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Start-up of partial nitrification-Anammox (PN/A) process treating piggery wastewater

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ABSTRACT

Nitrogen removal had become a major focus in piggery wastewater treatment since nitrogen was the nutrient causing eutrophication. Recently, partial nitrification-Anammox (PN/A) was a promising process for ammonia-rich wastewater. This study investigated the start-up and operation of PN/A process for nitrogen removal from piggery wastewater under oxygen-limited (0–0.2 mg/L). Firstly, the diluted piggery wastewater was used as the influent; results showed that excess of biodegradable organic carbon would threaten Anammox bacteria survive. Then, the anaerobic digestion of piggery wastewater with the gradient dilution was fed into the reactor, corresponding to gradually increasing the ratio of chemical oxygen demand (COD) to ammonia (COD/N) to 0.99, 1.37, and 1.61, the PN/A reactor could recover to good performance. And the ratio of COD removal value ($COD_{removed}$) to ammonia ($COD_{removed}$/N) closed to 0.17 averagely on phase III–V, which indicated that digested piggery wastewater which mainly consisted of refractory organics as the effluent did not affect the operation of the PN/A process. Using the high-throughput sequencing, the abundance of Candidatus Brocadiacese was decreased from 8.61% on day 125 to about 4.56% on day 173 and remained a stable proportion (3.41% on day 235). Our study provided a start-up strategy of the PN/A process for treating piggery wastewater.

Keywords: Piggery wastewater; Partial nitrification-Anammox; COD/N ratio; Refractory organics; Nitrite-oxidizing bacteria

1. Introduction

Piggery wastewater was mainly characterized by a high concentration of organic matter. The wastewater is subjected to treat by anaerobic digestion to remove the organic matter and recover biogas. However, after anaerobic digestion, a high concentration of ammonia is released from the digested organic matter. Part of piggery wastewater has been used for land application because of sufficient nutrients (i.e., ammonia) for crop yields [1]. However, the land application of piggery wastewater may cause health concerns, such as the residue of antibiotic resistance genes [2]. Even worse, the high concentration of ammonia in piggery wastewater is the key factor inducing eutrophication to the water bodies. The nitrification-denitrification process is typically used for biological nitrogen removal, in which the ratio of chemical oxygen demand (COD) to ammonia (COD/N) in piggery wastewater is far inadequate for denitrification process [3]. Therefore, external organic matter is required for complete denitrification, which consequently leads to additional

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operating costs. Considering the low profit of the piggery industry, the traditional method is not suitable for treating piggery wastewater.

Unlike nitrification–denitrification processes, anaerobic ammonium oxidation (Anammox) process is recognized as a more promising technology process due to less energy consumed and no need to add extra organic matter [4]. The Anammox process relies on the availability of nitrite and ammonia. However, piggery wastewater contains a high concentration of ammonia but few nitrite. The partial nitrification process can effectively produce nitrite. Therefore, the Anammox process is often combined with partial nitrification. Nevertheless, the separated operation of partial nitrification and the Anammox process requires a large footprint as well as the acid supplied for partial nitrification and the alkali supplied for Anammox. The partial nitrification-Anammox (PN/A) process, where partial nitrification and Anammox process is integrated into a single reactor, successfully overcomes these shortages.

As previously reported, organic matter is regarded to negatively affect the performance of the PN/A process [5,6]. Previous studies also indicated that the COD/N ratio for PN/A system should be less than 0.5 [7–9]. However, piggery wastewater is rich in various dissolved organic matter, including volatile fatty acids, protein, and humic substances. Even if after anaerobic treatment, most of the organic matter persists and the COD/N ratio in digested piggery wastewater remains in the range of 1–2 [10]. It seems to be a challenge to treat digested piggery wastewater using the PN/A process because of the high COD/N ratio. However, most of these investigations have been carried out using synthetic wastewater, in which COD is composed of easily biodegradable organic matter (e.g., acetate and glucose) [11]. COD composition in piggery wastewater is much complex and incorporates a large fraction of refractory organic matter, which might have little influence on the PN/A process. For example, real landfill leachate digested wastewater was reported to be successfully treated in PN/A process at a higher COD/N ratio (more than 1) [11,12]. Therefore, it is hypothesized that influent piggery wastewater as long as remain low concentration of biodegradable organic matter even if at a high COD/N ratio might also be successful in start-up PN/A process.

