Title:

Nitrous oxide emission in low-oxygen simultaneous nitrification and denitrification process: Sources and mechanisms

Author names:

Wenlin Jia[a,1], Shuang Liang[a,1], Jian Zhang[a,*], Huu Hao Ngo[b], Wenshan Guo[b], Yujie Yan[a], Yina Zou[a]

Author affiliations:

[a] Shandong Provincial Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, Jinan 250100, China

[b] School of Civil and Environmental Engineering, University of Technology Sydney, Broadway, NSW 2007, Australia.

*** Corresponding author:**

Tel: +86 531 88363015; fax: +86 531 88363015

E-mail address: zhangjian00@sdu.edu.cn (J. Zhang)

[1] The first two authors equally contributed.

Abstract

This study attempts to elucidate the emission sources and mechanisms of nitrous oxide (N_2O) during simultaneous nitrification and denitrification (SND) process under oxygen-limiting condition. The results indicated that N_2O emitted during low-oxygen SND process was 0.8±0.1mgN/gMLSS, accounting for 7.7% of the nitrogen input. This was much higher than the reported results from conventional nitrification and denitrification processes. Batch experiments revealed that nitrifier denitrification was attributed as the dominant source of N_2O production. This could be well explained by the change of ammonia-oxidizing bacteria (AOB) community caused by the low-oxygen condition. It was observed that during the low-oxygen SND process, AOB species capable of denitrification, i.e., *N. europaea* and *Nitrosomonas-like*, were enriched whilst the composition of denitrifiers was only slightly affected. N_2O emission by heterotrophic denitrification was considered to be limited by the presence of oxygen and unavailability of carbon source.

Keywords: Nitrous oxide; Nitrifier denitrification; Heterotrophic denitrification; Microbial community; Low-oxygen SND

1. Introduction

Nitrous oxide (N_2O) is considered as one of the critical greenhouse gas and the dominant ozone-depleting substance emitted in the 21st century (IPCC, 2007). Thus, the control of its emission has attracted increasingly more attentions over the past decade. It is generally accepted that biological wastewater treatment processes, especially those for enhanced nutrient removal, occupy an important position among the many sources of N_2O emission (Kampschreur et al., 2009; Foley et al., 2010). Recently, simultaneous nitrification-denitrification (SND) process under low oxygen condition has emerged as a promising process, due to its high nutrient removal efficiency and low energy consumption (Holman and Wareham, 2005; Liu et al., 2010; Hocaoglu et al., 2011). However, it was reported that a significant amount of N_2O may be produced during this process (Meyer et al., 2005).

Great efforts have been made to investigate N_2O emission during low-oxygen SND process. However, previous literatures mainly focused on the emission quantity (Zeng et al., 2003; Meyer et al., 2005) and influence factors such as electron acceptor (Lemaire et al., 2006), carbon source (Zeng et al., 2003; Zhu and Chen, 2011), and metal ion (Zhu and Chen, 2011). The sources and mechanisms of N_2O emission have not been seriously explored and remained unclear.

Although N_2O can be possibly produced via certain chemical pathways (e.g. hydroxylamine oxidation), nitrifier denitrification and heterotrophic denitrification are widely acknowledged to be the two main processes responsible for N_2O emission during low-oxygen SND process (Meyer et al., 2005; Wunderlin et al., 2012).

However, the individual contribution of the two important biological N_2O production processes has not been quantified. In addition, it is noteworthy that N_2O emission during low-oxygen SND process is significantly different from that in conventional nitrification and denitrification processes, taking into account the greatly intensified nitrifier denitrification and heterotrophic denitrification processes. The available results regarding N_2O emission source during traditional nitrification and denitrification process therefore may not be applicable to low-oxygen SND process.

N_2O emission during low-oxygen SND process is essentially a result of microbial metabolism. A detailed analysis of microbial community is therefore of great importance for better understanding of N_2O emission mechanisms. N_2O emission during nitrifier denitrification and heterotrophic denitrification is known to be executed and accomplished by certain bacteria species, mainly ammonia-oxidizing bacteria (AOB) and denitrifiers. However, to date, no published literature is available regarding the relationship between N_2O emission and its functional bacteria (i.e., AOB and denitrifiers) during low-oxygen SND process.

This study presented an initial attempt to determine the dominant source and mechanisms of N_2O emission during low-oxygen SND process. To this end, the contributions of nitrifier denitrification and heterotrophic denitrification to N_2O emission were evaluated by using batch experiments. Furthermore, the community structures of AOB and denitrifiers were investigated using polymerase chain reaction (PCR) - denaturing gradient gel electrophoresis (DGGE) technique, targeting ammonia monooxygenase submit A gene (*amoA*) and nitrous oxide reductase gene

(*nosZ*), respectively, to gain more detailed insights into the mechanisms of N_2O emission during low-oxygen SND process.

2. Material and Methods

2.1 SND bioreactor setup and operation

The experiments were conducted in a SND sequencing batch reactor (SBR) which was made of a transparent, rigid plexiglas cylinder with an effective volume of 15 L. The SBR was operated at room temperature (25±2°C) with a cycle time of 6 h, consisted of 6 min feeding, 90 min anaerobic stage, 180 min aeration, 70 min settling, and 14 min decant. In each cycle, 7.5 liters of wastewater was fed into the bioreactor and same amount of supernatant was withdrawn after settling, resulting in a hydraulic retention time (HRT) of 12 h. For better investigation on N_2O emission mechanisms, synthetic municipal wastewater instead of real wastewater was used in this study to eliminate the influence of water quality fluctuation. Glucose and sodium acetate was used as carbon source. NH_4Cl, KH_2PO_4 and K_2HPO_4 were added as nitrogen and phosphorus. Detailed information about SBR and the composition of synthetic municipal wastewater can be found in Jia et al. (Jia et al., 2012). The complete influent contained 350 mg COD/L, 50 mg NH_4-N/L, and 5 mg TP/L.

