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Coupling of sulfur(thiosulfate)-driven denitratation and anammox process to treat nitrate and ammonium contained wastewater

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ABSTRACT

This study investigated the feasibility of a new biological nitrogen removal process that integrates sulfur-driven autotrophic denitratation ($NO_3^- \rightarrow NO_2^-$) and anaerobic ammonium oxidation (Anammox) for simultaneous removal of nitrate and ammonium from industrial wastewater. The proposed sulfur(thiosulfate)-driven denitratation and Anammox process was developed in two phases: First, the thiosulfate-driven denitratation was established in the UASB inoculated with activated sludge and fed with ammonium, nitrate and thiosulfate for 52 days until the nitrite level in the effluent reached 32.1 mg N/L. Second, enriched Anammox biomass was introduced to the UASB to develop the integrated thiosulfate-driven denitratation and Anammox (TDDA) bioprocess (53–212 d). Results showed that nitrate and ammonium could be efficiently removed from synthetic wastewater by the integrated TDDA system at a total nitrogen (TN) removal efficiency of 82.5 ± 1.8% with an influent NH_4^+-N of 101.2 ± 2.2 mgN/L, NO_3^--N of 101.1 ± 1.5 mgN/L and thiosulfate of 202.5 ± 3.2 mg S/L. It was estimated that Anammox and autotrophic denitritation ($NO_2^- \rightarrow N_2$) contributed to about 90% and 10% of the TN removal respectively at stable operation. The established TDDA system was further supported by high-throughput sequencing analysis that sulfur-oxidizing bacteria (e.g., Thiobacillus and Sulfurimonas) coexisted with Anammox bacteria (e.g., Ca. Kuenenia and Ca. Anammoxoglobus) in this syntrophic biocenosis. Additionally, batch experiments were conducted to reveal the kinetic rates and to reconcile the stoichiometry of the electron donor/acceptor couples of the TDDA process. The results unraveled the mechanisms in the new bioprocess: i) sulfite and elemental sulfur (S^0) were initially generated from branched thiosulfate; ii) oxidation of sulfite and elemental sulfur coupled with fast and slow denitratation; iii) nitrite produced from denitratation together with ammonium were effectively converted to dinitrogen gas via Anammox.

1. Introduction

The anaerobic ammonium oxidation (Anammox) process is an energy-efficient and sustainable option for biological nitrogen removal (BNR). In this process, ammonium directly react with nitrite to produce dinitrogen gas under anoxic conditions (Kartal et al., 2011). The stable provision of nitrite for Anammox process is of crucial importance for its implementation. Nitrite produced in partial nitrification ($NH_4^+ \rightarrow NO_2^-$) can be utilized by Anammox bacteria. The combination of partial nitrification and Anammox (PN/A) process relies on stable cooperation between ammonium oxidizing bacteria (AOB) and Anammox bacteria. Although different process controls were applied to maintain the partial nitrification such as online monitoring and control (e.g., DO, pH, NH_4^+, NO_3^-) (Joss et al., 2011; Volcke et al., 2006; Wett, 2007), partial nitrification is still not always under control in long-term operation. For example, the produced nitrite in partial nitrification can be oxidized by

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nitrite-oxidizing bacteria (NOB), which resulted in nitrate build-up in the effluent and disruption of PN/A process. It significantly affects the performance of PN/A process, both in treating ammonium-rich wastewater (e.g., reject water, landfill leachate and food processing digestate) and municipal wastewater (Cao et al., 2017; Joss et al., 2011; Lackner et al., 2014).

Heterotrophic denitratation ($NO_3^- \rightarrow NO_2^-$) can also produce nitrite, which together with ammonium can be converted to dinitrogen gas by Anammox. The heterotrophic DEnitrifying AMmonium Oxidation (DEAMOX) process can be realized in a single reactor without complex controlling systems. However, the competition between heterotrophic denitrifying bacteria and Anammox bacteria is likely to occur in DEAMOX. The heterotrophic denitrifiers are more energetically active (- 427.0 kJ/mol), with higher growth rate (0.25/h) and biomass yields (1.1 gVSS/gNO_3^--N) (Henze, 2007; Nerenberg et al., 2002; Rittmann and McCarty, 2001) than Anammox bacteria (−355.0 kJ/mol, 0.0075−0.014/h and 0.1 gVSS/gN) (Jetten et al., 1998; Lotti et al., 2015; Zhang et al., 2017). The excessive growth of heterotrophic denitrifiers in DEAMOX system could result in: i) mass transfer limitation for Anammox biomass due to the formation of thick biofilms or aggregates, ii) reduction of the Anammox population and activity, and iii) deterioration of nitrogen removal performance (Du et al., 2017; Takekawa et al., 2014).

Autotrophic denitrifying sulfur-oxidizing bacteria (SOB) can use reduced sulfur compounds (e.g., S^{2-}, S^0, $S_2O_3^{2-}$) for chemolithotrophic growth with simultaneous nitrate reduction (Pokorna and Zabranska, 2015). Nitrite as an intermediate of the aforementioned nitrate reduction, its accumulation has been frequently observed in sulfur-driven autotrophic denitrification systems (An et al., 2010; Campos et al., 2008; Sun and Nemati, 2012), and this offers the opportunity to supply Anammox bacteria with nitrite. Moreover, due to the lower biomass yield of SOB (0.59−0.65 gVSS/gNO_3^--N) (Koenig and Liu, 2001; Mora et al., 2014), the risk is lower for Anammox bacteria to be overgrown by SOB. It is reported that the combination of sulfur-driven denitratation and anammox plays an important role for nitrogen cycle in marine systems (Canfield et al., 2010; Russ et al., 2014). However, little is known about using such combined process for wastewater treatment (Kalyuzhnyi et al., 2006; Van de Graaf et al., 1996).

The industrial wastewater containing both nitrate and ammonium (such as the effluents of mine and mill, fertilizer factory, Table S1) are generally treated in two-stage nitrification and denitrification with dosing external carbon source (Hirata et al., 2001; Shivaraman et al., 2001; Zaitsev et al., 2008). Compared with such conventional BNR, coupling of sulfur-driven denitratation and Anammox process could be an efficient and cost-effective approach. Therefore, we proposed to dose thiosulfate as external electron donor for developing a new thiosulfate-driven denitratation process and coupling with anammox for removal of nitrate and ammonium from wastewater.

In the present study, the thiosulfate-driven denitratation and Anammox (TDDA) system was developed in two phases. Thiosulfate-driven autotrophic denitratation was started up in an upflow anaerobic sludge bed (UASB) reactor in the first 52 days. Then Anammox biomass was inoculated into the UASB reactor and the reactor was continuously operated for another 160 days to investigate the feasibility and performance of the TDDA process. The detailed objectives of this study were to: i) investigate the performance and stability of the new combined bioprocess, ii) examine the stoichiometry and kinetics of both thiosulfate-driven denitratation and Anammox experimentally, iii) analyze the microbial communities in the reactor and iv) elucidate the mechanisms of thiosulfate oxidation and nitrogen removal in the TDDA system.

