Rapid start-up of the anammox process: Effects of five different sludge extracellular polymeric substances on the activity of anammox bacteria

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ABSTRACT

This study investigated the rapid start-up of the anaerobic ammonium oxidation (anammox) strategy by inoculating different biomass ratios of denitrifying granular sludge and anammox bacteria. The results demonstrated that two reactors (R1 and R2) were rapidly and successfully started-up on days 25 and 28 , respectively, with nitrogen removal rates (NRRs) of 0.70 kg/(m^3·d) and 0.72 kg/(m^3·d) at biomass ratios of 10:1 (R1) and 50:1 (R2). The explanation for rapid start-up was found by examining the effect of five different sludge extracellular polymeric substances (EPS) on the activity of anammox bacteria in the batch experiments. Batch experiments results first demonstrated that the denitrification sludge EPS (DS-EPS) enhanced the anammox bacteria activity the most, and NO_2^--N,

NH_4^+-N removal rates were 1.88- and 1.53-fold higher than the control with optimal DS-EPS volume of 10 mL. The rapid start-up strategy makes possible the application of anammox to practical engineering.

Keywords: Anammox; Denitrifying granular sludge; Start-up; Extracellular polymeric substances; Anammox bacteria activity.

1. Introduction

Since the first discovery of anaerobic ammonium oxidation (anammox) in a denitrifying fluidized bed reactor in the early 1990s (Mulder et al., 1995), the anammox process has become a promising technology for removing nitrogen contaminants. Anammox bacteria oxidize NH_4^+ under anaerobic conditions with NO_2^- as an electron acceptor to produce N_2. Compared to conventional biological processes, the anammox process can reduce aeration by 64%, exogenous electron donors by 100%, and sludge production by 80-90% (Chen et al., 2016).

However, a shortcoming in the full scale application of the anammox process is the requirement of a prolonged start-up time, which may be due to slow growth rates of anammox bacteria (the doubling time was reported to be approximately 11 days) (Strous et al., 1998). Various methods including reactor type, seed sludge and strategy have been tested for anammox rapid start-up (Tang et al., 2013). The selected reactor must be efficient in terms of biomass retention, and the loss of free bacteria should be avoided. In this case, upflow anaerobic sludge blanket (UASB) reactors can effectively retain biomass. In addition to UASB reactors, granulation assists in the enrichment of anammox bacteria to high concentrations. The biomass concentrations of anammox granular sludge in the reactors were 42.0-57.7 g-VSS/L (Tang et al., 2011). Moreover, several types of seed sludge,

including conventional activated sludge (Bi et al., 2014), denitrifying sludge (Tsushima et al., 2007; Chen et al., 2016), and nitrifying sludge (Wang et al., 2009), and methanogenic granules (Tang et al., 2009), have been tested to enrich anammox bacteria. It is widely known that EPS plays a significant role in adhesion phenomena, formation of matrix structure, granulation process and improvement of the long-term stability of granules (Schmidt and Ahring, 1994). In the rapid start-up anammox process, the structure of EPS can increase anammox bacteria resistance to adverse conditions. Cells are embedded in EPS, and this prevents anammox sludge from washing out of the reactor, and futhermore prolongs the sludge retention time to some extent. Tsushima et al. (2007) successfully started up anammox process by inoculating denitrifying sludge within 55 days. The start-up time was shortened to 40 days by using the denitrifying sludge as the seed sludge (Chen et al., 2016). The start-up time could be shorted greatly by using denitrifying sludge as seed sludge. And the anammox process was first discovered in a denitrifying fluidized bed reactor (Mulder et al., 1995). Prior to this study, the connection between the denitrifying bacteria and the anammox bacteria was unknown. Moreover, the reason why denitrifying sludge can shorten the start-up time and the effect of EPS on the activity of the anammox bacteria has not been investigated until now.

The objective of this study was to hasten the start-up of the anammox process by using the seed strategy (i.e. inoculating the denitrifying granular sludge mixed anammox bacteria) in upflow anaerobic sludge blanket (UASB) reactors. Two lab-scale UASB reactors with different inoculum biomass ratios were operated. The other major aim here was to explore the reason for rapid start-up by using the seed strategy. The effects of five different sludge EPS on anammox bacteria activity were investigated in the batch experiments.

