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Low-concentration methanol effect on the microorganisms, nitrogen removal, and recovery of the completely autotrophic nitrogen removal over nitrite

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ABSTRACT

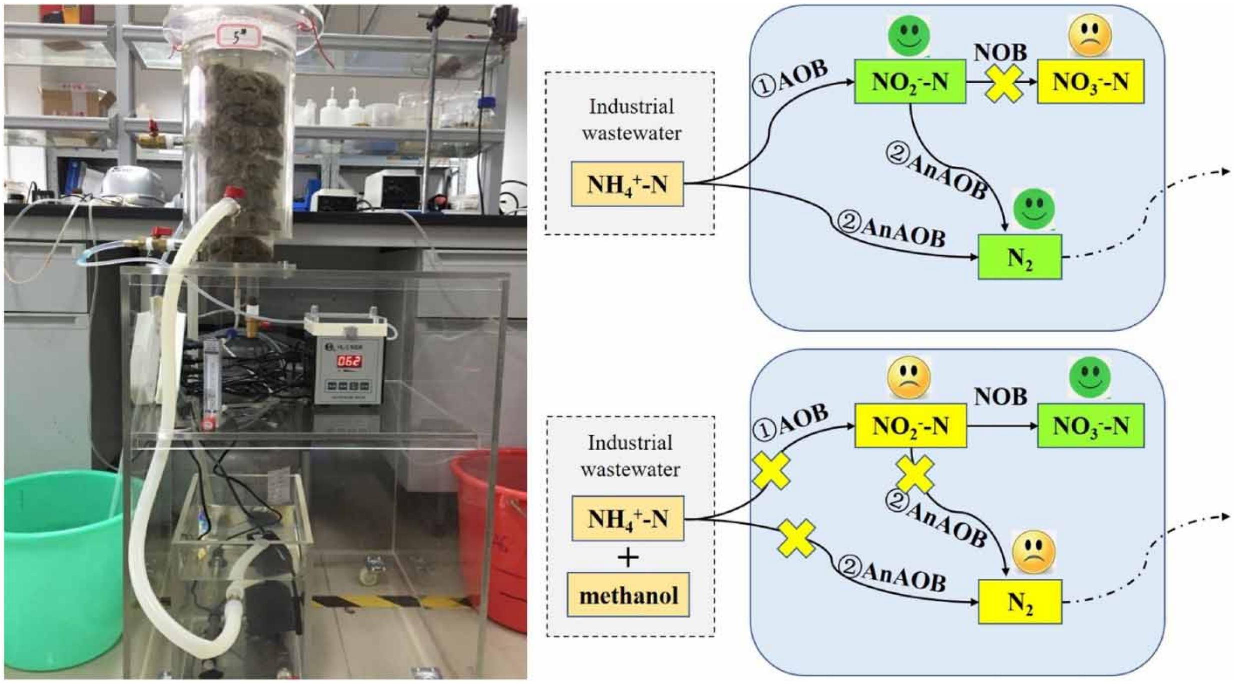
Methanol has a significant effect on the performance of the completely autotrophic nitrogen removal over the nitrite (CANON) process. In this research, the effect of low-concentration methanol on the functional microorganisms and nitrogen removal and recovery in the CANON system is investigated. The result shows that the anaerobic ammonium-oxidizing bacteria (AnAOB) was suppressed with low-concentration methanol addition, and the phylum *Planctomycetes* was hidden. The genus *Candidatus Brocadia* was restrained, and the relative abundances reduced from 25.5 to 15.0% in the upper biofilm and from 20.3 to 14.3% in the bottom biofilm, respectively. However, low-concentration methanol promoted the nitrifying oxidizing bacteria (NOB) activity. This phenomenon reduced the average ammonium nitrogen removal rate from 95.0 to 70.7%, and the average total nitrogen removal rate decreased from 81.3 to 43.6%, respectively. The results demonstrated that the low-concentration methanol as an organic carbon matter harmed the CANON process. Fortunately, the CANON system had an excellent self-healing ability when the methanol was stopped, with the average ammonium nitrogen removal rate and total nitrogen removal rate returning to 95.5 and 80.9%, respectively. This research supplies a reference for practical engineering design and application by improving the understanding of the effects of low-concentration methanol on CANON process performance.

Key words: anammox bacteria, completely autotrophic nitrogen removal over nitrite, low-concentration methanol effect, nitrifying oxidizing bacteria, nitrogen removal

HIGHLIGHTS

- Low-concentration methanol harmed the CANON process in a SABF system.
- The relative abundance of anammox bacteria reduced because of a low-concentration methanol.
- The genus Candidatus Brocadia was suppressed due to the low-concentration methanol.
- The relative abundance of nitrifying bacteria increased with a low-concentration methanol.
- The CANON system had an excellent self-healing ability without methanol addition.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The completely autotrophic nitrogen removal over nitrite (CANON) process is a novel biological ammonium nitrogen removal technology that can serve as a power- and cost-saving alternative to nitrification/denitrification. As Equation (1) shows, the reaction principle of this process is the ammonium-oxidizing bacteria (AOB) oxidizes the partial influent ammonium (NH_4^+-N) to nitrite (NO_2^--N), and then the anaerobic ammonium-oxidizing bacteria (AnAOB) reacts to the remaining NH_4^+-N with NO_2^--N production to form nitrogen gas (N_2) (Antwi *et al.* 2020). The advantages of the CANON process can reduce oxygen consumption by 62.5%, organic carbon source addition by 100%, and sludge production by 90% relative to the nitrification/denitrification process (Cho *et al.* 2020; Sanjaya *et al.* 2022):

$$NH_4^+ + 0.85O_2 \rightarrow 0.13NO_3^- + 0.435N_2 + 1.38H^+ + 1.31H_2O \quad (1)$$