2. Materials and methods

2.1. Reactor set-up and operation

The new TDDA system was developed in a double-walled lab-scale UASB reactor with an effective volume of 2.0 L, an inner diameter of 4.5 cm and a height of 130 cm. A three-phase (liquid-gas-solid) separator was installed at the top of the reactor, and the produced gas was continuously recirculated by a peristaltic pump to enhance mixing. The temperature of the reactor was maintained at 30 ± 1 °C by the recycling water from water bath to the inner and outer walls of the UASB. Details of the reactor set-up are shown in Fig. S1 of the supplementary information. The hydraulic retention time (HRT) of the reactor was set to 24 h before day 15, after which it was decreased to 12 h by doubling the influent flow rates for the rest of the investigation to day 212. The reactor was covered with aluminum foil to prevent growth of phototrophic bacteria and protect Anammox bacteria from light. The UASB reactor was operated for 212 days divided into two phases: Phase 1 for thiosulfate-driven denitratation (0−52 d) and Phase 2 for the TDDA (53−212 d). The operational conditions are summarized in Table 1.

2.2. Synthetic wastewater and sludge inoculum

The two synthetic wastewater streams fed to the UASB comprised solutions A and B fed in equal flows (1:1 v/v). Solution A contained $Na_2S_2O_3$ (200−400 mg S/L) and $NaHCO_3$ (4000 mg/L). Solution B contained $NaNO_3$ (100−200 mgN/L) and NH_4Cl (100-200mgN/L), $MgSO_4 \cdot 7H_2O$ (600 mg/L), KH_2PO_4 (54 mg/L), $CaCl_2 \cdot 2H_2O$ (360 mg/L). Two mL/L of each of trace element solutions I and II (Van de Graaf et al., 1996) were added to solution B. Detailed information about solutions A and B as well as the trace elemental solutions is shown in Table S2. The concentrations of nitrate and ammonium in the influent were designated at the moderate levels of the industrial wastewater (Table S1). Each prepared solution was purged with pure dinitrogen gas for 10 min to remove oxygen before it was connected to the influent pipes. The containers of the feeding solutions A and B and the reactor head space were connected to dinitrogen gas bags to maintain atmospheric pressure.

To initiate thiosulfate-driven autotrophic denitratation, the UASB was seeded with activated sludge taken from the Sha Tin Sewage Treatment Works in Hong Kong. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were 4.9 g/L and 4.4 g/L at the beginning of the operation (Fig. S2). The UASB was seeded with 600 mL of Anammox sludge on day 52 for developing TDDA process. The inoculated Anammox biomass was taken from an Anammox reactor. The Anammox system was an anaerobic sequencing batch reactor (SBR) with an effective volume of 4 L that was operated for more than 200 days and fed with nitrite and ammonium. The MLSS and MLVSS of Anammox sludge were 4.8 and 4.2 g/L. The performance of Anammox SBR is described in Fig. S3. On day 77, a further 400 mL of Anammox sludge was added to the UASB reactor.

2.3. Batch tests

After the UASB reactor achieved stability on day 129, three batch tests were performed on sludge harvested from the UASB reactor to investigate the stoichiometry and kinetics of sulfur and nitrogen conversions in the TDDA system. Batch test A (BT-A) was performed on day 142 to evaluate the thiosulfate-driven denitrification activity only. In BT-A, the initial concentrations of NO_3^- and $S_2O_3^{2-}$ were 50 mgN/L and 100 mg S/L respectively (without ammonium). Batch test B (BT-B) was conducted on day 144 to estimate the

Table 1
Operational conditions of the UASB at different phases.

Phase Time (d)	HRT (h)	Influent concentrations(mg/L)			N-loading kgN/(m^3·d)	S/N ratio
		NH_4^+-N	NO_3^--N	$S_2O_3^{2-}$-S		
Phase 1A (1–14)	24	50	50	175	0.1	3.5
Phase 1B (15–28)	12	50	50	100	0.2	2
Phase 1C (29–52)	12	100	100	200	0.4	2
Phase 2 (53–212)	12	100	100	200	0.4	2

Anammox activity only. The initial NH_4^+ and NO_2^- concentrations were both 40 mgN/L (without thiosulfate). Batch test C (BT-C) was performed on day 147 to simultaneously investigate autotrophic denitrification and Anammox activities. In BT-C, the initial concentrations of nitrogen and sulfur compounds were 50 mgN/L NO_3^-, 50 mgN/L NH_4^+ and 100 mg S/L $S_2O_3^{2-}$.

The batch tests were conducted in serum bottles with a total volume of 120 mL and 100 mL for liquid. The sludge taken from the UASB reactor was washed twice with mineral medium (same composition as synthetic wastewater without substrates) and centrifuged at 3500 rpm for 5 min at 25 °C, and resuspended in the serum bottles with mineral medium. The initial pH value was adjusted to 8.0 by adding 0.2M HCl or 0.2M NaOH. The serum bottles were flushed with pure dinitrogen gas for 10 min to remove the oxygen in the liquid and headspace, sealed with rubber stopper and aluminum cap, and then incubated on a thermostatic shaker operated at 220 rpm for 1 h at 31 °C to achieve stable condition. Then concentrated solutions ($NaNO_3$, $NaNO_2$, NH_4Cl, $Na_2S_2O_3$) were injected into serum bottles with a syringe to achieve the desired initial N and S concentrations. During the batch experiments, 2 mL of each sample was collected at appropriate time intervals (20–60 min) to measure the concentrations of thiosulfate, sulfate, nitrate, nitrite and ammonium. The concentrations of MLSS and MLVSS in serum bottles were determined at the end of the tests. All the tests were performed in duplicate. BT-A and BT-C were carried out for 480 min until the nitrate was completely consumed and BT-B was carried out for 150 min until the nitrite was depleted.

2.4. Calculations

2.4.1. Contributions of anammox and denitritation to the TN removal

As shown in Fig. 1b, Anammox and autotrophic denitritation are responsible for the TN removal in the TDDA system because both processes can produce nitrogen gas, which is then released from the system. In the TDDA system, ammonium uptake for bacterial synthesis was neglected because of low yield coefficients. Hence ammonium was assumed to be removed only via Anammox. The contributions from the Anammox (α_A) and the autotrophic denitritation (α_B) process to the TN removal were estimated by Eq. (1) and Eq. (2)

$$\alpha_A = 1.974^*[NH_4^+\text{-}N_{removal}]/[TN_{removal}] \times 100\% \quad (1)$$

$$\alpha_B = 100\%\text{-}\alpha_A \quad (2)$$

where $[NH_4^+\text{-}N_{removal}]$ and $[TN_{removal}]$ are the removed concentrations of ammonium (mgN/L) and total nitrogen (mgN/L) determined from routine data from the TDDA system. The 1.974 is the mass of produced dinitrogen gas per mass ammonium removed by Anammox ($mgN_2\text{-}N/mgNH_4^+\text{-}N$) (Lotti et al., 2014).

