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Nitrous oxide emissions from an anammox reactor from the startup to stable-running period

Yue Jin[a], Wenjie Zhang[b,*]

[a]*College of Civil Engineering and Architecture, Guilin University of Technology, Guilin 541004, China, email: 103375916@qq.com*
[b]*Guangxi Key Laboratory of Environmental Pollution Control Theory and Technology, College of Environmental Science and Engineering, Guilin University of Technology, Guilin 541004, China, Tel. +86 773 2536922; Fax: +86 773 2536922; email: 2010053@glut.edu.cn*

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ABSTRACT

N_2O emissions from anammox processes are a big challenge in full-scale application. This study investigated N_2O emissions from an anammox reactor from the startup to the stable-running period. A high N_2O emissions of 1.7% (as of nitrogen removal) were observed, which exceeded previously reported values by almost an order of magnitude. Furthermore, it was demonstrated that N_2O emissions could be reduced by increasing the nitrogen loading rate. The results showed that high N_2O emissions were positively correlated with the count of denitrifying bacterium enrichment culture clone NOA 1 C10 and *Defluviimonas denitrificans*, both of which coexist with anammox bacteria. Incomplete heterotrophic denitrification, which is attributed to the low chemical oxygen demand/nitrogen ratio, was considered the main cause of high N_2O emissions during the startup period.

Keywords: Nitrous oxide; Denitrification; Anammox; Startup; Greenhouse gas emission

1. Introduction

Anaerobic ammonium oxidation bacteria (AnAOB) were speculated to exist during the early 20th century [1]. They were observed in engineered reactors in the 1980s and reported in the literature in the 1990s [2,3]. The number of full-scale installations of anammox reactors has substantially increased in the past 10 years since the first full-scale partial nitrification–anammox reactor was installed in Rotterdam [4,5]. However, the practical implementation of anammox-based, full-scale treatment processes still involves some challenges, including a longer startup period, limited application to the mainstream municipal wastewater, and poor effluent-water quality [6].

To shorten the startup period, cultivated anammox seed sludge was used as inoculum, resulting in a relatively rapid startup compared with that observed with other kinds of seed sludge [4,7,8]. Inspired from the first full-scale anammox process, cultivated sludges might be used to initiate the anammox process. However, cultivated anammox sludge is not generally available when starting a new, full-scale treatment plant. Therefore, stored anammox sludge, which has been preserved at low temperatures, may be used when starting a new reactor. Wenjie et al. [8] accomplished the startup of an anammox reactor by using stored anammox sludge that had been refrigerated for 2 years. However, the activity of the stored anammox sludge was significantly low because of sludge lysis. Denitrification consumes a high amount of NO_2^-–N or NO_3^-–N owing to the excess chemical oxygen demand (COD) of sludge lyse during the startup period, and thus it is considered the most probable cause of N_2O emissions [9]. N_2O is a strong greenhouse gas, causes stratospheric ozone depletion, and is toxic to humans. Studies have shown that N_2 is the end

* Corresponding author.

product of the anammox processes [10,11]. However, N_2O emissions from anammox processes have also been reported [12–15]. To the best of our knowledge, there exist only a few reports on N_2O emissions from an anammox reactor during the startup period. Considering the high possibilities of N_2O emissions from anammox reactors, there is an urgent need to investigate the actual N_2O emissions from an anammox reactor during the startup period.

In this study, an anammox reactor seeded with cultivated anammox sludge (stored at 4°C for 90 d) was used to study the N_2O emissions during the startup period. Additionally, the microbial population of the anammox sludge was genetically characterized using the 16S rRNA gene.

2. Materials and methods

2.1. Anammox reactor and substrate

As shown in Fig. 1, the reactor had an inner diameter of 14 cm with a total liquid volume of 10 L, which included a reaction zone of 8 L and a settling zone of 2 L. The reactor was made of acrylic resin and had a water jacket for temperature control. Sampling ports were located at heights of 3, 17, 20 and 25 cm above the reactor bottom. The pH value was controlled between 7.4 and 7.6 and adjusted by using 0.5 mol L^{-1} H_2SO_4 via an online pH controller (TPH/T-10, Tengine, China). The outer jacket of the reactor was fed with heat-retaining circulating water to maintain the internal temperature at 32°C ± 1°C. The reactor was enclosed in a black vinyl sheet to prevent the growth of photosynthetic bacteria and algae. The hydraulic retention time (HRT) was 8 ± 1 h, and the ratio of the circulation flow rate to inlet flow rate was 2:1.

