Rapid start-up of the anammox process by denitrifying granular sludge and the mechanism of the anammox electron transport chain

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Abstract

This study investigated the rapid start-up of an anaerobic ammonium oxidation (anammox) reactor by inoculating denitrifying granular sludge mixed anammox bacteria. The mechanism of the anammox electron transport chain (AETC) was also studied using nine different inhibitors in batch tests. This is the first study that shortened the start-up anammox reactor time to 28 days. Nitrogen removal rates (NRRs) up to 0.72 kg/(m3 d) on day 28 were achieved. Each studied inhibitor had a different binding site in the AETC. The effect of each inhibitor was determined by comparing the total nitrogen removal efficiency between the presence of an inhibitor and an appropriate control. The results confirmed that each inhibitor had various inhibition degrees that distinctly affected the AETC. Finally, the AETC mechanism was explored in detail. These findings are important for developing fast start-up processes for anammox reactors.

Keywords: Anammox; Denitrifying granular sludge; Start-up; Up-flow anaerobic sludge blanket; Anammox electron transport chain

1. Introduction

Since the first discovery of anaerobic ammonium oxidation (anammox) in a denitrifying fluidized bed reactor in the early 1990's, the anammox process has become a promising technology for removing nitrogen contaminants [1]. Anammox bacteria oxidize NH_4^+ under anaerobic conditions using NO_2^- as an electron acceptor to produce N_2 [2]. Compared with conventional biological nitrogen removal processes, the anammox process can reduce aeration by 64%, exogenous electron donors by 100% and sludge production by 80–90% [3]. For these reasons, the anammox process is considered to be a novel and sustainable biological nitrogen removal technique.

More than 100 full-scale anammox plants exist around the world [4]. However, the adaption of the anammox process to the treatment of wastewaters has been difficult. A major obstacle for the application of the anammox process is the requirement of a long start-up time due to the relatively long doubling time of anammox bacteria. Therefore, methods for decreasing or shortening the start-up period of anammox reactors have been the subject of increasing interest. During the past few years, efforts to shorten the start-up time have focused on selecting suitable reactor types, trying different types of seed sludge, and using different types of bacteria carriers. Several types of seed sludge, including conventional activated sludge from municipal wastewater treatment plant (MWTP), denitrifying sludge, nitrifying sludge, and methanogenic granules, have been tested to enrich anammox bacteria. Denitrifying sludge and methanogenic granules have been shown to be more likely to achieve high anammox performance. However, the inoculation of methanogenic granules potentially leads to sludge breakdown in the initial start-up period [5]. For a rapid and successful anammox start-up process to work, a sufficient amount of seeding biomass is essential, and the inoculated biomass must be efficiently retained in the reactor. Anammox granular sludge exhibits significant advantages over flocculent sludge, especially in the aspects of enhancement of sludge retention and tolerance to fluctuation in external conditions [6].

Key enzyme activities and the growth rate of anammox bacteria are vital factors that affect the anammox process. Enhancing

the enzymatic activities could correspondingly increase anammox bacteria activities and expedite the start-up of an anammox reactor. The anammox process involves several key enzymes that convert NH_4^+ to N_2, including nitrate reductase (NAR), nitrite reductase (NIR), hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH), all of which are located inside anammoxosomes [7]. As respiratory chain blockers, inhibitors can block different sites for many biological processes. A number of studies have reported that inhibitors can affect several processes, including: iron (III) reduction [8], Cr (VI) degradation [9], denitrification [10], nitrogenase and bidirectional Hox-hydrogenase [11]. Several studies have focused on the key enzymes activities of anammox bacteria in attempts to increase their activity. However, the involvement of the anammox electron transport chain (AETC) and the connection between the AETC and key enzyme remain unclear. The effects of the AETC and those of the relationship between the AETC and key enzymes on a fast start-up anammox reactor should be investigated.

The objective of this study was to hasten the start-up the anammox process by inoculating the denitrifying granular sludge mixed anammox bacteria in an upflow anaerobic sludge blanket (UASB) reactor. The cultivation process of the denitrifying granular sludge was examined, and the characteristics of the sludge were analyzed. After mixing the anammox bacteria, concentrations of NH_4^+-N, NO_2^--N and NO_3^--N during the anammox performance were assessed to provide more useful information for the anammox reactor operation. The other main aim of this study was to investigate the AETC by using different inhibitors. These findings may provide details on improving the start-up of the anammox process.

