



High NO and N₂O accumulation during nitrite denitrification in lab-scale sequencing batch reactor: influencing factors and mechanism

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Abstract

Nitrous oxide (N₂O) accumulation in biological nitrogen removal has drawn much attention in recent years; however, nitric oxide (NO) accumulation in denitrification was rarely studied. In this study, NO and N₂O accumulation during nitrite denitrification in a lab-scale sequencing batch reactor (SBR) were investigated. Results showed that low pH (< 7) and high influent loading (> 360:90) (COD:NO₂⁻-N) caused serious NO and N₂O accumulation. The maximal NO accumulation of 4.96 mg L⁻¹ was observed at influent loading of 720:180 and the maximal N₂O accumulation of 46.29 mg L⁻¹ was found at pH of 6. The NO accumulation was far higher than the values reported in previous studies. In addition, the high NO accumulation could completely inhibit the activities of reductases involved in denitrification. High NO and N₂O accumulation were mainly caused by significant free nitrous acid (FNA) and NO inhibition at low pH and high influent loading. There were significant differences on NO and N₂O accumulation at different carbon to nitrogen (COD/N). Low COD/N (≤ 4) could mitigate NO accumulation, but led to high N₂O accumulation. It is speculated that NO accumulation is related to the rapid denitrification with accumulated electron in anaerobic stage at high COD/N. N₂O accumulation is attributed to intense electron competition at low COD/N. High dissolved oxygen (DO) of 4.04 mg L⁻¹ was detected during NO detoxification in this experiment, which is speculated to be partly caused by NO dismutation.

Keywords Nitrite denitrification · Nitric oxide · Nitrous oxide · NO inhibition · NO dismutation

Highlights

- High NO accumulation during nitrite denitrification can inhibit reductases completely
- The effects of COD/N on peaks of NO and N₂O accumulation were different.
- Low pH and high influent loading have serious effect on NO and N₂O reduction.
- High DO was detected during NO detoxification, it is partly caused by NO dismutation.

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Introduction

Biological nitrogen removal (BNR) is an effective and economic technology in wastewater treatment. Heterotrophic denitrification, as one of important process in BNR, has been widely investigated in the past few decades. The complete heterotrophic denitrification is the sequential reduction of nitrate (NO_3^- -N), nitrite (NO_2^- -N), nitric oxide (NO), nitrous oxide (N_2O), and finally to nitrogen gas (N_2) (Brotto et al. 2015). Sequential actions of several enzymes, such as nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos), are involved in this process (Pan et al. 2012). However, recent research showed that NO and N_2O were detected under certain conditions (Lu and Chandran 2010; Zhang et al. 2015). It is well known that NO and N_2O have adverse effects on the environment and living organisms. N_2O , as a potent greenhouse gas, can cause highly undesirable greenhouse effect even at a low emission rate. Meanwhile, N_2O is suggested as the dominant ozone depleting substance in the twenty-first century (Pan et al. 2012). NO is a potent cytotoxin, exerting severe biological impacts, such as being toxic to the metabolism of microorganism, including many groups of prokaryotes and eukaryotes (Wang et al. 2016b). Moreover, literature showed that NO also contributed to destruction of the ozone layer and to precursors of photochemical smog (Lu and Chandran 2010). Therefore, in order to mitigate the NO and N_2O production during heterotrophic denitrification, it is imperative to investigate the mechanism of NO and N_2O accumulation in this process.

Many studies reported the factors affecting N_2O production in denitrification. Pan et al. (2012) investigated the effect of pH on N_2O accumulation during denitrification. Results showed that substantial N_2O accumulation was observed at low pH levels (6.0–6.5). Kim and Bae (2000) noticed that 25–40% of the NO_3^- -N was converted to N_2O at influent concentration of 1500 mg L^{-1} , while no N_2O was accumulated when influent concentration was 750 mg L^{-1} (Kim and Bae 2000). Riya et al. (2015) also found that high nitrogen loading could lead to high N_2O emission. Wu et al. (2009) studied the effects of carbon to nitrogen (COD/N) on N_2O emission, showing that both high and low COD/N could cause high N_2O emission. Alinsafi et al. (2008) found that NO_2^- -N accumulation could inhibit Nos, leading to N_2O production. However, Zhou et al. (2008) believed that free nitrous acid (FNA), rather than NO_2^- -N, might be the true inhibitor on N_2O reduction.

Compared with N_2O , NO has received limited attention in recent researches. Adouani et al. (2015) investigated the impact of the temperature (5–20 °C) on NO emissions during heterotrophic denitrification, showing that NO emission increased when the temperature decreased. Wang et al. (2016a) studied the characterization of NO emission from a full-scale biological aerated filter, suggesting that influent

ammonia loading, NO_2^- -N concentration, and dissolved oxygen (DO) had significant effects on NO emission. Ribera-Guardia and Pijuan (2017) found that NO linearly correlated with the ammonia oxidation rate. In addition, results showed that NO is chemically produced when pH is decreased with HCl (Ribera-Guardia and Pijuan 2017). Schulthess et al. (1995) reported that NO accumulation occurred after a pulse of the nitrite, which caused a slight inhibition on reductases involved in denitrification. Ettwig et al. (2010) found that NO also was produced in nitrite-based anaerobic methane oxidation (n-DAMO), but the NO was decomposed into N_2 and O_2 instead of reducing to N_2O .

According to previous researches, most of the them were focused on NO production during ammonia oxidation. However, the factors and mechanisms responsible for NO accumulation as well as its relationship with N_2O during nitrite denitrification was not characterize well. In addition, high NO accumulation was observed in our pre-experiment, which has a significant on denitrification. Therefore, in order to better understand the process of nitrite denitrification, further research is needed regarding the NO and N_2O accumulation in this process.