As the functional bacteria of the CANON process, the AOB and AnAOB are the autotrophic microorganisms. Previous researches have investigated that several kinds of parameters, such as dissolved oxygen (DO) concentration, temperature and pH, could affect the activities and growths of AOB and AnAOB (Kim *et al.* 2020; Pedrouso *et al.* 2021; Hausherr *et al.* 2022), resulting in the fluctuation of the CANON process performance. In addition, organic matter is also a critical parameter of the CANON process (Wang *et al.* 2020). The presence of organic matter can inhibit the activity and growth of AnAOB. Thus, the CANON process is suitable for treating high-ammonium-concentration wastewaters with a low chemical oxygen demand/ammonium ratio owing to the lack of an organic carbon source. However, in reality, most ammonium nitrogen-containing wastewaters contain small numbers of naturally living organic materials or substances (Zhang *et al.* 2020a, 2020b). Therefore, it is necessary to study the effects of organic matter on nitrogen removal performance of the CANON process. Furthermore, different types of organic matters have diverse effects on the CANON process. Acetate and glucose are the commonly encountered organic matters. Low concentrations of these organic matters were found to not inhibit AnAOB and sustain a stable total nitrogen removal rate from the anammox granule biomass (Pereira *et al.* 2021). The extracellular polymeric substance contents of AnAOB tended to increase gradually. Protein and polysaccharide enrichment could be the ion-transport channel between the bulk liquid and AnAOB, thereby maintaining the 'lung-like breathing' behaviour and increasing the mass transfer efficiency (Chai *et al.* 2019; Li *et al.* 2021). At the optimum concentrations of these organic matters, AnAOB and denitrification bacteria can coexist in one reactor and enhance the nitrogen-removal ability of the CANON process (Huang *et al.* 2022; Sanjaya *et al.* 2022). However, in the presence of excessive organic matter, heterotrophic denitrifiers overgrow and compete with AnAOB for NO_2^--N, resulting in the elimination of AnAOB (Liu *et al.* 2021), and therefore, elimination of the CANON process. These results indicate that the types and concentrations of organic matter influence AnAOB and nitrogen removal in the CANON process, and therefore, further research on this topic is necessary.

Methanol is also a typical characteristic organic matter of industrial wastewaters. In nitrification and denitrification processes, methanol is usually used as an excellent organic carbon resource to provide nutrients for denitrifying bacteria, leading to improving the biological nitrogen removal performance of this process. Xu *et al.* (2018) confirmed that methanol could promote the AOB activity resulting in the improvement of nitrosation reaction. In addition, the phyla *Acidobacteria* and *Planctomycetes* were found to decrease in proportion in the presence of methanol. It is implied that methanol could have an important influence on partial nitrification reaction in the CANON process, which has few reports until now. Furthermore, some papers reported that sludge containing methanol could be suitable for AnAOB enrichment. The *Candidatus Brocadia* was the dominant population in the enriched biomass (Tang *et al.* 2010). However, others showed that methanol could almost completely inhibit AnAOB activity, and have an irreversible inhibitory effect on the anammox process, which can be caused by enzyme inactivation or cell death, wherein suppression of the threshold differed from studies (Kim *et al.* 2020; Isaka *et al.* 2021; Chen *et al.* 2022). The results signify that methanol has a marked impact on the CANON process performance, which needs further research, though it has not been reported. Furthermore, nitrifying oxidizing bacteria (NOB) inhibition is critical for stable CANON performance. Some previous research reported that NOB enrichment was destructive to the CANON process, as NOB competed with the AnAOB for NO_2^--N acting as an electron acceptor and inhibited the total nitrogen removal ability of the CANON system (Zhang *et al.* 2020a, 2020b; Yang *et al.* 2023). Organic carbon matter is one of the factors affecting NOB and different types of organic carbon matters have different effects on NOB (Chen *et al.* 2023). In particular, methanol can significantly affect the activity of NOB. Xu *et al.* (2018) reported that the methanol addition could suppress the phylum Nitrospirae (belonged to the NOB). Thus, the methanol effect on NOB needs further study to increase the stability of the CANON process for treating practical wastewaters.

This experiment studies the low-concentration methanol effect on the AnAOB growth, NOB elimination, and nitrogen removal of the CANON performance in a submerged aerated biological filter (SABF) system at a low DO level. The nitrogen removal ability and stability of this CANON process could indicate the AnAOB and NOB activities with low-concentration methanol. A 16S rDNA sequencing technology analyzed the CANON process's microorganisms and these relative abundances at phylum and genus levels, respectively, to demonstrate the low-concentration methanol effect on the AnAOB and NOB survivals. This research supplies a reference for practical engineering design and application by improving the understanding of the effects of low-concentration methanol on CANON process performance and is vital for optimizing CANON process performance in a SABF system.

2. MATERIALS AND METHODS

2.1. Reactor description

CANON systems with a 3.0-L practical volume were prepared from organic glass, as shown in Figure 1. The combined carriers were fixed in the SABF to increase the attachment of microorganisms and enhance their adsorption ability. The SABF reactor was heated using a water bath heater (PTC, Zhongshan Zhixin Electric Appliance Co., Ltd, China), and the temperature of the reactor was controlled and regulated using a temperature probe and temperature controller (PTC, Zhongshan Zhixin Electric Appliance Co., Ltd, China). Air was transported from the bottom of the reactor using an air pressure pump (LP20, Shenzhen Xing Risheng Industrial Co., Ltd, China), and the air supply was controlled and regulated with an air rotameter (LZB-3WB, Changzhou Shuanghuan Thermo-Technical Instrument Co., Ltd, China). The DO concentration and pH of the reactor were controlled using a DO meter (JPB-607A, Shanghai instrument electric science instrument Limited by Share Ltd, China) and a pH device (PHB-4, Shanghai instrument electric science instrument Limited by Share Ltd, China), respectively. The influent of the reactor was supplied using a peristaltic pump (BT100-2J, Longer Precision Pump Co., Ltd, China).

The combined carriers were fastened in the SABF, which consisted of plastic fibres, annulus, and a centre copper wire. Because of their high specific surface areas (1500 m^2/m^3), the combined carriers could improve the attachment of the

