JOURNAL OF ENVIRONMENTAL SCIENCES 124 (2023) 952–962

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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Metagenomic insights into responses of microbial population and key functional genes to fulvic acid during partial nitritation

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ARTICLE INFO

Article history:
Received 24 December 2021
Revised 1 March 2022
Accepted 1 March 2022
Available online 11 March 2022

Keywords:
Partial nitritation
Fulvic acid
Microbial community
Functional genes
Metagenomic

ABSTRACT

The long-term impact of fulvic acid (FA) on partial nitritation (PN) system was initially examined in this study. The obtained results revealed that the FA lower than 50 mg/L had negligible effect on the nitrite accumulation rate (NAR nearly 100%) and ammonium removal rate (ARR 56.85%), while FA over 50 mg/L decreased ARR from 56.85% to 0.7%. Sludge characteristics analysis found that appropriate FA (<50 mg/L) exposure promoted the settling performance and granulation of PN sludge by removing *Bacteroidetes* and accumulating *Chloroflexi*. The analysis of metagenomics suggested that the presence of limited FA (0-50 mg/L) stimulated the generation of NADH, which favors the denitrification and nitrite reduction. The negative impact of FA on the PN system could be divided into two stages. Initially, limited FA (50-120 mg/L) was decomposed by *Anaerolineae* to stimulate the growth and propagation of heterotrophic bacteria (*Thauera*). Increasing heterotrophs competed with AOB (*Nitrosomonas*) for dissolved oxygen, causing AOB to be eliminated and ARR to declined. Subsequently, when FA dosage was over 120 mg/L, *Anaerolineae* were inhibited and heterotrophic bacteria reduced, resulting in the abundance of AOB recovered. Nevertheless, the ammonium transformation pathway was suppressed because genes *amoABC* and *hao* were obviously reduced, leading to the deterioration of reactor performance. Overall, these results provide theoretical guidance for the practical application of PN for the treatment of FA-containing sewage.

Introduction

Anaerobic ammonium oxidation (anammox) is a promising autotrophic nitrogen removal process that has been developed as a cost-effective, energy-saving, and environmentally friendly alternative to traditional biological nitrogen removal approaches (Guo et al., 2022; Chen et al., 2019). In this process, anammox microbes use inorganic carbon as a carbon source to directly convert nitrite and ammonium to nitrogen gas in the absence of oxygen (Eq. (1)) (Strous et al., 1998). For Anammox, thus, maintaining a steady source of nitrite is key. At

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https://doi.org/10.1016/j.jes.2022.03.003
1001-0742/

present, common nitrite generation processes include partial nitritation (PN) and partial denitrification.

$$NH_4^+ + 1.32NO_2^- \rightarrow 1.02N_2 + 0.26NO_3^- + 2H_2O \quad (1)$$

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (2)$$

As one of the pretreatment processes of anammox, PN has also become a research hotspot (Cui et al., 2020a; Zhang et al., 2019). In this process, ammonia oxidizing bacteria (AOB) first converts ammonium to nitrite over hydroxylamine, and then nitrite oxidizing bacteria (NOB) further transfers nitrite to nitrate. Thus, the crucial feature of this technology is to accumulate or facilitate AOB and eliminate or suppress NOB to achieve the accumulation of nitrite according to their different physiological characteristics (Chu et al., 2020). In contrast to partial nitrification, PN in this study only converted 50% of the ammonium to nitrite (Eq. (2)), thus resulting in a nitrite/ammonium ratio that was close to that of the theoretical stoichiometry of the anammox reaction (1.32: 1). Therefore, it is important to achieve stable nitrite accumulation and to reduce nitrate generation in the PN system to allow for nitrogen removal in the subsequent anammox process (Huang et al., 2020).

In some practical projects, the composition of sewage is relatively complex and contains not only nitrogen but also organic matter (Sheng et al., 2018). Therefore, attention should be paid to the influence of organic matters in the PN process. Wang et al. (2020)have demonstrated that with chemical oxygen demand (COD: supplied with glucose) concentration from 0 to 150 mg/L, the mean ammonium removal efficiency and nitrite production rate reduced by 57.3% and 87.5%, respectively. Apart from the easily degradable organic compounds represented by glucose and acetate, refractory organics are also concerned. Early studies showed that the suppression range of phenol on nitrification process was 2.6-20 mg/L (Zhou et al., 2019). Li et al. (2020a) found that adding 100 mg/L tetracycline still had no significant inhibitory effect on PN system. Generally, the influence of organic matter on the PN system is linked to the matter type.

Humic substances (HS) are typical types of bio-refractory organic matter that not only exist in sewage but are also widely present in various aquatic systems such as freshwater (50% of dissolved organic matter) and groundwater (25% dissolved organic matter) (Zhang et al., 2020). HS are classified according to their molecular size, redox capacity, and solubility in liquid, and they include fulvic acid (FA), humic acid (HA), and humin (Martins et al., 2020). Among these, FA is the most abundant (accounting for 81%–95%) and dangerous of all HS, because it is soluble throughout the pH range (Linnik et al., 2013). Normally, surface water is dominated by FA, and the content is in the range of 1-100 mg/L (Łomińska-Płatek et al., 2018). Additionally, excessive amount of FA usually presents in industrial wastewater, such as landfill leachate. The content of organic matter (mg/L COD) in landfill leachate ranges from hundreds to tens of thousands (Fan et al., 2006), and FA represents 7%-72% (Artiola-Fortuny et al., 1982). Currently, in wastewater treatment plants (WWTPs), the sewage usually undergoes anaerobic digestion to remove degradable organic matter before entering the aerobic tank (such as PN). However, FA is produced during anaerobic digestion due to humification of organics (Guo et al., 2018). Therefore, PN process is always under the stress of FA.