An electric agitator with a rectangular paddle was used to keep the sludge suspended during anaerobic stage. During the subsequent aerobic stage, air supply was regulated by using an on/off control system to keep the dissolved oxygen (DO) level between 0.35-0.80 mg/L. Before settling, 0.75 L mixed liquor was wasted to keep the solids retention time (SRT) at approximately 20 days. The SBR was seeded

with the sludge from a local wastewater treatment plant, and the concentration of mixed liquor suspended solids (MLSS) was maintained at approximately 3000 -3300 mg/L. The pH value in the reactor was monitored in the range of 7.0-7.5 over the entire experimental period. The SBR was gastight and certain amount of off gases was collected into gas sampling bags at time intervals of 15 min to measure N_2O concentrations.

2.2 Batch experiments

The use of inhibitors can help to determine the magnitude of the various processes at the origin of nitrous oxide production (Tallec et al., 2006). Allythiourea (ATU) is a common inhibitor of the first step of nitrification (Hall, 1984), and the most efficient inhibitor of the second step of nitrification catalyzed by nitrite oxidoreductase is chlorate ($NaClO_3$) (Haider et al., 2003). Tallec et al. (16) demonstrated that N_2O emission by heterotrophic bacteria was not significantly affected in the presence of ATU and $NaClO_3$. Therefore, the amount of N_2O produced by heterotrophic denitrification alone and by the sum of nitrifier denitrification and heterotrophic denitrification can be respectively quantified by the batch experiment with or without the use of inhibitors.

After the stable effluent nutrients levels and high SND efficiency were achieved, which indicated that the SND SBR reached steady-state, a total of 3 liters of mixed liquor and sludge was taken from the parent SBR at the end of anaerobic stage and then was divided equally into three mini SBRs with working volume of 1 L. Three batch experiments were simultaneously conducted: (a) no addition of nitrite or

inhibitor, (b) with addition of nitrite, and (c) with addition of both nitrite and nitrification inhibitors (ATU and chlorate). The nitrite was added for heterotrophic denitrification with the presence of inhibitors. In addition, one liter of mixed liquor and sludge was taken and the sludge and supernatants were separated. After that, the batch experiments were conducted under the conditions of sludge resuspended with distilled water and with nitrite addition to evaluate the eliminated the effect of ammonium, and supernatants with nitrite and inhibitors addition. The nitrite, ATU and $NaClO_3$ were added at the start of experiment to have a concentration of 5.0 mg/L, 10.0 mg/L (Haider et al., 2003), and 1.0 g/L (Tallec et al., 2006), respectively.

A mixture of N_2 and air was supplied into the mini bioreactors with the ratio adjusted so as to best simulate the DO variation and hydrodynamic environment in the parent reactor. The off-gas during the experiments was collected into gasbags to quantify the emission amount of N_2O. Each experiment was triplicated.

2.3 Physicochemical analysis

The effluent COD and nutrients concentration of the bioreactor was monitored every five days during the start-up period until the SND efficiency stabilized at a high level (>85%). Nitrogen transformation, carbon conversion (COD and polyhydroxyalkanoates (PHA)) and N_2O emission were then evaluated.

The analysis of COD, NH_4^+-N, NO_3^--N, NO_2^--N, TN, TP and MLSS were conducted in accordance with the standard methods (APHA, 2001). DO was measured using a DO meter (HQ30d53LDO^{TM}, HACH, USA). N_2O concentration was determined using gas chromatography (SP-3410, China) with an electron capture

detector (ECD) and a Poropak Q column. PHA was measured using the gas chromatography with a flame ionization detector (FID) and a column DB-5.

The SND efficiency was calculated according to the equation described by Zeng et al. (2003). The emission rate and quantity of N_2O-N were calculated as described by Hu et al. (2010). N_2O-N conversion rate was calculated by N_2O-N/TN input.

2.4 Microbial analysis

Once the parent reactor reached steady-state, evidenced by the achieved stable satisfactory SND efficiency, the sludge sample was collected and centrifuged for DNA extraction. As control, the seed sludge in the wastewater treatment plant was also sampled before acclimation to the experimental operating conditions. The total genomic DNA was then extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA).

Partial gene fragments of *amoA* and *nosZ*, which represented AOB and denitrifiers, respectively, were amplified using primers *amoA-1F/amoA-2R* and *nosZ-F /nosZ-1622R*. The PCR was conducted as the protocol described by previous literature (Hu et al., 2011). The PCR product was used for DGGE analysis using the Bio-Rad Dcode system (Bio-Rad, USA). Electrophoresis was performed at 120 V for 7.5 h in 1×TAE buffer at a constant temperature of 60 °C.

Specific bands were excised, washed, and dissolved in sterile water. They were subsequently reamplified with appropriate primes. After being purified using the UNIQ-10 column PCR Purification Kit (Sangon Biotech., China), the PCR amplicons were used for sequencing (Sangon Biotech., Shanghai, China). The obtained sequences

were compared with the other available sequences in the GenBank by BLAST search. Phylogenetic trees were then conducted using the neighbor-joining method with a bootstrap of 1000 replications by using MEGA 4. All *amoA* and *nosZ* gene sequences determined in this study have been deposited in GenBank under the accession number from JQ731680 to JQ731700. The Shannon-Wiener index of species diversity (*H*) was calculated by the following equation:

$$H = -\sum_{i=1}^{s} p_i \log_e(p_i)$$

where, p_i represents the intensity proportion of band *i* in the DGGE profile and *s* is the total number of bands.

3. Results and discussion

3.1 Performances of the SND SBR

Fig. 1 shows the overall performance of the SND SBR during the startup period. COD was easily removed and the effluent concentration was kept in the range of 18.8-36.5 mg/L during the whole startup period (Fig. 1a). The removal of TP was unsatisfactory in the first two months. It was mainly because that the phosphorus accumulating organisms (PAOs) were not well enriched and the uptake of phosphorus was insufficient under low-oxygen condition. The effluent TP concentration decreased with the acclimation of activated sludge to low-oxygen condition as well as the enrichment of PAOs and possible denitrifying phosphorus accumulating organisms (DPAOs).