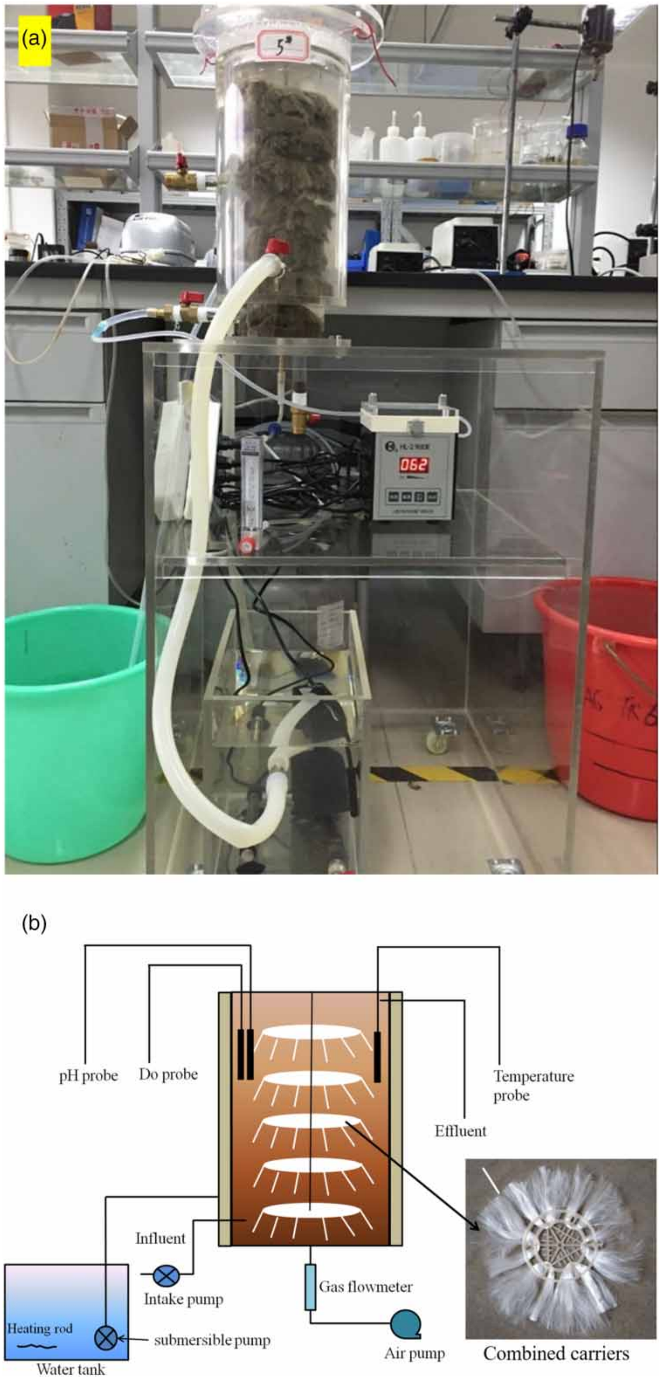


Figure 1 | Structure of this experiment system.

microorganisms to achieve a favourable adsorption capability. Moreover, AnAOB and AOB could be absorbed on the inside and outside of the biofilms, respectively, owing to the different concentrations of DO on the inside and outside of the biofilms.

Water distribution of the CANON system: In the system, the peristaltic pump transported the simulated wastewater to the bottom of the CANON system, after which the effluent was outflowed from the upper outlet of the system. The flow rate of the system was 2.5 mL·min^{-1}. To achieve a reactor's temperature of 30.1–31.5 °C, the submerged pump transported the heated water into the inlet of the water bath layer at the base of the reactors, after which the effluent was outflowed from the upper outlet of the water bath layer into the radiator.

2.2. Reactor performance

As Figure 2 shows, the CANON process in a SABF system had been successfully started up for 35 days and stably operated for 98 days. During the operation, theAOB enriched, and its activity was 2.25 mg·L^{-1}·h^{-1}, resulting in a high ammonium nitrogen removal rate of 95.0% in the CANON process. In addition, a suitable system environment prompted AnAOB enrichment in abundance, the average activity of which was 3.40 mg·L^{-1}·h^{-1}, leading to the stable total nitrogen removal rate of 81.6% in this system. However, the NOB was inhibited with its average activity of 0. The average practical NO_3^--N production to the NH_4^+-N consumption ratio (ΔNO_3^--N/ΔNH_4^+-N) standard for NOB overgrowth was 0.10, nearly 0.13 as a theoretical value. The experiment of this CANON process resulted in relatively few NOB in this system (Hausherr *et al.* 2022).

Beyond that, a temperature controller controlled the average temperature at 30.8 °C. This reactor was warmed by water insulation. An air pressure pump supplied the Dissolved Oxygen (DO), the concentration of which was controlled at 0.1–0.5 mg·L^{-1} in the start-up period and 0.1–0.3 mg·L^{-1} in the performance period. The hydraulic residence time (HRT) was 20 h. A temperature controller controlled the temperature at 30.1–31.5 °C. A constant flow pump pumped the influent from the bottom of the reactor.

2.3. Artificial wastewater

The ingredients of the artificial wastewater are shown in Tables 1 and 2. The influent water quality parameters of this experiment are shown in Table 3.

Effect of low-concentration methanol on nitrogen removal and microorganisms. This experiment added the methanol by 0, 20.0, 27.0 and 38 mg·L^{-1}, adjusting the average influent COD concentration to 0, 29, 39, and 58 mg·L^{-1}, respectively.

