N_2O emission in short-cut simultaneous nitrification and denitrification process: dynamic emission characteristics and succession of ammonia-oxidizing bacteria

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ABSTRACT

A sequencing batch airlift reactor was used to investigate the characteristics of nitrous oxide (N_2O) emission and the succession of an ammonia-oxidizing bacteria (AOB) community. The bioreactor could successfully switch from the complete simultaneous nitrification and denitrification (SND) process to the short-cut SND process by increasing the influent pH from 7.0–7.3 to 8.0–8.3. The results obtained showed that, compared with the complete SND process, the TN removal rate and SND efficiency were improved in the short-cut SND process by approximately 13 and 11%, respectively, while the amount of N_2O emission was nearly three times larger than that in the complete SND process. The N_2O emission was closely associated to nitrite accumulation. Analysis of the AOB microbial community showed that nitrifier denitrification by *Nitrosomonas*-like AOB could be an important pathway for the enhancement of N_2O emission in the short-cut SND process.

Key words | ammonia oxidizing bacteria, biological nitrogen removal, nitrous oxide, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE), short-cut simultaneous nitrification and denitrification

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INTRODUCTION

The increasing demand to achieve greater nitrogen removal efficiency with lower operation costs has resulted in the development of the short-cut simultaneous nitrification and denitrification (SND) process. The energy and carbon saving of the short-cut SND process, is due to the inhibition of nitrite oxidizing bacteria (NOB) by the uptake of nitrite as the electron acceptor for denitrification instead of nitrate (Peng & Zhu 2006). Numerous strategies for suppressing NOB to accumulate nitrite have been widely studied (Katsogiannis *et al.* 2003; Kim *et al.* 2006), such as short sludge retention time, high free ammonia (FA) concentrations and low dissolved oxygen (DO) concentrations. However, nitrite is known as a significant factor related to nitrous oxide (N_2O) emission from biological wastewater treatment (Kampschreur *et al.* 2009).

N_2O is one of the important greenhouse gases, having an atmospheric lifetime of about 114 years, and its global warming potential is 296 times that of carbon dioxide (CO_2) (IPCC 2007). N_2O also contributes to the depletion of stratospheric ozone because of the stratospheric reaction with atomic oxygen to give nitric oxide (NO) (Mosier 1998). Unfortunately, it is generally accepted that the biological nitrogen removal process in wastewater treatment occupies an extremely important position among the microbial processes of N_2O emission (Kampschreur *et al.* 2009). Thus, better understanding and controlling of N_2O emissions have become an urgent requirement of biological wastewater treatment.

Although N_2O can be formed during the incomplete oxidation of hydroxylamine to nitrite, nitrifier denitrification and heterotrophic denitrification are widely acknowledged to be the two main processes responsible for N_2O emission during the biological nitrogen removal process (Kim *et al.* 2010; Wunderlin *et al.* 2012). N_2O is one of the intermediates of denitrification and the amount of N_2O emission is relative to the activity of N_2O reductase (Kimochi *et al.* 1998). Alternatively, the activity of N_2O reductase depends on operational parameters such as DO concentration and nitrite

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concentration (Kampschreur *et al*. 2009). Some ammonia-oxidizing bacteria (AOB) can reduce NO_2^- and release N_2O under oxygen limiting conditions through nitrifier denitrification (Kim *et al*. 2010). In nitrifier denitrification, the oxidation of NH_3 to NO_2^- is followed by the reduction of NO_2^- to N_2O and N_2 (Kim *et al*. 2010). However, there is little literature about the succession of AOB and its contribution to N_2O production in the short-cut SND system.

In this study, we focused on comparing the differences of the characteristics of nitrogen removal and N_2O emission from a laboratory-scale sequencing batch airlift reactor (SBAR), operated sequentially in the complete SND process and the short-cut SND process. In addition, the correlation of succession of the AOB community to N_2O emission was also investigated. Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) technologies targeting the ammonia monooxygenase subunit A gene (*amoA*) were employed to track the succession of the AOB community. This study could be useful to control and minimize N_2O emission in the short-cut SND process.

