Bioresource Technology 342 (2021) 125917

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enhanced nitrogen removal by partial nitrification-anammox process with a novel high-frequency micro-aeration (HFMA) mode: Metabolic interactions among functional bacteria

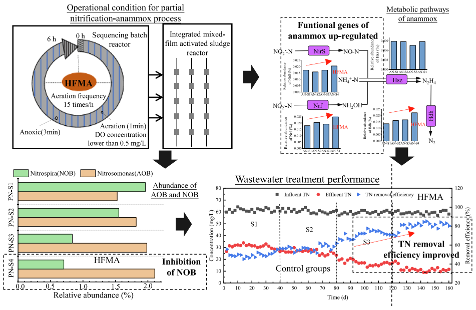
Yulei Chi[a,b,1], Xuan Shi[a,b,1], Pengkang Jin[a,b,*], Xiaochang C. Wang[b], Tong Ren[b], Bo Ren[b], Xin Jin[a,b]

[a] *School of Human Settlements and Civil Engineering, Xi'an Jiaotong University, Xi'an, Shaanxi Province 710049, China*
[b] *School of Environmental and Municipal Engineering, Xi'an University of Architecture and Technology, Xi'an, Shaanxi Province 710055, China*

HIGHLIGHTS

- HFMA mode with frequent aeration/anoxic alternation and low DO condition was proposed.
- The frequent aeration/anoxic alternation improved the NOB inhibition performance.
- HFMA strengthens the microbial metabolic interactions among functional bacteria.
- The interactions promoted the expression of genes related to anammox.
- The inhibited NOB and the enhanced anammox under HFMA improved the TN removal rate.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
High-frequency micro-aeration
Partial nitrification
Mainstream anammox
Domestic wastewater
Metabolic interaction

ABSTRACT

A novel high-frequency micro-aeration (HFMA) mode with aeration frequency of 15 times/h and DO concentration lower than 0.5 mg/L was proposed. Advanced partial nitrification-anammox (PN-A) performance was achieved in a two-stage sequencing batch reactor-integrated fixed-film activated sludge reactor with the HFMA mode. When treating wastewater with carbon/nitrogen ratio of 3, the abundance of NO_2^--N oxidation related genes decreased, and the genes carried out NO_3^--N reduction and carbon source consumption were up-regulated. These variations in microbial metabolism brought more NO_2^--N substrate for the subsequent anammox process, and consumed part of the accumulated organic matter and NO_3^--N. Thus, the HFMA conditions eventually promoted the expression of anammox bacteria with NH_2OH as an intermediate metabolite and the substance exchange activity of anammox bacteria. The changes in microorganisms lead to increase in the nitrite accumulation rate, nitrogen removal efficiency and abundance of anammox bacteria (16.34%, 18.71% and 5.92%, respectively).

---

* Corresponding author at: School of Human Settlements and Civil Engineering, Xi'an Jiaotong University, Xi'an, Shaanxi Province 710049, China.
E-mail address: pkjin@xjtu.edu.cn (P. Jin).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.biortech.2021.125917
Received 16 July 2021; Received in revised form 3 September 2021; Accepted 4 September 2021

1. Introduction

Faced with the increasingly stringent municipal wastewater treatment standards, together with new demands on energy consumption, partial nitrification-anammox (PN-A) has been extensively studied for municipal wastewater treatment (Li et al., 2020b). Maintaining high activity of ammonia oxidation bacteria (AOB) and anammox bacteria and fully inhibiting the activity of nitrite oxidation bacteria (NOB) are essential for implementing highly efficient nitrogen removal through PN-A (Chen et al., 2019). It is usually difficult to maintain long-term stable NOB inhibition since NOB could develop resistance to inhibition conditions (e.g., low DO environment) (Liu & Wang, 2013). The water quality of municipal wastewater with a Total nitrogen (TN) content usually in the range of 20–60 mg/L is disadvantageous to the retention of anammox bacteria (Li et al., 2021). Therefore, for the composition of municipal wastewater, it is still necessary to explore the process form and operation modes that are conductive to achieving highly efficient PN-A (Kowalski et al., 2019).

Environmental inhibitors are required in the partial nitrification stage of PN-A processes to fully suppress NOB activity (Laureni et al., 2019), and the changes in environmental conditions would affect the performance of the subsequent anammox stage (Agrawal et al., 2018). Therefore, many efforts have been made to improve the processing efficiency by optimizing the operation strategy in the partial nitrification stage (Choi et al., 2019), among which the intermittent aeration is one of the most common strategies (Xu et al., 2020). In the PN-A process with intermittent aeration mode, the activity of functional microorganisms carrying out nitrogen removal is affected by the DO concentration conditions and aeration frequency (Wang et al., 2018). Previous studies have found that in the mainstream PN-A process under intermittent aeration, the increase in aeration efficiency contributed to improve the nitrite accumulation efficiency (Chen et al., 2020). The increase in the aeration frequency not only directly affects the metabolism of functional microorganisms, but also causes a decrease in the duration of each aeration period and the average DO concentration (Guo et al., 2013), which is beneficial for maintaining efficient PN-A (Sliekers et al., 2005). Additionally, the method based on adjusting the aeration frequency has advantages such as no need for inhibitor addition and low aeration demand (Wang et al., 2017b). Therefore, it is still necessary to explore the aeration strategy for enhancing the municipal wastewater treatment using the PN-A process based on adjustment of aeration frequency.

The aeration frequency adopted in the PN-A process is usually lower than 3 times/h (Miao et al., 2017), and there has been little research on the processing efficiency under high-frequency aeration conditions. Under the conditions of high-frequency aeration, transient anoxic environment caused by frequent aeration/anoxic alternation is conductive to the achievement of partial nitrification (Wang et al., 2019). Moreover, a continuous micro-aeration condition, which refers to the operating conditions with low DO concentration and short aeration duration, can be easily attained under high-frequency aeration mode. The micro-aeration condition can enhance the efficiency of NOB inhibition. Therefore, there is a possibility that the High-frequency micro-aeration (HFMA) mode contributes to improving the municipal wastewater treatment efficiency of the PN-A process. Furthermore, few studies have explored the influence of aeration frequency on the microbial community structure and metabolic activity of anammox bacteria, while changes in the anammox metabolic characteristics are considered to be the key to improving the treatment performance of the PN-A process (Wang et al., 2020). Hence, it is necessary to use advanced biological information analysis technology (e.g., metagenomics sequencing) and conduct an in-depth analysis on the PN-A process for treating municipal wastewater (Li et al., 2020c).

To investigate the effect of the HFMA mode on the PN-A process treating municipal wastewater, a PN-A system with HFMA conditions was established on the basis of a two-stage sequencing batch reactor and integrated fixed-film activated sludge reactor (SBR-IFAS). During the operation of the PN-A process, parameters such as Nitrite accumulation rate (NAR) and substrate consumption ratio were compared between the HFMA and the control groups, which were operated under low DO (DO less than 0.9 mg/L) and low aeration frequency (lower than 3 times/h) conditions. The differences in community characteristics of nitrifying bacteria, denitrifying bacteria (DNB) and anammox bacteria between the HFMA system and other control groups were emphatically investigated. Finally, to establish a theoretical basis for the optimization of the PN-A process, the metabolic characteristics of microorganisms under HFMA were studied using metagenomic sequencing and analysis technology.

2. Materials and methods

2.1. Experimental device and reactor operation

A laboratory-scale two-stage configuration comprising a sequencing batch reactor (SBR) and an integrated mixed-film activated sludge (IFAS) reactor was used in this study. The working volumes of the SBR and IFAS reactors were 10 L. As is shown in Table 1, the operation of the SBR-IFAS was divided into four stages according to the discrepancy in aeration conditions (S1, S2, S3 and S4), the high-frequency micro-aeration (HFMA) mode was introduced in S4 stage, and the operating condition with aeration frequency lower than 3 times/h was adopted in stages S1-S3 (control groups).