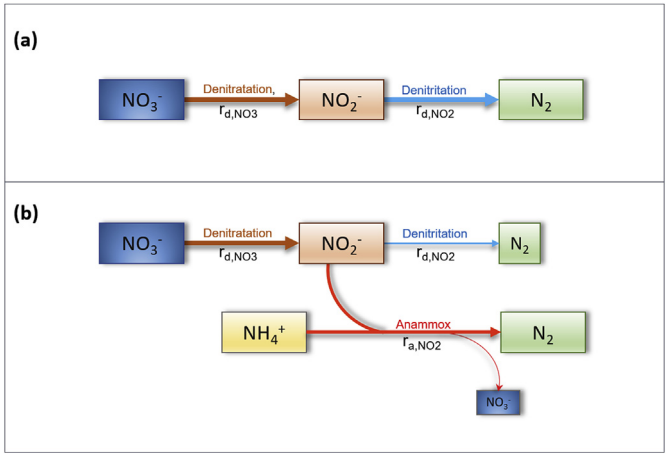

Fig. 1. Kinetics of nitrogen conversions in autotrophic denitrification (a) and the TDDA (b) systems.

2.4.2. Kinetics analysis of batch test

The two-step denitrification model was applied in this study to evaluate denitrification kinetics (Almeida et al., 1995; Matsui and Yamamoto, 1986). In the thiosulfate-driven denitrification system, nitrate is first reduced to nitrite by denitratation and then nitrite is in turn reduced to dinitrogen gas by denitritation, as illustrated in Fig. 1a. Denitratation and denitritation rates are calculated as Eq. (3) and Eq. (4) respectively. In the TDDA system, the nitrate is first reduced to nitrite by denitratation and the produced nitrite is utilized by Anammox and denitritation (Fig. 1b). The rates of autotrophic denitratation, denitritation and Anammox are determined by Eqs. (5)–(8).

For autotrophic denitrification (two-step denitrification):

$$r_{d,NO3} = -d[NO_3^-\text{-}N]/dt/VSS \quad (3)$$

$$r_{d,NO2} = r_{d,NO3} - d[NO_2^-\text{-}N]/dt/VSS \quad (4)$$

For TDDA (two-step denitrification and Anammox):

$$r_{a,NH4} = -d[NH_4^+\text{-}N]/dt/VSS \quad (5)$$

$$r_{a,NO2} = 1.146 \times r_{a,NH4} \quad (6)$$

$$r_{d,NO3} = -d[NO_3^-\text{-}N]/dt\ /VSS + 0.161 \times r_{a,NH4} \quad (7)$$

$$r_{d,NO2} = r_{d,NO3} - r_{a,NO2} - d[NO_2^-\text{-}N]/dt\ /VSS \quad (8)$$

where $r_{d,NO3}$ and $r_{d,NO2}$ are the nitrate and nitrite utilization rates through denitratation and denitritation; $r_{a,NH4}$ and $r_{a,NO2}$ are the ammonium and nitrite utilization rates through Anammox. $d[NH_4^+\text{-}N]/dt$, $d[NO_3^-\text{-}N]/dt$ and $d[NO_2^-\text{-}N]/dt$ are the slopes of the plots of ammonium, nitrate and nitrite concentrations versus time in the batch tests, which are obtained from linear regression of the measured data. The determined VSS is assumed to be the active biomass concentration in batch tests. It is recognized that this VSS

concentration comprises a number of different groups of functional organisms mediating the different bioprocesses in the TDDA system.

2.5. Analytical methods

The MLSS and MLVSS concentrations were determined according to standard methods (APHA, 2005). UASB influent and effluent samples were periodically collected to measure concentrations of nitrogen and sulfur compounds. NO_2^-, NO_3^-, $S_2O_3^{2-}$, and SO_4^{2-} were analyzed by ion chromatography (IC) using a Dionex Ionpac (AS19 columns). NH_4^+ was measured with a Lachat Quickchem 8000 (Milwaukee) flow-injection analyzer (FIA). The pH value was determined with a portable digital multi-parameter meter (WTW 3420).

2.6. Microbial community analysis

Microbial communities in the UASB reactor were analyzed by high-throughput sequencing analysis. Briefly, 20 mL of sludge were collected from the UASB reactor on day 52 (representing the thiosulfate-driven denitratation system) and day 140 (representing the TDDA system) and then stored in a freezer at −20 °C until DNA extraction. The DNA was extracted from the sludge sample using the E.Z.N.ATM Mag-Bind Soil DNA Kit (OMEGA, USA) according to manufacturer's instruction. Bacterial 16S rRNA genes (*Escherichia coli* position 341–805) were PCR-amplified with bacterial primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Eiler et al., 2012). Amplification was conducted with 15 μL of 2 × Taq master Mix, 1 μL of Bar-PCR primer 341F (10 μM), 1 μL of Primer 805R (10 μM), 10–20 ng of Genomic DNA with H_2O to 30 μL. Thermal program was 94 °C for 3 min, and 5 cycles of 94 °C for 30 s, 45 °C for 20 s and 65 °C for 30 s, and then 20 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Afterwards, the PCR products were then conducted by Illumina MiSeq platform at Sangon Biotech Co., Ltd, Shanghai, China.

3. Results and discussion

3.1. Reactor performance

3.1.1. Phase 1 of the UASB—thiosulfate-driven denitratation

The thiosulfate-driven denitratation process was established in 52 days, and it was divided into Phase 1A (0–14 d), Phase 1B (15–28 d), and Phase 1C (29–52 d). The influent concentrations of ammonium and nitrate on average were 50.9 ± 1.4 mgN/L and 51.6 ± 0.9 mgN/L in Phases 1A and 1B respectively, then increased to 100.6 ± 2.2 mgN/L and 100.2 ± 1.4 mgN/L in Phase 1C (Fig. 2a). The average nitrate removal efficiency was 74.3% in Phase 1A, dramatically decreased to 33.9% in Phase 1B, before gradually increasing to 62.5% at the end of Phase 1C (Fig. 2c). The concentration of nitrite in the effluent was below 0.2 mgN/L in Phase 1A, and then it was observed with a maximum concentration of 3.1 mgN/L in Phase 1B, and the nitrite accumulation ratio (NAR) varied from 7.2% to 18.6% calculated by Eq. (S1). The nitrite concentration in the effluent continuously increased to 32.1 mgN/L with a nitrite accumulation ratio of 52.2% at the end of Phase 1C (Fig. 2a). This result indicates that the thiosulfate-driven denitratation was successfully developed in the reactor. In contrast, ammonium cannot be removed in this phase with removal efficiency below 3.5%.

