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ENVIRONMENTAL TOXICOLOGY AND BIOGEOCHEMISTRY OF ECOSYSTEMS

N_2O profiles in the enhanced CANON process via long-term N_2H_4 addition: minimized N_2O production and the influence of exogenous N_2H_4 on N_2O sources

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Abstract
Production of the greenhouse gas nitrous oxide (N_2O) from the completely autotrophic nitrogen removal over nitrite (CANON) process is of growing concern. In this study, the effect of added hydrazine (N_2H_4) on N_2O production during the CANON process was investigated. Long-term trace N_2H_4 addition minimized N_2O production (0.018% ± 0.013% per unit total nitrogen removed) and maintaining high nitrogen removal capacity of CANON process (nitrogen removal rate and TN removal efficiency was 450 ± 60 mg N/L/day and 71 ± 8%, respectively). Ammonium oxidizing bacteria (AOB) was the main N_2O producer. AOB activity inhibition by N_2H_4 decreased N_2O production during aeration, and the N_2H_4 concentration was negatively correlated with N_2O production rate in NH_4^+ oxidation via AOB, whereas N_2O production was facilitated under anaerobic conditions because hydroxylamine (NH_2OH) production was accelerated due to anammox bacteria (AnAOB) activity strengthen via N_2H_4. Added N_2H_4 completely degraded in the initial aeration phases of the CANON SBR, during which some N_2H_4 intensified anammox for total nitrogen removal to eliminate N_2O production from nitrifier denitrification (ND) by anammox-associated, while the remaining N_2H_4 competed with NH_2OH for hydroxylamine oxidoreductase (HAO) in AOB to inhibit intermediates formation that result in N_2O production via NH_2OH oxidation (HO) pathway, consequently decreasing total N_2O production.

Keywords Completely autotrophic nitrogen removal over nitrite · N_2H_4 · Nitrous oxide · NH_2OH oxidation · Nitrifier denitrification · Anammox

Introduction

The completely autotrophic nitrogen removal over nitrite (CANON) (Sliekers et al. 2002) process combines nitritation and anaerobic ammonium (NH_4^+) oxidization (anammox) into a single process, in which aerobic ammonium oxidizing bacteria (AOB) partially oxidize NH_4^+ to nitrite (NO_2^-), while anammox bacteria (AnAOB) convert the resulting NO_2^- and any remaining NH_4^+ into nitrogen gas (N_2). The CANON process is a cost-effective and energy-efficient alternative to the conventional nitrogen removal process because of its reduced oxygen and carbon source demands (Sliekers et al. 2002; De Clippeleir et al. 2013; Joss et al. 2009), and it has been applied in full-scale wastewater treatment projects (Joss et al. 2009, 2011; Vázquez-Padín et al. 2009). Nitrous oxide (N_2O) is easily produced as an intermediate from incomplete nitrification and denitrification (Rassamee et al. 2011). Because N_2O is an ozone-depleting agent (Ravishankara et al. 2009) and a potent greenhouse gas with a global warming potential (GWP100) of 298 (IPCC 2011), N_2O produced by the CANON process is a subject of concern. Many researchers have assessed N_2O production and emission using different types of CANON reactors (Sliekers et al. 2002; Kampschreur et al. 2008, 2009a; Okabe et al. 2011; Xiao et al. 2014; Wang et al. 2017; Blum et al. 2018). These studies

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show that, irrespective of the operation mode or process conditions, controlling N_2O production and/or emissions during aerobic periods based on nitrification by AOB is advantageous because of reductions in the total N_2O emission from the system (Wunderlin et al. 2013a). A better understanding of the biological turnover processes of nitrogen compounds during N_2O production via aeration, as well as reactor operation parameters that can be adjusted to reduce N_2O production, is needed to improve process sustainability.

Hydroxylamine (NH_2OH) oxidation (HO) and nitrifier denitrification (ND) driven by AOB are the main biological pathways for N_2O production during aeration in the CANON process (Wunderlin et al. 2012, 2013a, b; Harris et al. 2015; Pocquet et al. 2016; Ali et al. 2016). The concentrations of NH_4^+, NO_2^- and dissolved oxygen (DO) in the reactor have direct effects on the two pathways by which N_2O is produced (Xiao et al. 2014; Schreiber et al. 2012; Peng et al. 2015, 2016). N_2O production by ND is favored at high NO_2^- concentrations and low DO concentrations (Wunderlin et al. 2012; Harris et al. 2015; Peng et al. 2015), whereas high NH_4^+ concentrations and a high NH_4^+ removal rate favor N_2O production by HO (Wunderlin et al. 2013a, b). When the NH_4^+ or DO concentrations in the reactor are elevated beyond a threshold, N_2O production by ND is enhanced and becomes the dominant source of N_2O. Moreover, N_2O production via HO is a relatively unimportant source of N_2O in CANON reactors under all conditions (Harris et al. 2015). Although N_2O is not a side-product of AnAOB metabolism (Kartal et al. 2011), AnAOB play an important role in N_2O production by providing NH_2OH as an electron donor for AOB, which reduce NO_2^- by ND in the CANON process (Xiao et al. 2014), and the existence of an unclear N_2O production pathway associated with anammox metabolism merits further study (Harris et al. 2015; Ali et al. 2016). Until recently, distinguishing the contributions of the HO, ND- and anammox-associated pathways to N_2O production during the CANON process has been difficult, and N_2O production has been regarded as either a single or synergistic effect of those pathways, depending on the specific operational state of the process.

