Article

Enhanced Nitrogen Removal from Domestic Wastewater by Partial-Denitrification/Anammox in an Anoxic/Oxic Biofilm Reactor

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Abstract: A partial-denitrification coupling with anaerobic ammonium oxidation (anammox) process (PD/A) in a continuous-flow anoxic/oxic (A/O) biofilm reactor was developed to treat carbon-limited domestic wastewater (ammonia (NH_4^+-N) of 55 mg/L and chemical oxygen demand (COD) of 148 mg/L in average) for about 200 days operation. Satisfactory NH_4^+-N oxidation efficiency above 95% was achieved with rapid biofilm formation in the aerobic zone. Notably, nitrite (NO_2^--N) accumulation was observed in the anoxic zone, mainly due to the insufficient electron donor for complete nitrate (NO_3^--N) reduction. The nitrate-to-nitrite transformation ratio (NTR) achieved was as high as 64.4%. After the inoculation of anammox-enriched sludge to anoxic zones, total nitrogen (TN) removal was significantly improved from 37.3% to 78.0%. Anammox bacteria were effectively retained in anoxic biofilm utilizing NO_2^--N produced via the PD approach and NH_4^+-N in domestic wastewater, with the relative abundance of 5.83% for stable operation. Anammox pathway contributed to TN removal by a high level of 38%. Overall, this study provided a promising method for mainstream nitrogen removal with low energy consumption and organic carbon demand.

Keywords: partial-denitrification/anammox (PD/A); anoxic/oxic (A/O) biofilm reactor; nitrite accumulation; domestic wastewater; nitrogen removal

1. Introduction

Nitrogen discharge to water bodies can cause eutrophication and brings serious threats to human health. Nitrogen removal is the major purpose in current wastewater treatment plants (WWTPs). Nitrification/denitrification are commonly employed as traditional biological nitrogen removal technologies in WWTPs. In the first step, ammonia (NH_4^+-N) can be oxidized to nitrite (NO_2^--N) by ammonia-oxidizing bacteria (AOB), and to nitrate (NO_3^--N) by nitrite-oxidizing bacteria (NOB) in the second step [1,2]. Finally, NO_3^--N is converted to nitrogen gas (N_2) by denitrification bacteria with organic carbon as the electron donor. However, this requires a carbon source and considerable consumption of oxygen, especially for meeting stricter standards [3].

The discovery of the anaerobic ammonium oxidation (anammox) process brought new insight into a promising alternative for nitrogen removal from wastewater. In the anammox reaction, NH_4^+-N and NO_2^--N can be converted into N_2 immediately under anoxic conditions [4]. Generally, partial nitrification of NH_4^+-N oxidation to NO_2^--N is needed to provide substrate for anammox bacteria. The combined process of partial nitrification and anammox has been successfully applied in high-strength wastewater treatment [5]. However, as for low-strength domestic wastewater, there are still challenges for engineering applications. Although increasing studies regarding the partial nitrification–anammox process in mainstream treatment have been carried out in recent years [6],

the stability of partial nitrification is the major challenge during long-term operation. Suppression on the activity of NOB needs more effort under the conditions of low nitrogen concentration, low temperature, and fluctuated influent quality [7].

Partial denitrification (PD) has been proposed as a new approach for producing NO_2^--N for anammox bacteria [8]. A previous study reported that high NO_2^--N accumulation could be achieved by seeding the fermentation and denitrification sludge [9]. The nitrate-to-nitrite transformation ratio (NTR) achieved a high level of 80% during long-term operation, demonstrating the feasibility of PD for providing NO_2^--N for anammox [10,11]. Compared to the traditional anammox-based process, integration with the PD process offers great advantages of low oxygen demands, reduced organic carbon consumption, and less sludge production [12]. Furthermore, nitrous oxide (N_2O) production was significantly reduced under mainstream conditions according to previous study [13]. There is sufficient NH_4^+-N in domestic wastewater, which would facilitate the anammox bacteria in mainstream conditions by combining with the PD process [14]. However, the sludge retention time of anammox bacteria is relatively long owing to their low growth rate [15]. Therefore, it is vital to maintain sufficient biomass and achieve desirable bacteria retention in the reactor treating actual domestic wastewater. Biofilms have been widely regarded as an efficient tool for the retention and enrichment of anammox bacteria [16,17]. As for the mainstream treatment, the moving bed biofilm reactor (MBBR) has been extensively applied for the upgrade of nutrient removal processes [18,19].

Although the MBBR reactor has been widely used in WWTPs since the end of the 1980s [20], more information is needed for the engineering application of anammox-related process in terms of the start-up, optimization, and control strategies. The biofilm method can not only improve the effluent quality since less biomass was present in the discharge water than that of the suspended activated sludge process [21,22], but it can also be operated with a short hydraulic retention time (HRT) [23]. The microbial aggregation in biofilm was closely related to the extracellular polymeric substances and secretory extracellular organic matter [24]. Typically, anammox bacteria have been reported to occur as an attached growth, and the interfaces of contrast phases were the hotspot of anammox reaction [25]. This would benefit its competition for substrate with denitrifying bacteria in the suspended sludge [26,27]. Therefore, the biofilm process is assumed to be more suitable for practical application of the anammox-related processes, i.e., PD combining with anammox (PD/A) process [28]. To date, most studies on PD/A were conducted in sequencing batch reactors (SBR) or fixed bed reactors [13,17]. Considering the wide application of the continuous-flow process, it would be necessary to investigate the performance and stability of the novel technology before its practical implementation. However, limited information was available especially for the start-up and long-term nitrogen removal performance when treating actual domestic wastewater.

Therefore, the current study aims to develop a PD/A followed by nitrification biofilms in a continuous-flow anoxic/oxic (A/O) system with carriers added in both aerobic and anoxic zones. This holds the advantage of efficient retention of slow-growth nitrifying bacteria in the aerobic zone and anammox bacteria in the anoxic zone, which differs from previous studies that applied the PD/A process in mainstream treatment [29]. Actual domestic wastewater with low C/N was used as influent during the whole experiment. The performance of PD in the anoxic zone without anammox was investigated during long-term operation. The PD/A process was then established and the enrichment of functional bacteria in biofilm was evaluated. Results are expected to provide new insight into the design, operation, and optimization of anammox-related mainstream treatment.

