Effects of SRT and DO on $N_2O$ reductase activity in an anoxic-oxic activated sludge system


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Abstract Nitrous oxide ($N_2O$) is emitted from wastewater treatment processes, and is known to be a greenhouse gas contributing to global warming. It is thus important to develop technology that can suppress $N_2O$ emission. The effects of sludge retention time (SRT) and dissolved oxygen (DO) on $N_2O$ emission in an anoxic-oxic activated sludge system were estimated. Moreover, the microbial community structure in the sludge, which plays an important role in $N_2O$ suppression, was clarified based on nitrous oxide reductase ($nos$Z) gene analysis by molecular biological techniques. The results showed that under low SRT conditions, nitrification efficiency was reduced and the $N_2O$ emission rate in the oxic reactors was increased. It was also observed that $N_2O$ emission was enhanced under low DO conditions, where the available oxygen is insufficient for nitrification. Moreover, molecular analysis revealed that the clones identified in this study were closely related to $Ralstonia eutropha$ and $Paracoccus denitrificans$. The fact that the identified sequences are not closely related to known cultivable denitrifier $nos$Z sequences indicates a substantial in situ diversity of denitrifiers contributing to $N_2O$ suppression, which are not reflected in the cultivatable fraction of the population. The further application of these new molecular techniques should serve to enhance our knowledge of the microbial community of denitrifying bacteria contributing to $N_2O$ suppression in wastewater treatment systems.

Keywords Denitrification; dissolved oxygen; global warming; nitrification; $nos$Z; sludge retention time

Introduction

Wastewater treatment processes produce three major greenhouse gases (CO$_2$, CH$_4$ and $N_2O$) that contribute to global warming. The contribution per molecule of $N_2O$ to the greenhouse effect is 200–300 times more than that of CO$_2$. The concentration of atmospheric $N_2O$ is estimated to be approximately 310 ppbv, which is about 8% greater than that during the pre-industrial era, and it is increasing at a rate of 0.2–0.3% per year. Although the concentration of $N_2O$ in the atmosphere is about a thousandth of that of CO$_2$, its large contribution to the greenhouse effect can by no means be neglected. It is therefore of great importance to develop technology that can suppress $N_2O$ emission from wastewater treatment processes.

Nitrous oxide is a byproduct of microbial nitrification and denitrification, which are the main mechanisms for nitrogen removal in wastewater treatment processes; therefore it is not surprising that high $N_2O$ emissions have been detected in a variety of wastewater treatment facilities. Zheng et al. (1994) reported that in anoxic treatment of wastewater, 0–19% of nitrogen was removed in the form of $N_2O$. Hanaki et al. (1990) observed a production of 24.1 mg of $N_2O$-N per m$^3$ of wastewater in an activated sludge system. It was reported that the availability of oxygen is a determining factor in $N_2O$ production under both oxic and
anoxic conditions (Kimochi et al., 1998). According to these reports, nitrification under strict aerobic condition results in negligible N\textsubscript{2}O production. Moreover, N\textsubscript{2}O is mainly formed at moderate O\textsubscript{2} concentrations. On the other hand, N\textsubscript{2}O is an intermediate of denitrification, which is finally reduced to N\textsubscript{2} gas by N\textsubscript{2}O reductase. N\textsubscript{2}O reductase has been reported to become unstable in the presence of oxygen (Okayasu et al., 1997). It should also be noted that N\textsubscript{2}O emission varies depending on the type of treatment process, the scale of the facilities, and other factors. Further investigation is therefore necessary to determine the optimal conditions for the suppression of N\textsubscript{2}O emission from an advanced wastewater treatment process.

There are many types of microorganisms that can reduce N\textsubscript{2}O (Anderson et al., 1993; Wu et al., 1995). However, little is known about the conditions leading to reduced N\textsubscript{2}O emission in complex ecological systems such as activated sludge. The knowledge of the microbial species that play dominant roles in reducing N\textsubscript{2}O emission is very important in order to modify their environment and to control their activity. Classical microbiological techniques are now considered insufficient for the natural study of bacterial assemblages, since the majority of bacteria are widely believed to be unculturable by traditional techniques (Amann et al., 1995). The current revolution in molecular biology has allowed microbiologists to overcome the limitations of culturability. However, molecular identification based on 16S rRNA (DNA) does not necessarily correspond to metabolic function such as N\textsubscript{2}O reduction. Therefore, to study a phylogenetically widespread process such as denitrification, analysis based on a functional gene is useful in determining the structure of a microbial community.

