) in biofilm PN/A reactors. A PN/A process was successfully start-up within 30 days in a sequencing batch biofilm reactor (SBBR) by applying intermittent aeration control (300 min for aeration/ 40 min for non-aeration) (Huang et al., 2021). The ammonia removal efficiency (ARE) of 98±2% was achieved at a low DO condition of about 0.2 mg L^{-1} in aeration period. Jia et al. (2023) used a lab-scale integrated fixed film activated sludge (IFAS) - PN/A reactor to treat a low-strength wastewater ($C/N > 5$) at 20 °C for more than 200 days. Under low DO-intermittent aeration conditions, NOB was suppressed via setting 30-min post-anoxic react phase after intermittent aeration. The ARE and NRE of 94±4%, 87±7% were achieved, respectively. From the mentioned information, intermittent aeration can inhibit NOB effectively and is easier to implement than other methods, such as FA and FNA, especially when treating mainstream wastewater via a single-stage PN/A process. In the aeration periods, AOB can generate nitrite, providing a substrate for the anammox process. Meanwhile, the non-aeration phase can limit the growth of NOB, thereby reducing the competition of NOB with AOB and anammox for oxygen demand and nitrite, respectively, in the same reactor. Therefore, oxygen is a key factor that can directly affect the treatment efficiency and long-term stability of operation in a single-stage PN/A process (Antwi et al., 2020). Up to now, few studies have indicated the suitable DO levels and the intermittent aeration time range in suppressing NOB for low-strength wastewater.

Table 1
Operation conditions of the single-stage PN/A.

Phase	I	II	III	IV
Days	1–7	8–14	15–19	20–61
$NH_4^+ - N_{inf}$ (mg L^{-1})	50 ± 1	50 ± 1	50 ± 1	50 ± 1
$NO_2^- - N_{inf}$ (mg L^{-1})	12.8 ± 2.7	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
NLR (kg N m^{-3} d^{-1})	0.16	0.12	0.12	0.12
Temperature (°C)	34 ± 1	34 ± 1	34 ± 1	34 ± 1
DO (mg L^{-1})	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Aeration/non-aeration duration (minutes)	40/0	40/0	10/30	20/40

This study aims to investigate the possibility of applying intermittent aeration in suppressing NOB and achieving high nitrogen removal in a PN/A reactor with fiber carriers to treat low-strength wastewater under mesophilic conditions, particularly suitable for developing the PN/A process for tropical regions. In previous studies, NOB communities (*Nitrospira*) have been successfully suppressed in a partial nitritation reactor (Le et al., 2020), and a high-efficient nitrogen removal was achieved by treating 50 mg $NH_4{}^+$–N/L wastewater in a biofilm anammox reactor (Le et al., 2022a). Thus, in this study, an intermittent aeration control was established to investigate the critical factor to inhibit NOB growth and long-term stable performance in a biofilm single-stage PN/A process base on the seed sludge of partial nitritation and anammox processes achieved before.

2. Materials and methods

2.1. Experimental setup and operation conditions

A laboratory scale 10 L continuous stirred-tank reactor was used in this study (Fig. 1) to evaluate the performance of the single-stage PN/A process for treating low-strength wastewater. This study used rope-type carriers (named fiber carriers) made of polypropylene and nylon with a specific surface area of 300 m^2 m^{-3} (Evak BC-50, SPT-Evak, Taiwan). The fiber carriers (2 cm wide and 15 cm long) are fastened horizontally at every 0.5 cm on the shaft of the stirrer. The PN/A reactor was an existing biofilm anammox reactor which cultured Anammox bacteria for more than four months which was mentioned in a previous study (Le et al., 2022b). Polyurethane foam balls (diameter of 1.5 mm) were floated on the surface of the PN/A reactor. A black cloth was covered outside the reactor to eliminate the impacts of light and algae on the growth of AOB and anammox bacteria. An aquarium heater (Aleas, China) was inserted into the reactor to maintain a temperature of 34 ± 1 °C. The reactor was stirred at about 40 rpm by an overhead stirrer. Air was supplied intermittently to create aerobic/anoxic conditions by an electric timer (SST-16C, Shin sung electric Co., LTD, Korea) to switch the on/off mode of the air compressor. The operating procedure was divided into 4 phases: phase I (day 1–7), phase II (day 8–14), phase III (day 15–19), and phase IV (20–61). Hydraulic retention time (HRT) was 10 h. The operation conditions are summarized in Table 1.