The wastewater was fed into the SBR, which was operated under alternating aeration and anoxic conditions to achieve nitrification, denitrification and organic removal. The four operation stages of SBR were defined as PN-S1, PN-S2, PN-S3 and PN-S4. As shown in Table 1, the aeration frequency of the four operational stages were 1, 2, 10 and 75 times/cycle, respectively. The duration of each typical cycle was 6 h, which consisted of a feeding period (20 min), alternating anoxic and aeration period (5 h), setting (20 min) and decanting period (20 min).

In the S1 stage (1–40 d), each typical cycle incorporates an anoxic period for 2 h and an aeration period of 3 h. In the S2 operational stage (41–80 d), aeration was conducted 2 times with the anoxic period and the aeration period alternated 2 times in each typical cycle; the duration of each anoxic and aeration periods were 1 h 20 min and 1 h 10 min, respectively. In the first two operation stages, the average and maximum

Table 1
Operational parameters.

Operation stage		Ordinary PN-A process			HFMA
		S1 1–40 d	S2 41–80 d	S3 81–120 d	S4 121–160 d
Typical cycle duration (h)		12	12	12	12
SBR	Feeding period (min)	20	20	20	20
	Alternating anoxic and aeration period (h)	5	5	5	5
	Aeration frequency (times/cycle)	1	2	10	75
	Duration of each aeration period (min)	180	80	10	1
	Duration of each anoxic period (min)	120	70	20	3
	Maximum of DO concentration during aeration (mg/L)	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.1
	Average of DO concentration during aeration (mg/L)	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
	Setting and decanting period (min)	40	40	40	40
IFAS	Feeding period (min)	20	20	20	20
	Anaerobic period (h)	5	5	5	5
	Setting and decanting period (min)	40	40	40	40

DO concentration during aeration was 0.6 ± 0.1 and 0.8 ± 0.2 mg/L, respectively. In the S3 operational stage (81–120 d), the aeration phase in each cycle was divided into 10 periods with the duration of each anoxic and aeration period being 20 min and 10 min, respectively, and the average and maximum DO concentration during aeration was 0.5 ± 0.1 and 0.6 ± 0.1 mg/L, respectively.

In the S4 operational stage (121–160 d), the HFMA mode was adopted with an aeration frequency during the alternating anoxic and aeration period of 75 times/cycle (15 times/h). To establish micro-aeration conditions, the duration of each aeration period was set as 1 min, and the average and maximum DO concentration during aeration was set as 0.3 ± 0.1 and 0.4 ± 0.1 mg/L, respectively. The low DO concentration condition was adopted in both HFMA system and control groups.

The SBR reactor was operated at 20–25 ℃ and the pH value was controlled at 7–8, and the sludge retention time was 18 d. The volume of effluent at the end of each cycle was 5 L, resulting in a hydraulic retention time (HRT) of 12 h. The aeration volume at each operational stages was 47.99 L/cycle, and the aeration rate in the four operational stages were 0.27, 0.40, 0.48 and 0.64 L/min. The supplied air was well-mixed during aeration period using microporous aeration device and constant velocity stirrer.

The produced water of the SBR was directed to an IFAS for advanced nitrogen removal by anammox microorganisms. A polyurethane sponge filler was used in the IFAS reactor with a filling ratio of 30%. Four operational stages of IFAS reactor were designed according to the discrepancy of influent, on the specific, the four stages are AN-S1 (1–40 d), AN-S2 (41–80 d), AN-S3 (81–120 d) and AN-S4 (121–160 d). A typical cycle of the IFAS reactor (6 h) incorporated the feeding period (20 min), anaerobic period (5 h), setting period (20 min) and decanting period (20 min). The HRT of the IFAS reactor was equal to 12 h. The pH value of IFAS reactor was controlled at 8–8.5, and the temperature was maintained at 30–35 ℃.

2.2. *Wastewater composition and seeding sludge*

The main characteristics of synthetic wastewater used in this research were: 179.34 ± 3.12 mg/L chemical oxygen demand (COD), 59.31 ± 1.61 mg/L total nitrogen (TN) and COD/TN ratio of appropriately 3. The detailed composition of influent wastewater was (one liter): 214 mg CH_3COONa, 229 mg NH_4Cl, 26 mg KH_2PO_4 and 1 ml trace element solution. The composition of trace element solution was as previous reported (Chi et al., 2021).

The seeding activated sludge for SBR was taken from a partial nitrification-denitrification municipal wastewater treatment reactor, which achieved a Nitrite accumulation rate (NAR) of 60%. The seeding sludge for the IFAS reactor was taken from an anammox process treating wastewater with a high NH_4^+-N concentration wastewater. Sludge accumulation (60 days) was introduced before the process started to enable microorganisms to adapt to the influent characteristics and operational conditions.

2.3. *Analysis and calculation methods*

The water samples were filtered using 0.45 μm filter paper, and the concentrations of COD, NH_4^+-N, NO_2^--N, NO_3^--N and TN in the collected samples were measured according to standard methods (APHA, 2005). The NAR was calculated to estimate the performance of partial nitrification, and its value was determined by the ratio of NO_2^--N concentration to the sum of NO_2^--N and NO_3^--N in the produced water of the SBR (Lv et al., 2019).

2.4. *Microbial analysis methods*

AOB, NOB, DNB and anammox bacterial community characteristics were analyzed according to the results of 16S rRNA high-throughput sequencing. Metagenomic sequencing technology was employed to explore the mechanisms of microbial metabolism. 16S rRNA high-throughput sequencing was conducted on the Illumina MiSeq platform at the Sangon Biotech (Shanghai, China), and the sequencing results were archived in the National Center for Biotechnology Information (NCBI) database with accession number PRJNA743151. The results of metagenomic sequencing were carried out on the Illumina Hiseq platform at the Sangon Biotech (Shanghai, China), and the accession number in the NCBI database was PRJNA743156. Gene function annotation was performed according to Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Statistical analysis (e.g., correlation coefficient) was performed using SPSS20.0 software.

3. Results and discussion

3.1. *Performance of the PN-A process under HFMA mode*

As is depicted in Fig. 1(a), the NAR was 52.24 ± 1.77 % (effluent NO_2^--N and NO_3^--N of 12.32 ± 0.51 and 11.26 ± 0.57 mg/L, respectively) in PN-S1 and increased to 58.37 ± 0.90 % (effluent NO_2^--N and NO_3^--N of 12.68 ± 0.21 and 9.05 ± 0.28 mg/L, respectively) in PN-S2. When the aeration frequency increased to 10 times/cycle in PN-S3 stage, the effluent NO_2^--N (12.50 ± 0.38 mg/L) concentration remained close to those in the PN-S1 and PN-S2 stages, the effluent NO_3^--N decreased to 5.75 ± 0.21 mg/L, and the NAR increased to about 70.46 ± 2.59 %. Stable partial nitrification performance was achieved in the control groups under low DO operating conditions.

The NAR reached the highest value of 77.46 ± 0.63 % in PN-S4 stage with HFMA mode, and the effluent NO_2^--N and NO_3^--N concentrations were 12.78 ± 0.14 mg/L and 3.76 ± 0.61 mg/L, respectively. Building a low DO environment is conductive to the realization of partial nitrification (Laureni et al., 2019), however, low DO was used in both the HFMA system and the control groups. Hence, the DO condition may not be the decisive reason for the difference in partial nitrification efficiency between the HFMA system and the control groups. Additionally, previous research has shown that a transient anoxic environment is formed in the aeration/anoxic alternating transition process, which can enhance the inhibition of NOB (Wang et al., 2019). Therefore, compared with the control groups, the enhancement in partial nitrification under HFMA was mainly due to the high-frequency aeration conditions.