The objective of this study is to investigate the start-up and operation of PN/A process for treating piggery wastewater in a sequencing batch reactor (SBR). In addition, its performance under different influent COD/N ratios was also evaluated. Furthermore, high-throughput sequencing was applied to investigate functional microbial species and population variety in PN/A process.

2. Materials and methods

2.1. Wastewater and inoculum

Three kinds of wastewater were used for the start-up of PN/A process treating piggery wastewater. One was the synthetic wastewater, which was used for the acclimation of PN/A sludge. The synthetic wastewater was prepared according to previous reports [13]. The second was the raw piggery wastewater, which was collected from a stabilization pond in a local pig farm (Hefei, China). The third was the digested piggery wastewater, which was the raw piggery wastewater being further treated by an upflow anaerobic sludge bed reactor to remove organic matter for reducing the COD/N ratio. The second and third wastewaters were used for the start-up of PN/A process treating piggery wastewater. The characteristics of synthetic wastewater, raw piggery wastewater, and digested piggery wastewater are listed in Table 1.

The inoculum sludge for the partial nitrification process was collected from a municipal wastewater treatment plant (Hefei, China). Granular Anammox sludge was collected from Anammox SBR in the laboratory, which has been stably operated for 400 d with a nitrogen loading rate of 0.35 kg N/m^3/d. The synthetic wastewater was fed into the SBR and operation parameters were set up according to previous reports. The Anammox process showed high removal efficiency of ammonia 99% and total nitrogen 80%.

2.2. Experimental design

An SBR with a working volume of 3 L and a total volume of 3.5 L was used in this experiment, as shown in Fig. 1. The SBR was made of plexiglass cylinder and an internal diameter of 100 mm, which operated at 35°C ± 3°C. And without pH control, that was, inside the reactor, around 7.7 ± 0.4. The start-up of PN/A process treating piggery wastewater was divided into five phases. In the first phase, the PN/A sludge was acclimated using synthetic wastewater. The start-up of PN/A process treating piggery wastewater had four phases using above acclimated PN/A sludge: (i) to investigate the necessity of further anaerobically treating piggery wastewater from stabilization pond before PN/A process,

Table 1
Characteristics of the wastewater used in this study

	Synthetic wastewater	Piggery wastewater	Digested piggery wastewater
NH_4^+–N (mg/L)	300.00–450.00	291.32 ± 35.26	301.43 ± 39.15
NO_3^-–N (mg/L)	–	35.25 ± 5.11	28.65 ± 3.77
SCOD (mg/L)	–	826.43 ± 155.27	494.42 ± 23.50
TP (mg/L)	–	26.51 ± 6.42	31.36 ± 7.20
COD/N ratio	–	2.84 ± 0.21	1.61 ± 0.14

Note: SCOD, soluble chemical oxygen demand; TP, total phosphorous.

the diluted raw piggery wastewater was used as the influent of PN/A and ammonium was added to decrease influent COD/N ratio averagely from 2.80 to 1.31 on phase II and (ii) to investigate the feasibility of starting PN/A process treating digested piggery wastewater, the digested piggery wastewater with the gradient dilution was fed into the reactor on phase III and IV, respectively. The influent COD/N ratio on phase III and phase IV was averagely controlled at 0.99 and 1.37 respectively, by adding ammonium to the diluted digested piggery wastewater. On phase V, piggery wastewater was no diluted as influent and the COD/N ratio was averaged 1.61. The detailed design is listed in Table 2.

SBR were used for the start-up of the PN/A process, which was operated with two cycles per day using the following model feeding for 5 min, intermittent aeration (following the interval of 8 min aeration and 22 min anaerobic phase) for 11 h, settling for 30 min, and drawing for 25 min. The oxygen-limited condition (0–0.2 mg/L) was controlled by a dissolved oxygen (DO) sensor (SJG-203A DO analyzer) in the reactor. The SBR volume exchange rate was 33.3%.