In the present study, a serial of tests were performed to investigate the NO and N_2O accumulation during nitrite denitrification. The main objectives were to (1) characterize NO and N_2O accumulation under different conditions; (2) clarify the mechanism of NO and N_2O accumulation; and (3) demonstrate the relationship between high NO accumulation and nitrite denitrification.

Materials and methods

Reactor setup and operation

A glucose utilizing denitrifying culture was enriched in an 8 L sequencing batch reactor (SBR), which was inoculated with activated sludge from the oxidation ditch of a domestic wastewater treatment plants in Xi'an, China. The SBR was operated at 30 ± 1 °C with a cycle time of 8 h, consisting of 5-min feeding, 60-min anaerobic reaction, 390-min anoxic reaction, 20-min setting, and 5-min decanting. The pH of the mixed liquor in reactor was 7.0 ± 0.1 after feeding. Three liters of synthetic wastewater (the composition is shown in the “Synthetic wastewater” section) was pumped into the reactor in each feeding period, resulting in a hydraulic retention time (HRT) of 21 h. Two hundred fifty milliliters of mixed liquor was wasted per day to achieve the solids retention time (SRT) of approximately 30 days. The SBR operation was automatically controlled by a programmable logic controller (PLC). The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were about 5000 and 3500 mg L^{-1} , respectively.

The SBR was operated about 6 months to achieve steady state, with > 99% removal of NO_2^- -N and chemical oxygen demand (COD). After that, the batch tests were performed. Before the tests, the active sludge was washed three times with tap water to remove residual COD, NO_2^- -N, and other substances, and then, sparged with N_2 for 5 min to ensure the anaerobic condition. Each test was performed in triplicates. After each test, the SBR was operated normally at least for 24 h before another test was performed.

Synthetic wastewater

The synthetic wastewater used as influent in SBR consisted of (per liter) 0.37 g $\text{C}_6\text{H}_{12}\text{O}_6$, 0.44 g NaNO_2 , 0.016 g KH_2PO_4 , 0.041 g CaCl_2 , and 1 mL trace element solution. The trace element solution contained (per liter) 0.15 g H_3BO_3 , 0.03 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.18 g KI, 0.12 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.06 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.12 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, and 10 g $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$.

Experimental strategy

Test 1: Effect of COD/N on NO and N_2O accumulation

To investigate the effect of COD/N on NO and N_2O accumulation, the tests were conducted at three different COD/N: 1, 4, and 6. The initial COD concentrations were set to 90, 360, and 540 mg L^{-1} , respectively. The initial pH of all tests was controlled at 6.9 ± 0.1 . After 60 min of anaerobic reaction, the NO_2^- -N of 90 mg L^{-1} was added to reactor. The tests were operated until NaNO_2 was exhausted when COD/N were 4 and 6. As to COD/N of 1, the test lasted for 540 min.

Test 2: Effect of initial pH on NO and N_2O accumulation

Five tests were performed on initial pH of 6, 7, 8, 9, and 10 to investigate the effect of pH on NO and N_2O accumulation. At the beginning of each test, the $\text{C}_6\text{H}_{12}\text{O}_6$ and other nutrient substances were added to the reactor, resulting in the initial COD of 360 mg L^{-1} . After 60 min of anaerobic reaction, the NaNO_2 was added to achieve an initial NO_2^- -N concentration of 90 mg L^{-1} . Each test was operated until NaNO_2 was exhausted.

Test 3: Effect of influent loading on NO and N_2O accumulation

Test 3 were carried out to study the effect of influent loading on NO and N_2O accumulation. The same initial pH for all tests is used in test 2. The initial COD concentrations were set to 90, 180, 360, and 720 mg L^{-1} . The NO_2^- -N concentrations of 22.5, 45, 90, and 180 mg L^{-1} were injected into the reactor after 60 min of anaerobic reaction to achieve same initial COD/N of 4. Each test was operated until NaNO_2 was exhausted.

Analysis and measurements

Ammonium nitrogen (NH_4^+ -N), NO_2^- -N, NO_3^- -N, COD, MLSS, and MLVSS were measured following Standard Method 5220 (APHA 2005). Total nitrogen (TN) was defined as the sum of NH_4^+ -N, NO_2^- -N, and NO_3^- -N concentrations. DO and pH were monitored by DO meter (HACH HQ30d, USA) and pH meter (PHS-3C, China) respectively. Dissolved NO and N_2O in the tests were continuously monitored using calibrated real-time online NO and N_2O microsensors (NO-500, Unisense A/S, Aarhus, Denmark; N_2O -500, Unisense A/S, Aarhus, Denmark) with a response time of 10 s. The absolute abundances of Nir, Nor, Nos, and NO dismutase (Nod) were quantified by flurogenic quantitative polymerase chain reaction. All statistical analyses were performed using SPSS 19.0 and Origin 8.0.

Results

Effect of COD/N on NO and N_2O accumulation

NO and N_2O accumulation under different COD/N are shown in Fig. 1. The peak of NO accumulation increased with the increase of COD/N. However, N_2O accumulation showed an opposite trend with NO. It suggests that the mechanism of NO accumulation under different COD/N may be different with that of N_2O .

