Effect of influent C/N ratio on N₂O emissions from anaerobic/anoxic/oxic biological nitrogen removal processes

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Abstract The problem of producing strong greenhouse gas of nitrous oxide (N₂O) from biological nitrogen removal (BNR) process in wastewater treatment plants (WWTP) has elicited great concern from various sectors. In this study, three laboratory-scale wastewater treatment systems, with influent C/N ratios of 3.4, 5.4, and 7.5, were set up to study the effect of influent C/N ratio on N₂O generation in anaerobic/anoxic/oxic (A²O) process. Results showed, with the increased influent C/N ratio, N₂O generation from both nitrification and denitrification process was decreased, and the N₂O-N conversion ratio of the process was obviously reduced from 2.23 to 0.05%. Nitrification rate in oxic section was reduced, while denitrification rate in anaerobic and anoxic section was elevated and the removal efficiency of COD, NH₄⁺-N, TN, and TP was enhanced in different extent. As the C/N ratio increased from 3.4 to 7.5, activities of three key denitrifying enzymes of nitrate reductase, nitrite reductase, and nitrous oxide reductase were increased. Moreover, microorganism analysis indicated that the relative abundance of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria

(NOB) were positively correlated with N₂O generation, which was reduced from (8.42 ± 3.65) to (3.61 ± 1.66)% and (10.38 ± 4.12) to (4.67 ± 1.62)%, respectively. *NosZ* gene copy numbers of the A²O system were increased from (1.19 ± 0.49) × 10⁷ to (2.84 ± 0.54) × 10⁸ copies/g MLSS with the influent C/N ratio elevated from 3.4 to 7.5. Hence, appropriate influent C/N condition of A²O process could optimize the microbial community structure that simultaneously improve treatment efficiency and decrease the N₂O generation.

Keywords Biological nitrogen removal · Greenhouse gas · Nitrous oxide · C/N ratio · Anaerobic/anoxic/oxic process · *NosZ* gene

Introduction

The strong greenhouse gas nitrous oxide (N₂O) is an important cause of the global greenhouse effect and ozone depletion in the stratosphere (Rodriguez-Caballero et al. 2015). The 100-year global warming potential of N₂O is approximately 300 times stronger than that of carbon dioxide (CO₂), and its emission amount continuously increases at a rate of 0.25% per year (Kishida et al. 2004; Marques et al. 2016). Biological nitrogen removal (BNR) processes in wastewater treatment plants (WWTPs) are considered a significant artificial N₂O emission source, as they emit 3.2% of the total anthropogenic N₂O emissions globally (Kampschreur et al. 2009; Wang et al. 2016). As the phenomenon of eutrophication occurring in rivers and lakes becomes increasingly serious, the effluent quality standards (especially for nitrogen levels) for discharge wastewater become more stringent (Zhang et al. 2016a), which leads to extensive application of BNR techniques and increasing N₂O generation from WWTPs. Therefore,

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studying the mechanism of N_2O production and designing an effective mitigation approach to employ during BNR processes is desperately needed.

Generally, BNR involves nitrification and denitrification processes, both of which can lead to N_2O generation. The autotrophic organisms, ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), predominantly perform the nitrification process, as AOB convert ammonia ($\text{NH}_4^+\text{-N}$) to nitrite ($\text{NO}_2^-\text{-N}$) and NOB convert $\text{NO}_2^-\text{-N}$ to nitrate ($\text{NO}_3^-\text{-N}$) (Lu et al. 2014). $\text{NH}_4^+\text{-N}/\text{NO}_2^-\text{-N}$ and CO_2 are the energy and carbon sources, respectively (Kampschreur et al. 2009). During this process, N_2O is recognized generally as a by-product and two pathways for its generation have been found (Wei et al. 2017). One pathway is hydroxylamine oxidation. During this pathway, intermediates of biological hydroxylamine oxidation are the source for N_2O generation, which is possibly participant to the obvious disbalances in nitrifier metabolic activity, or to the chemical decomposition of hydroxylamine and miscellaneous reactions of the intermediates constituted in $\text{NH}_4^+\text{-N}$ oxidation. The other one is nitrifier denitrification, in which N_2O is produced primarily from the reduction of $\text{NO}_2^-\text{-N}$ by AOB, especially under the environmental conditions of low dissolved oxygen (DO) or high $\text{NO}_2^-\text{-N}$ concentrations (Lv et al. 2016; Kool et al. 2010).

In the denitrification process, N_2O is recognized as an obligatory intermediate. Incomplete denitrification can result in N_2O generation, which can be caused by insufficient carbon source amount or improper DO conditions (Wunderlin et al. 2012; Ribera-Guardia et al. 2016). This process is performed by a physiologically diverse group of bacteria, which convert $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ to gaseous products of nitric oxide (NO), N_2O , and dinitrogen (N_2) in a stepwise manner under anoxic or anaerobic conditions (Li et al. 2017). In the following order, four different reductases, nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos), catalyze the consecutive denitrification reactions (Zhang et al. 2016b). Among them, Nos, which is encoded by the *nosZ* gene and exists in the bacterial periplasm, is closely related to N_2O generation. Generally, the *nosZ* gene is used to investigate the DNA of particular denitrification microorganisms and measure the capacity of microbes that convert N_2O to N_2 (Zhu and Chen 2011).