METHODS

Reactor operation

The experiment was conducted in a laboratory-scale SBAR with a working volume of 5.3 L. The SBAR was operated three cycles every day, and each cycle was 8 h, consisting of 420 min aeration (including feeding during the first 10 min), 5 min settling, 10 min decant and 45 min idling. During the feeding period, 3.8 L of synthetic wastewater was fed into the reactor, the synthetic wastewater being composed of: chemical oxygen demand (COD) 250 mg L^{-1} (prepared with glucose); NH_4^+-N 50 mg L^{-1} (prepared with NH_4Cl); $NaHCO_3$ 550 mg L^{-1}; Na_2CO_3 100 mg L^{-1}; KH_2PO_4 37.5 mg L^{-1}; $MgSO_4{\cdot}7H_2O$ 100 mg L^{-1}; $FeSO_4{\cdot}7H_2O$ 15 mg L^{-1}; $CaCl_2$ 50 mg L^{-1} and other nutrient solution. The influent was adjusted to appropriate pH with 0.5 M Na_2CO_3 and 0.5 M HCl solution.

The SBAR was operated at 25–30 °C, and maintained the DO at 0.5–0.8 mg/L. The mixed liquor suspended solids (MLSS) concentration was maintained at 4500 ± 500 mg L^{-1}, and a certain amount of excess sludge was disposed to control the sludge retention time at 20–25 days. The system was operated in four stages: the set up stage, the complete SND stage, the transition stage and the short-cut SND stage. In the set up and complete SND stages, the pH was initially maintained at 7.0–7.3. After stable running for 20 days in the complete SND process, the target pH was increased to 8.0–8.3 so as to promote the short-cut SND process, and other operational conditions remained unchanged.

Analysis methods

The reactor performance was monitored once every 2 days by measuring COD, ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N) and MLSS in accordance with the standard methods (APHA 2005). DO and pH were monitored by DO and pH meters (YSI-550, USA and PHS-3C, China), respectively. The special oxygen uptake rate of ammonium ($SOUR_{NH_4^+}$) and nitrite ($SOUR_{NO_2^-}$) were used to characterize the activity of AOB and NOB respectively, and were measured by the method described by Madoni *et al*. (1999). The concentration of FA, SND efficiency and the nitrite accumulated rate (NAR) were calculated through the equation described by Shi *et al*. (2011).

The N_2O concentration was determined by gas chromatography (GC7890II, ShangHai, China) with an electron capture detector and a Poropak *Q* column, and measured once every 2 days from the 56th d. The temperature of the detector, injector and oven were set at 280, 130 and 45 °C, respectively, and the carrier gas was high purity nitrogen with the flow rate about 30 mL• min^{-1}. The quantity of the N_2O emission and the N_2O conversion rate were calculated according to the following equations.

The N_2O emission rate, N (mg/min) was calculated from Equation (1) (Park *et al*. 2000):

$$N = qc\rho \tag{1}$$

where q is the volumetric flow rate of the off gas (m^3/min); c is the N_2O concentration in the syringe (μL/L); and ρ is the density of N_2O at room temperature.

The time-weighted average emission rate of N_2O, N_{avg} (mg/min) was calculated using Equation (2) (Park *et al*. 2000):

$$N_{avg} = \frac{\sum_{k=1}^{n}[(N_{k-1} + N_k)\Delta t_k]}{2\sum_{k=1}^{n}\Delta t_k} \tag{2}$$

where n is the number of sampling points, N_k is the emission rate of N_2O at the sampling point, and $\triangle t_k$ is the time interval between each sampling point (min).

The cumulative emission amount of N_2O-N, Q (mg) was expressed as

$$Q = N_{avg} \cdot t \tag{3}$$

where t is the duration of each cycle (min).