Fig. 1b shows the nitrogen removal performance during the startup period. The effluent ammonium and nitrite concentration was very low (<1 mg/L) during the

whole startup period. It was mainly due to the complete nitrification caused by sufficient aeration time. The effluent nitrate concentration decreased from 26.4 mg/L to approximately 5.0 mg/L gradually with the operating time, indicating that the denitrification was enhanced during the startup period, which was also confirmed by the increase of SND efficiency. After running for about four months, the effluent TN concentration was below 6.0 mg/L, and the SND efficiency was above 90%. Stable effluent contaminant levels and high SND efficiency confirmed that the bioreactor performance was in steady state and the simultaneous removal of organic carbon and nutrients was achieved.

During the steady-state period of the SND SBR, the contaminants removal efficiencies and N_2O yield were studied and the results are shown in Table 1. The COD and TP removal efficiencies were high, just as that during startup period. The SBR gained satisfactory nitrogen removal efficiency for the enhanced simultaneous nitrification and denitrification in the low-oxygen aeration stage. Nearly all NH_4^+-N was removed and little NO_x-N was accumulated. The average TN removal efficiency reached to 92.5%. Moreover, the N_2O emission rate during one cycle was 0.8±0.1 mg N/gMLSS on average, and the conversion rate of N_2O-N to TN input was 7.7%.

Table 2 shows the TN removal efficiency and N_2O conversion rate under different operating conditions treating municipal wastewater in recent literatures. The TN removal efficiency during traditional anaerobic-aerobic wastewater treatment in other literatures was 45-70%, which was lower than that in the present study, no matter under high or low DO condition. Meanwhile, the N_2O conversion rate in this study

was also higher than that of other reactors which was operated under high DO condition (0.2-5.3%). The result was consistent with the study of Zhu and Chen [10]. Compared with the conventional nitrification and denitrification process, although the removal of nitrogen was enhanced simultaneously, the low-oxygen SND process stimulated the N_2O emission.

The time courses of nitrogen transformation and N_2O emission rate were investigated and the results are shown in Fig. 2. The N_2O emission rate was approximately zero during the anaerobic stage. The highest emission rate occurred at 195 min and the emission rate was 6.88±0.65 μg N/gMLSS/min. Meanwhile, the NO_2^- concentration reached to the highest (0.96±0.48 mg N/L). The profile of N_2O emission rate was in accordance to the change of nitrite concentration in the SBR. It was because that the nitrite could stimulate the emission of N_2O. The majority of N_2O emission occurred during the aerobic stage. It was mainly caused by the following reasons. Firstly, the low-oxygen condition favors nitrifier denitrification, of which the product is mainly NO and N_2O (Colliver and Stephenson, 2000). Secondly, the presence of oxygen inhibits the activity of nitrous oxide reductase, leading to the accumulation of N_2O during heterotrophic denitrification. In addition, in the parent SND reactor, the COD was nearly completely consumed for denitrification and hydrolysis of intracellular stored polyphosphate at the first 30 min of anaerobic stage, leading to the low C/N ratio (< 2 before 195 min) in the aerobic stage (Fig. 2). The PHA was synthesized in the anaerobic stage, and then it was degraded in the aerobic stage using for the phosphorus uptake and denitrification. The denitrification driven by

PHA could stimulate the N_2O emission (Meyer et al., 2005).

3.2 Respective contribution of nitrifier denitrification and heterotrophic denitrification to N_2O emission

Table 3 shows the nitrogen transformation in each batch experiment. It can be seen that, compared with the control experiment (a), the oxidization rate of NH_4^+ slightly decreased by about 8% with the addition of nitrite alone (the batch experiment (b)). However, in contrast, the average conversion rate of NO_2^- and NO_3^- significantly increased during experiment (b). The nitrite added during experiment (b) was consumed by nitrifier denitrification and heterotrophic denitrification and no nitrite remained at the end of experiment, causing a disappearance rate of 0.52 mgN/gMLSS/h. The oxidation of added nitrite during the batch experiment (b) also led to the increase of NO_3^- conversion rate (from 0.47 to 0.66 mgN/gMLSS/h).

No considerable conversion of NH_4^+ and NO_3^- were observed with the addition of inhibitors (experiment c). It appeared that nitrification did not occur in the presence of inhibitors. The lack of nitrification implies that the removed NO_2^- almost fully came from the added nitrite, which was used only for heterotrophic denitrification. During experiment (d) most of added nitrite was reduced by denitrification and only a small amount of nitrate was produced.

The N_2O emission rate significantly increased from 0.31 (the batch experiment (a)) to 0.48 mgN/gMLSS/h (the batch experiment (b)) due to the addition of nitrite. Nitrite proved to stimulate the emission of N_2O (Schulthess et al., 1995; Colliver and Stephenson, 2000). During experiment (b) the N_2O was produced through both

nitrifier denitrification and heterotrophic denitrification, and the N_2O-N came from the conversion of ammonium and nitrite addition. However, the N_2O emission rate decreased to merely 0.15 mgN/gMLSS/h when inhibitors were present (experiment (c)), despite the addition of nitrite. It appeared that the use of inhibitors decreased about 69% of N_2O emission (0.48 mgN/gMLSS/h of experiment (b) *vs.* 0.15 mgN/gMLSS/h of Experiment (c)). The N_2O produced during the batch experiment (c) came from the conversion of nitrite addition by heterotrophic denitrification. The N_2O emission rate of experiment (d) was 0.44 mgN/gMLSS/h, which was similar with that of experiment (b). This result showed that the presence of ammonium in the liquor had little impact on N_2O emission when the nitrite was added. Thus, it can be concluded that the reduced 69% of N_2O during the batch experiment (c) was mainly caused by the inhibition of AOB. The denitrification of AOB, i.e. nitrifier denitrification, was shown to be the dominant source of N_2O emission during low-oxygen SND process, and the N_2O yield of this process was more than two times higher than that of heterotrophic denitrification.