The concentration of thiosulfate in the influent was maintained at 175.4 ± 5.4 mg S/L to achieve a S/N mass ratio of 3.5 in Phase 1A (Fig. 2b). When the S/N ratio was decreased to 2.0 in Phase 1B, the influent thiosulfate was 101.6 ± 1.9 mg S/L. Afterwards, it was increased to 201.2 ± 2.0 mg S/L in Phase 1C to keep the S/N ratio at 2.0 when the influent ammonium and nitrate concentrations were doubled. Thiosulfate was completely consumed throughout Phases 1A-1C and it could not be detected in the effluent of the UASB reactor. The concentration of sulfate in the influent was kept at 42.6 ± 1.4 mg S/L throughout Phases 1A-1C (from the $MgSO_4 \cdot 7H_2O$ in the influent). The average concentration of sulfate in the effluent was 216.7 mg S/L in Phase 1A, and it decreased to 147.3 mg S/L in Phase 1B before rising to 239.7 mg S/L in Phase 1C. During Phases 1A-1C, the sulfur mass balance between the influent and the effluent exceeded 95.5% ($|\Delta SO_4^{2-}\text{-S}/\Delta S_2O_3^{2-}\text{-S}| \times 100\%$). Therefore, all of the thiosulfate was oxidized to sulfate with no accumulation of sulfur intermediate in the reactor, and this result was in line with the previous studies using thiosulfate for autotrophic denitrification (Manconi et al., 2007; Matsui and Yamamoto, 1986; Qian et al., 2018).

The level of nitrite in the effluent of the UASB reactor was negligible in Phase 1A, suggesting that the consumed nitrate was completely reduced to dinitrogen gas. This finding was similar with previous studies that nitrate could be completely reduced to dinitrogen gas without nitrite accumulation in the effluent of the reactor, when the S/N ratio in the influent was higher than 4.3 (Yamamoto-Ikemoto et al., 2000; Zou et al., 2016). However, the significant increase of nitrite concentration in the effluent of the UASB reactor was observed in Phases 1B and 1C as the S/N ratio decreased to 2.0. Matsui and Yamamoto (1986) observed that over 30% of nitrite accumulation ratio in the fluidized bed reactor, when the S/N ratio was below 2.0 in the influent. Qian et al. (2018) also reported that when the UASB reactor conducted with S/N of 1.5, nitrite accumulation ratio increased to 80% in the effluent. As a result, the S/N ratio is a critical parameter for determining the final products in autotrophic denitrification. When the system was operated under S/N of 3.5, formed nitrite can be further reduced to dinitrogen gas and negligible nitrite was detected in the effluent. However, when S/N ratio was decreased to 2.0, accumulated nitrite cannot be further reduced due to thiosulfate limitation and nitrite was retained in the effluent.

3.1.2. Phase 2 of the UASB—thiosulfate-driven denitratation and anammox

To develop the proposed TDDA system, the enriched Anammox biomass was introduced to the UASB reactor on day 52. During Phase 2, the UASB was continuously operated for more than 5 months (160 days) to investigate the performance of the TDDA bioprocess. Phase 2 was comprised of Phase 2A (TDDA start-up, 53–129 d) and Phase 2B (TDDA stable operation, 130–212 d). The concentrations of nitrate and ammonium in the influent were kept at 101.1 ± 1.5 and 101.2 ± 2.2 mgN/L respectively in Phase 2 (Fig. 3a). The UASB reactor immediately showed ammonium removal capability after inoculation of Anammox biomass, since the ammonium concentration in the effluent decreased to 75.6 mgN/L on day 53 (Fig. 3a) with an ammonium removal efficiency of 24.2% (Fig. 3c). Thereafter, the ammonium removal efficiency gradually increased and reached 71.8% at the end of Phase 2A. The ammonium removal efficiency and the effluent ammonium concentration during Phase 2B remained constant at 75.7 ± 2.1% and 24.6 ± 2.3 mgN/L respectively. The concentration of nitrate in the effluent decreased along with the ammonium—it decreased to 11.1 mgN/L at the end of Phase 2A, and then remained at 9.6 ± 1.5 mgN/L throughout Phase 2B, with a nitrate removal efficiency exceeding 90.0%. The concentration of nitrite in the effluent was low with an average of 0.7 mgN/L during Phases 2A and 2B. As the concentrations of nitrate and ammonium in the effluent continuously decreased, the TN removal efficiency increased from 39.5% on day 53–80.3% at the end of Phase 2A, and thereafter remained constant at 82.5%

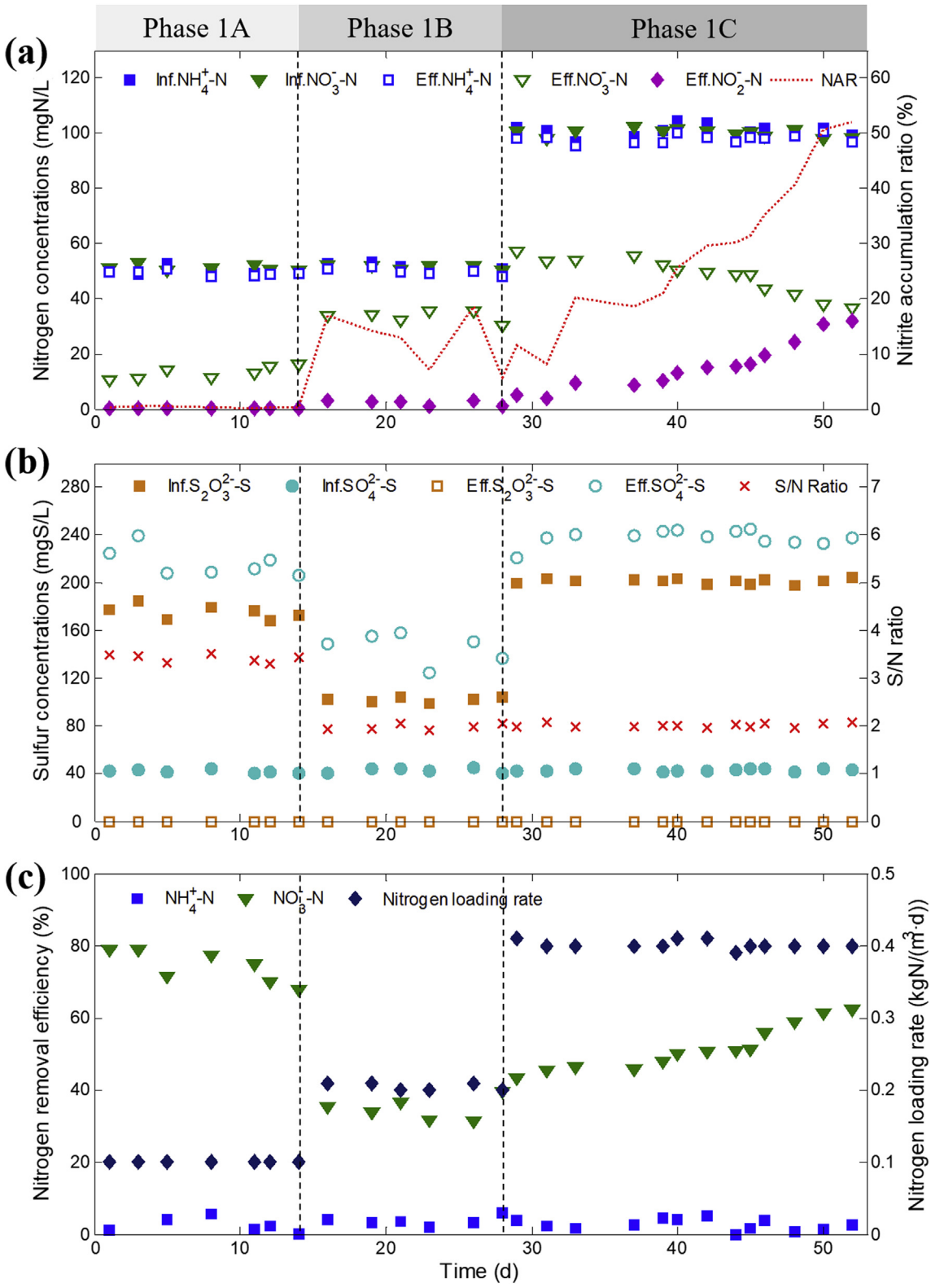

Fig. 2. Performance of the UASB in Phase 1: (a) influent and effluent nitrogen concentrations and the nitrite accumulation ratio (NAR), (b) influent and effluent sulfur concentrations and the S/N ratio, and (c) removal efficiencies of ammonium and nitrate and the nitrogen loading rate.