2.3. Analytical methods and calculation procedure

The concentrations of COD, NH_4^+–N, NO_2^-–N, NO_3^-–N, mixed liquid suspended solids and mixed liquid violated suspended solids were measured according to the Standard Methods [14]. Nitrite and nitrate production to ammonium conversion ratio (η) was calculated according to a previous study [15], as listed in Eq. (1).

$$\eta = \frac{\left[NO_3^- - N\right]_{eff} - \left[NO_3^- - N\right]_{inf} + \left[NO_2^- - N\right]_{eff}}{\left[NH_4^+ - N\right]_{inf} - \left[NH_4^+ - N\right]_{eff} + \left[NO_2^- - N\right]_{inf}} \times 100\% \quad (1)$$

2.4. Microbial activities

The specific ammonium uptake rate was determined in 250 mL flasks with magnetic stirrer at 30°C ± 2°C. The sludge sample was collected from the operating SBR and washed with tap water, and then diluted with sodium phosphate buffer from 0.01 mol/L to 2 mg VSS/L. The initial ammonium concentration of 40 mg/L was added to the flasks. The DO concentration was controlled to beyond 2 mg/L by aeration. Samples were collected every 20 min to determine the residual ammonia concentration.

The specific Anammox activity was also determined in 250 mL flasks in a shaker at 30°C ± 2°C. The sludge was sampled from SBR and washed and diluted with tap water to 2 mg VSS/L. Ammonium of 30 mg/L and nitrite of 36 mg/L were added to the flasks. Then the flasks were purged with nitrogen for 3 min to create anaerobic condition. Samples were collected every 40 min to determine residual ammonia concentration.

2.5. Microbial communities

The microbial communities during the start-up of the PN/A process were analyzed using high-throughput sequencing to quantify the 16S-rDNA of the sludge. Three sludge samples on the days of 125, 173 and 235 (corresponding to the first phase, fourth phase, and fifth phase) were collected during SBR operation. According to the manufacturers' instructions, the DNA was isolated from sludge samples with an extraction kit (E.Z.N.ATM. Mag-Bind® Soil DNA Kit, OMEGA, USA) and DNA integrity was detected by Agarose gel electrophoresis. The primer pair for sequencing the V4 and V5 region of 16S-rDNA

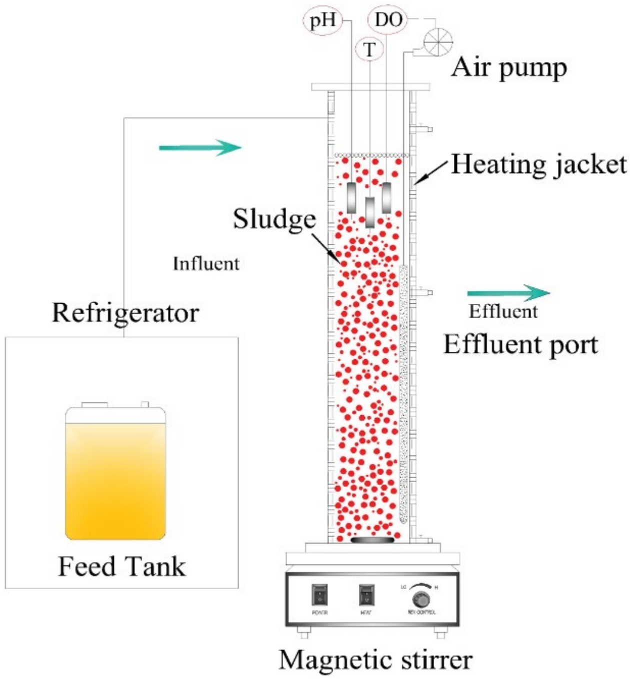

Fig. 1. Schematic diagram of sequencing batch reactor (SBR).