2. Materials and Methods

2.1. Reactor and Operation

The continuous-flow A/O biofilm reactor was made of transparent plexiglas, which was divided into nine compartments (Figure 1). The first four compartments were set as anoxic zones, while the latter four were aerobic zones, resulting in the volume ratio of the

anoxic to the aerobic zone of 1:1. The last zone was an effluent discharging compartment. Each compartment possessed a working volume of 3 L, resulting in the total working volume of the whole reactor of 24 L. In each compartment, 100 polyethylene carriers were added with a filling ratio of around 33%. The carriers in the four anoxic zones were fixed by string and the diving mixer was installed in each compartment to guarantee the complete mixture. The carriers used in this study were made of polyethylene with a specific surface area of 500 m^2/m^3. In aerobic zones, aeration was implemented by using an air pump to supply a porous stone diffuser at the bottom of the reactor. The dissolved oxygen (DO) was in range of 1.5~2.0 mg/L. Influent and effluent recycling were controlled using a peristaltic pump.
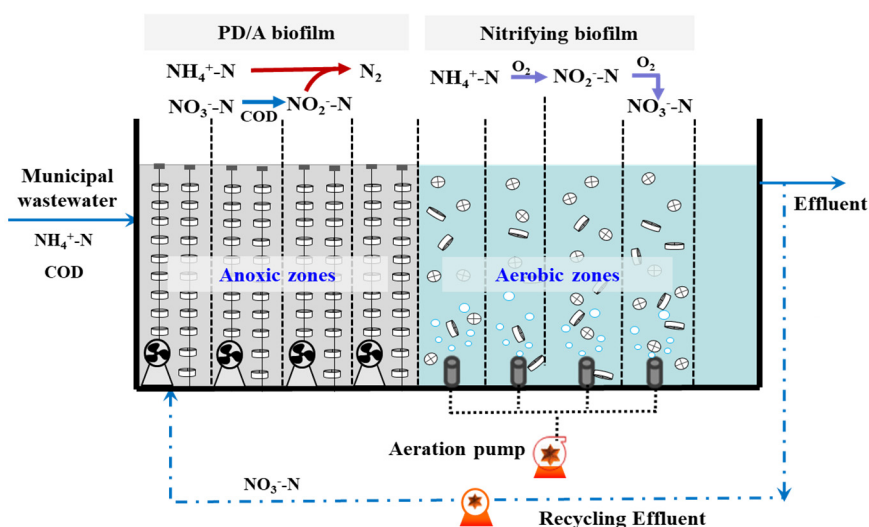
Figure 1. Schematic diagram of the PD/A anoxic/oxic biofilm reactor.

The seeding sludge was taken from a laboratory-scale endogenous post-denitrification and phosphorus removal system treating actual domestic wastewater [30]. The domestic wastewater was collected from a residential area of Beijing University of Technology (Beijing, China). The detailed characteristics of domestic wastewater are presented in Table 1. The influent flow rate was constant at 2 L/h, resulting in a total HRT of 12 h. During the operation process, the effluent recycling ratio was maintained at 200%. The reactor was operated at room temperature, which varied with seasonal change. The whole operation process could be divided into three phases as follows.

Table 1. The influent characteristics of domestic wastewater.

Concentration	COD (mg/L)	NH_4^+-N (mg/L)	NO_3^--N (mg/L)	TN (mg/L)	C/N	Temperature (°C)
Range	99–250	41–80	0–1.1	41–81	1.5–4.9	13.5–29.4
Mean value	148 ± 37	55 ± 9.0	0.4 ± 0.3	56 ± 9.1	2.68 ± 0.7	21.8

Phase I: Biofilm formation (day 1–58). New carriers were added into both the anoxic and aerobic zone. It was expected that the heterotrophic denitrifying organisms and nitrifying bacteria could be enriched by biofilm formation [31]. The recycling effluent contained NO_3^--N and the carbon source in raw water could provide favorable conditions for denitrifying bacteria in anoxic zones.

Phase II: NO_2^-N accumulation during denitrification (day 59–106). With the denitrifying bacteria growth attached to the anoxic biofilm and in suspended sludge, NO_2^--N accumulation occurred, which was also observed in a previous study on partial denitrification [32]. Biofilms were relatively thin at this stage [33].

Phase III: Addition of anammox sludge (day 107–199). The anammox sludge was collected from a pilot-scale reactor treating anaerobic digestion filtrate and was inoculated

to the anoxic zones [34]. A filter screen was added to the outlet of the latter two anoxic compartments of anoxic zones. The nitrogen removal performance and the stability of PD/A and the nitrification biofilm system were evaluated in this stage.

2.2. Batch Tests for NO_3^--N Reduction and NO_2^--N Accumulation Capacity of Anoxic Biofilms

The NO_3^--N reduction and NO_2^--N accumulation capacity of denitrification in anoxic biomass was evaluated on day 102. The carriers in the anoxic zones were taken out and transferred to four SBR with a working volume of 1.0 L. The feeding wastewater used in batch tests was similar to the influent of the reactor, which was mixed with the domestic wastewater and effluent of the A/O reactor using a volume ratio of 1:2. Subsequently, 33 carriers were removed from each anoxic compartment in the reactors. Nitrogen gas was purged into the reactors for 10 min to drive out DO before the reaction. The reaction lasted for 6 h during each test. A total of eleven samples were taken for nitrogen concentration measurement, including the first four samples taken in the first hour, another four samples taken in the next two hours, and the last three samples taken in the last three hours. The pH was maintained at 7.0–7.5 during the test by adding HCl or NaOH solution. The batch tests were repeated three times.