A synthetic solution was used to simulate wastewater influent in this study. The inorganic synthetic wastewater contained 504 mg L^{-1} $NaHCO_3$, 27 mg L^{-1} KH_2PO_4, 100 mg L^{-1} $MgCl_2{\cdot}6H_2O$, 176 mg L^{-1} $CaCl_2{\cdot}2H_2O$ and 1 mL/L each of trace elements solutions (van de Graaf et al., 1996). $NH_4{}^+$–N was added to the synthetic wastewater in the form of NH_4Cl (Table 1) . pH was adjusted to be pH 7.8 ± 0.2 by using 1M NaOH and 1M HCl.

2.2. Batch experiments

The *ex-situ* batch experiments were conducted to determine the specific activities of AOB (S_{AOB}) and anammox bacteria (SAA). The mixer gradually increased the agitation to 100 rpm to mix the suspended biomass in the reactor, and then 200 mL of suspended sludge and a 5 cm length carrier, which included the biomass, were taken out of the reactor for specific activities experiments. The mixed liquor suspended solids (MLSS) was approximately 2.0 ± 0.1 g L^{-1}. The biomass was washed with tap water two times until $NH_4{}^+$–N, $NO_2{}^-$–N, and $NO_3{}^-$–N contents were undetected. A 63 μm aperture sieve (Chunggye Sanggong Sa, Korea) was used for recovering sludge.

For determining S_{AOB}, the washed biomass was transferred to a sealed 1 L Erlenmeyer flask in which a DO probe and a syringe were inserted. Mixed liquor, which contained 350 mL synthetic wastewater, was agitated at 35 °C and 120 rpm by a thermostatic magnetic stirring bath (WCL-3, SciLab, Korea). The mixed liquor was purged with air or nitrogen gas through a mass flow controller (MFC) (TSC-220, MPK Company, Korea) until a desired concentration of DO was reached. The influent pH was adjusted at pH 8.0 ± 0.2 by adding 1M NaOH. The initial $NH_4{}^+$–N of 50 ± 0.5 mg L^{-1} was used for AOB reaction. The activity of NOB and anammox bacteria were blocked by adding 24 μmol sodium azide (NaN_3) (Ginestet et al., 1998) and 0.5 mmol methanol (NH_3OH) (Isaka et al., 2008).

For SAA test, the washed biomass was transferred to a 1L glass bottle sealed with a stainless steel 2-ports connector cap (one for purging N_2 and another for sampling), which contained 350 mL synthetic wastewater. The wastewater was adjusted to pH 8.0 ± 0.2 and purged with nitrogen gas for 15 min to remove DO. The batch cultivations were carried out

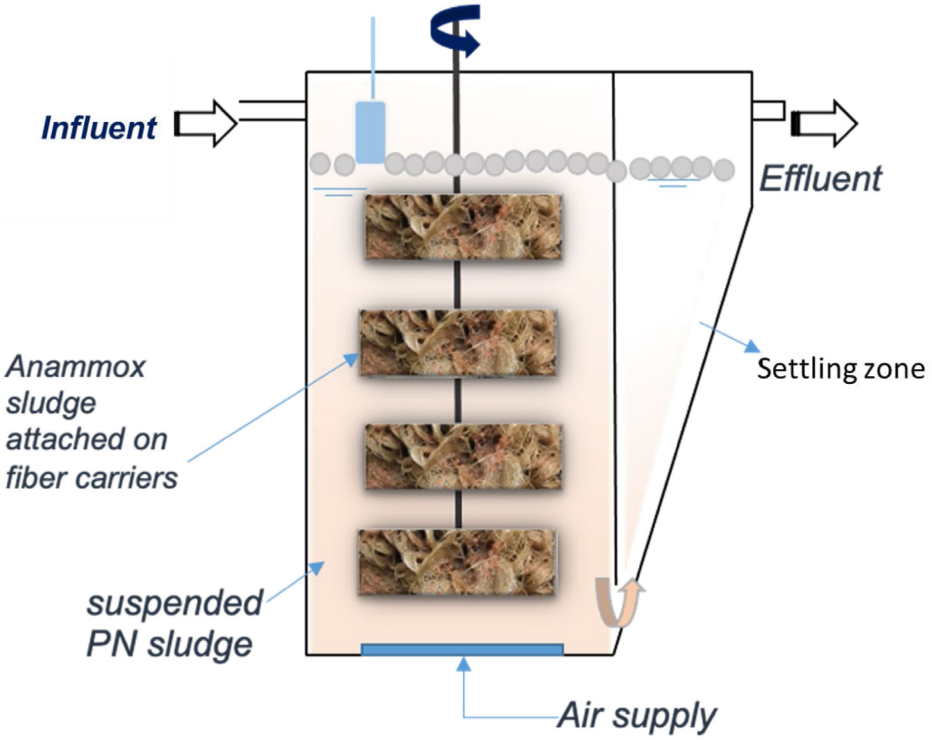

Fig. 1. The schematic diagram of the single-stage partial nitritation/anammox reactor.