In recent years, many studies have been undertaken to identify the emissions values and generation mechanism of N_2O in BNR processes at both the lab- and full-scales. A huge variation in N_2O emissions data was found, with the conversion rate of $\text{N}_2\text{O}\text{-N}$ widely ranging from 0 to 95% of the influent nitrogen load (Wang et al. 2016; Kampschreur et al. 2009). Several factors benefiting N_2O emissions from BNR were found, such as low influent C/N ratio (Hu et al. 2013), low pH (Hynes and Knowles 1984), short sludge retention time (SRT) (Hanaki et al. 1992), low DO concentration (Kampschreur et al. 2008), presence of toxic compounds

(Mannina et al. 2016), and carbon source shock (Wang et al. 2011b). Particularly, influent C/N ratio is considered one of the most significant parameters because it can markedly affect the N_2O emissions from both the nitrification and denitrification processes. He et al. (2016) found N_2O emission at the C/N ratio of 2 was much stronger than that at the ratios of 5 and 8 during nitrification process in a biological aerated filter reactor. In a SBR reactor, N_2O emission in the condition of BOD_5/TN of 2.6 was about 270 times greater than that in BOD_5/TN of 4.5 (Kishida et al. 2004). During the denitrification process, the N_2O conversion rate was 0.005% for optimal C/N ratio, when the carbon source became insufficient the conversion rate increased to 0.5% (Chung and Chung 2000).

Among multiple types of BNR processes, the anaerobic/anoxic/oxic (A^2O) process is one of the most widely used (accounting for 24% of all BNR processes in WWTPs in China (Xie and Wang 2011)). Some studies have investigated N_2O emissions from the full-scale WWTPs with an A^2O process and identified the emission characteristics (Yan et al. 2014; Wang et al. 2011a). Ren et al. (2015) studied the direct emissions of N_2O from an A^2O system under different influent C/N ratios, but the N_2O generation pathway and function microorganism for this process was not illuminated (Ren et al. 2015). In fact, microbes are the functional core of wastewater treatment systems and major producers of N_2O emissions. To date, few studies have focused on the effect of influent C/N ratio on N_2O generation and the functional bacterial content of AOB and denitrifiers for this widely implemented treatment process (Ren et al. 2015). Thorough study about the influence mechanism of influent C/N ratio on N_2O generation for A^2O process was greatly needed.

The purpose of this study was to reveal the effect of influent C/N ratio on N_2O production and the related functional bacteria in the A^2O treatment process. Three parallel A^2O processes were set up in a laboratory with influent C/N ratios of 3.4, 5.4, and 7.5. Under the different influent C/N ratios, the characteristics of N_2O emissions and generation, nitrification and denitrification rates, and activities of key denitrifying enzymes (Nar, Nir, and Nos) during the A^2O processes were investigated. Furthermore, relative abundances of AOB and NOB in the oxic section were analyzed by fluorescence in situ hybridization (FISH), and gene copy numbers of *nosZ* in the anoxic section were analyzed using real-time quantitative PCR (qPCR).

Materials and methods

Experimental setup and operation

Three lab-scale A^2O treatment systems were parallelly kept with the influent C/N ratios of 3.4, 5.4, and 7.5. A schematic diagram and operating parameters of the process are shown in

Fig. 1 Schematic diagram of the experimental equipment and process

Fig. 1 and Table 1, respectively. Except for influent C/N ratio, the three parallel treatment processes were under identical operation conditions (Table 1). The hydraulic retention time (HRT) for the anaerobic tank, anoxic tank, oxalic tank, and secondary sedimentation tank was 1.4, 2.8, 8.5, and 1.4 h, respectively. The seeding sludge for the treatment processes was from oxalic section of the Luotuowan WWTP (Xinxiang, China).

To prevent the sedimentation of sludge in anaerobic and anoxic tanks, magnetic stirrers (85–2, Sile Shanghai) were fixed and kept with the rotational speed of 500 rpm. DO concentration in the oxalic section was kept at approximately 2.0 mg/L, by adjusting the aeration rate. A mechanical stirrer was fixed at the bottom of the secondary sedimentation tanks and operated at a very low rotating speed of 30 rpm to prevent the concentrated activated sludge from forming massive flocs, which is favorable for sludge recycling. After 2 months of sludge acclimation, the treatment processes stabilized, indicated by the stable COD, $\text{NH}_4^+\text{-N}$, TN, and TP concentrations observed in the effluents. Then, gas and water samples were taken simultaneously every 3 days for 1 month. The gas samples were used to determine the N_2O concentration; the details of which are described in “Analysis procedures” section. Liquid samples were used to determine water quality parameters.