In this study, we investigated the effects of sludge retention time (SRT) and dissolved oxygen (DO) on N\textsubscript{2}O emission and then clarified the microbial community structure in the sludge based on nitrous oxide reductase (\textit{nosZ}) gene analysis using molecular biology techniques.

**Materials and methods**

**Wastewater treatment system**

Three series of anoxic-oxic activated sludge systems, which treated domestic wastewater, were used to examine the effects of SRT on N\textsubscript{2}O emission. These systems each consisted of three anoxic and two oxic reactors. The SRTs were controlled at 7 (Run 1), 10 (Run 2) and 20 days (Run 3) with a constant DO concentration in the oxic reactors of 2.0 mg·l\textsuperscript{-1}. The volume of each reactor was 6 l. Treated water was pumped up through a membrane (Pore size: 4.0 µm, Yuasa Co. Japan) sunk into the second oxic reactor. The hydraulic retention time (HRT) through these systems was 8 h. The liquid recirculation ratio was 400% for each system.

To examine the effect of DO concentration, three series of anoxic-oxic activated sludge systems composed of two anoxic, two oxic reactors and one sedimentation tank were used. The volume of each reactor was 6 l. The DO concentrations in the oxic reactors were controlled at 0.3 (Run 4), 2.0 (Run 5) and 4.0 (Run 6) mg·l\textsuperscript{-1}, respectively, with a constant SRT of 13 days. The hydraulic retention time (HRT) through these systems was 8 h. The sludge and liquid recirculation ratios were 50% and 350%, respectively.

**Analytical methods for water and gas samples**

The concentrations of nitrogenous compounds (ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, and total nitrogen) in the influent and effluent of the reactors were analyzed by automated colorimetric methods using a TRAACS-2000 (Bran+Luebbe Inc., Japan) instrument. The concentration of N\textsubscript{2}O gas was determined by a gas chromatograph equipped with an electron-capture detector (ECD) and a packed Poropak Q column (Shimadzu Co.,
The temperature of the detector and column were 340°C and 80°C, respectively. Argon containing methane (5%) was supplied as a carrier gas and the flow rate was 40 ml·min^{-1}.

The nitrification efficiency $Ne$ (%) was calculated as follows:

$$Ne = \frac{(TN_{in} - NOx_{in}) - (TN_{out} - NOx_{out})}{TN_{in} - NOx_{in}} \times 100$$

where $TN_{in}$ and $NOx_{in}$ are the total nitrogen and $NOx$ concentration (mg·l^{-1}) in the influent, respectively; $TN_{out}$ and $NOx_{out}$ are the total nitrogen and $NOx$ concentration (mg·l^{-1}) in the effluent, respectively.

The nitrogen removal ratio $Nr$ (%) was calculated as follows:

$$Nr = \frac{(TN_{in} - TN_{out})}{TN_{in}} \times 100$$

The symbols are the same as those in Eq. (1).

**Calculation of $N_2O$ emission rate and dissolved $N_2O$ concentration**

The $N_2O$ emission rate from an oxic reactor was measured by a sampling bag technique and the $N_2O$ emission rate from an anoxic reactor was measured by a gas replacement technique using a syringe according to Kimochi et al. (1998). The emission rate of $N_2O$ from the oxic reactor, $F_{oxic}$ (mg·m^{-3}·min^{-1}) was calculated as follows:

$$F_{oxic} = \frac{Q \cdot \omega_{air} \cdot M \cdot P}{R \cdot T \cdot V_1}$$

where $Q$ is the volumetric flow rate of aeration (min^{-1}); $\omega_{air}$ is the $N_2O$ concentration in the aerated gases (V/V) analyzed by ECD-GC; $M$ is the molecular weight of $N_2O$ (44.02); $P$ is the atmospheric pressure (1 atm); $R$ is the gas constant (0.082 l·atm·K^{-1}·mol^{-1}); $T$ is the temperature (K); and $V_1$ is the volume of the aeration reactor (m^{3}).