The reactor was operated in the up-flow mode, with the influent introduced at its bottom using a peristaltic pump (BT100-2J, Longer Pump, China). A biocarrier, which was used as a bacterial carrier, comprised activated carbon particles with the internal filling diameter in the range of 1–3 mm, which was used as the bacterial carrier. The cultivated anammox sludge used in the reactor was taken from a pilot-scale anammox reactor, and had been preserved by refrigeration at 4°C for 90 d. Granule activated carbon granules with a settling velocity of over 150 m h^{-1} (4.2 cm s^{-1}) were used as the seed sludge [16]. The initial seeding concentration (mass of mixed liquor suspended solids [MLSS] per liter) was set to 4 g MLSS L^{-1}.

As shown in Table 1, the reactor was fed with synthetic wastewater, referring to the compositions described by Wenjie et al. [16]. The NO_2^-–N:NH_4^+–N molar ratios were set at 1.2. The influent storage tank was flushed with nitrogen gas to bring the dissolved oxygen (DO) at zero.

2.2. Analytical methods

NO_2^-–N, NH_4^+–N and total nitrogen (TN) were measured according to Standard Methods [17]. NO_3^-–N was determined by calculation of the difference of TN and the sum of NO_2^-–N and NH_4^+–N. The pH was measured with a pH meter (9010, Jenco, USA) and DO was measured with a DO meter (6010, Jenco, USA).

2.3. Gas collection and analysis

Gas was collected through a gas collector [18–20], and its volume was measured using an inverted cylinder, which contained tap water with the pH lowered to 3 using

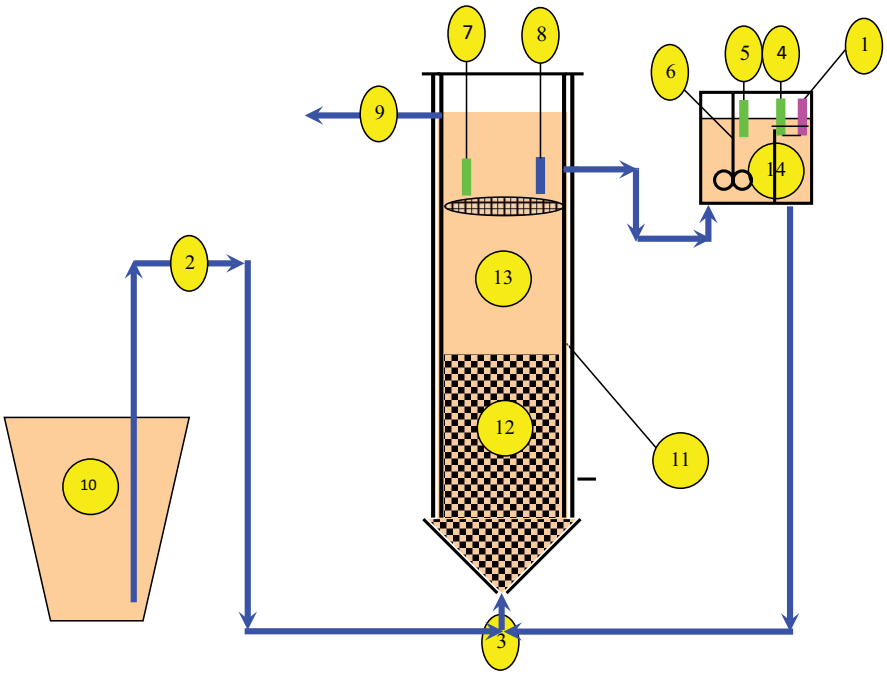

Fig. 1. Diagram of the reactor ((1) water level sensor; (2) influent pump; (3) recirculation pump; (4) heater; (5) pH control; (6) mixer; (7) pH recorder; (8) temperature recorder; (9) effluent; (10) influent tank; (11) water jacket; (12) biocarrier; (13) reactor; (14) pH adjustment tank).

