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Fast startup of one-stage nitritation-ANAMMOX reactor for high-rate nitrogen removal from mature landfill leachate

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ABSTRACT

Fast startup of one-stage partial nitritation (PN)-anaerobic ammonium oxidation (ANAMMOX) process for the treatment of nitrogenous wastewater is still a challenge that remains to be solved. In this study, rapid establishment of one-stage PN-ANAMMOX column reactor inoculated with the mixture of dispersed biomass from a partial nitritation (PN) reactor and granular biomass from an ANAMMOX reactor was investigated. Excellent reactor performance was shown during adaptation first to synthetic inorganic wastewater and thereafter to mature landfill leachate. Combining online pH and DO monitoring for ensuring a stable pH level and oxygen limitation was the key to maintaining stable reactor performance. Nitrogen removal up to 1.7 kg N m^{-3} d^{-1} from raw mature landfill leachate was eventually obtained, indicating the effectiveness in the operational strategy on high rate and efficient nitrogen removal from landfill leachate via one-stage PN-ANAMMOX process. Nitrogen mass balance analysis indicates that ANAMMOX and heterotrophic denitrification contributed to 78.7% and 20.7% of the nitrogen removal, respectively.

Keywords: Partial nitritation; ANAMMOX; Landfill leachate; Fast startup; Nitrogen removal; Denitrification

1. Introduction

Mature landfill leachate is a highly nitrogenous wastewater with a low carbon/nitrogen (C/N) ratio, which requires high cost for nitrogen removal using conventional nitrification and denitrification process [1–4]. In the last decade, the development of the combined partial nitritation (PN) and anaerobic ammonium oxidation (ANAMMOX) process [5,6], either in two-stage or in single reactor system, provides a novel and cost-effective alternative to mature landfill leachate treatment [2,7,8]. The PN-ANAMMOX process features substantial reduction in oxygen demand and no need of external organic carbon source, and has attracted a great deal of attention of environmental researchers [9,10].

It has been pointed out that the one-stage ANAMMOX system is more compact and flexible than the two-stage

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one [11]. Moreover, it has been shown that the one-stage PN-ANAMMOX system is feasible to treat some real nitrogenous wastewater, such as sludge digester liquid [12], swine wastewater [13] and landfill leachate [7] with a low C/N ratio. However, due to the slow growth rate of both ammonium oxidizing bacteria (AOB) and ANAMMOX bacteria (AnAOB), it usually requires several months or more to establish a one-stage PN-ANAMMOX process [14]. Therefore, the technological strategy for a fast startup of the one-stage ANAMMOX reactor should be investigated.

Currently, to our knowledge, there have been several strategies for startup of one-stage PN-ANAMMOX reactor. The first involved the use of both nitrifying biomass and ANAMMOX sludge to establish the one-stage PN-ANAMMOX system [15]. A second strategy that was proven feasible had the inoculation of ANAMMOX biomass for the reactor started initially as an ANAMMOX reactor and then to a one-stage PN-ANAMMOX reactor [16–18]. In a third strategy, an alternative was the inoculation of activated sludge for the up-flow biofilm reactor, which started initially as a PN reactor and then to a one-stage PN-ANAMMOX reactor [16]. Another strategy is the use of conventional activated sludge in a sequencing batch reactor (SBR) which requires 4 months for startup [14]. The above-mentioned results show that the inoculation of ANAMMOX sludge can lead to a faster startup, however, a proper technological control strategy is also essential to establish the one-stage PN-ANAMMOX process.

Theoretically, the one-stage PN-ANAMMOX process entails the proper cooperation of AOB at the outer layer of the granules or biofilm and the AnAOB at the inner layer, thereby the AOB partially oxidize ammonia to nitrite while the AnAOB consume the resulted ammonia and nitrite to form nitrogen gas [17,19,20]. Therefore, by adding the scattered/dispersed AOB and granular ANAMMOX biomass at the same time could very likely trigger a rapid establishment of the aforementioned typical spatial distribution for one-stage PN-ANAMMOX granules, thereby resulting in a fast reactor startup. Moreover, a decreasing small amount of nitrite in the feed [21] could possibly provide an adaption of AnAOB to oxygen limited conditions during the initial startup period.

Therefore, in this study, fast startup of the one-stage ANAMMOX reactor was conducted by inoculating a mixture of dispersed AOB cells from a PN-SBR and granular ANAMMOX biomass from an ANAMMOX reactor and by adding a decreasingly small amount of nitrite in the feed. The adaption to mature landfill leachate after startup from synthetic inorganic wastewater was also carried out by stepwise increments in the percentage of raw leachate in the feed and by the aid of online pH and dissolved oxygen (DO) monitoring. Furthermore, the quantitative PCR (qPCR) was conducted to provide insight into the dynamics of bacterial community and nitrogen removal rate (NRR).

2. Materials and methods

2.1. Wastewater characteristics

During Phase 1 (days 1–53 d), the reactor was fed with synthetic inorganic wastewater which had characteristics as listed in Table 1. The influent also contained other mineral mediums consisting of $NaHCO_3$, $CaCl_2$, $MgSO_4$ and KH_2PO_4, and trace element mediums as described elsewhere [22]. It is noted that, during the initial 44 d, a small amount of NO_2^-–N from 50 mg L^{-1} to zero was added, aiming to adapt the AnAOB to oxygen limitation.