The N_2O conversion rate, $R_{N_2O\text{-}N}$ (%) was calculated by Equation (4) (Shi *et al.* 2011):

$$R_{N_2O\text{-}N} = \frac{Q}{\Delta TN} \times 100 \quad (4)$$

Microbial analysis

The sludge samples collected from the reactor were washed with TE buffer and centrifuged at 8,000 rpm before stored being at −20 °C. Total genomic DNA was extracted using the Soil DNA Isolation Kit (Songon Biotech, China) according to the manufacturer's instructions. The primer set amoA-1F and amoA-2R with a GC-clamp was used to amplify partial gene fragments of amoA (Ibekwe *et al.* 2003). PCR amplification was performed in a thermocycler (Bio-Rad, USA). The amplified products were separated by DGGE on a BioRad DCode system (Bio-Rad, USA). The polyacrylamide gels were made with 8% (wt/vol) and denaturing gradients ranging from 30%–50%. The gel was electrophoreses in 1 × TAE buffer at 60 °C and at 120 V for 7 h.

A portion of individual DGGE bands were excised, washed sequentially with ethanol and 1 × TE buffer, and then transferred to 10 μl of sterile water and stored at −20 °C. The bands were reamplified with free GC-clamp former primers and sequenced. The obtained amoA partial gene sequences were compared with sequences in GenBank by the BLAST Search program, and phylogenetic trees were constructed using MEGA 4.

RESULTS AND DISCUSSION

Reactor performance

A laboratory-scale SBAR was carried out by maintaining short sludge settling times to create selective stress on the microbial community and aerobic granules. An aerobic granular sludge was successfully developed at the end of 30 days of acclimation, which had irregular shape, was grey in colour, and with a granule size of 0.45–0.9 mm. The performance of the bioreactor is shown in Figure 1(a). To investigate the dynamic changes of nitrogen composition during the steady operation period, typical cycle studies within the aerated period (0–420 min) were carried out on different days (72th day of the complete SND process (Figure 1(b)) and 110th day of the short-cut SND process (Figure 1(c)).

After cultivation for 56 days, the complete SND process was achieved, whereupon SOUR, N_2O and molecular monitoring commenced. During the complete SND stage (from the 56th to 76th days), the SND efficiency was stable (SND = 71.1 ± 1.6%), and the NAR was low (NAR = 1.6 ± 0.7%). After switching the influent pH to 8.0–8.3 on the 78th day, it took 20 days to reach the short-cut SND process in the bioreactor. During the short-cut SND stage from 98th to 120th day, ammonia was predominately oxidized to nitrite (NAR = 97.1 ± 1.5%) rather than to nitrate, and the average TN removal and SND efficiency were almost 13 and 11% higher, respectively, than in the complete SND process.

The different performance of the bioreactor, operated sequentially in complete SND and short-cut SND, was mainly owing to the inhibition of FA for NOB. The calculated influent FA concentration was increased from 0.5 to 4.7 mg L^{-1}, as respond to the pH was changed from 7.0–7.3 to 8.0–8.3, which was above the inhibition concentration for NOB activity (0.1–1.0 mg L^{-1}), but below the inhibition concentration for AOB activity (10–150 mg L^{-1}) (Anthonisen *et al.* 1976). This suggested that appropriately controlling the influent FA concentration (1.0–10.0 mg L^{-1}) by increasing the influent pH was an effective strategy for switching the bioreactor from the complete SND process to the short-cut SND process.

AOB and NOB activity

The performance of nitrogen removal in the bioreactor was closely relative to the microbial species and activity. AOB and NOB activity can be measured by the special oxygen uptake rate of ammonium ($SOUR_{NH_4^+}$) and nitrite ($SOUR_{NO_2^-}$), respectively (Zou *et al.* 2009), and $SOUR_{NO_2^-}/SOUR_{NH_4^+}$ can give information about the relative activities of NOB and AOB in the bioreactor. Approximately 500 mL MLSS were separated from the bioreactor to determine the $SOUR_{NH_4^+}$ and $SOUR_{NO_2^-}$ every 2 days from the 56th day.