Table 2
Operating parameters of PN/A on different phases

Phase	Time (d)	Inf. NH_4^+–N (mg/L)	Inf. SCOD (mg/L)	COD/N ratio
I: Synthetic wastewater	0–125	300.00–450.00	–	–
II: 40% diluted raw piggery wastewater	126–149	285.12 ± 22.16	388.22 ± 20.45	1.31
III: 40% diluted digested piggery wastewater	149–173	287.22 ± 30.25	288.36 ± 16.50	0.99
IV: 70% diluted digested piggery wastewater	174–189	287.26 ± 19.50	393.54 ± 13.50	1.37
V: 100% digested piggery wastewater	190–237	306.27 ± 21.50	494.42 ± 23.50	1.61

Note: 50 mg/L of nitrite was added into the SBR on phase III–V (165th–194th day).

gene was 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (5′-GGACTACHVGGGTATCTAATCC-3′). To quantify the total 16S-rDNA, Qubit 3.0 DNA Detection Kit was used. Afterward polymerase chain reaction technology was used to amplify DNA. Then 16S-rDNA genes were sequenced on the Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA). All of these procedures were conducted by Sangon Biotechnology Incorporated Company (Shanghai, China).

3. Result and discussion

3.1. Acclimation of activated sludge in the PN/A process using synthetic wastewater

The acclimation of the PN/A process was composed of two steps the acclimations of partial nitrification process and the PN/A process. The acclimation of partial nitrification process was performed through intermittent aeration to control DO at low concentration because low DO concentration inhibited nitrite-oxidizing bacteria (NOB) activity and intermittent aeration suppressed NOB growth. As shown in Fig. 2a, in the beginning, less nitrite was accumulated in the reactor, and the nitrate concentration gradually increased and reached a maximum of 78.70 mg/L on day 10. However, the nitrite began to accumulate in the reactor from day 10 and continuously increased to 168.47 mg/L on day 37, while nitrate concentration decreased to 6.45 mg/L, and the ratio of NH_4^+/NO_2^- was around 1:1 in the effluent, confirming that intermittent aeration and low DO concentration successfully inhibited NOB activity and growth, and the partial nitrification process was achieved [16,17].

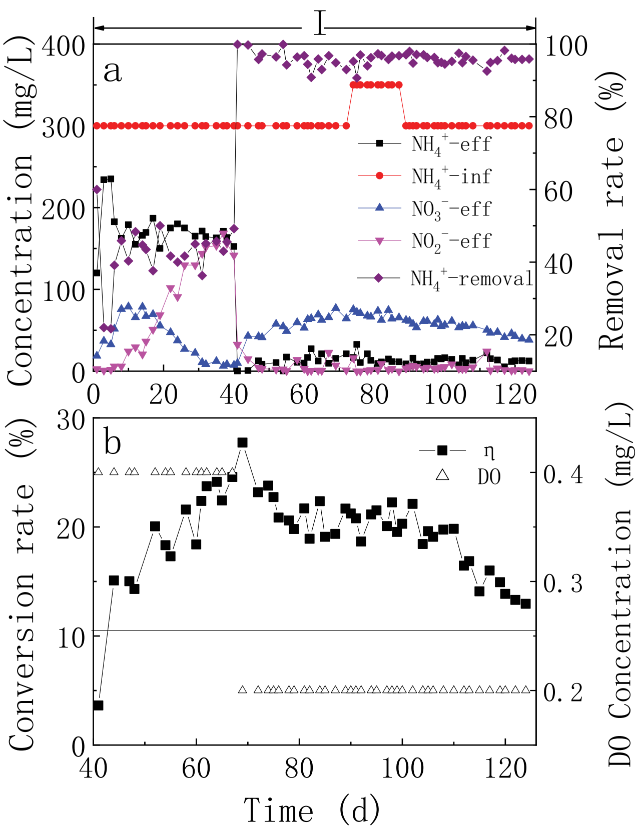

Fig. 2. (a) Profiles of nitrogen compounds in the influent and effluent, and nitrogen removal efficiencies and (b) temporal variation of nitrite and nitrate production to ammonium conversion efficiency (η) in the reactor.