Various studies have demonstrated that FA has a significant impact on biological technology, especially nitrogen removal processes. Dang et al. (2016) found that increasing FA influent concentration resulted in a decrease in biomethane production. In the research of Li et al. (2016), FA promoted nitrate reduction by stimulating the activity of key enzymes which related to metabolism of a carbon source and nitrogen recycle. In addition, Zhang et al. (2020) found that *Candidatus* Brocadia bacteria decreased, while *Candidatus* Jettenia and *Candidatus* Kuenenia bacteria were enriched after adding FA, resulting in changes in performance of anammox system. Furthermore, FA acts as an electron acceptor or electron shuttle to participate in some microbial oxidation processes (Luo et al., 2019). Therefore, it can be speculated that the occurrence of FA in PN reactors would also potentially shift the microbial composition structure and functional genes, resulting in fluctuation of the PN reactor performance. The unstable production or accumulation of nitrite will directly determine the efficiency of subsequent nitrogen removal processes, such as anammox or denitrification. Thus, revealing the interactions between FA and PN system would be of significance for the application of PN-based technology. However, to date the long-term effects of FA on the PN process and the potential mechanisms remain unclear. Therefore, to address this issue, metagenomic analysis was applied to offer comprehensive information of community structure, functional potentials, and key genes in complex microbial systems in this study.

Thus, the purposes of this study were to (1) comprehensively evaluate the effects of various concentrations of FA on the nitrogen transformation performance and sludge characteristics of PN and to (2) clarify the response of microbial community and functional genes related to nitrogen metabolism during PN process to FA. This study is the first to investigate the long-term effects of FA on PN systems and to examine the underlying mechanisms from gene level using metagenomic analysis. These results provide theoretical guidance for the practical application of PN for the treatment of wastewater containing various FA content.

1. Materials and methods

1.1. *Experimental setup and operation*

The experiment was conducted in a sequencing batch reactor (SBR) with a working volume of 8 L and a discharge ratio of 50%. The reactor configuration is presented in **Appendix A Fig. S1**. The SBR treatment cycle consisted of six operating stages that included filling (20 min), standing (40 min), reacting (120 min), settling (45 min), drainage (2 min), and idling (13 min), and this cycle was run six times on a typical day. The temperature was maintained at 30±2°C, and the dissolved oxygen (DO) content was 0.4±0.1 mg/L. $KHCO_3$ and NaOH were used to maintain an influent pH of 8.3. The excess sludge was discharged from the reactor for sampling. The experiment was conducted for 120 days and divided into Stage I (start-up) and

Table 1 – Operating conditions of the PN reactor.

Parameter	Stage I	Stage II			
		Stage II-1			Stage II-2
	Day 1-20	Day 21-28	Day 29-35	Day 36-50	Day 51-120
HRT (days)	8-12	8	8	8	8
DO (mg/L)	0.40±0.10	0.40±0.10	0.32-0.92	0.40±0.10	0.40±0.10
Influent ammonia content (mg NH_4^+-N/L)	150-300	290±10	290±10	290±10	290±10
Influent alkalinity (mg $CaCO_3$/L)	1000	3000	3000	3000	3000
FA (mg/L)	0	5-15	15-30	30-50	50-385
NAR (10^2%)	0.10-1	1	1	1	1
ARR (10^2%)	0.05±0.10	0.55±0.05	0.65±0.05	0.55±0.05	0-0.55

Note: HRT, NAR, and ARR represent the hydraulic retention time, ammonia transformation rate, and nitrite accumulation rate, respectively.

Stage II (impact). The detailed operating parameters are listed in Table 1.

1.2. *Sewage and seeding sludge*

In this study, wastewater was simulated by adding the following chemicals to deionized water: $(NH_4)_2SO_4$ (300 mg/L), $KHCO_3$ (3000 mg/L), KH_2PO_4 (260 mg/L), $MgSO_4•5H_2O$ (90 mg/L), $CaCl_2$ (11 mg/L), trace element I (2 mL/L), and trace element II (1 mL/L). Trace element I is composed of EDTA (15000 mg/L) and $FeSO_4$ (5000 g/L). Trace element II is composed of $ZnSO_4•7H_2O$ (430 mg/L), $MnCl_4•H_2O$ (990 mg/L), $CoCl_2•6H_2O$ (240 mg/L), $CuSO_4•5H_2O$ (250 mg/L), $NiCl•6H_2O$ (190 mg/L), $NaMoO_4•2H_2O$ (220 mg/L), H_3BO_4 (14 mg/L), and $NaSeO_4$ •$10H_2O$ (210 mg/L). All of the above chemicals were obtained from Sinopharm. FA was purchased from BASF Biotechnology (Beijing, China). Inoculated activated sludge was collected from the secondary sedimentation tank of the Beijing Gaobeidian Wastewater Treatment Plant (WWTP). The suspended matter concentration of the activated sludge-wastewater mixture was 2700 mg/L, and the ρ(VSS)/ρ(SS) was approximately 0.7.

1.3. *Analysis methods*

A 100-mL reactor supernatant was obtained during the sedimentation phase. Samples were collected every two days and tested after filtration through a 0.45-μm membrane. The NH_4^+-N content was determined using the indophenol blue spectrophotometric method, and the NO_3^--N and NO_2^--N contents were measured according to the colorimetric method (APHA, 2012). The DO content and pH were measured using a portable water quality analyzer (Mu 3620 IDS, WTW, Germany), and the organic carbon (TOC) concentrations were determined using a TOC analyzer (TOC-L CPH/CPN, Shimadzu, Japan). The 30-min sludge volume index (SVI_{30}) and mixed-liquor suspended solids concentration (MLSS) were determined regularly according to a standard method (APHA, 2012). A laser particle size analyzer (Microtrac S3500, USA) was used to measure the sludge particle size distribution.