The focus of aeration strategies, especially during NH_4^+ oxidation, is to mitigate N_2O emission. In an earlier study, optimization of the DO concentration and dynamic co-optimization of DO and pH were found to be effective methods of controlling N_2O production (Kampschreur et al. 2009a, b). Nevertheless, a single change in either of these operating conditions did not significantly reduce the amount of N_2O emission from the CANON process (Weissenbacher et al. 2011). Later, the NO_2^- concentration and NH_2OH oxidation activity were both found to be positively correlated with N_2O production (Okabe et al. 2011; Wunderlin et al. 2012). Maintaining a low concentration of dissolved NO_2^- and a low level of NH_2OH oxidation activity are key requirements for minimizing N_2O production during the CANON process (Wunderlin et al. 2013a). Aerobic NH_4^+ oxidation activity influences NH_2OH oxidation and the NO_2^- concentration (Xiao et al. 2014; Wunderlin et al. 2013a, b). Increasing the frequency of aeration cycling to stimulate aerobic NH_4^+ oxidation activity significantly minimizes N_2O production while maintaining maximum liquid phase nitrogen removal efficiency (Domingo-Félez et al. 2014). However, operating conditions are very difficult to control during frequent aeration cycling because long-term adjustments and fine control of the process (Domingo-Félez et al. 2014) are required. Maintaining a low rate of NH_4^+ oxidation has become the central focus of strategies aimed at reducing N_2O production from the CANON process. Taken together, the previous study has shown that, although a low NH_4^+ oxidation rate can negatively influence denitrification activity, high-frequency intermittent aeration with short cycle times, low volumetric N loading, and maintaining an extremely low NH_4^+ oxidation rate are effective strategies for reducing N_2O emission by the CANON process (Blum et al. 2018). Methods of rapidly and efficiently minimizing N_2O production and emissions while maintaining the stability and performance of the CANON process are an important research focus.

The role of hydrazine (N_2H_4) as an intermediate product in anammox (Kartal et al. 2011) has received a significant attention. The addition of N_2H_4 to a CANON reactor can simultaneously enhance the total nitrogen (TN) removal performance and reactor stability (Yao et al. 2013; Xiao et al. 2015). However, the development of an improved "green" CANON process with a reduced environmental impact is hindered by a lack of knowledge regarding the effect of N_2H_4 on N_2O production. In the present work, a lab-scale CANON granular sequencing batch reactor (SBR) with alternating oxygen-limited/anaerobic conditions was implemented to study the effect of exogenous N_2H_4 on N_2O production. The influence of exogenous N_2H_4 on sources of N_2O was investigated using batch experiments, and the mechanism of N_2O reduction was studied during one cycle of SBR operation. The results of this study provide a strategy for tracing the effects of added N_2H_4 in a CANON reactor to rapidly achieve minimal N_2O production while maintaining stable and effective nitrogen removal.

Materials and methods

Operation of the CANON SBR

A continuously running CANON SBR with enriched granular sludge was utilized in this study. A schematic diagram of the CANON SBR, as well as its working volume, operating parameters, and components, with related concentrations of synthetic wastewater and the method of long-term N_2H_4 (4 mg/L) addition was reported by Xiao et al. (2014, 2015).

Batch tests

Batch tests were conducted to investigate the influence of exogenous N_2H_4 (in the form of $N_2H_4 \times H_2SO_4$) on sources of N_2O in the CANON process. How the batch tests are prepared and conducted has been reported by Xiao et al. (2014) .The experimental conditions are shown in Table 1.

Under oxygen-limited conditions, batch tests A and B focused on the influence of exogenous N_2H_4 on N_2O production in the CANON process. Different concentrations of N_2H_4 were added in batch tests C, D, and E (with methanol added to inhibit AnAOB activity) to investigate the effect of exogenous N_2H_4 on N_2O production during aerobic NH_4^+ oxidation. Batch tests H, I, and J were conducted to distinguish the contributions of HO and ND during aerobic NH_4^+ oxidation. Allylthiourea (ATU) and methanol were added in batch tests H and I to impede oxidation of NH_4^+ to form NH_2OH (Xiao et al. 2014). Diethyldithiocarbamate (DDC), an inhibitor of copper-containing nitrite reductase (NirK), was added in batch test I to block reduction of NO_2^- to form N_2O by the ND pathway (Ravishankara et al. 2009). It has been reported that a small amount of N_2O may be formed from chemical oxidation of NH_2OH (Shapleigh and Payne 1985). Therefore, batch test J (aerobic conditions without sludge) was designed to investigate N_2O production via chemical oxidation of NH_2OH in the CANON process. Batch tests K and L were conducted to investigate the influence of the addition of a trace amount of N_2H_4 on N_2O production under anaerobic conditions. Batch tests F, G, and M were conducted to investigate the production of N_2O via heterotrophic denitrification (HD).