2. Materials and methods

2.1 Synthetic wastewater

The synthetic wastewater was used to fast start-up the anammox reactor. The compositions of synthetic wastewater was simulated the real wastewater. And the characteristic of the synthetic wastewater was similar with the real wastewater. The composition of the anammox synthetic wastewater was contained as the following (all measured in g/L, except for 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 the following (in g/L): 5.00 EDTA, 5.00 $FeSO_4$, 0.43 $ZnSO_4 \cdot 7H_2O$, 0.014 H_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 $CoCl_2 \cdot 6H_2O$ (van de Graff et al., 1996).

2.2 Reactor operation

Two identical up-flow anaerobic sludge blanket (UASB) reactors (R1 and R2) were utilized to investigate the rapid start-up process in this study. The working volume of the two reactors was approximately 3.5 L with an internal diameter of 6.8 cm and a height of 71 cm. The denitrifying granular sludge was sourced from a lab-scale mature denitrification reactor while anammox bacteria were sourced from a lab-scale anammox reactor cultured for over 2 years. The volatile suspended solids (VSS) of denitrifying granular sludge and anammox bacteria were 24.53 g/L and 16.89 g/L, respectively. R1 was seeded with 900 mL denitrifying granular sludge and 130 mL anammox bacteria, while R2 was seeded with 1000 mL denitrifying granular sludge and 30 mL anammox bacteria. The biomass ratio of denitrifying granular sludge and anammox bacteria for R1 and R2 was 10:1 and 50:1, respectively. The two reactors were 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.

2.3 Batch experiments

In order to evaluate the rapid start-up strategy by inoculating the denitrifying granular sludge and anammox bacteria, five different sludge EPS (denitrification sludge [DS-EPS], nitrification sludge [NS-EPS], activated sludge [AS-EPS], perchlorate reducing bacteria [PCRB-EPS] and S-driven perchlorate reducing bacteria [S-PCRB-EPS]) were used in the batch experiments. The EPS was extracted from the same biomass of five different sludge. Moreover, the activated sludge was sourced from a municipal wastewater treatment plant. The nitrification sludge was sourced from a lab-scale reactor. The PCRB was sourced from a lab-scale reactor by using acetate as electron donors. The S-PCRB was derived from a lab-scale reactor by using S^0 as electron donors. These sludge EPS were originated from bacterial secretion, cell lysis and hydrolysis, leakage of exocellular constituents, and adsorbed organic matter from the surrounding wastewater. EPS consist of polysaccharides, proteins, nucleic acids, and lipids (Adav et al., 2008).

The experiments were carried out in 300 ml serum bottles containing synthetic wastewater of 100 mg/L NH_4^+-N and 100 mg/L NO_2^--N, 20 g (wet weight) anammox bacteria and five different sludge EPS. The 20g anammox bacteria collected from R1 were washed three times with phosphate buffer solution to remove organic matter and then washed three times with synthetic wastewater to remove any residual nitrogen. Serum bottles were flushed with N_2 for 5 min and sealed tightly with rubber caps to maintain anaerobic conditions. The synthetic wastewater mainly comprised NH_4^+-N and NO_2^--N in the form of $(NH_4)_2SO_4$ and $NaNO_2$. 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 synthetic wastewater was adjusted to 7-7.5 with 1 M HCl or 1 M NaOH. The effluent 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.

2.4 Analytical methods

The EPS were extracted from the sludge samples using a heat technique (Morgan et al., 1990). The polysaccharides content of the EPS was measured by the anthrone-H_2SO_4 method using glucose as the standard. The protein content of the EPS was measured by the Bradford method using bovine serum albumin (BSA) as the respective standard. The effluent samples were centrifuged for 15 min at 8,000 r/min to remove the insoluble particles from the supernatant. The concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N were measured according to APHA (1995) standard methods. A digital pH meter (Delta-320, China) was used for pH measurements.