1.4. *Calculations*

The ammonia removal rate (ARR), nitrite accumulation rate (NAR), and nitrogen loss efficiency (NLE) during PN were calculated using Eqs. (3) - (5).

$$ARR = (NH_4^+ - N_{Inf} - NH_4^+ - N_{Eff})/NH_4^+ - N_{Inf} \times 100\% \quad (3)$$

$$NAR = (NO_2^- - N_{Eff} - NO_2^- - N_{Inf})/(NH_4^+ - N_{Inf} - NH_4^+ - N_{Eff}) \times 100\% \quad (4)$$

$$NLE = (TN_{Inf} - TN_{Eff})/TN_{Inf} \times 100\% \quad (5)$$

where NH_4^+-N_{Inf} and NH_4^+-N_{Eff} are the influent and effluent ammonia concentrations, respectively, NO_2^--N_{Inf} and NO_2^--N_{Eff} are the influent and effluent nitrite concentrations, respectively, and TN_{Inf} and TN_{Eff} are the influent and effluent total nitrogen concentrations, respectively.

1.5. *Metagenomic sequencing analysis*

Samples from the PN reactor were collected on day 20 (FA0), day 50 (FA50), day 85 (FA200), and day 120 (FA385), and sample DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). A TBS-380 and a NanoDrop2000 were used to detect the concentration and purity of the extracted DNA, respectively. Covaris M220 (Gene Company Limited, China) was used to construct a paired library of DNA extracts with an average size of approximately 400 bp. Next, the paired-end library was constructed using NextFLextM Rapid DNA-seq (Bio Scientific, Austin, TX, USA). An adapter containing the complete sequencing primer hybridization site was attached to the blunt end of the fragment. Paired-end sequencing was performed on an Illumina Hiseq Xten (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Clean reads were generated by adaptor sequences, pruning, and removing low-quality raw reads from metagenomic sequencing using FASTP19 (V0.20.0) on the free online MajorBio cloud platform (cloud.majorbio.com). Then, these high-quality reads were assembled into contigs by Megahit

(V1.1.2)[20] utilizing a concise de Bruijn graph. Contigs possessing lengths of over 300 bp were selected as the final assembly results.

Open reading frames (ORFs) were identified using MetaGene. ORFs possessing predicted lengths of greater than or equal to 100 bp were transcribed into amino acid sequences using the NCBI translation table. CD-HIT (V4.6.1) was used to construct the non-redundant gene catalog with a sequence identity and coverage of 90%. SOAPaligner (V2.21) was used to map quality-controlled reads to a non-redundant gene list with 95% identity, and the gene abundance in all samples was then evaluated.

KEGG annotation was performed according to the Kyoto Encyclopedia of Genes and Genomes Database (V94.2) using Diamond (V0.8.35), and the e-value was truncated by $1e^{-5}$. Functional genes related to nitrogen metabolism were identified by searching for the KO number within the functional annotation results. The relative abundance of functional genes refers to the proportion of matched reads within the total number of effective reads.

2. Results and discussion

2.1. *Effect of FA on PN performance*

The transformation of nitrogen and the performance of nitrite accumulation and ammonia removal are presented in Fig. 1a-b. The first 20 days were defined as Stage I and included FA free in the PN reactor. As presented in Fig. 1b, an ammonium removal rate (ARR) of 59.96% and a nitrite accumulation rate (NAR) of nearly 100% were obtained during days 12-20, thus indicating that the PN process was established.

During Stage II-1 (days 21-50), an increasing content of FA (5 to 50 mg/L) was added to the PN reactor. Initially, fluctuations were observed on days 21-35. From day 36 to day 50, the PN reactor exhibited a stable running state where the average effluent nitrite concentration and ARR were 137.52 mg/L and 56.85%, respectively. During this period, the AOB in the PN-SBR gradually adapted to the environment and FA ranged from 30 to 50 mg/L. Furthermore, the NAR was almost 100%, as there was almost no NO_3^- produced and an appropriate Eff NO_2^-/Eff NH_4^+ ratio of approximately 1.2 was maintained for subsequent anammox processes (Zhang et al., 2011). These results imply that PN system possesses a certain tolerance to low-dose FA (5–50 mg/L). Luo et al. (2019) also reported that the presence of HS (0–50 mg/L) in sewage streams did not significantly impact nitrite products during the long-term test; however, fluctuations were observed due to sudden humic substance loads.

During Stage II-2 (days 51–120), the PN system was destroyed in a step-by-step manner. Compared to the levels on day 50 (FA 50 mg/L), the effluent nitrite and ARR suddenly decreased to 95.53 mg/L and 38.30%, respectively, when the FA addition was 60 mg/L on day 51. These results indicated that FA addition worsened the ammonium conversion performance, which is exacerbated with increasing FA. When the dosage of FA finally peaked at 385 mg/L from days 118 to 120, there was no conversion of ammonium, and this suggested that the reactor was completely destroyed.