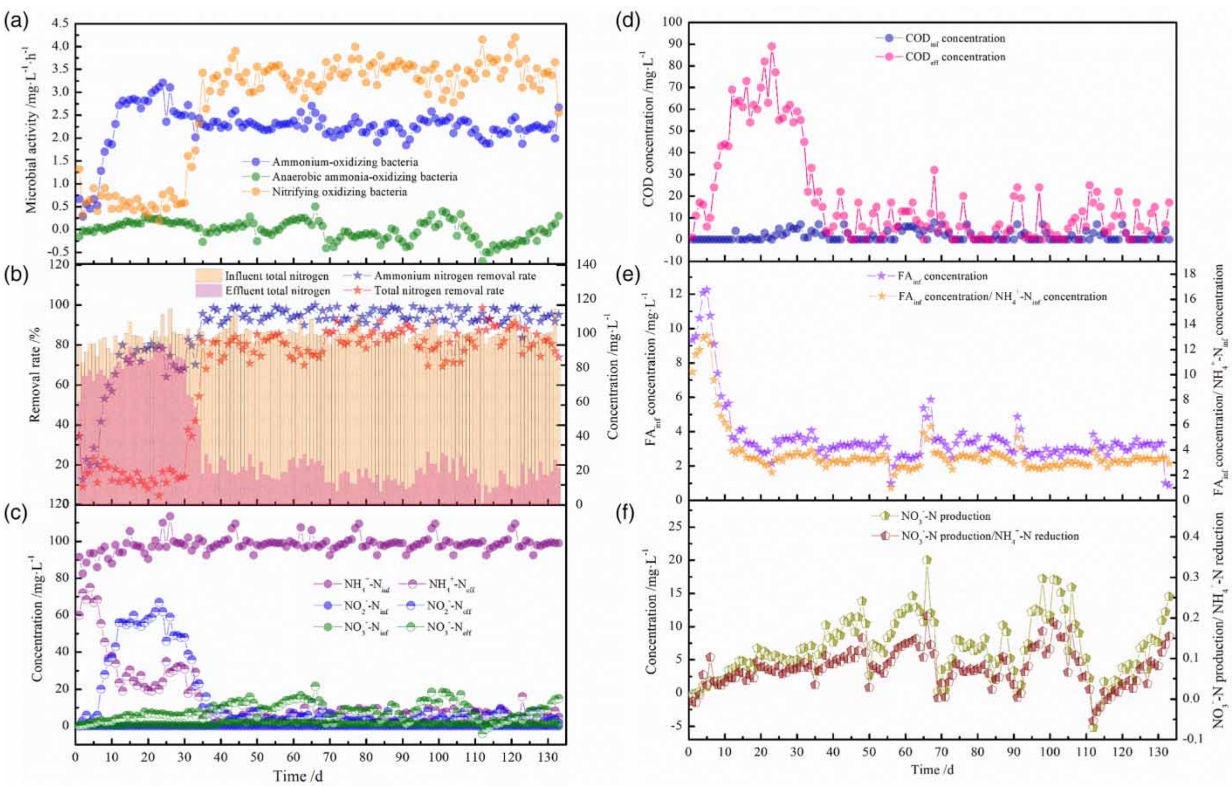


Figure 2 | Start-up and performance of this experiment system (NH_4^+-N_{inf} was represented influent NH_4^+-N. NH_4^+-N_{eff} was represented effluent NH_4^+-N. NO_2^--N_{inf} was represented influent NO_2^--N. NO_2^--N_{eff} was represented effluent NO_2^--N. NO_3^--N_{inf} was represented influent NO_3^--N. NO_3^--N_{eff} was represented effluent NO_3^--N. TN_{inf} was represented influent TN. TN_{eff} was represented influent TN. COD_{inf} concentration was represented influent COD. COD_{eff} concentration was represented effluent COD. FA_{inf} concentration was represented influent free ammonia concentration. FA_{inf} concentration/NH_4^+-N_{inf} concentration was represented the influent free ammonia concentration to the influent NH_4^+-N concentration ratio.).

Table 1 | The compositions of the artificial wastewater

Item	Unit	Value
NH_4Cl	$g{\cdot}L^{-1}$	0.38–0.41
KH_2PO_4	$g{\cdot}L^{-1}$	0.03
$NaHCO_3$	$g{\cdot}L^{-1}$	1.00
$CaCl_2$	$g{\cdot}L^{-1}$	0.03
$MgSO_4$	$g{\cdot}L^{-1}$	0.01
Trace element solution	$ml{\cdot}L^{-1}$	0.30

Table 2 | The compositions of the trace element solution

Item	Unit	Value
$MnCl_2{\cdot}4H_2O$	$g{\cdot}L^{-1}$	0.36
$FeCl_3{\cdot}6H_2O$	$g{\cdot}L^{-1}$	3.60
$ZnSO_4{\cdot}7H_2O$	$g{\cdot}L^{-1}$	0.40
$CuSO_4{\cdot}5H_2O$	$g{\cdot}L^{-1}$	0.10
$CoCl_2{\cdot}6H_2O$	$g{\cdot}L^{-1}$	0.40

Table 3 | Main quality parameters of artificial wastewater

Item	Unit	Value
pH	–	7.91–8.06
COD	$mg{\cdot}L^{-1}$	0–61
NH_4^+-N	$mg{\cdot}L^{-1}$	99.0–106.5
NO_2^--N	$mg{\cdot}L^{-1}$	0.0–1.0
NO_3^--N	$mg{\cdot}L^{-1}$	0.0–2.1

2.4. Microbial community in the sludge samples

The samples of sludge were analysed by a 16S rRNA gene sequencing technology (Guangzhou RiboBio Co., Ltd). The process of detection is described in supplementary material A.

2.5. Analytical methods

The water samples of the CANON biofilm system were filtered through a qualitative filter paper. The detections of the COD, NH_4^+-N, NO_2^--N, and NO_3^--N concentrations were based on the instructions of the Standard Methods (APHA) (APHA 2005).

The integrity of the nucleic acids in the sludge was measured using a TapeStation 2200 instrument (Agilent Technologies Co. Ltd, America), and the concentration and mass of the nucleic acids were measured using a Qubit 2.0 fluorimeter (Life Technologies Co. Ltd, America) and an ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific Co. Ltd, America). The Q30 ratio and total data were detected using a Miseq sequencer (Illumina Ltd, America).

2.6. Calculations

For Equations (2)–(7), influent total nitrogen concentration, effluent total nitrogen concentration, ammonium nitrogen removal rate, total nitrogen removal rate, NO_3^--N production ($\triangle NO_3^-$-N) and NO_3^--N production to the NH_4^+-N consumption ratio ($\triangle NO_3^-$-N/$\triangle NH_4^+$-N) were calculated, respectively. The activity values of AnAOB, NOB, and AOB were calculated according to Equations (8)–(10) (Antwi *et al.* 2020). In these equations, NH_4^+-N_{inf} was represented influent NH_4^+-N. NH_4^+-N_{eff}

was represented effluent NH_4^+-N. NO_2^--N_{inf} was represented influent NO_2^--N. NO_2^--N_{eff} was represented effluent NO_2^--N. NO_3^--N_{inf} was represented influent NO_3^--N. NO_3^--N_{eff} was represented effluent NO_3^--N. TN_{inf} was represented influent TN. TN_{eff} was represented influent TN:

$$\text{Influent total nitrogen}(mg \cdot L^{-1}) = NH_4^+ - N_{inf} + NO_3^- - N_{inf} \quad (2)$$

$$\text{Effluent total nitrogen}(mg \cdot L^{-1}) = NH_4^+ - N_{eff} + NO_2^- - N_{eff} + NO_3^- - N_{eff} \quad (3)$$

$$\text{Ammonium nitrogen removal rate}(\%) = \frac{NH_4^+ - N_{inf} - NH_4^+ - N_{eff}}{NH_4^+ - N_{inf}} \times 100 \quad (4)$$