The effluent COD concentrations of PN-S1 and PN-S2 stage were 29.11 ± 3.34 mg/L, and the COD concentrations of PN-S3 and PN-S4 stage decreased to less than 20 mg/L with the increase in aeration frequency. Insufficient carbon sources in the domestic wastewater treatment system will cause disintegration of microorganisms, and lead to an increase in effluent COD concentration (Zubrowska-Sudol & Walczak, 2014). Herein, the decrease in SBR effluent COD concentration under the HFMA mode indicated a more stable growth state of microorganisms, and that the application of HFMA mode is beneficial for anammox microorganisms in the subsequent reactor.

During the operation of SBR, the effluent TN concentration was decreased from 33.90 ± 1.06 (PN-S1 stage) to 27.32 ± 0.65 mg/L (PN-S4 stage) Fig. 1(b). Furthermore, the proportion of NO_3^--N in the effluent of SBR with HFMA mode (PN-S4) was 14.35%, which was 19.78% lower than that in other operational stages, indicating that the reduction in NO_3^--N was the main reason for the increase in TN removal performance. There are several possible reasons for the reduced NO_3^--N content: 1) the NO_3^--N reduction activity of DNB was strengthened with the increase in aeration frequency; 2) the NO_2^--N reduction activity of DNB was improved after the stabilization of partial nitrification, and the competition between DNB and NOB under the low DO conditions resulted in a decrease in NO_3^--N formation (Li et al., 2020a).

In the AN-S4, the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N in the produced water of IFAS decreased to the lowest values of 3.31 ± 0.77, 1.50 ± 0.31 and 6.32 ± 0.64 mg/L, respectively. Meanwhile, the effluent TN concentration of the PN-A process under the HFMA mode

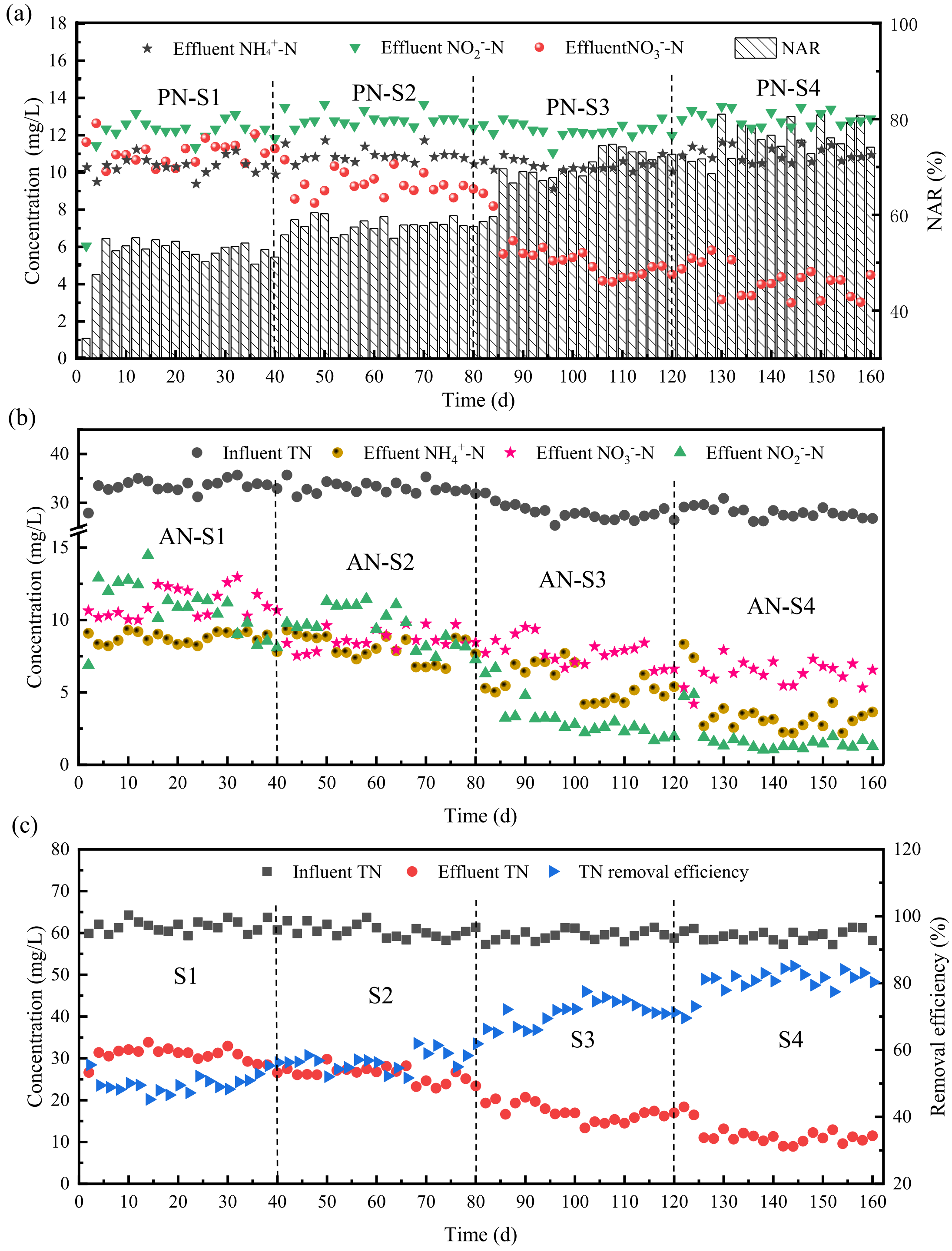


Fig. 1. Nitrogen composition in the influent and effluent water of SBR (a) and IFAS (b), and the TN removal efficiency of SBR-IFAS reactor (c).

(S4) was 11.12 ± 1.24 mg/L, and TN removal efficiency reached the highest value of 81.29 ± 2.59 %. In conclusion, the PN-A process had higher anammox activity and nitrogen removal efficiency with the HFMA conditions. The application of HFMA mode led to a change in the partial nitrification efficiency and the substrate conditions in anammox reaction phase, which was one of the main reasons for the change in anammox and TN removal performance.

3.2. Effects of the HFMA mode on nitrifying, denitrifying and anammox bacteria

3.2.1. Nitrifiers and DNB community structure

The results of nitrifier community analysis (general level) are shown in Fig. 2 (a). Nitrosomonas was the identified AOB, and its relative abundance showed a slight increase along with the increase in aeration frequency (from 1.52% to 2.16%). Nitrospira was the core genus of NOB and varied significantly between different aeration conditions. The relative abundances of NOB in PN-S1 and PN-S2 were 1.96% and 1.55%, respectively, and the abundance decreased to 0.71% after HFMA