Table 1
Characteristics of the experimental wastewater

Parameter	Phase 1	Phase 2
NH_4^+–N (mg L^{-1})	500–550	550→1,300
NO_2^-–N (mg L^{-1})	50→0	–
TN (mg L^{-1})	550	550–1,300
COD (mg L^{-1})	–	100–1,000
BOD_5 (mg L^{-1})	–	20–150

During Phase 2 (days 54–118 d), the reactor was fed with landfill leachate, and the percentage of leachate in the influent was progressively increased until 100% of raw landfill leachate was reached. The characteristics of raw leachate was as follows, pH 8.01–8.45, alkalinity 2,000–2,500 mg $CaCO_3$ L^{-1}, COD 850–1,500 mg L^{-1}, BOD_5 50–150 mg L^{-1}, NH_4^+–N 1,000–1,200 mg L^{-1}, TN 1,150–1,250 mg L^{-1}, NO_2^-–N < 1 mg L^{-1} and NO_3^-– N < 20 mg L^{-1}.

2.2. Experimental setup

A lab-scale column reactor with a working volume of 2.4 L and a height to diameter ratio of around 7 was used in this study. The influent was continuously fed from the bottom of the reactor, then mixed with the biomass in the reactor and filtered by a stainless steel sieve (pore size of 0.15 mm) at the outlet of the reactor. The effluent was further clarified in the settler, and the settled sludge was manually recycled to the reactor on a daily basis. Reaction temperature was controlled at 28°C ± 1°C, aeration was conducted by an aquarium air pump and a fine bubble stone. Online pH and DO meters were used to continuously monitor the pH and DO of the mixed liquor, respectively (Fig. 1). The pH of the mixed liquor was controlled at 7.55–7.60 by dosing bicarbonate buffer ($NaHCO_3$, 1 mol L^{-1}), and the DO was controlled at 0–0.3 mg L^{-1} by adjusting the air flow rate (AFR).

2.3. Inoculum and startup procedure

Activated sludge (around 2 g VSS) from a lab-scale PN-SBR [23,24] and granular sludge (around 5 g VSS) from an ANAMMOX reactor [25] were used as inocula. AOB species of *Nitrosomonas* sp. IWT514 (JX898090, 99%), *Nitrosomonas eutropha* (CP000450, 96%) and *Nitrosomonas eutropha* (NR_027566, 96%) were detected in the biomass from the PN-SBR [24], while the dominant AnAOB species of *Kuenenia stuttgartiensis* (CT573071, 100%) was detected in the biomass from the ANAMMOX-UASB [24]. Initially, the reactor was operated with relatively high HRT and low DO conditions. After the observation of improvement in nitrogen removal, the HRT was decreased in a stepwise manner and the AFR was progressively increased to elevate the treatment capacity. From day 53, the reactor was fed with diluted landfill leachate, and the percentage of raw leachate in the feed was increased until 100% of raw leachate was fed.

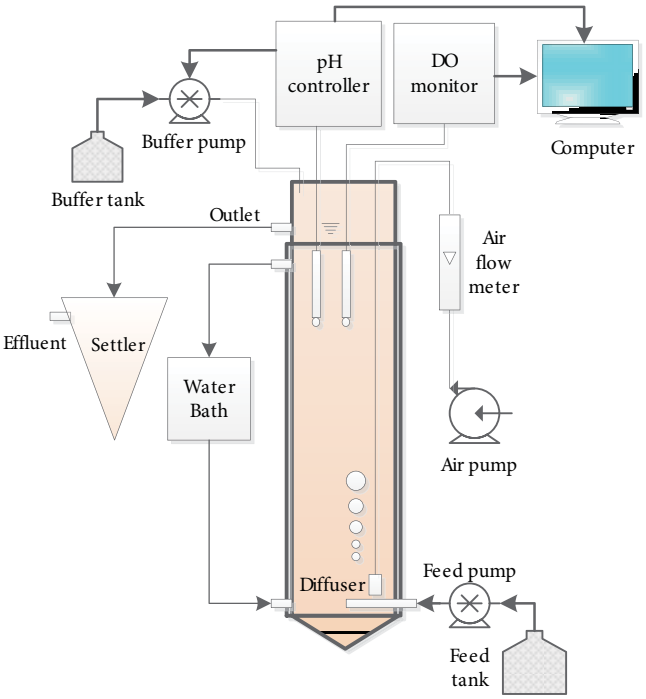

Fig. 1. Schematic diagram of the one-stage PN-ANAMMOX system.

2.4. Analytical methods

Measurements of COD, ammonium, nitrate, nitrite and alkalinity were carried out according to standard methods [26]. The COD values contributed by organics were corrected according to the fact that nitrite exerts a COD of 1.1 mg O_2 mg^{-1} NO_2^-–N. Total inorganic nitrogen (sum of the NH_4^+–N, NO_2^-–N and NO_3^-–N) was calculated to assess the nitrogen removal performance.

For morphological observation, the granules obtained from the reactor were screened by a stainless steel sieve with pore size of 1.00 mm and then were selected for appearance observation by digital camera (CANON, 500D, Japan) and scanning electron microscopy (SEM, ZEISS, Germany). The pre-treatment of biomass samples was carried out by modification of the methods described elsewhere [27]. In brief, the collected granules were prepared for eventual SEM observation by paraformaldehyde fixation, phosphate buffer washing, ethanol dehydration, lyophilization and gold coating.

DNA extraction of biomass from the one-stage PN-ANAMMOX reactor was carried out by PowerSoil® DNA Isolation Kit (MOBIO, USA), following the manufacturer's instructions. qPCR was conducted to quantify the related three groups of functional bacteria, including AOB, nitrite oxidizing bacteria (NOB) and AnAOB. The primers used and the related literature are listed in Table 2, the related qPCR conditions are the same to a previous study [24]. In brief, the qPCR reagents were as follows: 2 × TransStartTM qPCR SuperMix 12.5 μL; primer F/R (5 μM) 1 μL; passive reference dye 0.5 μL; DNA 2 μL; dd H_2O 9 μL. PCR sequences were as follows: 95°C pre-denaturation 4 min; 95°C denaturation 30 s, anneal (EUB 57°C, AOB 55°C, *Nitrobacter* 50°C, ANAMMOX 65°C) 30 s, 72°C extension 30 s, 30 cycles; final extension 72°C for 10 min.