As shown in Fig. 1a, NO accumulation increased quickly as soon as NaNO_2 was added in the reactor at COD/N of 1, which decreased gradually after reaching the peak of 0.14 mg L^{-1} . NO accumulation lasted for about 110 min. Interestingly, two different stages were observed in N_2O accumulation. In the first stage, N_2O increased rapidly for about 96 min, and then decreased in a short time. After that, N_2O increased again, but the accumulation rate (0.03 $\text{mg L}^{-1} \text{min}^{-1}$) was lower than the first one (0.041 $\text{mg L}^{-1} \text{min}^{-1}$). N_2O accumulation decreased gradually after the peak was achieved. The decrease rate of N_2O was closely equal to emission rate, which indicated no N_2O reduction occurred during this period.

At COD/N of 4, NO accumulation lasted for about only 2 min, which was much shorter than that COD/N of 1. Two different stages of N_2O accumulation also were observed at COD/N of 4. In the first stage, N_2O accumulation increased quickly at beginning of anoxic stage, and then decreased to a low level in a few minutes. In the second stage, N_2O accumulation showed a similar variation, but the N_2O accumulation rate and reduction rate were lower than that in the first stage. Furthermore, a higher peak of N_2O accumulation was found in the second stage.

When COD/N was 6, the variation of NO accumulation was similar to COD/N of 1, but its duration is relatively short. N_2O accumulation and its duration were much smaller than

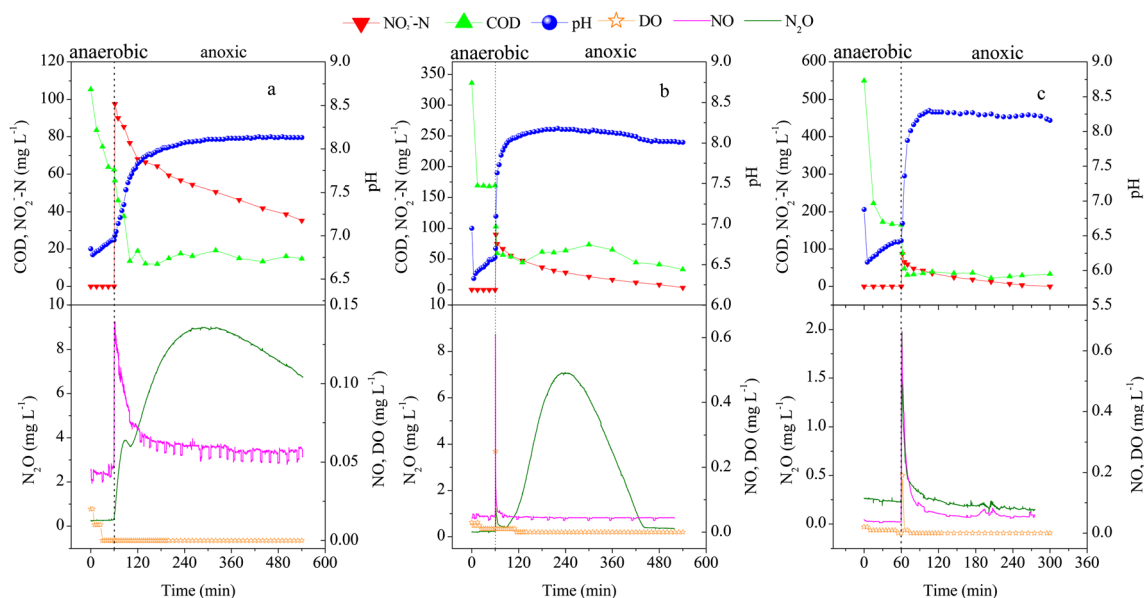


Fig. 1 Dynamic transformations of COD, NO_2^- -N, pH, DO, NO, and N_2O at different COD/N (a) COD/N = 1, (b) COD/N = 4, (c) COD/N = 6

that COD/N of 1 and 4. Zhao et al. (2013) also found that a low COD/N could stimulate more N_2O accumulation, which was similar with present test.

Effect of initial pH on NO and N_2O accumulation

NO and N_2O accumulation at different initial pH are shown in Fig. 2. The peak of NO and N_2O accumulation decreased with the increase of initial pH, and the maximal NO and N_2O accumulation were 3.58 and 46.29 mg L^{-1} , respectively. At initial pH of 6, NO and N_2O concentration increased immediately as soon as NaNO_2 was added in the reactor. However, N_2O did not increase when NO increased to the peak, which maintained at a constant level until NO reduced to a very low level. After that, N_2O increased again, and then reduced to N_2 after the peak was achieved. Unexpectedly, high DO concentration (4.04 mg L^{-1}) was observed during NO accumulation in this test. When the initial pH was higher than 6, NO decreased quickly after an increase. It was different with that initial pH of 6, and the peaks of NO were also significantly lower than pH of 6. As shown in Fig. 2, the peak of N_2O accumulation at different initial pH showed a similar trend with NO; the maximal N_2O accumulation was achieved at initial pH of 6. However, when the initial pH was higher than 7, little N_2O was detected, and the duration of N_2O accumulation also is shorten simultaneously.

Effect of influent loading on NO and N_2O accumulation

As shown in Fig. 3, the maximal NO accumulation of 4.96 mg L^{-1} was observed with the influent loading of 720:180. There was a slight decrease when influent loading decreased from

180:45 to 90:22.5. The duration of NO accumulation increased with the increase of influent loading except for 360:90. Interestingly, Fig. 3b showed that NO increased rapidly as soon as NaNO_2 was added in the reactor and then decreased quickly after reaching the peak. After a few minutes decrease, the NO decrease rate slowed down gradually, which was different with other influent loadings. N_2O accumulation increased with the increase of influent loading, and the maximum of N_2O accumulation was 20.43 mg L^{-1} . The duration of N_2O accumulation increased when influent loading increased from 90:22.5 to 360:90. However, the duration of N_2O accumulation decreased when influent loading was 720:180, and the variation of N_2O accumulation was similar to that at pH of 6 (Fig. 1).