The major contribution of nitrifier denitrification to N_2O emission was contradictory to the previous results reported in various nitrification and denitrification processes. Hu et al. (2011) showed that heterotrophic denitrification contributed much more than nitrifier denitrification to N_2O emission in traditional nitrification and denitrification process at low oxygen condition. More recently, Wunderlin et al. (2012) found that heterotrophic denitrification dominated the N_2O production during SND at high DO and organic carbon load conditions. Moreover, the

present research results showed that nitrifier denitrification contributed much more than heterotrophic denitrification to N_2O emission during low-oxygen SND process, inconsistence with the previous study that nitrifier denitrification represented no more than 60% of N_2O production when DO concentration was lower than 1.0 mg/L (Tallec et al., 2006).

The dominance of nitrifier denitrification in N_2O emission during low-oxygen SND process can be partially explained from the following two aspects:

Firstly, denitrification of AOB would be more favorable at low-oxygen condition. Oxygen stress is important for nitrifier denitrification. Colliver and Stephenson (2000) found that the N_2O yield under oxygen limiting conditions by *N. europaea*, which was probably the most representative nitrifier, was 3-5 times higher than that at fully aerated conditions. As delineated and discussed in details later (Section 3.3), the low DO concentration in this study resulted in a substantial change of AOB community and thus an enhanced nitrifier denitrification.

Secondly, heterotrophic denitrification could be partially inhibited due to the limited carbon source in the aerobic stage. As shown in Fig. 2, COD concentration was less than 36.5 mg/L over the aerobic stage. Although intracellular storage compounds could be used as carbon source for heterotrophic denitrification, the efficiency of heterotrophic denitrification would be greatly limited because most of the PHA was used for phosphorus uptake firstly.

It was noteworthy that there was no nitrogen transformation caused by chemical reaction during the batch experiment (e). This suggested that the inhibitors had no

impact on the solution, and thus could effectively differentiate the nitrifier denitrification and heterotrophic denitrification.

The N_2O emission was 0.01 mgN/gMLSS during the batch experiments (e). This negligible part of N_2O may be produced by the aeration through the air pump since no chemical or biological nitrogen transformation occurred in the supernatant. Although it was reported that possible chemical pathways could lead to N_2O formation by the reaction between nitrite and hydroxylamine and nitrite reductions with organic or inorganic compounds (Cleemput, 1998), it did not occur in this study. This may be because the supernatant in this study had no Fe^{2+} and neutral pH therefore chemical denitrification was not induced.

3.3 Microbial source of N_2O emission during low-oxygen SND process

Fig. 3 shows the DGGE patterns of the denitrifiers and AOB based on *nosZ* and *amoA* gene. It can be seen that the community of denitrifiers in the seed sludge (control) was similar to that in the low-oxygen SND reactor (Fig. 3A). The Shannon-Wiener index (*H*) was calculated to be 2.5 and 2.4 for control and SND sample, respectively, indicating that the species diversity of two samples was similar. The phylogenetic tree of denitrifiers based on the *nosZ* gene showed that all the sequences belonged to the alpha-proteobacteria and beta-proteobacteria (Fig. 4). The community of denitrifiers was similar to the previous reported sequences retrieved from common activated sludge or traditional nitrification denitrification bioreactors (Hu et al., 2011; Srinandan et al., 2011).

The results indicated that low-oxygen condition had no significant impact on

denitrifier community. It was because most of the denitrifiers can cope with low oxygen condition by using NO_3^- as an alternative electron acceptor in respiration instead of oxygen. Moreover, denitrifiers mainly locate inside of activated sludge flocs, usually facing oxygen-transfer limitation. They are thus less sensitive to the decrease of oxygen.

Compared with the unchanged denitrifier community, the composition of AOB community was found to be significantly affected by the oxygen level. It can be seen from Fig. 3B that the AOB community in the low-oxygen SND sample was more complex than that in the control sample. The diversity of AOB (H = 2.9) in the low-oxygen SND sample was higher than that in the control sample (H = 2.1). Apparently, some new AOB sequences were enriched under low-oxygen condition due to the different oxygen affinity of each AOB species.

Based on the *amoA* gene sequencing, the phylogenetic tree of AOB communities was constructed (Fig. 5). A total of 12 partial *amoA* sequences were successfully identified from the *amoA* DGGE gels. The results illustrated that all of these bacterial *amoA* sequences were affiliated to *Nitrosomonas* genus, with *N. ureae*, *N. oligotropha*, and *N. europaea* being the three most dominant species. It could be found that the species affiliated to *N. europaea* and *Nitrosomonas-like* were abundant in the SND sample. This was probably because the *Nitrosomonas*-like AOB had higher affinity for oxygen (Hu et al., 2011). *Nitrosomonas*-like AOB was widely reported to be capable of denitrification (Colliver and Stephenson, 2000; Shrestha et al., 2002) It appeared that the higher N_2O emission of nitrifier denitrification in low-oxygen

condition was mainly due to the enrichment of certain AOB species capable of denitrification.

4. Conclusions

During low-oxygen SND process, the nitrogen input was converted to higher amount of N_2O than that in the conventional nitrification and denitrification processes. Nitrifier denitrification was identified to be the dominant source of N_2O emission (e.g. more than two times higher than that of heterotrophic denitrification). The dominant role of nitrifier denitrification was caused by the change of AOB community. The microbial community composition of AOB was affected significantly by the low oxygen condition and AOB species which were capable of conducting denitrification, i.e., *N. europaea* and *Nitrosomonas-like*, were enriched during low-oxygen SND process.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 21177075 and 50908133), Program for New Century Excellent Talents in University (NCET-10-0554) and Natural Science Foundation for Distinguished Young Scholars of Shandong province (JQ201216).

References

1. APHA-AWWA-WPCF, 2001. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC.

2. Colliver, B.B., Stephenson, T., 2000. Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers, Biotechnol. Adv. 18, 219–232.

3. Foley, J., de Haas, D., Yuan, Z., Lant, P., 2010. Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. Water Res. 44, 831–844.

4. Haider, S., Svardal, K., Vanrolleghem, P.A., Kroiss, H., 2003. The effect of low sludge age on wastewater fractionation (S_s, S_I). Water Sci. Technol. 47, 203–209.

