Isotopocule characterization of N₂O dynamics during simulated wastewater treatment under oxic and anoxic conditions

AZZAYA TUMENDELGER,1,2+ SAKAE TOYODA,3 NAOHIRO YOSHIDA,1,4 HIROSHI SHIOMI5 and RINA KOUNO5,6

1Department of Environmental Chemistry and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan
2Institute of Chemistry and Chemical Technology, Mongolian Academy of Science, MAS 4th Building, Peace Avenue, Bayanzurkh District, Ulaanbaatar 13330, Mongolia
3Department of Environmental Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan
4Earth-Life Science Institute, Tokyo Institute of Technology, Meguro-ku, Tokyo 152-8551, Japan
5Bureau of Sewerage, Tokyo Metropolitan Government, 2-8-1 Nishi-shinjyuku, Shinjyuku-ku, Tokyo 163-8001, Japan
6Bureau of Waterworks, Tokyo Metropolitan Government, 2-8-1 Nishi-shinjyuku, Shinjyuku-ku, Tokyo 163-8001, Japan

*Corresponding author (e-mail: azzaya@icct.mas.ac.mn; azzaya.usb@gmail.com)

Copyright © 2016 by The Geochemical Society of Japan.

**INTRODUCTION**

Since the Industrial Revolution, concentrations of atmospheric nitrous oxide (N₂O), a powerful greenhouse gas, have increased by 20% from 271 ppb in pre-industrial era to 324 ppb at present as a result of anthropogenic activity (IPCC, 2007). It adversely affects the stratosphere, where it breaks down and acts as a catalyst in ozone layer destruction (Ravishankara et al., 2009). Although it is not as abundant in the atmosphere as carbon dioxide (CO₂), its radiative forcing is about 300 times greater on a per-molecule basis than that of CO₂ (IPCC, 2007). Different microbial pathways involved in biological nitrogen removal processes in wastewater treatment plants (WWTPs) can produce N₂O as well as more favorable gaseous product, N₂ (Colliver and Stephenson, 2000; Kimochi et al., 1998). During nitrification, N₂O is produced from hydroxylamine (NH₂OH) as a byproduct of ammonium (NH₄⁺) oxidation to nitrite (NO₂⁻) by ammonia oxidizing bacteria. The concentration of dissolved oxygen (DO) should be maintained at an appropriate level because nitrifying bacteria require oxygen as an electron acceptor. During denitrification, N₂O is produced as an intermediate during nitrate (NO₃⁻) reduction to dinitrogen (N₂) by heterotrophic denitrifying bacteria in the absence of oxygen. Organics are used as electron donors in denitrification. Therefore, a sufficient amount of carbon
is necessary to achieve effective nitrogen removal (Hanaki et al., 1992; Itokawa et al., 1996). Moreover, N\textsubscript{2}O is producible through the nitrifier-denitrification pathway, which is the oxidation of NH\textsubscript{4}+ to NO\textsubscript{2}- followed by the reduction of NO\textsubscript{2} to N\textsubscript{2}O by autotrophic nitrifiers under insufficient oxygen conditions (Colliver and Stephenson, 2000; Wrage et al., 2001).

Global N\textsubscript{2}O emissions from WWTPs are estimated as 0.22 Tg-N yr\textsuperscript{-1}, which accounts for approximately 2.8% of all anthropogenic sources (IPCC, 2007). To control and reduce N\textsubscript{2}O emissions from WWTPs, key factors leading to N\textsubscript{2}O production should be well managed during the biological nitrogen removal process. To date, key factors enhancing N\textsubscript{2}O production were reportedambiguously in the literature as low DO, high NO\textsubscript{2}- accumulation or low carbon/nitrogen (C/N) ratios during denitrification (Toyoda et al., 2001; Kampeschreur et al., 2008, 2009; Tallec et al., 2006a; Wunderlin et al., 2012) as well as short solid retention time (SRT) (Zheng et al., 1994; Noda et al., 2003). Therefore, identification of main N\textsubscript{2}O production pathway and key factors leading to its production is necessary to develop wastewater treatment processes with low N\textsubscript{2}O emission.