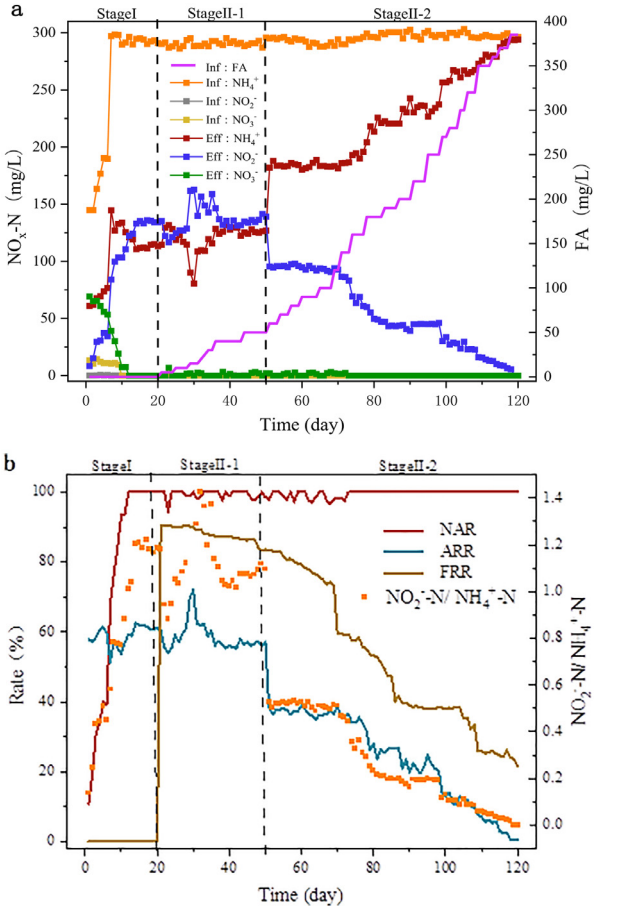

Fig. 1 – Measurement results during the operation of the SBR: (a) influent and effluent ammonium, nitrate, nitrite, and FA concentrations; (b) ARR, NAR, FRR, and effluent NO_2^--N/NH_4^+-N ratios.

Vadivelu et al. (2006) observed that when the concentration of free ammonia reached 16 mg/L, there was still no inhibitory effect on AOB. However, in this experiment, the average free ammonia concentration was just 14.4 mg/L, and this was lower than the inhibition content. Thus, the deterioration of ammonium removal could be attributed to FA-induced changes in bacterial activity, community composition, or functional genes. These possibilities are discussed further in the following sections.

Fig. 1b also shows the (FA) removal rate (FRR) at each stage. When the FA concentration less than 120 mg/L, the FRR reached 80%±3%, indicating that the majority of the FA was degraded. When FA concentration exceeded 120 mg/L, FRR plummeted to 60% and continued to decrease, suggesting that FA accumulated in the reactor.

In conclusion, the influence of FA on the performance of the PN system was linked to the dosage. For example, 50 mg/L of FA is the threshold for normal operation of the PN reactor. In contrast, when the FA content reached 385 mg/L, ammonium transformation was completely prevented. Furthermore, FA possesses the potential to inhibit NOB and maintain a steady NAR during PN.

2.2. Variations in microbial abundance and diversity

A metagenomic sequencing was used to elucidate the diversity, structure, and functional genes of the microorganic community within the PN reactor. Four sludge samples (FA0, FA50, FA200, and FA385) were selected based on mutation of nitrite concentration in effluent.

Appendix A Table S1 displays the abundance and diversity indices of the bacterial populations within the sludge samples from the PN system. The high coverage index of all samples was over 0.99, and this suggests that the high-throughput results possess high veracity and reliability. Based on the Chao1 indices, the abundance of microbial species was first increased from FA0 to FA50, and then decreased from FA50 to FA385. These results suggested that the presence of limited FA (< 50 mg/L) was beneficial to microbial enrichment, but unlimited FA (> 50 mg/L) lowered bacterial richness. The Simpson index values from 0.03 (FA0) to 0.22 (FA 385) and the variation in microbial diversity were consistent with the abundance. In general, the effects of FA on microbial abundance and diversity are dose-dependent, with low concentrations beneficial and high concentrations harmful.

2.3. Response of the microbial population to FA

2.3.1. Changes in the microbial community at the phylum level

The relative abundance of the microbic composition at the phylum levels is presented in Fig. 2a. In all samples, *Proteobacteria* (43.04%–60.59%) was the dominant population, and this was followed by the sequences affiliated with *Bacteroidota* (6.59%–26.81%) and *Chloroflexi* (3.9%–23.94%). *Bacteroidetes* and *Proteobacteria* were the most common phyla in sewage treatment processes, regardless of the environment (with or without FA) (Liu et al., 2018). The phylum *Proteobacteria* that contained the majority of the nitrogen transformation species (such as AOB and NOB) exhibited a decrease in abundance from 58.67% (FA0) to 43.04% (FA50) and then increased to 51.35% (FA385). This indicated that *Proteobacteria* had a certain tolerance to FA.

Bacteroidetes shared 10.62% (FA0), 6.59% (FA50), 20.35% (FA200), and 26.81% (FA385), respectively. Earlier studies have demonstrated that *Bacteroidetes* could act as a consumer of dissolved organic matter such as EPS that is secreted by nitrifying bacteria and metabolites generated by decayed biomass (Yang et al., 2017; Tang et al., 2018). Thus, the pressure exerted by FA resulted in the death of a large number of microbes, and decomposition was responsible for the augmentation of *Bacteroidetes*. Additionally, as they are filamentous bacteria, the quantity of *Bacteroidetes* is linked to the sludge bulking (Du et al., 2017). **Appendix A Fig. S2a** presents the changes in the sludge volume index (SVI) and mixed liquor suspended solids (MLSS) of PN sludge during the treatment of wastewater with FA. The continuously smaller SVI (from 102.95 to 76.47 ml/g) represented greater settleability on days 20-50 (FA 0–50 mg/L). Accordingly, the percentage of *Bacteroidetes* dropped from 10.62% (FA0) to 6.59% (FA50). Nevertheless, with the augment of FA (50-385 mg/L), the percentage of *Bacteroidetes* also increased from 6.59% to 26.81%, resulting in the deterioration of sludge settling performance and biomass washout (Table 2).