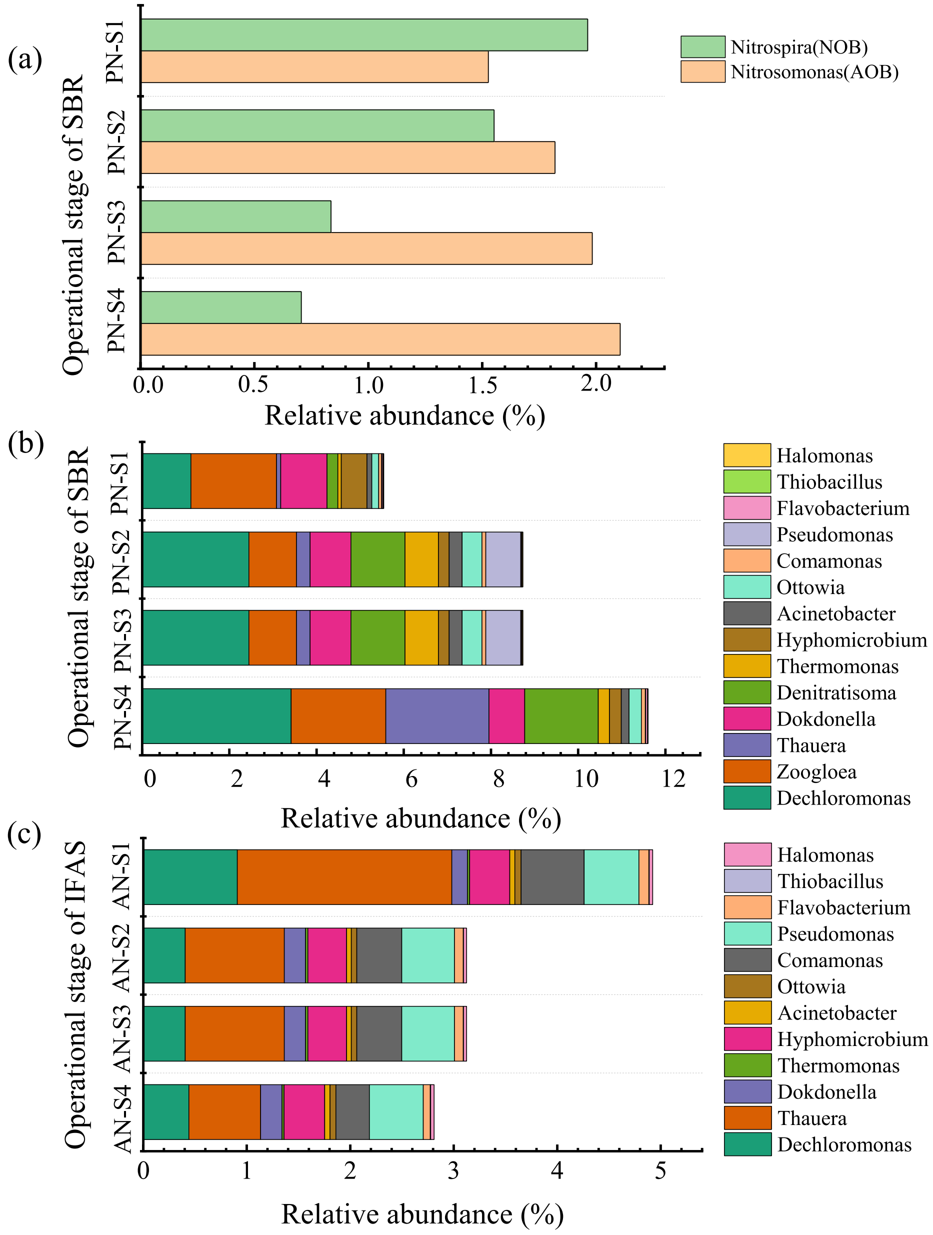


Fig. 2. Relative abundance of nitrifying bacteria (a) and DNB in SBR (b) and IFAS reactor (c).

application (PN-S4). The Pearson correlation coefficient of relative abundance of NOB and NAR was −0.984, indicating that the decrease in NOB population determined the partial nitrification efficiency of HFMA. Previous studies demonstrated that the inhibition of NOB could be enhanced by utilizing the activity discrepancy between AOB and NOB (Ge et al., 2014). The low DO concentration condition adopted in HFMAs system and control groups was efficient in widened the discrepancy in AOB and NOB activity. However, the average DO concentration during aeration for the four operational stages was much lower than the O_2 saturation constant of NOB (1.2–1.5 mg/L). Therefore, the decrease in DO concentration after the application of HFMA was not the core reason for the difference between the HFMA and the control groups. Meanwhile, the alternating aeration/anoxic condition determined by the parameter of aeration frequency is conductive in NOB inhibition. Hence, the difference in NOB inhibition between the HFMA system and the control groups was mainly attributed to the selective pressure on NOB proliferation caused by the continuous aeration/anoxic alternation under high aeration frequency conditions.

The microbial community structure of DNB in the SBR is shown in Fig. 2(b). In the PN-S1, DNB accounted for 5.54% of the total population, and Dechloromonas, Zooglea and Dokdonella were the dominant DNB genera (Zheng et al., 2019). The proliferation of DNB was enhanced following an increase in aeration frequency. In the PN-S4, the relative abundance of DNB reached its maximum value (12.6%), and the microbial community structure of DNB also changed significantly. Furthermore, the consumption of NO_2^--N by DNB did not lead to a reduction in the NO_2^--N concentration in the produced wastewater of SBR. In contrast, with an increase in the activity and abundance of DNB, the NAR of the effluent gradually increased. In the current study, the NO_2^--N consumption by DNB may exert influence on NOB proliferation and promoted the efficient and stable partial nitrification.

The microbial community structure of DNB in IFAS is shown in Fig. 2 (c). Integrating with the results of the microbial community structure of DNB in SBR Fig. 2(b), it can be noted that the relative abundance of DNB in IFAS decreased gradually with the enrichment of DNB in SBR. The main reason for the changes in DNB is that the application of HFMA mode in the SBR prompted DNB in the reactor to make full use of easily consumable substrates (e.g., biodegradable organic matter), resulting in a decrease in the substrate concentration in the IFAS influent, which led to a decrease in the abundance of DNB. The downward trend of DNB abundance in IFAS consequently provided a more suitable growth environment for anammox microorganisms at low substrate concentrations. In general, the application of HFMA mode in the SBR exerts an influence on the growth of DNB, and ultimately affects the growth of anammox and nitrogen removal efficiency.

3.2.2. *Community structure of anammox bacteria*

The identified anammox species included Candidatus Brocadia, Candidatus Kuenenia and Candidatus Jettenia. Candidatus Brocadia was dominant in the anammox microbial community, and its relative abundance was increased from 15.35% to 21.27% after the application of HFMA condition (Table 2). The Pearson correlation coefficient of relative abundance of Anammox bacteria and TN removal efficiency was 0.976, indicating that the enrichment of Anammox bacteria under HFMA contributed to the improvement in TN removal. Candidatus Kuenenia was the second most abundant species, and its population reached the highest (2.16%) in the stage AN-S4 with a HFMA condition. Previous studies demonstrated that the Candidatus Kueneni genus has a smaller half-saturation index than other anammox microorganisms, which is conductive to its growth under the conditions with low NH_4^+-N concentrations (Oshiki et al., 2016). Additionally, some studies have pointed that there is a symbiotic relationship between Candidatus Brocadia and Candidatus Kuenenia, and the growth of the former can provide secondary metabolites for the latter (Yue et al., 2019). Therefore, in AN-S4 stage, the enrichment of Candidatus Brocadia promoted the proliferation of Candidatus Kuenenia.

As previously mentioned in Section 3.2.1, the HFMA strategy employed in SBR could exerted influence on the growth of anammox bacteria in IFAS reactor. In order to explore the potential influence of aeration conditions, the correlation between DNB and anammox community in IFAS was analyzed using the correlation analysis method. As is shown in Fig. 3, there was a significantly negative correlation between the pre-dominant DNB genera (including Dechloromonas and Thauera) and anammox bacteria (correlation coefficient less than −0.6). The results proved that the decrease of the DNB population in IFAS ultimately contributed to the enhancement of anammox and wastewater treatment performance. It was discussed in section 3.2.1 that the relative abundance of DNB in IFAS decreased gradually with the enrichment of DNB in SBR, which was caused by the application of HFMA mode. Therefore, the results of correlation analysis confirmed that the application of HFMA improved the growth of DNB in SBR, and thereby enabled the anammox bacteria in the HFMA system has a better performance in coping with low NH_4^+-N concentration conditions.