Table 1
Composition of synthetic wastewater

Composition	Concentration (mg L^{-1})
Nitrogen loading rate (NLR, g-N L^{-1} d^{-1})	0.15–9
$(NH_4)_2SO_4$, $NaNO_2$ (as mg N L^{-1})	38.1–650.0, 46.4–780.0
$NaHCO_3$	1,000
NaH_2PO_4	50
$CaCl_2{\cdot}2H_2O$	100
$MgSO_4{\cdot}7H_2O$	50

1 N H_2SO_4. The specific operations were as follows: completely close the reactor; the gas in the reactor enters the gas collection tank from the vent hole on the top of the reactor, and the gas from the upper space of the gas collection tank is extracted from the valve on the gas collection tank into a gas-sampling bag. Notably, the gas-sampling bag was rinsed twice with the gas in the tank before being collected for determination. Samples were taken once a day, and the sampling time was marked. Gas analyses were performed by using a GC-112A gas chromatograph with a thermal conductivity detector (INESA INSTRUMENT, China). The column used was JN PorapaKQ with a length of 2 m. The chromatographic conditions were as follows: high-purity hydrogen (purity > 99.999%) used as a carrier gas at a gas flow rate of 4.1 mL min^{-1}; the column box temperature was 50°C; injector temperature was 80°C; detector temperature was 120°C; injection volume was 1 mL.

2.4. Microbial population analysis

Considering the change in N_2O emissions from the reactor, sludge samples were collected from the reactor at 15 d intervals for 4 months. The microbial population in the samples was analyzed using the method described by Wenjie et al. [16]. The PCR-denaturing gradient gel electrophoresis (DGGE), a molecular biology technique, was used to study the changes in the microbial population structure in the reactor during the anammox initiation. Relevant microorganisms that released N_2O during the initiation were found.

2.5. SEM analysis

Because most biological samples contain water and are significantly soft, they should be treated before performing scanning electron microscopy. The main requirement is to keep the surface structure of the sample as well as possible, free from deformation and contamination, dry, and conductive. The scanning electron microscope model used was JSM-6380.

3. Results

3.1. Reactor performance

Considering the amount and activity of the cultivated anammox seed sludges, a nitrogen-loading rate (NLR) of 0.15 g-N L^{-1} d^{-1} was adopted. The removal performance is shown in Fig. 2. During the startup period, with the influent NH_4^+–N and NO_2^-–N concentrations of 38.1 and 46.4 mg L^{-1}, respectively, the effluent NH_4^+–N and NO_2^-–N concentrations of 17.1 and 5.3 mg L^{-1} were obtained, respectively, with a TN removal rate of 61%. Subsequently, the influent NH_4^+–N and NO_2^-–N concentrations were increased to 56.4 and 61.0 mg L^{-1}, respectively, and consequently the effluent NH_4^+–N concentrations gradually decreased to 5.1 mg L^{-1} with a TN removal rate of 84% over a 13-d period. These results indicated that the cultivated anammox seed sludge could restore this activity in a short time. An NLR of approximately 1.0 g-N L^{-1} d^{-1} was maintained to study the N_2O emission during days 14–70. However, from day 70, the NLR was increased by adjusting the influent nitrogen concentration and HRT. The NLR was increased to 9 g-N L^{-1} d^{-1} during 3 months and the influent TN concentration reached 780 mg L^{-1}, the highest concentration level used in this study. Under these conditions, an average TN removal efficiency of 83% was achieved. For the operation period during days

