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Impact of internal recycle ratio on nitrous oxide generation from anaerobic/anoxic/oxic biological nitrogen removal process

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ABSTRACT

To evaluate the effects of internal recycle ratio on nitrous oxide (N₂O) generation, we set up three laboratory-scale anaerobic–anoxic–oxic (A²O) wastewater treatment processes, with internal recycle ratios of 100%, 200%, and 300%, respectively. Total nitrogen (TN) removal was markedly enhanced from 45.8% to 61.9%, as the internal recycle ratio increased from 100% to 300%. N₂O generation was increased from 3.47 to 9.81×10^{-2} mg/L during the treatment process, with the anoxic section showing the largest N₂O increment from denitrification. This phenomenon is attributed to the increased amount of nitrate (NO₃[−]-N) substrate available for denitrification, due to the increased volume of internal recycle liquid, as well as the increased amount of oxygen, which could restrain the activity of nitrous oxide reductase brought to the anoxic section. Nitrous oxide reductase was more sensitive to oxygen than nitrate and nitrite reductases. Microorganism analysis indicated that the population of *nosZ* gene-containing bacteria was only slightly affected by the recycle ratio. However, the number of *nosZ* gene copies decreased as the internal recycle ratio increased from 100% to 300%; this result reveals noticeable decreases in the denitrification capacity of the system for reducing N₂O to N₂.

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1. Introduction

The contribution of greenhouse gases (GHGs) to global warming has elicited great concern from various sectors in the recent decades. Wastewater treatment plants (WWTPs) are considered artificial GHG emission resources because of nitrous oxide (N₂O) emissions during the biological nitrogen removal (BNR) process. It is arrestive that N₂O is about 300 times more greenhouse effective than carbon dioxide (CO₂), and its emission increased with a high rate of around 0.3% per year [1]. About 3.2% of all global anthropogenic N₂O emissions originate from the BNR processes of WWTPs [2]. Considering the widespread use of BNR processes, as well as the hazards of N₂O, investigation of the effect of key operating parameters and sources of N₂O emissions on the overall BNR process is an important undertaking.

N₂O can be produced from both nitrification and denitrification in BNR processes. Nitrification refers to the stepwise autotrophic oxidation of ammonia (NH₄⁺) to nitrite (NO₂[−]) by ammonia-oxidizing bacteria (AOB), and then to nitrate (NO₃[−])

by nitrite-oxidizing bacteria (NOB). During nitrification, N₂O is produced through two pathways. The first is the aerobic hydroxylamine oxidation pathway, in which N₂O is produced from intermediates of biological hydroxylamine oxidation, probably related to significant imbalances in the metabolic activity of AOB, or by chemical decomposition of hydroxylamine and chemical oxidation with NO₂[−] as an electron acceptor (chemo-denitrification). The second pathway is nitrifier denitrification, which involves reduction of NO₂[−] by AOB in combination with ammonia, hydrogen, or pyruvate as electron donors, for example, at oxygen-limited conditions or elevated nitrite concentrations [3,4].

Denitrification, on the other hand, refers to the reduction of NO₃[−] to atmospheric nitrogen (N₂) by heterotrophic denitrifiers, with NO₂[−], nitric oxide (NO) and N₂O as obligatory intermediates [5]. N₂O, a well-known intermediate emitted in this process, is released in high quantities in environments with high dissolved oxygen (DO) and NO₂[−] concentrations and low C/N water quality [3,6,7].

In present studies, the amounts of N₂O emitted during wastewater treatment process are reported with high range of variation. Kampschreur et al. [2] concluded that 0.0–95.0% and 0.0–14.5% of the influent nitrogen could be removed through N₂O generation during lab-scale and full-scale wastewater treatment, respectively.

