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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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\textbf{A B S T R A C T}

To evaluate the effects of internal recycle ratio on nitrous oxide (N\textsubscript{2}O) generation, we set up three laboratory-scale anaerobic–anoxic–oxic (A\textsuperscript{2}O\textsubscript{3}) 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%. N\textsubscript{2}O generation was increased from 3.47 to 9.81 × 10\textsuperscript{-2} mg/L during the treatment process, with the anoxic section showing the largest N\textsubscript{2}O increment from denitrification. This phenomenon is attributed to the increased amount of nitrate (NO\textsubscript{3}\textsuperscript{-}–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 N\textsubscript{2}O to N\textsubscript{2}.

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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 (N\textsubscript{2}O) emissions during the biological nitrogen removal (BNR) process. It is arrestive that N\textsubscript{2}O is about 300 times more greenhouse effective than carbon dioxide (CO\textsubscript{2}), and its emission increased with a high rate of around 0.3% per year [1]. About 3.2% of all global anthropogenic N\textsubscript{2}O emissions originate from the BNR processes of WWTPs [2]. Considering the widespread use of BNR processes, as well as the hazards of N\textsubscript{2}O, investigation of the effect of key operating parameters and sources of N\textsubscript{2}O emissions on the overall BNR process is an important undertaking.

N\textsubscript{2}O can be produced from both nitrification and denitrification in BNR processes. Nitrification refers to the stepwise autotrophic oxidation of ammonia (NH\textsubscript{4}\textsuperscript{+}) to nitrite (NO\textsubscript{2}\textsuperscript{-}) by ammonia-oxidizing bacteria (AOB), and then to nitrate (NO\textsubscript{3}\textsuperscript{-}) by nitrite-oxidizing bacteria (NOB). During nitrification, N\textsubscript{2}O is produced through two pathways. The first is the aerobic hydroxylamine oxidation pathway, in which N\textsubscript{2}O 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 NO\textsubscript{2}\textsuperscript{-} as an electron acceptor (chemo-denitrification). The second pathway is nitrifier denitrification, which involves reduction of NO\textsubscript{2}\textsuperscript{-} 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 NO\textsubscript{3}\textsuperscript{-} to atmospheric nitrogen (N\textsubscript{2}) by heterotrophic denitrifiers, with NO\textsubscript{2}\textsuperscript{-}, nitric oxide (NO) and N\textsubscript{2}O as obligatory intermediates [5]. N\textsubscript{2}O, a well-known intermediate emitted in this process, is released in high quantities in environments with high dissolved oxygen (DO) and NO\textsubscript{2}\textsuperscript{-} concentrations and low C/N water quality [3,6,7].

In present studies, the amounts of N\textsubscript{2}O emitted during wastewater treatment processes are reported with high range of variation. Kampshure et al. [2] concluded that 0.0–95.0% and 0.0–14.5% of the influent nitrogen could be removed through N\textsubscript{2}O generation during lab-scale and full-scale wastewater treatment, respectively.

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To reduce N\textsubscript{2}O emissions, in recent years, significant efforts have been made to understand the effect of various WWTP operating parameters on N\textsubscript{2}O production [8–10]. As a result, several parameters favoring N\textsubscript{2}O 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\textsubscript{2}O generation. At present, however, the effects of internal recycle ratio changes on N\textsubscript{2}O 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\textsubscript{2}O emission characteristics and production mechanisms in BNR process. Three parallel laboratory-scale A\textsuperscript{2}O 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\textsubscript{2}O generation in each unit of the A\textsuperscript{2}O 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\textsuperscript{2}O 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\textsuperscript{2}O process had a total effective volume of 50L (Fig. 1). The volumes of the anaerobic section, anoxic section, and aerobic sections were 5, 10, 30, and 5L, respectively. The treatment system was seeded with sludge from the aerobic tank of Luotouwan 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\textsubscript{4}+ -N 38.7 ± 3.2 mg/L, NO\textsubscript{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 aerobic, anoxic andoxic 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 85L/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 500r/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 anoxic 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 sedimentation tank (US-52 Oteli Beijing) and operated at a rotating speed of 30r/min. Gas and liquid samples were obtained at the same time; liquid-phase samples were used to measure water quality parameters of COD, NH\textsubscript{4}+ -N, NO\textsubscript{3}− -N, NO\textsubscript{2}− -N, TN and TP. All measurements were carried out after the A\textsuperscript{2}O 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\textsubscript{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/m\textsuperscript{2} min), from the liquid surface in the static chamber was calculated using the equation:

\[ F = \frac{V}{A} \rho \left( \frac{\Delta c}{\Delta t} \right) \]