in a dark environment at 35 °C on a shaking incubator (SJ-804M2, Sejong Technology Co. LTD, Korea). Initial NH_4^+–N and NO_2^-–N concentrations were 22 ± 1 mg N L^{-1} and 28 ± 1 mg N L^{-1}, respectively.

Besides, the *in-situ* cycle experiment was directly carried out in the 10 L PN/A reactor to investigate the effects of intermittent aeration on autotrophic nitrogen removal. The PN/A reactor was changed from a continuous to batch mode. The mixer and supplied air in the PN/A reactor were turned off, and a settling period of 30 min was applied. An amount of 8L supernatant was pumped out by a pump. Next, the same volume of synthetic medium with an initial NH_4^+–N of 30 ± 1 mg N L^{-1} was pumped into the reactor. The 20 min aeration period started, and subsequently, the anoxic period began within 40 min. The samples were taken at the end of the aeration and anoxic periods until the substrate reached a limited value.

During the experiments, 5 mL of aliquots were taken, filtered through a 0.45 μm Whatman membrane (GE Healthcare, Chicago, IL, USA), and filtrates were kept on ice to determine concentrations of NH_4^+–N, NO_2^-–N, and NO_3^-–N. Rates of ammonia removal were calculated from linear regressions on time-course data. The specific activity of AOB (S_{AOB}), anammox activity (SAA), PN/A (S_{PNA}) were described as the mass of consumed nitrogen per g MLSS per hour (mg N mg^{-1} MLSS h^{-1}).

2.3. Analytical methods

All samples were filtered through 0.45 μm Whatman membranes before analytical procedures. The concentrations of ammonium nitrogen (Phenate method 4500–NH_3 F) and nitrite nitrogen (Colorimetric method 4500–NO_2^-B) were determined following standard methods (APHA, 1998). Nitrate nitrogen was measured by following chromotropic acid method (Hach method 10020). MLSS was measured using Standard methods 2540 D (APHA, 1998). DO and pH were measured using an Orion star A223 DO meter and an Orion star A211 pH meter (Thermo Fisher Spectronic, Waltham, MA, USA), respectively. Biomass extraction for protein (PN) and polysaccharide (PS) analysis was done according to Le et al. (2022b). PN and PS concentrations were determined according to Lowry method (Lowry et al., 1951) and Anthrone method (Grandy et al., 2000).

2.4. Calculations

The ratio of NO_3^-–$N_{produced}$/ NH_4^+–$N_{removed}$ were calculated using Eq. 4 as below:

$$NO_3^-–N_{produced}/NH_4^+–N_{removed} = (NO_3^-–N_{eff}–NO_3^-–N_{inf})/(NH_4^+–N_{inf}–NH_4^+–N_{eff}) \quad (4)$$

where, $NH_4{}^+$–N_{inf}, $NO_3{}^-$–N_{inf}, $NH_4{}^+$–N_{eff}, and $NO_3{}^-$–N_{eff} were the concentrations of ammonium nitrogen, nitrate nitrogen in the influent and effluent.

The nitrogen removal rate (NRR) and nitrogen removal efficient (NRE) were calculated by Eqs. (5) and (6):

$$\text{NRR}\ (\text{kgNm}^{-3}\text{d}^{-1}) = \frac{(\text{TN}_{\text{inf}} - \text{TN}_{\text{eff}})}{1000 \times \text{HRT}} \tag{5}$$

$$\text{NRE}(\%) = \frac{(\text{TN}_{\text{inf}} - \text{TN}_{\text{eff}}) \times 100}{\text{TN}_{\text{inf}}} \tag{6}$$

where, total nitrogen concentration (TN) (mg L^{-1}) = $NO_2{}^-$–N + $NH_4{}^+$–N