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To reduce N_2O emissions, in recent years, significant efforts have been made to understand the effect of various WWTP operating parameters on N_2O production [8–10]. As a result, several parameters favoring N_2O production have been identified: low dissolved oxygen concentration [11–13], accumulation of nitrite [3], rapidly changing (dynamic) conditions [14], a low ratio of chemical oxygen demand (COD) to N-compounds during heterotrophic denitrification [15,16], sludge retention time (SRT) [17], pH [18], or toxic compounds [19].

The internal recycle ratio in BNR WWTPs performs an important function in the nitrogen removal process [20]. For example, increasing the internal recycle ratio from 100% to 400% decreases the nitrate concentration in the effluent and, hence, improves the nitrogen removal efficiency of the system [21]. The environmental conditions in the nitrification and denitrification sections of WWTP systems are also influenced by the internal recycle ratio. Therefore, our hypothesis is that a close relationship exists between internal recycle ratio and N_2O generation. At present, however, the effects of internal recycle ratio changes on N_2O emissions in BNR wastewater treatment processes have not been extensively studied.

The purpose of this study is to investigate the effect of internal recycle ratio on N_2O emission characteristics and production mechanisms in BNR process. Three parallel laboratory-scale A^2O wastewater treatment processes were acclimated under different internal recycle ratios of 100%, 200%, and 300%, and the effect of changes in internal recycle ratio on N_2O generation in each unit of the A^2O process was studied. Denitrifier community compositions and gene copy numbers in each unit under different internal recycle ratios were analyzed using a clone library and real-time quantitative PCR (qPCR), targeting functional genes of *nosZ*.

2. Materials and methods

2.1. Reactor setup and operation

Three parallel laboratory-scale A^2O wastewater treatment processes were operated under different internal recycle ratios of 100%, 200%, and 300%. Except for the internal recycle rate, three parallel processes were carried out under same operating parameters, as follows. The A^2O process had a total effective volume of 50 L (Fig. 1). The volumes of the anaerobic section, anoxic section, oxic section, and secondary sedimentation tank were 5, 10, 30, and 5 L, respectively. The treatment system was seeded with sludge from the aerobic tank of Luotuowan Wastewater Treatment Plant (Xinxiang, China). Influent wastewater from a residential area in Henan Normal University was loaded at a rate of 85 L/d. Water quality results showed the following contents: COD 175.6 ± 30.2 mg/L, NH_4^+-N 38.7 ± 3.2 mg/L, NO_3^--N 1.2 ± 0.2 mg/L, TN 39.5 ± 3.3 mg/L, TP 7.2 ± 2.4 mg/L. The water temperature was kept constant at 25 °C by three temperature controllers in anaerobic, anoxic and oxic sections throughout the research period. The SRT of system and mixed liquor suspended solid (MLSS) concentration of the water was maintained at 15 d and approximately 3000 mg/L, by controlling the emission amount of excess sludge. The sludge recycle ratio of 100% was controlled by a peristaltic pump, which brought the sludge from secondary sedimentation tank to anaerobic section with the flow of 85 L/d (Fig. 1).

The anaerobic and anoxic sections of the experimental process were stirred by a magnetic stirrer (85-2 Sile Shanghai) at a rotating speed of 500 r/min to keep the activated sludge suspended in the water. Two dismountable sealing covers were on the top of anaerobic and anoxic section to maintain the anaerobic and anoxic conditions, respectively. The DO of the oxic section was maintained at 2 mg/L by an air pump with an adjustable air flow meter. A mechanical stirrer was installed in the secondary sedi-

mentation tank (US-52 Oteli Beijing) and operated at a rotating speed of 30 r/min. Gas and liquid samples were obtained at the same time; liquid-phase samples were used to measure water quality parameters of COD, NH_4^+-N , NO_3^--N , NO_2^--N , TN and TP. All measurements were carried out after the A^2O process had been acclimated under each internal recycle rate (100%, 200%, and 300%) for over 2 months and reached stable performance, as indicated by the stable COD, NH_4^+-N , TN and TP concentrations observed in the effluents.