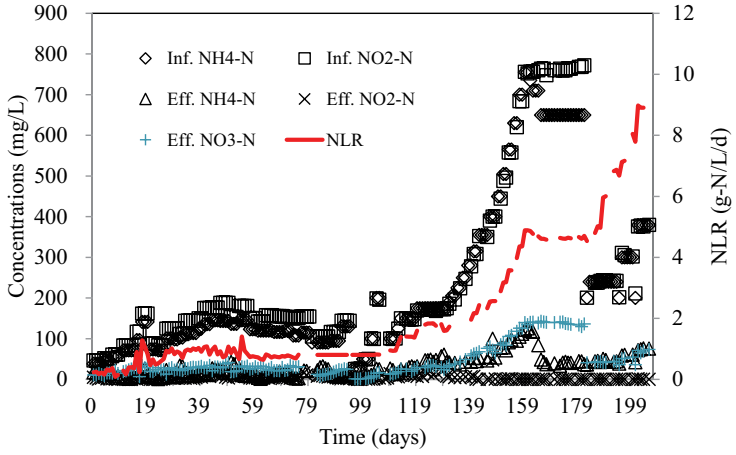

Fig. 2. Reactor performance during the study (Inf. NH_4^+–N indicates influent NH_4^+–N; inf. NO_2^-–N indicates influent NO_2^-–N; eff. NH_4^+–N indicates effluent NH_4^+–N; eff. NO_2^-–N indicates effluent NO_2^-–N; eff. NO_3^-–N indicates effluent NO_3^-–N; NLR, nitrogen loading rate).

139–159, the effluent NH_4^+–N concentration also increased because of the increase in the inflow NH_4^+–N concentration. However, with subsequent reduction in the inflow NH_4^+–N concentration, the effluent NH_4^+–N concentration too gradually decreased in the following days. Overall, the reactor could operate at a stable nitrogen-removal rate.

Fig. 3 shows both the effluent NO_2^-–N removal/NH_4^+–N removal ratio and the effluent NO_3^-–N production/NH_4^+–N removal ratio. Initially, the effluent NO_2^-–N removal/NH_4^+–N removal ratio, and the effluent NO_3^-–N production/NH_4^+–N removal ratio were 2.0 and 0.5, respectively. The additional NO_2^-–N removal was attributed to denitrification. The source of NO_3^-–N might be the cultivated anammox seed sludge, which was used to treat high-strength wastewater [16]. The effluent NO_2^-–N removal/NH_4^+–N removal ratio and the effluent NO_3^-–N production/NH_4^+–N removal ratio were close to the reported values [2] during the first 13 operation days, although they were unsteady. However, both a stable effluent NO_2^-–N removal/NH_4^+–N removal ratio, and a stable effluent NO_3^-–N production/NH_4^+–N removal ratio could be obtained only after almost 3 months of operation. Therefore, the startup period was considered to exist from day 0 to 90.

3.2. N_2O emission

The N_2O emissions from the reactor over the course of this study are shown in Fig. 4. From the figure, it is evident that the N_2O emissions initially increased, then decreased, with peaks at days 30 and 70. Emissions with N_2O concentration of 0.3%–1.7% were detected during days 0–90. Emissions with N_2O concentration up to 1.7%, which is almost 10 times the previously reported value [13] were detected in the gas emission. To the best of our knowledge, such high N_2O emissions have never been reported. After the 90-d startup period, N_2O concentration of 0.04%–0.30% was detected in the gas emission. Although NLR of 9 g-N L^{-1} d^{-1} was achieved during the stable-running period (Fig. 2), N_2O emissions were still detected from the anammox reactor, suggesting that N_2O emissions were unavoidable in the anammox reactor used in this study. Additionally, the amount of the N_2O emissions was unstable even during the stable-running period, thereby agreeing with other results [12].

3.3. SEM results

The morphology of the sludge was analyzed by SEM, as shown in Fig. 5. Granular sludges were sampled on day 1 and 200. Spherical bacteria were observed on the surface of the granules on days 1 and 200, indicating that the anammox particles were spherical. At the beginning of the experiment, the anammox particles were scattered on the surface of the granules. At the end of the experiment also, considerable amount of anammox particles were observed to be distributed on the surface of the granules. And the AnAOB activity in the reactor was significantly high. However, the AnAOB activity was reduced in the inoculated sludge and could increase well during the experiment. The granular sludge could be restructured to strike a new balance between microorganisms. This may be related to the low N_2O emission in the later experimental period.