As observed in Figure 2, $SOUR_{NO_2^-}$ (0.70 ± 0.07 mgO_2·$(g{\cdot}min)^{-1}$) was larger than $SOUR_{NH_4^+}$ (0.22 ± 0.03 $mgO_2(g{\cdot}min)^{-1}$) in the complete SND stage, and $SOUR_{NO_2^-}/SOUR_{NH_4^+}$ was 3.27 ± 0.4, which was consistent with the above result that the intermediate of the complete SND process was nitrate rather than nitrite. During the short-cut SND stage, $SOUR_{NH_4^+}$ (0.21 ± 0.03 $mgO_2(g{\cdot}min)^{-1}$) was statistically similar to the complete SND process, implying that increasing the influent pH to 8.0–8.3 barely had effect on the activity of AOB. However, $SOUR_{NO_2^-}$ decreased drastically from the 78th day, and $SOUR_{NO_2^-}/SOUR_{NH_4^+}$ was only 0.013 ± 0.0006 in the short-cut SND stage. The low level of

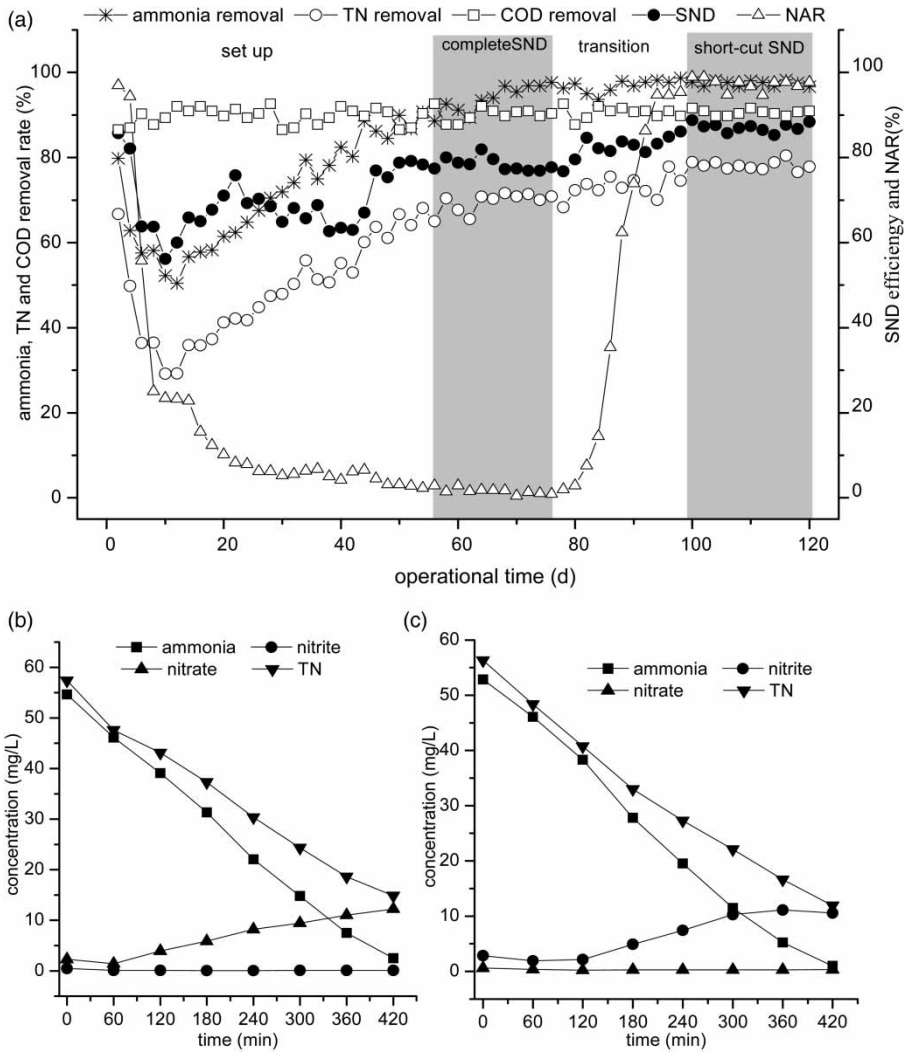

Figure 1 | The overall performance of the reactor: (a) ammonia, TN and COD removal rates; SND efficiency, NAR of the reactor during different stages; (b) typical changes of nitrogen compounds during the stable complete SND period on 72th d; (c) typical changes of nitrogen compounds during the stable short-cut SND period on 110th d.