3. Results and discussion

3.1. Nitrogen removal of the single-stage PN/A (PN/A)

At the beginning of the PN/A process, continuous low DO conditions were applied (DO of 0.3 ± 0.1 mg L^{-1}), and the feed medium contained both $NH_4{}^+$–N and $NO_2{}^-$–N on the early seven days (period I) for avoiding shocked cultured environment when immediately switched from the anammox conditions of the previous study (Le et al., 2022b) to single-stage PN/A conditions (Table 1). In period II (day 8–14), only $NH_4{}^+$–N (50 mg L^{-1}) was used for initiating simultaneous PN and anammox reactions (Fig. 2). The PN/A reaction occurred with average NRE of 69 ± 1.6%, effluent $NO_2{}^-$–N and $NO_3{}^-$–N was 0.8 ± 0.04 mg N L^{-1} and 19.5 ± 4.7 mg N L^{-1}. However, the $NO_3{}^-$–N production increased by 29% after 5 days. Besides, the FA concentration in the reactor was 0.6 mg N L^{-1}, which was far from the inhibit range of NOB (3.5–60 mg N L^{-1}) (Yamamoto et al., 2011; Le et al., 2020). It was thought that at a nitrogen loading rate (NLR) of 0.16 kg N m^{-3} d^{-1}, the PN/A process lacked mechanisms for NOB inhibition when only low DO was used.

Therefore, in period III, an intermittent aeration strategy applied at low DO of 0.4 ± 0.1 mg L^{-1} to make an anoxic condition for suppressing NOB in the PN/A reactor. Because of 30 min non-aeration in a cycle of 40 min (Table 1), NOB lacked oxygen for nitratation, resulting in the effluent $NO_3{}^-$–N concentration reduced to 16.2 mg N L^{-1} (Fig. 2). In contrast, only 10 min aeration might be insufficient of oxygen demand for nitritation; subsequently, the anammox reaction lack of electron donor ($NO_2{}^-$–N). The average effluent $NH_4{}^+$–N increased from 14.5 ± 0.6 to 17.2 ± 0.4 mg N L^{-1}. The NRE reduced by 4%, the NRR of 0.1 kg N m^{-3} d^{-1}.

In period IV, the aeration and non-aeration durations were extended to 20 min and 40 min, respectively, which led to better ammonia removal, and inhibition of NOB activity in PN/A under low DO conditions. The PN/A reactions well occurred and stable with the highest NRE of 84.3% after 50 days at DO concentration of approximately 0.4 mg L^{-1}(Table 1). These results were in line with the study of Daverey et al. (2015), which achieved a stable biofilm PN/A process in about 51 days. Rong et al. (2022b) applied a pilot single-stage PN/A process to treat mainstream wastewater. The IFAS-PN/A reactor was stable at NRE of 82% under intermittent aeration and 25 °C conditions.

The average $NO_3{}^-$–$N_{produced}$ to $NH_4{}^+$–$N_{removed}$ ratio in the present study of approximately 30% was attained in period IV, which was higher than the theoretical value that presented Eq. (3) for the PN/A reaction (Third et al., 2001). The higher-than-expected increase in nitrate levels for the single-stage PN/A reaction could be induced by part of NOB activity. This phenomenon was reported by Chen et al. (2021) with a nitrate production rate increase of over 20%. It has been suggested that the multilayer structure of the biofilm may enable some individuals in the NOB community to adapt to intermittent aeration in the PN/A reactor and live together with anammox and AOB but not affect nitrogen removal efficiency. Moreover, Malovanyy et al. (2015) and Li et al. (2021) indicated that a successful operating of the single-stage PN/A led to a low concentration of $NO_2{}^-$–N in the effluent. From the above-mentioned information, the single-stage PN/A process in this study was successfully achieved through intermittent aeration conditions (20 min aerobic/40 min anoxic) under limited oxygen conditions of about 0.4 mg L^{-1} for 61 days. The average NRE, NRR, effluent $NH_4{}^+$–N, $NO_2{}^-$–N, and $NO_3{}^-$–N concentrations in the present study were 80.6 ± 2.4%, 0.14 ± 0.004 kg N m^{-3} d^{-1}, 9.6 ± 1.2 mg N L^{-1}, 0.05 ± 0.02 mg N L^{-1}, and 14.5 ± 1.5 mg N L^{-1}, respectively (Fig. 2).