3.3. *The linkage between HFMA mode and microbial metabolic interactions*

3.3.1. *Overview of microbial metabolism*

As is shown in Fig. 4(a), for the samples from SBR, gene counts for the majority of metabolic pathways were increased after the application of HFMA mode. The significantly upregulated genes were mainly associated with basic metabolism of microorganisms, including energy metabolism, amino acid metabolism and carbohydrate metabolism pathways. It has been well recognized that energy and amino metabolism are the basis for the proliferation of nitrifiers and DNB, and the activity of carbohydrate metabolism determines the efficiency of denitrification. Previous studies also demonstrated that in the partial nitrification-anammox process treating municipal wastewater, a moderate increase in denitrification performance is conducive to the growth of anammox bacteria (Li et al., 2020a). Generally, in the HFMA system, the enhancement in carbohydrate metabolism promoted the interactions between DNB and other functional microorganisms that carry out nitrogen removal, thus improving the overall nitrogen removal performance.

Table 2
Relative abundance of anammox bacteria (general level).

Operational stage	AN-S1	AN-S2	AN-S3	AN-S4
Relative abundance of Candidatus Brocadia (%)	15.35	17.76	18.73	21.27
Relative abundance of Candidatus Kuenenia (%)	0.75	0.91	1.16	2.16
Relative abundance of Candidatus Jettenia (%)	0.31	0.41	0.65	0.71
Relative abundance of anammox bacteria (%)	16.41	19.09	21.55	24.15

The results of differential metabolic pathway analysis also demonstrated that multiple significantly upregulated genes for the sample from SBR were involved in membrane transport and environmental information transduction processes, and the upregulation of these two functional genes is conducive to the realization and stable operation of partial nitrification (Wang et al., 2017b). Fig. 4(b) shows the differential metabolic pathways of microorganisms in the anammox reactor under different operational conditions. The abundance of genes in volved in substance metabolism and environmental information transduction pathways reached the highest value in AN-S4. This indicates that the interactions between anammox bacteria and other functional bacteria were strengthened under the HFMA conditions.

3.3.2. *Effects of HFMA mode on functional genes and metabolic interactions*

Partial nitrification and denitrification were carried out simultaneously in the SBR because the municipal wastewater contained a certain number of organic substances. Fig. 5(a) shows the functional genes in SBR microorganisms associated with nitrification and denitrification. The amoA is functional gene that carry out ammonia oxidation (Limpiyakorn et al., 2011), and the relative abundance of the amoA did not change significantly. The relative abundance of NxrA, which is the functional gene for NO_2^--N oxidation (Wang et al., 2015a), decreased by 0.03% in the HFMA system, and the variation trend of NxrA was consistent with that of the NOB population shown in Section 3.2.1. For the denitrification process, NarG is a key functional gene for the reduction of NO_3^--N to NO_2^--N (Jiang et al., 2019), and its abundance increased by 0.02% in the HFMA system. NirK is the gene responsible for NO_2^--N to N_2 transformation (Huang et al., 2017), and the increase in the relative abundance of NirK (0.04%) was 0.02% higher than that of NarG. This indicates that the application of HFMA mode selectively promotes the utilization of NO_2^--N by DNB, which could consume the substrate of NOB. Hence, the changes in functional genes in the HFMA system regulate the metabolic interactions among nitrifying and denitrifying microorganisms, which promotes the microbial community more favorable for partial nitrification (Fig. 5(d)).

According to information from the KEGG database, nitrogen metabolism pathways are directly connected with arginine metabolism, glutamate metabolism and carbon fixation pathways, which have a great influence on nitrogen transformation. The gene abundance in the metabolic pathways of the SBR microorganisms is shown in Fig. 5(c). The increase in the abundance of genes in the arginine and glutamate metabolism pathways promotes the occurrence of partial nitrification (Lv et al., 2019). Hence, in the HFMA system, the improvement in arginine and glutamate metabolic activity was one of the reasons for the enhancement in growth rate and activity of microorganisms carry out partial nitrification and denitrification (Fig. 5(d)).

Moreover, as pointed out in Section 3.3.1, the improvement in the caron source utilization capacity of DNB can promote the stability of the partial nitrification performance. Therefore, analyses were conducted on the pathways associated with carbon source metabolism to explore

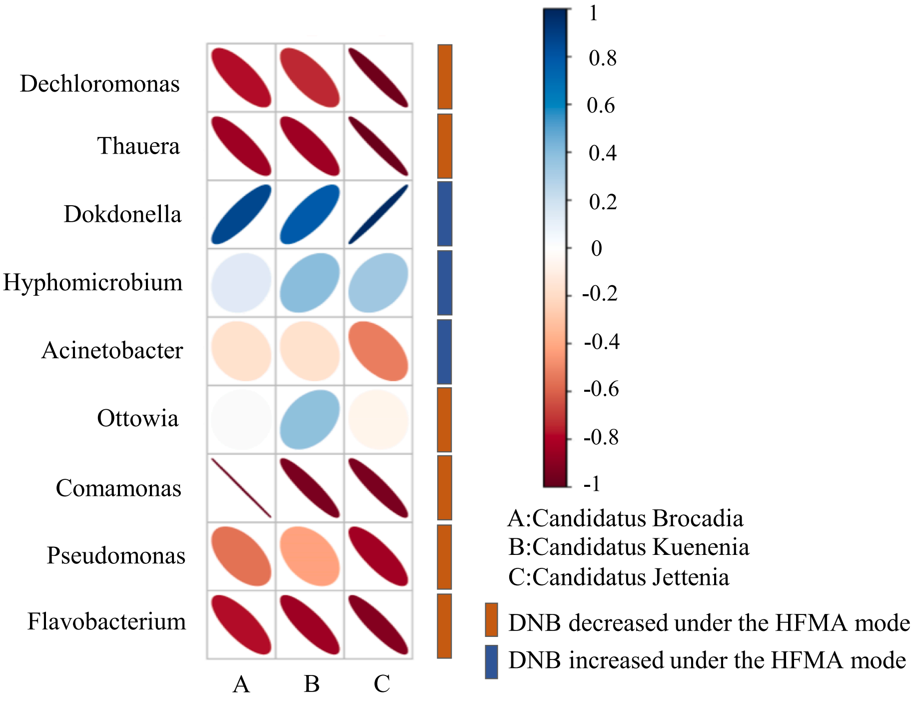


Fig. 3. The correlation of DNB with anammox bacteria community in IFAS.