5. Hall, G.H., 1984. Measurement of nitrification rates in lake sediments: comparison of the nitrification inhibitors nitrapyrin and allylthiourea. Microb. Ecol. 10, 25–36.

6. Hocaoglu, S.M., Insel, G., Cokgor, E.U., Orhon, D., 2011. Effect of low dissolved oxygen on simultaneous nitrification and denitrification in a membrane bioreactor treating black water. Bioresour. Technol. 102, 4333-4340.

7. Holman, J.B., Wareham, D.G., 2005. COD, ammonia and dissolved oxygen time profiles in the simultaneous nitrification/denitrification process. Biochem. Eng. J. 22, 125–133.

8. Hu, Z., Zhang, J., Li, S., Xie, H., Wang, J., Zhang, T., Li, Y., Zhang, H., 2010. Effect of aeration rate on the emission of N_2O in anoxic–aerobic sequencing batch reactors (A/O SBRs). J. Biosci. Bioeng. 109, 487–491.

9. Hu, Z., Zhang, J., Xie, H., Li, S., Zhang, T., Wang, J., 2011. Identifying sources of nitrous oxide emission in anoxic/aerobic sequencing batch reactors (A/O SBRs) acclimated in different aeration rates. Enzyme Microb. Technol. 49, 237–245.

10. IPCC, 2007. Changes in atmospheric constituents and in radiative forcing, in: Solomon, S., Qin, D., Manning, M., Miller, H.L. (Eds.), Climate change 2007: the physical science basis. Contribution of working group I to the fourth assessment

report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 114–143.

11. Jia, W., Zhang, J., Xie, H., Yan, Y., Wang, J., Zhao, Y., Xu, X., 2012. Effect of PHB and oxygen uptake rate on nitrous oxide emission during simultaneous nitrification denitrification process. Bioresour. Technol. 113, 232–238.
12. Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S.M., van Loosdrecht, M.C.M., 2009. Nitrous oxide emission during wastewater treatment. Water Res. 43, 4093–4103.
13. Lemaire, R., Meyer, R., Taske, A., Crocetti, G.R., Keller, J., Yuan, Z., 2006. Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. J. Biotechnol. 122, 62–72.
14. Liu, X., Peng, Y., Wu, C., Akio, T., Peng, Y., 2008. Nitrous oxide production during nitrogen removal from domestic wastewater in lab-scale sequencing batch reactor. J. Environ. Sci. 20, 641–645.
15. Liu, Y., Shi, H., Xia, L., Shi, H., Shen, T., Wang, Z., Wang, G., Wang, Y., 2010. Study of operational conditions of simultaneous nitrification and denitrification in a Carrousel oxidation ditch for domestic wastewater treatment. Bioresour. Technol. 101, 901–906.
16. Meyer, R.L., Zeng, R.J., Giugliano, V., Blackall, L.L., 2005. Challenges for simultaneous nitrification, denitrification, and phosphorus removal in microbial aggregates: mass transfer limitation and nitrous oxide production. FEMS Microbiol.

Ecol. 52, 329–338.

17. Noda, N., Kaneko, N., Mikami, M., Kimochi, Y., Tsuneda, S., Hirata, A., Mizuochi, M., Inamori, Y., 2003. Effects of SRT and DO on N_2O reductase activity in an anoxic-oxic activated sludge system. Water Sci. Technol. 48, 363–370.
18. Park, K.Y., Inamori, Y., Mizuochi, M., Ahn, K.H., 2000. Emission and control of nitrous oxide from a biological wastewater treatment system with intermittent aeration. J. Biosci. Bioeng. 90, 247–252.
19. Schulthess, R.V., Kühni, M., Gujer, W., 1995. Release of nitric and nitrous oxides from denitrifying activated sludge. Water Res. 29, 215–226.
20. Shrestha, N.K., Hadano, S., Kamachi, T., Okura, I., 2002. Dinitrogen production from ammonia by *Nitrosomonas europaea*. Appl. Catal. A- Gen. 237, 33–39.
21. Srinandan, C.S., Shah, M., Patel, B., Nerurkar, A.S., 2011. Assessment of denitrifying bacterial composition in activated sludge. Bioresour. Technol. 102, 9481–9489.
22. Tallec, G., 2005. Nitrous oxide emissions during nitrogen treatment in wastewater treatment plants–Paris conurbation. Doctoral dissertation, Ecole Nationale des Ponts et Chaussées (in French).
23. Tallec, G., Garnier, J., Billen, G., Gousailles, M., 2006. Nitrous oxide emissions from secondary activated sludge in nitrifying conditions of urban wastewater treatment plants: Effect of oxygenation level. Water Res. 40, 2972–2980.
24. van Cleemput, O., 1998. Subsoils: chemo- and biological denitrification, N_2O and

N_2 emissions. Nutr. Cycl. Agroecosys. 52, 187–194.

25. Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., Siegrist, H., 2012. Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions. Water Res. 46, 1027–1037.

26. Zeng, R.J., Lemaire, R., Yuan, Z., Keller, J., 2003. Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. Biotechnol. Bioeng. 84, 170–178.

27. Zhu, X., Chen, Y., 2011. Reduction of N_2O and NO generation in anaerobic-aerobic (low dissolved oxygen) biological wastewater treatment process by using sludge alkaline fermentation liquid. Environ. Sci. Technol. 45, 2137–2143.

Figure captions:

Fig. 1 Performances of SND reactor during start-up period. Variation of (a) COD and phosphorus concentration, and (b) nitrogen concentration and SND efficiency as a function of operating time.

Fig. 2 COD and nitrogen transformation during a typical cycle in stabilized parent SND-SBR. The dash line indicates the transition from the anaerobic to the aerobic stage.

Fig. 3 DGGE profiles of (A) denitrifiers and (B) AOB in control and SND samples, respectively, based on *nosZ* and *amoA* fragments.

Fig. 4 Neighbor-joining phylogenetic tree based on bacterial *nosZ* gene sequences. Sequences obtained in this study are shown with "nosZ" in the names. Other sequences were obtained from GenBank.