3. Results and discussion

3.1. Fast startup of one-stage ANAMMOX reactor fed with synthetic inorganic wastewater

During Phase 1, the reactor was operated with a relatively long HRT (36–40 h) under oxygen limitation (DO below 0.3 mg L^{-1}). A decreasing addition of NO_2^-–N in the feed from 50 mg L^{-1} on day 1 to almost zero on day 44 was conducted to adapt the AOB and AnAOB under aeration condition. As expected, a decrease in NH_4^+–N and NO_2^-–N concentrations and a slight increase in NO_3^-–N concentration in the effluent (Fig. 2(a)) along with the increase in nitrogen removal (Figs. 2(b) and (c)) were observed, indicating a prompt establishment of synergism between AOB and AnAOB. Ammonium removal can be gradually improved upon the increase in AFR (Figs. 2(b) and (d)), meantime maintaining a low DO condition (DO < 0.3 mg L^{-1}). This implies that the increase in air supply was balanced by the increased consumption of oxygen due to enhanced nitritation via AOB and simultaneous removal of nitrogen via AnAOB. Therefore, it is important to keep the reactor at the oxygen availability rate limiting condition for suppressing the NOB [34]. Despite there are controversial opinions over the DO control of the one-stage ANAMMOX process, controlling low DO in the reactor is usually recommended [10,35,36].

Table 2
Primers used in the qPCR in this study

Primers	Sequence	Target	Literature
Pla46F	GGATTAGGCATGCAAGTC	AnAOB	[28,29]
Amx667R	ACCAGAAGTTCCACTCTC		
CTO189fA/B	GGAGRAAAGCAGGGGATCG	AOB	[30,31]
CTO189fC	GGAGGAAAGTAGGGGATCG		
RT1r	CGTCCTCTCAGACCARCTACTG		
FGPS872-f	CTAAAACTCAAAGGAATTGA	NOB (Nitrobacter)	[30,32]
FGPS1269-r	TTTTTTGAGATTTGCTAG		
121f	AGGAAGGTGGGGATGAC	EUB	[33]
238r	CGGCTTTCTGGGATTGG		

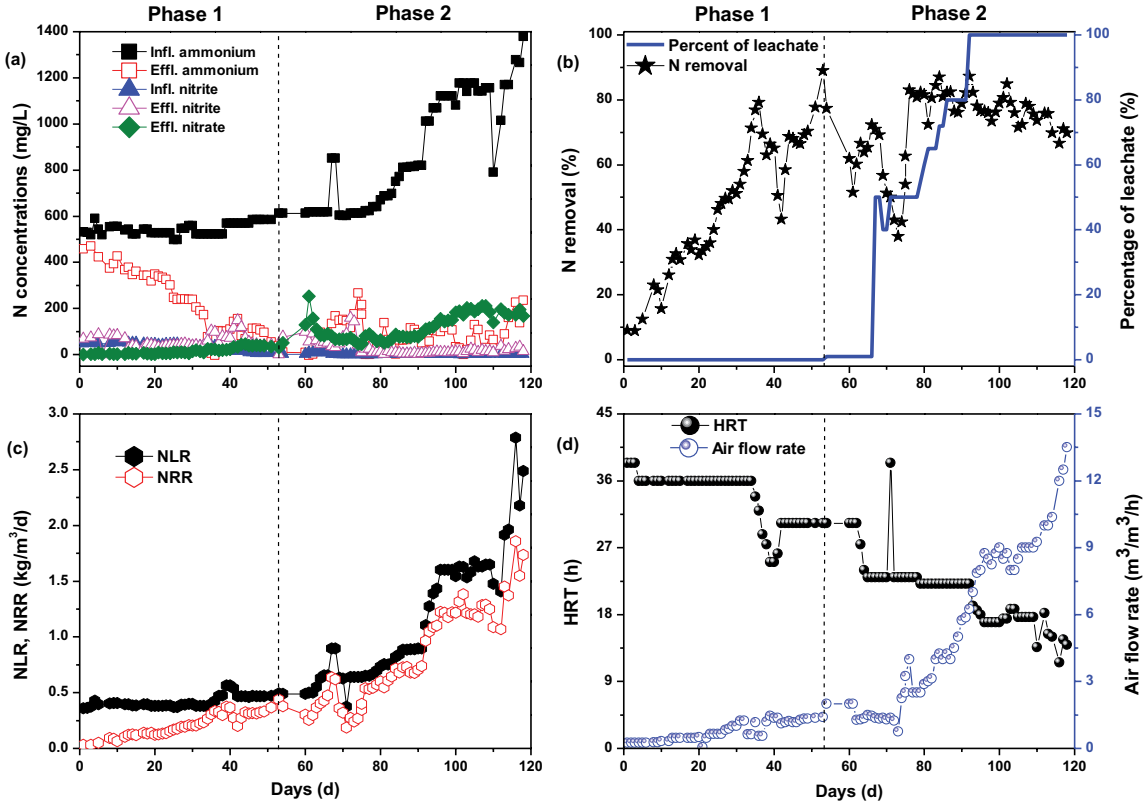

Fig. 2. N removal performance of the one-stage PN-ANAMMOX reactor: (a) N concentrations in the influent and the effluent, (b) N removal efficiency and percentage of raw landfill leachate in the feed, (c) NLR and NRR, and (d) HRT and air flow rate.