3. Results and discussion

3.1 Rapid start-up of the anammox process

Fig. 1. Start-up performance of R1. (a) Influent concentrations of ammonium (Inf NH_4^+) and nitrite (Inf NO_2^-), effluent concentrations of ammonium (Eff NH_4^+), nitrite (Eff NO_2^-), and nitrate (Eff NO_3^-); (b) nitrogen loading rates (NLRs) and nitrogen removal rates (NRRs).

Fig. 2. Start-up performance of R2. (a) Influent concentrations of ammonium (Inf NH_4^+) and nitrite (Inf NO_2^-), effluent concentrations of ammonium (Eff NH_4^+), nitrite (Eff NO_2^-), and nitrate (Eff NO_3^-); (b) nitrogen loading rates (NLRs) and nitrogen removal rates (NRRs).

The biomass ratio of denitrifying granular sludge and anammox bacteria of R1 and R2 was 10:1 and 50:1, respectively. Two reactors were started up by increasing stepwise the influent NH_4^+-N and NO_2^--N concentrations as shown in Figs. 1 and 2. Based on the amount of ammonium removed, the start-up of the anammox process was divided into four phases as follows: a cell lysis phase (I), a lag phase (II), an activity elevation phase (III), and a stationary phase (IV) (Chamchoi and Nitisoravut, 2007).

At the beginning of the anammox reactors' start-up, NH_4^+ and organic matter were released from the denitrifying bacteria autolysis due to the initial adverse conditions of the reactor (Chamchoi and Nitisoravut, 2007). Bi et al. (2014) named this period the cell lysis phase (the effluent ammonium was higher than the influent one), which was commonly found in many other studies (Wang et al., 2009; Tang et al., 2009). The cell lysis phase of R1 lasted 10 days (day 1-10), while the R2 lasted 13 days (day 1-13). However, due to the presence of anammox bacteria the effluent NH_4^+-N concentration did not exceed that of the influent in this study, indicating the superiority of our start-up strategy. The results were same with those of Li (2012). Rapid start-up anammox strategy by inoculating the mixed sludge from a partial nitration reactor and an anammox reactor was successful on day 35, and furthermore there was no sign of the cell lysis phase (Li et al., 2012). The NO_2^--N of R1 and R2 was completely consumed in this phase, which could be attributed to the prevalence of denitrification. The remaining denitrifying bacteria reduced NO_3^--N and NO_2^--N with the released organic matter from autolysis acting as electron donors.

The lag phase lasted for 6 days (day 11-16) and 10 days (day 14-23) for R1 and R2, respectively. In this phase, the effluent average ammonium removal efficiency was 75.0% and 80.8% of R1 and R2, respectively. Similarly, the compete removal of NO_2^--N confirmed that denitrification as well as cell lysis phase still continued in this phase. Moreover, the effluent NO_3^--N concentration of R1 and R2 increased progressively. The effluent NO_3^--N concentration was 13.44 mg/L and 19.98 mg/L of R1 and R2 on day 16 and 23, respectively, which revealed itself to be juvenile anammox activity in both reactors (Fig. 1, 2).

Continuous increase of ammonium and nitrate removal was observed during the activity elevation phase. Take R1 for example (Fig. 1), from day 17 to 25, the NRRs increased from 0.23 kg/(m^3·d) to 0.70 kg/(m^3·d), which suggested that anammox performance improved progressively. The reactor was successfully started up on day 25 according to the start-up standard for the anammox process proposed by Jin et al. (2008), which was 0.50 kg/(m^3·d) for NRRs. After 25 days of cultivation, the average stoichiometric molar ratio of nitrite consumption versus ammonium was calculated as 1.29, and the stoichiometric molar ratio of nitrate production versus ammonium consumption was calculated as 0.22-0.29, which was similar to the theoretical stoichiometric ratio of 1.32 and 0.26 through the anammox process (Strous et al., 1998). These results suggested that the anammox performance progressively improved. R2 was successfully started-up with 0.72 kg/(m^3·d) NRRs on day 28.

During the stationary phase, the reactors' influent NO_2^- and NH_4^+ concentrations were further improved to 300 mg/L and 230 mg/L, respectively. The maximum effluent NO_3^--N concentration was 65.05 mg/L and 66.93 mg/L of R1 and R2, which was a slightly higher than the theoretical value as calculated by stoichiometric ratio. The high effluent nitrate concentration indicated that the

denitrification disappeared in the stationary phase. The anammox bacteria were dominant in reactors.