Fig. 5 Neighbor-joining phylogenetic tree based on bacterial *amoA* gene sequences. Sequences obtained in this study are shown with "amoA" in the names. Other sequences were obtained from GenBank.

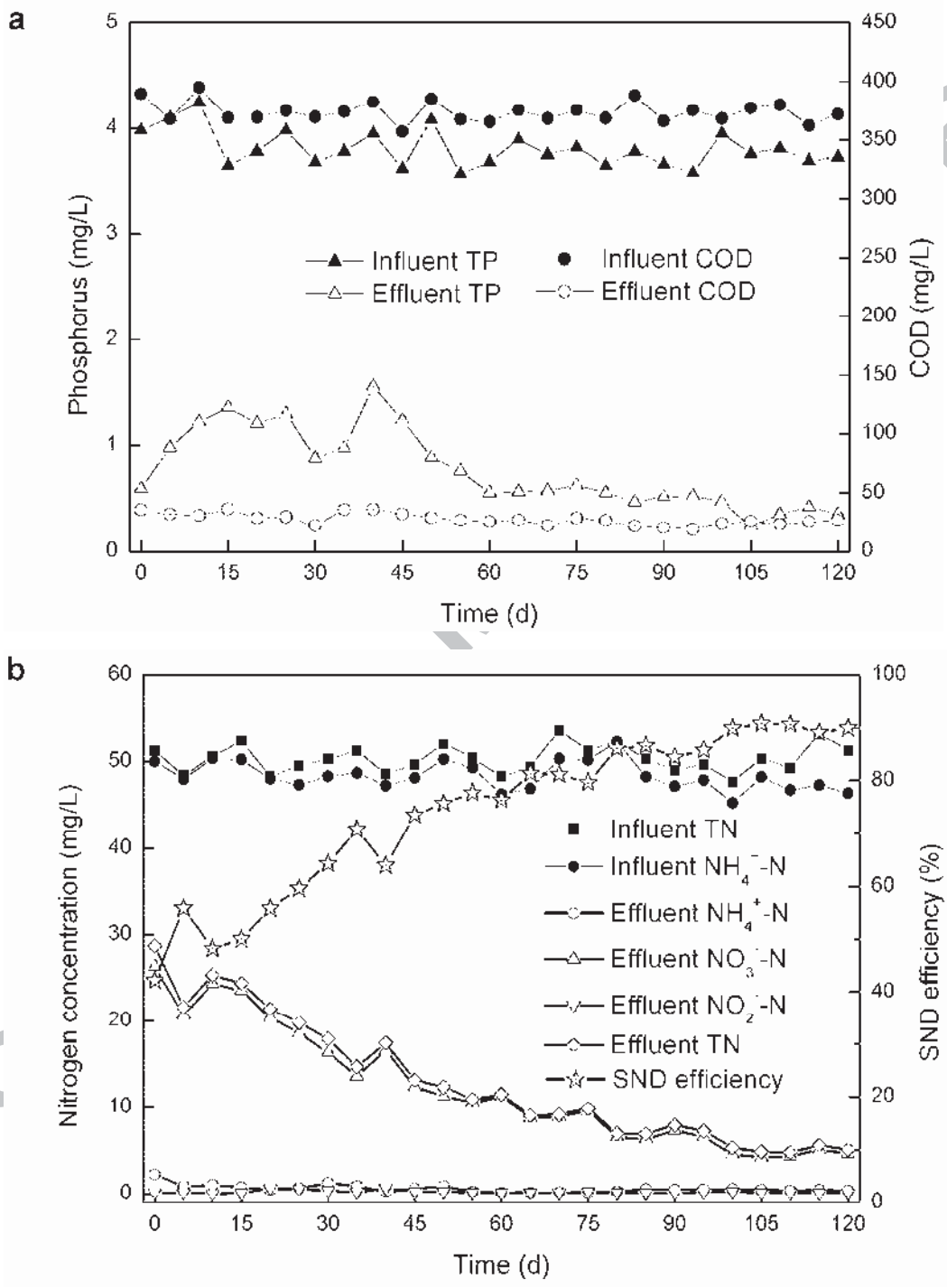

Fig. 1

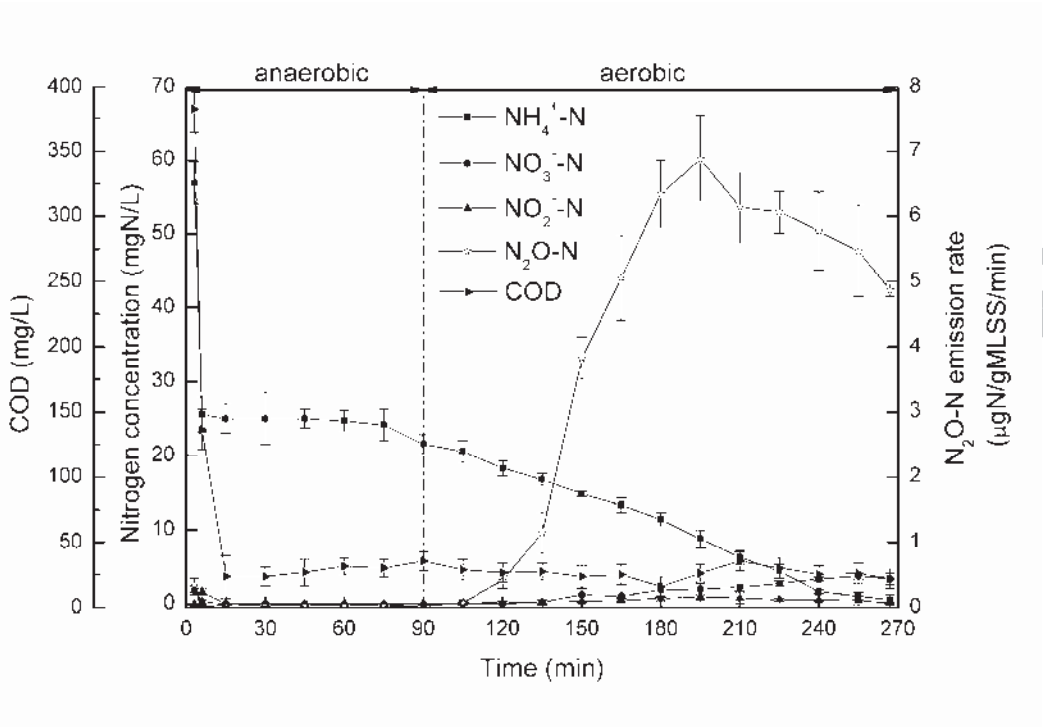

Fig. 2

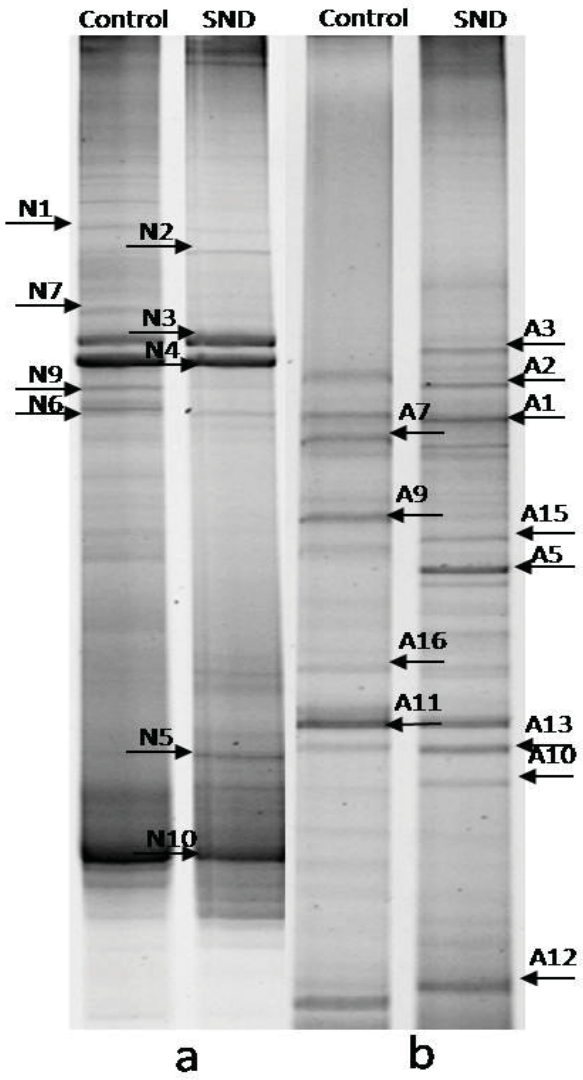

Fig. 3

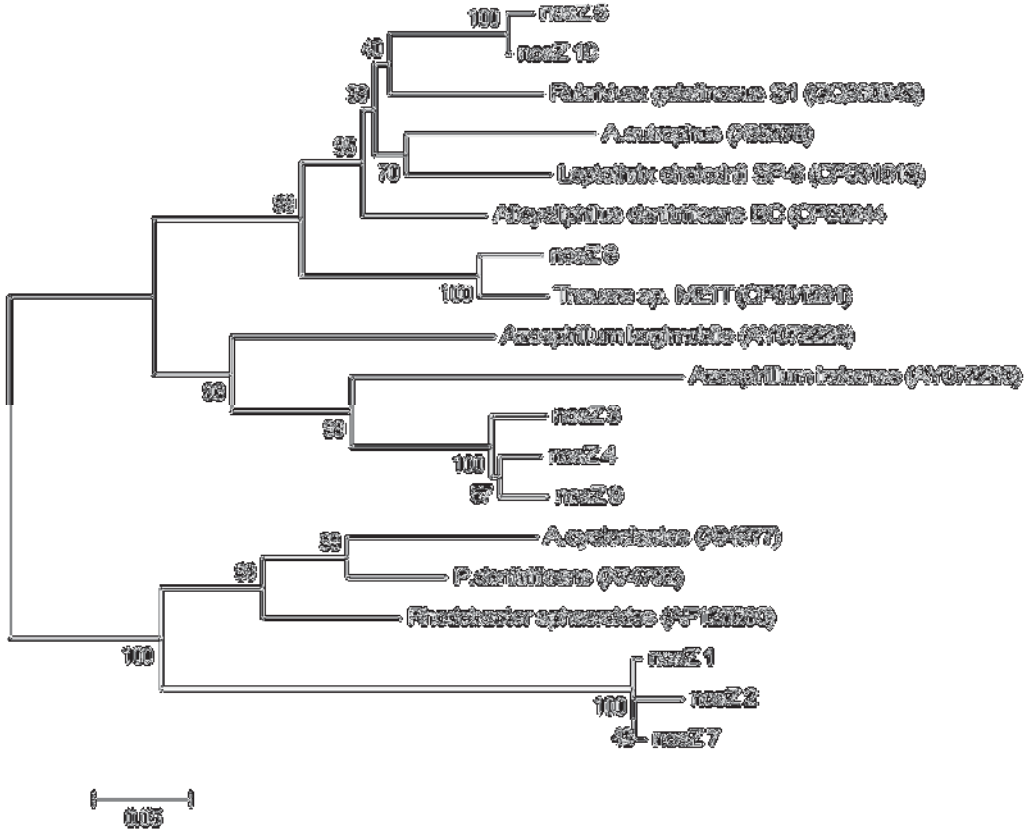

Fig. 4

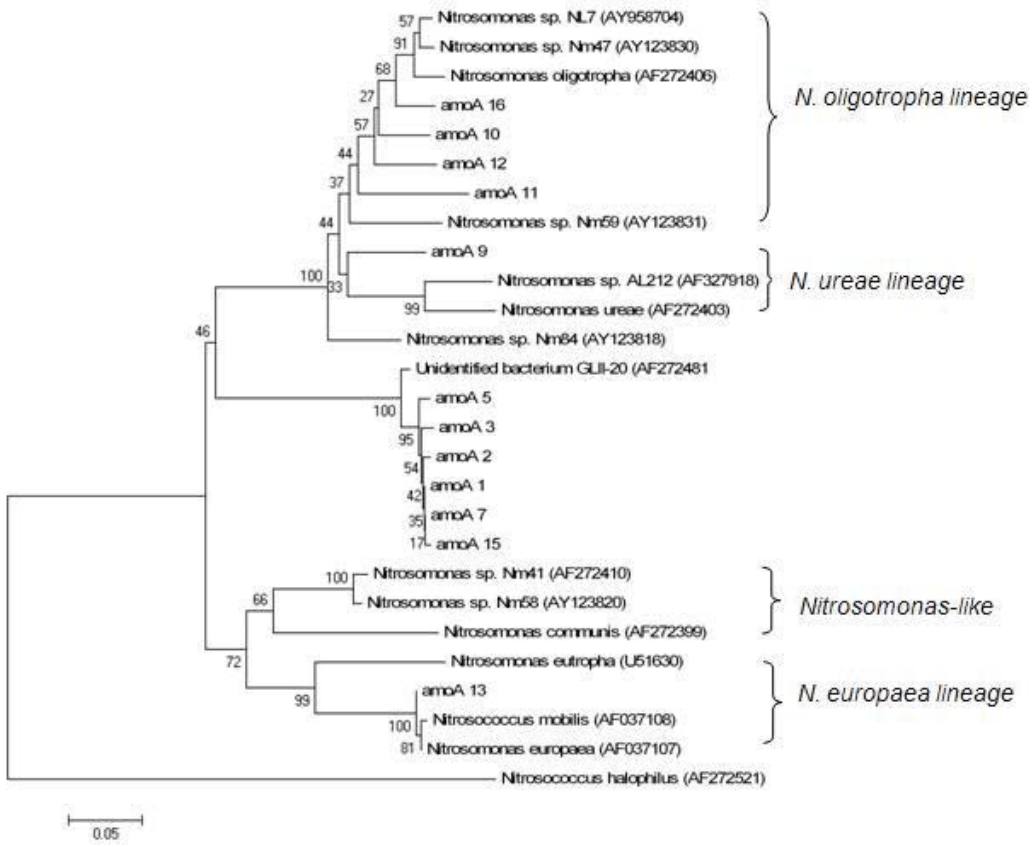

Fig. 5

Table 1 Removal performances of the SND SBR, N_2O yield (Y_{N2O-N}) and conversion rate. Values represent the mean±SD (n = 10).

Variables	COD	TP	NH_4^+-N	NO_3^--N	NO_2^--N	TN	Y_{N_2O-N}	N_2O conversion
Influent (mg/L)	373.8 ± 9.1	3.8 ± 0.2	51.9 ± 2.9	1.8 ± 0.8	0	53.7 ± 3.4	—	—
Effluent (mg/L)	25.9 ± 4.7	0.7 ± 0.1	0.6 ± 0.2	3.3 ± 0.8	0.1 ± 0.1	4.1 ± 0.5	0.8 ± 0.1[a]	—
E[b] (%)	93.1	82.9	98.9	—	—	92.5	—	7.7[c]

[a] The unit of N_2O yield is "mg N/gMLSS".

[b] Removal efficiency calculated as: E = (Influent Con.-Effluent Con.)/Influent Con. × 100%.