2.2. Sampling and analysis methods

2.2.1. Sampling for anaerobic section, anoxic section, and secondary sedimentation tank

A modified closed-chamber technique was used to measure fluxes in non-aerated surfaces (anaerobic section, anoxic section and secondary sedimentation tank) [22]. There were three closed chambers for each one of the non-aerated surfaces. Each tank and its constant chamber had the same diameter. There were flanges at the lower rim of the chamber and the upside of tanks. During gas sampling, closed chambers were fixed on the top of tanks, by joining the flanges. The pressure of the sampling device and joint was checked at each sampling. Four samples were collected from the headspace of the chambers through polytetrafluoroethylene tubes, into 50-mL polypropylene syringes, at 3-min intervals. The gas flux, F (mg/m² min), from the liquid surface in the static chamber was calculated using the equation:

$$F = \left(\frac{V}{A} \right) \rho \left(\frac{\Delta c}{\Delta t} \right) \quad (1)$$

where V (m³) is the volume from the liquid level to the top of chamber, A (m²) is the enclosed surface area, ρ (mg/m³) is the density of the gas at the temperature recorded in the chamber, and Δc (v/v)/ Δt (min) is the change of gas concentration in the chamber per unit time during sampling. The gas density (ρ) is calculated by the equation:

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

where M is the molecular weight of N_2O (44.02 g/mol), P is the atmospheric pressure (1 atm), R is the gas constant (0.082 L atm/K mol), T is the temperature (K).

2.2.2. Oxic section sampling

During gas sampling, a closed gas-tight chamber with a gas-sampling outlet was fixed to the tank. The pressure of the sampling device and joint was checked at each sampling. The entire system was gas-tight and the effluent air was collected into gas sampling bags. Three parallel samples were taken at each sampling, and the gas flux, F (mg/m² min), was calculated by

$$F = \frac{\rho c Q_a}{A} \quad (3)$$

where ρ (mg/m³) is the density of the gases at the sampling temperature, c (v/v) is the sample gas concentration, Q_a (m³/min) is the aeration rate, and A (m²) is the total surface area.

2.2.3. Dissolved gas sampling

To collect gas samples dissolved in wastewater, the headspace gas method described by Kimochi et al. [23] was used. Thirty milliliter each of water and argon gas were sealed in a 50-mL syringe; 1 mL of 20 mmol/L mercury(II) chloride was added to this mixture to prevent biological degradation. After vigorous shaking, the syringe was left at room temperature for 1 h without moving. The resulting gas phase in the syringe was collected as a gas sample.

Fig. 1. Schematic of the experimental process.

Dissolved gas concentrations can be calculated using Henry's Law through the equilibrium headspace gas concentrations.

2.2.4. N_2O and water quality analysis

A gas chromatograph (HP-Chemstation 5890, Agilent Co., Ltd., USA) with an electron capture detector (ECD) was used to analyze N_2O concentrations. COD, NH_4^+-N , $NO_2^- -N$, $NO_3^- -N$, and TN were analyzed according to standard methods [24]. The measurements of oxidation–reduction potential (ORP) and DO of wastewater were conducted using WTW3110 ORP (made in Germany) and WTW-Multi 340i DO (made in Germany), respectively.

2.2.5. Activity measurements

The activities of nitrate reductase, nitrite reductase, and nitrous oxide reductase were determined anaerobically at 25 °C. Activated sludge was washed in 25 mmol/L HEPES buffer (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid], pH 7.2). TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine [Sigma]) and ascorbate (Sigma) were used as the electron donor couple [6]. EDTA (1.0 mmol/L) was added to prevent possible chemical reduction [25]. The amount of chemical reduction was estimated by control experiments, using pasteurized (2 min, 95 °C) activated sludge. Nitrate and nitrite disappearance was followed by taking samples at 2-min intervals from a reaction chamber (100 mL). Nitrous oxide disappearance was determined by measuring the dissolved N_2O by the method described in Section 2.2.3. At the initiation of the reactions, the concentrations of $NO_3^- -N$, $NO_2^- -N$ and dissolved N_2O were 25, 5 and 2.0×10^{-2} mg/L, respectively.