2. Materials and methods

2.1. Materials

All chemicals reagents were of analytical grade. Metabolic inhibitors were purchased from Sigma (St. Louis, MO), and all other reagents were purchased from Xiandai, Ltd. (Shijiazhuang, China). The inhibitors included carbonyl cyanide 3-chlorophenyl hydrazone (CCCP), sodium azide (NaN_3), capsaicin, N,N'-Dicyclohexylcarbodiimide (DCCD), quinacrine dihydrochloride (QDH), dicumarol, cupricchloride ($CuCl_2$), rotenone, and antimycin A. The inhibitors concentrations were selected according to other papers [8,10–13]. The inhibition site and concentration of the inhibitors are shown in Table 1.

2.2. Methods

2.2.1. Reactor operation

A Plexiglas upflow sludge blanket (USB) reactor with a height of 60 cm, an internal diameter of 6.8 cm and a working volume of 3.5 L was used. First, the reactor was inoculated with an activated flocculent sludge to culture denitrifying granular sludge. The activated flocculent was sourced from a municipal wastewater treatment plant located in Shijiazhuang, China. This process began with denitrified synthetic wastewater containing CH_3OH, $NaNO_3$ and KH_2PO_4. CH_3OH acted as the organic carbon, NO_3^- acted as the electron acceptor, and KH_2PO_4 acted as the anammox growth element. The C:N:P ratio was maintained at 40:10:1. Second, after the denitrifying granular sludge was matured, anammox bacteria from another anammox reactor were added to accelerate the start-up process. The biomass ratio of anammox bacteria and denitrifying granular sludge was 50:1. The reactor was operated at 35 °C. The influent pH was controlled within the 7–7.5 range. An opaque, black plastic film enclosure served to inhibit the growth of photosynthetic bacteria. The composition of the anammox synthetic wastewater was modified according to Trigo [14] (the followings are all measured in g/L, except for the trace element solution): 0.48–1.44 $(NH_4)_2SO_4$, 0.48–1.44 $NaNO_2$, 0.136 $CaCl_2$, 0.1 $MgSO_4$, 0.03 KH_2PO_4, and 0.3 $NaHCO_3$. Additionally, 1 mL/L of a trace element solution was added, which contained (in g/L) 5.00 EDTA, 5.00 $FeSO_4$, 0.43 $ZnSO_4{\cdot}7H_2O$, 0.014H_3BO_4, 0.99 $MnCl_2{\cdot}4H_2O$, 0.25 $CuSO_4{\cdot}5H_2O$, 0.19 $NiCl_2{\cdot}6H_2O$, and 0.24 $CoCl_2{\cdot}6H_2O$ [15].

2.2.2. Batch test

To ascertain the effects of different inhibitors on the anammox process, the anammox bacteria were used in batch tests with different inhibitors. The anammox bacteria were sourced from another lab-scale anammox UASB reactor with an NRRs of 6.7 kg/(m^3 d) cultured for over 2 years, which was also started-up using the same method of mixing denitrifying granular sludge. And the anammox bacteria volatile suspended solid (VSS) was 30.07 g/L, which was used to show the biomass of anammox bacteria. The tests were carried out in 300 mL serum bottles containing 300 mL media with 100 mg/L NH_4^+-N, 100 mg/L NO_2^--N, 20 g anammox bacteria (wet weight) and inhibitors at different concentrations. Serum bottles were flushed with N_2 for 5 min and sealed tightly with rubber caps to maintain anaerobic conditions. The media mainly comprised NH_4^+-N and NO_2^--N in the form of $(NH_4)_2SO_4$ and $NaNO_2$.

Biomass samples collected from the UASB reactor were washed three times with phosphate buffer solution to remove organic matter and were then washed three times with media to remove any residual nitrogen. Because the initial amounts of NH_4^+-N and NO_2^--N were consumed within 12 h (i.e., 1 cycle), biomass samples collected after the first cycle were washed three times in phosphate buffer solution and media to remove inhibitors.

All serum bottles were wrapped in an opaque, black plastic film to prevent the growth of photosynthetic bacteria. The bottles were incubated on a shaker at 35 °C. The pH of the media was adjusted to 7–7.5 with 1 M HCl or 1 M NaOH. The water samples were collected every 2 h using a needle and syringe to monitor the concentrations of NO_3^--N, NH_4^+-N and NO_2^--N, and the total nitrogen removal efficiency was calculated. The inhibitory effect of each inhibitor was determined by comparing the total nitrogen removal efficiency between the samples with an inhibitor and an appropriate control (i.e., samples containing the media without an inhibitor). Two other control tests were used in the batch test: firstly, control test 1 contained inactivated anammox bacteria and the media, and secondly, control test 2 contained only the media.

2.2.3. Analytical methods

The water samples were centrifuged for 15 min at 8000 r/min to remove the insoluble particles from the supernatant. The COD, NO_3^--N, NO_2^--N and NH_4^+-N concentrations were measured according to APHA (1995) standard methods [16]. A digital pH metre (Delta-320, China) was used for pH measurements.