3.4. Bacterial community analysis

The results of the sequence analysis of major DGGE bands are presented in Table 2. Nine samples were obtained from the same reactor and were obviously similar in terms of community structure. *Kuenenia stuttgartiensis* and *Planctomycete* KSU-1, both of which are anammox strains, prevailed as the majority of the clones following cultivation. From the 16S rRNA analysis, denitrifying bacterium enrichment culture clones NOA 1 C10 and *Defluviimonas denitrificans*, both identified as denitrifiers, showed a similar trend for N_2O emissions (Fig. 4); both NO_2^-–N and NO_3^-–N are substrates for denitrifiers. In contrast with Fig. 2, N_2O might be produced as an intermediate of incomplete heterotrophic denitrification due to the low COD of sludge lysis. During the startup and stable process conditions, denitrifying bacterium enrichment culture clones NOA 1

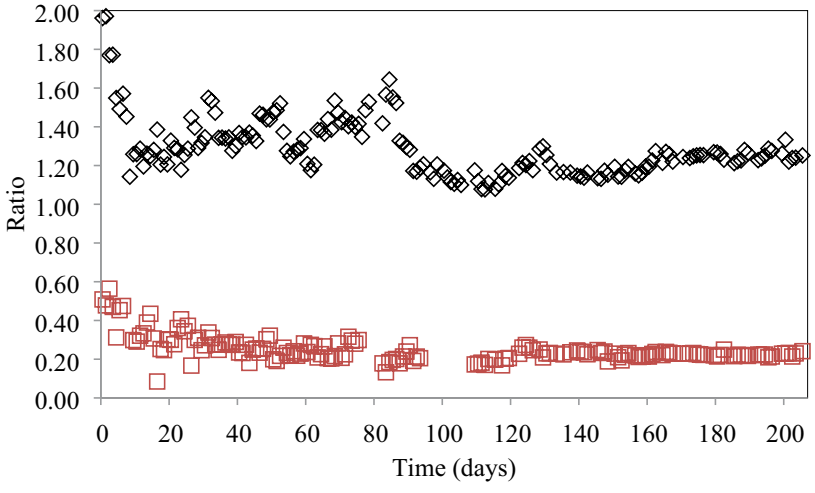

Fig. 3. Effluent NO_2–N removal/NH_4–N removal ratio and the effluent NO_3–N production/NH_4–N removal ratio.

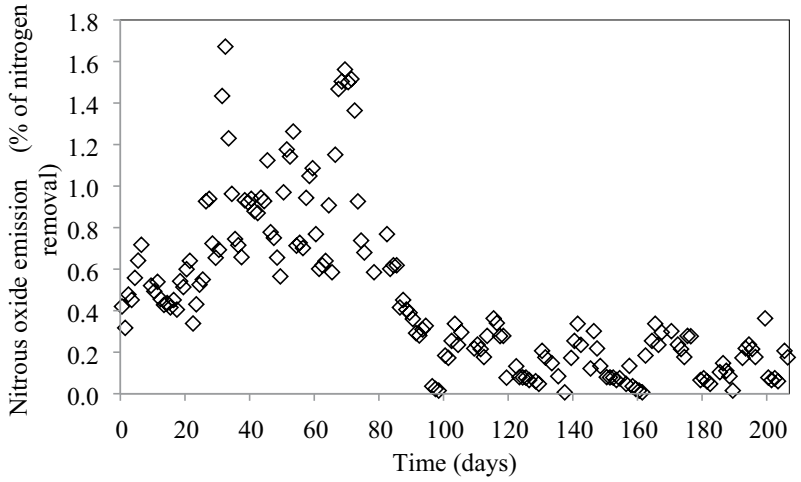

Fig. 4. N_2O emissions during the course of this study.