Discussion

Mechanism of NO and N_2O accumulation under different COD/N

Results showed that NO increased rapidly at the beginning of anoxic stage, which may be related to the quantity of electron accumulation during the anaerobic stage. Pan et al. (2013b) found that COD could be converted to reduced mediator (Mred) in the absence of electron acceptors. When electron acceptor (such as NO_2^- -N) was added in the system, Mred would be quickly oxidized to oxidized mediator (Mox) ($\text{NO}_2^- + 1/2\text{Mred} + \text{H}^+ \rightarrow \text{NO} + 1/2\text{Mox} + \text{H}_2\text{O}$). The total concentration of electron carriers (Mtot) was defined as the sum of Mred and Mox. Therefore, it can be speculated that the number of Mred is approximately equal to Mtot during anaerobic stage, namely most of the electron mediators are reduced form. The accumulated Mred made the conversion rate of

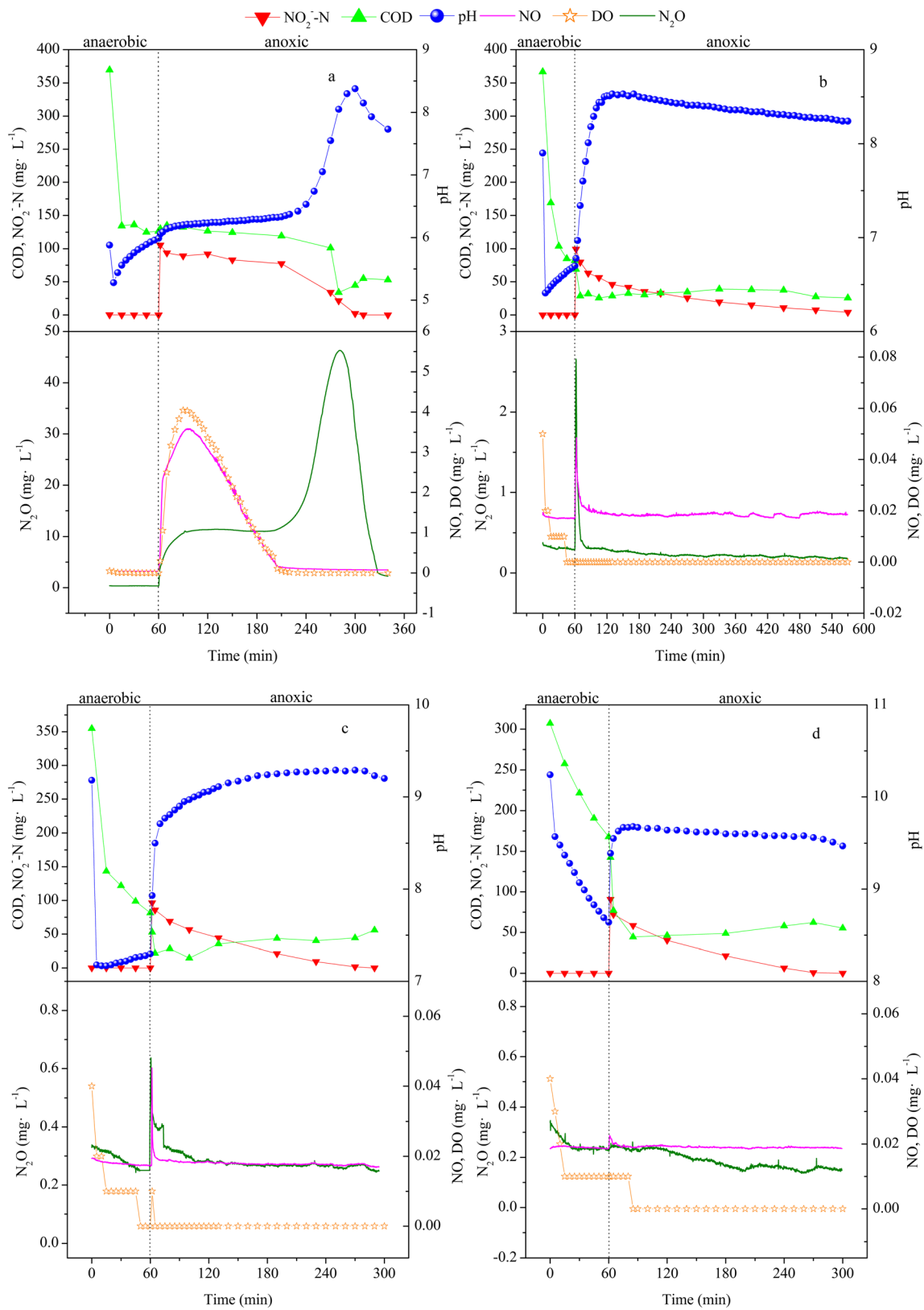


Fig. 2 Dynamic transformations of COD, NO_2^- -N, pH, DO, NO, and N_2O at different pH (a) pH = 6, (b) pH = 8, (c) pH = 9, (d) pH = 10

reductases in optimal state. When the NO_2^- -N was added in the reactor, the accumulated Mred could react with NO_2^- -N

immediately due to the high activity of Nir, which resulted in rapid NO accumulation at beginning of anoxic stage.