On day 41, the reactor was inoculated with Anammox sludge to initiate the acclimation of the PN/A process. After being inoculated with Anammox sludge, ammonia removal efficiency remained around 98% and the value of η reached 20%, which was close to the theoretical value of 11%. However, the value of η gradually increased to 28% on day 69 (Fig. 2b), indicating that NOB activity was partly recovered, and nitrite was partially utilized by NOB to produce nitrate. Therefore, the suppression of NOB was very necessary. Because low DO could inhibit the growth of NOB, the DO value was decreased from 0.40 to 0.2 mg/L through controlling the aeration on day 69. As seen in Fig. 2b, the value of η was decreased to 14% on day 115, which was very close to the theoretical value of 11% and lasted for one week, indicating that the acclimation of the PN/A process was complete.

3.2. Start-up of PN/A process treating piggery wastewater

After the completion of the acclimation of PN/A sludge, the PN/A process treating piggery wastewater was started up on day 126 in phase II. In the beginning, the diluted piggery wastewater collected from a stabilization bond was used as the influent. The COD/N ratio of the diluted piggery wastewater was about 1.31. During the 126th–136th day, the PN/A process showed high removal efficiency of ammonia 98% and total nitrogen 84%. However, the ammonia and total nitrogen removal efficiencies gradually decreased to 78% and 52%, respectively on day 149. The activities of Anammox and ammonia oxidizing bacteria (AOB) were decreased from 47.89 and 108.23 mg NH_4^+–N/g VSS/d on day 125 to 25.81 mg NH_4^+–N/g VSS/d and 76.63 mg NH_4^+–N/g VSS/d on day 149 (Fig. 3e), respectively. These results showed that PN/A process was subjected to inhibition on phase II. The reason account for the deterioration of the PN/A system in phase II was explained that the presence of biodegradable organic matter in influent. Firstly, the organic matter could promote the growth of heterotrophic bacteria, which compete with AOB for oxygen and thus reduced AOB activity [11]. Additionally, the influent COD/N ratio was a key parameter to affect the performance PN/A process and a previous study revealed that a significant reduction of the nitrogen removal efficiency for treating piggery wastewater when influent COD/N ratio reached 1.24 [18]. Furthermore, the decline of AOB activity caused to its reduced oxygen depletion rate, and when oxygen supply exceeded the oxygen depletion rate of the AOB, residual oxygen more likely utilized by NOB, thus leading to facilitate recovery of NOB activity [16]. NOB would compete with Anammox for nitrite and further inhibit its activity.

To avoid further deterioration of PN/A system and facilitate start-up of PN/A process treating piggery wastewater, two measures would be implemented; firstly, piggery wastewater through anaerobic digestion to reduce biodegradable organic matter. That means the residual organic matter in piggery wastewater was difficult to biodegrade. Then, the influent COD/N ratio reduced to 0.99 was introduced to PN/A process on the phase III. Although these measures were implemented to restore the stability of the PN/A process, the ammonia and total nitrogen removal efficiency decreased to 73.03% and 50.08%, respectively on day 156. That meant the PN/A system was not well recovered at once. The declined AOB activity might cause insufficient nitrite produced in PN/A reactor, therefore influent was added 50 mg/L nitrite on day 165 which can promote the growth of Anammox bacteria [12]. By the way, due to the digested piggery wastewater contained less nitrite, the addition of nitrite was terminated on day 194. As can be seen from Fig. 3a, after this approach had been implemented, the ammonium removal performance was improved within one week. The ammonia and total nitrogen removal efficacy increased to 90.58% and 70.74% on day 173, respectively.

To investigate the feasibility of starting PN/A process treating digested piggery wastewater, the digested piggery wastewater with the gradient dilution was fed into the reactor, corresponding to gradually increasing the ratio of COD/N to 1.37 and 1.61 on the phase IV (173^{th}–189^{th}) and V (190^{th}–237^{th}), respectively. On phase IV and V, the ammonia and total nitrogen removal efficiency were above 90% and 70%, respectively. Compared on day 180, the Anammox activity slightly decreased to 32.01 mg NH_4^+–N/g VSS/d and AOB activity slightly increased to 93.36 mg NH_4^+–N/g VSS/d on day 235, as shown in Fig. 3e. Nevertheless, the average value of η (14%) was much closer to the theoretical value, which indicated the dominant route for ammonia removal was mainly through PN/A process. Furthermore, the performance of ammonia, nitrite, nitrate, and COD in a typical cycle in phase V proved that the PN/A process achieved a