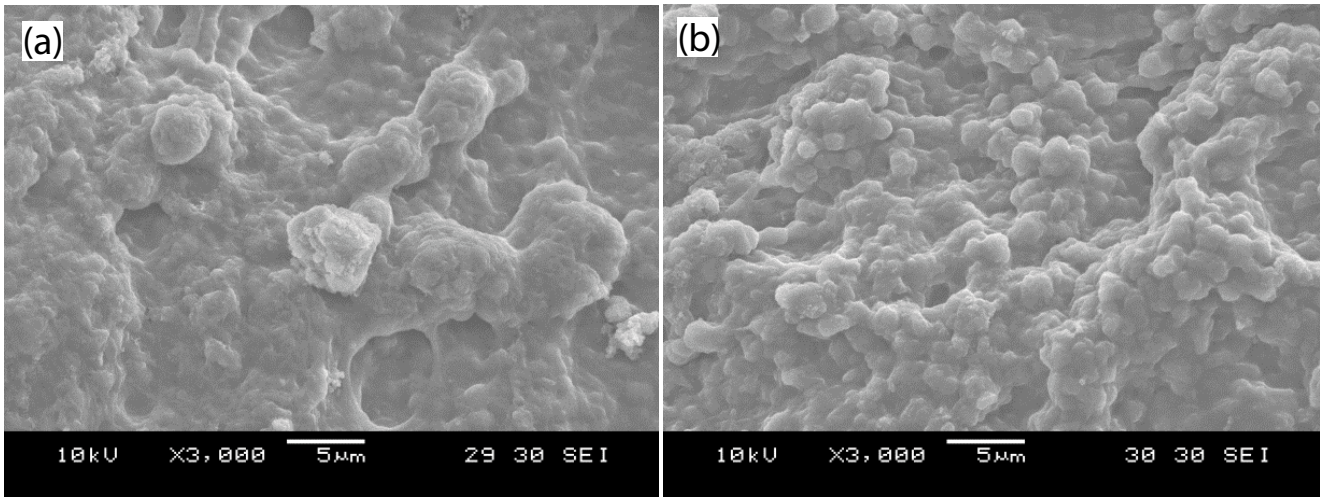

Fig. 5. SEM images ((a) day 1 and (b) day 200).

C10 and *D. denitrificans*, as co-existent microbes, were still present in the anammox sludge. Their functions need to be further distinguished.

Fig. 6 shows the phylogenetic tree of the sludges sampled from the reactor. According to the phylogenetic tree constructed by 16S rRNA, these strains were divided into the following three genotypes: *Planococcus*, *Pseudomonas*, *Chryseobacterium*, *Candidatus Chloracidobacterium* in group 1; *Psychrobacter* and *Paenisporosarcina* in group 2; *Nitrosomonas* and *Arenimonas* in group 3. Figs. 7 and 8 show the share in the microbial community on day 1 and day 120 for group 1. At the beginning of the experiment, group 2 was the dominant strain, whose *Psychrobacter* strain accounted for 36.07% of the microbial composition, and *Candidatus Chloracidobacterium* accounted for 9.35% ranking third in the total microbial share. On day 120, group 1 was the dominant strain, whose *Candidatus Chloracidobacterium* strain accounted for 40.19% of the microbial composition. The results showed that *Candidatus Chloracidobacterium* gradually replaced *Psychrobacter* as the dominant strain. It was preliminarily inferred that the microbial group that released N_2O was *Psychrobacter*.

4. Discussions

The N_2O emissions exceeded the reported values during the startup period. Okabe et al. [13] reported emissions with N_2O concentration of only 0.05%–0.23% with a nitrogen removal rate of 7.5–15 g-N L^{-1} d^{-1}. Ali et al. [21] reported that emissions with N_2O concentration of 0.2%–0.4% were contributed by anammox granules in a one-stage reactor. In a full-scale plant, a slightly higher emission of 0.6% was reported [22]. However, the highest N_2O concentration (1.7%) was measured during the startup period in this study, compared with the results of other studies presented in Table 3. From the table, it is evident that increasing the NLRs resulted in decreasing N_2O concentrations, also confirmed by the results of this study. Thus, increasing the NLR is effective in reducing N_2O emissions; however, N_2O emissions are unavoidable in an anammox

Table 2
Homology search results for the 16S rRNA gene sequences of the main bacterial members in the microbial community

Taxon	Identity (%)	Day								
		0	15	30	45	60	75	90	105	120
Denitrifying bacterium enrichment culture clone NOA 1 C10	96–97	6	5	5	4	4	3	2	2	2
Uncultured bacterium clone KIST-JJY030	98	2	4	4	3	4	2	2	3	3
*Defluviimonas denitrifica*ns	97	10	10	9	7	5	4	2	1	1
Uncultured bacterium clone 80	88	1	2	2	3	2	1	2	2	2
Kuenenia stuttgartiensis	96–100	2	4	5	7	8	10	12	16	20
Planctomycete KSU-1	99	2	1	3	4	4	3	4	5	5
Uncultured bacterium clone 37	95	0	0	2	3	3	3	4	3	3
Uncultured bacterium clone Dok04	96	1	1	2	2	3	4	3	3	3
Uncultured *Chloroflexi bacterium* clone ST01-SN2H	93	2	2	3	2	1	1	1	1	0
Uncultured bacterium clone AA102	88	1	1	2	1	2	3	3	4	3
Uncultured bacterium clone Dok53	99	0	2	1	2	3	4	3	3	4