3. Results and discussion

3.1. Cultivation of denitrifying granular sludge

The process of removing nitrogen and carbon from the cultivated denitrifying granular sludge is shown in Fig. 1. This process began with the denitrified synthetic wastewater containing CH_3OH, $NaNO_3$ and KH_2PO_4. CH_3OH acted as the organic carbon, NO_3^- acted as the electron acceptor and KH_2PO_4 acted as the anammox growth element. The upflow velocity of the UASB reactor was 0.71 m/h. The nitrogen loading rates (NLRs) increased from 0.69 kg/($m^3{\cdot}$d) to 2.06 kg/($m^3{\cdot}$d) as the influent NO_3^--N concentration gradually increased from 100 mg/L to 300 mg/L. Initially the nitrate removal efficiency was 55.9% and the COD removal efficiency was 66.9%, i.e., the activity of the denitrifying bacteria in

Table 1
Inhibitors used in this study.

Chmical inhibitor	Site of inhibition	Concentration (mM)	Reference
Capsaicin	NADH dehydrogenase (Complex I)	0, 0.2, 0.5, 0.8	[8]
Rotenone	NADH dehydrogenase (Complex I)	0, 0.04, 0.1, 0.2	[8]
$CuCl_2$	The prosthetic group (Fe-S protein) of NADH dehydrogenase	0, 0.02, 0.1, 0.2	[13]
QDH	FAD dehydrogenase (Complex II)	0, 0.02, 0.2, 0.4	[8]
Dicumarol	Quinone loop	0, 0.02, 0.04, 0.08	[8]
Antimycin A	Complex III	0, 0.004, 0.02, 0.04	[8]
NaN_3	Cytochrome oxidase (Complex IV)	0, 0.04, 0.2, 0.4	[10]
DCCD	ATPase	0, 0.2, 0.5, 0.8	[12]
CCCP	Proton transport	0, 0.01, 0.02, 0.04	[11]

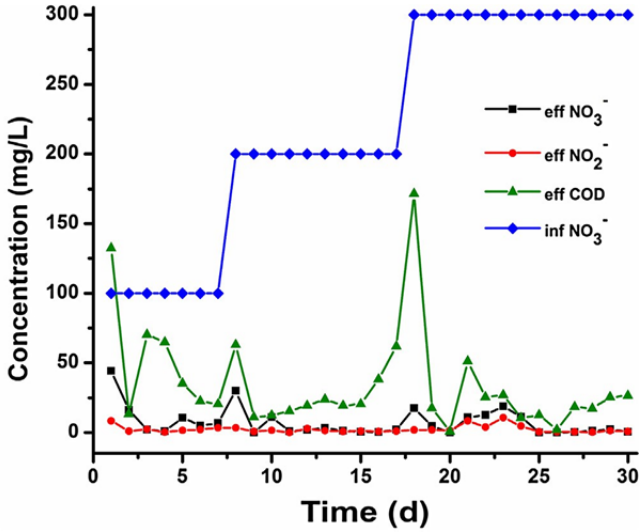

Fig. 1. The performance of nitrogen and carbon removal on the cultivation denitrifying granular sludge process in UASB reactor.

the seed sludge was poor. On day 4, the nitrate and COD removal efficiency increased to 90% and 92.5%, respectively, which indicated that the amount and activity of the denitrifying bacteria had increased. On days 4–30, the effluent NO_3^- concentration was well below the established US drinking water standard of 10 mg/L [17], and there was no accumulation of NO_2^-.

The characteristics of the denitrifying granular sludge are listed in Supporting information Fig. S1 in the online version at DOI: 10.1016/j.bej.2016.09.001. The seed activated sludge was flocculent with a dark brown color. The flocculent sludge formed finer sand-like particles with a pale brown color on day 12, and the mean diameter of the sludge in the reactor was 1–1.5 mm. After 30 days of cultivation, the granular diameter was approximately 2–4 mm with a white color and the wet density was 1.695 g/cm^3. Due to the high wet density, the settling velocity of the mature denitrifying granular sludge was 173.5 ± 15.5 m/h. This was higher than the values of 18–100 m/h reported in the literature [18]. The large granular diameter and good settling velocity indicated that the cultivation of denitrifying granular sludge was successfully achieved, and the denitrifying granular sludge was mature.