The durations of every phase for either reactor during the start-up period is summarized in Table 1. In this study the successful start-up time of R1 and R2 took 25 and 28 days, respectively, and the NRRs were 0.70 kg/(m^3·d) and 0.72 kg/(m^3·d), also respectively. According to Chen et al. (2016), the anammox process was successfully started up by mixing denitrifying-anammox at a 3:1 volume ratio on day 40, and the NRRs were 0.55 kg/(m^3·d). These findings indicated that the denitrifying sludge accelerated the anammox start-up process. However, the reason why the denitrifying sludge can accelerate the anammox start-up process was not investigated.

Table 1 The duration of every phase for R1 and R2 during the start-up period.

3.2 The reason for rapid start-up of the anammox process using the seed strategy

3.2.1 The effects of DS-EPS on the activity of the anammox bacteria

Fig. 3 The activity of the anammox bacteria with different volumes of denitrification sludge EPS (DS-EPS). (a) The concentration of NO_2^-; (b) the concentration of NH_4^+; (c) the concentration of NO_3^-; (d) the removal rate of NO_2^- and NH_4^+.

Fig. 3 depicts the effects of different DS-EPS volumes on the activity of anammox bacteria in the batch experiments. Five DS-EPS volumes (1 mL, 5 mL, 10 mL, 20 mL, and 30 mL) were selected for this experiment. Excepting the 30 mL, the anammox activity was enhanced in all other volumes. The activity of anammox bacteria tended to increase when the DS-EPS volume ranged from 0 to 10 mL. The NH_4^+-N and NO_2^--N showed peak removal performance with maximum removal rates of 11.70 mg/L-h and 8.11 mg/L-h, and the NO_3^- concentration reached its maximum with 10 mL DS-EPS at 8 h. Following this the NO_2^--N and NH_4^+-N removal rates fell to 7.72 mg/L-h

and 6.33 mg/L-h with 20 mL DS-EPS, which was still higher than the control. The NH_4^+-N and NO_2^--N removal rates with 30 mL DS-EPS were almost same as the control. The control NO_2^--N removal rate was 6.21mg/L-h, while the NO_2^--N removal rates with 1 mL, 5 mL, 10 mL, and 20 mL DS-EPS were 7.56 mg/L-h, 9.33 mg/L-h, 11.70 mg/L-h, and 7.72 mg/L-h, respectively, these were 1.22-, 1.50-, 1.88- and 1.24-fold higher than the control experiment. Moreover, the NH_4^+-N removal rate was 1.09-, 1.29-, 1.53-, and 1.11-fold higher than the control.

Based on the batch experiments it can be concluded that the anammox activity was greatly enhanced with the DS-EPS, especially with 10 mL DS-EPS. The start-up time obtained using the denitrifying granular sludge mixed with anammox as the inoculated sludge was abbreviated to 25 days and 28 days, which also proved that the denitrifying sludge had a positive effect on the anammox bacteria activity. In this way the start-up strategy proved to be advantageous.

3.2.2 The effect of other four sludge EPS on the activity of the anammox bacteria

Fig. 4 The activity of the anammox bacteria with activated sludge EPS (AS-EPS), S-driven perchlorate reducing bacteria EPS (S-PCRB-EPS), nitrification sludge EPS (NS-EPS) and perchlorate reducing bacteria EPS (PCRB-EPS) volumes of 10 mL. (a) The concentration of NO_2^-; (b) the concentration of NH_4^+; (c) the concentration of NO_3^-".