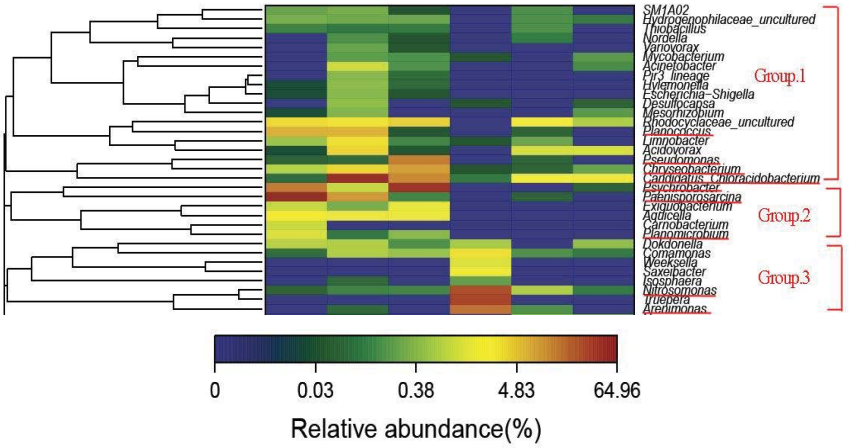

Fig. 6. Hierarchical cluster analysis of the bacterial communities (left to right, day 1, 15, 30, 60, 90 and 120).

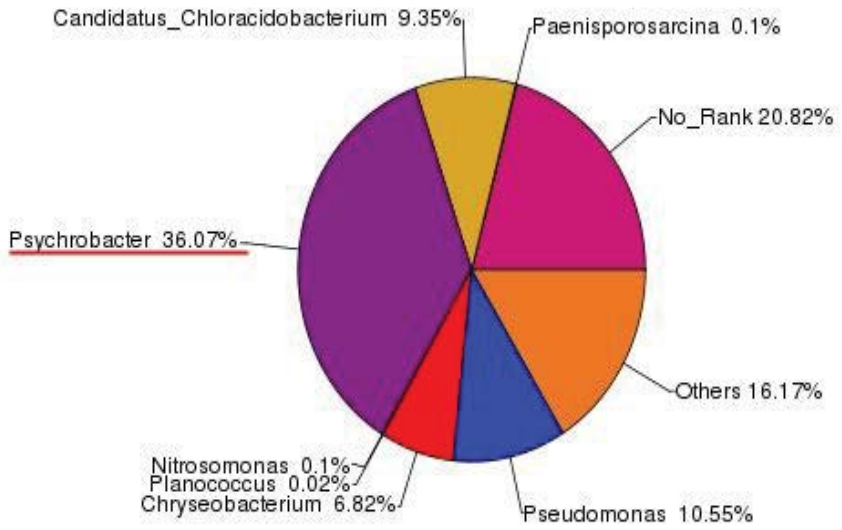

Fig. 7. Microbial diversity on day 1.

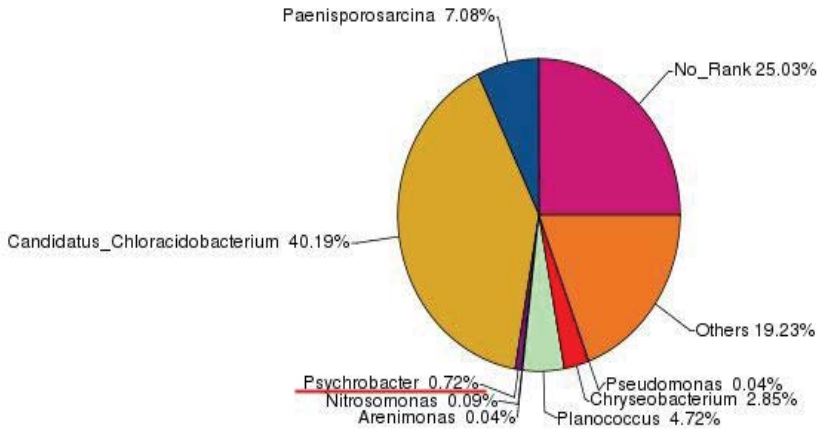

Fig. 8. Microbial diversity on day 120.