throughout Phase 2B, with a TN removal rate of 0.33 kg N/(m^3·d). The ratio of consumed NO_3^--N to consumed NH_4^+-N was maintained at 1.20 during stable operation of the TDDA system, and ratio of consumed $S_2O_3^{2-}$-S to consumed NO_3^--N was 2.21.

The influent concentration of thiosulfate was maintained at 202.5 ± 3.2 mg S/L in Phases 2A and 2B. This thiosulfate was completely oxidized to sulfate in the UASB, and together with the influent sulfate concentration of 42.1 ± 2.3 mg S/L, resulted in an

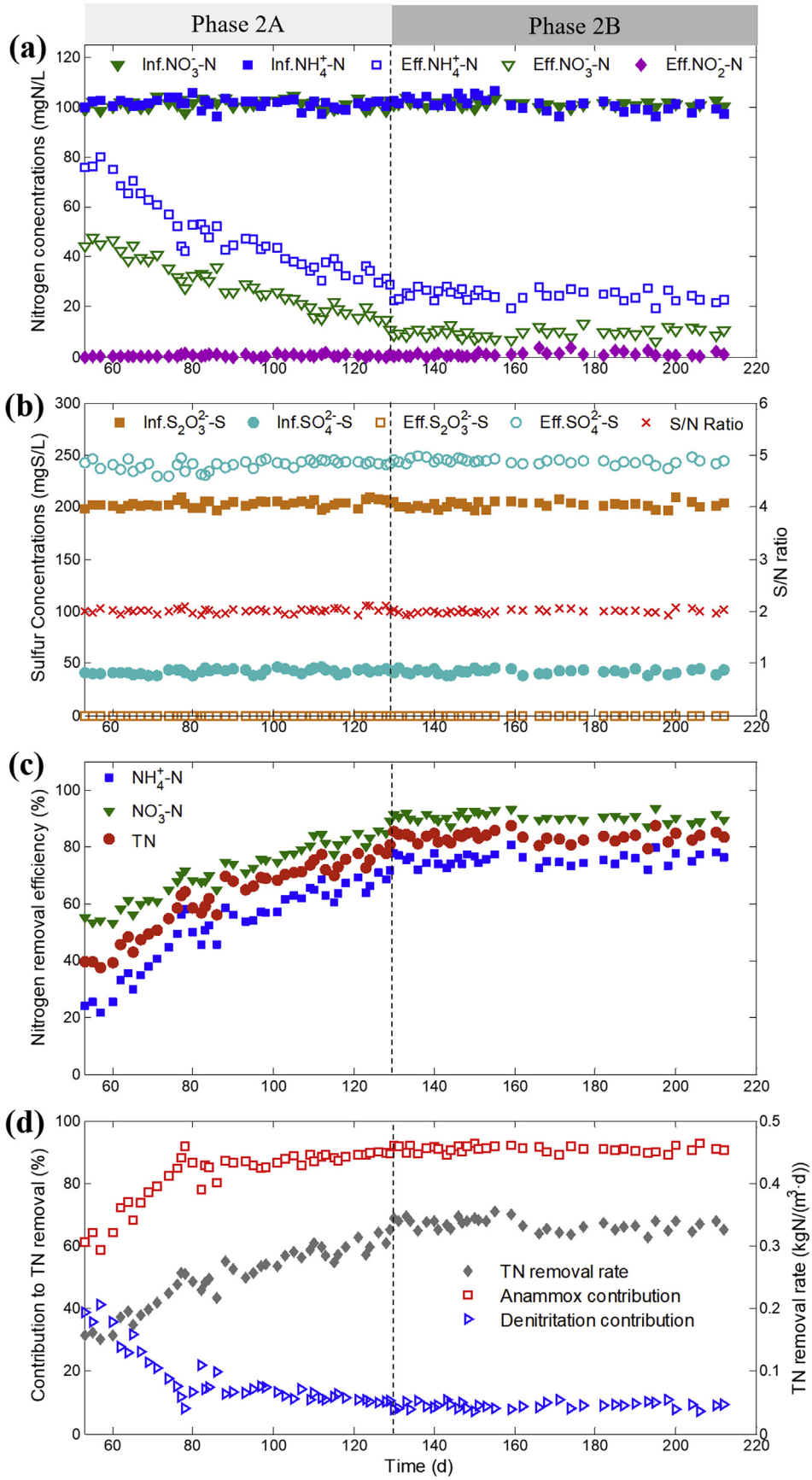

Fig. 3. Performance of the UASB in Phase 2: (a) influent and effluent nitrogen concentrations, (b) influent and effluent sulfur concentrations and the S/N ratio, (c) ammonium, nitrate and the TN removal efficiencies, and (d) contribution of Anammox and autotrophic denitritation to total nitrogen removal.

effluent sulfate concentration of 242.0 ± 4.2 mg S/L. The variations of sulfur compounds in the TDDA system were similar to those in the thiosulfate-driven denitratation system, in which thiosulfate was fully oxidized to sulfate and the sulfur mass balance exceeded 95%.

In order to examine the effectiveness of Anammox in this system, the contributions of Anammox and autotrophic denitritation to the TN removal were estimated by Eq. (1) and Eq. (2). At the beginning of Phase 2A, autotrophic denitritation comprised 38.9% of the TN removal, but it decreased to 10.1% (Fig. 3d) at the end of Phase 2A and remained at about 10% throughout Phase 2B. In contrast, Anammox accounted for 61.1% of the TN removal at the beginning of Phase 2A, then it increased to 89.9% at the end of Phase 2A, and remained at about 90% throughout Phase 2B. These outcomes confirmed that about 90% of dinitrogen gas was generated via Anammox in the combined system, while the autotrophic denitrification was mainly responsible for nitrate reduction to nitrite, and only 10% of dinitrogen gas was produced through autotrophic denitrification. This finding is consistent with the performance in DEAMOX that Anammox constituted over 80–94% of the TN removal (Cao et al., 2016; Du et al., 2017). Consequently, the efficient nitrogen removal in the TDDA system was attributed to the cooperative action between autotrophic denitratation and Anammox.