In order to further confirm the effect of DS-EPS on the activity of the anammox bacteria, other four sludge EPS including AS-EPS, NS-EPS, PCRB-EPS and S-PCRB-EPS were also investigated. Fig. 4 illustrates the effects of these four sludge EPS on the activity of the anammox bacteria with other four sludge EPS volumes of 10 mL. It can be seen that NO_2^--N was completely consumed at 8 h with existing PCRB EPS. The control NO_2^--N and NH_4^+-N removal rates were 10.21 mg/L-h and 7.81 mg/L-h, respectively. The NO_2^--N and NH_4^+-N removal rates were 11.82 mg/L-h and 8.19

mg/L-h with 10 mL PCRB-EPS, which was 1.15- and 1.05-folds higher than the control, respectively. The NO_2^--N and NH_4^+-N removal rates with the AS-EPS were almost same as the control. The NO_2^--N and NH_4^+-N removal rates were inhibited compared to the control with the S-PCRB-EPS and NS-EPS. The NO_2^--N and NH_4^+-N removal rates were enhanced by 1.88- and 1.53-fold with the 10 mL DS-EPS. It was only the PCRB-EPS that slightly accelerated the NO_2^--N and NH_4^+-N removal rates, but was still lower than the removal rates with 10 mL DS-EPS.

Many studies have confirmed that nitrate as an alternative electron acceptor can be reduced by PCRB (Xiao and Roberts, 2013). Research has also suggested that a potential exists for shared enzymes in the reduction pathway of perchlorate and nitrate for some PCRB (Xu et al., 2004). Therefore the PCRB and denitrifying bacteria may secrete the same substances or enzymes on their EPS, which increases the anammox activity. PCRB could accomplish perchlorate reduction using both organic (including lactate, pyruvate, methanol, ethanol, and acetate) and inorganic electron donors (including H_2, Fe^0, S^0, $S_2O_3^{2-}$) (Shrout and Parkin, 2006), of which acetate was the most commonly used. In this study, the PCRB and the S-PCRB were cultured by using acetate and S^0 as the electron donors, respectively. The PCRB used the organic matter as the electron donor, which was the same as the denitrifying bacteria. However, the autotrophic S-PCRB used S^0 as the electron donor, which was different from the heterotrophic denitrifying bacteria. Subsequently the PCRB increased the activity of the anammox bacteria, but the S-PCRB did not.

In this study, the EPS contents of the different sludge were also analyzed. The EPS contents were shown in Table 2. It can be seen that the proteins of DS-EPS and PCRB-EPS was higher than that of others sludge. Thus, it is worth speculating that the proteins of the EPS may be increase the anammox activity. However, this assumption needs to be investigated in the future.

Table 2 The EPS contents of different sludge

From the above results, it can be seen that the DS-EPS was the most effective on increasing the activity of anammox bacteria. It was worth noting that the DS-EPS increased the anammox activity progressively. Proof emerged that the start-up strategy using denitrifying granular sludge mixed anammox bacteria was successful. However, further research is required to verify which substance affects anammox bacteria activity.

4. Conclusion

In this study, the successful start-up times of reactors R1 and R2 took 25 and 28 days with 10:1 and 50:1 biomass ratios of denitrifying granular sludge and anammox bacteria, respectively. The start-up time was in fact substantially shortened when the seed strategy was applied. The five different sludge EPS batch experiments proved that the anammox bacteria activity reached its peak with 10 mL DS-EPS. The batch experiments also verified that the strategy of rapidly starting up the anammox reactors by denitrifying granular sludge mixed anammox bacteria was successful and beneficial.

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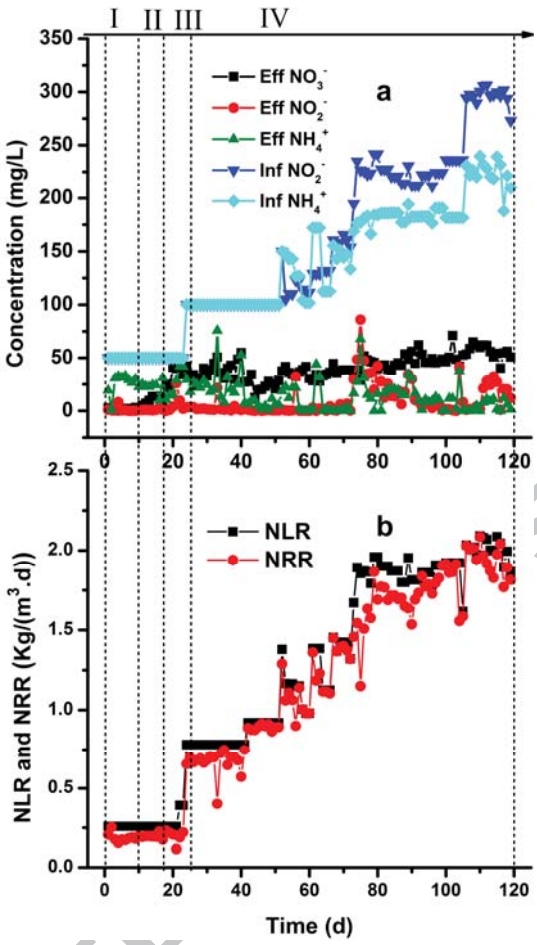