3.2. Fast start-up of the anammox process

The use of mature denitrifying granular sludge expedited the start-up of the anammox process in the UASB reactor. The biomass ratio of denitrification and anammox was 50:1. The reactor was initiated by increasing stepwise the influent NH_4^+-N and NO_2^--N concentrations as shown in Fig. 2. Based on the ammonium removal, the start-up of the anammox process was divided into

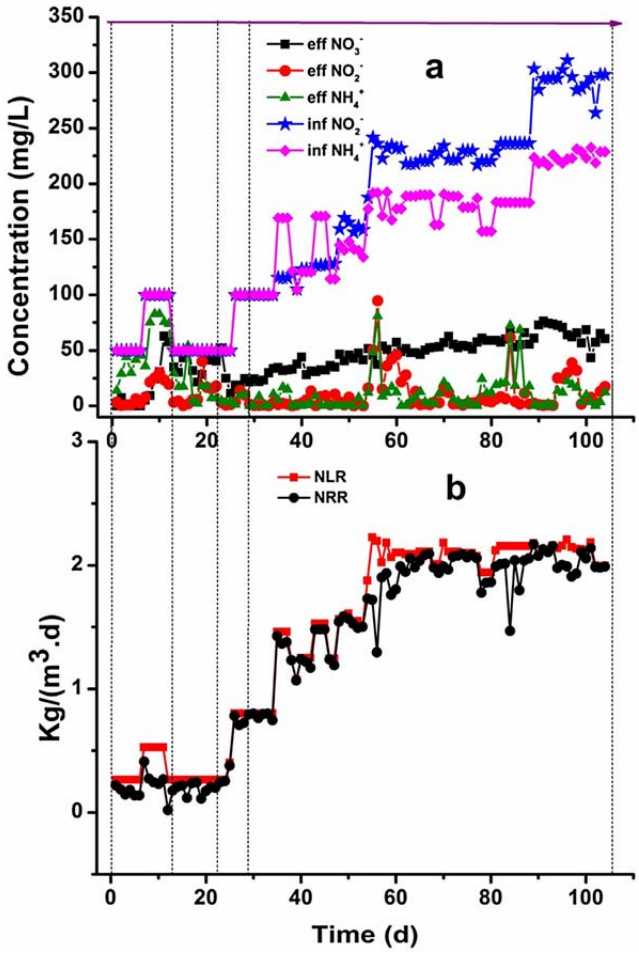

Fig. 2. Start-up performance of the anammox reactor. (a) Influent concentrations of ammonium and nitrite, effluent concentrations of ammonium, nitrite and nitrate; (b) nitrogen loading rates (NLRs) and nitrogen removal rates (NRRs).

four phases: a cell lysis phase, a lag phase, an activity elevation phase and a stationary phase [19].

Low ammonium removal was observed during the cell lysis phase (days 1–13). After mixing, the reactor was continuously fed with anammox synthetic wastewater to enrich the anammox bacteria. NH_4^+ and organic matter were released from the autolysis of the denitrifying bacteria due to the initial adverse conditions of the reactor [20]. Thus, the NH_4^+ removal was extremely low during the cell lysis phase. However, because of the presence of mixed anammox bacteria, which were able to consume some of the released NH_4^+, the effluent NH_4^+ concentration did not exceed that of the influent, indicating the superiority of our start-up strategy. The remaining denitrifying bacteria reduced NO_3^- and NO_2^- with the released organic matter from autolysis acting as electron

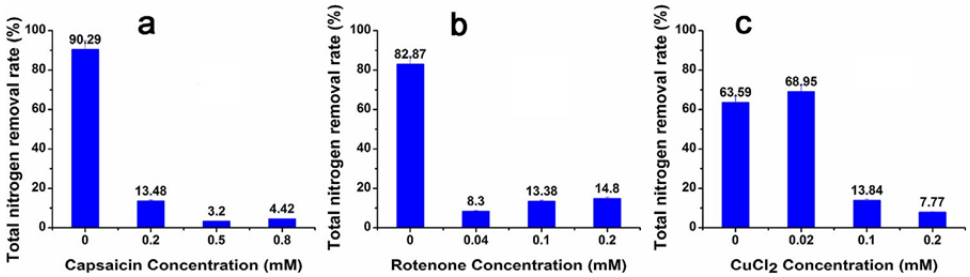

Fig. 3. Effects of inhibitors on Complex I. (a) Capsaicin; (b) rotenone; (c) $CuCl_2$.

donors. NO_2^- molecules were also reduced by the anammox bacteria. The effluent NO_2^--N concentration was lower than 5 mg/L in this phase. The concentration of the produced NO_3^--N was nearly 0. On day 14, the ammonium removal efficiency increased to 66.15% which suggested that the activity of anammox bacteria increased. From days 7–11, the influent NH_4^+-N and NO_2^--N concentrations increased to 100 mg/L. The weak activities of the anammox bacteria resulted in high effluent NH_4^+-N and NO_2^--N concentrations; thus, the influent NH_4^+-N and NO_2^--N concentrations decreased to 50 mg/L.