A few studies have provided information related to sources and production and consumption processes of N\textsubscript{2}O using isotope ratios. By observations in full-scale WWTPs, nitrifier-denitrification was found to be the main contributor to N\textsubscript{2}O production in an oxic tank, whereas NO\textsubscript{2}- reduction by heterotrophic denitrifier was the main source of N\textsubscript{2}O in an anoxic tank (Townsend-Small et al., 2011; Toyoda et al., 2011a; Tumendelger et al., 2014). Batch incubation experiments conducted under specific conditions demonstrated that nitrifier-denitrification dominantly produced N\textsubscript{2}O in a multistep process of NH\textsubscript{4}+ oxidation (Wunderlin et al., 2013). However, more isotopic studies must be done to elucidate the relative contributions from each N\textsubscript{2}O production pathway and the occurrence of N\textsubscript{2}O reduction in wastewater treatment operated under different conditions. For instance, no reports describe studies examining isotopic fractionation during N\textsubscript{2}O reduction by activated sludge of a WWTP.

Natural abundance ratios of isotopocouples (=moleculare species that only differ in either the number or positions of isotopic substitutions, Coplen, 2011) of N\textsubscript{2}O are useful tools for elucidating N\textsubscript{2}O dynamics because they reflect the isotopic composition of the precursor materials (Kim and Graig, 1993; Yoshida et al., 1989). In addition, analytical methods for determining the intramolecular \textsuperscript{15}N distribution in the asymmetric N\textsubscript{2}O molecule have been developed by Toyoda and Yoshida (1999) and Waechter et al. (2008). The \textsuperscript{15}N-site preference, SP (difference in \textsuperscript{15}/\textsuperscript{14}N isotope ratio between central (\textalpha{}) and terminal (\textbeta{}) N), provides a new parameter to interpret N\textsubscript{2}O production mechanisms and to estimate the global N\textsubscript{2}O budget (Toyoda et al., 2015; Yoshida and Toyoda, 2000). Earlier studies show that SP is independent of the substrate’s isotopic signature, and unique value reflecting microbial production pathways (Sutka et al., 2003, 2004, 2006; Toyoda et al., 2005). For example, the SP of N\textsubscript{2}O produced by NH\textsubscript{2}OH oxidation is around +33%, whereas the SP of N\textsubscript{2}O produced by NO\textsubscript{2}- reduction is around 0%. This significant difference enables SP to be used to distinguish production pathways in the environment. Therefore, isotopocouple analysis can provide qualitative information that supplements the quantitative information produced by concentration analysis alone.

This study was conducted to elucidate the dependence of production and consumption mechanisms of N\textsubscript{2}O during wastewater treatment on controlling factors such as DO, the C/N ratio, and water temperature. To accomplish this, we set up a series of oxic or anoxic batch-scale experiments with activated sewage sludge taken from a full-scale WWTP under variable experimental conditions. We first measured temporal changes in concentrations and isotope ratios of N\textsubscript{2}O during its decomposition to reveal the effects of mixed liquor suspended solids (MLSS) on the N\textsubscript{2}O reduction process and to estimate isotopic enrichment factors (\textepsilon{}) during N\textsubscript{2}O reduction by activated sludge. Then we made time series measurements of concentrations and isotope ratios of N\textsubscript{2}O and potential substrates (NH\textsubscript{4}+ and NO\textsubscript{3}-) to identify N\textsubscript{2}O production pathways and to examine the occurrence of N\textsubscript{2}O reduction.