Winkler et al. [21,37] suggested that adding decreased amount of nitrite in the feed was effective to establish a one-stage PN-ANAMMOX process initiated with ANAMMOX granules. In our study, the reactor was inoculated with dispersed AOB and granular AnAOB biomass, and operated with nitrite during the initial startup period. This strategy is shown to be successful to trigger a fast establishment of one-stage PN-ANAMMOX process without a pre-culture step that uses the operational condition similar to the ANAMMOX reactor. The oxygen limitation may impair the AOB activity in the initial startup period, thereby may affect the formation of nitrite that should penetrate the granular outer to reach the ANAMMOX zone. The addition of nitrite in the feed can directly provide NO_2^-–N and NH_4^+–N for the AnAOB in the anoxic zone of the granular sludge, facilitating the establishment of one-stage PN-ANAMMOX process.

On day 33, a failure in the feeding pump occurred at night, resulting in a temporary stop of feeding for 8–12 h. Interestingly, the performance measurement in the following day showed that NH_4^+–N was depleted and effluent NO_2^-–N concentration increased but NO_3^-–N remained unchanged (Fig. 2(a)). Upon the depletion of NH_4^+–N in the mixed liquor, the DO concentration increased to over 4 mg L^{-1}, and later the pH also increased to around 8 due to excess aeration and thus CO_2 stripping (Fig. 3(a)). Temporary excess aeration only led to accumulation of NO_2^-–N but not NO_3^-–N, this suggests that NOB was severely inhibited. After replacing with a new pump at 14:25 (hh:mm), the DO and pH of the mixed liquid decreased quickly and recovered to normal conditions within 2 h. Interestingly, the sensitivity of the DO profile is greater than that of the pH profile, while a combination of both led to a clear diagnosis of the state of the reactor, ensuring a timely alert and then a remediation of the operational problem. The accumulation of nitrite was eliminated after around 10 d, suggesting again that the nitrite accumulation is a tricky problem in the one-stage PN-ANAMMOX process [34]. On day 53, the NRR and nitrogen removal efficiency (NRE) reached 0.43 kg N m^{-3} d^{-1} and 89%, respectively, which are better than those reported by Cho et al. [16] using other operational strategies for the one-stage PN-ANAMMOX reactor, indicating a successful startup in this study.

3.2. Adaptation to mature landfill leachate

During Phase 2, a variation in AFR due to an excess aeration (2 m^3 m^{-3} h^{-1}, Fig. 2(d)) during the vacation period (days 54–59) resulted in a DO concentration higher than 7 mg L^{-1} (Fig. 3(b)), which could cause severe inhibition to AnAOB and thus activation of NOB [34]. The following performance measurement did show a depletion of NH_4^+–N but a simultaneous accumulation of NO_2^-–N (55 mg L^{-1}) and NO_3^-–N (254 mg L^{-1}) in the effluent (Fig. 2(a)), resulting in a significant decrease in nitrogen removal (Figs. 2(b) and (c)). To avoid the excess aeration and thus to inhibit NOB activity, therefore, the AFR was decreased to 1.3 m^3 m^{-3} h^{-1} at which the DO of the mixed liquid was maintained below 0.3 mg L^{-1} during the following few days (days 62–68). Consequently, the nitrogen removal progressively improved along with the decrease in

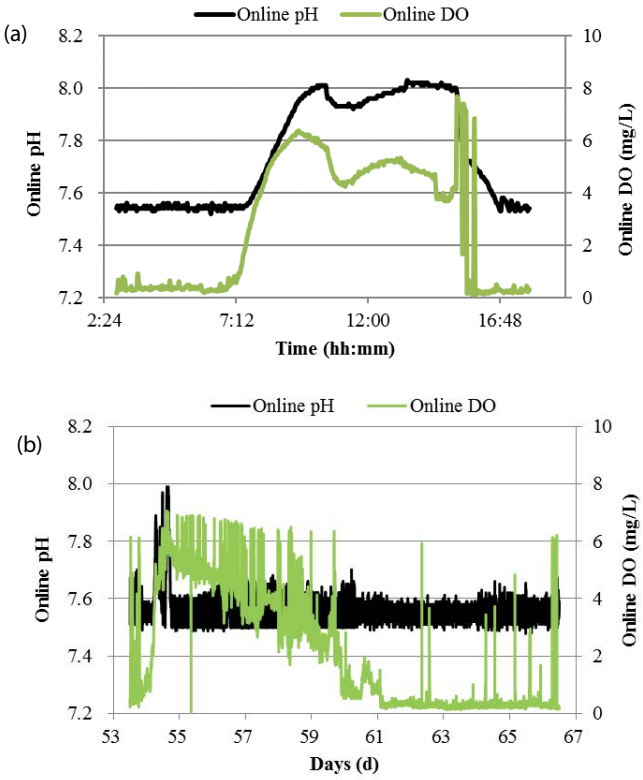

Fig. 3. Online pH and DO profiles during the period of (a) the pump failure on day 33 and (b) excess aeration during days 53–66.

effluent NO_3^-–N concentration (Figs. 2(b) and (c)). It is shown that once the excess oxygen problem is eliminated, the activity of AnAOB can be recovered immediately even after over-aeration for several days. The significantly higher affinity for oxygen of AOB compared with NOB (half-saturation constant of 0.6 compared with 2.2 mg O_2 L^{-1}) may be responsible for this competition [34,38]. It should be noted that in this study, the oxygen inhibition on AnAOB is reversible for a long exposure of high DO over five consecutive days. This interesting observation suggests that close monitoring and the operational strategy on balancing the oxygen supply and NH_4^+–N loading rate are essential to improve the stability of the one-stage PN-ANAMMOX reactor.