The lag phase (days 14–23) was characterized by a fitful decrease in the effluent NH_4^+ concentration. Overall, the average effluent NH_4^+ concentration was lower than that of the influent. During the lag phase, the average NH_4^+ removal was 80.8%. The high removal efficiency of NO_2^- and low production efficiency of NO_3^- confirmed that the denitrification process and cell lysis continued in the lag phase. On day 22, the average NRRs was 0.20 kg/(m^3·d) and the effluent NO_3^- reached 40.31 mg/L, i.e., a moderate level of anammox activity.

During the activity elevation phase (days 24–28), a continuous and increasing ammonium removal rate was observed. After 28 days of cultivation, the effluent NH_4^+-N and NO_2^--N concentrations were consistently lower than 10 mg/L. The anammox bacteria became the predominant bacteria in the consortia, and the NO_2^- was almost completely consumed by anammox. In this phase, the average effluent ammonium removal efficiency increased from 80.8% to 92.7% as the influent NH_4^+ concentrations rose from 50 mg/L to 100 mg/L. The stoichiometric molar ratio of NO_3^- production to NH_4^+ consumption was 0.20–0.27, which was similar to the theoretical stoichiometric ratio of 0.26 through the anammox process [4]. The NRRs in the effluent was 0.72 kg/(m^3 d) on day 28. The standard start-up NRRs for the anammox process was 0.50 kg/(m^3·d) [21]. These results suggested that the anammox performance progressively improved, and that the anammox reactor was successfully started up on day 28.

During the stationary phase, the influent NH_4^+-N and NO_2^--N concentrations were further improved to 220 mg/L and 300 mg/L, respectively. The average effluent NH_4^+-N concentration was maintained at 10.3 mg/L, and the ammonium removal efficiency was stably maintained at 94.29%. The NO_2^- concentration remained steady and lower than 10 mg/L. NO_2^- was effectively reduced by the anammox bacteria. The production of NO_3^- increased stepwise, and the maximum NO_3^- concentration in the effluent was 76.32 mg/L. The NH_4^+, NO_2^- and NO_3^- stoichiometric ratio was 1:1.36:0.32, i.e. nearly the same as the theoretical ratio of 1:1.32:0.26. These results indicated that the anammox reactor had reached a steady state.

Based on the above results, the start-up time of the anammox process in the UASB reactor was shortened to 28 days, and the NRRs reached 0.72 kg/(m^3 d) by using denitrifying granular sludge mixed with anammox bacteria as the inoculum. Li et al. [5] demonstrated that an anammox reactor was successfully started-up on day 35. Tsushima et al. [22] observed anammox reactions after a 55-day the start-up process. Chen et al. [23] reported that an anammox reactor was successfully started-up on day 40. In this study, an NRRs of 0.72 kg/(m^3 d) was achieved on day 28 during the start-up of the reactor. The short start-up time indicated the superiority of our start-up strategy.

3.3. Effects of inhibitors on the AETC mechanism

Several studies have focused on increasing the enzymatic activity of anammox bacteria and the fast start-up of anammox reactors. However, the mechanism of the AETC has not been clearly established. One of the aims of this study was to determine the effects of inhibitors on the AETC and its enzymes. The investigation of rapid start-up of anammox reactors using the AETC should be explored in future studies. The accelerating mechanism for redox mediators was explored using the denitrifying electron transport chain (ETC) in the denitrifying process [10]. The key enzymes activity in the anammox process was enhanced by redox mediators [24]. Thus, the anammox accelerating mechanism could also be investigated using the AETC.

In this study, the total nitrogen removal efficiency was used to investigate an influence of the inhibitors on the anammox process. Antimycin A was investigated based on the ammonium and nitrite removal efficiency. Additionally, the NH_4^+-N and NO_2^--N concentrations were kept constant in the two control tests. The results indicated that the inactivated bacteria and the media-only controls had no effects on the batch test. In the following discussion, the two control tests results are not shown in the figures.

3.3.1. Effects of inhibitors on complex I

The results in Fig. 3a reveal that the anammox process was inhibited by capsaicin even at the lowest tested concentration (0.2 mM, resulting in a total nitrogen removal efficiency of 13.48%). Capsaicin inhibited the active site of Complex I (NADH dehydrogenase). The total nitrogen removal efficiency was 3.2% and 4.42% at 0.5 mM and 0.8 mM, respectively. There was no obvious difference between the degrees of inhibition at 0.5 mM and 0.8 mM. Complex I catalyzed the electron transfer from NADH to ubiquinone (CoQ) with the transfer of $4H^+$ and the release of energy. Complex I acts as a "proton pump" driven by electron transfer. Capsaicin inhibited Complex I by binding to the active site of NADH and prevented the transfer of electrons from NADH to CoQ. This led to the inhibition of NH_4^+ and NO_2^- removal. These results suggest that Complex I plays an important role in the anammox process.