2.3. Ex-situ Activity of Denitrification Coupling with Anammox

Ex-situ activity of denitrification coupled with anammox was investigated on day 180 and the tests were conducted three times. Specifically, 33 carriers were removed from each of the four anoxic compartments and transferred to the SBRs. The feeding water was prepared by mixing actual domestic wastewater and effluent of the reactor at a volume ratio of 1:2. Nitrogen gas was purged into the reactors for 10 min to drive out DO before the reaction. The pH was maintained at 7.0–7.5 during the test by adding HCl or NaOH solution. The ex situ activities of denitrification coupling anammox were determined by the NO_2^--N reduction rates and NH_4^+-N removal rates during the reaction.

2.4. Oxygen Uptake Rate (OUR) of AOB, NOB, and Ordinary Heterotrophic Organisms (OHO)

Batch tests were conducted to evaluate the NH_4^+-N removal pathway in anoxic biofilm on day 192. Aerobic OUR was measured to detect the possible nitrification activity of AOB, NOB, and oxygen consumption by OHO using a respirometer (Strathtox, Strathkelvin, England). Taking into consideration that DO was very limited in the third and fourth anoxic, the aerobic NH_4^+-N removal capacity could be negligible. Therefore, ten carriers were taken from the first two compartments in anoxic zones for the OUR measurement. The samples were washed three times to remove the residual substrate and the biomass attached to the carriers was scraped off and equally added into three flasks with a working volume of 500 mL. The four groups of batch tests consisted of the control group without biomass and three tests for the OUR of AOB, NOB, and OHO. DO probes were connected to the flasks for real-time recording of DO concentration. The batch tests were conducted three times.

2.5. Analytical Methods

All samples were collected using 0.45 μm filter paper before analysis. NH_4^+-N, NO_2^--N, and NO_3^--N were analyzed using the Lachat Quik-Chem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee, WI, USA). Chemical oxygen demand (COD) was analyzed using the COD quick-analysis apparatus (Lian-Hua Tech. Co., Ltd., Beijing, China). Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solid (MLVSS) were analyzed by standard methods [35]. Moreover, temperature, pH, and DO were measured using a detector of Multi 3420 meter (WTW Company, Munich, Germany).

2.6. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

The abundances of AOB, Nitrospira, Nitrobacter, anammox bacteria, and total bacteria were determined by real-time qPCR using an MX3000P Real-Time PCR system

(Stratagene, La Jolla, CA, USA) with fluorescent SYBR-Green. In addition, the genomic DNA of the sludge sample was extracted using the Fast DNA SPIN kits for soil (Bio 101, Vista, CA, USA). All assays were performed in 20 μL reaction mixtures consisting of 10 μL SYBR Green exTaq (Takara, Dalian, China), 7 μL deionized water, 0.4 μL ROX Reference Dye50, 0.3 μL of each primer (10 mmol/L), and 2 μL DNA template (1–10 ng). To be specific, such a process consisted of the following steps: 3 min at 95 °C, followed by 40 cycles of the 30 s at 95 °C, 30 s at corresponding annealing temperature, and 45 s at 72 °C. Notably, the corresponding annealing temperatures are listed in Table 2. The qPCR was repeated three times for each group of bacteria.

Table 2. The primers and the annealing temperature.

Specificity	*AOB* amo A	*Nitrospira* 16S rRNA	*Nitrobacter* 16S rRNA	*Anammox bacteria* 16S rRNA	*Total bacteria* 16S rRNA
Former Primer	amoA-1F	338F	FGPS872F	Amx368F	341F
Reverse Primer	amoA-2R	685R	FGPS1269R	Amx820R	534R
Annealing temperature (°C)	55	53	51	56	55

2.7. Calculations

The $NO_3^- $-N-to-$NO_2^-$-N transformation ratio in the anoxic zone (NTR_{an}) was calculated according to Equation (1):

$$NTR_{an} = (\text{Terminal } NO_2^- \text{-N}_{an} - \text{Initial } NO_2^- \text{-N}_{an}) \times 100 / (\text{Initial } NO_3^- \text{-N}_{an} - \text{Terminal } NO_3^- \text{-N}_{an}) \quad (1)$$

where Terminal NO_2^--N_{an} and Initial NO_2^--N_{an} represent the NO_2^--N concentration at the end and beginning of the entire anoxic zone, respectively; similarly, Terminal NO_3^--N_{an} and Initial NO_3^--N_{an} represent the NO_3^--N concentration at the end and beginning of the entire anoxic zone, respectively.

The contribution of anammox pathway to TN removal was calculated by Equations (2)–(4) [17]:

$$\text{Contribution of anammox} = (\Delta NH_4^+ \text{-N} + 1.32 \, \Delta NH_4^+ \text{-N} - 0.26 \, \Delta NH_4^+ \text{-N}) \times 100\% / \text{TN removal} \quad (2)$$

$$\Delta NH_4^+ \text{-N} = \text{Theoretical } NH_4^+ \text{-N}_{an} - \text{Measured } NH_4^+ \text{-N}_{an} \quad (3)$$

$$\text{TN removal} = TN_{effluent} - TN_{influent} \quad (4)$$

where $\triangle NH_4^+$-N refer to the NH_4^+-N loss in anoxic zones, determined by theoretical concentration (Theoretical NH_4^+-N_{an}) and measured concentration of NH_4^+-N (Measured NH_4^+-N_{an}) at the end of anoxic zones; specially, theoretical NH_4^+-N_{an} was calculated according to the influent NH_4^+-N of the domestic wastewater by applying the recycling rate in this study. TN removal was determined by influent and effluent TN concentration in the system.