Thereafter, the percentage of leachate in the feed was further increased (Fig. 2(b)). It is noted that on day 73 the bubble stone was found to float to the middle height of the reactor, causing a settling of the granules to the bottom of reactor thus leading to a decline in nitrogen removal performance during the prior days (days 69–73). After reinserting the bubble stone to the bottom of the reactor, the red granules were re-suspended and re-mixed in the reactor. As a result, the nitrogen removal performance was quickly improved (Figs. 2(a)–(c)). Afterwards, to enhance the treatment capacity, the percentage of leachate in the feed (Fig. 2(b)) was further increased and the hydraulic retention time (Fig. 2(d)) was progressively decreased. Correspondingly, the AFR was increased to provide enough oxygen for ammonium oxidation to nitrite but not to an excess extent that the AnAOB would be inhibited. On day 101, under the conditions of nitrogen loading rate (NLR) of 1.6 kg N m^{-3} d^{-1}, AFR of 8.8 m^3 m^{-3} h^{-1} and HRT of 18 h, the NRR and NRE reached 1.3 kg N m^{-3} d^{-1} and 81%, respectively, which is the highest nitrogen removal for raw landfill leachate treatment using the ANAMMOX process. Upon further increasing the NLR, the NRR kept increasing to 1.7 kg N m^{-3} d^{-1} on day 118, however, the NRE slightly decreased to around 70%. Therefore, further increase in NLR over 1.6 kg N m^{-3} d^{-1} could increase the risk of instability of the one-stage ANAMMOX reactor for landfill leachate treatment. An NLR lower than 1.6 kg N m^{-3} d^{-1} is suggested for nitrogen removal from landfill leachate via one-stage PN-ANAMMOX process.

During Phase 2, the influent COD concentration increased from 200 mg L^{-1} on day 65 to 1,120 mg L^{-1} on day 118 (Fig. 4) due to the higher stepwise increase in percentage of leachate in the feed. Because of the poor biodegradability in landfill leachate, COD removal efficiency was only at the range of 15%–40%, and the effluent COD concentration was found to increase along with the increase in influent COD concentration (Fig. 4). On day 118, the COD removal rate and efficiency were 0.79 kg COD m^{-3} d^{-1} and 39%, respectively. It should be noted that the high COD concentration of the mixed liquid up to 1,100 mg L^{-1} did not impair the efficient nitrogen removal in this one-stage PN-ANAMMOX reactor. This is because that the major organic matter in the biologically treated leachate is aquatic humic substance [39,40], which is highly biologically inert and may not inhibit the AnAOB. Wang et al. [7] reported the successful one-stage PN-ANAMMOX treatment of mature landfill leachate with an NRE of 76% and a calculated NRR of 0.38 kg N m^{-3} d^{-1} under a low influent COD concentration of 554 mg L^{-1}. In our study, the one-stage PN-ANAMMOX process is also demonstrated feasible to remove the nitrogen at a very high loading rate from the mature landfill leachate with a COD concentration as high as 1,100 mg L^{-1} and a C/N ratio of around 0.9.

3.3. Appearance and structure of the one-stage ANAMMOX granules

The biomass on day 102 was collected for morphological observation. Because the AOB and AnAOB are

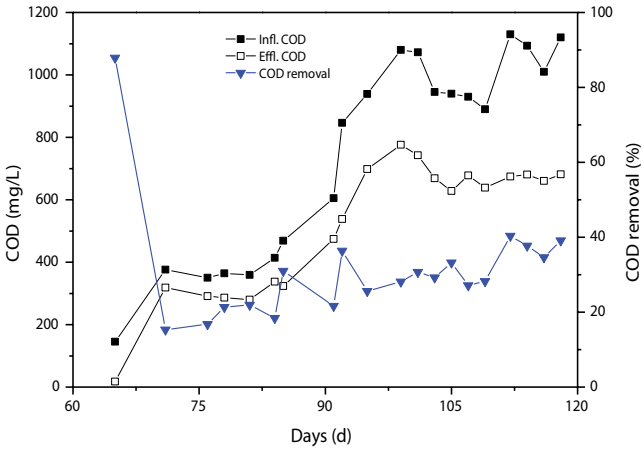

Fig. 4. COD removal of the one-stage PN-ANAMMOX reactor during the experimental period.

both red in color, the obtained granules in the one-stage PN-ANAMMOX reactor were also reddish in color (Fig. 5(a)). The one-stage PN-ANAMMOX granules were compact and irregular in shape (Fig. 5(a)). The SEM image demonstrates that there was a large amount of coccus-like and rod-shaped bacteria distributed compactly on the surface of the granules (Figs. 5(c) and (d)). The AnAOB and *Nitrosococcus*-like AOB are coccus-like bacteria, while most of the *Nitrosomonas*-like AOB are rod-shaped [41,42]. Interestingly, there were also some cavities, seemingly the passages for gas released by AnAOB in the granules (Fig. 5(b)). In addition, there were some mesh-like extracellular polymeric substances [43] for combination of the different groups of bacteria to form the granules (Fig. 5(d)).