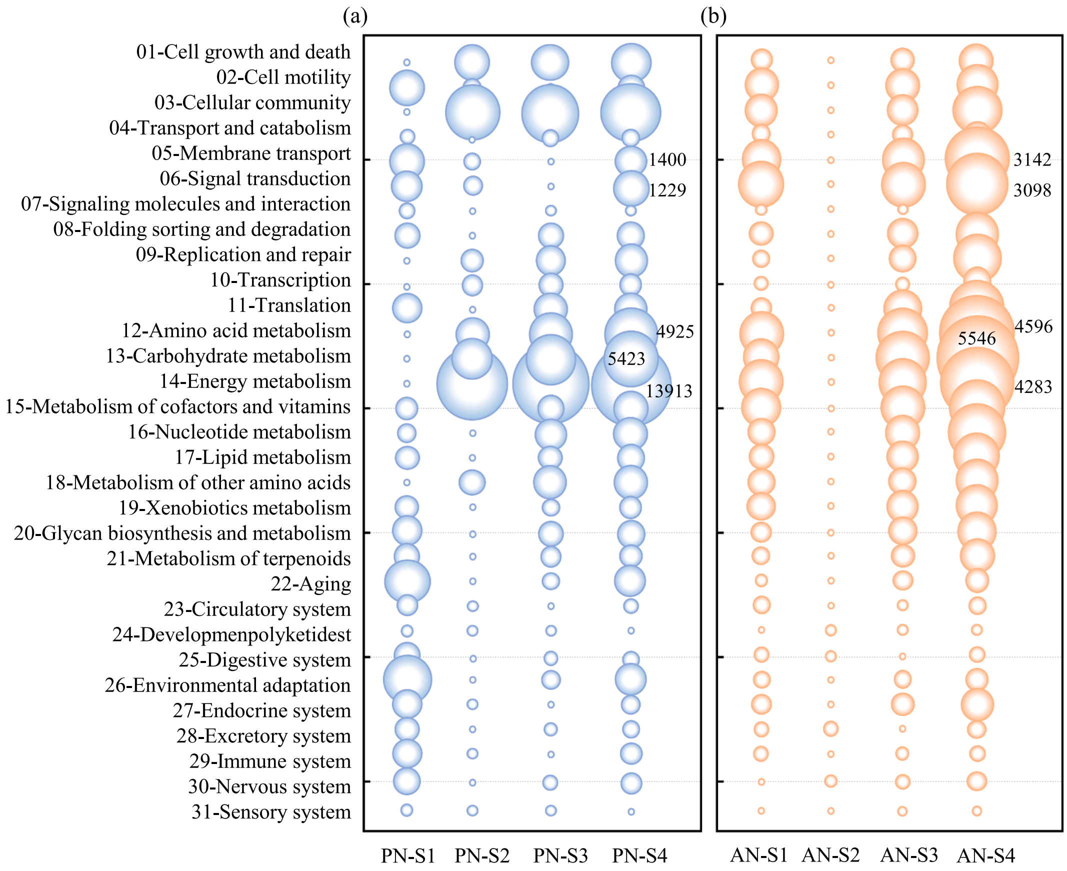


Fig. 4. Overview of microbial metabolism in (a) SBR and (b) IFAS. The area of circle represents difference value between the abundance of genes in the sample and the lowest value of the abundance of these genes in four groups.

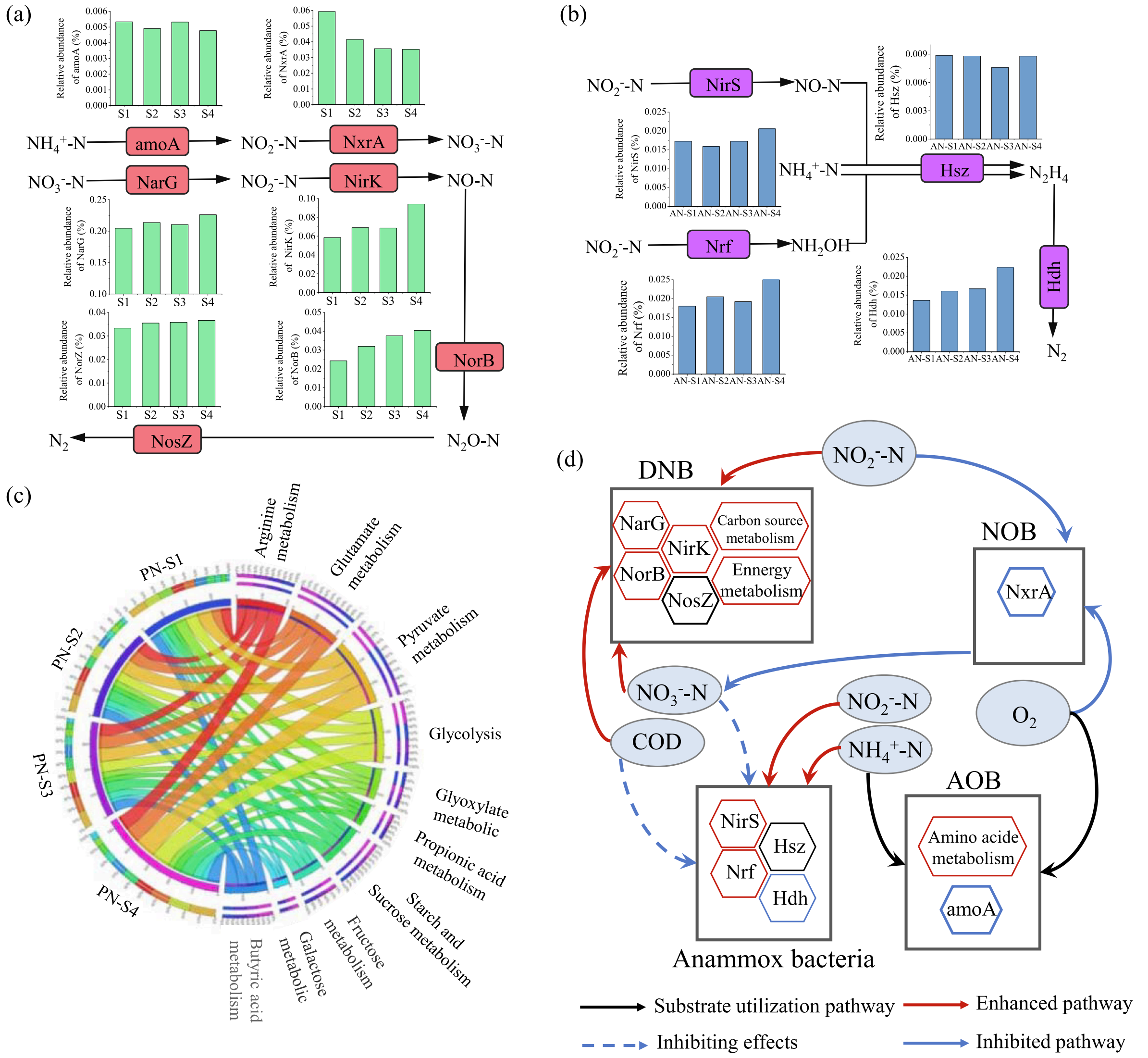


Fig. 5. Metabolic pathways related to nitrogen removal in SBR (a) and IFAS (b). The amino acid metabolism and carbohydrate metabolism pathway in SBR (c). Schematic diagram of microbial metabolic interactions under the HFMA mode (d).

the internal mechanism causing changes in the metabolic characteristics of DNB. As is shown in Fig. 5(c), pyruvate metabolism, glycolysis, butyric acid metabolism and six other carbon source metabolic pathways showed an up-regulated trend. The results confirmed that the improvement in DNB metabolic activity under the HFMA mode was caused by the variety of carbon source utilization, and finally promoted the competition between DNB and NOB (Fig. 5(d)).

The metabolic mode of functional microorganisms in the anammox reactive stage is shown in Fig. 5(b). To adapt to the fluctuating external environment, there are two main patterns of nitrogen metabolism in the anammox community (Yang et al., 2020). Part of the anammox (i.e., Candidatus Kuenenia) uses NO_2^--N as a substrate and form NO under the catalysis of nitrite reductase (NO-forming) (NirS). The produced NO binds with NH_4^+N to form hydrazine (N_2H_4) under the action of the hydrazine synthase subunit (Hsz). Finally, N_2H_4 was gradually transformed into N_2 under the action of hydrazine dehydrogenase (Hdh). Other kinds of anammox (i.e., Candidatus Brocadia) prefer to convert NO_2^--N into hydroxylamine (NH_2OH) catalyzed along by Nitrite reductase (cytochrome *c*-552) (Nrf) (Wang et al., 2021). The results of metagenomic sequencing and analysis showed that the relative abundance of Nrf and Hdh showed an upward trend. This also indicated that the activity of functional genes carrying out anammox was enhanced when the HFMA mode was employed. In addition, the gap between the relative abundance of NirK and Nrf was increased after the application of HFMA mode. The changes in these two functional genes suggested that the HFMA mode promoted the activity of anammox with NH_2OH as the intermediate metabolite, thereby causing changes in the microbial community structure of the anammox community.