3. Results and Discussion

3.1. Performance of A/O Biofilm System for Domestic Wastewater Treatment

3.1.1. Start-Up and Biofilm Formation

The long-term performance of A/O biofilm with carriers in both anoxic and the following aerobic zones was evaluated. The entire operation could be divided into three phases and the conditions are summarized in Table 1. The nitrogen and COD removal were tracked during the 199 day operation (Figure 2). It showed that the effluent NH_4^+-N was at a high level in the initial operation, indicating insufficient nitrification performance (Figure 2a). It decreased gradually with the acclimatization of bacteria. After 50 days of operation, the NH_4^+-N oxidization efficiency increased from below 1% to over 95% with the effluent NH_4^+-N below 5 mg/L. This was maintained stably during the following operation, indicating the complete recovery of nitrification activity.
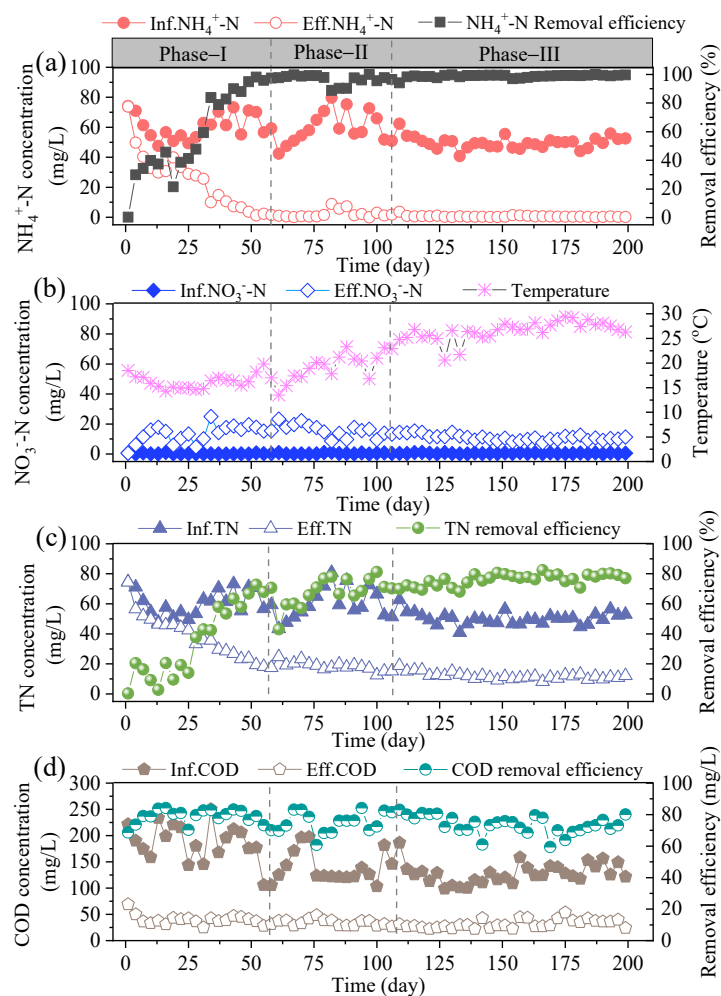
Figure 2. Long-term performance of nitrogen and COD removal in a continuous-flow A/O biofilm system: (**a**) influent and effluent NH_4^+-N concentration and removal efficiency; (**b**) NO_3^--N concentration and temperature; (**c**) influent and effluent TN concentration and removal efficiency; (**d**) influent and effluent TN concentration and removal efficiency.

Moreover, the formation of biofilm in the aerobic zone was observed after 59 days of operation, which was assumed to benefit the attached growth and retention of the nitrifying bacteria. This was supported by a previous study showing that the biofilm played a major role in nitrification, as it predominated over the suspended solids at higher media filling fractions [36]. Notably, the nitrifying bacteria (AOB and NOB) were chemoautotrophic and required no organic substances. Specifically, the heterotrophic bacteria would become the dominant bacteria using the organic substances in raw wastewater. This would possibly lead to competition between heterotrophic bacteria and nitrifying bacteria for DO and other nutrients. In this study, although the domestic wastewater was used as the influent containing a certain amount of organic matter, it would have little negative effect on the rapid recovery of nitrification and biofilm formation. This was mainly due to the consumption of COD in the former anoxic zone.

In comparison, more rapid recovery of COD removal was obtained, which was demonstrated by the increasing COD removal efficiency from 68.7% to 79.1% after 7 days of operation (Figure 2d). The effluent COD concentration was maintained below 50 mg/L during this phase. This suggested that the heterotrophic bacteria could be recovered more quickly than nitrifying bacteria. Therefore, nitrification became the limited step in the start-up of the biofilm system treating actual domestic wastewater. However, it could be seen that the relatively high effluent TN, greater than 20 mg/L, was eventually discharged due

to the high NH_4^+-N residual (Figure 2c). With the recovery of nitrification activity, effluent NH_4^+-N decreased gradually while the TN remained above 15 mg/L in phase I. There was up to 19 mg/L NO_3^--N residual in the effluent, indicating insufficient denitrification performance due to the limited organic carbon in raw wastewater (Table 3).

Table 3. Performance of A/O biofilm system during three operation phases.

| Phase	Time (day)	Influent Concentration (mg/L)			Effluent Concentration (mg/L)			
		NH_4^+-N	NO_3^--N	COD	NH_4^+-N	NO_2^--N	NO_3^--N	COD
I	1–58	61 ± 8.4	0.25 ± 0.30	184 ± 39	24 ± 19	0.44 ± 0.25	13 ± 5.8	40 ± 9.3
II	59–106	60 ± 11	0.41 ± 0.39	141 ± 29	2.3 ± 2.7	0.41 ± 0.27	16 ± 4.3	35 ± 6.1
III	107–199	50 ± 4.1	0.48 ± 0.30	128 ± 19	0.64 ± 0.6	0.38 ± 0.24	11 ± 2.0	32 ± 8.2

After 58 days, it was considered that the reactor was successfully started since the NH_4^+-N removal efficiency was achieved above 90% and maintained for 5 days, with the bacterial growth observed on the carriers. It was assumed that the nitrifying bacteria could grow attached to the carriers but with a low abundance due to its slow growth rate. Although the denitrifying bacteria exhibit much higher growth rates than the autotrophic bacteria, efficient retention of nitrifying bacteria was speculated to be achieved and attributed to the biofilm formation. The ammonium transport was reported to be related to the substrate concentration, and the oligotrophic environments would result in a decrease in cell growth of AOB, since more cellular energy was consumed in ammonia loss and recovery [2]. In this case, growth on the biofilm would provide a more suitable environment for AOB to mass transfer between cells. However, further improvement of the nitrogen removal performance needs to be considered.