Granular sludge taken from the CANON SBR was washed three times with nutrient solution before the batch tests. The concentrations of trace elements and $NaHCO_3$ in the batch tests were consistent with those observed in the operation of the CANON SBR. Mineral medium and inhibitors were added into 500-mL batch reactors according to the experimental conditions shown in Table 1. Initial concentrations of NH_4^+-N, NO_2^--N, NH_2OH-N, and N_2H_4-N were measured. DO was measured with a DO meter (BANTE820, Shanghai Bant Instrument Company, China). A gas flow meter (95 mL air/min) was used to control the aeration rate and maintain the DO level at 0.35 ± 0.05 mg/L under oxygen-limited conditions. The batch reactors were flushed with nitrogen gas (99.99%) at 30 mL N_2/min to create anaerobic conditions in batch tests K, L, and M. The pH was measured using a pH electrode (HI991002, Hanna Instruments, Italy) and adjusted to 7.8 by adding 1 mol/L HCl and 1 mol/L NaOH. The temperature of the batch reactors was maintained at 31 ± 1 °C using a water bath.

Sampling and analysis

Batch test samples (10 mL) were taken from the reactors every hour using syringes. According to previously reported protocols (APHA 1998), NH_4^+-N, NO_2^--N, and chemical oxygen demand (COD) were analyzed using colorimetric methods, whereas nitrate (NO_3^-) was measured spectrophotometrically. Mixed liquor suspended solids (MLSS) and mixed liquor

Table 1 Experimental conditions for each batch test

Batch tests	NH_4^+-N (mg N/L)	NO_2^--N (mg N/L)	NH_2OH (mg N/L)	N_2H_4 (mg N/L)	ATU (mg/L)	CH_3OH (mM)	DDC (mM)	Remarks
A	87.23	/	/	/	/	/	/	Oxygen-limited conditions
B	85.90	/	/	3.2	/	/	/	
C	83.24	/	/	/	/	10	/	
D	85.31	/	/	3.04	/	10	/	
E	87.08	/	/	7.16	/	10	/	
F	/	67.4	/	/	/	/	/	
G		67.66	/	/	/	10	/	
H	/	/	82.08	/	20	10	/	
I	/	/	83.43	/	20	10	10	
J	/	/	84.24	/	/	/	/	Without sludge, aerobic conditions
K	66.09	62.47	/	/	/	/	/	Anaerobic conditions
L	70.52	62.51	/	3.02	/	/	/	
M		62.19	/	/	/	10	/	

volatile suspended solids (MLVSS) were measured using standard methods (2540). The NH_2OH concentration was measured using a spectrophotometric method as previously described (Frear and Burrell 1955). The N_2H_4 concentration was measured using a spectrophotometric method as previously described (Watt and Chrisp 1952), and the interference of NO_2^- was eliminated by the addition of 0.5% sulfamic acid (George et al. 2008). Total N_2O, consisting of the N_2O in the emission gas and the N_2O dissolved in the mixed liquid, was analyzed in triplicate using gas chromatography (2010-plus, Shimadzu Scientific Instruments, Japan). The experimental conditions for the GC measurements and the methods used for measurement and calculation of the total concentration and production rate of N_2O-N were described (Xiao et al. 2014).

Results

CANON SBR performance and N_2O production

The N_2O production and nitrogen removal performance of the CANON SBR used in this study (with N_2H_4 addition) from days 505 to 525 are shown in Fig. 1.

From days 505 to 509, the total nitrogen removal rate (NRR) and TN removal efficiency of the reactor gradually increased (Fig. 1 panel a). In contrast, from days 505 to 509, the average N_2O production rate sharply decreased from 0.20 to 0.064 mg N/L/day (Fig. 1 panel b). The NRR and TN removal efficiencies reached 450 ± 60 mg N/L/day and 71 ± 8% (average value from days 509 to 525), respectively, which were nearly equal to the results (420 ± 40 mg N/L/day and 72 ± 9%, respectively) previously reported for the reactor (Xiao et al. 2015). From days 509 to 525, the average N_2O production rate was relatively low (0.066 ± 0.047 mg N/L/day) and represented only approximately 0.018 ± 0.013% of the NRR.

Batch tests

The nitrogen conversion and N_2O average production rates of the batch tests are shown in Table 2.

Under oxygen-limited conditions

In batch test A (without N_2H_4 addition), the NRR was 213.30 mg N/g VSS/day, and the average N_2O production rate (0.078 mg N/g VSS/day) was only 0.037% of the NRR. With the addition of 3.2 mg N/L N_2H_4 in batch test B, the NRR increased slightly to 286.86 mg N/g VSS/day, whereas the average N_2O production rate decreased to 0.032 mg N/g VSS/day, which was only 0.012% of the NRR and 41% of that in batch test A.