(1)

where V (m\textsuperscript{3}) is the volume from the liquid level to the top of chamber, A (m\textsuperscript{2}) is the enclosed surface area, \( \rho \) (mg/m\textsuperscript{3}) is the density of the gas at the temperature recorded in the chamber, and \( \Delta c (v/v)/\Delta t \) (min) is the change of gas concentration in the chamber per unit time during sampling. The gas density (\( \rho \)) is calculated by the equation:

\[ \rho = 10^{-6} \frac{MP}{RT} \]

(2)

where M is the molecular weight of N\textsubscript{2}O (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. Oxid 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/m\textsuperscript{2} min), was calculated by

\[ F = \frac{\Delta c d}{\Delta t A} \]

(3)

where \( \rho \) (mg/m\textsuperscript{3}) is the density of the gases at the sampling temperature, c (v/v) is the sample gas concentration, Q\textsubscript{a} (m\textsuperscript{3}/min) is the aeration rate, and A (m\textsuperscript{2}) 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.
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-Chemistation 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$^2$O process can be calculated using Eqs. (4)–(7).

\[ \begin{align*}
G_{An} &= E_{An} + C_{An} \times (1 + R_1) Q - C_{In} \times Q - C_{SST} \times R_1 Q \\
G_{Ax} &= E_{Ax} + C_{Ax} \times (1 + R_1 + R_2) Q - C_{An} \times (1 + R_1) Q - C_{Ax} \times R_2 Q \\
G_{Ae} &= E_{Ae} + C_{Ae} \times (1 + R_1 + R_2) Q - C_{Ax} \times (1 + R_1 + R_2) Q \\
G_{SST} &= E_{SST} + C_{SST} \times (1 + R_1) Q - C_{Ax} \times (1 + R_1) Q
\end{align*} \]

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; $C_{In}$, $C_{An}$, $C_{Ax}$, 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-GACGACGCTATCGACA) and nosZ-R (TCCATGGTGAACCGTGGTGT) 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 μL; 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 DH5α 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.
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-AGAACGACAGCTGATGCAGA-3 and nosZ-R: 5-TCCATGTTGACCGTGGTGGT-3 [26]. For nosZ gene PCR, 2 μL of extracted DNA was added to a PCR reaction mixture containing 12.5 μL of SYBR Premix Ex Taq (TaKaRa, Japan), 0.5 μL of nosZ-F (10 μM each), 0.5 μL of nosZ-R (10 μM each), and 9.5 μL of dH2O. qPCR was performed using a TaKaRa PCR Thermal Cycler Dice real-time PCR system (TaKaRa Code: TP8000) 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 (R² = 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 A^2^O 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 A^2^O 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 the A^2^O process. Variations in COD, NH₄⁺-N, NO₂⁻-N, TN and TP concentration detected among the units showed the distinctive functions of the anaerobic, anoxic, andoxic sections in the A^2^O process. While theoxic 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 inoxic 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). NO₂⁻-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, probably because more electron donors are needed during NO₃⁻ (NO₂⁻ and NO₃⁻) 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 efficiencies under higher internal recycle ratios (Fig. 2). The economic cost is directly related to the internal recycle rate; an increase in recycle 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%.

Table 1

| ORP (mV) | Anaerobic section | Anoxic section | Oxidation 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     |

3.2. N₂O generation and emission of A^2^O under different internal recycle ratios

The N₂O mass balances of dissolved liquid and gas phase N₂O obtained during A^2^O under different internal recycle ratios are shown in Fig. 3.

In this study, N₂O emission was detected in all units of the A^2^O treatment process studied (Fig. 3). Under each operation mode, maximal N₂O emission was detected inoxic section, whereas minimal N₂O emission was found in thesecondary 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 0.13g/l. 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 A^2^O 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 A^2^O treatment process, the N₂O generated in each unit was calculated according to Eqs. (4)–(7) (Table 2).

Table 2

| N₂O generation under the three operating modes studied. |
|--------------------------------------------------------|
| Parameters                                   | 100%  | 200%  | 300%  |
| Anoxic 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 |
| Oxidation 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 N₂O generation (mg/L)                  | 3.47 × 10⁻² | 6.59 × 10⁻² | 9.81 × 10⁻² |
| N₂O-N conversion ratio (%)                   | 0.14   | 0.20   | 0.21   |

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With the increased internal recycle ratio, \( \text{N}_2\text{O} \) emission increments were highest in the anoxic section among the units observed (Table 2), but part of this \( \text{N}_2\text{O} \) is released in the oxic section (Fig. 3). Denitrification mainly occurs in the anoxic section, and \( \text{N}_2\text{O} \) is produced as an intermediate product of this process [2]. As the internal recycle ratio increased, more \( \text{NO}_3^- \)-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 \( \text{NO}_3^- \)-N concentration and \( \text{NO}_2^- \)-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.

| Activity                          | 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$_2$O 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%.