Chloroflexi in the biological nitrogen removal process was observed to complete the degradation of complex compounds. Nevertheless, the abundance of *Chloroflexi* was decreased from 11.83% at 0 mg/L FA to 4.62% at 385 mg/L FA, indicating that *Chloroflexi* does not possess the ability to decompose high concentrations of FA. Furthermore, the accumulation of *Chloroflexi* is beneficial for sludge granulation (Miura et al., 2007). The mean particle diameter and the distribution of various granule sizes during the experiment are presented in **Appendix A Fig. S2b**. The initial average sludge diameter in Stage I (days 1-20) was 93.40 μm, and the mean diameter was increased to 101.4 μm (day 21) with a granulation rate of 0.40 μm/day. When FA was added into the reactor, the average granular size became obviously increased to 122.6 μm on day 50 with a rate of 0.71 μm/d and an FA content of 50 mg/L. However, the granulation rate declined to 0.29 μm/day during days 50-90 (FA 50-200 mg/L), and the granulation rate was only 0.11 μm/day at the end of the experiment (FA385). As discussed above, the appropriate FA concentration (<50 mg/L) strengthened the settling performance and accelerated the granulation rate of the PN sludge by accumulating *Chloroflexi* and suppressing *Bacteroidetes* (Table 2).

2.3.2. Changes in the microbial community at the genus level

Functional bacterial groups were investigated at the genus level (Fig. 2b). The results confirmed that FA concentration was the primary contributor to the evolution of microbial communities during PN. The sole AOB genus (*Nitrosomonas*) was observed in the PN reactor. In regard to the relative abundances of 6.29% in FA0, the relative abundance was 0.12%, 4.52% and 7.40% for *Nitrosomonas* in FA50, FA200 and FA385, respectively. These results implied that FA inhibited AOB enrichment at low concentration (50 mg/L), but slightly promoted it at high concentration (385 mg/L). Interestingly, at FA385, the nitrogen conversion performance of the reactor was the worst, but the proportion of AOB reached the peak. Therefore, it can be inferred that the influence of FA on the nitrogen transformation performance of the PN system is not only a result of a simple effect on AOB richness. In order to further explore the influence mechanism of FA on PN system, changes in the abundance of functional genes were discussed in section 3.4. Meanwhile, *Nitrospira*-related NOB was not identified in FA0-385, implying that NOB was completely suppressed in this system. Based on this, a high NAR (96.62%-100%) was obtained from FA0 to FA385 (days 20-120), implying that FA may possess the potential to inhibit NOB. *Thauera* is a common denitrifying bacteria that exhibited proportions of 3.19%, 12.87%, 0.94%, and 1.70% in FA0, FA50, FA200, and FA385, respectively. Therefore, the presence of the *Thauera* was responsible for nitrogen loss in the PN system.

Members of the genus *unclassified_c Anaerolineae* were detected and maintained a relative abundance of 0.17%, 1.98%, 0.35%, and 0.09% in FA0, FA50, FA200, and FA385, respectively. Early studies have demonstrated that *Anaerolineae* is a vital member of the microbial community that is required for the degradation of refractory organic matter (Zhang et al., 2018). Thus, the presence of bacteria *Anaerolineae* led to the degradation of FA into small molecules of organic matter for use by other heterotrophic microorganisms in FA 50. This was consistent with the FA removal rate (FRR) as high as 85%±5% during

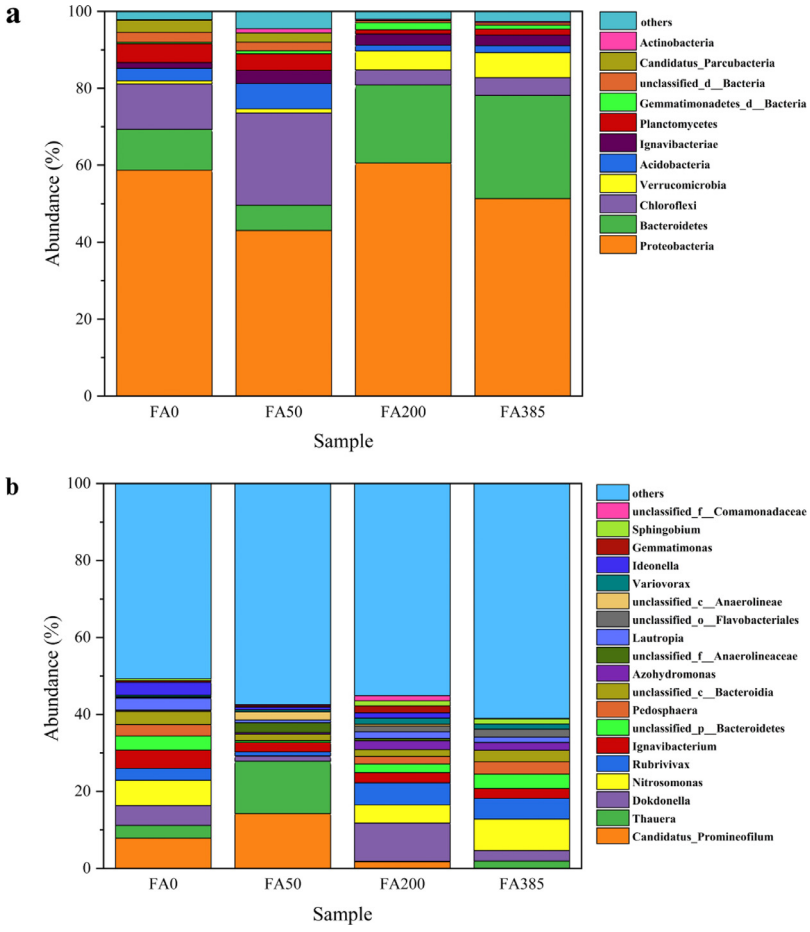

Fig. 2 – Microbial community structure at (a) the pum and (b) genus levels under different FA concentrations.