3.2. The role of intermittent aeration with low DO for inhibit NOB growth in the single-stage PN/A process

The *in-situ* cycle experiment was carried out directly in the PN/A reactor on day 60 to investigate the possibility of NOB suppressing and achieving high nitrogen removal in the single-stage PN/A process (Fig. 3). The alternate aeration and non-aeration periods at low DO concentrations were set up in this experiment. As known, NOB utilizes nitrite for nitratation. Nitrite was not only a substrate of nitratation but also the product of ammonia oxidation. Therefore, we could see that nitratation always occurred behind the ammonia oxidation. The mechanisms for NOB inhibition in this experiment are based on the hypothesis that under the aerobic condition and low DO, $NO_2{}^-$–N quickly accumulated due to the specific growth rate of AOB (μ_{AOB}) higher than that of NOB (μ_{NOB}) (Shah and Rodriguez-Couto, 2022). When $NO_2{}^-$–N accumulated, the aeration was turned off, and the non-aeration period was applied, enabling anammox bacteria to consume $NO_2{}^-$–N for anammox reaction. After many cycles of aeration and non-aeration, the NOB communities lack of substrate for growing under limited oxygen conditions of non-aeration periods. Therefore, NOB could be outcompeted efficiently and reduce its potential inhibition of anammox bacteria in the PN/A reactor.

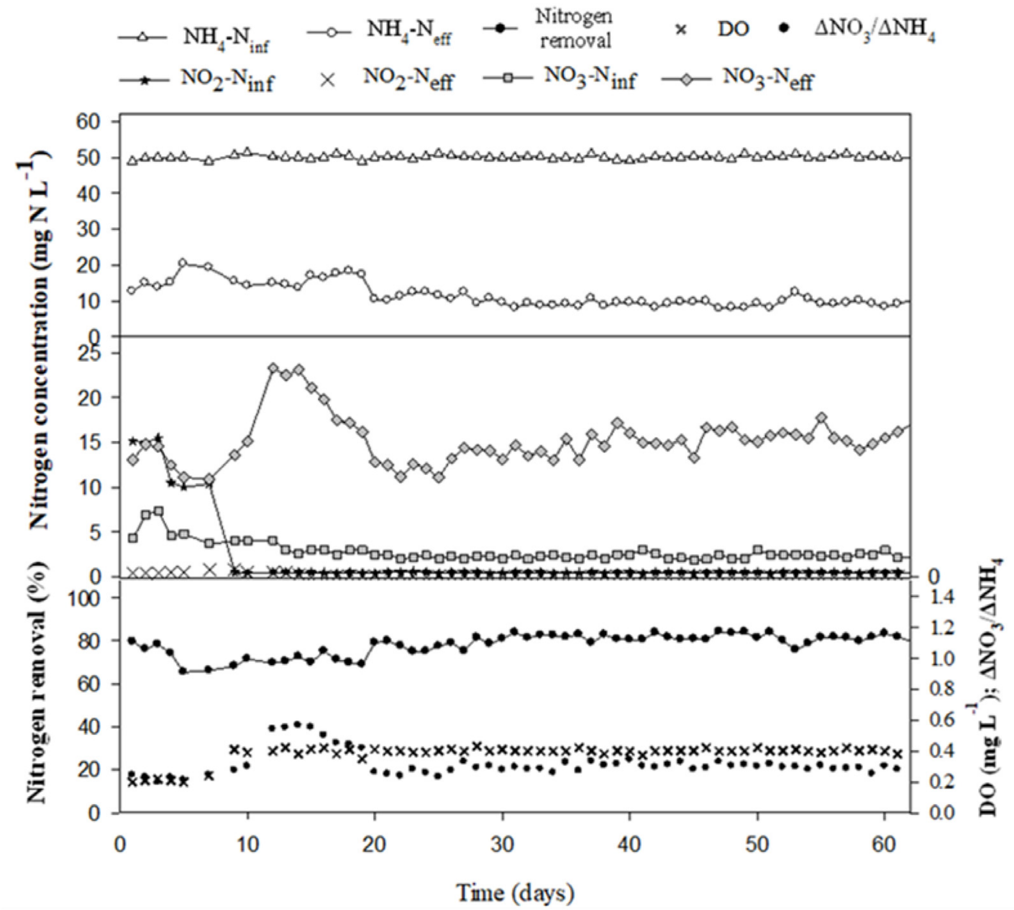

Fig. 2. Nitrogen removal of the single-stage nitritation/anammox process, including (a) NH_4^+–N, (b) NO_2^-–N, and NO_3^-–N, (c) Nitrogen removal, DO and NO_3^-–N/NH_4^+–N ratio, during 61 days of operation.