Table 1 Operation parameters of parallel A²O treatment processes

Parameters	Value
Total effective volume (L)	50
Total inflow (L/day)	85
Water temperature (°C)	25 ± 1
SRT (day)	15
Mixed liquor suspended solid (MLSS) (mg/L)	3000 ± 200
Internal recycle ratio (%)	200
Sludge recycle ratio (%)	100

Influent preparation

The influent wastewater for the A²O processes was obtained from the rotational flow grit chamber of the Luotuowan WWTP. As seen in Fig. 2, the effluent quality of the rotational flow grit chamber exhibited approximately the same diurnal trend. According to the effluent quality data shown in Fig. 2, the wastewater was taken at a set time, so the C/N ratio of the wastewater met the demand of this study. Occasionally, the C/N ratio of the obtained wastewater was far from the demanded ratio, so carbon or nitrogen sources or running water was increased to adjust the water quality. $\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$ and NH_4Cl were used as carbon and nitrogen source, respectively.

Sampling and analytical methods

Gas sampling of nonaerated surfaces

For the nonaerated surfaces of anaerobic tank, anoxic tank, and secondary sedimentation tank, the sampling method was according to our previous publication (Yan et al. 2016). A closed gas-tight chamber was installed on the top of the tanks,

Fig. 2 Diurnal variation in effluent quality from the rotational flow grit chamber based on the data from October to December 2015

and the air tightness was checked during each sampling event. Four gas samples were taken at 3 min intervals for 9 min. Each gas sample was collected into a 50-ml sampling bag (Delin, China) through polytetrafluoroethylene tubes using a polypropylene syringe. The gas flux of the nonaerated surface was calculated by the equation:

$$F = (V/A)\rho(\Delta c/\Delta t) \quad (1)$$

where F (mg/m² min) is the gas flux, V (m³) is the volume of the upper-water space, A (m²) is the liquid surface area, ρ (mg/m³) is the concentration of N₂O, and Δc (v/v)/ Δt (min) is the change in N₂O concentration per minute during sampling time. The N₂O density (ρ) was calculated using the equation:

$$\rho = 10^{-6}MP/RT \quad (2)$$

where M is the molecular weight of N₂O (44.02 g/mol), P is the atmospheric pressure (1 atm), R is the gas constant (0.082 L atm/K mol), and T is the temperature (K).

Gas sampling of aerated surfaces

The gas sampling method used in the oxic section was according to our recent publications (Yan et al. 2016). During the sampling period, a closed gas-tight chamber was installed on the top of the tank. The air tightness was tested during each sampling event. The effluent air was obtained through polytetrafluoroethylene tubes using a polypropylene syringe and then kept in 50-ml sampling bags. The gas flux of the aerated surface was calculated using:

$$F = \rho cQ_a/A \quad (3)$$

where c (v/v) is the N₂O concentration, Q_a (m³/min) is the aeration intensity of air pump, and A (m²) is the liquid surface of the oxic tank.

Dissolved gas sampling

The detection method used for dissolved N₂O in wastewater was according to Kimochi et al. (1998).

Analysis procedures

Gas N₂O concentrations were analyzed using a gas chromatograph (HP-Chemistation 5890, Agilent Co. Ltd., USA), equipped with an electron capture detector (GC-ECD). The oxidation-reduction potential (ORP) of the water was measured by a WTW3110 ORP (WTW, Germany) meter. The water quality parameters of COD, NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, TN, and TP were measured using standard methods (CEPB 2004). COD was detected by closed reflux and colorimetric method.

Nesslerization, colorimetric method, ultraviolet spectrophotometric screening method, and persulfate method were used in the analyses of NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, and TN, respectively. Vanadomolybdophosphoric acid colorimetric method was adopted in TP measurement. The DO were measured by WTW-Multi 340i DO (WTW, Germany) meters. The activity detection methods of Nar, Nir, and Nor were according to our previous publication (Yan et al. 2016). The preparation of cell extracts was according to Kristjansson and Hollocher (1980).