3.2. Kinetics analysis through batch experiments

3.2.1. Evaluation of thiosulfate-driven denitrification activity

The first batch test (BT-A) was performed to determine autotrophic denitrification activity (without Anammox), as shown in Fig. 4a. The test was divided into Period-1 (0–20 min) and Period-2 (20–480 min) based on different conversion rates of nitrogen and sulfur compounds. The thiosulfate was rapidly depleted in Period-1 with the initial concentration of 105.7 mg S/L, and the consumption rate was 141.0 mg S/(gVSS·h) (Table 2). The sulfate concentration rapidly increased from 57.9 mg S/L to 111.5 mg S/L at a generation rate of 68.5 mg S/(gVSS·h) in Period-1. It then increased more gradually to 154.4 mg S/L at the end of Period-2 at a constant rate of 2.6 mg S/(gVSS·h). Unlike present study, previous experiments described that the amount of thiosulfate removed was in agreement with the amount of sulfate generated in thiosulfate-driven denitrification (Campos et al., 2008; Mora et al., 2014). This phenomenon implies that the thiosulfate was not directly oxidized to sulfate, but with sulfur intermediates formation (to be discussed in Section 3.3).

Coupled with the oxidation of thiosulfate and its intermediates, the concentration of nitrate quickly declined from 55.2 mgN/L to 34.6 mgN/L at a rate of 26.3 mgN/(gVSS·h) in Period-1 (Table 2), then gradually decreasing to zero at a removal rate of 2.1 mgN/(gVSS·h) in Period-2. The concentration of nitrite quickly increased to 19.9 mgN/L at a nitrite accumulation rate of 25.4 mgN/(gVSS·h) in Period-1, before rising slowly and continuously to 42.3 mgN/L at a rate of 1.6 mgN/(gVSS·h) in Period-2. The nitrite accumulation ratio reached 81.7% by the end of test.

BT-A revealed nitrite accumulation in TDDA system resulting from a higher reduction rate of nitrate than nitrite. As the nitrate reduction rate was 29.2 and 4.2 times higher than the nitrite reduction rate in the first and second periods (Table 2), nitrite concentration was continuously increasing during experiment. From the two-step denitrification analysis (Fig. 1a), 25% of the electrons transferred (2.67 mmol e^-/L) from thiosulfate oxidation was used in nitrite reduction to dinitrogen gas, while 75% (7.88 mmol e^-/L) was consumed in nitrate reduction to nitrite.

3.2.2. Evaluation of anammox activity

The second batch test (BT-B) was performed to determine Anammox activity (without thiosulfate-driven denitrification), as shown in Fig. 4b. The initial nitrite and ammonium concentrations were 41.3 mgN/L and 42.6 mgN/L, respectively. The concentrations of nitrite and ammonium simultaneously decreased at the rates of 7.6 and 5.5 mgN/(gVSS·h) respectively, while the concentration of nitrate slowly increased at a rate of 1.4 mgN/(gVSS·h). The stoichiometric ratio of the ammonium consumption rate to the nitrite consumption rate was 1.38, and it was slightly higher than 1.32–1.33 obtained from enriched Anammox culture in SBR and UASB reactors (Jetten et al., 1998; Tang et al., 2011).

3.2.3. Evaluation of thiosulfate-denitrification and anammox activities

The third batch test (BT-C) was carried out to simultaneously investigate the autotrophic denitrification and Anammox activities, described in Fig. 4c. The conversions of thiosulfate and sulfate were similar to observation in the BT-A. In Period-1, thiosulfate was completely consumed in 20 min at a rate of 133.7 mg S/(gVSS·h), and about half of thiosulfate was converted to sulfate at a production rate of 70.7 mg S/(gVSS·h) (Table 2). In Period-2, the sulfate increased slowly to 162.9 mg S/L at the end of experiment, with a sulfate generation rate of 3.3 mg S/(gVSS·h).

Nitrite increased to its peak of 20.3 mgN/L at the end of Period-1. The accumulated nitrite was then entirely consumed within 150 min, afterwards, the nitrite cannot be detected in the Period-2. The concentration of nitrate dropped from 55.3 mgN/L to 32.5 mgN/L at a removal rate of 29.5 mgN/(gVSS·h) in Period-1. Nitrate then dropped more gradually at 3.0 mgN/(gVSS·h) in Period-2, and it was depleted within 420 min. The level of ammonium dropped from 54.5 mgN/L to 52.9 mgN/L in Period-1 at a consumption rate of 2.0 mgN/(gVSS·h). In Period-2, the ammonium removal rate stayed at 5.3 mgN/(gVSS·h) (from 20 to 150 min) in the presence of nitrite in the bulk liquid. The ammonium removal rate then decreased and remained at 1.4 mgN/(gVSS·h) after the formed nitrite concentration decreased to zero in the rest of Period-2 (from 150 to 420 min). The ammonium concentration was finally reduced to 11.2 mgN/L at the end of Period-2. About 19.6% of the produced nitrite from nitrate reduction was reduced to dinitrogen gas by denitritation in the TDDA system, whereas 80.4% of the produced nitrite from denitratation was utilized by Anammox. And this was in agreement with nitrite accumulation ratio of 81.7% in BT-A. The ratio of removed NO_3^--N to removed NH_4^+-N and removed $S_2O_3^{2-}$-S was 1:0.78:1.89. The stoichiometric value of the S/N ratio for full denitrification was the 3.68 (Mora et al., 2014). Both nitrate and nitrite were entirely removed in this test, and this result proved that TDDA can largely reduce the thiosulfate (electron donor) demand by 49% compared with complete thiosulfate-driven denitrification.

3.3. Speculated mechanisms in TDDA system

As mentioned above, denitritation was less competitive than denitratation and Anammox in the TDDA system. The reaction rates of denitritation, denitratation and Anammox could be obtained from the third batch test (BT-C). The denitratation rate was about 22.7 times higher than the denitritation rate in Period-1, and it was 2.1–4.4 times higher than denitritation in Period-2. The electrons consumption in nitrate reduction (9.05 mmol e^-/L) were 3.35 times higher than nitrite reduction (2.70 mmol e^-/L) calculated from the two-step denitrification analysis (Fig. 1b). The outcomes confirmed that denitratation has a higher activity than denitritation in the TDDA system.