The $N_2O$ emission rate from the anoxic reactor was calculated as follows:

$$F_{anox} = \frac{V_2 \cdot \omega_{air} \cdot M \cdot P}{(R \cdot T \cdot V_1 \cdot \Delta t)}$$

where $F_{anox}$ is the emission rate of $N_2O$ from the anoxic reactor; $V_2$ is the head space volume of the anoxic reactor (l); $\omega_{air}$ is the $N_2O$ concentration of the head space (V/V); $\Delta t$ is the time between argon gas replacement and gas sampling (min). All other symbols are the same as those in Eq. (3).

The dissolved $N_2O$ concentration was calculated as follows:

$$C = \frac{(1+\beta) \cdot \omega_{dis} \cdot M \cdot P}{(R \cdot T)}$$

where $C$ is the $N_2O$ concentration dissolved in a unit volume of the water sample (g·m^{-3}); $\omega_{dis}$ is the $N_2O$ concentration in the gas phase in the syringe (V/V); and $\beta$ is Ostwald’s solubility coefficient for $N_2O$.

**DNA extraction and PCR amplification**

DNA extraction was performed according to the methods of Smalla (1995) with slight modification. The oligonucleotide primers Nos1527F and Nos1773R were used to selectively amplify nosZ genes (Scala and Kerkhof, 1998). Amplification was performed with a model 9700 thermal cycler (Applied Biosystems, USA). The mixtures used for PCR amplification of bacterial sequences contained 25 ng of extracted DNA, 0.5 U/µmol l^{-1} of each
primer, 200 µmol l–1 of each deoxynucleoside triphosphate, 1.5 mmol l–1 MgCl₂, 0.05 U/µl of TaKaRa Taq polymerase (TAKARA Co., Ltd., Japan), 5 µl of 10 × PCR buffer for TaKaRa Taq and sterile water to a final volume of 50 µl. PCR amplification was carried out using the following program: 94°C for 5 min; 30 cycles of denaturation at 94°C for 0.5 min, annealing at 51°C for 0.5 min, and extension at 72°C for 0.5 min; followed by a single final extension at 72°C for 5 min. The presence of PCR products was confirmed by analyzing 8 µl of product on 2% agarose gels stained with ethidium bromide.

Cloning and sequencing of PCR products

PCR products were cloned using the PCR cloning plus Kit (QIAGEN Inc., USA) according to the manufacturer’s instructions. The transformed clones were checked by Insert Check PCR Mix (TAKARA Co., Ltd., Japan), and were reamplified in accordance with the procedure described above. After reamplification of the transformed clone, the PCR products were purified using a PCR purification kit (QIAGEN Inc., USA), and used as template DNA in a cycle sequencing reaction with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer’s instructions. Sequencing of nosZ fragments was carried out with an ABI PRISM 377 DNA Sequencing System (Applied Biosystems).

Phylogenetic analysis

Database searches were conducted using the sequencing match program of the RDP II (Ribosomal Database Project II) (Maidak et al., 2001). The sequences determined in this study and those retrieved from the databases were aligned by Clustal W (Thompson et al., 1994) with the DDBJ (DNA Data Bank of Japan). Neighbor-joining trees (Saitou and Nei, 1987) were constructed by Clustal W with the DDBJ and Tree View.

Results and discussion

Influence of SRT on nitrogen removal

The anoxic-oxic activated sludge systems were operated for a period of approximately 2 months. The influent nitrogen composition in the treatment systems was 25–30 mg·l–1 total nitrogen, with 20–25 mg·l –1 NH₄-N and a negligible concentration of NOₓ-N. The water qualities of Runs 1, 2 and 3 in terms of nitrogen removal are shown in Figures 1 and 2. After 11 days, the nitrification efficiencies in the oxic reactor were about 80% in Run 3. However, in Runs 1 and 2, they were only about 50%. Moreover, the nitrogen removal ratio of the total system was approximately 65% in Run 3, while only about 45% in Runs 1 and 2. These findings indicate that a longer SRT, such as 20 days, at start-up will result in early stabilization of nitrogen removal.