Fig. 1. Start-up performance of R1. (a) Influent concentrations of ammonium (Inf NH_4^+) and nitrite (Inf NO_2^-), effluent concentrations of ammonium (Eff NH_4^+), nitrite (Eff NO_2^-), and nitrate (Eff NO_3^-); (b) nitrogen loading rates (NLRs) and nitrogen removal rates (NRRs).

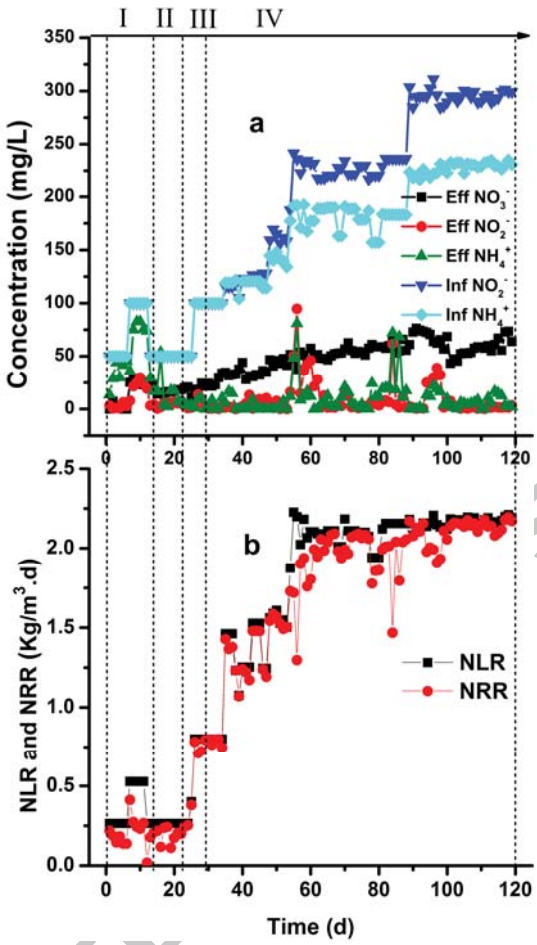


Fig. 2. Start-up performance of R2. (a) Influent concentrations of ammonium (Inf NH_4^+) and nitrite (Inf NO_2^-), effluent concentrations of ammonium (Eff NH_4^+), nitrite (Eff NO_2^-), and nitrate (Eff NO_3^-); (b) nitrogen loading rates (NLRs) and nitrogen removal rates (NRRs).