Rotenone affected the anammox process as shown in Fig. 3b. Rotenone and capsaicin have been used as respiratory chain Complex I inhibitors (at the Q site) [25]. The total nitrogen removal efficiency gradually declined from 82.87% to 14.80% to 13.38% to 8.3% with increasing rotenone concentration. Rotenone significantly inhibited the AETC at all concentrations, and the variations in the degrees of inhibition were negligible. This outcome demonstrated that rotenone inhibited Complex I and prevented electron transfer from NADH to CoQ. Like capsaicin, rotenone disrupted the

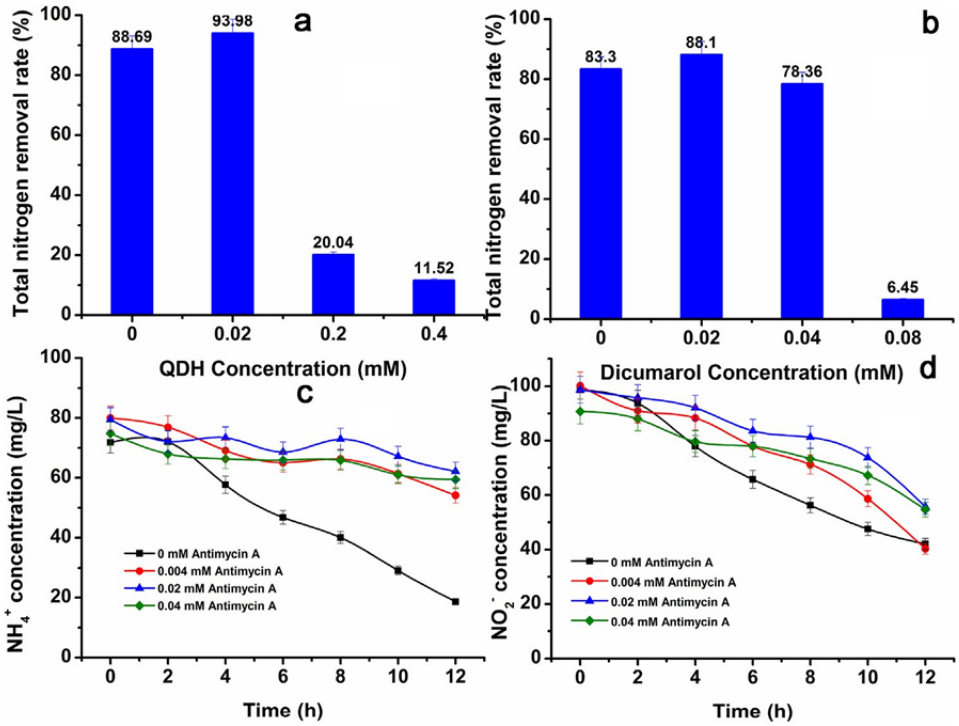

Fig. 4. Effects of inhibitors on Complex II and Complex III. (a) QDH; (b) dicumarol; (c) antimycin A NH_4^+; (d) antimycin A NO_2^-.

AETC and energy release, thereby decreasing the total nitrogen removal efficiency. These results were the same as those for the capsaicin experiment.

Cu^{2+} affected the anammox process as shown in Fig. 3c. At the lowest tested Cu^{2+} concentration of 0.02 mM, there was a slight increase in the total nitrogen removal efficiency, which was 5.36% higher than that of the control. This increase in the removal efficiency was due to the action of Cu^{2+} as an electron redox centre for shuttling electrons between different protein subunits. Many investigators have suggested that Cu^{2+} plays an important role as a prosthetic group in reducing enzymes [26]. The total nitrogen removal efficiency decreased to 13.84% and 7.77% as the concentration of Cu^{2+} increased. Thus, at higher Cu^{2+} concentrations, the activity of the anammox bacteria was inhibited. Heavy metals cannot be used as electron donors because they decrease the activity of the soluble enzymes [27]. When the Cu^{2+} concentration was 0.1 mM and 0.2 mM, the total nitrogen removal efficiency decreased. Based on these results, it can be concluded that Complex I plays a key role in the AETC.

3.3.2. Effects of inhibitors on complex II

QDH affected the anammox process as shown in Fig. 4a. QDH inhibited FAD dehydrogenase. The experimental results showed that QDH had an inhibitory effect on the anammox process. However, at a QDH concentration of 0.02 mM, the total nitrogen removal efficiency was enhanced by 5.29% compared with that of the control. The total nitrogen removal efficiency was 20.04% and 11.52% at the concentrations of 0.2 mM and 0.4 mM, respectively. The inhibition degree was more prominent at QDH concentrations of 0.2 mM and 0.4 mM than at 0.02 mM.