$SOUR_{NO_2^-}$ (0.0032 ± 0.0001 $mgO_2(g{\cdot}min)^{-1}$) suggested that most of the NOB microbial community was inhibited in the short-cut SND stage, which was similar to the results observed and discussed by Villaverde *et al.* (1997).

Characteristics of N_2O emission

In the biological nitrogen removal process, there are two forms of N_2O emissions: the off-gas form and the effluent form. While more than 99.5% of total N_2O production is stripped out to the off-gas, and few remain in the effluent (Itokawa *et al.* 1996). Thus, in this context, the off-gas form of N_2O emission was detected every 2 days from the 56th d (Figure 3(a)), and the dynamic changes of N_2O concentration during the steady operation period were measured (Figure 3(b)). The N_2O concentration was detected once every 15 min, and each tested sample of N_2O was measured three times.

Figure 3(a) clarifies that the amount of N_2O emission during the complete SND stage (from the 56 to 76th d) was 26.7 ± 3.3 mg N_2O-N/cycle, and 12.8% of the total nitrogen input was emitted as N_2O-N. Meanwhile, a dramatic increase of the N_2O emission and conversion rate was observed as a response to elevating the influent pH to 8.0–8.3. This was likely due to the effect of variable operational conditions by AOB (Kampschreur *et al.* 2008). However, the amount of N_2O emission (111.9 ± 6.4 mg N_2O-N/cycle) during the short-cut SND stage (from the 98 to 120th d) was nearly three times higher than that in the complete SND process and the conversion rate was 49.1%.

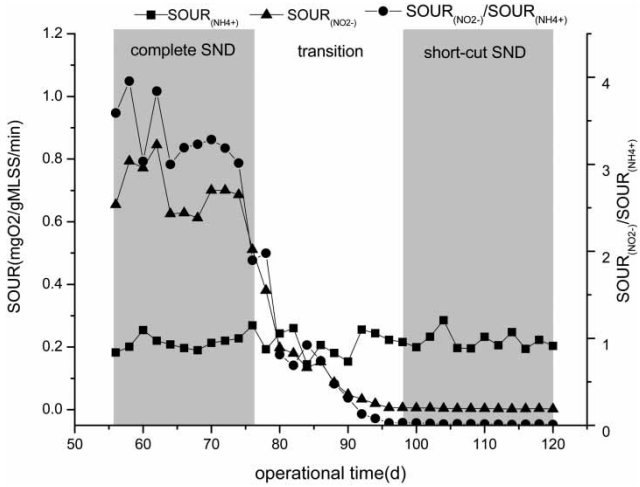

Figure 2 | The specific oxygen uptake rates associated with ammonia and nitrite oxidation with operating time.