2.3. N_2O mass balance calculation

The N_2O generated in each unit of the A^2O process can be calculated using Eqs. (4)–(7).

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

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

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

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

where G_{An} , G_{Ax} , G_{Ae} , and G_{SST} (mg/L) are the amounts of N_2O generated in the anaerobic section, anoxic section, oxic section, and secondary sedimentation tank, respectively; E_{An} , E_{Ax} , E_{Ae} , and E_{SST} (mg/d) are the amounts of N_2O emitted from the anaerobic section, anoxic section, oxic section, and secondary sedimentation tank, respectively; C_{In} , C_{An} , C_{Ax} , C_{Ae} , and C_{SST} (mg/L) are the concentrations of dissolved N_2O in influent, anaerobic section, anoxic section, oxic section, and secondary sedimentation tank, respectively; R_1 (%) is the sludge return ratio; and R_2 (%) is the internal recycle ratio; Q (L/d) is the influent flow rate.

2.4. DNA extraction, PCR amplification, and sequencing

Isolation of total DNA was accomplished with a Magnetic System-16 (TanBead, Taiwan) setup. Primers nosZ-F (AGAAC-GACCAGCTGATCGACA) and nosZ-R (TCCATGGTGACG CCG TGGTTG) were used to amplify the gene segment of nosZ encoding nitrous oxide reductase [26]. The PCR amplification reaction was performed using a Gene AmpR PCR system (9700, AB, USA) at a final volume of 50 μ L; the PCR program was as follows: followed by 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s. After purification by an agarose-gel extraction kit (Dingguo, China), the PCR products were ligated and transformed into *Escherichia coli* DH5 α competent cells. The positive clones were selected cultured on LB medium with X-gal, IPTG and Amp to submit for sequencing using the ABI 3730DXL DNA sequencer (AB, USA).

2.5. Clone library construction and phylogenetic analysis of sequences in the clone libraries

All sequences were manually checked and trimmed to exclude vector sequences and were then checked for chimeras using Bellerophon on the Greengenes website (<http://greengenes.lbl.gov/>). After excluding chimeras and false-positive clones, other sequences of each sample were aligned and were classified into universal operational taxonomic units (OTUs) at a threshold of 97% minimum similarity. Individual sequences were also assigned into OTUs to identify bacterial populations in sludge from different sampling sites. The representative sequence of each OTU in group-specific libraries was aligned to the NCBI database by BLAST. To identify the phylogenetic affiliation of all OTUs, a phylogenetic tree including representative sequences of each OTU and related sequences from the previous NCBI database was constructed using the neighbor joining algorithm with MEGA version 6.1.

Table 1
ORPs in each unit under three operation modes.

	ORP (mv)		
	Anaerobic section	Anoxic section	Oxic section
100%	−213.2 ± 30.5	−82.3 ± 21.3	123.4 ± 32.3
200%	−209.2 ± 25.8	−74.2 ± 19.2	119.6 ± 29.1
300%	−198.4 ± 27.1	−67.8 ± 15.2	116.7 ± 38.4