The 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$_2$O reductase is more sensitive to oxygen than other enzymes, increased N$_2$O 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$_2$O generation in the anoxic section increased with increasing internal recycle ratio.

N$_2$O 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$_2$O (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$_2$O 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$_2$O is not present as an intermediate in the main catabolic pathway of nitrification, AOB are known to produce N$_2$O, which is predominantly associated with the nitrifier denitrification pathways [28]; N$_2$O 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$_2$O production from chemical reactions in active sludge systems is of minor importance. Thus, N$_2$O 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$_2$O emissions observed at higher internal recycle ratios [32,33]. Several researchers have found that N$_2$O 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$_2$O production. This hypothesis may also explain why the N$_2$O generated in every unit increased with increasing internal recycle ratio.

In contrast to expectations, the secondary settling tank produced N$_2$O during wastewater treatment; 32.9%, 23.4%, and 19.1% of the total N$_2$O were generated under internal ratios of 100%, 200% and 300%, respectively. This N$_2$O contribution is speculated to originate from the denitrification process because AOB and denitrification bacteria could denitrify NO$_3$ to N$_2$O or N$_2$ at low DO conditions [2]. High NO$_3$−N and low COD concentrations, which benefit N$_2$O 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 N₂O to N₂. To investigate the mechanisms of N₂O 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 A²0 system decreased sharply from over 1.0 × 10⁶ to less than 6.0 × 10⁵. However, nosZ gene copies between internal recycle ratios of 200% and 300% showed only slightly 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 A²0 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 N₂O to N₂ is decreased. This hypothesis may explain why N₂O 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 N₂O emission reductions could be achieved by regulating the expression of functional genes under different operating parameters. Practically, several strategies have been reported to mitigate N₂O emissions from wastewater denitrification [35]. Based on this research, ensuring sufficient anoxic HRT, bioaugmentation with the N₂O-reducing denitrifier *Pseudomonas stutzeri*, choosing methanol over ethanol and adding copper ions (10–100 mg/L) may reduce N₂O generation and simultaneously enhance TN removal under high internal recycle rate for A₂O process [34,36,37].

### 4. Conclusion

Internal recycle ratio has a significant impact on nitrogen removal and N₂O production in A²O treatment process. The N₂O generation and nitrogen removal rate both increased significantly.
with increasing internal recycle ratio from 100% to 300%, due to more NO\textsubscript{3}−−N and oxygen brought to anoxic section. In this study, improvements in pollutant removal rate and N\textsubscript{2}O reduction revealed apparently a contradiction. In WWTPs, balance must be established between TN removal effects and N\textsubscript{2}O production. New evaluation systems of wastewater treatment processes considering water quality and N\textsubscript{2}O emissions may be considered in future research. Moreover, N\textsubscript{2}O reductase is closely related with N\textsubscript{2}O generation and more sensitive to oxygen than nitrate reductase and nitrite reductase. Higher internal recycle ratio could inhibit the nosZ gene-containing bacteria in treatment process, thereby the system revealed decreased ability to reduce N\textsubscript{2}O to N\textsubscript{2}. 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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References