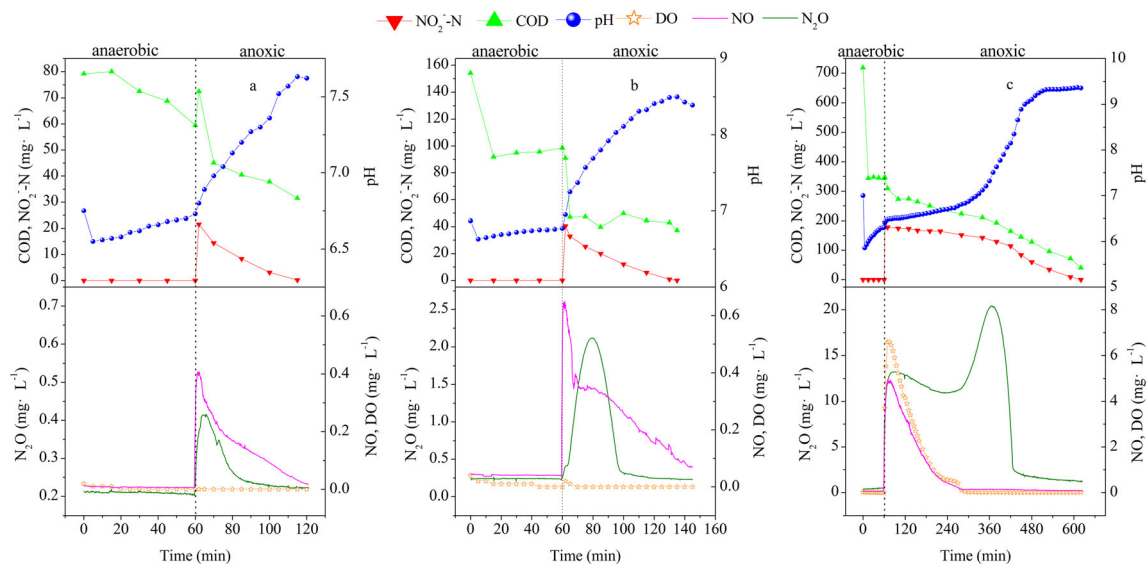


Fig. 3 Dynamic transformations of COD, NO_2^- -N, pH, DO, NO, and N_2O at different influent loadings (a) influent loading = 90:22.5, (b) influent loading = 180:45, (c) influent loading = 720:180

In addition, result showed that high COD/N could cause a serious effect on NO accumulation. It is known that NO_2^- -N is first converted to NO, then to N_2O , and finally to N_2 during nitrite denitrification. Therefore, NO accumulation was determined by nitrite reduction rate. As shown in Table 1, NO reduction rate was slower than the nitrite reduction rate under all COD/N at beginning of anoxic stage, which resulted in NO accumulation. Furthermore, nitrite reduction rate decreased with the decrease of COD/N due to the lack of sufficient electron donor, leading to low NO accumulation occurred at low COD/N. Similar result was found during nitrate to nitrite (Li et al. 2013). However, the duration of NO accumulation showed a different trend. The duration of NO accumulation under different COD/N may be caused by following reasons: when COD/N was 1, the serious shortage of carbon source may result in a slower NO reduction rate during the stage of NO decrease. Subsequently, a long duration of NO accumulation was observed at COD/N of 1. In addition, previous report showed that high NO accumulation was harmful to microbial activities (Schulthess et al. 1995). When NO accumulation reached to a relatively high level (but the microbial activities was not inhibited completely), microorganisms would reduce NO to N_2O immediately to mitigate the NO toxicity. Therefore, fast NO reduction was found at high NO accumulation. However, in present experiment, the shortest duration of NO accumulation occurred when the COD/N was 4. The possible reason is that the parent reactor was operated at COD/N of 4 during normal operation; microorganisms had adapted this condition for a long time, which may lead to a faster NO reduction rate than other COD/N.

As shown in Fig. 1, the peak of N_2O accumulation decreased with the increase of COD/N, which was similar with the result reported by Yan et al. (2017). Pan et al. (2013a) reported that

electron competition among four reductases would occur during denitrification, and low COD/N could potentially intensify the electron competition through their possible effect of slowing down the electron supply rate. It is known that Nos has lower affinity for electron than other reductases, resulting in more serious N_2O accumulation at low COD/N. Thus, it indicates that the different N_2O accumulations may be caused by different electron competition among denitrification reductases.

Table 1 The peaks of NO/ N_2O and the reduction rates of NO_2^- -N, NO and N_2O at different COD/N

	C/N			
	1	4	6	
NO_2^- - $\text{N}_{\text{red}}(\text{inc})$ ($\text{mg h}^{-1} \text{vss}^{-1}$)	0.016	0.087	0.121	
$\text{NO}_{\text{red}}(\text{inc})$ ($\text{mg h}^{-1} \text{vss}^{-1}$)	0.013	0.082	0.116	
NO_2^- - $\text{N}_{\text{red}}(\text{dec})$ ($\text{mg h}^{-1} \text{vss}^{-1}$)	0.007	0.087	0.021	
$\text{NO}_{\text{red}}(\text{dec})$ ($\text{mg h}^{-1} \text{vss}^{-1}$)	0.008	0.088	0.022	
NO_{max} (mg L^{-1})	0.140	0.61	0.665	
$\text{N}_2\text{O}_{\text{red}}$ ($\text{mg h}^{-1} \text{vss}^{-1}$)	$\text{N}_2\text{O}_{\text{red}}(\text{dec1})$	0.0099	0.0497	0.0214
	$\text{N}_2\text{O}_{\text{red}}(\text{dec2})$	0.00099	0.0017	
$\text{N}_2\text{O}_{\text{max}}$ (mg L^{-1})	9.028	7.098	1.976	
FNA (mg L^{-1})	0.00084	0.00040	0.00619	