$$\text{Total nitrogen removal rate}(\%) = \frac{TN_{inf} - TN_{eff}}{TN_{inf}} \times 100 \quad (5)$$

$$\Delta NO_3^- - N(mg \cdot L^{-1}) = NO_3^- - N_{eff} - NO_3^- - N_{inf} \quad (6)$$

$$\frac{\Delta NO_3^- - N}{\Delta NH_4^+ - N} = \frac{NO_3^- - N_{eff} - NO_3^- - N_{inf}}{NH_4^+ - N_{eff} - NH_4^+ - N_{inf}} \quad (7)$$

$$\text{AnAOB activity}(\text{mg} \cdot L^{-1} \cdot h^{-1}) = \frac{\Delta TN}{24} \quad (8)$$

$$\text{NOB activity}(\text{mg} \cdot L^{-1} \cdot h^{-1}) = \frac{\Delta NO_3^- - N - \frac{\Delta TN}{2.04} \times 0.26}{24} \quad (9)$$

$$\text{AOB activity}(\text{mg} \cdot L^{-1} \cdot h^{-1}) = \frac{\Delta NH_4^+ - N - \frac{\Delta TN}{2.04}}{24} \quad (10)$$

3. RESULTS AND DISCUSSION

3.1. Low-concentration methanol effect on microbial activities and nitrogen removal

As shown in Figure 3, low-concentration methanol significantly impacted this CANON process in a SABF system. The gas flowmeter maintained DO_{sys} at 0.1–0.3 mg·L^{-1}, and pH_{sys} and temperature_{sys} were held at 7.21–7.53 and 30.3–31.8 °C, respectively (DO_{sys} was represented DO of this system. pH_{sys} was represented pH of this system. Temperature_{sys} was represented Temperature of this system).

When the low-concentration methanol increased from 0 to 38.0 mg·L^{-1} in this CANON process, the AOB was suppressed with its average activity reducing from 2.42 to 2.09 mg·L^{-1}·h^{-1} (Figure 3(a)), causing the average NH_4^+-N_{eff} concentration collection from 3.5 to 30.0 mg·L^{-1} (Figure 3(c)) and the average ammonium nitrogen removal rate reduction from 96.6 to 70.7% (Figure 3(b)). Low-concentration methanol also inhibited the AnAOB activity, with the average value from 3.48 mg·L^{-1}·h^{-1} to 1.89 mg·L^{-1}·h^{-1} (Figure 3(a)), resulting in the total nitrogen removal rate reduction from 80.6 to 43.6%, as Figure 3(b) shows (the average FA_{inf} concentration and average ratio of FA_{inf} concentration/NH_4^+-N_{inf} concentration were 2.57 mg·L^{-1} and 2.53, respectively, which were not enough to affect the CANON reaction system). The reason for this phenomenon is that low-concentration methanol as a toxicant could cause the microbial toxication or enzyme deactivation of anammox bacteria, which was named formaldehyde inhibition (Huang *et al.* 2022). As a critical anammox enzyme, a hydroxylamine oxidoreductase might change methanol into intracellular formaldehyde, which cross-linked the peptide chains to damaged enzyme and protein activity. These results were similar to that of previous studies (Xu *et al.* 2018; Isaka *et al.* 2021). In addition, the average COD consumption in this system increased to 57.2 mg·L^{-1} (Figure 3(d)), which might be due to heterotrophic microorganism accumulation (Cao *et al.* 2020) and competed with the anammox bacteria for space and electron acceptor (NO_2^--N) (Du *et al.* 2020; Li *et al.* 2020). Thus, low-concentration methanol eliminated the anammox bacteria and reduced the CANON process's total nitrogen removal ability in a SABF system.

Furthermore, NOB suppression is vital to the CANON process performance. As Figure 3(a) shows, when the low-concentration methanol was added, the average NOB activity increased from 0 to 0.10 mg·L^{-1}·h^{-1}, and the average NO_3^--N_{eff} concentration increased from 20.1 to 58.6 mg·L^{-1}, as Figure 3(c) shows. It was because the NOB inhibited the AnAOB,