3.1.2. NO_2^--N Accumulation in Anoxic Zone

To investigate the development and efficiency of partial denitrification in the continuous-flow biofilm system, NO_2^--N and NH_4^+-N concentrations in the anoxic zone were measured, as shown in Figure 3. It should be noted that the obvious accumulation of NO_2^--N occurred in the anoxic zones after 67 days of stable operation, with the NO_2^--N_{an} concentration in anoxic effluent of 1.1 mg/L (Figure 3a). Subsequently, consecutive NO_2^--N accumulation was identified and slightly increased in the anoxic zones, which peaked at 106 days with 6.0 mg/L and NTR_{an} of 64%. According to the best of our knowledge, this was at a relatively high level of NO_2^--N accumulation in the continuous plug-flow reactor. A previous study also reported a similar phenomenon of NO_2^--N accumulation in the anoxic zone of a modified UCT (University of Cape Town) step-feed reactor treating municipal wastewater, with the greatest value of about 2.5 mg/L [37]. The level NO_2^--N accumulation in this study was higher than that in the previous study, and it was detected from the first anoxic compartment to the end of the anoxic compartment during phase II. This mainly resulted from insufficient electron donor in the domestic wastewater for complete NO_3^--N reduction. Meanwhile, the effluent concentration of NO_3^--N decreased. This most likely occurred because the electron donor for reducing NO_3^--N was saved due to the NO_2^--N accumulation; more organic carbon source could be used to reduce NO_3^--N, resulting in the lower effluent NO_3^--N.

According to previous studies, high NO_2^--N accumulation could be achieved in a sequencing batch reactor with the NTR above 70% [38], while the stable NO_2^--N accumulation in the anoxic zone at this high level in a continuous-flow biofilm reactor for actual municipal wastewater treatment has been rarely reported. In this study, the NO_2^--N concentration at the end of the anoxic zone increased from 0.2 to 6.0 mg/L, with the initial NO_2^--N concentration of the anoxic stage maintained at a very low level of 0.2 mg/L on average. During this phase, the NTR_{an} increased from 0.97% to 64.4%, which was comparable to the value in previous studies with synthetic wastewater and an external carbon source [39]. Therefore, this study offered an effective approach to achieve anammox

by providing a stable substrate in an A/O biofilm system. Moreover, the NH_4^+-N was abundant in the domestic wastewater, and it maintained sufficient concentration at the end of anoxic, as shown in Figure 3a. It was assumed that the NO_2^--N accumulation and NH_4^+-N residue in the anoxic zones provided favorable conditions for the growth of anammox bacteria.
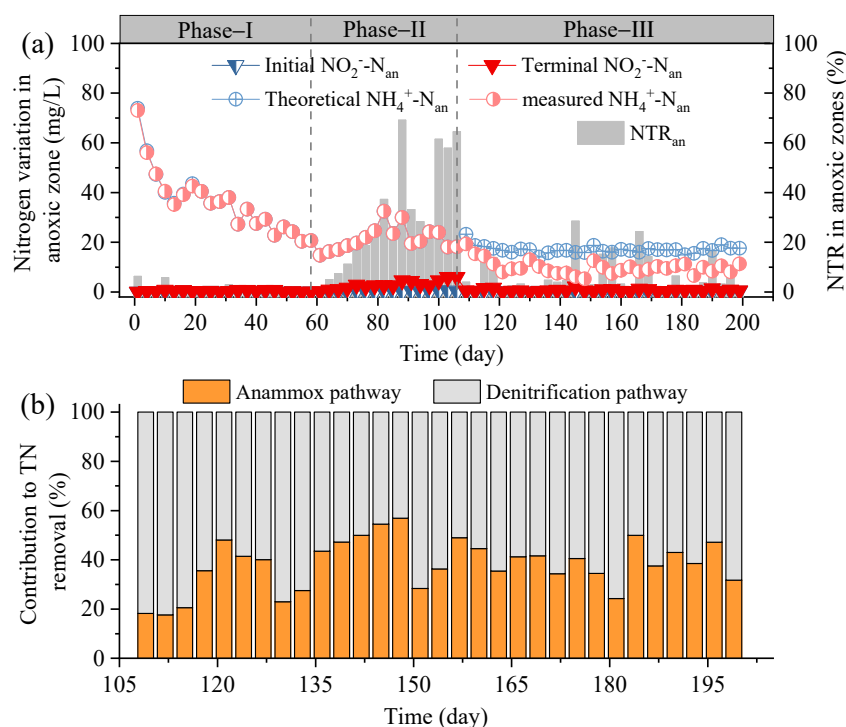

Figure 3. (**a**) Variation of NO_2^--N and NH_4^+-N concentration during long-term operation of the anoxic zone of the A/O biofilm reactor. Typically the initial and terminal NO_2^--N concentration in the anoxic zone (initial NO_2^--N_{an}, terminal NO_2^--N_{an}) was tracked. The theoretical and measured value of NH_4^+-N concentration at the end of anoxic zone (initial NH_4^+-N_{an}, terminal NH_4^+-N_{an}) were analyzed, and NTR_{an} was referred to the nitrate-to-nitrite ratio during the anoxic reaction period. (**b**) Contribution of anammox and denitrification pathways to TN removal of the entire system in phase III.

To evaluate the feasibility of advanced nitrogen removal by the PD/A process, the NO_2^--N production capability of anoxic biofilm was evaluated by batch test with the biofilm taken from the four anoxic compartments.