NO_2^- - $\text{N}_{\text{red}}(\text{inc})$, NO_2^- -N reduction rate until the peak of NO reached; $\text{NO}_{\text{red}}(\text{inc})$, NO reduction rate until the NO peak reached; NO_2^- - $\text{N}_{\text{red}}(\text{dec})$, NO_2^- -N reduction rate when NO decreased from peak to stable level; $\text{NO}_{\text{red}}(\text{dec})$, NO reduction rate when NO decreased from peak to stable level; NO_{max} , the peak of NO accumulation; $\text{N}_2\text{O}_{\text{red}}$, N_2O reduction rate when N_2O decreased from peak to stable level; $\text{N}_2\text{O}_{\text{red}}(\text{dec1})$, N_2O reduction rate in exogenous denitrification when N_2O decreased from peak to stable level; $\text{N}_2\text{O}_{\text{red}}(\text{dec2})$, N_2O reduction rate in endogenous denitrification when N_2O decreased from peak to stable level; $\text{N}_2\text{O}_{\text{max}}$, the peak of N_2O accumulation

Results showed that there were two different N_2O accumulation stages at COD/N of 1 and 4. Figure 1 a and b showed that COD was not stored completely during anaerobic stage. Thus, two different reduction stages were observed in this test: denitrification in the presence of external carbon source (exogenous denitrification) and denitrification without an external carbon source (endogenous denitrification). As shown in Fig. 1 a and b, COD concentration did not decrease after the first N_2O accumulation was reduced completely. It indicates that the external carbon source had been consumed exhaustively during this stage. Meanwhile, the pH increased rapidly until N_2O accumulation increased again. It is widely known that exogenous denitrification rate is faster than endogenous denitrification. Therefore, a faster pH variation was observed in exogenous denitrification. According to the variation of COD and pH, it can be inferred that the two different N_2O accumulation stages at COD/N of 1 and 4 may be mainly attributed to the two stages of denitrification. In addition, literature showed that more N_2O accumulation occurred with intracellular polymer as electron donor compared with external carbon source. Therefore, a higher N_2O accumulation was observed in the second stage (Wu et al. 2014).

Mechanism of NO and N_2O accumulation under different pH

Ribera-Guardia and Pijuan (2017) suggested that there was a close correlation between NO accumulation and pH. However, they found that NO was produced by chemodenitrification, but not by biodenitrification. In their experiment, when HCl was added in the reactor, NO increased rapidly to a peak and then decreased sharply. Thus, they believed that it could be caused by the deprotonation of HNO_2 ($2HNO_2 \leftrightarrow NO + NO_2 + H_2O$). Compared with previous study, there were some different results in the present tests. As shown in Fig. 2, high NO accumulation was achieved at initial pH of 6, which lasted for about 200 min. It was different with the result that reported by Ribera-Guardia and Pijuan (2017). Therefore, it indicates that NO accumulation may be produced biologically in the present test. In addition, literature showed that nitrite could inhibit the Nor activity, leading to NO accumulation (Schulthess et al. 1995). In test 1, the same initial NO_2^- -N concentration was supplied, but the different NO accumulations were achieved. It indicates that NO accumulation is not caused by NO_2^- -N concentration in the present test. However, Fig. S1 showed that there was a good correlation between NO and FNA at different initial pH; the peak of NO accumulation increased with the increase of FNA. Therefore, it can be speculated that NO accumulation is mainly caused by high FNA. In addition, literature showed that pH could affect the conversion rates of enzymes involved in NO production (Nir) and reduction (Nor), leading to NO accumulation (Blum et al. 2018).

Figure 2 a showed that high NO accumulation caused a significant inhibition on reductases during denitrification. At the beginning of the anoxic stage, Nor and Nos were inhibited due to the low pH and high FNA, resulting in rapid increase of NO and N_2O . When NO concentration reached to the maximum of 3.57 mg L^{-1} , there were no obvious change in NO_2^- -N and N_2O until NO reduced to a low level. It is possible that Nir and Nor were inhibited completely by NO. The decrease of NO during this period may be attributed to emission and detoxification by another way. It is known that dissolved NO in the liquid phase could emit to atmosphere through air stripping and diffusion. Therefore, when NO was accumulated in the reactor, a part of NO would emit to atmosphere, resulting in the decrease of NO. However, result showed that the NO decline rate was higher than that of emission rate, indicating that the decline of NO was not only contributed to emission but also related to biodegradation. Ettwig et al. (2010) found that NO could be decomposed into N_2 and O_2 by Nod during n-DAMO ($2NO = N_2 + O_2$), which may result in the decrease of NO. Results showed that DO was detected during NO accumulation, and the more NO accumulated, the higher the DO detected. Therefore, it is possible that NO dismutation occurs in this experiment. However, literature showed that NO could cause a positive inference with some models of optical DO probes (Klaus et al. 2017), including the HACH DO used in this experiment. Therefore, the interference caused by NO may be an important reason for the increase of DO. In order to determine if DO was produced during NO reduction, an abiotic test was performed. In this test, NO was bubbled into the reactor with no other substances were added; DO and NO were recorded simultaneously until no DO was detected. The correlation equation between NO and DO was obtained by regression analysis. Then, the real DO could be calculated by the correlation equation. As shown in Fig. S2a, the detected DO was higher than interfering DO, which indicates that DO was produced in this test. Similar result was observed at influent of 720:180 (Fig. S2b). In addition, in order to further determine the possibility of NO dismutation, the absolute abundances of functional genes in this system were identified (Table 2). Results showed that high Nod abundance of $(5.94 \pm 0.74) \times 10^5 \text{ copies} \cdot \text{g}^{-1}$ was detected, which was higher than that of Nor. This confirmed that NO dismutation could occur in this experiment. According to the results and literatures, it can be inferred that NO dismutation may be another important way to cause the decrease of NO in the present study. When NO decreased to the low level, the inhibitions of Nir and Nor were mitigated gradually, but Nos was still inhibited by FNA. Therefore, the end product was mainly N_2O rather than N_2 . After a period of operation, Nos activity was recovered gradually with the decrease of FNA; the N_2O was transformed into N_2 , and it was stabilized at low level during the rest period of the cycle.