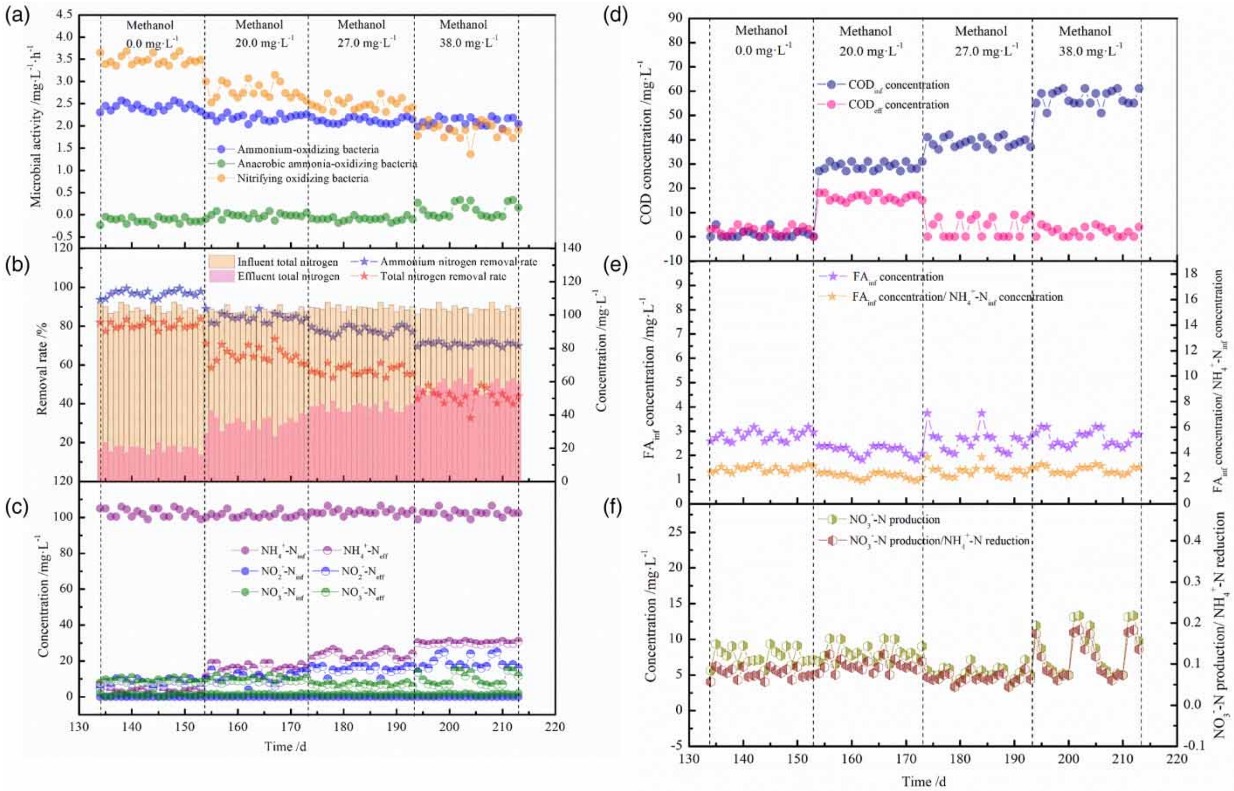


Figure 3 | Low-concentration methanol effect in this system (NH_4^+-N_{inf} was represented influent NH_4^+-N. NH_4^+-N_{eff} was represented effluent NH_4^+-N. NO_2^--N_{inf} was represented influent NO_2^--N. NO_2^--N_{eff} was represented effluent NO_2^--N. NO_3^--N_{inf} was represented influent NO_3^--N. NO_3^--N_{eff} was represented effluent NO_3^--N. TN_{inf} was represented influent TN. TN_{eff} was represented effluent TN. COD_{inf} concentration was represented influent COD. COD_{eff} concentration was represented effluent COD. FA_{inf} concentration was represented influent free ammonia concentration. FA_{inf} concentration/ NH_4^+-N_{inf} concentration was represented the influent free ammonia concentration to the influent NH_4^+-N concentration ratio).

obsolete at lower DO concentrations without mass transfer limitation in biofilms when organic matter existed (Yang *et al.* 2023). Thus, the low-concentration methanol facilitated the NOB survival, inhibiting ammonium nitrogen oxidation and total nitrogen removal ability of the CANON process, resulting in the deterioration of this SABF system. Beyond that, as Figure 3(f) shows, the ratio of $\triangle NO_3^-$-N/$\triangle NH_4^+$-N maintained at 0.07–0.11, signifying no denitrification occurred. This result was different from other studies reporting anammox and denitrification coexisting in a one-stage system with organic matters (Peng *et al.* 2020; Chen *et al.* 2023). The reason for this phenomenon was likely that the low-concentration methanol could suppress the denitrifying bacteria enrichment (Zhang *et al.* 2022). Furthermore, the low-concentration methanol was challenging to diffuse into the thick biofilm for denitrifying growing bacteria and therefore inhibited the nitrogen removal of this system.

3.2. Low-concentration methanol effect on the functional microorganisms

The microorganisms of this system were examined by a 16S rDNA sequencing analysis method under the condition of low-concentration methanol. The results of this experiment are summarized in Figure 4.

When the CANON process in a SABF system was stably performed without methanol, the biofilm's dominant microorganisms were phyla *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*, accounting for >77.6% of all microorganisms detected. Among these microbes, the phyla *Planctomycetes* and *Proteobacteria* were the critical actors in this CANON system's adequate biological nitrogen removal. The phylum *Proteobacteria* attached to AOB, NOB and denitrifiers, and the relative abundances were in the upper biofilm by 21.4% and in the bottom biofilm by 18.0%. The phylum *Planctomycetes* belonged to the AnAOB (Chen *et al.* 2022) with relative abundances in the upper biofilm by 31.0% and in the bottom biofilm by 38.1%. Moreover, the phylum *Chloroflexi* enrichment often appeared in the CANON process, and