In conclusion, during the partial nitrification-denitrification reaction phase, the application of HFMA mode strengthened the selective suppression of NO_2^--N oxidation activity and the metabolic interactions between NOB and DNB, and further provided a foundation for the subsequent anammox reaction phase by improving the consumption activity of NO_3^--N and organic substrates (Fig. 5(d)). The influence of the influent composition, which includes both nitrogen composition and microbial metabolites, on the growth of anammox bacteria has been widely recognized in many studies(Zhang et al., 2021). Therefore, in the current study, the effect of HFMA on the microbial metabolic characteristics and the corresponding nitrogen conversion pathways in the partial nitrification phase eventually affected the metabolic activity and pattern of anammox bacteria, leading to changes in the growth of functional microorganisms and the overall anammox performance.

3.4. Potential of PN-A with HFMA mode for advanced nitrogen removal in municipal wastewater treatment

The current study provides a novel approach to achieve high-efficiency partial nitrification and anammox in municipal wastewater treatment processes by introducing HFMA conditions. Previous studies have demonstrated that intermittent aeration conditions are conducive to partial nitrification (Xu et al., 2020), but due to the characteristics of municipal wastewater, it is usually necessary to combine with other inhibiting factors to improve partial nitrification (Wang et al., 2017a). Compared with the addition of inhibitors, the optimal operating conditions, which were proposed based on revealing of the effect of aeration frequency on partial nitrification performance, reduced the operational complexity and broadened the thought for optimization of the partial nitrification system. Moreover, the low strengthening environment in municipal wastewater treatment systems is a disadvantage for the growth of anammox bacteria, and they become more sensitive to the influent components and have more complex metabolic and regulatory mechanisms (Ji et al., 2018). In this study, based on the results of metagenomic sequencing, it was found that the application of HFMA mode and the corresponding changes in SBR effluent caused changes in microbial metabolism during the subsequent anammox period, especially with respect to nitrogen metabolism and environmental perception. The exploration of the relationship between the operating conditions and metabolic characteristics of functional microorganisms is beneficial for improving anammox efficiency.

At present, many municipal wastewater treatment plants in China are faced with the situation of low influent carbon source concentration, (Wang et al., 2015b) and the potential for energy recovery is limited (Lu et al., 2019). Therefore, the carbon sources in municipal wastewater are mainly consumed during the biological nitrogen removal process and cause changes in the DNB community. Under the HFMA mode, succession in DNB leads to a decrease in COD and NO_3^--N concentrations in the IFAS, thereby promoting the propagation of anammox bacteria. Moreover, since the metabolite of DNB has the potential to promote the growth of anammox microorganisms (Zhang et al., 2021), the effects of carbon sources exert on DNB will eventually affect the basic metabolism of anammox microorganisms, thus improving the stability of anammox performance. Last but not the least, due to the high O_2 transfer efficiency under the micro-aeration conditions, the demand for O_2 in the high-frequency micro-aeration system was reduced by approximately 32.5%, and the energy consumption was significantly reduced. In conclusion, the high-frequency micro-aeration PN-A system has the advantages of improving the metabolic activity of functional microorganisms, low energy consumption and low carbon source demand, which has the potential for advanced nitrogen removal in municipal wastewater treatment, especially for treatment of municipal wastewater with a low carbon/ nitrogen ratio.

4. Conclusions

In this study, NOB inhibition and anammox bacteria proliferation were improved by employing the HFMA mode with aeration frequency of 15 times/h and DO concentration lower than 0.5 mg/L. The metabolic activity related to NO_2^--N oxidation decreased, and the functional genes carried out NO_3^--N reduction and carbon source consumption up-regulated. These variations in microbial metabolism strengthened the expression of anammox bacteria with NH_2OH as an intermediate metabolite and the metabolic interactions between functional microorganisms, which improved NAR, nitrogen removal efficiency and abundance of anammox bacteria of HFMA process (an increase of 20.88%, 20.11% and 5.92%, respectively).

CRediT authorship contribution statement

Yulei Chi: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Xuan Shi:** Conceptualization, Data curation, Investigation, Methodology, Visualization. **Pengkang Jin:** Funding acquisition, Project administration, Resources, Supervision. **Xiaochang C. Wang:** Conceptualization, Validation, Visualization. **Tong Ren:** Formal analysis, Software, Visualization. **Bo Ren:** Software, Investigation. **Xin Jin:** Investigation, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the Key Research and Development Project of Shaanxi Province (2021ZDLSF05-06), the National Key Research and Development Program (2016YFC0400701), and the Shaanxi Science & Technology Co-ordination & Innovation Project (2016TZC-S-19-3).

References

Agrawal, S., Seuntjens, D., Cocker, P.D., Lackner, S., Vlaeminck, S.E., 2018. Success of mainstream partial nitritation/anammox demands integration of engineering, microbiome and modeling insights. Curr. Opin. Biotechnol. 50, 214–221.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.

Chen, R., Ji, J., Chen, Y., Takemura, Y., Liu, Y., Kubota, K., Ma, H., Li, Y.Y., 2019. Successful operation performance and syntrophic micro-granule in partial nitritation and anammox reactor treating low-strength ammonia wastewater. Water Res. 155, 288–299.

Chen, Y., Zhao, Z., Liu, H., Ma, Y., An, F., Huang, J., Shao, Z., 2020. Achieving stable two-stage mainstream partial-nitrification/anammox (PN/A) operation via intermittent aeration. Chemosphere 245, 125650.

Chi, Y., Zhang, X., Shi, X., Ren, T., Jin, X., Jin, P., 2021. Quick start-up of partial nitrification in a novel anaerobic/ pulse washout (APW) process for treating municipal wastewater. J. Cleaner Prod. 285, 124850.

Choi, D., Cho, K., Jung, J., 2019. Optimization of nitrogen removal performance in a single-stage SBR based on partial nitritation and ANAMMOX. Water Res. 162, 105–114.

Ge, S., Peng, Y., Qiu, S., Zhu, A., Ren, N., 2014. Complete nitrogen removal from municipal wastewater via partial nitrification by appropriately alternating anoxic/ aerobic conditions in a continuous plug-flow step feed process. Water Res. 55, 95–105.

Guo, C., Fu, W., Chen, X., Peng, D., Jin, P., 2013. Nitrogen-removal performance and community structure of nitrifying bacteria under different aeration modes in an oxidation ditch. Water Res. 47 (11), 3845–3853.

Huang, X., Dong, W., Wang, H., Jiang, S., 2017. Biological nutrient removal and molecular biological characteristics in an anaerobic-multistage anaerobic/oxic (A-MAO) process to treat municipal wastewater. Bioresour. Technol. 241, 969–978.

Ji, J., Peng, Y., Mai, W., He, J., Wang, B., Li, X., Zhang, Q., 2018. Achieving advanced nitrogen removal from low C/N wastewater by combining endogenous partial denitrification with anammox in mainstream treatment. Bioresour. Technol. 270, 570–579.