Denitrification bacteria content was measured via qPCR based on the nosZ gene, which was performed according to our recent publication (Yan et al. 2016). The primers used in the measurement were nosZ-F 5-AGAACGACCAGCTGATCGACA-3 and nosZ-R 5-TCCATGGTGACGCCGTGGTTG-3 (Chon et al. 2011). The distribution of functional bacteria in the oxic section under different influent C/N ratios was monitored by the fluorescence in situ hybridization (FISH) technique with 16S rRNA-targeted oligonucleotide probes. The activated sludge flocs from the oxic section after 2 months of operation under each influent C/N ratio were fixed with 4% paraformaldehyde at 4 °C for about 12 h for the FISH experiments. These fixed flocs were washed three times with phosphate-buffered saline (pH 7.2) and then were spotted on gelatin-coated glass slides, air-dried, and consecutively dehydrated in 50, 80, and 100% ethanol. A hybridization solution containing 5 ng/μl of probe was applied to the slide. Table 2 lists the oligonucleotide probes used for FISH in this study. In situ hybridization was performed at 46 °C for 3 h. The excess probes were washed off by heating at 48 °C for 15 min with washing buffer containing Tris-HCl (20 mM, pH 7.2), NaCl (70 mM), EDTA (5 mM), and SDS (0.01%). The NaCl concentration was fixed by experiments. The images of the FISH were captured by confocal laser scanning microscopy (CLSM) and analyzed with LAS AF Lite software (Leica Microsystems, Inc., USA).

Calculation of N₂O generation

The calculation of N₂O generation amount from units of the A²O system was using Eqs. (4)–(7).

$$G_{An} = (E_{An} + C_{An} \times (1 + R_1)Q - C_{If} \times Q - C_{SST} \times R_1Q)/Q \quad (4)$$

$$G_{Ax} = (E_{Ax} + C_{Ax} \times (1 + R_1 + R_2)Q - C_{An} \times (1 + R_1)Q - C_{Ox} \times R_2Q)/Q \quad (5)$$

$$G_{Ox} = (E_{Ox} + C_{Ox} \times (1 + R_1 + R_2)Q - C_{Ax} \times (1 + R_1 + R_2)Q)/Q \quad (6)$$

$$G_{SST} = (E_{SST} + C_{SST} \times (1 + R_1)Q - C_{Ox} \times (1 + R_1)Q)/Q \quad (7)$$

where G_{An} (mg/L) is the N₂O generation from anaerobic tank, G_{Ax} (mg/L) is the N₂O generation from anoxic tank, G_{Ox} (mg/

Table 2 Oligonucleotide primer, fluorescently labeled, and hybridization conditions (Zhu and Chen et al. 2011; Han et al. 2012)

Probe	Sequence (5'-3')	Fluorescein	Color	Specificity	Formamide solution concentration (%)
EUB338	GCTGCCTCCCGTAGGAGT	Texas Red	Red	Bacteria	20
Nit3	CCTGTGCTCCATGCTCCG	FITC	Green	<i>Nitrobacter</i> spp.	40
Ntspa662	GGAATTCGCGCTCCTCT	FITC		Genus <i>Nitrospira</i>	40
Nsr1156	CCCGTTCTCCTGGGCAGT	FITC		Genus <i>Nitrospira</i>	30
Nso190	CGATCCCCTGCTTTTCTCC	Cy5	Yellow	Ammonium-oxidizing β -Proteobacteria	55
Nso1225	CGCGATTGTATTACGTGTGA	Cy5		Ammonium-oxidizing β -Proteobacteria	35

L) is the N_2O generation from oxic tank, and G_{SST} (mg/L) is the N_2O generation from secondary sedimentation tank; E_{An} (mg/L) is the N_2O emission from anaerobic tank, E_{Ax} (mg/L) is the N_2O emission from anoxic tank, E_{Ox} (mg/L) is the N_2O emission from oxic tank, and E_{SST} (mg/L) is the N_2O emission from secondary sedimentation tank; C_{IF} is the dissolved N_2O concentration in influent, C_{An} (mg/L) is the dissolved N_2O concentration in anaerobic tank, C_{Ax} (mg/L) is the dissolved N_2O concentration in anoxic tank, C_{Ox} (mg/L) is the dissolved N_2O concentration in oxic tank, and C_{SST} (mg/L) is the dissolved N_2O concentration in secondary sedimentation tank; and R_1 (%), R_2 (%), and Q (L/day) are the sludge return ratio, internal recycle ratio, and influent flow rate, respectively.

Calculation of nitrification and denitrification efficiency

In an A²O treatment process, the nitrification rate in the oxic area and the denitrification rate in both the anaerobic and anoxic areas can be calculated using Eqs. (8)–(10).

$$R_{n-oxic} = \frac{([NH_4^+-N]_{anoxic} - [NH_4^+-N]_{oxic}) \cdot (F_I + F_{IR} + F_{ER})}{V_{oxic} \cdot VSS_{oxic}} \quad (8)$$

where R_{n-oxic} (g/g volatile suspended solid (VSS) day) is the nitrification rate in the oxic area, $[NH_4^+-N]_{anoxic}$ (mg/L) is the NH_4^+-N concentration in the anoxic area, $[NH_4^+-N]_{oxic}$ (mg/L) is the NH_4^+-N concentration in the oxic area, F_I (85 L/day) is the influent flow, F_{IR} (170 L/day) is the internal recycle flow, F_{ER} (85 L/day) is the external recycle flow, V_{oxic} (L) is the volume of the oxic area, and VSS_{oxic} (mg/L) is the VSSs in the oxic area.