At 0.02 mM, QDH acted as either a medium (redox partner) or as an enhancer of FAD dehydrogenase in the transfer of electrons, as indicated by the relatively higher total nitrogen removal efficiency compared with the control. As the concentration increased, the ability for FAD dehydrogenase to transfer electrons was adversely inhibited by QDH. The inhibition of FAD dehydrogenase via QDH blocked the electron transfer channel, leading to a decreased total nitrogen removal efficiency. These results indicate that FAD dehydrogenase participates in the AETC.

3.3.3. Effects of inhibitors on complex III

Dicumarol, a quinone analogue inhibitor, blocks the transfer of electrons to menaquinone and is often used as an inhibitor of the quinone loop [28]. However, as shown in Fig. 4b, there were no obvious differences between the total nitrogen removal efficiency at the 0.02 mM and 0.04 mM compared with the control. Consequently, there was likely an alternative electron transport pathway separate from the quinone loop to the next electron carrier of NAR. The degree of inhibition was the most severe at 0.08 mM. These data indicate that the quinone loop participates in the AETC.

The antimycin A experiments showed that antimycin A slightly inhibited NO_2^- reduction (Fig. 4d) and heavily inhibited NH_4^+ oxidation (Fig. 4c). The ammonium and nitrite removal efficiency were further explored. Antimycin A inhibited the active site of Complex III and blocked electron transport from cytochrome b to cytochrome c [29]. Moreover, the NIR reduced NO_2^- to NO, and the HZS oxidized NH_4^+ to N_2H_4. When the antimycin A was added to the system, the enzymes located in front of Complex III were slightly inhibited. Thus, Complex III is located in front of the HZS and after the NIR. The findings of Kartal [30] also show that Complex III is located after the HZS, similar to the results of this study.

3.3.4. Effects of inhibitors on complex IV

Sodium azide (NaN_3) affected the anammox process as shown in Fig. 5a. Sodium azide inhibited Complex IV (cytochrome oxidase) [8]. The total nitrogen removal efficiency was 90.02%, 49.55%, 39.7% and 26.72% as the NaN_3 concentration increased. The degree of inhibition increased with increasing NaN_3 concentration. Complex IV catalyzed the transfer of electrons from cytochrome c to O_2. For each oxygen molecule reduced by cytochrome c oxidase, $4H^+$ were transported across the membrane to the intermembrane space. Sodium azide has been shown to be an inhibitor of cytochrome oxidase and has also been linked with preventing aerobic oxidation [31]. Due to the inhibition of electron transfer at the terminal

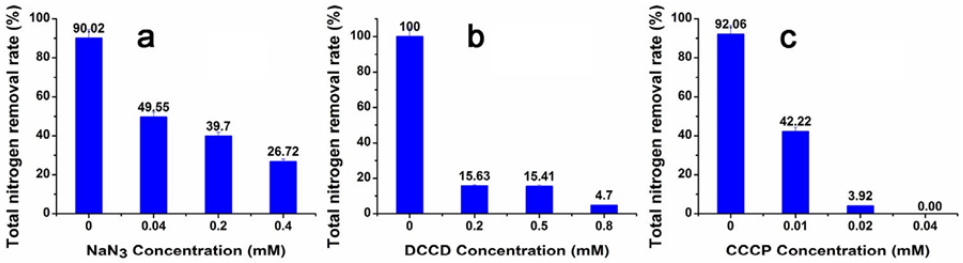

Fig. 5. Effects of inhibitors on Complex IV and other sites. (a) NaN_3; (b) DCCD; (c) CCCP.

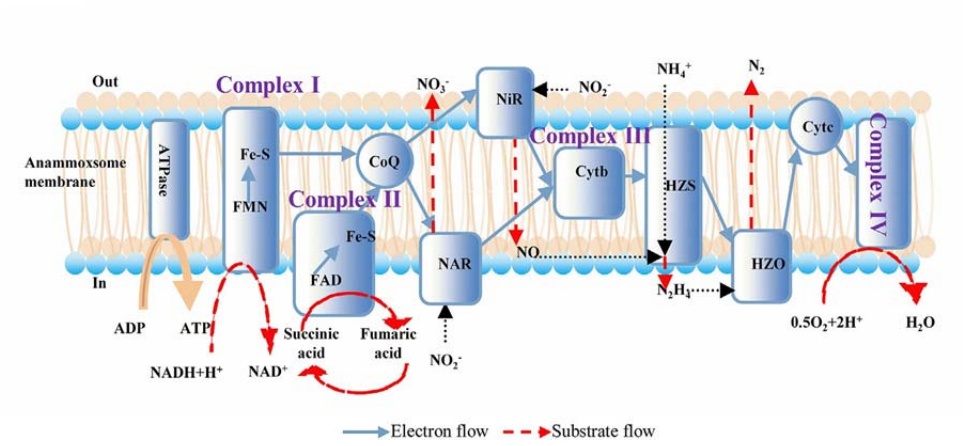

Fig. 6. The supposed mechanism of the AETC.