Results showed that pH had a significant effect on N_2O accumulation. There are three possible reasons for this phenomenon. Firstly, pH may have direct effect on denitrifiers

Table 2 Abundances of nitrogen functional genes in SBR

Functional gene	Abundance (copies·g ⁻¹)
Nir	$(3.36 \pm 0.14) \times 10^7$
Nor	$(1.32 \pm 0.12) \times 10^4$
Nos	$(8.36 \pm 0.56) \times 10^6$
Nod	$(5.94 \pm 0.74) \times 10^5$

and activities of four reductases involved in denitrification. It has been previously suggested that a slower turnover of Nos at low pH as compared with Nar, Nir, and Nor, resulting in N₂O as the end product of denitrification (Bergaust et al. 2010). Liu et al. (2014) also reported that the Nos activity declined dramatically when pH was lower than 6.1, which was similar to the present test. In addition, as shown in Table 3, N₂O reduction rate increased with the increase of initial pH. Therefore, it can be speculated that the high N₂O accumulation is attributed to low Nos activity and turnover at low pH. Secondly, denitrification involves carbon oxidation to supply electrons and nitrogen oxides reduction to consume electrons. When electron production rate cannot meet the electron consumption, electron competition among different reductases would occur. Literature showed that low pH had adverse impact on carbon oxidation rate, which would intensify electron competition (Pan et al. 2012). Therefore, it could be inferred that high N₂O accumulation at low pH was possibly related to the reduced carbon oxidation rate. Thirdly, it has been proven that FNA could bind to the active sites of copper-contained enzymes, resulting in N₂O accumulation, and the FNA inhibition concentration was 0.0007–0.001 mg L⁻¹ (Zhou et al. 2008). However, in this experiment, the minimal FNA inhibition concentrations at pH of 6 and 7 (Table 3) were lower than the inhibition threshold reported in previous study. It may be caused by the different culture systems and microbial structures. According to present study and previous reports, it can be concluded that FNA played an important role in N₂O accumulation during denitrification.

Table 3 The peaks of NO/N₂O and the reduction rates of NO₂⁻-N, NO, and N₂O at different pH

	pH			
	6	7	8	9
NO ₂ ⁻ -N _{red (inc)} (mg h ⁻¹ vss ⁻¹)	0.009	0.087	0.041	0.061
NO _{red (inc)} (mg h ⁻¹ vss ⁻¹)	0.005	0.085	0.040	0.059
NO ₂ ⁻ -N _{red (dec)} (mg h ⁻¹ vss ⁻¹)	0.0017	0.087	0.0188	0.0601
NO _{red (dec)} (mg h ⁻¹ vss ⁻¹)	0.0018	0.088	0.01911	0.0618
NO _{max} (mg L ⁻¹)	3.586	0.61	0.0485	0.045
N ₂ O _{red} (mg h ⁻¹ vss ⁻¹)				
	N ₂ O _{red (dec1)}	0.0188	0.0497	0.045
	N ₂ O _{red (dec2)}		0.0017	0.062
N ₂ O _{max} (mg L ⁻¹)	46.291	7.098	2.657	0.7339
FNA (mg L ⁻¹)	0.00041	0.00040	0.0080	0.00245

Mechanism of NO and N₂O accumulation under different influent loadings

Based on Michaelis-Menten kinetics, a higher substrate concentration could result in a faster reaction rate. Subsequently, the faster reaction rate might lead to more NO accumulation when NO₂⁻-N was used as electron acceptor. In addition, Castro-Barros et al. (2016) reported that nitrite could improve the expression of Nir, which stimulated the NO production. Therefore, the more NO was accumulated with the increase of influent loading. As shown in Table 4, nitrite reduction rate increased with the increase of influent loading at the beginning of anoxic stage except for at 720:180. The peak of NO accumulation followed with the trend of nitrite reduction rate. However, when influent loading was 720:180, the nitrite reduction rate decreased remarkably, but NO accumulation with influent loading of 720:180 was much higher than other influent loadings. As discussed in the “Mechanism of NO and N₂O accumulation under different pH” section, high FNA was harmful to the activities of reductases involved in denitrification, resulting in an inhibition on denitrification. Compared with Nir, Nor was easier to be inhibited when suffering high FNA concentration. In this experiment, FNA concentration increased with the increase of nitrite in the influent. The NO reduction would be inhibited significantly when FNA concentration was 0.12 mg L⁻¹, but nitrite reduction was not inhibited obviously. Therefore, it can be inferred that the NO accumulation with influent loading of 720:180 may be mainly attributed to FNA inhibition, when NO concentration reached to a high level, which would cause a more serious inhibition on Nor, resulting in a higher NO accumulation. In order to mitigate NO toxicity, microorganisms would convert NO to other substances. The mechanism of NO detoxification at influent loading of 720:180 was similar with that of at pH of 6 (Mechanism of NO and N₂O accumulation under different pH).