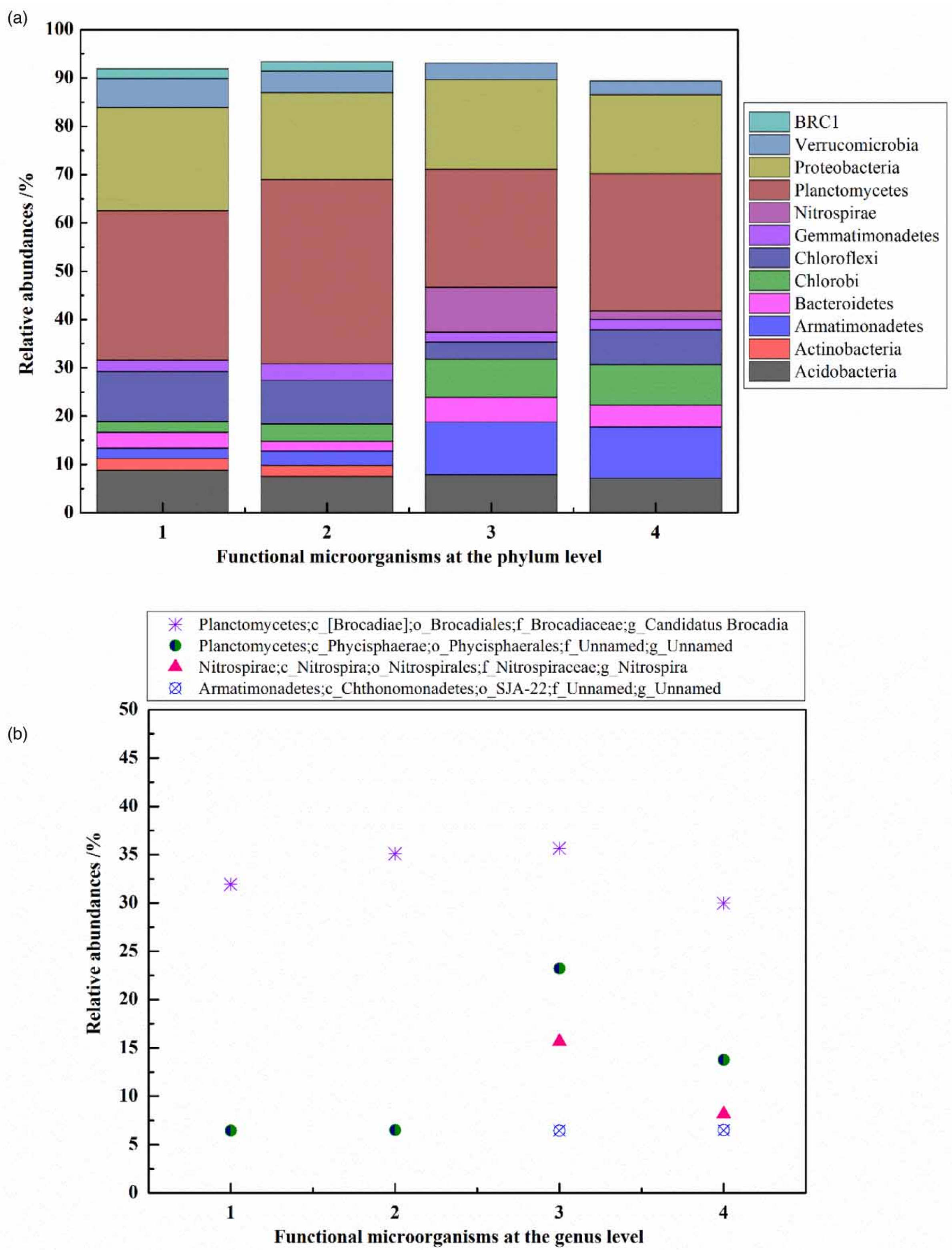


Figure 4 | Effect of low-concentration methanol on the functional microorganisms of the CANON process (A-1: upper biofilm without methanol; A-2: bottom biofilm without methanol; A-3: upper biofilm with low-concentration methanol of 38.0 mg·L^{-1}; A-4: bottom biofilm with low-concentration methanol of 38.0 mg·L^{-1}; B-1: upper biofilm without methanol; B-2: bottom biofilm without methanol; B-3: upper biofilm with low-concentration methanol of 38.0 mg·L^{-1}; B-4: bottom biofilm with low-concentration methanol of 38.0 mg·L^{-1}).

its relative abundances were 10.4% in the upper biofilm and 9.1% in the bottom biofilm. The reason was that the autotrophic phyla *Planctomycetes* and *Proteobacteria* grew well, and their metabolic products provided enough nutrients for phylum *Chloroflexi* accumulation (Qian *et al.* 2022). Furthermore, as the conventional microorganisms, the phyla *Acidobacteria* and *Verrucomicrobia* often exist in wastewater treatment plants. This stable microbial community resulted in a regular CANON operation in a SABF system.

When the low-concentration methanol increased from 0 to 38.0 $mg \cdot L^{-1}$, the CANON's microbial community in a SABF system was varied. The enzyme of phylum *Planctomycetes* converted methanol into formaldehyde, resulting in the phylum *Planctomycetes* suppression with relative abundances from 31.0 to 24.4% in the upper biofilm and from 38.1 to 28.5% in the bottom biofilm. The result agreed with some reports on low-concentration methanol inhibiting the phylum *Planctomycetes* as an AnAOB, leading to the anammox inhibition (Qian *et al.* 2022). Moreover, the phylum *Proteobacteria* inhibition occurred due to the low-concentration methanol. Its relative abundances decreased in the upper biofilm from 21.4 to 18.5% and in the bottom biofilm from 18.0 to 16.3%, resulting in ammonium nitrogen removal rate reduction in the CANON process. Furthermore, the phyla *Planctomycetes* and *Proteobacteria* inhibition resulted in fewer metabolic products, sufficient for the phylum *Chloroflexi* survival. Thus, the phylum *Chloroflexi* was suppressed with relative abundances in the upper biofilm by 3.6% and in the bottom biofilm by 7.2%. However, associated with NOB, the phylum *Nitrospirae* accumulated relative abundances of 9.3% in the upper biofilm and 1.7% in the bottom biofilm. The reason was that the surplus DO due to the AOB inhibition possibly promoted the NOB growth, which competed with the AnAOB for survival space and the AOB for the electron acceptors (O_2). Thus, the low-concentration methanol adversely influenced the stable CANON process' functional microorganisms in a SABF system.

As shown in Figure 4(b), the genus *Candidatus Brocadia*, belonging to the phylum *Planctomycetes*, was the nitrogen removal contributor to the CANON process. Its relative abundances were in the upper biofilm by 25.5% and in the bottom biofilm by 28.6% without methanol addition. When the low-concentration methanol increased from 0 to 38.0 $mg \cdot L^{-1}$, the genus *Candidatus Brocadia* was restrained with relative abundances in the upper biofilm by 12.5% and in the bottom biofilm by 16.2%. This result was similar to other research that reported that methanol feeding significantly limits genus *Candidatus Brocadia* activity and accumulation (Isaka *et al.* 2021).

The genus *Nitrospira*, which belonged to the phylum *Nitrospirae* (NOB), was enriched because of the low-concentration methanol addition, with relative abundances of 9.2% in the upper biofilm and 1.7% in the bottom biofilm. The genus *Nitrospira* enrichment harmed the CANON process. One reason was that the genus *Nitrospira* accumulation led to the AnAOB elimination at lower DO concentrations with no mass transfer limitation in the biofilm in the presence of methanol. Another reason was that the NOB enrichment could compete with the genus *Candidatus Brocadia* for nitrite nitrogen as the electron acceptor, inhibiting the system's total nitrogen removal ability (Wang *et al.* 2021). Therefore, the low-concentration methanol promoted the NOB enrichment and AnAOB elimination, breaking down the stability of the CANON process (Wu *et al.* 2021).

Furthermore, some researches showed that the methanol addition generally promoted the growth of denitrifying microorganisms, such as genera *Azospira, Dechloromonas, Denitratisoma, Flavobacterium, Longilinea*, *Ornatilinea*, *Pseudomonas Thermomarinilinea*, *Thauera*, *unclassified Chlorobiales*, *Zoogloea*, etc. Partial nitrification, anammox, and denitrification were also reported to exist in a single reactor depending on the organic matter concentration (Zhang *et al.* 2020a, 2020b). However, as Table 4 shows, these denitrifying microorganisms were virtually absent in this system. The reason was that the low-concentration methanol did not provide sufficient organic carbon sources for denitrifying microorganisms' growth. Therefore, low-concentration methanol negatively affected the dominant CANON process' microorganisms in a SABF system.