In batch test C, with methanol added to inhibit AnAOB activity and without the addition of N_2H_4, the NRR and average N_2O production rate were 24.85 mg N/g VSS/day and 0.06 mg N/g VSS/day, respectively, which were 11.7% and 76.9%, respectively, of the values measured in batch test A. In batch test D, with the addition of 3.04 mg N/L N_2H_4, the TN removal and average N_2O production rate decreased to 19.09 mg N/g VSS/day and 0.02 mg N/g VSS/day, respectively. The average N_2O production rate of batch test D was 33% of that of batch test C, whereas it was 62.5% of that of batch test B. In batch test E, with the addition of 7.16 mg N/L N_2H_4, the NRR decreased to 9.59 mg N/g VSS/day, and the average N_2O production rate was lower than the detection limit. In batch tests C, D, and E, N_2O production decreased in parallel with the added concentrations of N_2H_4 during aerobic NH_4^+ oxidation. The conversion rates at different concentrations of N_2H_4 in batch tests D and E were nearly equivalent, which indicated that the maximum aerobic degradation rate of N_2H_4 was reached when approximately 3 mg N/L N_2H_4 was added to the reactor. It has been reported that N_2H_4 might have the

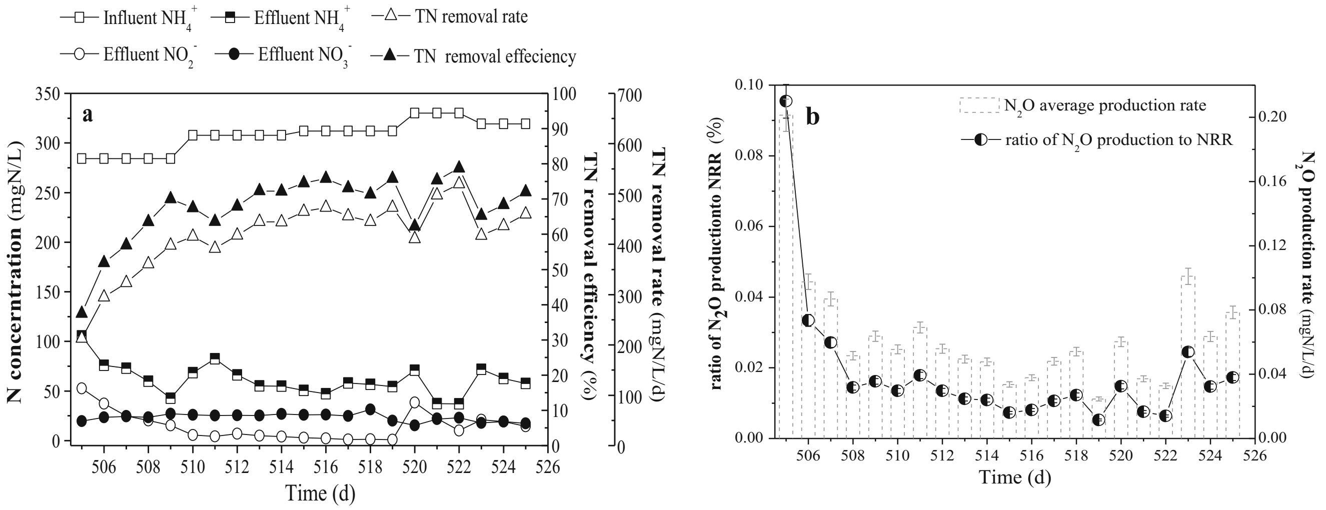

Fig. 1 Operation performance (panel **a**) and N_2O production (panel b) of the CANON SBR with N_2H_4 addition (days 505 to 525)

Table 2 Nitrogen removal and N_2O production in batch tests

Experiments conditions	Numbers	NH_4^+-N[c] Removal rate (mgN/gVSS/day)	NH_2OH[c] Removal rate (mg N/gVSS/day)	NO_2^--N[c] Removal rate (mg N/g VSS/day)	NO_3^--N[c] Removal rate (mgN/gVSS/day)	N_2O average Production rate (mgN/gVSS/day)	TN[a] Removal rate (mgN/gVSS/day)	N_2H_4 Removal rate (mgN/gVSS/day)	N_2O production rate/NRR (%)
Oxygen limited	A	− 231.17[b]	/	+ 3.13[b]	+ 14.64	+ 0.078	− 213.30	/	0.037
	B	− 286.86	/	+ 5.49	+ 9.53	+ 0.032	− 273.60	/[d]	0.012
	C	− 167.74	/	+ 124.93	+ 18.88	+ 0.060	− 24.85	/	0.24
	D	− 105.75	/	+ 81.79	+ 13.82	+ 0.020	− 19.09	− 2.23	0.10
	E	− 63.41	/	+ 47.62	+ 5.52	+ 0	− 9.59	− 2.35	0
	F	/	/	− 38.99	+ 38.74	/	/	/	/
	G	/	/	− 30.11	+ 30.11	/	/	/	/
	H	/	− 175.68	+ 147.65	+ 8.97	+ 0.074	− 18.84	/	0.39
	I	/	− 170.10	+ 147.63	+ 4.5	+ 0.046	− 17.89	/	0.26
Aerobic without sludge	J	/	/	/	/	/	/	/	0
Anaerobic	K	− 142.36	/	− 165.97	+ 3.17	+ 0.024	− 305.06	/	0.008
	L	− 143.46	/	− 182.34	+ 3.02	+ 0.094	− 322.67	/[d]	0.029
	M	/	/	/	/	/	/	/	0

[a] TN is consisted of NH_4^+-N, NO_2^--N and NO_3^--N

[b] "+" in front of numerical values meant production, and "–" meant removal.