Jiang, C., Xu, S., Wang, R., Feng, S., Zhou, S., Wu, S., Zeng, X., Wu, S., Bai, Z., Zhuang, G., Zhuang, X., 2019. Achieving efficient nitrogen removal from real sewage via nitrite pathway in a continuous nitrogen removal process by combining free nitrous acid sludge treatment and DO control. Water Res. 161, 590–600.

Kowalski, M.S., Devlin, T.R., di Biase, A., Oleszkiewicz, J.A., 2019. Effective nitrogen removal in a two-stage partial nitritation-anammox reactor treating municipal wastewater - piloting PN-MBBR/AMX-IFAS configuration. Bioresour Technol 289, 121742.

Laureni, M., Weissbrodt, D.G., Villez, K., Robin, O., de Jonge, N., Rosenthal, A., Wells, G., Nielsen, J.L., Morgenroth, E., Joss, A., 2019. Biomass segregation between biofilm and flocs improves the control of nitrite-oxidizing bacteria in mainstream partial nitritation and anammox processes. Water Res. 154, 104–116.

Li, J., Li, J., Peng, Y., Wang, S., Zhang, L., Yang, S., Li, S., 2020a. Insight into the impacts of organics on anammox and their potential linking to system performance of sewage partial nitrification-anammox (PN/A): a critical review. Bioresour. Technol. 300, 122655.

Li, J., Peng, Y., Zhang, L., Gao, R., Yang, L., Liu, Q., Zhang, Q., Li, X., Wang, S., 2020b. Enhanced nitrogen removal assisted by mainstream partial-anammox from real sewage in a continuous flow A^2/O reactor. Chem. Eng. J. 400, 125893.

Li, S., Li, J., Yang, S., Zhang, Q., Li, X., Zhang, L., Peng, Y., 2021. Rapid achieving partial nitrification in domestic wastewater: Controlling aeration time to selectively enrich ammonium oxidizing bacteria (AOB) after simultaneously eliminating AOB and nitrite oxidizing bacteria (NOB). Bioresour. Technol. 328, 124810.

Li, W., Zhuang, J.L., Zhou, Y.Y., Meng, F.G., Kang, D., Zheng, P., Shapleigh, J.P., Liu, Y. D., 2020c. Metagenomics reveals microbial community differences lead to differential nitrate production in anammox reactors with differing nitrogen loading rates. Water Res. 169, 115279.

Limpiyakorn, T., Sonthiphand, P., Rongsayamanont, C., Polprasert, C., 2011. Abundance of amoa genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. Bioresour. Technol. 102 (4), 3694–3701.

Liu, G., Wang, J., 2013. Long-term low DO enriches and shifts nitrifier community in activated sludge. Environ. Sci. Technol. 47 (10), 5109–5117.

Lu, J.Y., Wang, X.M., Liu, H.Q., Yu, H.Q., Li, W.W., 2019. Optimizing operation of municipal wastewater treatment plants in China: the remaining barriers and future implications. Environ. Int. 129, 273–278.

Lv, Y., Pan, J., Huo, T., Zhao, Y., Liu, S., 2019. Enhanced microbial metabolism in one stage partial nitritation-anammox system treating low strength wastewater by novel composite carrier. Water Res. 163, 114872.

Miao, Y., Zhang, L., Li, B., Zhang, Q., Wang, S., Peng, Y., 2017. Enhancing ammonium oxidizing bacteria activity was key to single-stage partial nitrification-anammox system treating low-strength sewage under intermittent aeration condition. Bioresour. Technol. 231, 36–44.

Oshiki, M., Satoh, H., Okabe, S., 2016. Ecology and physiology of anaerobic ammonium oxidizing bacteria. Environ. Microbiol. 18 (9), 2784–2796.

Sliekers, A.O., Haaijer, S.C., Stafsnes, M.H., Kuenen, J.G., Jetten, M.S., 2005. Competition and coexistence of aerobic ammonium- and nitrite-oxidizing bacteria at low oxygen concentrations. Appl. Microbiol. Biotechnol. 68 (6), 808–817.

Wang, D., Zheng, Q., Huang, K., Springael, D., Zhang, X.-X., 2020. Metagenomic and metatranscriptomic insights into the complex nitrogen metabolic pathways in a single-stage bioreactor coupling partial denitrification with anammox. Chem. Eng. J 398, 125653.

Wang, H., Ji, G., Bai, X., He, C., 2015a. Assessing nitrogen transformation processes in a trickling filter under hydraulic loading rate constraints using nitrogen functional gene abundances. Bioresour. Technol. 177, 217–223.

Wang, H., Xu, G., Qiu, Z., Zhou, Y., Liu, Y.J.C., 2019. NOB suppression in pilot-scale mainstream nitritation-denitritation system coupled with MBR for municipal wastewater treatment. Chemosphere 216, 633–639.

Wang, Q., Duan, H., Wei, W., Ni, B.J., Laloo, A., Yuan, Z., 2017a. Achieving stable mainstream nitrogen removal via the nitrite pathway by sludge treatment using free ammonia. Environ. Sci. Technol. 51 (17), 9800–9807.

Wang, S., Deng, L., Zheng, D., Wang, L., Zhang, Y., Yang, H., Jiang, Y., Huang, F., 2018. Control of partial nitrification using pulse aeration for treating digested effluent of swine wastewater. Bioresour. Technol. 262, 271–277.

Wang, S., Ishii, K., Yu, H., Shi, X., Smets, B.F., Palomo, A., Zuo, J., 2021. Stable nitrogen removal by anammox process after rapid temperature drops: Insights from metagenomics and metaproteomics. Bioresour. Technol. 320, 124231.

Wang, X., Wang, S., Xue, T., Li, B., Dai, X., Peng, Y., 2015b. Treating low carbon/nitrogen (C/N) wastewater in simultaneous nitrification-endogenous denitrification and phosphorous removal (SNDPR) systems by strengthening anaerobic intracellular carbon storage. Water Res. 77, 191–200.

Wang, Z., Liu, X., Ni, S.Q., Zhang, J., Zhang, X., Ahmad, H.A., Gao, B., 2017b. Weak magnetic field: a powerful strategy to enhance partial nitrification. Water Res. 120, 190–198.

Xu, Z., Zhang, L., Gao, X., Peng, Y., 2020. Optimization of the intermittent aeration to improve the stability and flexibility of a mainstream hybrid partial nitrification-anammox system. Chemosphere 261, 127670.

Yang, Y., Pan, J., Zhou, Z., Wu, J., Liu, Y., Lin, J.G., Hong, Y., Li, X., Li, M., Gu, J.D., 2020. Complex microbial nitrogen-cycling networks in three distinct anammox-inoculated wastewater treatment systems. Water Res. 168, 115142.

Yue, H., Zhang, Y., He, Y., Wei, G., Shu, D., 2019. Keystone taxa regulate microbial assemblage patterns and functional traits of different microbial aggregates in simultaneous anammox and denitrification (SAD) systems. Bioresour. Technol. 290, 121778.

Zhang, T., Yin, Q., Shi, Y., Wu, G., 2021. Microbial physiology and interactions in anammox systems with the intermittent addition of organic carbons. Bioresour. Technol. 319, 124226.

Zheng, Z., Huang, S., Bian, W., Liang, D., Wang, X., Zhang, K., Ma, X., Li, J., 2019. Enhanced nitrogen removal of the simultaneous partial nitrification, anammox and denitrification (SNAD) biofilm reactor for treating mainstream wastewater under low dissolved oxygen (DO) concentration. Bioresour. Technol. 283, 213–220.

Zubrowska-Sudol, M., Walczak, J., 2014. Effects of mechanical disintegration of activated sludge on the activity of nitrifying and denitrifying bacteria and phosphorus accumulating organisms. Water Res. 61, 200–209.