Table 2 – Changes of sludge characteristics and corresponding microbial abundance.

Sample	Grannulation rate (μm/day)	Relative abundance of *Chloroflexi* (%)	Sludge settling property (mL/g)	Relative abundance of *Bacteroidetes* (%)
FA 0	0.40	11.83	129.63	10.62
FA 50	0.71	23.94	86.14	6.59
FA 200	0.29	3.92	75.00	19.19
FA 385	0.11	4.61	103.92	26.81

this period (Fig. 1b). *Nitrosomonas*, as an autotroph, neither metabolized FA directly nor utilized decomposed small molecular organic matter. The existence of biodegradable organic matter was the primary reason for the high abundance of *Thauera* (12.87%) during this stage. The enrichment of *Thauera* resulted in competition with AOB for dissolved oxygen (DO), thus leading to a relatively low abundance of AOB in FA 50 and drop of ARR (Huang et al., 2019; Li et al., 2019). Subsequently, as the FA concentration increased, the *Anaerolineae* was inhibited, ultimately resulting in a reduction in the amount of degradable organic matter within the system. Thus, numerous heterotrophic bacteria died due to the absence of organics (FRR from 85% to 23%), leading to the relative abundance of AOB in FA50-385 increased, but the microbial enzymes were inhibited due to the accumulation of FA (Dang et al., 2016).

Overall, FA affected the abundance of functional bacteria (AOB) by affecting the heterotrophic bacteria. First, a low dose of FA (under 50 mg/L) was conducive to the growth and reproduction of heterotrophic bacteria, and this resulted in a low proportion of AOB (*Nitrosomonas*). At this point, certain denitrifying bacteria (e.g., *Thauera, Dokdonella*) were involved in nitrogen removal, and this resulted in a loss of nitrogen in the PN system. Subsequently, a high dose of FA (over 50 mg/L) suppressed the enrichment of heterotrophic bacteria (*Thauera*), ultimately leading to the accumulation of AOB (*Nitrosomonas*) due to a lack of competitors. Furthermore, no NOB was observed to be responsible for the high NAR present during the entire experiment.

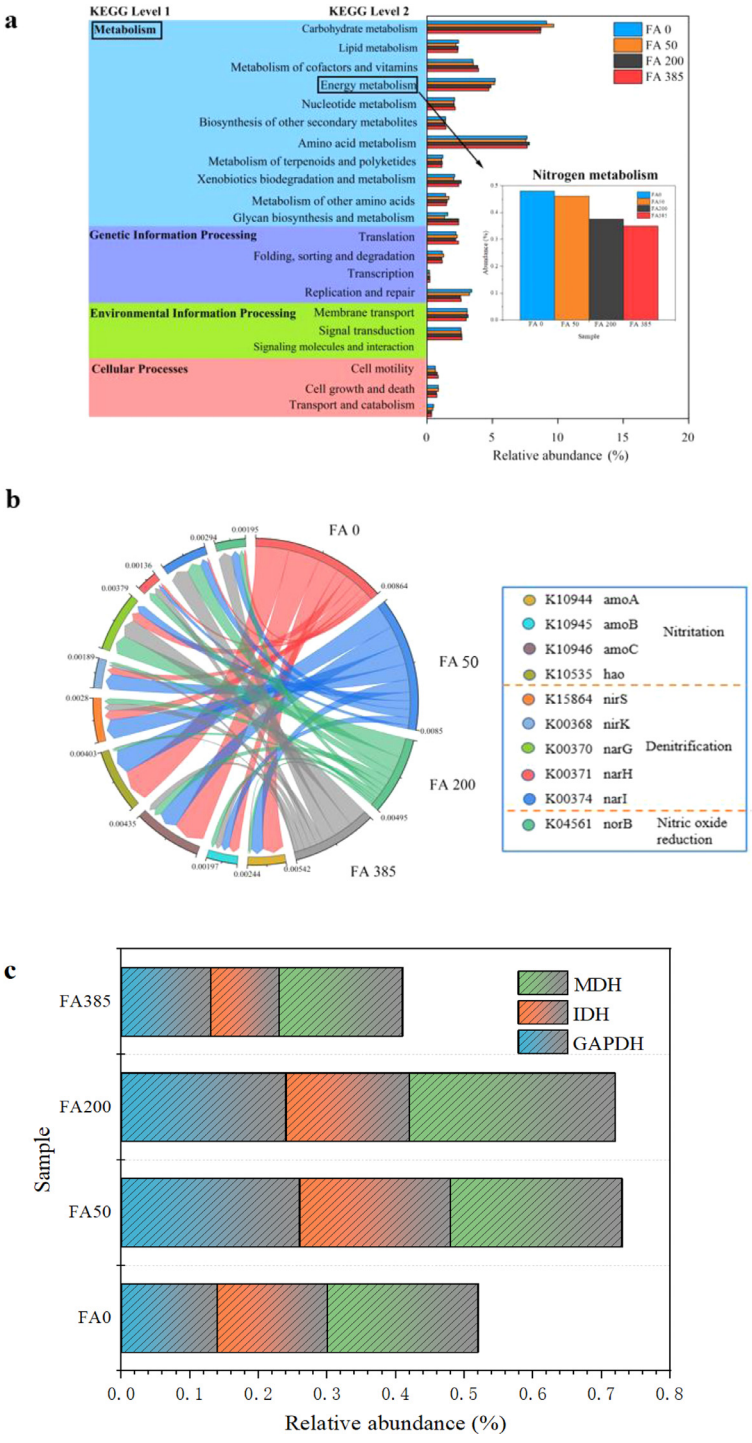

Fig. 3 – The relative abundance of (a) functional categories of metabolic pathways; (b) functional genes for nitrogen metabolism and (c) functional genes for NADH generation identified by metagenome analysis