Gradual NO_2^--N accumulation along with NO_3^--N reduction was observed (Figure 4). The NO_2^--N accumulation rates were much higher during the first three hours and then decreased. The accumulated NO_2^--N concentration achieved a steady level after four hours. The NO_2^--N accumulation efficiency in the second anoxic zone was the highest, and the last anoxic zone showed the lowest value, corresponding to the NTR in the four anoxic zones of 63.8%, 71.7%, 68.9%, and 59.3%, respectively. This would be related to the higher microbial community for NO_3^--N reduction when the water flows into anoxic-2 after anoxic-1, which would improve the denitrification activity of the biomass in anoxic-2. Moreover, the organic carbon source decreased to the lower level in anoxic-3 and anoxic-4 compared to anoxic-2, resulting in a decreased NO_3^--N reduction efficiency and NO_2^--N generation. This indicated that the HRT and organic carbon source could play important roles in the development of the PD/A process. Results of this study proved that the partial denitrification with stable NO_2^--N generation could be achieved successfully in a continuous-flow biofilm system, thus providing a meaningful method to achieve mainstream anammox in practical application.
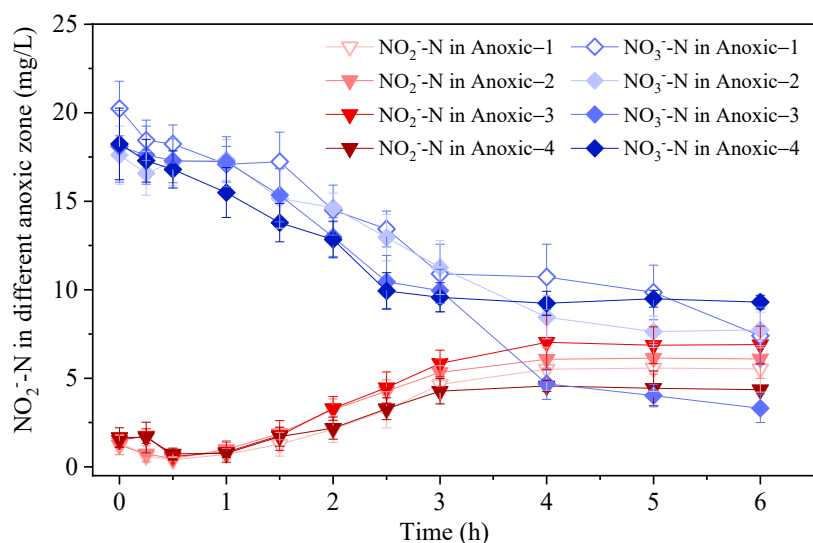
Figure 4. Evaluation of NO_2^--N accumulation capacity during NO_3^--N reduction of biofilms taken from the anoxic zones.

### 3.2. Development of Partial Denitrification/Anammox in A/O Biofilm System

Based on the results described above, stable and efficient NO_2^--N accumulation in the anoxic zone was observed and assumed to provide a suitable environment for the growth of anammox bacteria. Therefore, during phase III, the anammox-enriched sludge was inoculated on day 109 to investigate the feasibility and performance of the PD/A process in this system.

Results showed that there was an obvious gradual decrease in NH_4^+-N concentration in the anoxic zones, as shown in Figure 3, which declined from 19 to 11 mg/L at the end of the anoxic stage after the addition of anammox sludge. It indicated that the anammox activity occurred by consuming NH_4^+-N and NO_2^--N under this condition. This was also illustrated by the decrease of NO_2^--N accumulation in the anoxic zone, which declined from 6.0 to 0.5 mg/L at the end of the operation. Moreover, the effluent NO_3^--N concentration decreased gradually in the final effluent, corresponding to the effluent TN decreasing to 11 mg/L on average during the stable operation from day 154 to 199. The TN removal efficiency increased to 78.0% and was much higher than that in phase I (37.3% on average). This demonstrated that the PD/A could be developed successfully in the A/O biofilm system for advanced nitrogen removal from actual domestic wastewater. It should be noted that very limited growth of biomass was found in the biofilm system. The average MLSS in anoxic zones was about 1200 mg/L, which was about 700 mg/L in aerobic zones at the end of the operation. This suggested that the novel PD/A biofilm system holds great advantages in achieving substantially large nitrogen removal loading rates with reducing sludge production.

On the other hand, results in this study implied that the PD and anammox bacteria could be efficiently retained in an anoxic zone via rapid biofilm formation. A previous study also reported that the PD/A process could be achieved in a step-feed anoxic–oxic (A/O) system by adding the carriers containing anammox bacteria [40], while different results showed that the activity of anammox was lower under the carriers-only condition, compared to that under the hybrid condition of carriers and flocs. In this study, blank carriers were added into both the anoxic and aerobic zone, indicating that the rapid formation of PD/A biofilm played a key role in enhanced nitrogen removal performance. Therefore, it provided an efficient strategy to improve the nitrogen removal performance in mainstream treatment without mature anammox biofilm and low biomass concentration.