$$R_{dn-anaerobic} = \frac{[NO_x^- - N]_{influent} \cdot F_I + [NO_x^- - N]_{ER} \cdot F_{ER} - [NO_x^- - N]_{anaerobic} \cdot (F_I + F_{ER})}{V_{anaerobic} \cdot VSS_{anaerobic}} \quad (9)$$

where $R_{dn-anaerobic}$ (g/g VSS day) is the denitrification rate in the anaerobic area, $[NO_x^- - N]_{influent}$ (mg/L) is the sum of the $NO_3^- - N$ and $NO_2^- - N$ concentrations in the influent, $[NO_x^- - N]_{ER}$ (mg/L) is the sum of the $NO_3^- - N$ and $NO_2^- - N$ concentrations in the external flow, $[NO_x^- - N]_{anaerobic}$ (mg/L) is the

sum of the $NO_3^- - N$ and $NO_2^- - N$ concentrations in the anaerobic area, $V_{anaerobic}$ (L) is the volume of the anaerobic area, and $VSS_{anaerobic}$ (mg/L) is the VSS concentration in the anaerobic area.

$$R_{dn-anoxic} = \frac{[NO_x^- - N]_{anaerobic} \cdot (F_I + F_{ER}) + [NO_x^- - N]_{oxic} \cdot F_{IR} - [NO_x^- - N]_{anoxic} \cdot (F_I + F_{IR} + F_{ER})}{V_{anoxic} \cdot VSS_{anoxic}} \quad (10)$$

where $R_{dn-anoxic}$ (g/g VSS day) is the denitrification rate in the anoxic area, $[NO_x^- - N]_{oxic}$ (mg/L) is the sum of the $NO_3^- - N$ and $NO_2^- - N$ concentrations in the oxic area, $[NO_x^- - N]_{anoxic}$ (mg/L) is the sum of the $NO_3^- - N$ and $NO_2^- - N$ concentrations in the anoxic area, V_{anoxic} (L) is the volume of the anoxic area, and VSS_{anoxic} (mg/L) is the VSS concentration in the anoxic area.

Results and discussion

Comparison of the performance between different C/N ratios

The COD, $NH_4^+ - N$, TN, TP, $NO_3^- - N$, and $NO_2^- - N$ concentrations in each tank of the A²O treatment system under

Fig. 3 Pollutant concentrations in each unit under different influent C/N ratios

different C/N ratios are shown in Fig. 3. High removal efficiency of COD, $\text{NH}_4^+\text{-N}$, and TP occurred under all operation modes in this work. As the C/N ratio increased from 3.4 to 7.5, the TN removal efficiency increased dramatically from 21.69 to 61.56%, which revealed the denitrification reactions had been enhanced. The removal rates of COD, $\text{NH}_4^+\text{-N}$, and TP were also elevated slightly. To understand better the N_2O generation mechanisms in this A²O system, the ORP and DO in each unit were investigated (Fig. 3 and Table 3).

The changes in the influent C/N ratio showed a marked impact on nitrogen removal. As $\text{NH}_4^+\text{-N}$ concentrations in the influent were almost constant under the three operation modes, the differences in TN removal rate were caused by increased carbon source in the influent. The carbon source plays a significant role in denitrification processes, as limited biodegradable

organic carbon leads to incomplete denitrification and low TN removal efficiency because of the absence of an electron donor (Chung and Chung 2000; Itokawa et al. 2001). The denitrification rates of the anaerobic and anoxic tanks under different C/N ratios were analyzed using Eqs. (9)–(10) (Table 4). The denitrification rates in the anaerobic section were higher than those in the anoxic section under the influent C/N ratios of 3.4 and 5.4 in this work. When the C/N ratio was 7.5, the available carbon source in the anoxic section increased, which led to an increase in the denitrification rate of the anoxic section. The changes in ORP in the anoxic section also reflected that the denitrification had been promoted with increased COD in the influent (Table 3). However, the indication of this result was not the $\text{NO}_x^-\text{-N}$ removal mainly processed in the anaerobic section. The $\text{NO}_x^-\text{-N}$ removal amount was decided mainly by the

Table 3 ORP and DO in anaerobic, anoxic, and oxic section under different influent C/N ratios

	ORP (mV)				DO (mg/L)			
	Anaerobic section	Anoxic section	Oxic section	Secondary sedimentation tank	Anaerobic section	Anoxic section	Oxic section	Secondary sedimentation tank
C/N = 3.4	-185.28 ± 32.96	-23.60 ± 64.47	161.90 ± 29.92	10.32 ± 18.84	0.20 ± 0.03	0.27 ± 0.05	2.01 ± 0.15	0.52 ± 0.11
C/N = 5.4	-195.43 ± 92.73	-99.91 ± 27.60	86.91 ± 33.24	-16.46 ± 14.22	0.17 ± 0.02	0.25 ± 0.02	2.03 ± 0.17	0.47 ± 0.15
C/N = 7.5	-226.26 ± 42.66	-147.08 ± 44.60	51.98 ± 15.28	-22.76 ± 10.60	0.16 ± 0.02	0.24 ± 0.03	1.98 ± 0.12	0.48 ± 0.08