2.4. *Functional genes involved in the PN process incorporating FA*

Functional gene analysis was performed on four samples to provide more information regarding the changes in microbial metabolic functions. As presented in Fig. 3a, the functional gene cluster includes metabolism, genetic information processing, environmental information processing, human diseases, cellular processes, and organismal systems on KEGG Level 1. Metabolism was the dominant functional subsystem and accounted for 75.96%, 76.44%, 77.36%, and 77.43% in FA0, FA50, FA200, and FA385, respectively (Fig. 3a). Additionally, the energy metabolic pathway plays a vital role in nitrogen transformation, energy transport (e.g., ATP metabolism), and

electron transport (e.g., NADH metabolism) in the PN process (Li et al., 2018), and it decreases from 5.21% (FA 0) to 4.73% (FA385). Above them, the relative abundance of nitrogen metabolism continued to decrease after reaching the maximum value in FA0 (0.48%). Especially, when FA increased from 50 to 200 mg/L, the relative abundance of nitrogen metabolism dropped sharply from 0.47% to 0.38%. These results demonstrated that the microbial nitrogen metabolic activity of the PN system was suppressed under long-term high FA dosages.

The abundances of various key genes were calculated to better understand the functional diversity of the nitrogen metabolism pathways (Fig. 3b). The nitrogen metabolism pathway of AOB includes two steps: the oxidation of ammonium and the oxidation of hydroxylamine. First, with the participation of enzymes ammonia monooxygenase (*amoABC*), ammonium was oxidized to hydroxylamine with O_2 as the final electron acceptor and CO_2 as the sole carbon source. This process is the single way for AOB to obtain energy and requires the participation of two external electrons. Subsequently, hydroxylamine is further oxidized to nitrite with hydroxylamine dehydrogenase (*hao*) and releases four electrons, two of which are transferred to *amoABC* for ammonium oxidation. In general, the relative abundance of genes *amoABC* and *hao* were related to FA dose in this study. FA feeding increased the abundance of the *amoABC* gene from 0.42% (FA 0) to 0.64% (FA50) and then to 0.14% (FA200) and finally to 0.08% (FA385). The relative abundance of *hao* in FA385 was just 0.022 folds that in FA0. These observations indicated that the AOB nitrogen metabolism pathway is affected by FA, ultimately leading to changes in ARR. Specially, high concentrations of FA reduced the abundance of genes related to ammonia oxidation, especially *hao*, impairing the performance of the PN system. Luo et al. (2019) demonstrated that the effect of humus (0-50 mg/L) on the abundance of *amoABC* was negligible, while the abundance of *hao* decreased from 0.3% to 0.03%, which was similar to our conclusion. The trend of relative abundance of key genes was inconsistent with AOB, which may be affected by other microorganisms (such as heterotrophic bacteria) in the system.

Numerous subunits of genes encoding enzymes involved in denitrification (*narG, narH, narI, nirS, nirK*, and *norB*) were annotated, and all of them exhibited relatively high abundance. This observation suggests that denitrification was present in the PN reactor and responsible for nitrogen loss. Different from AOB, nicotinamide adenine dinucleotide (NADH) produced by carbon metabolism (glycolysis and TCA cycle) is the main electron donor for heterotrophic denitrifying bacteria, and the NADH will determine the performance of denitrification (Su et al., 2015). Therefore, several key functional genes involved in NADH generation (*GAPDH, IDH*, and *MDH*) were evaluated in this study (Dong et al., 2017). As shown in the Fig. 3c, limited FA was degraded into small molecular organics to stimulate the generation of NADH, which enhanced the denitrification process. Accordingly, genes related to nitrite reduction (*nirSK*) increased from 0.11% (FA 0) to 0.24% (FA 50). This validated the conclusion that there are abundant denitrifying bacteria and significant nitrogen losses in the system with limited FA (< 50 mg/L). The presence of a small amount of FA had no direct effect on AOB metabolism, but the enhancement of denitrification leads to the partial nitrite consumption, indirectly favoring the PN process. Subsequently, with the accumulation of FA, the proportion of *nirSK* decreased to 0.07% at FA385. Correspondingly, the nitrogen loss of the whole system reached its nadir at FA385, and the nitrite production in the PN process was negatively affected. Notably, key functional genes related to the reduction of nitrate to nitrite (*narGHI*) were observed in the all samples. However, as no NOB was detected and the influent without nitrate, it is unlikely that abundant nitrate was present in the system. Thus, nitrate reduction was not the primary pathway of nitrite accumulation due to lack of substrates. A reasonable explanation is that these genes were assigned to other microbes that were not part of the denitrification function (Luo et al., 2019).

In summary, low concentrations of FA (< 50 mg/L) had no direct effect on the AOB metabolic pathway, but stimulated the denitrification process. In response to an increase in FA concentration, the abundance of functional genes related to the nitrogen metabolism became decreased, particularly the *hao*. This is the core reason for the decrease in the ARR in the PN reactor. Additionally, increases in FA also reduced the abundance of functional genes related to denitrification (*nirSK*), and this was related to the observed decrease in heterotrophic denitrifying bacteria. This was also responsible for the gradual reduction in nitrogen loss within the reactor.

2.5. *Implication of this study*

In this study, the influence of FA on the PN system was identified and other valuable results were obtained through long-term experiments. Generally speaking, the negative effect of FA on the nitrogen conversion performance of the PN reactor was related to the dose of FA (Fig. 4). On one hand, when the FA concentration was between 50-120 mg/L, the FA removal rate (FRR) was as high as 80%±3%. At this time, FA was degraded into small molecular organic matter to stimulate the growth and propagation of heterotrophic bacteria, resulting in the deterioration of nitrogen conversion performance of the PN system due to the decline of AOB proportion. On the other hand, when the FA concentration was higher than 120 mg/L, the FRR dropped below 60%, causing a large amount of FA to accumulate in the reactor. Meanwhile, FA bound to enzyme proteins in microorganisms and affected the activity and expression of related enzymes (Dang et al., 2016). The biological toxicity of FA was responsible for the suppression of microbial energy metabolism and the decrease of gene *hao*, which ultimately led to further deterioration of PN performance. In addition, it should be noted that the PN reactor maintained a high NAR throughout the experimental period, and this was primarily due to the absence of NOB.