Fig. 3 showed that the accumulated NO_2^-–N and removed NH_4^+–N concentrations were 1.52 mg N L^{-1} and 26.4 mg N L^{-1}, respectively, after the first aeration period. Subsequently, in anoxic period, the NO_2^-–N and NH_4^+–N concentrations decreased by 1.15 mg N L^{-1} and 2.39 mg N L^{-1}, respectively. These results presented that NH_4^+–N could be removed in both aeration and non-aeration periods. The amount of NH_4^+–N decreased under aerobic conditions was mainly carried out by AOB and anammox bacteria through simultaneous nitritation and anammox reactions (Yang et al., 2015). Anammox bacteria consumed a part of NH_4^+–N under anoxic conditions in the non-aeration period. Indeed, the NH_4^+–N removal rate in the aeration period (0.14 mg N L^{-1} min^{-1}) was higher than that in the non-aeration period (0.09 mg N L^{-1} min^{-1}) (data not shown). These results indicated that AOB and anammox bacteria were well symbiotic in the PN/A reactor under intermittent aeration and low DO concentration conditions.

In addition, nitrate accumulated in the effluent is a common phenomenon when applying the single-stage PN/A process to treat mainstream wastewater (Li et al., 2021; Liu et al., 2021). Nitrate can be produced through NOB and anammox bacteria activities (Yang et al., 2015). According to the PN/A stoichiometry reaction (Eq. (3)), approximately 11% of nitrogen as a substrate is converted to nitrate. Therefore, a NO_3^-–N/NH_4^+–N ratio higher than 11% showed that NOB growth and activity in the PN/A reactor. In this study, the NO_3^-–$N_{produced}$ to NH_4^+–$N_{removed}$ ratio of aeration and non-aeration periods were 0.3 and 0.26, respectively. Similarly, a NO_3^-–N/NH_4^+–N ratio higher than 20% was also reported in the research of Li et al. (2021) when treating municipal wastewater by using a biofilm PNA-IFAS reactor under SBR mode. It was thought that NOB is responsible for a part of NO_3^-–$N_{produced}$ concentration, which was higher than the theoretical NO_3^-–N/NH_4^+–N ratio as showed in Eq. (3). As known, NOB used oxygen and mainly developed under aerobic conditions. Nonetheless, NO_2^-–N, the energy source of NOB for nitratation, only accumulated at the end of the aeration period (Fig. 3).

Besides, various studies showed that NO_2^-–N half-saturation concentration (K_S) of *Nitrospira* and *Nitrobacter* (typical NOB species) were in a wide range of 0.4–0.98 mg L^{-1} and 0.7–7.6 mg L^{-1}, respectively (Tangkitjawisut et al., 2016; Kunapongkiti et al., 2019); Sharif (Shourjeh et al., 2021)). According to the qPCR results of Le et al. (2020), the major NOB species presented in the PN seed used for the present study was *Nitrospira*. From the information mentioned earlier, the lowest NO_2^-–N concentration of about 0.5 mg L^{-1} in the anoxic period could reduce NOB activity. Indeed, the NO_3^-–N

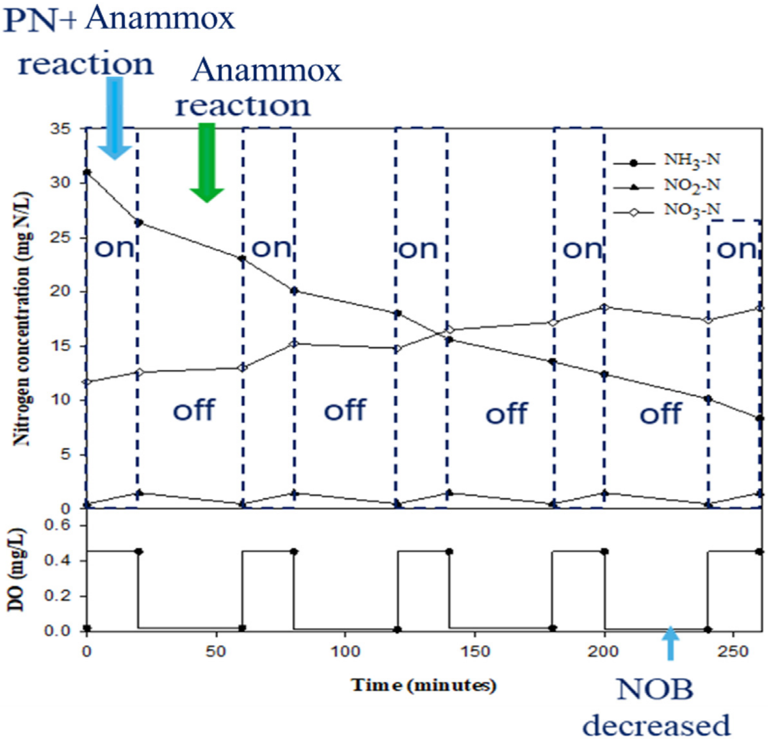

Fig. 3. The *in-situ* intermittent aeration cycles experiment of the single-stage nitritation/Anammox process.