[c] The conversion rate of NH_4^+-N, NO_2^--N, NO_3^--N , NH_2OH, N_2H_4, and TN was got via dividing the slope of linear fitting from the experimental results (measured concentrations) by MLVSS, respectively; the related linear regression coefficient of the experimental results was all over 90%

[d] Concentrations of added N_2H_4 in batch tests B (under oxygen-limited conditions) and L (under anaerobic conditions) completely decreased in the first 1 h

capacity to impact the growth of AOB (Choi et al. 2013), and AOB can degrade N_2H_4 (Yao et al. 2013) .Therefore, the half-saturation coefficient of N_2H_4 in aerobic NH_4^+ oxidation was likely less than 3 mg N/L, which was lower than that previously reported for anammox (10.42 mg N/L) (Yao et al. 2015).

On the basis of the results of batch tests A and C without N_2H_4 addition, the contributions of AOB and AnAOB to the total N_2O production were determined to be 76.9% and 23.1%, respectively. When a similar concentration of exogenous N_2H_4 was added in batch tests B and D, the contributions of AOB and AnAOB to total N_2O production were 62.5% and 38.5%, respectively. These results demonstrate that exogenous N_2H_4 slightly increased the contribution of AnAOB to total N_2O production during aeration.

In the presence of only NO_2^- in batch test F, very little TN was removed, and only a small amount of NO_3^- was produced by nitrite oxidization bacteria (NOB). With the addition of NO_2^- and methanol together in batch test G, very little TN was removed and only slight variation in COD was observed, while only a small amount of NO_3^- was produced (Fig. 2). N_2O production was not detected in batch test F or batch test G. These results indicate that HD did not contribute to nitrogen removal and N_2O production under oxygen-limited conditions.

In the presence of NH_2OH only, no significant change in the concentration of NH_2OH or N_2O was observed after 4 h of continuous aeration under aerobic conditions without sludge in batch test J. Therefore, the N_2O production pathway from NH_2OH chemical oxidation in the CANON process could be ignored. With the addition of 20 mg/L ATU to inhibit NH_4^+ oxidation and 10 mM methanol to restrict AnAOB activity in batch test H, the NH_2OH removal rate was 175.68 mg N/g VSS/day, and the average N_2O production rate was 0.074 mg N/g VSS/day, which was greater than that of batch test C. With the addition of 20 mg/L ATU, 10 mM methanol, and 10 mM DDC in batch test I, the NH_2OH removal rate marginally decreased to 170.10 mg N/g VSS/day, while the average N_2O production rate was 0.046 mg N/g VSS/day, which was 62.1% of that of batch test H. In batch test I, NirK activity was restrained by DDC to block N_2O formation from NO_2^- reduction by ND. HO and ND are pathways for N_2O production in the CANON process under oxygen-limited conditions. The contributions of HO and ND to total N_2O production during aeration were 62.1% and 37.9%, respectively.

Under anaerobic conditions

In batch test K without N_2H_4 addition, the NRR was 305.06 mg N/g VSS/day and the N_2O average production rate was 0.024 mg N/g VSS/day, which was 0.008% of the NRR. With the addition of N_2H_4 in batch test L, the NRR slightly increased to 322.67 mg N/g VSS/day, and the average N_2O production rate was 0.094 mg N/g VSS/day, which was 0.029% of the NRR and 3.9 times that of batch test K. Exogenous N_2H_4 effectively increased N_2O production via anammox under anaerobic conditions in the CANON process.

With the addition of both NO_2^- and methanol in batch test M, very little COD variation or nitrogen removal was observed, and no N_2O production was detected (Fig. 2). These results demonstrate that HD was still not observable under anaerobic conditions.

Dynamic N_2O production in one cycle of the CANON SBR operation

The dynamic concentration profiles during one cycle of CANON SBR operation (day 518) are illustrated in Fig. 3. NH_4^+-N decreased, whereas NO_3^--N production by NOB increased slightly at the beginning of the aeration phase and then