Table 4 Nitrification rate in oxic section and denitrification rate in anaerobic and anoxic section under different influent C/N ratio

	Nitrification rate in oxic section (mg NH ₄ ⁺ -N/g VSS day)	Denitrification rate in anaerobic section (mg NO _x ⁻ -N/g VSS day)	Denitrification rate in anoxic section (mg NO _x ⁻ -N/g VSS day)
C/N = 3.4	77.54 ± 19.85	27.59 ± 11.51	18.71 ± 7.07
C/N = 5.4	68.10 ± 19.77	78.59 ± 36.13	53.54 ± 26.10
C/N = 7.5	50.70 ± 13.97	81.87 ± 61.76	83.58 ± 38.13

denitrification rate and retention time of the section. The retention times were 2.8 and 1.4 h for the anoxic and anaerobic sections, respectively. The NO_x⁻-N removal amounts in the anaerobic and anoxic sections were 314.5 ± 131.3 and 426.7 ± 161.2 mg/day, 895.9 ± 411.8 and 1220.6 ± 595.0 mg/day, and 933.3 ± 704.1 and 1905.7 ± 869.4 mg/L under the influent C/N ratios of 3.4, 5.4, and 7.5, respectively. This result indicated the denitrification reactions occurred primarily in the anoxic section, although a relatively high reaction rate was observed in the anaerobic section.

In addition, heterotrophic microorganisms could decrease the TN concentration by assimilating inorganic nitrogen, which is a potential approach for nitrogen removal in the anoxic section (Yan et al. 2016). At an elevated influent C/N ratio, adequate organic matter enhanced the assimilation of microorganisms. Thus, with the influent C/N ratio increased, the nitrification rate in the oxic section decreased (Table 4), and COD and NH₄⁺-N showed higher removal effects (Fig. 3).

N₂O generation and emissions under different C/N ratios

Table 5 shows the N₂O emissions and generation amount in each tank of the A²O system under different C/N ratios. N₂O emissions were calculated according to Eqs. (1)–(3), and the generation amount was calculated using Eqs. (4)–(7). The comparison of N₂O emission between this study and other researchers was showed in Table 6. The activities of the three

key denitrifying enzymes, Nar, Nir, and Nos, in the anoxic section are shown in Fig. 4.

Several researchers have studied the effect of C/N ratio on N₂O emission in different wastewater treatment processes. Results showed a huge variation in the fraction of nitrogen that was emitted as N₂O (Table 6). The N₂O conversion ratio of this work was at a low level compared with others. In this treatment process, N₂O emissions and generation, as well as the total N₂O-N conversion ratio, decreased distinctly as the influent C/N ratio increased. Due to dissolved N₂O could be brought by internal recycle liquid, sludge recycle liquid, and wastewater flow between units of the treatment process, N₂O generation and emissions usually processed in different unit (Yan et al. 2014; Park et al. 2000). In this study, N₂O emissions were primarily from the oxic section, as 59.56, 53.51, and 55.03% of the totals were emitted from this unit under the C/N ratios of 3.4, 5.4, and 7.5, respectively. The anoxic and anaerobic sections generated the most N₂O under the three influent modes (Table 5), but much lower emissions were observed in these two units. This was ascribed to the mechanical aeration and stirring in the oxic section, which promoted the stripping of the dissolved N₂O from the liquid by diffusion (Czepiel et al. 1995).

N₂O can be generated in both nitrification and denitrification processes (Kampschreur et al. 2009). In this study, N₂O was generated mainly from the denitrification process in the anaerobic and anoxic tanks (Table 5). N₂O is an intermediate in the denitrification process, lacking for electron donor of carbon source often result in incomplete denitrification which can lead to N₂O generation. The NO₂⁻-N and NO₃⁻-N concentrations in the anaerobic and anoxic sections reflected the status of the denitrification processes (Fig. 3). When the influent C/N ratio was 3.4, denitrification reactions hardly occurred, which was reflected by the relative high NO₃⁻-N and low NO₂⁻-N concentrations. Under the C/N ratios of 5.4 and 7.5, denitrification increased but incomplete denitrification also existed, as the NO₃⁻-N concentration decreased in the

Table 5 N₂O emission and generation in each unit of A²O process under different influent C/N ratios