Since activated sludge was flowed out from day 21 in Run 1 due to unexpected system trouble, the concentration of MLSS decreased with time. Therefore, both nitrification efficiency and the nitrogen removal ratio began to decrease. On the other hand, high nitrification efficiency and nitrogen removal ratio were maintained in Runs 2 and 3. It was suggested that longer SRT resulted in increased nitrification efficiency and nitrogen removal ratio due to higher MLSS concentrations.

Influence of SRT on N₂O emission

The rate of N₂O emission into the atmosphere and the concentration of dissolved N₂O in the oxic and anoxic reactors on day 21 and 50 are shown in Table 1. It was found that as the SRT was reduced, the emission rate of N₂O, and the concentration of dissolved N₂O became higher in the oxic reactor. In particular, the N₂O emission rate from the oxic reactor in Run 1 was approximately 10 times as high as that in Run 3. In the anoxic reactor, the N₂O
emission rate increased with decreasing SRT. Moreover, the concentration of dissolved 
\( \text{N}_2\text{O} \) in Run 1 was the highest of all runs, and it gradually decreased as the SRT was extended. It was thus observed that the \( \text{N}_2\text{O} \) emission rate and concentration of dissolved \( \text{N}_2\text{O} \) in both oxic and anoxic reactors decreased with increasing SRT.

The conversion ratio of influent nitrogen to \( \text{N}_2\text{O} \) is shown in Figure 3. The conversion ratio of influent nitrogen to \( \text{N}_2\text{O} \) in Run 1 increased rapidly from day 35, induced by the reduction of MLSS concentration due to system trouble. On the other hand, the conversion ratios of influent nitrogen to \( \text{N}_2\text{O} \) in Runs 2 and 3 were kept below 0.2% over the entire operating period. Therefore, for the sake of reducing \( \text{N}_2\text{O} \) emission, it is important to keep sufficient MLSS concentration in both the oxic and anoxic reactors.

**Influence of DO concentration on nitrogen removal**

The nitrification efficiencies and nitrogen removal ratio of Runs 4, 5 and 6 are shown in Figures 4 and 5. In order to estimate the influence of DO concentration on nitrogen removal

<table>
<thead>
<tr>
<th>Day</th>
<th>Run</th>
<th>21</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SRT</td>
<td>(7 days)</td>
<td>(10 days)</td>
</tr>
<tr>
<td>N(_2)O emission rate ((10^{-3} \text{ mg·min}^{-1}))</td>
<td>Anoxic reactor</td>
<td>0.0437</td>
<td>0.0380</td>
</tr>
<tr>
<td></td>
<td>Oxic reactor</td>
<td>1.72</td>
<td>1.00</td>
</tr>
<tr>
<td>Dissolved (\text{N}_2\text{O}) concentration ((10^{-4} \text{ mg·l}^{-1}))</td>
<td>Anoxic reactor</td>
<td>2.05</td>
<td>0.730</td>
</tr>
<tr>
<td></td>
<td>Oxic reactor</td>
<td>1.35</td>
<td>0.568</td>
</tr>
</tbody>
</table>

**Figure 3**  The conversion ratio of influent nitrogen to \( \text{N}_2\text{O} \)
and \( \text{N}_2\text{O} \) emission, the DO concentration of all systems was controlled from day 32. After this operation, both the nitrification efficiency and nitrogen removal ratio in Run 4 immediately began to decrease and, 76 days after this operation, dropped to 36% and 35%, respectively. In contrast, the nitrification efficiency in Runs 5 and 6 was stable at a high value of 95%, and the nitrogen removal ratios in those were also high, at a value of 75%. It was suggested that the low nitrification efficiency in Run 1 was caused by the reduction of activity and quantity of nitrifying bacteria due to insufficient oxygen supply.