2.6. Real-time quantitative PCR

Denitrifying bacterial counts were determined by qPCR quantification based on the *nosZ* gene, using the primers *nosZ*-F: 5-AGAAGACACGCTGATCGACA-3 and *nosZ*-R: 5-TCCATGGTGACGCCGTGGTTG-3 [26]. For *nosZ* gene PCR, 2 μ L of extracted DNA was added to a PCR reaction mixture containing 12.5 μ L of SYBR Premix Ex Taq (TaKaRa, Japan), 0.5 μ L of *nosZ*-F (10 μ M each), 0.5 μ L of *nosZ*-R (10 μ M each), and 9.5 μ L of dH₂O. qPCR was performed using a TaKaRa PCR Thermal Cycler Dice real-time PCR system (TaKaRa Code: TP800) with an initial denaturation step of 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s and 65 °C for 30 s. Denitrifying bacterial *nosZ* from the samples was calculated using the quantitative software available with the PCR instrument using serially diluted PCR products to generate a standard curve. Sample concentrations of *nosZ* were determined by comparison of the crossing threshold cycle (Ct), against the standard curve crossing threshold (Ct) produced to count copies of target DNA per sample. The numbers of standard DNA for the primer set were adjusted as a series of tenfold dilutions ranging from 1.0×10^2 (*nosZ* gene) copies mL^{−1} to 1.0×10^7 (*nosZ* gene) copies mL^{−1}. In the standard curve, the linear correlation ($R^2 = 0.999$) was observed for six orders of magnitude ranging from 10^2 to 10^7 gene copies per mL of standard DNA.

3. Results and discussion

3.1. Performance of A²O process with different internal recycle ratios

Fig. 2 shows COD, NH₄⁺-N, NO₂[−]-N, NO₃[−]-N, TN and TP concentrations in each unit of the A²O process with internal recycle ratios of 100%, 200%, and 300%. Table 1 shows the ORP of sludge mixtures in each unit.

High COD, NH₄⁺-N and TP removal were observed among the three operating modes evaluated in this work, and the removal characteristics in each operating mode were similar (Fig. 2). COD and NH₄⁺-N removal rates slightly improved as the internal recycle ratio increased from 100% to 300%. These results reveal that the internal recycle ratio could influence organic matter oxidation in A²O process. Variations in COD, NH₄⁺-N, NO₃[−]-N, TN and TP concentration detected among the units showed the distinctive functions of the anaerobic, anoxic, and oxic sections in an A²O process. While the oxic section is assumed responsible for organic matter oxidation and NH₄⁺-N nitrification, denitrification mainly occurs in the anoxic section. Phosphorus uptake and release was processed in oxic and anaerobic section, respectively.

Denitrification effects in the anoxic section were enhanced as the internal recycle ratio increased from 100% to 300%; the TN decreased amount was increased from 1.5 to 7.8 mg/L in this unit. Thus, the TN removal rate significantly increased from 45.8% to 61.9% (Fig. 2). NO₃[−]-N concentrations in each unit decreased with the increased internal recycle rate in this work (Fig. 2). On one hand, this phenomenon may be ascribed to the fact that increase in recycle liquid ratio provides more nitrates to the denitrification reactor and thus improves overall nitrogen removal rates. COD in the anoxic section also decreased with increasing internal recycle ratio,

Table 2
N₂O generation under the three operating modes studied.

Parameters	100%	200%	300%
Anaerobic section (mg/L)	0.39×10^{-2}	1.05×10^{-2}	1.94×10^{-2}
Anoxic section (mg/L)	0.60×10^{-2}	1.92×10^{-2}	3.35×10^{-2}
Oxic section (mg/L)	1.34×10^{-2}	2.08×10^{-2}	2.65×10^{-2}
Secondary sedimentation tank (mg/L)	1.14×10^{-2}	1.54×10^{-2}	1.87×10^{-2}
Total N ₂ O generation (mg/L)	3.47×10^{-2}	6.59×10^{-2}	9.81×10^{-2}
N ₂ O-N conversion ratio (%)	0.14	0.20	0.21

probably because more electron donors are needed during NO_x (NO₃[−] and NO₂[−]) denitrification. On the other hand, assimilation of dissolved inorganic nitrogen by heterotrophic microorganisms is a potentially large contributor to TN removal during the anoxic phase of treatment, and adequate NO₃[−]-N provided by high internal recycle ratios enhances cell assimilation during this phase. Thus, COD and NH₄⁺-N show higher removal rates under higher internal recycle ratios (Fig. 2). The economic cost is directly related to the internal recycle rate, as an increase of the ratio from 1 to 5 implies approximately a five times higher energy consumption [27]. Therefore, to ensure a cost efficient project, we did not conduct experiments with recycle rates higher than 300%.