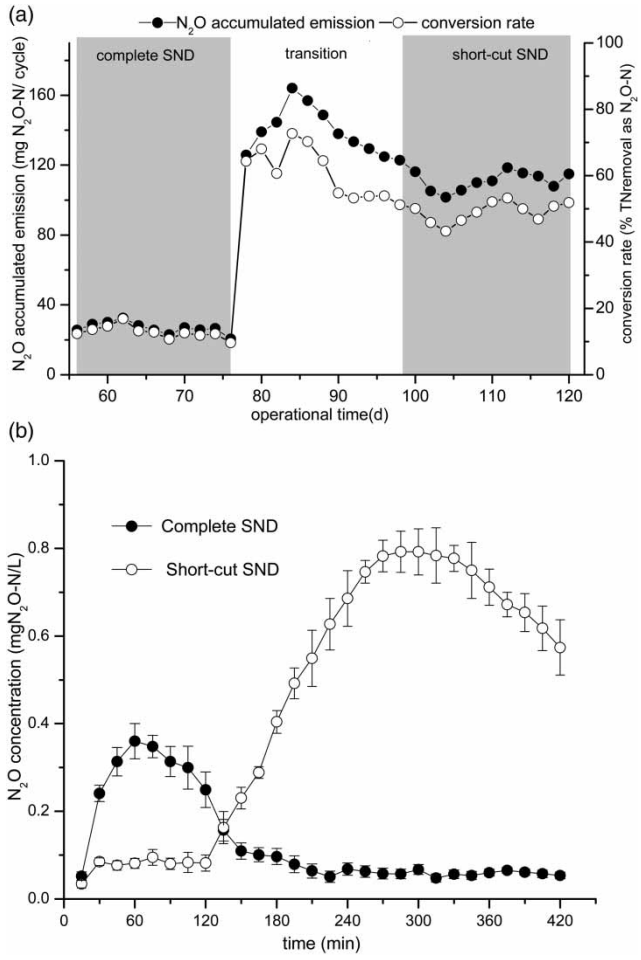

Figure 3 | N_2O emission of the reactor: (a) the accumulated amount and conversion rate of N_2O-N emission with operating time; (b) the dynamic changes of N_2O concentration during the steady state operation period (72th day of the complete SND process and 110th day of the short-cut SND process).

From the dynamic changes of N_2O concentration in Figure 3(b), one N_2O concentration peak was observed after incubation for 60 min and then decreased in the complete SND process, while in the short-cut SND process, the N_2O concentration started to increase at 150 min and reached the maximum value at 270–330 min after which it decreased.

From a comparison of Figures 1(c) and 3(b), a close relationship between N_2O emission and nitrite accumulation ($r = 0.924$, Pearson product-moment correlation coefficient) was found during the short-cut SND process. These results suggested that nitrite accumulation would be a trigger for N_2O emission in the short-cut SND process. Zeng *et al.* (2008) demonstrated that nitrite was a well-known inhibitor to N_2O reductase. NO_2^- reduction by AOB under aerobic conditions is known to lead to the production of NO, N_2O and N_2 (Kim *et al.* 2010).

Analysis of AOB community

A succession analysis of the *amo-A* containing AOB community was conducted on all samples using DGGE (Figure 4(a)). The phylogenetic analysis was constructed by using MEGA 4, and the result is shown in Figure 4(b).

Comparison of DGGE profiles showed that the AOB community fingerprints were apparently altered from the complete SND stage to short-cut SND stage. During the complete SND stage (56, 66 and 76th d), both *Nitrosomonas*-like AOB (bands 4, 7, 8 and 9) and *Nitrospira*-like AOB (bands 10, 11 and 12) were the dominant organisms. However, *Nitrospira*-like AOB (bands 10, 11 and 12) were inhibited gradually in the short-cut SND stage (100, 110 and 120th d), which was likely because *Nitrospira*-like AOB was sensitive to nitrite (Blackburne *et al.* 2007). Beaumont *et al.* (2004) discovered that the presence of NO_2^- could force *Nitrosomonas europaea* to regulate particular enzymes to defend against NO_2^- toxicity, so this might explain why *Nitrosomonas europaea* (bands 1, 2, and 3) was enriched in the short-cut SND process.

The research of Kim *et al.* (2010) indicated that nitrite can be used as a terminal electron acceptor by *Nitrosomonas*-like AOB, namely nitrifier denitrification. In nitrifier denitrification, N_2O is produced by nitrite reduction similar to the process of reduction by heterotrophic denitrifying bacteria, and then N_2O could be reduced to N_2 by N_2O reductase. However, N_2O reductase is more sensitive to oxygen than nitrate and nitrite reductase (Schulthess *et al.* 1994) and, as a result, the final product of nitrifier