[1] IPCC, Climate Change 2007: Synthesis Report, IPCC, Geneva, 2007.
[2] M.J. Kampf, B. van der Star, H.A. Wieders, J.W. Mulder, M.M. Jetten, M.C.M. van Loosdrecht, Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment, Water Res. 42 (2008) 812–826.
[3] R.Y. Schultheiss, M. Kurni, W. Gujer, Release of nitric and nitrous oxides from denitrifying activated sludge, Water Res. 29 (1995) 215–226.
[4] K. Hanaki, Z. Hong, T. Matsu, Production of nitrous oxide gas during denitrification of wastewater, Water Sci. Technol. 26 (1992) 1027–1036.
[5] R.K. Hynes, R. Knowles, Production of nitrous oxide by nitrosomonas europaea—effects of acetylene, pH, and oxygen, Can. J. Microbiol. 30 (1984) 1397–1404.
[6] B. Schonharting, R. Rehner, J.W. Metzger, K. Krauth, M. Rizzi, Release of nitrous oxide (N\textsubscript{2}O) from denitrifying activated sludge caused by H\textsubscript{2}S-containing wastewater: quantification and application of a new mathematical model, Water Sci. Tech. 38 (1998) 237–246.
[7] Y.T. Ahn, S.-T. Kang, S.R. Chae, J.L. Lim, S.H. Lee, H.S. Shin, Effect of internal recycle ratio on the high-strength nitrogen wastewater treatment in the combined UBF/MBR system, Water Sci. Tech. 51 (2005) 241–247.
[8] P. Fongratikul, D.G. Wareham, P. Elefentisiotis, P. Charoenruk, Treatment of a sludge-water wastewater: effect of internal recycle ratio on effluent COD and the oxygen demand, total Kjeldahl nitrogen and total phosphorous removal, Environ. Technol. 33 (2011) 1755–1759.
[9] Y. Kimochi, Y. Inamori, M. Mizuoku, K. Xu, M. Matsumura, Nitrogen removal and N\textsubscript{2}O emission in a full-scale domestic wastewater plant with intermittent aeration, J. Ferment. Bioeng. 86 (1998) 202–206.
[10] CEBP, Standard Methods for Examination of Water and Wastewater (2002), 4th ed., China Environmental Science Press, Beijing, 2004.
[11] W.G. Zumft, F. Frunze, Discrimination of ascorbate-dependent nonenzymatic and enzymatic, membrane-bound reduction of nitric oxide in denitrifying Pseudomonas putrefaciens, Biochim. Biophys. Acta 681 (1982) 459–468.
[12] K. Chon, J.S. Chang, E. Lee, J. Lee, J. Ryu, J. Cho, Abundance of denitrifying genes coding for nitrate (nirG), nitrite (nirK), and nitrous oxide (nosZ) reductases in estuarine versus wastewater effluent-fed constructed wetlands, Ecol. Eng. 37 (2011) 64–69.
[13] J.A. Baeva, D. Gabriel, J. Lafuente, Effect of internal recycle on the nitrogen removal efficiency of an anoxic/anoxic/aerobic (A\textsuperscript{2}O\textsubscript{2}) wastewater treatment process (WWTP), Process Biochem. 39 (2004) 1615–1624.
[14] Z. Hu, J. Zhang, X. Xie, S. Liang, S. Li, Minimization of nitrous oxide emission from anoxic-oxic/biological nitrogen removal process: effect of influent COD/NH\textsubscript{4}\textsuperscript{+} ratio and feeding strategy, J. Biosci. Bioeng. 115 (2013) 272–278.
[15] J.H. Ahn, S.K. Kim, H. Park, B. Rahm, K. Papilla, K. Chandran, N\textsubscript{2}O emissions from activated sludge processes, 2008–2009: results of a national monitoring survey in the United States, Environ. Sci. Technol. 44 (2010) 4505–4511.
[16] S. Toyoda, Y. Suzuki, S. Hattori, K. Yamada, A. Fujii, N. Yoshida, R. Rouko, K. Murayama, H. Shimoi, Isotopomer analysis of production and consumption mechanisms of N\textsubscript{2}O and CH\textsubscript{4} in an advanced wastewater treatment system, Environ. Sci. Technol. 45 (2011) 917–922.
[17] F. Wunderlin, J. Mohn, A. Joss, L. Emmenegger, H. Siegrist, Mechanisms of N\textsubscript{2}O production in biological wastewater treatment under nitriying and denitrifying conditions, Water Res. 46 (2012) 1027–1037.
[18] J.M. Garrido, J. Moreno, R. Mendez-Pampin, J.M. Lema, Nitrous oxide production under toxic conditions in a denitrifying anoxic filter, Water Res. 32 (1998) 2550–2552.
[19] W.G.J.M. Benthum, Nitrogen removal in intermittently aeration biofilm airlift reactor, J. Environ. Eng. 128 (2002) 279–284.
[20] X.Y. Zhu, Y.G. Chen, Reduction of N\textsubscript{2}O and NO generation in anaerobic–aerobic (low dissolved oxygen) biological wastewater treatment process by using sludge alkaline fermentation liquid, Environ. Sci. Technol. 45 (2011) 2137–2143.
[21] H. Lu, K. Chandran, D. Stensel, Microbial ecology of denitrification in biological wastewater treatment, Water Res. 46 (2014) 237–254.
[22] K. Chandran, Factors promoting emissions of nitrous oxide and nitric oxide from denitrifying sequencing batch reactors operated with methanol and ethanol as electron donors, Biotechnol. Bioeng. 106 (2010) 390–398.
[23] J. Desloover, S.E. Vlaeminck, P. Claeure, W. Verstraete, N. Boon, Strategies to mitigate N\textsubscript{2}O emissions from biological nitrogen removal systems, Curr. Opin. Biotechnol. 23 (2012) 474–482.

Fig. 5: N\textsubscript{2}O gene copy numbers in each A\textsuperscript{2}O unit under different internal recycle rates.