		C/N = 3.4	C/N = 5.4	C/N = 7.5
Emission	Anaerobic section (mg/L)	(12.87 ± 7.41) × 10 ⁻²	(1.26 ± 0.90) × 10 ⁻²	(0.85 ± 0.79) × 10 ⁻²
	Anoxic section (mg/L)	(12.14 ± 7.30) × 10 ⁻²	(1.32 ± 1.21) × 10 ⁻²	(0.51 ± 0.46) × 10 ⁻²
	Oxic section (mg/L)	(38.20 ± 6.88) × 10 ⁻²	(3.28 ± 1.65) × 10 ⁻²	(1.75 ± 1.23) × 10 ⁻²
	Secondary sedimentation tank (mg/L)	(0.93 ± 0.46) × 10 ⁻²	(0.27 ± 0.05) × 10 ⁻²	(0.07 ± 0.02) × 10 ⁻²
	Total N ₂ O emission (mg/L)	(64.14 ± 16.45) × 10 ⁻²	(6.13 ± 2.24) × 10 ⁻²	(3.18 ± 1.23) × 10 ⁻²
Generation	Anaerobic section (mg/L)	(18.29 ± 9.53) × 10 ⁻²	(1.42 ± 1.06) × 10 ⁻²	(0.94 ± 0.56) × 10 ⁻²
	Anoxic section (mg/L)	(25.06 ± 12.03) × 10 ⁻²	(2.54 ± 1.77) × 10 ⁻²	(1.39 ± 0.65) × 10 ⁻²
	Oxic section (mg/L)	(16.76 ± 7.15) × 10 ⁻²	(1.32 ± 0.77) × 10 ⁻²	(0.67 ± 0.26) × 10 ⁻²
	Secondary sedimentation tank (mg/L)	(9.89 ± 4.73) × 10 ⁻²	(1.37 ± 0.60) × 10 ⁻²	(0.33 ± 0.24) × 10 ⁻²
	Total N ₂ O generation (mg/L)	(70.00 ± 18.47) × 10 ⁻²	(6.65 ± 1.96) × 10 ⁻²	(3.33 ± 0.74) × 10 ⁻²
N ₂ O-N conversion ratio (%)		2.23	0.12	0.05

Table 6 Comparison of N₂O emission between this study and other researchers

Type of process	C/N ratio	N ₂ O emission	Remarks	Reference
A/A/O	3.5	9.18% of TN _{removed}	Real municipal wastewater	Ren et al. (2015)
	7.5	0.41% of TN _{removed}		
	10.8	0.04% of TN _{removed}		
Biological nitrogen removal process	2.4–3.5	20–30% of TN _{load}	High-strength wastewater (1700–1800 mg NH ₄ ⁺ -N/L and 4100–9900 mg COD/L)	Itokawa et al. (2001)
	5.0–5.5	Less than 1% of TN _{load}		
Nitrogen removal process of swine wastewater treatment	2.6	17.70% of TN _{load}	Swine waste was added to adjust the C/N ratio	Kishida et al. (2004)
	4.5	1.71% of TN _{load}		
Denitrification process	2.5–5.6	0.005%–0.5% of TN _{removed}	Real municipal wastewater	Chung and Chung (2000)
	3.4	2.23% of TN _{removed}		
A/A/O	5.4	0.12% of TN _{removed}	Real municipal wastewater	This study
	7.5	0.05% of TN _{removed}		

anaerobic and anoxic sections and relatively high NO₂⁻-N concentrations were observed. Moreover, the activities of Nar, Nir, and Nos in the anoxic section, which were positively correlated with denitrification rate and TN removal rate, obviously increased as the C/N ratio increased. As a result, as the carbon source increased in the influent, the denitrification increased and N₂O generation decreased observably (Tables 4 and 5). The decrease in ORP in anaerobic and anoxic tanks also reflected that denitrification reactions were enhanced with the increased carbon source (Table 3). Ren et al. (2015) and Itokawa et al. (2001) also found a similar phenomenon in aerobic treatment processes and a lab-scale sequencing batch reactor (SBR), respectively.

N₂O generated from nitrification processes have been observed in several full-scale WWTPs (Yan et al. 2014; Ahn et al. 2010; Foley et al. 2010), which was also found in this study. Of the total N₂O production, 23.94, 19.85, and 20.12% were generated from the oxic section under the C/N ratios of 3.4, 5.4, and 7.5, respectively. Moreover, the N₂O generation from this process decreased from $(16.76 \pm 7.15) \times 10^{-2}$ to $(0.67 \pm 0.26) \times 10^{-2}$ mg/L as the C/N ratio increased from

3.4 to 7.5. During the nitrification process, when N₂O was not an intermediate product, nitrifier denitrification by AOB was considered the primary N₂O production source (Kampschreur et al. 2009). Even though N₂O production in pure culture experiments has been observed from chemical reactions of unstable biological intermediates (Colliver and Stephenson 2000), this pathway is of minor importance in active sludge systems (Wunderlin et al. 2012). Consequently, the N₂O generation from the nitrifier denitrification pathway also showed negative correlation with the influent C/N ratio.