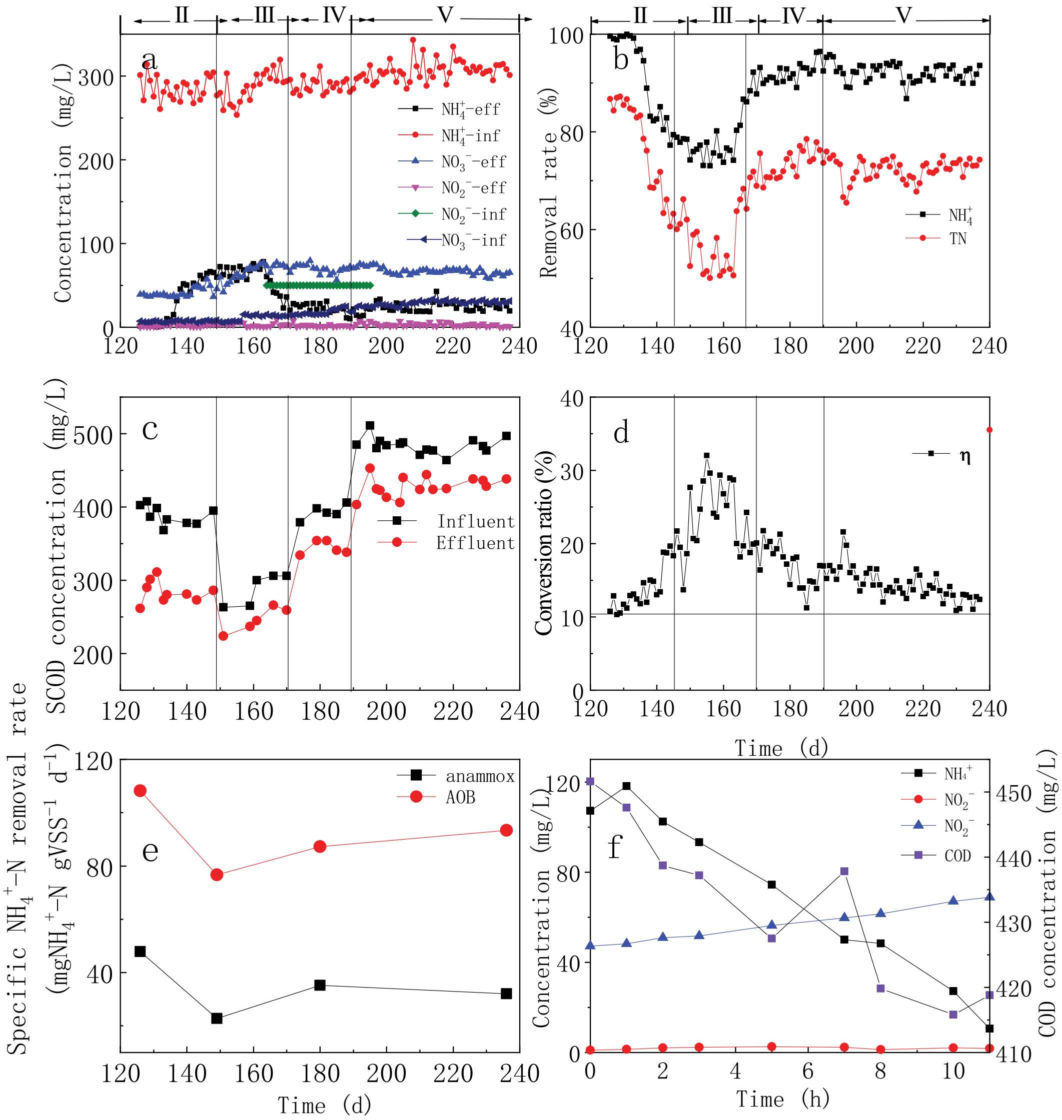

Fig. 3. (a) Profiles of nitrogen compounds in influent and effluent in the reactor, (b) total nitrogen and nitrogen removal efficiencies in the reactor, (c) profiles of COD in influent and effluent in the reactor, (d) temporal variation of nitrite and nitrate production to ammonium conversion efficiency (η) in the reactor, (e) profiles of microbial activity test parameters obtained in different phases, and (f) concentrations of ammonia, nitrite, nitrate, and COD in a typical cycle.