Figure 3 b showed that two different NO decrease rates were observed. The possible reasons are as follows. The activities of Nir, Nor, and Nos were inhibited mostly when

NO reaches to the peak. In order to mitigate the NO inhibition, most of the NO might be converted to other substances in a short time through another pathway, namely NO dismutation (as discussed in “Mechanism of NO and N₂O accumulation under different pH” section). NO decreased rapidly during this stage. The inhibition on denitrification was relieved, and the activities of Nir and Nor recovered gradually when NO decreased to a low level. After that, NO₂⁻-N and NO were reduced by normal denitrification, which led to NO decrease rate slowed down.

Results showed that high influent loading was more likely to lead to N₂O accumulation, which was similar with Kim and Bae (Kim and Bae 2000). They found that 25–40% of the nitrate in the influent was converted to N₂O at a nitrate concentration of 1500 mg L⁻¹. However, no N₂O was produced when the nitrate concentration decreased to 750 mg L⁻¹. In this experiment, two main reasons were responsible for the different N₂O accumulations. FNA concentration increased with the increase of nitrite in the influent, resulting in higher N₂O accumulation at high influent loading. In addition, NO inhibition might be another possible reason for N₂O accumulation. It is known that NO is a toxic substance, which can inhibit the reductases in denitrification. Ni and Yuan found that NO inhibit constants for the reductions of nitrite, NO, and N₂O were 0.5, 0.3, 0.075, respectively (Ni and Yuan 2013). It suggests that Nos is most susceptible to NO inhibition, which may be inhibited by a relative low NO concentration compared with other reductases. Therefore, it can be speculated that the higher the NO concentration, the stronger the Nos inhibited, and the more N₂O accumulation was observed in this test (Table 4).

Implications to the operation of nitrite denitrification process

Wastewater treatment plants are always subjected to large fluctuations in water quality, which would result in NO and N₂O accumulation. It is known that NO and N₂O have adverse

effect on environment, human, and microorganism. Therefore, in order to reduce NO and N₂O production during nitrite denitrification, it is significant to investigate the mechanism of NO and N₂O production under different conditions.

According to the results of this study, there were mainly three aspects that should be cautious to avoid high NO and N₂O production in nitrite denitrification. Firstly, results suggest that low COD/N could cause high N₂O accumulation, but it is conducive to mitigate NO accumulation. Thus, an appropriate COD/N should be controlled to reduce NO and N₂O accumulation simultaneously. Based on the above results, the COD/N should be controlled in 4–6. Secondly, results showed that NO and N₂O accumulation was much high at low pH conditions (Fig. 2). Literature showed that strong alkalizer carbon source, such as CH₃COONa, could potentially reduce the risk of NO and N₂O accumulation due to the high increase of pH in denitrification (Li et al. 2014). In addition, the alkaline matter (e.g., calcium hydroxide) can be added for pH adjustment. Thirdly, the high influent loading could cause a significant NO and N₂O accumulation (Fig. 3). Therefore, it is important to reduce influent loading as much as possible in the practical operation. The addition of regulating pool would be an optimal choice.

Conclusion

The NO and N₂O accumulation during nitrite denitrification were investigated in this study. The main conclusions are as follows:

- (1) High NO accumulation of 4.96 mg L⁻¹ was detected in this experiment, which was far higher than the values reported in previous studies. The nitrite denitrification could be inhibited completely by the high NO accumulation.
- (2) NO accumulation increased with the increase of COD/N due to the rapid denitrification with accumulated electron

Table 4 The peaks of NO/N₂O and the reduction rates of NO₂⁻-N, NO, and N₂O at different influent loadings

	Influent loading			
	90:22.5	180:45	360:90	720:180
NO ₂ ⁻ -N _{red (inc)} (mg h ⁻¹ vss ⁻¹)	0.016	0.043	0.087	0.0107
NO _{red (inc)} (mg h ⁻¹ vss ⁻¹)	0.014	0.041	0.085	0.005
NO ₂ ⁻ -N _{red (dec)} (mg h ⁻¹ vss ⁻¹)	0.005	0.007	0.087	0.0019
NO _{red (dec)} (mg h ⁻¹ vss ⁻¹)	0.004	0.008	0.088	0.0023
NO _{max} (mg L ⁻¹)	0.408	0.65	0.61	4.955
N ₂ O _{red} (mg h ⁻¹ vss ⁻¹)				
	N ₂ O _{red (dec1)}	0.0112	0.0102	0.0497
	N ₂ O _{red (dec2)}			0.0017
N ₂ O _{max} (mg L ⁻¹)	0.416	2.117	7.098	20.43
FNA (mg L ⁻¹)	0.0075	0.0011	0.00040	0.0064

in anaerobic stage at high COD/N. However, N₂O accumulation showed an opposite trend with NO, which is related to the intense electron competition at low COD/N.

- (3) Low pH and high influent loading could result in serious NO and N₂O accumulation. The maximal NO and N₂O accumulation of 3.58 and 46.29 mg L⁻¹ were observed at pH of 6. When influent loading was 720:180, the maximums of NO and N₂O accumulation were 4.96 and 20.43 mg L⁻¹, respectively. High NO and N₂O accumulation are mainly attribute to the significant FNA and NO inhibition at low pH and high influent loading.
- (4) High DO of 4.04 mg L⁻¹ was detected during NO accumulation, which is presumed to be partly caused by NO dismutation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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