production rate in the aeration period (0.035 mg N L^{-1} min^{-1}) was higher than that in the non-aeration period (0.02 mg N L^{-1} min^{-1}) (data not shown). These results induced that under the limited oxygen concentrations of non-aeration period, NOB might be inhibited due to a lack of oxygen and NO_2^-–N substrate for growing (Pérez et al., 2014). Subsequently, anammox bacteria had an obvious benefit in competing with the substrate (NO_2^-–N) with NOB under the intermittent aeration strategy in the PN/A reactor. These results indicated that the intermittent strategist with 20 min aeration and 40 min non-aeration in this study has a positive effect on NOB suppression, which was in line with Malovanyy et al. (2015). They applied an intermittent aeration strategy to treat municipal wastewater in an IFAS reactor. The intermittent aeration strategy was used with the DO concentration switched from 0.28 mg L^{-1} (15 minute-aerobic) to 1.5 mg L^{-1} (45 minute-anoxic). They successfully achieved the nitrogen removal of 70% in a mainstream PN/A process.

In short, the effluent concentration of NH_4^+–N, NRE, NNR in the *in-situ* experiment were 9.8 mg N L^{-1}, 73.2%, 0.14 kg N m^{-3} d^{-1} for 260 min. Besides, the intermittent aeration strategy had a positive effect on NOB suppression, with the nitrate production rate in the anoxic periods was 0.6 times lower than that in the aerobic periods. These results were suitable for the long-term performance of the PN/A in this study. Besides, to confirm that PN and anammox reactions occurred well in the PN/A reactor under intermittent low DO conditions, *ex-situ* batch experiments were carried out separately. Fig. 4 showed that the specific activities of anammox bacteria (SAA) of 0.006 mg N mg^{-1} MLSS h^{-1} were 1.5 times higher than that (S_{AOB}) of AOB. Finally, the results mentioned above indicated that the alternately switched between aeration/non-aeration periods had a positive effect on NOB inhibition and achieving high nitrogen removal in the PN/A process for low strength wastewater. DO concentrations and aeration time were critical factors for controlling the microorganism (anammox, AOB, and NOB) activity for a single-stage PN/A process.

3.3. Biomass characteristics

So far, various studies have applied different carriers for retaining biomass in biological treatment processes, such as sponge, biochar, nonwoven fabric material, or some trademark products (i.e., K5, K3, Biochip) (Gu et al., 2020; Wu et al., 2016).

This study used novel fiber carriers mentioned elsewhere (Le et al., 2022b) to retain the biomass in the PN/A reactor. Digital photos of the seed anammox granule sludge on day one and the PN/A sludge were taken on the last day of the operating period. The most significant dimension of dark red granular anammox sludge in the suspended mixture was approximately 3–4 mm observed in the bottom of the PN/A reactor (Fig. S1). The fiber carrier can effectively retain the biomass in the PN/A reactor due to tightly packed fibrous bundle structure. The brown-red anammox biofilm attached to

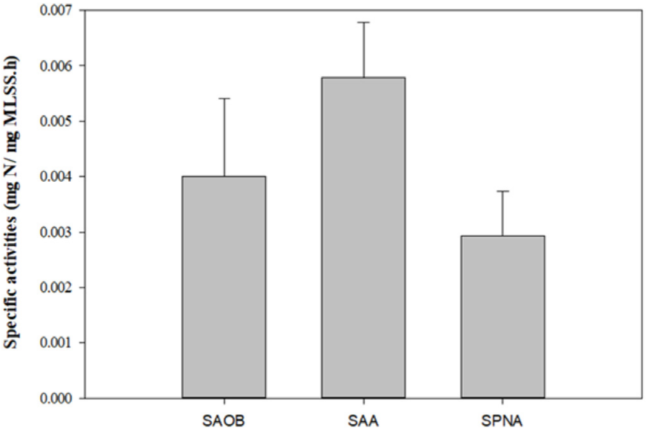

Fig. 4. The specific activities of Ammonium oxidation bacteria, anammox, and single-stage partial nitritation/anammox process.

the carriers proliferation day by day together with a dense granular red anammox sludge attached to the yarn bundles of the carriers.