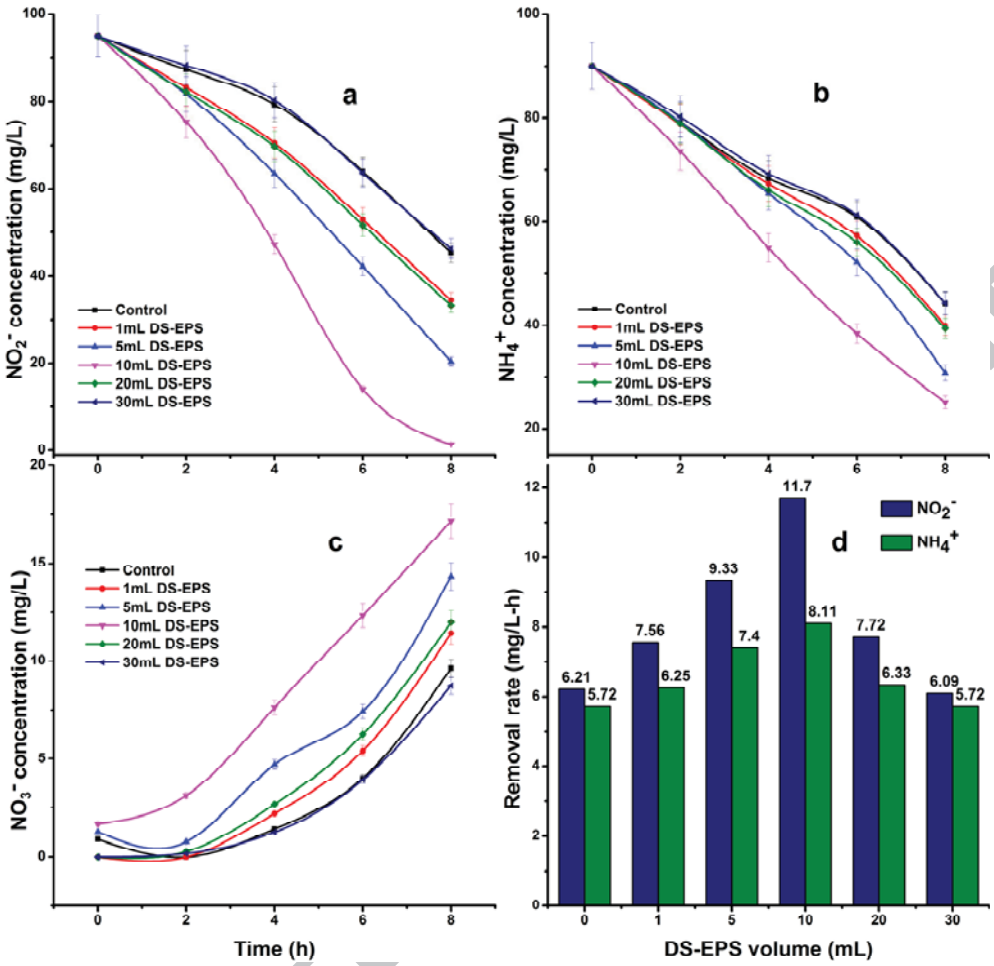


Fig. 3 The activity of the anammox bacteria with different volumes of denitrification sludge EPS (DS-EPS). (a) The concentration of NO_2^-; (b) the concentration of NH_4^+; (c) the concentration of NO_3^-; (d) the removal rate of NO_2^- and NH_4^+.

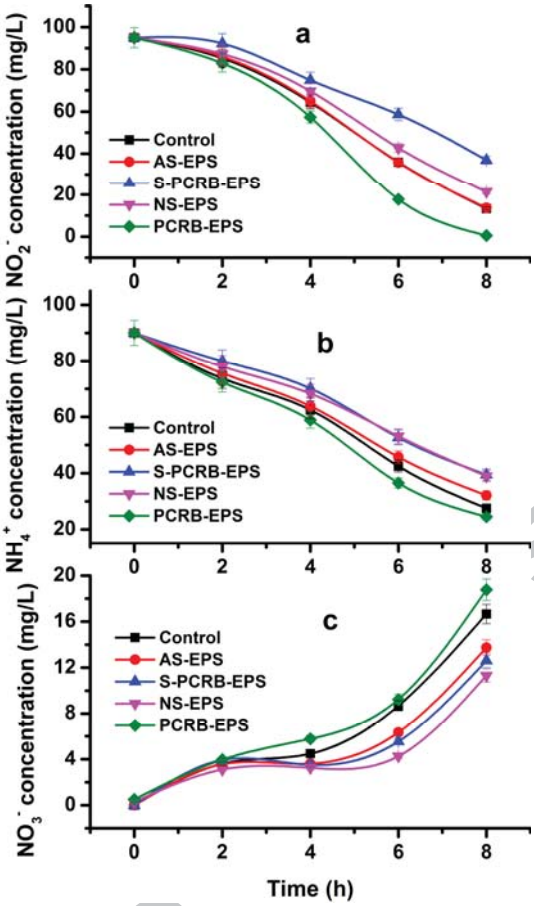


Fig. 4 The activity of the anammox bacteria with activated sludge EPS (AS-EPS), S-driven perchlorate reducing bacteria EPS (S-PCRB-EPS), nitrification sludge EPS (NS-EPS) and perchlorate reducing bacteria EPS (PCRB-EPS) volumes of 10 mL. (a) The concentration of NO_2^-; (b) the concentration of NH_4^+; (c) the concentration of NO_3^-".