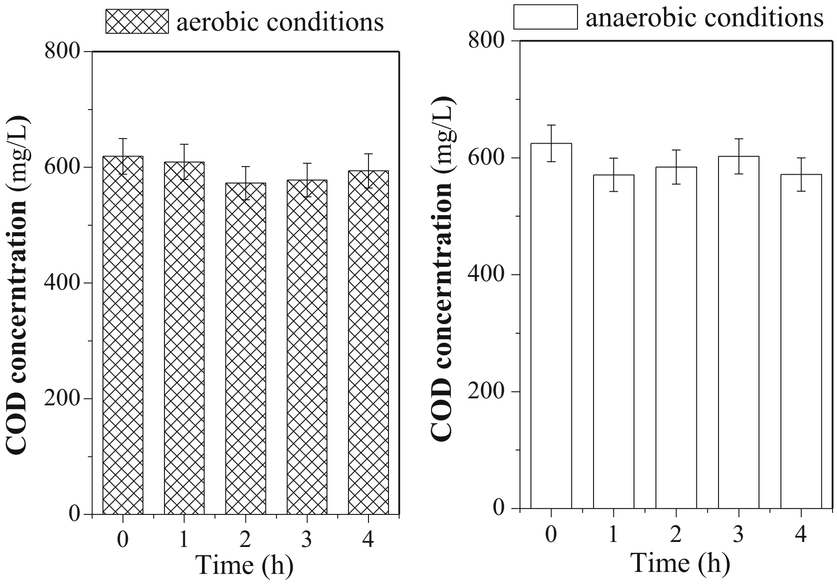

Fig. 2 COD variation with methanol addition in batch tests F, G, and M

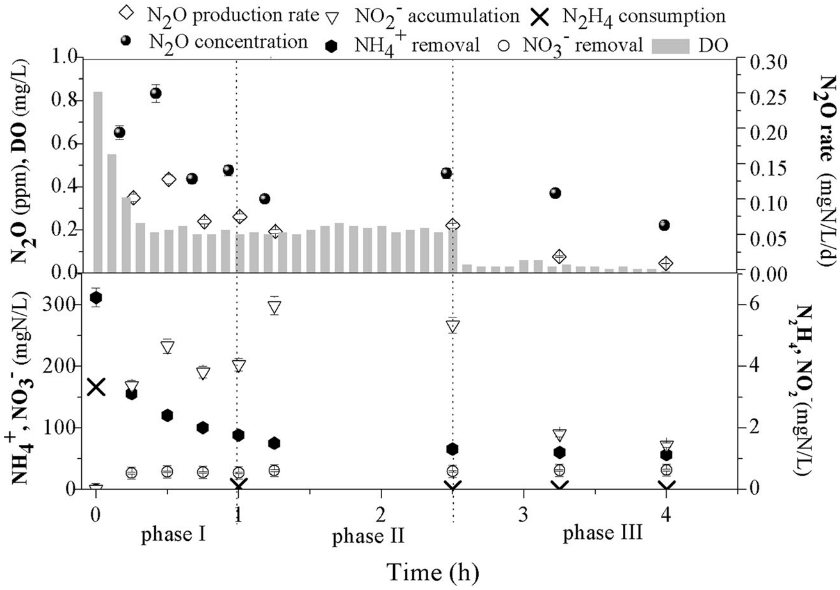

Fig. 3 N_2O production and nitrogen removal in one cycle of CANON SBR operation

remained unchanged. Exogenous N_2H_4 decreased during the first hour of the cycle. In aeration phase I (0–1 h, shown in Fig. 3), NO_2^- gradually accumulated during the first half hour, after which the amount of NO_2^- decreased. The N_2O concentration and average production rate peaked at 0.84 mg N_2O-N/L and 0.15 mg N_2O-N/L/day during the first half hour and then decreased. During aeration phase II (1–2.5 h, shown in Fig. 3), the NO_2^- concentration rapidly increased, and peak NO_2^- concentration occurred at 1.25 h. The N_2O concentration and average production rate slightly increased in aeration phase II. In anaerobic phase III (2.5–4 h, shown in Fig. 3), the NO_2^- concentration rapidly decreased, and the N_2O concentration and average production rate decreased (to 0.009 mg N/L/day) at the end of the anaerobic period.

Discussion

Minimized N_2O production with long-term N_2H_4 addition

Information regarding N_2O production observed in various studies during autotrophic nitrogen removal over nitrite is summarized in Table 3. In this study, the enhanced lab-scale CANON granular system with exogenous N_2H_4 was maintained stable, high-rate nitrogen removal performance as previously reported (Rassamee et al. 2011). The average N_2O production rate was 0.066 ± 0.047 mg N/L/day, which accounted for only 0.018% ± 0.013% of the NRR, which was a minimal contribution in comparison with the corresponding results from other reactors (Table 3).

Alternatively, with regard to aeration control strategies aimed at achieving minimal N_2O production (0.011% ± 0.004% per unit NH_4^+ removed) during autotrophic nitrogen removal over nitrite, shorter cycle times (160 min), lower volumetric N loading (less than 100 mg NH_4^+-N/L), and a low rate of aerobic NH_4^+ oxidation (5 mg N/g VSS/h) (Blum et al. 2018) have been reported to effective. As shown in Table 2, the results obtained in the batch experiments in this study regarding N_2O production with the addition of trace N_2H_4 (0.011% per unit NH_4^+removed) are in agreement with previously reported results from experiments conducted with a similar cycle time (240 min) and volumetric N loading (85.9 mg NH_4^+-N/L), but with a higher NH_4^+ oxidation rate (11.95 mgN/gVSS/h). Consequently, these results indicate that exogenous N_2H_4 minimized N_2O production by the CANON process while retaining stable, high-rate nitrogen removal performance.

Effect of N_2H_4 on N_2O production pathways

Under oxygen-limited conditions in the CANON system, HD had no impact on TN removal or N_2O production in batch tests F and G. The activity level of heterotrophic bacteria was extremely low, as previously reported (Xiao et al. 2014). Whether anammox activity was inhibited or not (in batch tests A and C), the average N_2O production rates of batch tests B and D (with the same added N_2H_4 concentration) were reduced by 67% and 59%, respectively, in comparison with those of batch tests A and C (without N_2H_4 addition), respectively. Hydroxylamine oxidoreductase (HAO) plays a key role in production of NO and NO_2^- (Poughon et al. 2001), which play an important role in the HO and ND pathways of N_2O production by AOB (Wunderlin et al. 2012). N_2H_4 competes strongly with NH_2OH for HAO in AOB (Hollocher et al. 1981); therefore, the reduction in total N_2O production in the CANON process under oxygen-limited conditions was primarily caused by inhibition of AOB activity by exogenous

Table 3 Nitrous oxide production rates observed in various studies during autotrophic nitrogen removal over nitrite