ORP monitoring results, which increased from the anaerobic to the oxic section (Table 1) under all operating modes, also revealed the distinct functions of each of the units in the A²O process. NO₂[−]-N accumulation occurred in the anaerobic and anoxic sections, but not in the oxic section, under the three operating conditions (Fig. 2). As the internal recycle ratio increased, so did the NO₂[−]-N concentrations. NO₂[−]-N was produced in denitrification process for the nitrate reductase is more competitive in the competition of electron donor between nitrate reductase and nitrite reductase [28]. As the recycle liquid brought more NO₃[−]-N to anoxic section under high internal recycle ratios, the first step of denitrification was enhanced, which was shown as decreased TN concentration.

3.2. N₂O generation and emission of A²O under different internal recycle ratios

The N₂O mass balances of dissolved liquid and gas phase N₂O obtained during A²O under different internal recycle ratios are shown in Fig. 3.

In this study, N₂O emission was detected in all units of the A²O treatment process studied (Fig. 3). Under each operation mode, maximal N₂O emission was detected in oxic section, whereas minimal N₂O emission was found in the secondary settling tank. Dissolved N₂O concentration among the units varied considerably with variations in internal recycle ratios. At an internal recycle ratio of 100%, the secondary settling tank reflected the highest concentration of dissolved N₂O (1.13 g/d). As the internal recycle ratio increased, the internal recycle liquid flow increased and the anoxic section showed the highest concentration of dissolved N₂O. Dissolved N₂O amounts taken up by the effluent under internal recycle ratio of 100%, 200%, and 300% respectively totaled 19.3%, 12.0%, and 9.4% of the total N₂O generation. Most of this dissolved N₂O is probably released to the atmosphere because the average background N₂O concentration was 319 ppb [1].

N₂O production in the A²O process generally increased with increasing internal recycle ratio (Table 2). During wastewater treatment process, N₂O is first generated in the liquid phase and then stripped from water to the atmosphere in the gas phase by mechanical stirring and aeration. Considering differences in agitation conditions, N₂O production and emission may not occur in the same unit. To identify the increased N₂O emission source in A²O treatment process, the N₂O generated in each unit was calculated according to Eqs. (4)–(7) (Table 2).

Fig. 2. Pollutant concentrations in each unit under different internal recycle ratios: (A) internal recycle ratio of 100%, (B) internal recycle ratio of 200%, and (C) internal recycle ratio of 300%.

With the increased internal recycle ratio, N_2O emission increments were highest in the anoxic section among the units observed (Table 2), but part of this N_2O is released in the oxic section (Fig. 3). Denitrification mainly occurs in the anoxic section, and N_2O is produced as an intermediate product of this process [2]. As the internal recycle ratio increased, more NO_3^- -N was brought to the anoxic section (Fig. 2), and the availability of substrates for denitrification

increased. More DO from the oxic section is also brought to the anoxic section. DO of 0.24 ± 0.04 , 0.37 ± 0.07 , and 0.46 ± 0.06 mg/L were detected in the anoxic section at internal recycle ratios of 100%, 200%, and 300%, respectively. Oxygen inhibits both the synthesis and activity of denitrification enzymes [2]. Coupled with the variation of NO_3^- -N concentration and NO_2^- -N accumulation phenomenon in anoxic section, the impact from increase of internal

Table 3

Activities of nitrate reductase, nitrite reductase and nitrous oxide reductase in anoxic section under the three operating modes.