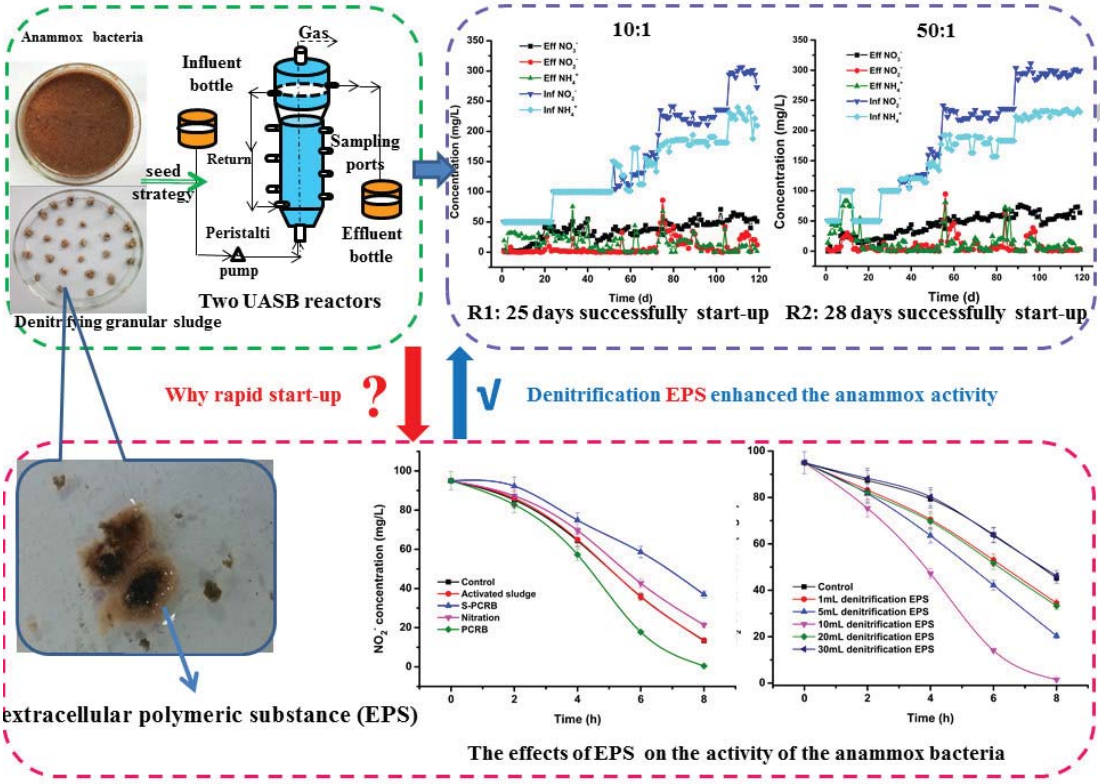
Table 1 The duration of every phase for R1 and R2 during the start-up period.

Reactor	Cell lysis phase (days)	lag phase (days)	activity elevation phase (days)	Total start-up period (days)
R1	10 (day 1-10)	6 (day 11-16)	9 (day 17-25)	25
R2	13 (day 1-13)	10 (day 14-23)	5 (day 24-28)	28

Table 2 The EPS contents of different sludge

Sludge	Extracellular Polymers Substances (mg/L)	
	proteins	Polysaccharides
Denitrifying granular sludge	178.3647	136.3197
PCRB	111.0356	38.0709
S-PCRB	0.3153	41.6923
Activated sludge	0.6839	59.0118
Nitration sludge	47.1442	69.7186

Graphical Abstract



Highlights

- The activity of the anammox bacteria was greatly enhanced with 10 mL denitrification EPS.
- The activity of the anammox bacteria was slightly enhanced with 10 mL PCRB EPS.
- The anammox reactor was fast start-up by mixture of denitrifying-anammox bacteria.
- The start-up time of R1 and R2 was shortened to 25 and 28 days at biomass ratios of 10:1 and 50:1.