3.4. Microbial populations

In order to examine the dynamics of the bacterial community of the sludge in the one-stage PN-ANAMMOX reactor, biomass samples from days 23, 45, 51, 66, and 91 were collected and quantified by qPCR (Table 3). Three groups of bacteria including AOB, NOB and AnAOB were analyzed. It is shown that the abundance of total bacteria was increasing in the same order of magnitude overall during the operation of the reactor. The abundance of AOB was also increased (except on day 51) along with the increase in NRR, but still proliferated with the same order of magnitude. The abundance of AnAOB was observed in a random trend regardless of the increase in NRR. The biomass-based ANAMMOX activity of the mixed granules was not intentionally measured in this study. The volumetric nitrogen loading (Fig. 2(c)) clearly indicates the substantial increase in volumetric activity of AnAOB, which may reveal that the improvement in nitrogen removal is due to the increase in activity of AnAOB rather than the abundance. In terms of the percentage of the three groups of functional bacteria out of total bacteria, AOB only accounted for 1%–3% of the total bacteria, while far less of AnAOB (<0.3%) were detected in the qPCR analysis. No *Nitrobacter* were detected, however, the over-aeration did cause a temporary activation of NOB activity during days 54–58. This reveals that other types of NOB may exist but are suppressed in the reactor subject to

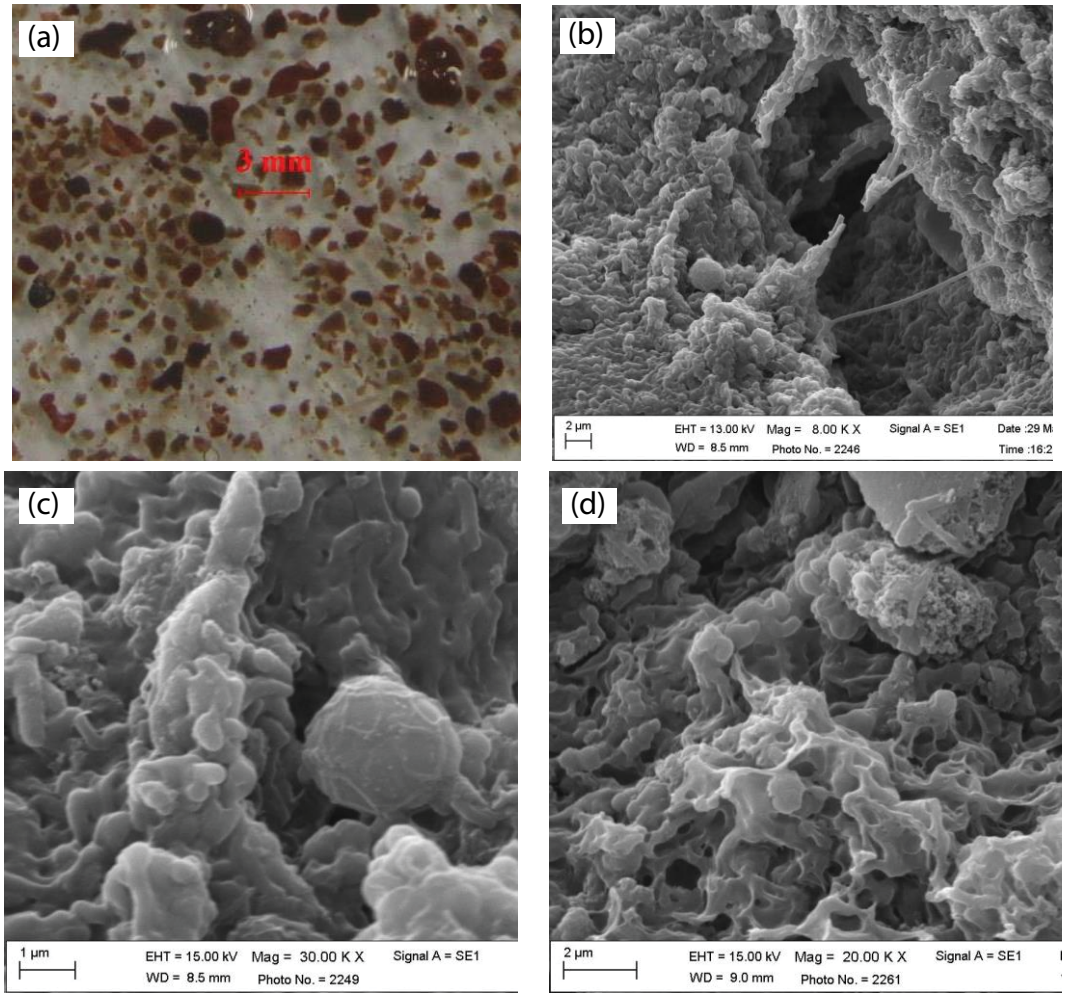

Fig. 5. Appearance of the granules in the one-stage PN-ANAMMOX reactor: (a) image of the granules collected in the petri dish, the marker length is 3 mm, (b) SEM image of the granules, the marker length is 2 μm, (c) enlarged SEM image of the small granules, the marker length is 1 μm, and (d) SEM image of the large granules, the marker length is 2 μm.

Table 3
qPCR analysis of the bacteria in the one-stage PN-ANAMMOX reactor during the operating period

Parameter		EUB	AOB	NOB	AnAOB
Abundance	Day 23	4.40E+10	4.47E+08	n.d[a].	1.30E+08
(copies/g VSS)	Day 45	2.86E+10	5.16E+08	n.d.	2.85E+06
	Day 51	8.70E+10	2.77E+09	n.d.	3.95E+07
	Day 66	9.12E+10	9.76E+08	n.d.	2.89E+06
	Day 95	9.70 E+10	9.92E+08	n.d.	1.5 E+07
Percentage (%)	Day 23	100	1.02	n.d.	0.295
	Day 45	100	1.80	n.d.	0.010
	Day 51	100	3.18	n.d.	0.045
	Day 66	100	1.07	n.d.	0.003
	Day 95	100	1.02	n.d.	0.015

[a]Not detected.

oxygen limitation. Therefore, this indicates that in a bioreactor treating a complex real wastewater, high bacterial diversity is usually observed, which is in line with other studies [41,44].