**Influence of DO concentration on \( \text{N}_2\text{O} \) emission**

The \( \text{N}_2\text{O} \) emission rate into the atmosphere and the concentration of dissolved \( \text{N}_2\text{O} \) in the oxic and anoxic reactors on day 38 and 55 are shown in Figure 6. In the first oxic reactor of Run 5, the \( \text{N}_2\text{O} \) emission rate was significantly increased compared with that of Runs 4 and 6. On day 38, the \( \text{N}_2\text{O} \) emission rate of Run 5 was 4.5 times as much as that of Run 4, and 2.6 times as much as that of Run 6. At the first oxic reactor of Run 5, nitrification did not progress sufficiently, and thus accumulation of ammonia and/or nitrite was observed. In contrast, no accumulation of ammonia or nitrite was observed in Run 6. It was thus observed that \( \text{N}_2\text{O} \) emission is accelerated under conditions where DO is so low as to result in insufficient nitrification.

![Figure 4 Time courses of nitrification efficiency](image1)

![Figure 5 Time courses of nitrogen removal ratio](image2)

![Figure 6 \( \text{N}_2\text{O} \) emission rate and dissolved \( \text{N}_2\text{O} \) concentration](image3)
On the other hand, the concentration of dissolved N$_2$O in the first anoxic reactor of Run 6 was much higher than that of Runs 4 and 5 on day 38 and 55. The DO concentration in the oxic reactor of Run 6 was the highest of all runs. Moreover, the ORPs in the first anoxic reactors of Run 4, 5 and 6 on day 38 were $-189$, $-165$ and $-88$ mV, respectively. It was suggested that DO transferred by water recirculation from the oxic to the anoxic reactors inactivated nitrous oxide reductase in the anoxic reactor. These findings indicated that an increase in N$_2$O emission was induced by lower DO concentration in the oxic reactors, and a high DO concentration in the anoxic reactors. The conversion ratio of influent nitrogen to N$_2$O after DO control is shown in Figure 7. While the conversion ratio of influent nitrogen to N$_2$O rapidly increased, initially, it then dropped to under 0.05%, similar to Run 6. On the other hand, the conversion ratio of influent nitrogen to N$_2$O in Run 6 was kept under 0.05% during the entire DO-control period.

**Sequence analysis of nosZ gene fragment**

Amplification of the nosZ gene from genomic DNA was attempted for two activated sludge samples collected from the first anoxic reactor of Runs 5 and 6. Twenty-two unique nosZ gene sequences were obtained in this study. After phylogenetic analysis, these unique clones were separated into three groups. The first was the group clustered with *Ralstonia eutropha*. Thirteen clones (8 from Run 5 and 5 from Run 6) belonged to this group, which clearly dominated in Runs 5 and 6. The second was the group clustered with *Paracoccus denitrificans*. Six clones (1 from Run 5 and 4 from Run 6) belonged to this group. None of the sequences registered in the database were clustered with the third group. This group was composed of 2 clones from Run 5 and one clone from Run 6.

Scala and Kerkhof (1998) reported that the nosZ gene sequences from continental shelf sediments were closely related to those of *Paracoccus denitrificans* or *Rhizobium meliloti*. In this study, most of the identified clones were closely related to *Ralstonia eutropha*. It was thus observed that unique nosZ sequences in our activated sludge were different from those present in DDBJ, including those identified from continental shelf sediments. Moreover, the fact that the identified sequences are not closely related to known culturable denitrifier nosZ sequences, indicates a substantial in situ diversity of denitrifiers contributing to N$_2$O suppression, which are not reflected in the cultivatable fraction of the population. The further application of these new molecular techniques should serve to enhance our knowledge of the microbial community structure of denitrifying bacteria contributing to N$_2$O suppression in wastewater treatment systems.

![Figure 7](https://iwaponline.com/wst/article-pdf/48/11-12/363/421947/363.pdf)
Conclusions
The effects of SRT and DO on N₂O emission and the diversity of the gene coding N₂O reductase in the anoxic-oxic activated sludge system were investigated, and the following results were obtained.

1. Under low SRT conditions, nitrification efficiency was decreased and N₂O emission rate in the oxic reactors was increased. The conversion ratios of influent nitrogen to N₂O in Runs 2 and 3, which were operated with long SRT values, were kept under 0.2% during the entire operating period.

2. It was revealed that N₂O emission was accelerated under low DO conditions insufficient for nitrification.

3. Molecular analysis revealed that the 22 unique nosZ gene sequences obtained in this study and identified clones were closely related to Ralstonia eutropha and Paracoccus denitrificans.

References