Furthermore, Anammox was more competitive for nitrite utilization than denitritation: the nitrite consumption rate of Anammox

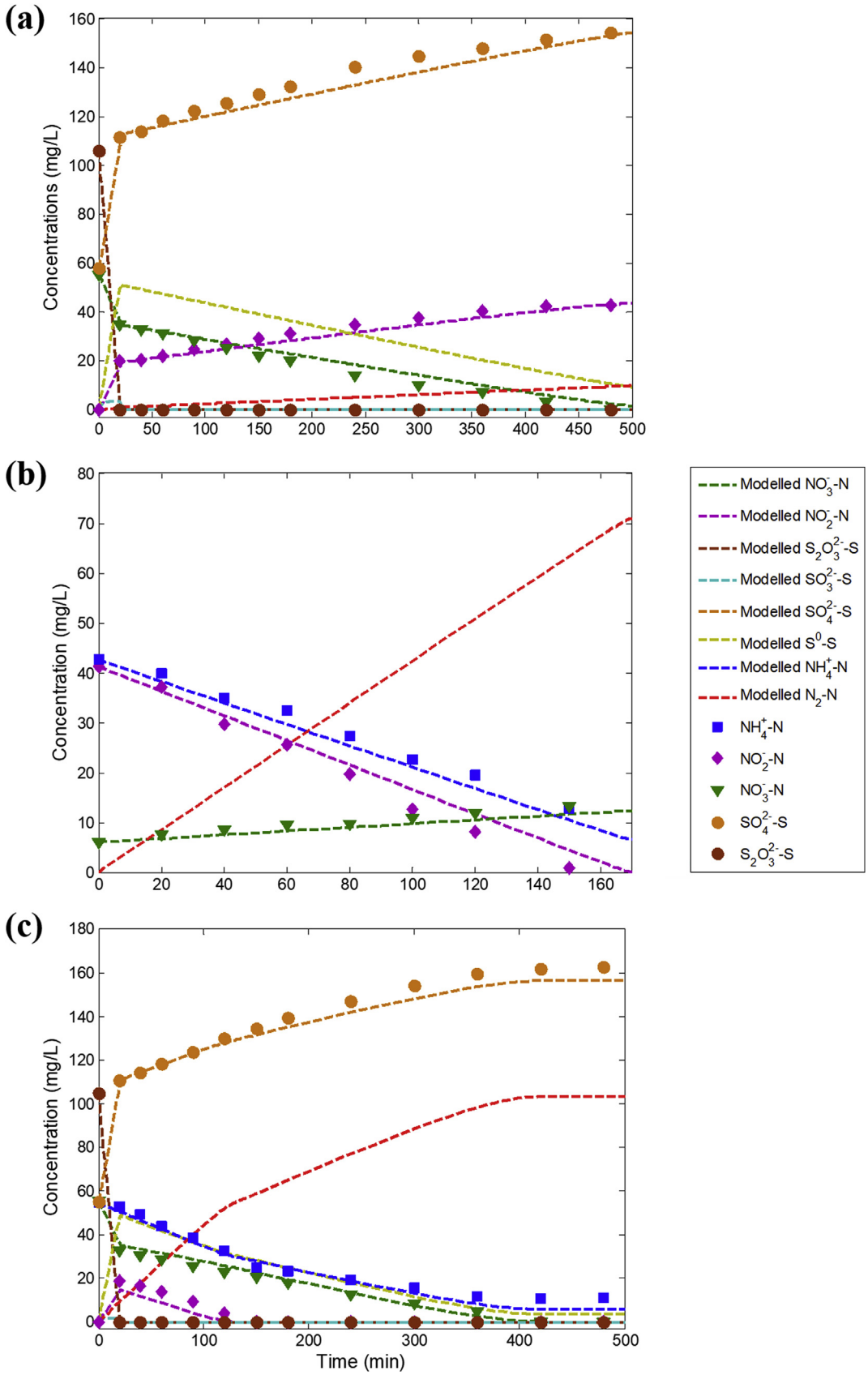

Fig. 4. Nitrogen and sulfur conversions in three batch tests: (a) BT-A with nitrate and thiosulfate concentrations of 50 mgN/L and 100 mg S/L, (b) BT-B with nitrite and ammonium of 40 mgN/L and 40 mgN/L, and (c) BT-C with 50 mgN/L of nitrate, 50 mgN/L of ammonium, and 100 mg S/L of thiosulfate. (Solid symbols present measured data and dashed lines present modelling results.)

Table 2
Kinetic rates in batch tests (unit: mgN/(gVSS·h) or mgS/(gVSS·h)).

		Autotrophic denitrification				Anammox	
		$r_{d,S2O3}$	$r_{d,SO4}$	$r_{d,NO3}$	$r_{d,NO2}$	$r_{a,NO2}$	$r_{a,NH4}$
A	Period-1	141.0 ± 3.1	68.5 ± 1.5	26.3 ± 0.5	0.9 ± 0.1	–	–
	Period-2	0	2.6 ± 0.1	2.1 ± 0.1	0.5 ± 0.1	–	–
B	–	–	–	–	–	7.6 ± 0.6	5.5 ± 0.3
C	Period-1	133.7 ± 2.8	70.7 ± 1.5	29.5 ± 0.6	1.3 ± 0.1	2.3 ± 0.1	2.0 ± 0.2
	Period-2	0	3.3 ± 0.2	3.0 ± 0.1	1.4 ± 0.0	6.2 ± 0.3	5.3 ± 0.2
		0	3.3 ± 0.2	2.2 ± 0.2	0.5 ± 0.0	1.7 ± 0.1	1.4 ± 0.1

(6.2 mgN/(gVSS·h)) was 4.4 times higher than the rate of denitritation (1.4 mgN/(gVSS·h)) when nitrite was present in the bulk liquid. When the accumulated nitrite in the bulk liquid was completely depleted in Period-2, the nitrite generated through the slow denitratation was consumed by Anammox at 1.7 mgN/(gVSS·h), which was still 3.4 higher than the denitritation rate (0.5 mgN/(gVSS·h)). The Anammox has a higher activity than denitritation, possibly due to its higher affinity to nitrite than denitritation. As reported in the literature, Anammox bacteria have a high affinity for nitrite because of their unique Anammoxosome membrane and nitrite transporters (Damste et al., 2002; van Niftrik and Jetten, 2012). For example, the half-saturation constants (Ks) for nitrite of Anammox were 0.2–35 μg/L (Awata et al., 2013; Lotti et al., 2014), while thiosulfate-driven denitritation has a relatively low affinity for nitrite with a Ks of 2.3 mgN/L (Mora et al., 2015).

Based on the observations in BT-A and BT-C, the sulfate production rates were about half of the thiosulfate removal rate (Table 2) in Period-1, then sulfate generation rate dramatically dropped by about 95% in Period-2. Also, the medium became milky during the experiments. Based on the formation of the suspended material and sulfur unbalance between thiosulfate and sulfate, it was postulated that sulfite and elemental sulfur were formed as intermediates during thiosulfate oxidation in batch tests. Schedel and Truper (1980) studied the anaerobic oxidation of thiosulfate with Thiobacillus denitrificans by radioactively labelled sulfur atoms, and results revealed that thiosulfate oxidation started with splitting between sulfane and sulfone sulfur, and then produced sulfite and elemental sulfur were separately oxidized (branched thiosulfate oxidation). As reported by Hooper and Dispirito (1985) that sulfite can be directly and rapidly oxidized to sulfate by the sulfite: acceptor oxidoreductase inside the periplasm, while the accumulated elemental sulfur was less active than produced sulfite (Schedel and Truper, 1980). Hence, it was speculated that this branched thiosulfate oxidation process ($S_2O_3^{2-} \rightarrow SO_3^{2-} + S^0$) occurred firstly in the TDDA process, followed by the SO_3^{2-} and S^0 oxidation coupled with the fast and slow denitratation respectively. The different kinetic results between this experiment and previous studies (Campos et al., 2008; Mora et al., 2014; Qian et al., 2018) were attributed to the multiple pathways of thiosulfate oxidation in SOB, as it was proposed that thiosulfate could be oxidized to sulfate by three completely different pathways (Lens and Pol, 2000).