[c] N_2O conversion rate calculated as : E=N_2O-N/TN input× 100%.

Table 2 Summary of TN removal efficiency and N_2O emission in different reactors.

Reactor	Operation condition	TN removal efficiency (%)	N_2O emission (% of N input)	Reference
SBR	Anaerobic-aerobic (low DO)	92.5%	7.7%	This study
SBR	Anaerobic-aerobic, DO >2 mg/L	66.3%	2.1%	Jia et al. (2012)
SBR	Anaerobic-aerobic (low DO), acetate as carbon source	65.3%	28.2%	Zhu and Chen (2011)
SBR	Anaerobic-aerobic (low DO), sludge Fermentation Liquid as carbon source	77.5%	10.4%	Zhu and Chen (2011)
SBR	Anaerobic-aerobic, DO >2 mg/L	51.0%	5.3%	Hu et al. (2010)
SBR	Aerobic-anaserobic, methanol addition, DO=1.5-2.0	76.0%	2.7%	Liu et al. (2008)
Anoxic-oxic activated sludge reactor	Anoxic-oxic, DO=2.0 mg/L	45.0%-65.0%	<0.2%	Noda et al. (2003)
Continuous nitrifying and denitrifying reactor	Intermittent aeration, DO>2 mg/L; methanol addition for denitrification	>90%	0.2-4.5%	Park et al. (2000)