3.3. Removal Pathway of NH$_4$$^+$-N in Anoxic Stage

It should be noted that the DO in recycling nitrification effluent of A/O was at a relatively high level. There might be simultaneous nitrification and denitrification responsible for NH$_4$$^+$-N removal in anoxic zones apart from the anammox process. Consequently, it was necessary to verify whether autotrophic (anammox) or heterotrophic (complete denitrification) pathways would be responsible for the NH$_4$$^+$-N loss in the anoxic zone. The maximum OUR of AOB, NOB, and OHO were measured with biofilm samples taken from anoxic zones for estimation of the nitrification activity. Results showed that the activity of OHO was far greater than those of AOB and NOB in the case of organic compounds (Figure 5). Meanwhile, OHO consumed oxygen at a rate of 21.23 mgO$_2$/(gVSS·h) in the first anoxic zone, which was much higher than the 4.04 mgO$_2$/(gVSS·h) of AOB and 2.41 mgO$_2$/(gVSS·h) of NOB. In addition, OHO consumed oxygen at a rate of 13.39 mgO$_2$/(gVSS·h) in the latter anoxic zones, which were 1.44 mgO$_2$/(gVSS·h) and 2.16 mgO$_2$/(gVSS·h) for AOB and NOB, respectively. Moreover, since there was sufficient carbon source in anoxic zones, DO was preferentially consumed by OHO due to the rapid reaction rates, indicating that the nitrification in anoxic zones would be very limited. It should be noted that there is a difference in aerobic respiration rates of the biomass on anoxic carriers between values in the batch tests and actual operation in the reactor due to the substrate transfer limitation in biofilm. Moreover, it was difficult to maintain the DO and substrate concentration at the same level. Therefore, more detailed evidence and thus analysis of microbial abundance was needed to confirm the contribution of the anammox pathway on nitrogen removal in anoxic zones, which is discussed in the following section. This agreed well with a previous study on the enhanced NH$_4$$^+$-N removal that occurred in a full-scale wastewater treatment plant by adding moving carriers into the anoxic zone, with the anammox contribution accounting for 15.9% due to PD providing substrate [41]. Therefore, the PD/A is a promising and feasible method for mainstream treatment.
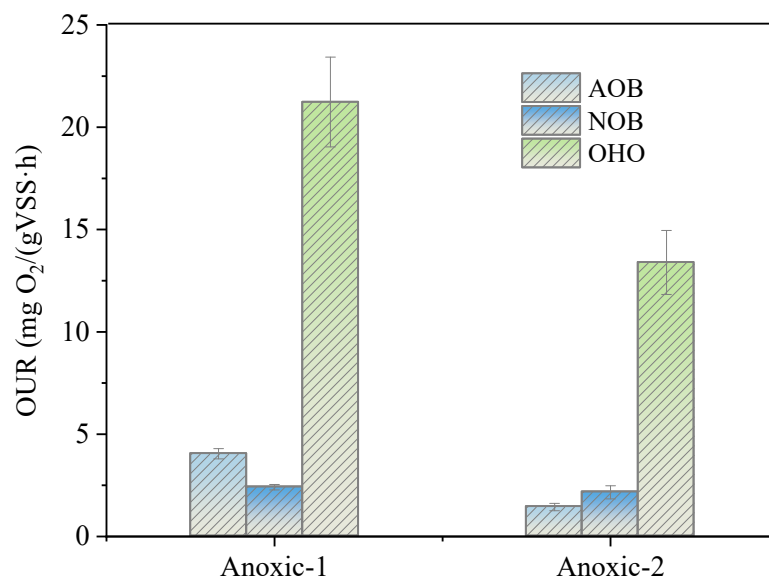
Figure 5. Oxygen uptake rates of AOB, NOB, and OHO in the biomass taken from anoxic biofilms.

Furthermore, the ex-situ activities of PD/A were detected in the anoxic biofilm after 147 days of operation. Results showed that the NO$_3$$^-$-N concentration decreased rapidly at the beginning of the reaction, along with the increased NO$_2$$^-$-N (data not shown). Anammox occurred after 30 min when higher NO$_2$$^-$-N was accumulated. The carbon source from the actual domestic wastewater provided sufficient electron donor during the first 30 min, resulting in faster denitrification activity and the accumulation of NO$_2$$^-$-N. At the same time, higher activity of anammox was observed with the simultaneous decrease of NH$_4$$^+$-N, NO$_2$$^-$-N, and NO$_3$$^-$-N concentration. This suggested that the organic matter

concentration in domestic wastewater played an important role in anammox performance in this system, which was consistent with previous studies [42,43]. In this case, it should be taken into consideration that the excess COD concentration has a negative effect on anammox activity and efficient strategies were needed to optimize the carbon utilization by heterotrophic bacteria in practical application.

It can be seen that NH_4^+-N appeared to decrease at the beginning of the second anoxic zone. Since there was much more carbon source than usual in the reactor, the accumulation of NO_2^--N was also observed in the experiment, and the anammox activity was lower than the denitrification activity at the beginning. The anammox activity in the second anoxic zone was higher than that in the first zone, which could be attributed to the decreasing denitrification activity due to less organic carbon source in the second compartment.

3.4. COD Removal during Stable Operation

The COD removal was improved after the addition of anammox sludge. As a result, the effluent COD was kept at <50 mg/L, with a removal rate of >75%. The average influent COD was around 172 mg/L, which dropped to 50–60 mg/L at the end of anoxic zones. This could be due to the dilution by recycling effluent; the COD concentration in the final effluent decreased to 35–45 mg/L.

On the other hand, there was no external COD addition during the whole operation. This was mainly attributed to the denitrification coupling with anammox in anoxic zones in the reactor, which saves the electron donor for complete reduction of NO_3^--N to N_2. As for the treatment of wastewater with low C/N, insufficient carbon sources would limit the denitrification efficiency, resulting in insufficient effluent quality. In this study, more efficient use of carbon sources could be achieved in the anoxic stage for partial reduction of NO_3^--N to NO_2^--N, which was consumed by anammox bacteria as electron donor. Therefore, desirable TN removal performance could be achieved despite influent C/N as low as 2.65. Furthermore, the aeration energy could also be saved due to the NH_4^+-N removal via the anammox pathway in anoxic zones, thus the NH_4^+-N flowing to the aerobic zone would decrease.

3.5. Effect of DO on Nitrogen Removal of A/O Biofilm System

In the anoxic zones of the A/O biofilm reactor, denitrification and anammox was responsible for the NO_3^--N and NH_4^+-N removal in the anoxic zone, and complete nitrification for NH_4^+-N oxidation to NO_3^--N was carried out by AOB and NOB in the aerobic zones. The NO_3^--N remaining at the end of the anoxic zones would affect directly the TN concentration in the final effluent. Elevated NO_3^--N concentration at the end of anoxic zones was 0–7 mg/L during phases I and II, indicating that the reaction in the anoxic zones was not optimal. The biomass in the aerobic zones in a biofilm reactor was less than that of activated sludge, possibly resulting in a high effluent DO level. It should be noted that part of the organic matter would be consumed by OHO under aerobic conditions rather than for NO_3^--N reduction in anoxic zones, if a certain amount of DO was introduced into the anoxic zones from the cycling effluent. In this study, DO concentration in recycling effluent ranged from 4 to 8 mg/L. However, the DO of the first two anoxic zones always remained below 0.1 mg/L. As a result, fewer electron donor could serve for denitrification, resulting in lower TN removal before the PD/A development.