In order to further prove elemental sulfur formation in thiosulfate oxidation pathway, a simplified kinetic model was developed to reveal the mechanisms of thiosulfate oxidation and nitrogen removal in the TDDA system. The model stoichiometric matrix is summarized in Table S3 and the kinetic parameters are shown in Table S4. The modelled results are presented in Fig. 4. The coefficients of determination (R^2) for ammonium, nitrate, sulfate and thiosulfate all exceeded 0.98 for the model of BT-C (Table S5), indicating that the proposed model is able to describe the kinetic behavior of nitrogen and sulfur compounds. Moreover, a check of the mass and element balance of the stoichiometry using the Excel spreadsheet of the Gujer Matrix showed that all of the elements balanced. Therefore, elemental sulfur was considered as an intermediate during thiosulfate oxidation in this study. Intermediates in thiosulfate oxidation will be quantified and the thiosulfate oxidation pathway will be determined in future studies.

3.4. Microbial community analysis

The phylogenetic classification of bacterial sequences was conducted at genus levels (Fig. 5). The Shannon index for the first and second samples were 4.11 and 3.21, and the Simpson's diversity Index were 0.06 and 0.22 respectively, suggesting higher evenness and diversity in the thiosulfate-driven denitratation system than the TDDA system. At the phylum level, Proteobacteria (41.33–68.33%), Planctomycetes (0.69–3.31%), Ignavibacteriae (0.85–3.24%),

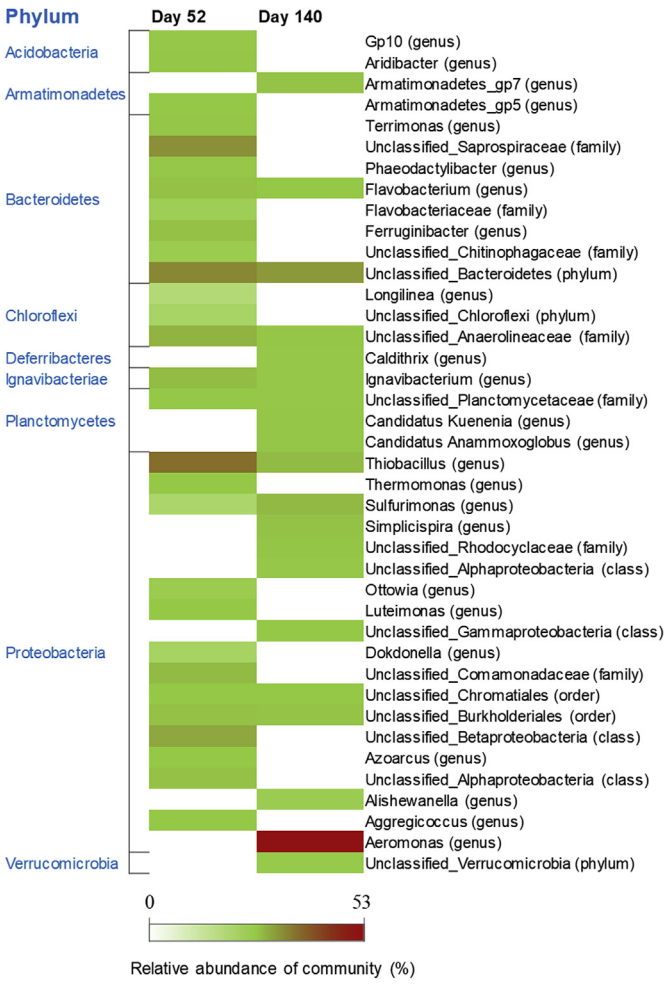

Fig. 5. Heat map of bacterial communities from the UASB reactor on day 52 and 140.

Chloroflexi (1.07–6.00%), Bacteroidetes (11.69–35.70%) and Armatimonadetes (0.68–1.65%) were dominant in both microbial communities.

In the first sludge sample, the functional bacteria in genus level were Thiobacillus (21.53%), Thermomonas (0.54%), Ottowia (0.51%), and Sulfurimonas (0.41%) (Fig. 5). These four strains all belong to the Proteobacteria phylum accounting for 41.33% of total relative abundance in the thiosulfate-driven denitratation system. The reduced sulfur compounds can be used by these four groups as electron donor to reduce nitrate or nitrite, and hydrogen can also be used by Sulfurimonas (Han and Perner, 2015), and Ottowia is capable of utilizing organics for facultative anaerobic growth (Spring et al., 2004). Although no organics were added in the influent of the UASB reactor in this study, heterotrophic denitrifying bacteria were still detected in the communities such as Flavobacterium (2.18%), Terrimonas (1.29%) and Phaeodactylibacter (0.82%). These heterotrophic denitrifying bacteria may also contribute to nitrate reduction in the reactor, which could use organic matter liberated from the endogenous metabolism, lysis and death of autotrophic bacteria (Okabe et al., 2011). No strains belonging to the Anammox genus were detected in the first sludge sample, in accordance with negligible ammonium removal efficiency of the reactor in Phase 1.

In the second sludge sample, the relative abundance of Thiobacillus decreased to 3.46%, while the number for Sulfurimonas increased to 3.92%. Thermomonas and Ottowia strains disappeared from the TDDA system. Denitrifying bacteria using organic carbon were also detected in TDDA system such as Simplicispira (1.94%) and Flavobacterium (0.71%). The high abundance of Aeromonas was observed in the second sludge sample, and it was reported that these bacteria are fermentative and facultative anaerobic and plays an important role in biofilm formation (Talagrand-Reboul et al., 2017). Meanwhile, two groups belonging to the Planctomycetes phylum and representing two Anammox genera were detected in the TDDA system with Ca. Kuenenia (1.27%) and Ca. Anammoxoglobus (1.12%). It has been reported that Ca. Kuenenia are r-strategists with a high affinity for ammonium and nitrite (Oshiki et al., 2016) and Ca. Anammoxoglobus are capable of co-oxidation of propionate and ammonium in the presence of ammonium, nitrite and nitrate (Kartal et al., 2007). The relative abundance of Anammox bacteria in the TDDA system was in the similar range of other Anammox based processes (1.7–3%) (Du et al., 2017; Xie et al., 2018). Such community composition confirmed that SOB can coexist with Anammox bacteria in one reactor.

4. Conclusions

The feasibility of developing a thiosulfate-driven denitratation and Anammox (TDDA) process was investigated in a lab-scale UASB reactor. The following conclusions could be drawn from this study:

- The TDDA process can be stably operated to treat nitrate and ammonium contained wastewater with TN removal efficiency of 82.5%.
- Anammox and denitritation were responsible for about 90% and 10% of the TN removal respectively.
- Compared with complete thiosulfate-driven denitrification, the TDDA process can reduce the electron consumption by about 49%.
- The branched thiosulfate oxidation generated sulfur intermediates—sulfite and elemental sulfur for 'fast' and 'slow' denitratation.
- SOB and Anammox bacteria functioned symbiotically together.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.114854.

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