donor, less energy was produced by ATPase, and the activity of the anammox bacteria decreased. These results showed that Complex IV is involved in the AETC. NaN_3 prevented the transfer of electrons from cytochrome c to the final electron acceptor (oxygen). However, because the anammox process converts NH_4^+ into N_2 and the process can alternatively use NO_2^- as the terminal electron acceptor in the absence of O_2, nitrogen was still removed even at the highly inhibiting sodium azide concentration of 0.4 mM.

3.3.5. Effects of inhibitors on other sites

As illustrated in Fig. 5b, the total nitrogen removal efficiency sharply decreased with increasing DCCD. DCCD inactivated the function of ATPase by inhibiting proton translocation through the F_0 subunit of the enzyme [32]. As the DCCD concentration increased, the total nitrogen removal efficiency decreased. The lowest total nitrogen removal efficiency was 4.7% at 0.8 mM DCCD. Racker proved [33] that ATP synthase consists of F_0 and F_1 subunits. When H^+ traverses the proton channel and is returned to the mitochondrial matrix, the released energy promotes the synthesis of ATP synthase. DCCD, a fat soluble carboxyl reagent, combines with the F_0 subunit to block the proton channel and inhibit the activity of ATPase. The distinct inhibition of the total nitrogen removal efficiency in the presence of DCCD indicates the involvement of ATPase in the anammox process. In the Van Niftrik et al. [34] study, the ATPase was associated with the anammox membranes which the result was same with our investigation.

The CCCP impact on the anammox process is depicted in Fig. 5c. CCCP uncoupled the proton pump mechanism from the mitochondrial membrane, resulting in a disruption of proton transport across the membrane [35]. The total nitrogen removal efficiency fell sharply to 42.22% at 0.01 mM CCCP. The total nitrogen removal efficiency was 3.92% at 0.02 mM CCCP. The anammox process was completely inhibited at 0.04 mM. A chemiosmotic hypothesis was proposed by Mitchell [36], which suggested that most ATP synthesis in respiring cells originates from an electrochemical gradient across the inner membranes of the mitochondria through the use of NADH and $FADH_2$ and forms from the breakdown of energy-rich molecules such as glucose. The spent energy produces a proton gradient based on the electron transfer process. The proton gradient across the membrane produces a "proton-motive force" (pmf), and ATP synthase uses the pmf across the membrane to drive the synthesis of ATP from ADP. CCCP disrupts this pmf, resulting in less ATP synthesis [37]. In this experiment, the reduced enzyme activity inhibited the anammox process.

3.3.6. Electron transport chain

The supposed mechanism of the AETC and the key enzymes involved were explored using the different inhibitors that inhibited different sites in the anammox process. Inhibitory experiments with capsaicin, rotenone and $CuCl_2$ demonstrated that Complex I plays an important role in the AETC. The total nitrogen removal efficiency decreased at high QDH concentrations, which indicated that Complex II was inhibited and took part in the AETC. Complex III and Complex IV were shown to participate in the anammox process using antimycin A, dicumarol and sodium azide. Moreover, in the antimycin A experiments, the inhibition of NH_4^+ was higher than the inhibition of NO_2^-. This indicated that Complex III was located in front of the HZS and after the NIR. The DCCD experiment indicated that ATPase played an important role in the anammox process. Based on this study and previous investigations [30,38–40], a schematic of the mechanism of the AETC is shown in Fig. 6. These key enzymes are located in the anammoxosome membrane. The anammoxosome, an intracytoplasmic bounded compartment, is the locus of anammox catabolism. N_2H_4 was produced from ammonium and nitrite in the anammox substrates. In the anammox reaction, NO_2^- was first reduced to nitric oxide (NO) by NIR. NO then reacts with NH_4^+ to yield N_2H_4 by HZS. Finally, N_2H_4 was oxidized to N_2 by HZS. ATPase catalyzed the

reaction of ADP to ATP. Complex I, Complex II, Complex III and Complex IV took part in the AETC.

4. Conclusions

In this study, rapid start-up of an anammox reactor can be triggered by inoculating the denitrifying granular sludge mixed with some anammox biomass. This represents the first time that the start-up time of an anammox reactor was shortened to 28 days. The NRRs reached 0.72 kg/(m^3·d) on day 28. The supposed mechanism of the AETC was explored. The mechanism further elucidated the anammox process. Finally, according to the mechanism, electron transfer could be accelerated by increasing the key enzymatic activities.

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