Table 3 Nitrogen transformation and N_2O emission rate (r) in the batch experiments under various conditions. Values represent the mean±SD (n = 3)[a].

Experiment number	Experimental conditions	rNH_4^+ (mgN/gMLSS/h)	rNO_2^- (mgN/gMLSS/h)	rNO_3^- (mgN/gMLSS/h)	rN_2O (mgN/gMLSS/h)
a	Mixed liquor and sludge	-1.99 ± 0.21	0	0.47 ± 0.12	0.31 ± 0.18
b	(a)+nitrite	-1.83 ± 0.13	-0.52 ± 0.14	0.66 ± 0.15	0.48 ± 0.12
c	(a)+nitrite+ inhibitors	0	-0.22 ± 0.07	0	0.15 ± 0.07
d	Sludge+nitrite	0	-0.55±0.06	0.08±0.01	0.44±0.05
e	Supernatant+nitrite+ inhibitors	0	0	0	0

[a] Positive and negative values represent appearance and disappearance rates, respectively.

Highlights

- The sources and mechanisms of N_2O emission in low-oxygen SND process were studied.
- Nitrifier denitrification turned out to be the dominant N_2O emission source.
- Nitrifier denitrification was enhanced due to enrichment of denitrification AOB.
- Heterotrophic denitrification was limited by oxygen and the lack of carbon source.