Type of reactor	NRR (mg N/L/day)	Ratio of N_2O production to NRR (%)	References
Full-scale N&A	1350	0.89	Joss et al. 2009
Full-scale N&A	1480	1.67	Kampschreur et al. 2009a
Full-scale N&A	1310	2.69	Castro-Barros et al. 2015
Pilot-scale N&A	470–1310	11.1–19.1	De Clippeleir et al. 2013
Lab-scale N&A	250–610	0.48–2.44	Yang et al. 2013
Lab-scale N&A	21	2.4	Hu et al. 2013
Lab-scale N&A	660	1.7–70	Domingo-Félez et al. 2014
Lab-scale N&A	400–600	1.35 ± 0.72	Ali et al. 2016
Lab-scale N&A	700–900	1.1 ± 0.5	Blum et al. 2018
Lab-scale CANON	110	0.11	Sliekers et al. 2002
Lab-scale CANON	480	0.41–1.53	Xiao et al. 2014
Lab-scale CANON	450 ± 60	0.018 ± 0.013	This study

N_2H_4, which likely influenced the HO and/or ND pathways of N_2O formation.

Moreover, the total N_2O production rate was reduced by 67% with inhibition of AnAOB activity, whereas it was reduced by 59% without inhibition of AnAOB activity, suggesting that the inhibitory effect of by N_2H_4 on N_2O production from AOB coexisting with AnAOB was less severe than its effect on N_2O production from AOB only. N_2H_4 significantly increases the specific growth rate (Yao et al. 2013) and abundance of AnAOB (Xiao et al. 2015), as well as specific anammox activity (Ma et al. 2018). Thus, exogenous N_2H_4 was likely utilized by AnAOB to accelerate anammox activity for TN removal during aeration, which weakened the inhibitory effect on N_2O formation from ND by AOB.

In this research, batch tests H, I, and J in the presence of NH_2OH under oxygen-limited conditions showed that N_2O is produced by the HO and ND pathways during aeration in the CANON process. The contributions of HO and ND to total N_2O production during aeration with inhibition of AnAOB activity were 62.1% and 37.9%, respectively. Similar results have been reported in a partial nitrification granular reactor in which the HO pathway accounted for 65% of total N_2O production (Rathnayake et al. 2013). These findings demonstrate that N_2O production mechanisms are complex and could involve multiple N_2O production pathways in CANON process. The variation in the N_2O production rate in batch tests H and I is shown in Fig. 4. The N_2O production rate in batch test I, in which the ND pathway was inhibited, was lower than that of batch test H during the reaction. HO and ND were both involved in N_2O production, as NH_2OH was the sole electron donor. With the addition of methanol to inhibit AnAOB activity, the N_2O production rate in the presence of NH_4^+ (batch test C) was slower than that of NH_2OH (batch test H). When NH_2OH replaces NH_4^+ as the only substrate, more electrons are released from NH_2OH oxidation to NO_2^- for the respiratory chain of AOB because of a lack of ammonia monooxygenase (AMO) activity (Chandran and Smets 2008).

Under anaerobic conditions, HD by heterotrophic bacteria (Ali et al. 2016; Li et al. 2017) and ND by AOB in the anammox process (Blum et al. 2018; Xiao et al. 2014) were primary pathways for N_2O production in the one-stage autotrophic nitrogen removal process. The results of batch tests K, L, and M showed that the contribution of heterotrophic bacteria to N_2O production in non-aerated conditions could be ignored. ND via AOB in the anammox process was primarily responsible for N_2O generation as previously reported, which NH_4^+ had no impact on N_2O production, yet NH_2OH was the pivotal electron donor involved in the reduction of NO_2^- to N_2 and N_2O via ND in the anammox process (Xiao et al. 2014). N_2H_4 was synthesized from NH_4^+ and NO by hydrazine synthase (HZS) (Kartal et al. 2011) and could convert to NH_2OH