Table 3
Comparison of N_2O emissions at different NLRs in the anammox reactor

Reactor type	Reactor volume (L)	Removal rate (g-N L^{-1} d^{-1})	N_2O emission (%)	References
Granules-based	0.15	7.5–15	0.05–0.23	[13]
Granules-based	2	0.6	0.2–0.4	[21]
Granules-based	70,000	7	0.6%	[22]
Granules-based	10	0.15–1.0 (startup period)	0.3–1.7 (startup period)	Present work
		1.0–9 (stable-running period)	0.04–0.3 (stable-running period)	

reactor (Fig. 4). Therefore, reducing the N_2O emissions still remains a concern for anammox-based applications.

N_2O is a strong greenhouse gas. Its radiation intensity of N_2O is 300 times higher than that of CO_2, and it can react with stratospheric O_3. The United Nations Intergovernmental Panel on Climate Change has specifically calculated the contribution of N_2O toward the greenhouse gas emissions from wastewater treatment processes [23]. Therefore, N_2O emissions become particularly important for calculating the "carbon footprint" of wastewater treatment plants [24,25]. In wastewater treatment systems, the following three N_2O generation pathways exist [9]: ammonia monooxygenase (AMO) based oxidation of NH_2OH, hydroxylamine redox enzyme (HAO) based reduction of NH_2OH in the nitrification process, and N_2O release based on nitrous oxide reductase (NOS) activity inhibition in the denitrification process. In a two-stage anammox process, the nitrification part is the main source of N_2O production [21], as 65% of the total N_2O is generated by the AMO-based oxidation of NH_2OH. However, in a one-stage anammox process, the information on the N_2O generation pathway is significantly limited. According to the lab-scale, in-situ analysis results of an SBR reactor, NH_2OH oxidation, and the incomplete NO_2^-–N denitrification based on nitrite reductase and nitrite oxidoreductase are essentially the same, accounting for approximately 70% of the total N_2O production. However, the anoxic zone dominated by anammox bacteria accounts for approximately 30% of the total production [21]. The percentage of N_2O emissions vary with the anammox process, with a maximum of more than 20% and a minimum of only 0.11% [21]. Additionally, the operational parameters of the process [26,27], such as the influent substrate concentration (IC, Ca^{2+}, Mg^{2+}, etc.), will affect the microbial population, thereby changing the spatiotemporal distribution of autotrophic, and heterotrophic bacteria, affecting the N_2O emission. A previous research [28] by our group also confirmed that Fe^{2+}, as an important component of the heme proteins in anammox bacteria, can also generate N_2O by reducing NO_2^- (see the following formula), thereby revealing another source of N_2O from anammox. One has the following:

$$NO_2^- + Fe^{2+} + 2H^+ \rightarrow Fe^{3+} + NO + H_2O \quad (1)$$

$$NO + Fe^{2+} + 1H^+ \rightarrow Fe^{3+} + 0.5N_2O + 0.5H_2O \quad (2)$$

The above-mentioned data showed that although the N_2O emission from the anammox process was unavoidable, the minimum N_2O emission could be achieved through reasonably constructing the reactor and optimizing the operational conditions. Therefore, in the case of full-scale processes, we can reduce the N_2O emissions by reducing the concentrations of Ca^{2+}, Mg^{2+}, IC in the wastewater, shortening the HRT, and maintaining an appropriate pH.

5. Conclusions

An anammox reactor was used to investigate N_2O emissions from the startup to stable-running period. N_2O concentrations of 0.3%–1.7% were observed in the gas emission during this study. N_2O concentrations of up to 1.7%, which is almost 10 times the previously reported values

during the stable-running period, were also observed in the gas emission. These results suggest that N_2O was mainly a product of heterotrophic denitrification during the startup period.

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