A portion of N₂O generation by the treatment system originated from the secondary sedimentation tank by the denitrification pathway, which was inferred by the difference in TN concentration between the secondary sedimentation tank and the oxic section. High NO_x⁻-N, low DO, and insufficient COD were also detected in this unit (Table 3 and Fig. 3), which all benefited N₂O generation (Kampschreur et al. 2009).

Microbial community characteristics under different C/N ratios

Activated sludge was taken from the A²O process after continuous operation for 2 months. The FISH images from the oxic section of the process are shown in Fig. 5. To quantify the microorganisms' ability to convert N₂O to N₂, a qPCR assay targeting the *nosZ* gene was carried out. The *nosZ* gene copy numbers in the anoxic section under different influent C/N ratios are presented in Fig. 6.

The characterization of the microbial communities in the oxic section revealed that AOB accounted for (8.42 ± 3.65) , (4.93 ± 2.27) , and $(3.61 \pm 1.66)\%$ and NOB accounted for (10.38 ± 4.12) , (5.79 ± 2.08) , and $(4.67 \pm 1.62)\%$, under the influent C/N ratios of 3.4, 5.4, and 7.5, respectively (accounting for the domain bacteria). This result showed that with the

Fig. 4 Nar, Nir, and Nor activities in the anoxic section

Fig. 5 Microscope images of sludge from the oxic section of the A₂O process as visualized by FISH. A1–A3, B1–B3, and C1–C3 were the images under the influent C/N ratio of 3.4, 5.4, and 7.4, respectively. Domain bacteria that were dyed DAPI displayed red, the green pixels in images represented NOB, and the yellow pixels represented AOB

increased carbon source from the influent, the relative abundances of AOB and NOB in the systems decreased, as did the nitrification rate observed in the oxic section (Table 4).

Compared with aerobic heterotrophs, the growth rate and competitive ability for oxygen of autotrophic nitrifying bacteria are lower; therefore, nitrification is difficult to maintain in WWTPs (Lee et al. 2011). Moreover, AOB are known to

produce N₂O via nitrifier denitrification under oxic conditions (Colliver and Stephenson 2000). This decrease in relative abundance of AOB was probably the main reason for the reduction in N₂O generation from the oxic section as the influent C/N ratio increased.

The *nosZ* gene, which encodes Nos, is well known for catalyzing the reduction from N₂O to N₂ and usually has been employed for detecting denitrifier-specific DNA (Zhu and Chen et al. 2011). In BNR processes, the existence of the *nosZ* gene implies that the treatment biosystem has the ability to convert N₂O to N₂ (Lu et al. 2014). In this study, the *nosZ* gene copy numbers markedly increased as the C/N ratio increased from 3.4 to 7.5 (Fig. 6), revealing the importance of the influent nutritional condition for *nosZ*-containing bacteria. In addition, the *nosZ* gene copy numbers showed a positive correlation with the activities of the denitrifying enzymes and denitrification effect but negative correlation with N₂O generation. The increase in *nosZ* gene copy number means the denitrification ability of N₂O increased (Yan et al. 2016). Hence, nitrogen removal and N₂O emissions reduction effects were simultaneously improved as the C/N ratio increased in this treatment process.

Fig. 6 *NosZ* gene copy numbers in the anoxic section under different influent C/N ratios

The removal of pollutants and N_2O generation from wastewater treatment is determined primarily by the microorganismal community composition and activity of the activated sludge (Kampschreur et al. 2009). Sun et al. (2012) observed how the changes in initial biodegradable COD/ NO_3^- -N ratio affected the nitrate reduction rate and the denitrifier microbial communities. Zhu and Chen (2011) increased *nosZ* gene copy numbers by using waste-activated sludge alkaline fermentation liquid as the synthetic wastewater-carbon source in an anaerobic-aerobic treatment process. As a result, the N_2O generation decreased by 68.7% and the removal efficiencies of TP and TN also improved. In an A^2O wastewater treatment process, Yan et al. (2016) found an increase in internal recycle ratio from 100 to 300% could decrease the *nosZ* gene copy numbers dramatically, which led to an increase in N_2O production. As shown from this work, changes in influent C/N ratio influenced the relative abundances of AOB and NOB and the *nosZ* gene copy numbers, which resulted in N_2O generation variation. These results reveal that in wastewater treatment systems, appropriate influent C/N conditions can enhance nitrogen removal efficiency and simultaneously decrease N_2O generation.

Conclusions

Changes in influent C/N ratio showed obvious effects on BNR and N_2O generation in the A^2O treatment process. As the influent C/N ratio increased from 3.4 to 7.5, the TN removal rate increased from 21.69 to 61.56%, N_2O generation in both nitrification and denitrification processes decreased, and the N_2O conversion ratio of the treatment system decreased evidently from 2.23 to 0.05%. The primarily cause of this phenomenon was the variation of microbial community, as increased influent C/N ratio reduced the relative abundance of AOB and NOB and promoted the development of *nosZ*-containing bacteria.

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