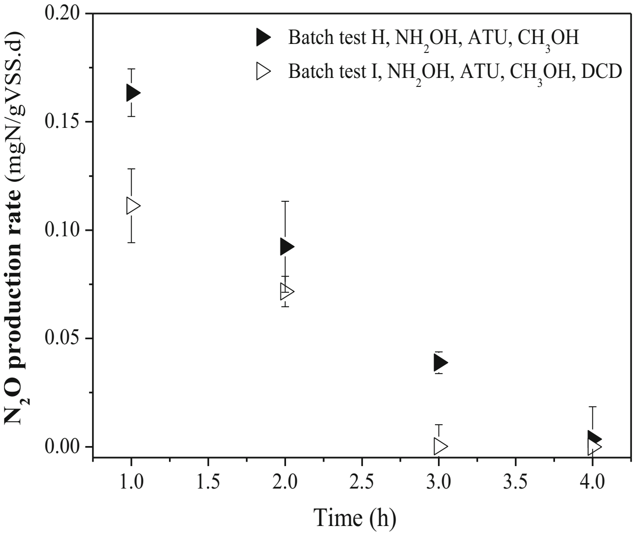

Fig. 4 Variation in the N_2O production rate in the presence of NH_2OH only

by disproportionation during anammox metabolism (van der Star et al. 2008). The added N_2H_4 can be oxidized by AnAOB using hydrazine dehydrogenase (HDH) to release electrons (Yao et al. 2013, 2015, 2016), which are passed to HZS through the electron transport chain to facilitate N_2H_4 synthesis (Ma et al. 2018). In addition, the conversion of N_2H_4 to NH_2OH accelerated N_2O production because NH_2OH enhanced N_2O emission (Ritchie and Nicholas 1972). Furthermore, some other unknown pathways associated with anammox metabolism might be involved in N_2O production in the CANON process (Harris et al. 2015; Ali et al. 2016). The metabolic diversity of AnAOB (Kartal et al. 2011; Oshiki et al. 2016) suggests that different species could have different effects on N_2O production. The community structure of AnAOB in the CANON granular system enhanced by exogenous N_2H_4 has been reported (Xiao et al. 2015) to be approximately 85.75% *Candidatus Scalindua* and 14.25% *Ca. Brocadia anammoxidans*. The effects of N_2H_4 addition on the metabolism of AnAOB of different candidate genera (e.g., *Ca. Scalindua*), especially with regard to putative N_2O production pathway(s), merit further investigation.

Potential biochemical mechanism of N_2O reduction

In the present study, exogenous N_2H_4 was completely degraded during aeration phase I (0–1 h, Fig. 3). NH_4^+ abundance rapidly decreased during this period, whereas the NO_2^- concentration increased in the initial 0.5 h, after which it decreased to a low concentration. This result was in good agreement with batch tests A, B, C, and D, which suggested that some of the added N_2H_4 was utilized by AnAOB to accelerate anammox activity during aeration.

The peak average N_2O production rate was reached during the initial 0.5 h of aeration (Fig. 3). This finding basically coincided with a previous report, in which the peak average N_2O production rate was reached in the initial 40 min (approximately 0.67 h) of the aeration period, and the HO pathway was the primary mechanism of N_2O production (Rathnayake et al. 2013). N_2O production by HO is favored at high NH_4^+ concentrations and NH_4^+ removal rates (Wunderlin et al. 2013a, b). Sudden fluctuations in the DO concentration, NH_4^+ concentration, and pH during reactor start-up generally facilitate HO (Rathnayake et al. 2013). N_2O production by ND has been reported to be favored by a high NO_2^- concentration and a low DO concentration (Wunderlin et al. 2013a; Peng et al. 2015), as well as by elevating the NH_4^+ and DO concentrations in CANON reactors (Harris et al. 2015). Although NO_2^- accumulated in the initial 0.5 h, the concentration of NO_2^- was quite low, and it decreased rapidly in the latter part of aeration phase I. Decreasing the NH_4^+ concentration and maintaining low concentrations of NO_2^- and DO in the initial 1 h of operation inhibited the formation of N_2O by the ND pathway. Hence, the HO pathway, in which N_2O is formed from intermediates (e.g., NOH or $N_2O_2H_2$) (Hollocher et al. 1981) of HAO via NH_2OH oxidation (Poughon et al. 2001), is more likely to dominate N_2O production in the first hour of SBR operation in the presence of exogenous N_2H_4, whereas N_2O produced via ND is relatively unimportant. Batch tests A, B, C, and D suggested that N_2H_4 could inhibit AOB activity to reduce total N_2O production in the CANON process under oxygen-limited conditions. As expected, the N_2O concentration and production rate were quite low (0.8 ppm and 0.15 mgN/L, respectively) even during a single cycle of SBR operation. Consequently, in addition to accelerating anammox activity, exogenous N_2H_4 competed with NH_2OH for HAO in AOB to inhibit the formation of intermediates and facilitate N_2O reduction by the HO pathway within the first hour of SBR operation.

During aeration phase II (1–2.5 h, Fig. 3), without N_2H_4, the NH_4^+ concentration slowed decreased, but NO_2^- accumulated rapidly. Increasing the NO_2^- concentration and decreasing the NH_4^+ concentration with a low DO level in the reactor could facilitate NO_2^- reduction to increase the rate of N_2O production by the ND pathway (Harris et al. 2015; Peng et al. 2015). In addition, N_2O production by HO was gradually inhibited. Enhancement of the ND pathway and weakening of the HO pathway could slightly elevate N_2O production during aeration phase II. Therefore, after N_2H_4 degradation, the contribution of the ND pathway to N_2O production was comparable with that of the HO pathway in the latter aeration phase. This finding is similar to that reported for a PN granular reactor (Rathnayake et al. 2013).

Finally, in anaerobic phase III (2.5–4 h, Fig. 3), declining N_2O production due to low HD activity during the entire anaerobic phase of SBR operation showed that the ND pathway associated with anammox activity did not form N_2O, because exogenous N_2H_4 added during the aeration phase was completely degraded.

Conclusion

Exogenous N_2H_4 minimized N_2O production from the CANON SBR and retaining high nitrogen removal efficiency. N_2O production rate during aeration was mitigated because AOB activity inhibited by N_2H_4, but N_2O production rate under anaerobic conditions was increased by accelerated NH_2OH production due to anammox activity enhanced by N_2H_4. AOB was the main producer of N_2O during the enhanced CANON process. A potential biochemical mechanism of N_2O reduction in the CANON process enhanced by N_2H_4 could be explained as follows: added N_2H_4 completely degraded in the initial aeration period of the SBR to facilitate anammox activity, and meanwhile, compete with NH_2OH for HAO in AOB to inhibit intermediates forming of HO pathway to significantly reduce N_2O production.

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