3.5. Nitrogen balance analysis

Nitrogen balance analysis based on the results of reactor operation on day 101 was conducted and is illustrated in Table 4. The nitrogen conversion due to ammonia stripping and physiochemical adsorption was relatively insignificant [45]. The organic nitrogen conversion was also not considered, because the related data were not determined in this study. The yield of NOB was selected to be 0.07 g cell g^{-1} N [46]. A fraction of NO_3^-–N was considered to be removed by complete heterotrophic denitrification, and the detailed calculation was described in the supplementary section (Table S1). Taking the cell synthesis into account, most (78.7%) of the nitrogen removal is due to the ANAMMOX pathway, while denitrification contributed to 20.7% of nitrogen removal.

3.6. Rapid startup mechanism

The mechanism for rapid startup is to properly control the quantity, activity and synergism between AOB and AnAOB. Consequently, three strategies can be concluded from the experience in this study. First, the co-seeding of dispersed AOB and AnAOB provides the related bacteria for synergetic nitrogen conversion, greatly reducing the time span for enrichment of these two bacteria. AnAOB should account for a major biomass fraction in the seed since they have a longer doubling time, while AOB biomass in the seed can have a lower percentage. The suggested total biomass concentration in inocula should be higher than 2.9 g VSS L^{-1} (Section 2.3), which has been shown successful in this study. Second, the addition of small amount of NO_2^-–N (<50 mg L^{-1}) in the feed can directly offer nitrite for the AnAOB in the anoxic zone of the granular sludge, lowering the initial inhibition on AnAOB and thus facilitating the establishment of one-stage PN-ANAMMOX process. The influent concentration of NO_2^-–N should gradually decrease to zero after progressive establishing synergism between AOB and AnAOB. Third, the online pH and DO monitoring is visualized to guide the reactor operation, promoting the diagnosis of the reactor state and thereby helping the stable maintenance of the reactor. The set-point of pH and DO should be designed in both optimum ranges. In this study, pH was controlled at 7.55–7.60, while the DO was controlled at lower than 0.3 mg L^{-1}. Any operation beyond these optimum ranges should be alarmed and treated immediately. The key point lies in proper control of the supply of oxygen and substrate to balance the synergism between AOB and AnAOB. The reversible inhibition on AnAOB due to over-aeration further verifies this viewpoint.

3.7. Implications and future study

This study indicates that co-seeding of dispersed AOB and granular AnAOB can lead to fast startup of the one-stage PN-ANAMMOX reactor. However, in-depth investigation into microbial ecology and its link to real-time control of reactor should be conducted in the future. The dynamics of the size distribution, amount, activity, abundance of dispersed activated sludge and granular sludge in the one-stage nitritation-ANAMMOX reactor during the whole startup period should be systematically analyzed. Most importantly, the specific microbial ecology needs to be correlated with the real-time monitoring of the online pH and DO of the reactor. Elucidation of the nexus between these two issues is the key to realizing precise control and thus establishing the strategy for automatic operation of the one-stage PN-ANAMMOX reactor.

Table 4
Nitrogen balance analysis of the one-stage PN-ANAMMOX reactor on day 101 (unit: g d^{-1})

Components	Influent (g d^{-1})	Effluent (g d^{-1})	N-Conversion			
			Cell synthesis	Yield-related N conversion (g d^{-1})	Bioprocess	Bio-N removal via N_2 (g d^{-1})
NH_4^+–N	3.884	0.092	AOB	0.122		
NO_2^-–N	0.003	0.040	NOB	0.069		
NO_3^-–N	0.030	0.624	AnAOB	0.130	ANAMMOX-N-removal	2.331
COD	3.538	2.574	Denitrification	0.145	Denitrification N-removal	0.511

4. Conclusions

By using dispersed AOB and granular AnAOB as seeds, and adding a small amount of nitrite in the feed during the first 45 d, fast startup of a one-stage PN-ANAMMOX reactor can be achieved. High rate and efficient nitrogen removal from raw landfill leachate (COD of 1,100 mg L^{-1} and C/N ratio of 0.80–0.95) can be maintained by use of online monitoring of pH and DO. The qPCR analysis showed that AOB accounted for only 1%–3% of the total bacteria in the one-stage PN-ANAMMOX system, AnAOB accounted for a lower proportion (0.3%) of total bacteria. Nitrogen mass balance analysis indicates that ANAMMOX and heterotrophic denitrification contributed to 78.7% and 20.7% of the nitrogen removal, respectively. The mechanism for rapid startup is to properly control the quantity, activity and synergism between AOB and AnAOB. The key point lies in proper control of the supply of oxygen and substrate to balance the synergism between AOB and AnAOB.