Besides, polysaccharide (PS) and protein (PN) were measured during the operating period. PN and PS were ingredients of extracellular polymer substrates (EPS), responsible for structural and formation of biofilm. In fact, the anammox granules formation in this study was closely related to EPS secretion. This is also consistent with previous study conclusions (Ren et al., 2018). The protein and polysaccharide ratio increased from 1.34 to 2.1 and 2.8 on day 22, day 40, and day 61, respectively, showing that PN/A biofilm developed well (Wang et al., 2019) on the surface of the fiber carriers, promote the growth of particle size and make system more stably. From these results, it was thought that the PN/A biomass could adapt well under intermittent aeration conditions on the rope-type structure of fiber carriers.

3.4. Future perspective for implementation of a biofilm single-stage PN/A to treat mainsteam wastewater

In practice, the biofilm single-stage PN/A process was considered the most promising process to treat mainstream wastewater (C/N > 4) of full-scale WWTPs by applying intermittent aeration. The intermittent aeration can inhibit NOB more effectively and is easier to apply than other methods such as high substrate concentrations, high temperature, FA and/or FNA. The fiber carriers can be easily moved when needed, such as renovating or upgrading the reactor. In addition, the fibers carriers can help start up the PN/A system quickly, keep the anammox and AOB biomass efficiently, and maintain the long-term stability of the PN/A operation.

However, the disadvantage of the biofilm single-stage PN/A process is that it can generate nitrate when treating mainstream wastewater (C/N > 4). Nitrate can be produced as a result of NOB growth and are also products of the anammox reaction. Besides, the high COD concentration in mainstream wastewater can facilitate the overgrowth of heterotrophs, inhibiting the activity of AOB and anammox. Therefore, when applying single-stage biofilm PN/A process, it is necessary to minimize nitrate formation and reduce the influence of COD on the growth of anammox bacteria.

It is assumed that a typical COD concentration of about 500 mg L^{-1} in mainstream wastewater could affect the growth of AOB and anammox communities. Therefore, organic matters need to be pre-treated via the A- stage before going to B - stage by various processes, such as high-rate activated sludge (Wu et al., 2016; Cao et al., 2017, 2022), chemically enhanced primary treatment (CEPT) (Shewa and Dagnew, 2020), anaerobic process (Wan et al., 2016). It is noteworthy that among different processes used for the A-stage, the anaerobic fermentation process was considered ideal because methane production could help decrease the energy demand of WWTPs and provide energy-efficient wastewater treatment.

In the B-stage, nitrogen content in the effluent of the A-stage above will be treated by a biofilm single-stage PN/A process under intermittent aeration conditions. Thanks to the intermittent aeration conditions, NOB growth was inhibited effectively and improved nitrogen and organic matter removal of the PN/A process. Under aerobic conditions, AOB and anammox bacteria simultaneously removed a significant amount of ammonium nitrogen through the PN and anammox reactions (Eq. (3)). According to Eq. (3), the amount of nitrate produced from the activity of anammox bacteria could be used by heterotrophic bacteria to oxidize organic carbon in the non-aerated phase of the PN/A process (Eq. (7)). Most of the nitrogen content in the mainstream of the WWTP can be removed by the symbiosis of AOB, anammox, and heterotrophic bacteria in a mainstream biofilm single-stage PN/A reactor. Therefore, the nitrogen emission concentration will be negligible, and the nitrogen concentration of discharged limit standards could be satisfied.

$$NO_3{}^- + 0.108\ C_6H_{19}O_3N \rightarrow CO_2 + 0.5\ N_2 + 0.3\ H_2O + OH^- \tag{7}$$

4. Conclusions

This study investigated the feasibility of using intermittent aeration with low dissolved oxygen (DO) to suppress nitrite oxidizing bacteria (NOB) to achieve high nitrogen removal by the biofilm single-stage PN/A process. The NOB activities were successfully suppressed by applying intermittent aeration cycle with 20 min on/ 40 min off at DO concentrations as low as 0.3–0.4 mg L^{-1}. Nitrogen removal of 80% was consistently achieved by the biofilm single-stage PN/A process. Ammonia oxidizing bacteria (AOB) and anammox bacteria were to be successfully co-cultured in the PN/A reactor with the specific activities of anammox was 1.5 higher than that of AOB.

CRediT authorship contribution statement

Linh-Thy Le: Investigation, Formal analysis, Visualization, Writing – original draft, Review & editing. **Long D. Nghiem:** Review & editing. **Xuan-Thanh Bui:** Review & editing. **Deokjin Jahng:** Supervision, Conceptualization, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2023.103078.

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