Nevertheless, compared to common organic matter, biorefractory organic matter exerts the opposite effect on autotrophic nitrogen removal (conversion) systems. In the study by Luo et al. (2019), although the addition of HS reduced the relative abundance of *Nitrosomonas*, it enriched other AOB (*Nitrosospira*). This indicated that a high concentration of biorefractory organic matter will not drop the abundance of autotrophs. Additionally, according to the data presented in **Appendix A Table S2**, the nitrogen loss efficiency (NLE) of the PN system incorporating bio-refractory organic matter (FA, HS) is less than 20%, while that of PN incorporating ordinary organic

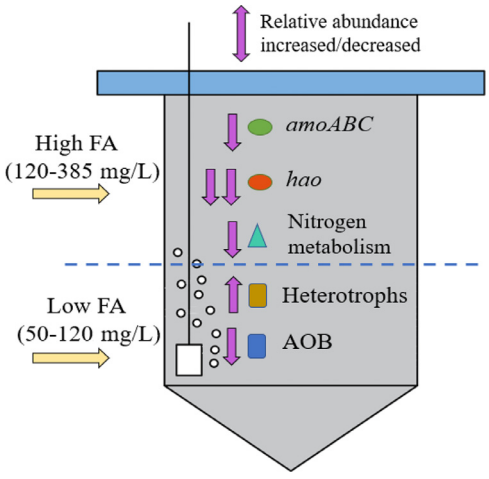

Fig. 4 – Inhibition mechanism of FA on PN systemSeveral studies have shown that with an increase in degradable organic matter concentration, the heterotrophic bacteria increased sharply (Liu et al.,2016; Wang et al., 2020). As we all know, increasing heterotrophs will compete with autotrophic bacteria (AOB) for space, DO, and other survival resources, leading to the elimination of autotrophs. The reduction of AOB causes the deterioration of the nitrogen conversion performance and the complete collapse of the entire PN system. In addition, the loss of nitrogen also results from the augment of heterotrophic denitrifying bacteria in the PN system.

matter is greater than 20% and even as high as 65.2%. Therefore, compared to the degradable organics, the existence of FA in the PN reactor is conducive to the enrichment of AOB, the elimination of heterotrophic bacteria, and a reduction of nitrogen loss.

Furthermore, creating stable nitrite accumulation in PN systems through the suppression of NOB has always been a research hotspot. Various strategies have been reported to maintain high NAR for PN. However, certain traditional strategies such as low sludge retention time (SRT), low DO, high free ammonia, and high free nitrous acid (FNA) exhibit a number of limitations in regard to maintaining the stable operation of PN in wastewater treatment. First, low SRT and DO levels lead to continuous washout of functional biomass and sludge bulking (Cui et al., 2020b; Bao et al., 2009). Second, over time, NOB will adapt to free ammonia and FNA, thus resulting in fluctuation of NAR (Jiang et al., 2021; Li et al., 2020b). Therefore, novel methods have been used to maintain the stable operation of PN. Huang et al. (2019) reported that AOB accumulated and NOB was eradicated in response to the application of ultrasound. However, if this method is used in actual engineering, the cost cannot be ignored. Additionally, some chemical products (such as hydroxylamine and benzethonium chloride) also have the potential to inhibit NOB (Zhao et al., 2020; Cui et al., 2020b), but their toxicity and price have become obstacles to engineering applications. Thus, it is necessary to develop an effective, environmentally friendly, safe, economical, and sustainable NOB inhibitor.

In this study, substances that inhibit NOB include free ammonia and FA. Early studies revealed that the inhibition range of free ammonia against NOB was 0.1-1 mg/L, and this was much lower than the 14.4 mg/L concentration used in this study. Therefore, free ammonia was the major factor that inhibited NOB enrichment in this experiment. However, in other studies using high free ammonia but without FA, the NAR was only approximately 92% (Wang et al., 2017), and this was lower than the NAR (96.62%-100%) obtained in this study. Thus, the presence of FA is helpful for promoting the stability of nitrite accumulation in the PN system.

Li et al. (2016) demonstrated that various types of FAs exert different effects on the same biological denitrification process. Thus, it is necessary to focus on the influence of different types of FAs on the PN process in the future. Furthermore, the inhibitory mechanism of FA on NOB needs to be further explored under the premise of excluding the influence of free ammonia.

3. Conclusion

The results showed that the effect mechanism of FA on PN system was closely related to concentration. First of all, low concentration FA (< 50 mg/L) has inapparent effect on the ammonium transformation, but promotes the denitrification, accelerates sludge granulation and improves sedimentation performance. Secondly, when FA in the range of 50-120 mg/L, AOB is eliminated due to the proliferation of heterotrophic bacteria, which affects nitrogen conversion efficiency. Ultimately, high concentration of FA (> 120 mg/L) significantly reduced the abundance of functional genes responsible for ammonia nitrogen oxidation (*amoABC* and *hao*), leading to further deterioration of PN performance.

Acknowledgments

This research was supported by the Key Research & Developmental Program of Shandong Province (Nos. 2021CXGC011202; 2020CXGC011404), the Beijing Natural Science Foundation (No. 8192004), and the Beijing Major Science and Technology Projects (No. Z181100005318001).

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2022.03.003.

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