3.3. Recovery of this system

As shown in Figure 5, this experiment stopped the methanol supply from recovering in this system. The influent COD concentration was attributed to COD components in the tap water. Suitable experimental condition parameters recovered the CANON process within 46 days with an ammonium nitrogen removal rate of 97.1% and a total nitrogen removal rate of 80.8%. Then the system was in the stable operation stage for 259–361 days. When the concentration of NH_4^+-N_{inf} was 90.5–116.5 $mg \cdot L^{-1}$, the average concentrations of NH_4^+-N_{eff} and NO_2^--N_{eff} were kept in 4.4 and 4.7 $mg \cdot L^{-1}$, respectively. Besides, in this system, the average ammonium and total nitrogen removal rate were 95.5 and 80.9%, respectively.

Table 4 | Denitrifying microorganisms of this system at a genus level

Denitrifying microorganisms	Relative abundance (%) Upper biofilm without methanol	Bottom biofilm without methanol	Upper biofilm with methanol of 38.0 mg/L	Bottom biofilm with methanol of 38.0 mg/L
Azospira	0	0	0.0023	0.0023
Denitratisoma	0.0378	0.0371	0	0
Dechloromonas	0.0031	0.0068	0.1267	0.0991
Flavobacterium	0.0062	0.0332	0.0461	0.0161
Longilinea	0.1837	0.2746	0.0092	0.0184
Ornatilinea	0	0	0	0
Pseudomona	0.0463	0.0547	0	0
Thauera	0.1837	0.2746	0.0023	0.0023
Thermomarinilinea	0	0	0	0
Unclassified Chlorobiales	0	0.0010	0	0
Zoogloea	0.1304	0.1515	0	0.0023

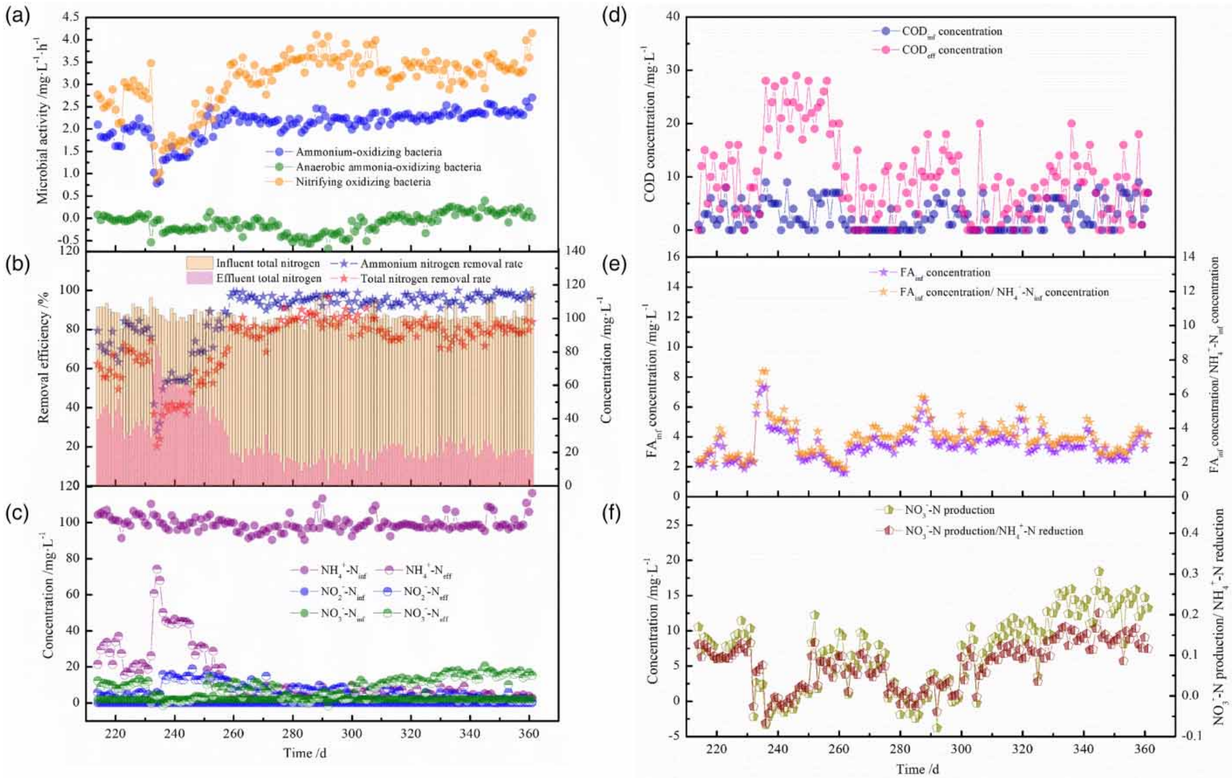


Figure 5 | Recovery of this system.

Furthermore, in the stable CANON process performance, the NOB is a potential spoilage organism to compete for O_2 with AOB and NO_2^--N with AnAOB, thus needs to be restrained. Generally, the experiment used ΔNO_3^--N/ΔNH_4^+-N as a standard for NOB overgrowth of the CANON system stability. As Figure 5(f) shows, the average practical ΔNO_3^--N/ΔNH_4^+-N standard for NOB overgrowth was 0.10, nearly 0.13 as a theoretical value (Zhao *et al.* 2021). The result implied that relatively few NOB existed in this system.

From the above, through the adverse effects of low-concentration methanol, the CANON process operation in a SABF system recovered rapidly, and the system maintained a stable total nitrogen removal capacity. The results verified that the CANON process in a SABF system had an excellent self-healing ability, which could upgrade the future CANON process's development.

4. CONCLUSIONS

This study demonstrated that the low-concentration methanol harmed the CANON process in a SABF system. The AOB and AnAOB of the CANON process were suppressed with the low-concentration methanol addition, respectively. In particular, the genus *Candidatus Brocadia* (belonging to phylum *Planctomycetes*) was obviously restrained. However, low-concentration methanol promoted the enrichment of NOB. The genus *Nitrospira* (belonging to phylum *Nitrospirae*) rapidly grew with the low-concentration methanol addition. These results led to the average ammonium nitrogen removal rate reduction from 95.0 to 70.7% and the average total nitrogen removal rate decreased from 81.3 to 43.6%. It is demonstrated that the low-concentration methanol harmed the CANON process. Fortunately, when the methanol was stopped, the CANON system had an excellent self-healing ability with the average ammonium and total nitrogen removal rate returning to 95.5 and 80.9%, respectively.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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