	100%	200%	300%
Nitrate reductase activities (mg N/g MLSS min)	$38.1 \pm 3.0 \times 10^{-2}$	$37.7 \pm 2.3 \times 10^{-2}$	$36.3 \pm 1.5 \times 10^{-2}$
Nitrite reductase activities (mg N/g MLSS min)	$33.4 \pm 1.3 \times 10^{-2}$	$29.3 \pm 1.8 \times 10^{-2}$	$25.1 \pm 1.3 \times 10^{-2}$
Nitrous oxide reductase activities (mg N/g MLSS min)	$9.8 \pm 0.5 \times 10^{-2}$	$5.3 \pm 0.6 \times 10^{-2}$	$2.2 \pm 0.2 \times 10^{-2}$

Fig. 3. Schematic of N_2O mass balances under different internal recycle ratios: (A) internal recycle ratio of 100%, (B) internal recycle ratio of 200%, and (C) internal recycle ratio of 300%.

recycle rate on denitrification enzymes was in this order: nitrous oxide reductase > nitrite reductase > nitrate reductase (Table 3). This result is in agreement with the results reported by Otte et al. [6], showing that N_2O reductase is more sensitive to oxygen than other enzymes, increased N_2O emissions during denitrification may be observed when low concentrations of oxygen are present in the anoxic section. This inference may explain why both nitrogen removal rates and N_2O generation in the anoxic section increased with increasing internal recycle ratio.

N_2O was observed at concentrations of 1.34×10^{-2} and 2.08×10^{-2} mg/L during oxic production at internal recycle ratios of 100% and 200%, respectively, these values being highest under their operation modes. In addition, when the internal recycle ratio was 300%, the oxic section produced the second highest amount of N_2O (2.65×10^{-2} mg/L) among all of the units (anoxic section was 3.35×10^{-2} mg/L). Several researchers have shown that N_2O emissions in full-scale WWTPs primarily occur in the oxic section [10,29,30]. The phenomenon observed in this study agrees with these results. While N_2O is not present as an intermediate in the main catabolic pathway of nitrification, AOB are known to produce N_2O , which is predominantly associated with the nitrifier denitrification pathways [28]; N_2O emissions due to chemical reactions of unstable biological intermediates have also been observed (in pure culture experiment) [3]. Wunderlin et al. [31] reported that N_2O production from chemical reactions in active sludge systems is of

minor importance. Thus, N_2O generation in the oxic section may be attributed to nitrifier denitrification produced by AOB.

Rapid changes in environmental conditions may also explain the higher N_2O emissions observed at higher internal recycle ratios [32,33]. Several researchers have found that N_2O generation increases markedly when the environmental conditions change. High internal recycle ratios accelerate liquid recycle between units and promote changes in other process conditions, especially DO, thereby leading to increased N_2O production. This hypothesis may also explain why the N_2O generated in every unit increased with increasing internal recycle ratio.

In contrast to expectations, the secondary settling tank produced N_2O during wastewater treatment; 32.9%, 23.4%, and 19.1% of the total N_2O were generated under internal ratios of 100%, 200% and 300%, respectively. This N_2O contribution is speculated to originate from the denitrification process because AOB and denitrification bacteria could denitrify NO_x to N_2O or N_2 at low DO conditions [2]. High NO_3^- -N and low COD concentrations, which benefit N_2O production, were also detected in the secondary settling tank (Fig. 2).

3.3. Analysis of *nosZ*-containing microbial communities

The *nosZ* gene encoding nitrous oxide reductase is known to be unique to denitrifying bacteria; the presence of this gene indi-

cates that the bacterial species under consideration is capable of reducing N_2O to N_2 . To investigate the mechanisms of N_2O emissions, the compositions of *nosZ*-containing bacterial communities in each unit under three operating modes were analyzed using clone libraries (Fig. 4). qPCR assay targeting the *nosZ* gene was also conducted (Fig. 5).