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Author contributions

Huosheng Li and Shaoqi Zhou conceived and designed the experiments; Huosheng Li performed the experiments; Jianyou Long, Yujie Qin, Hongguo Zhang and Fanson Zheng analyzed the data and contributed to the analysis tools; Huosheng Li and Shaoqi Zhou and Jianyou Long wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary information

Nitrogen balance analysis

i. Preconditions

The nitrogen conversion due to ammonia stripping and physiochemical adsorption was relatively insignificant [45]. The organic nitrogen conversion was also not considered, because the related data were not determined in this study. Therefore, the nitrogen removal is assumed to be via ANAMMOX pathway, heterotrophic denitrification pathway and bacterial synthesis. Unless instructed specifically, the unit used is volumetric mass concentration (mg L^{-1}).

ii. Yield selection

The yield of NOB was selected to be 0.07 g cell g^{-1} N [46].

iii. Bio-reaction formula selection

The ANAMMOX (Eq. (1)) and denitrification (Eq. (2)) reactions were referenced to Strous et al. [S1] and Chamchoi et al. [S2], respectively.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

$$NO_3^- + 0.29CH_3CH_2CH_2COOH + H_2CO_3 \rightarrow 0.034C_5H_7O_2N + HCO_3^- + 1.54H_2O + 0.986CO_2 + 0.483N_2 \quad (2)$$

iv. Nitrogen removal estimation

The nitrogen removal ($N_{removed}$):

$$\begin{aligned} N_{removed} &= NH_4^+ - N_{inf} + NO_2^- - N_{inf} + NO_3^- - N_{inf} - \\ &\quad NH_4^+ - N_{eff} - NO_2^- - N_{eff} - NO_3^- - N_{eff} \\ &= 1{,}177 + 1 + 9 - 28 - 12 - 189 = 958 \end{aligned} \quad (3)$$

v. Denitrification estimation

Assuming that all the COD removal is due to complete heterotrophic denitrification via Eq. (2), then the nitrogen removal can be calculated according to Eq. (2).

$$\begin{aligned} &NO_3^- - N \sim 0.29CH_3CH_2CH_2COOH \sim 0.034C_5H_7O_2N \sim 0.483N_2 \\ &14 \sim 0.29 \times 88 \sim 0.034 \times 113 \sim 0.483 \times 28 \\ &COD_{inf} - COD_{eff} = \sim \text{Cell Synthesis} \sim NO_3^- - \text{N removal} \\ &1{,}072 - 780 = 292 \end{aligned} \quad (4)$$

Then the cell synthesis due to denitrifying bacteria is estimated to be 43.96 (292 × 0.034 × 113 ÷ 0.29 ÷ 88 = 43.96), and the denitrification mediated nitrogen removal via N_2 gas ($N_{denitrification}$) is estimated to be 154.74 (292 × 0.483 × 28 ÷ 0.29 ÷ 88 = 154.74).

vi. ANAMMOX's first estimation

Because the cell synthesis is very low, therefore, we consider that the other nitrogen removal is due to ANAMMOX pathway ($N'_{ANAMMOX}$).

$$N'_{ANAMMOX} = N_{removed} - N_{denitrification} = 958 - 154.74 = 803.26 \quad (5)$$

vii. ANAMMOX's yield and actual nitrogen removal estimation

Based on Eq. (1), the NH_4^+–N used for ANAMMOX pathway is estimated to be 346.23 (803.26 × (1 ÷ (1 + 1.32)) = 346.23).

$$\begin{aligned} &NH_4^+ - N \sim 0.066CH_2O_{0.5}N_{0.15} \sim 1.02N_2 \\ &14 \sim 0.066 \times 24.1 \sim 1.02 \times 28 \\ &346.23 \sim \text{Cell Synthesis} \sim \text{ANAMMOX} - \text{N removal} \end{aligned} \quad (6)$$

Then the AnAOB synthesis is estimated to be 39.34 (346.23 × 0.066 × 24.1 ÷ 14) = 39.34), and the actual removal via N_2 due to ANAMMOX pathway ($N_{ANAMMOX}$) is estimated to be 706.31 (346.23 × 1.02 × 28 ÷ 14) = 706.31).

viii. NOB synthesis estimation

The NOB synthesis estimation (NOB_{syn}) can be obtained through the following equation.

$$\begin{aligned} NOB_{Syn} &= \left(NO_3^- - N_{eff} - NO_3^- - N_{inf} - NO_3^- - N_{ANAMMOX} + NO_3^- - N_{denitrification} \times NOB_{Yield}\right) \\ &= \left(189 - 9 - \left(39.34 + 706.31\right) \times 0.11 + 154.74 + 43.96\right) \\ &= 20.77\left(mgL^{-1}\right) \end{aligned} \quad (7)$$

Where NO_3^-–$N_{ANAMMOX}$ is the formation of NO_3^-–N from ANAMMOX pathway, which is about 11% of the nitrogen removal via ANAMMOX pathway (including the cell synthesis). NO_3^-–$N_{denitrification}$ is the covert removal of NO_3^-–N during denitrification (including the cell synthesis).

ix. AOB synthesis estimation

The rest of the nitrogen removal is due to the AOB cell synthesis, which is estimated to be 36.84. Therefore, the whole nitrogen balance is listed below (Table S1).

x. Conversion to mass balance

The volume of the reactor is 2.4 L, and the HRT is 17.45 h, therefore, all the nitrogen concentration (mg L^{-1}) listed in Table S1 could be converted to mass balance per day (mg d^{-1}), and is listed in Table 4.

Supplementary References

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Table S1
Nitrogen balance analysis of the one-stage PN-ANAMMOX reactor (unit: mg L^{-1})

Components	Influent (mg L^{-1})	Effluent (mg L^{-1})	N-Conversion			
			N-Synthesis	Yield-related N conversion (mg L^{-1})	Bioprocess	Bio-N Removal via N_2 (mg L^{-1})
NH_4^+–N	1,177	28	AOB synthesis	36.84		
NO_2^-–N	1	12	NOB synthesis	20.77		
NO_3^-–N	9	189	AnAOB synthesis	39.34	ANAMMOX-N-removal	706.31
COD	1,072	780	Denitrification synthesis	43.96	Denitrification N-removal	154.74