The negative effect of DO on denitrification in anoxic conditions could be alleviated in the stable operation of the PD/A biofilm system. As observed from Figure 3a, there was a difference between the theoretical concentration and the measured value of NH_4^+-N concentration after mixing the recycling effluent with influent wastewater. This indicated that the nitrogen removal in the anoxic stage could be significantly improved by introducing the anammox process. However, a high DO should be avoided since it had a negative effect on anammox activity. The average contribution of the anammox pathway to TN removal achieved 38% in the stable operation. This was comparable with a previous study, which found that the anammox could account for 32–47% of TN removal in a step-feed

AO system [41]. However, this was lower than that in two-stage nitrification and PD/A SBRs treating domestic wastewater, in which the anammox could account for 72% of TN removal [13]. This was possibly due to the negative effect of DO being much less in a separated reactor for PD/A. Further optimization is needed to improve the anammox contribution in the continuous-flow systems.

These results indicated that enhanced nitrogen removal from low-carbon wastewater could be achieved by PD/A in a continuous-flow biofilm system without the addition of an external organic carbon source. Moreover, the aeration energy could be significantly saved by up to 19% compared to the traditional nitrification and denitrification process, attributed to 38% of TN removal by the PD/A process. According to the previous study, the PD/A-based process in theory has the potential for reducing oxygen and organic carbon demands by 50% and 80% [13]. Therefore, it is a promising and effective alternative in a continuous-flow biofilm reactor to promote anammox contribution and reduce energy consumption. On the other hand, we investigated the N_2O production in the PD process with high NO_2^--N accumulation in a previous study, which indicated very limited N_2O production during the reduction from NO_3^--N to NO_2^--N process [10]. This was explicable since the NO_3^--N was converted to NO_2^--N without further reduction. Additionally, the N_2O production in the PD/A process under mainstream conditions was explored in our previous study, which illustrated that the maximum N_2O accumulation in the PD/A process could be as little as 0.7% of influent nitrogen and was much lower than that previously reported for conventional nitrification–denitrification or single-stage PN/A, and such a small amount of N_2O could be depleted during the following reaction [13]. This clearly suggested that the N_2O emission could be mitigated in this process. It was mainly attributed to the transition step from NO_3^--N to NO_2^--N, followed by NH_4^+-N oxidation via anammox that produced little N_2O due to the microbial metabolism. Further investigation is necessary to explore the potential risk of N_2O production in the whole process of the continuous-flow PD/A biofilm system.

3.6. Abundance of Functional Bacteria in PD/A Biofilm

The abundance of functional bacteria including AOB, NOB, and anammox bacteria were analyzed by qPCR. In addition, the functional bacteria in suspended biomass returned to anoxic zones by recycling biomass were also investigated.

Results showed that anammox bacteria were enriched in the anoxic biofilms with abundance of 8.69×10^8 and 8.82×10^8 copies/gSS in the biofilms of the first and second anoxic zone, accounting for 5.83% and 4.41% of total bacteria, respectively (Figure 6). In comparison, it was relatively lower for AOB and two types of NOB in the same biofilms. The abundance of AOB was 1.34×10^5 and 3.15×10^5 copies/gSS. A higher abundance of *Nitrospira* was detected: 6.46×10^6 and 9.16×10^6 copies/gSS, respectively. The abundance of *Nitrobacter* was slightly lower, 1.41×10^6 and 2.17×10^6 copies/gSS, respectively. In addition, the abundance of AOB and NOB was still low in recycling biomass, indicating that the nitrification by nitrifying bacteria returned from the aerobic zone to the anoxic zone would be very limited. This confirmed that the partial denitrification coupling with anammox was the dominant contributor to NH_4^+-N loss rather than the simultaneous nitrification and denitrification process. The anammox bacteria in recycling biomass would derive from the flocs growth or the fall-off biofilms. Overall, results in this study demonstrated that the anammox bacteria could be retained in the anoxic biofilm community by utilizing NH_4^+-N from domestic wastewater and NO_2^--N from NO_3^--N reduction via the PD approach.
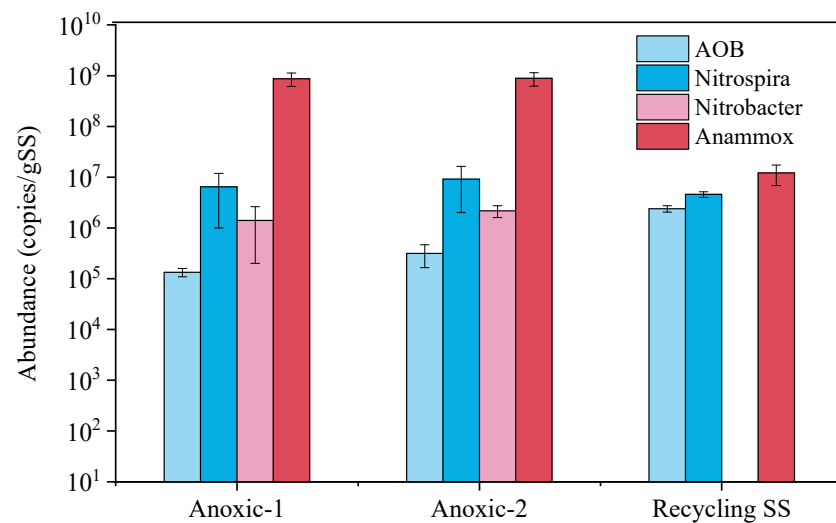
Figure 6. Abundance of nitrifying and anammox bacteria in biofilms.

4. Conclusions

This study proposed and demonstrated a novel PD/A biofilm system in a continuous-flow reactor for low-carbon domestic wastewater treatment. Satisfactory NH_4^+-N oxidation efficiency above 95% was achieved due to the rapid biofilm formation in the aerobic zone. High NO_2^--N accumulation occurred in anoxic zones due to the insufficient organic carbon for complete denitrification, with a peak NO_2^--N value of 6.0 mg/L and NTR_{an} achieving 64.4%. The TN removal was significantly improved from 37.3% to 78.0% despite a low COD/TN of 2.65 when anammox sludge was added to the anoxic zones. A high level of anammox contribution of 38% to TN removal was achieved in the PD/A system. Anammox bacteria could be efficiently retained in the anoxic biofilm and played a key role in advanced nitrogen removal from the domestic wastewater without external organic carbon addition.

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