Based on the phylogenetic tree obtained, similarities among denitrifying bacteria containing the *nosZ* gene were observed under different internal recycle ratios. *Pseudomonas* sp., *Azospirillum* sp., *Mesorhizobium* sp., *Pseudomonas lini*, and other uncultured bacteria were detected. These results imply that bacterial populations in the bioreactor are only slightly affected by changes in recycle ratio when the influent quality and inoculated sludge applied are held constant.

qPCR showed distinct differences in *nosZ* gene copy number with variations in internal recycle ratio. When recycle ratio increased from 100% to 200%, *nosZ* gene copies in A^2O system decreased sharply from over 1.0×10^6 to less than 6.0×10^5 . However, *nosZ* gene copies between internal recycle ratios of 200% and 300% showed only slight disparities. *nosZ* gene copy numbers among the three operating modes were as follows: anaerobic section > anoxic section > oxic section. Therefore, decreases in *nosZ* gene copy number during A^2O with increasing internal recycle ratio are closely related to the DO environment of each unit during treatment. It is known that *nosZ* gene encoding nitrous oxide reductase is largely unique to denitrifying bacteria and has recently been used for the detection of denitrifier-specific DNA [34]. Oxygen inhibits denitrification by providing a better electron acceptor for denitrifying populations to generate energy, resulting in deteriorated denitrification bacteria at high DO concentrations [35]. High internal ratios bring about significant changes in the process conditions between the anaerobic and anoxic sections [33]. In this system, DO of anoxic section was increased from 0.24 ± 0.04 to 0.46 ± 0.06 mg/L with internal recycle rate elevated from 100% to 300%, and the transition of activity sludge between anoxic and oxic section (DO was 2.0 mg/L) became more continually, which was disadvantageous for *nosZ* gene-containing bacteria. Although increasing internal recycle ratios brought more substrates for denitrification to the anaerobic and anoxic sections, DO circumstance affecting *nosZ* gene-containing microorganisms was observable. Reductions in *nosZ* gene copy numbers reveal that the denitrification capacity of the system for reducing N_2O to N_2 is decreased. This hypothesis may explain why N_2O generation increases with increasing internal recycle ratio.

In this study, functional gene expression was affected largely than the bacterial community composition by variations in operating parameters of internal recycle rate. The bacterial population did not change significantly, but the numbers of functional genes were obviously altered under different internal recycle ratios. These results demonstrate that N_2O emission reductions could be achieved by regulating the expression of functional genes under different operating parameters. Practically, several strategies have been reported to mitigate N_2O emissions from wastewater denitrification [35]. Based on this research, ensuring sufficient anoxic HRT, bioaugmentation with the N_2O -reducing denitrifier *Pseudomonas stutzeri*, choosing methanol over ethanol and adding copper ions (10–100 mg/L) may reduce N_2O generation and simultaneously enhance TN removal under high internal recycle rate for A^2O process [34,36,37].

4. Conclusion

Internal recycle ratio has a significant impact on nitrogen removal and N_2O production in A^2O treatment process. The N_2O generation and nitrogen removal rate both increased significantly

Fig. 4. Neighbor-joining phylogenetic tree based on partial sequences of *nosZ*: (A) internal recycle ratio of 100%, (B) internal recycle ratio of 200%, and (C) internal recycle ratio of 300%.

Fig. 5. NosZ gene copy numbers in each A²O unit under different internal recycle ratios.

with increasing internal recycle ratio from 100% to 300%, due to more NO₃⁻-N and oxygen brought to anoxic section. In this study, improvements in pollutant removal rate and N₂O reduction revealed apparently a contradiction. In WWTPs, balance must be established between TN removal effects and N₂O production. New evaluation systems of wastewater treatment processes considering water quality and N₂O emissions may be considered in future research. Moreover, N₂O reductase is closely related with N₂O generation and more sensitive to oxygen than nitrate reductase and nitrite reductase. Higher internal recycle rate could inhibit the nosZ gene-containing bacteria in treatment process, thereby the system revealed decreased ability to reduce N₂O to N₂. Functional gene expression of nosZ was affected largely than the bacterial community composition by variations in operating parameters of internal recycle rate.

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