good performance treating digested piggery wastewater (Fig. 3d). These results shown that the influent COD/N ratio increased from 0.99 to 1.67 did not affect reactor performance. And after a period of domestication of the microbial community, the PN/A system had been successfully started. However, its results were significantly different from phase II (COD/N ratio 1.31). The previous study had pointed that organic carbon was not as an inhibitor to Anammox bacteria and when organic matter was utilized by heterotrophic bacteria could prevent Anammox bacteria uptake of inorganic carbon, thus the growth potential of Anammox bacteria would be decreased significantly [19]. Otherwise, the ratio of organic matter in terms of COD removed ($COD_{removed}$) by the PN/A reactor and ammonia ($COD_{removed}$/N) was also an important parameter on PN/A process [20]. In phase II, the $COD_{removed}$/N ratio was averagely closed to 0.38 which indicated the diluted piggery wastewater contained much biodegradable organic matter and affected the performance of the reactor. So it was necessary to further degrade organic matter. On phase III–V, the value of the $COD_{removed}$/N ratio decreased to 0.17 averagely and the performance of the PN/A process was recovered. As previously reported in the literature, this $COD_{removed}$/N ratio in our study did not negatively effect either AOB or Anammox bacteria [7,20–22]. The deterioration of PN/A process on phase II could be attributed to longer exposure to biodegradable organic matter which effected the growth of Anammox. When diluted digested piggery wastewater was introduced into PN/A process on the phase III–V, refractory organics did not affect the growth and activity of Anammox. That could be explained the reason why the PN/A process could be started successfully treating digested piggery wastewater with the same or beyond COD/N ratio compared to raw piggery wastewater. Which indicated even if the influent with high COD/N ratio, the PN/A system still could maintain good nitrogen removal performance as long as the low $COD_{removed}$/N ratio.

3.3. Microbial community evolution in PN/A system

To investigate the microbial community evolution during the start-up of PN/A process treating piggery wastewater, the sludge samples in different phases on day 125, 173, and 235 for the high-throughput sequencing analysis. The results of phylogenetic classification confirmed the presence of AOB, Anammox bacteria and denitrifiers. The Candidatus Brocadiacese was the major Anammox bacteria species in the system, as shown in Fig. 4a. The genus Candidatus Anammoxoglobus and Candidatus Brocadia belonged to Candidatus Brocadiacese. The abundance of Candidatus Brocadiacese decreased from 8.61% on day 125 to about 4.56% on day 173 and to 3.41% on day 235. A similar study also found a decrease abundance of Anammox bacteria due to piggery wastewater introduced into PN/A reactor when increasing the COD/N ratio from 0.6 to 1.24 [18]. It confirmed that organic matter had a negative effect on the growth of Anammox bacteria. Previous studies found that Candidatus Brocadia shown a higher potential growth rate than other Anammox bacteria in the presence of acetate and Candidatus Anammoxoglobus could out-compete other Anammox bacteria in the presence of propionate [23,24]. The abundance of Candidatus Anammoxoglobus and Candidatus Brocadia varies in PN/A process might depend on the types of organic matter in digested piggery wastewater. The Candidatus Anammoxoglobus decreased to 2.64% on day 173 compared to day 125 (6.88%) and remained 2.55% on day 235. That indicated the abundance of Candidatus Anammoxoglobus had