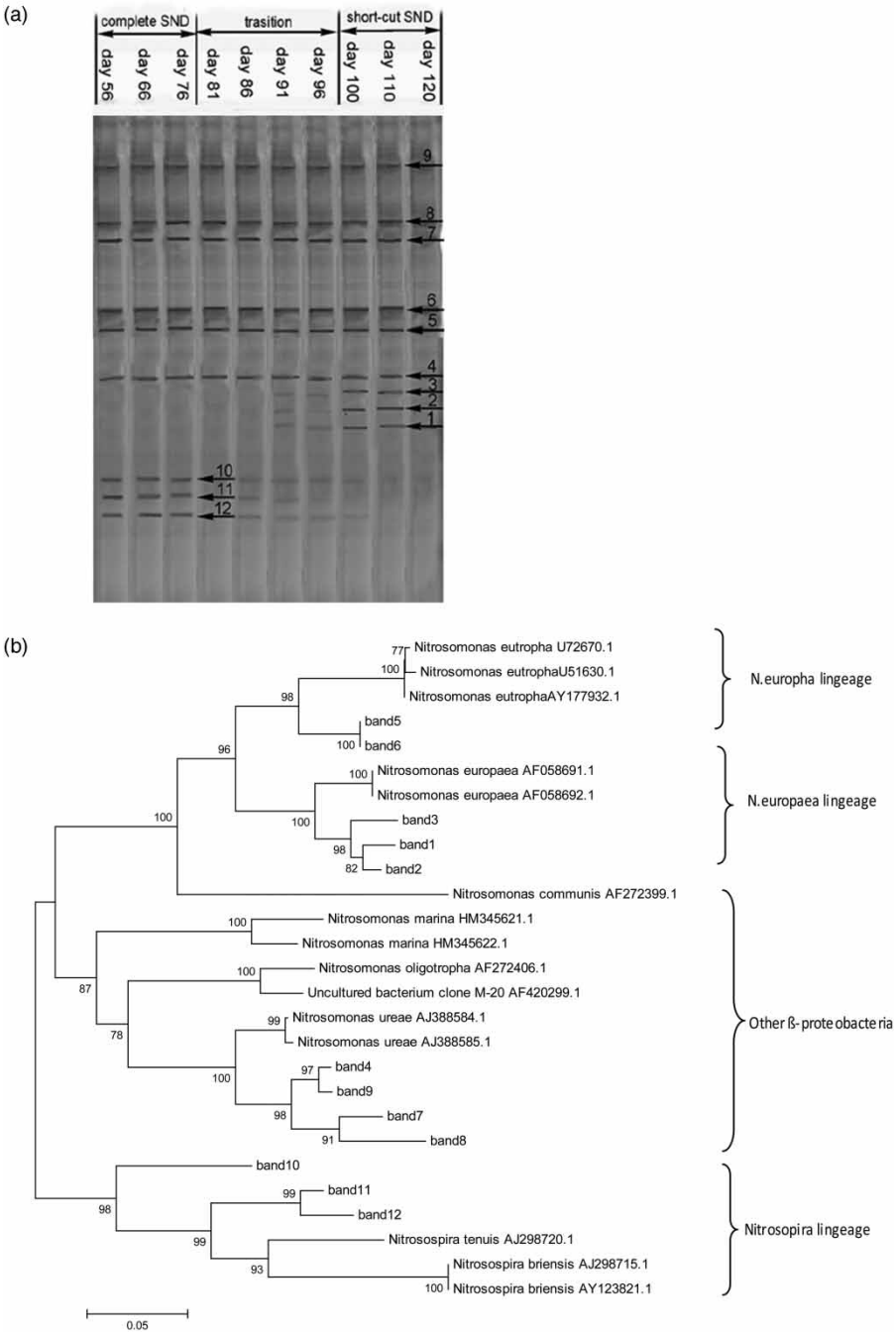

Figure 4 | The evolution of the AOB community with operating time: (a) DGGE profiles of *amoA* fragments of sludge samples; (b) neighbour-joining phylogenetic tree based on partial sequences of *amoA*.

denitrification under oxygen limited conditions is N_2O (Bonin *et al*. 2002). In this study, although *Nitrosomonas*-like AOB existed in the complete SND process, the production of N_2O was relatively insignificant. This was because the intermediate of nitrification was nitrate rather than nitrite in the completed SND process.

CONCLUSIONS

An SBAR was applied to investigate the characteristics of nitrous oxide emission and the succession of the AOB community in the short-cut SND process. Compared with the complete SND process, the TN removal rate and SND

efficiency were improved by approximately 13 and 11%, respectively. Nevertheless, the amount of N_2O emission was nearly three times higher than that in the complete SND process, and the N_2O emission was closely relative to the nitrite accumulation. The analysis of the microbial community of AOB showed that the nitrifier denitrification by *Nitrosomonas*-like AOB would be an important pathway for the enhancement of N_2O emission in the short-cut SND process.

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