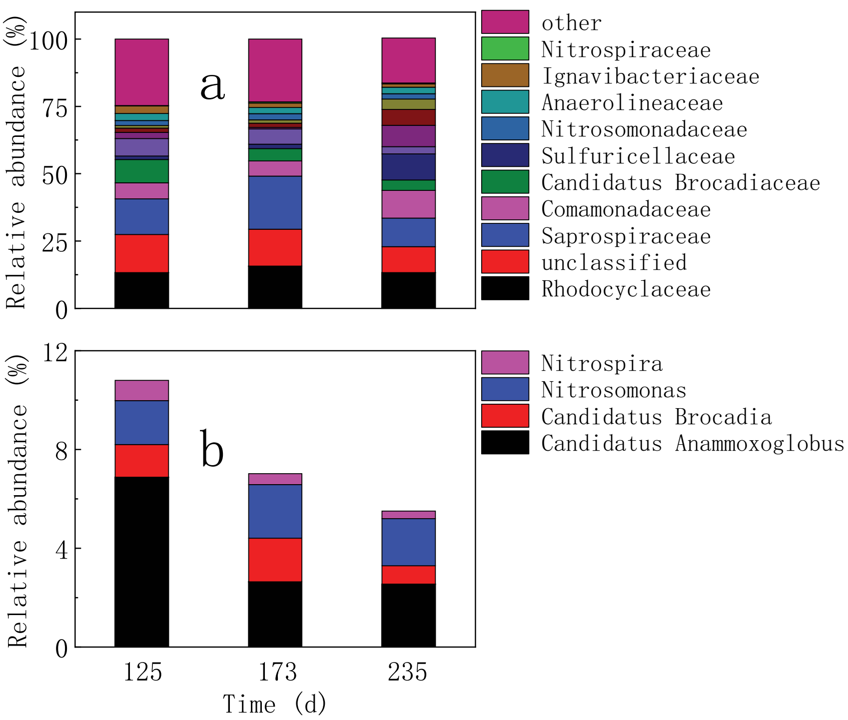

Fig. 4. Profiles of microbial community evolution obtained in different phases (a) family and (b) genus.

no change when the piggery wastewater at a high COD/N ratio introduced to PN/A process and Anammox bacteria had adapted to the presence of organic matter. However, the decreased abundance of Anammox bacteria was consistent with the decline of Anammox activity in the PN/A system. Furthermore, the dominant route for ammonia removal was mainly through PN/A process though the Anammox abundance was much lower than the heterotrophic bacteria. Similar results were also reported that the high efficiency of the ammonia removal rate was achieved through the dominant Anammox species (Candidatus Brocadiacese) with low proportion [25,26].

Additionally, Nitrosomonadaceae bacteria remained a stable proportion (approximate 2%) in PN/A reactor all the time (Fig. 4b). Even if the activity of AOB was reduced on day 149 and 180 compared to day 125, it had no significant effect on its abundance. Moreover, the abundance of Nitrospira decreased from 0.85% on day 125 to 0.32% on day 235. Nitrospira was considered to threaten the stability of PN/A system by competing with Anammox bacteria for nitrite and AOB for DO. Limited amount of DO and intermittent aeration could effectively restrain the growth of Nitrospira. That could be explained that value of η was much closed to the theoretical value (11%). Notably, as shown Fig. 4a, the family Comamonadaceae slightly increased from 6.01% on day 125 to 10.33% on day 235. Comamonadaceae frequently detected in many wastewater treatment plants, which reported to be responsible for denitrifying processes [27].

However, organic carbon was required for denitrifying bacteria as energy sources, but denitrifying bacteria was detected even if no organic matter was supplemented on phase I (0–125 days). Therefore, the carbon source of Comamonadaceae might be from cellular compounds released from the endogenous metabolism of microorganisms [28]. Importantly, due to the limitation of biodegradable organic carbon, the abundance of Comamonadaceae remained relatively stable ratio in PN/A system.

4. Conclusion

In this study, it was demonstrated that PN/A process in a single reactor was started for nitrogen removal from piggery wastewater. The presence of biodegradable organic matter would favor the growth of heterotrophic bacteria and threaten Anammox bacteria to survive. To facilitate the start-up of the PN/A process, effective anaerobic digestion to reduce influent biodegradable organic carbon was necessary. The 16S-rDNA gene technology results showed that when digested piggery wastewater which mainly consisted of refractory organics as effluent introduced into PN/A reactor, the growth of heterotroph would be suppressed which thus resulted in stability the abundance of AOB and Anammox bacteria. Our study showed that the PN/A process was a promising alternative way to remove nitrogen of piggery wastewater.

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