**Experimental**

**Lab-scale experimental reactor**

A laboratory-scale incubation reactor with working volume of 30 L (Fig. 1) was filled with activated sludge.
taken from a municipal WWTP located in eastern Tokyo. The head space in the incubation vessel (about 7 L) was purged continuously with N2 gas flow of about 4 L min⁻¹ using a flow controller to monitor the N2O concentration. Air was supplied from the vessel bottom using three flow controllers to adjust the oxygen concentrations in oxic (nitrification) experiments. Measurements of DO, pH, and oxidation-reduction potential (ORP) were taken, respectively, using oxygen, pH, and ORP electrodes (DO-31P, HM-31P; TOA-DDK Corp., Tokyo, Japan). The pH was adjusted using sodium bicarbonate (NaHCO3) at around 7.0 in an oxic experiment.

**Batch incubation experiments**

Each set of experiments was conducted at 25°C with activated sludge samples obtained in autumn (October–November, 2010) and replicated at 18°C with activated sludge samples obtained in winter (February, 2011) from biological oxic and anoxic reaction tanks at the WWTP. For N2O decomposition experiments, activated sludge was collected in March, 2011.

**N2O decomposition experiments (R1 and R2)**

Activated sludge collected from the anoxic reaction tank was kept under anoxic conditions until all the NO3⁻ and NO2⁻ were completely consumed. Then it was diluted to 30 L with distilled water and was put into the incubation reactor. The experiments were conducted respectively at different MLSS concentrations: 189.2 mg L⁻¹ (R1) and 94.6 mg L⁻¹ (R2). After confirming that all DO was consumed and anoxic condition was established, the dissolved N2O concentration was adjusted to approximately 30 μmol L⁻¹ by bubbling with N2O standard gas (1000 ppm) for about 1 hour at the rate of 4 L min⁻¹. Then the N2O supply was stopped and experiments were started by adding organic carbon (100 mL of 22.2 g L⁻¹, CH3COONa). Samples for dissolved N2O analysis were collected immediately after bubbling with N2O standard, 1 min after the addition of organic carbon (t = 0), t = 15 and 30 min. They were transferred into 125 ml glass vials (Maruemu Corp. Co. Ltd., Osaka, Japan), sterilized with 5 ml of saturated HgCl2 solution to prevent microbial N2O production or consumption, and then sealed with butyl rubber stoppers and aluminum caps with special care taken to exclude air bubbles. They were stored at 4°C and were analyzed within four weeks. Results confirmed that no significant change occurred in the dissolved concentration or isotopocule ratios of N2O (data not shown).

**Anoxic experiments (D1–D4)**

The experimental conditions of the anoxic N2O production experiments are presented in Table 1. Activated sludge was collected from an anoxic reaction tank and was diluted to 30 L. Then it was put into the incubation reactor. After confirming that all DO was consumed and that anoxic condition was established, experiments were started by adding nitrate (KNO3; 3.8 g·NL⁻¹) and organic carbon (CH3COOH; 22.2 g L⁻¹) as substrates to adjust the initial NO3⁻ concentration and C/N ratio. The water temperatures were set at 25°C and 18°C in autumn and winter experiments, respectively. Anoxic conditions were confirmed by the ORP measurement. They were negative at both temperatures. The pH levels were, respectively 7–9 and 7–8.5 at 25°C and 18°C. Experiments (D1–D4) were conducted under

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Key controlling factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DO⁺ [mg L⁻¹]</td>
</tr>
<tr>
<td></td>
<td>(25°C)</td>
</tr>
<tr>
<td>Oxic</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>0.5</td>
</tr>
<tr>
<td>N2</td>
<td>1.0</td>
</tr>
<tr>
<td>N3</td>
<td>2.0</td>
</tr>
<tr>
<td>N4</td>
<td>3.0</td>
</tr>
<tr>
<td>Anoxic</td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>na</td>
</tr>
<tr>
<td>D2</td>
<td>na</td>
</tr>
<tr>
<td>D3</td>
<td>na</td>
</tr>
<tr>
<td>D4</td>
<td>na</td>
</tr>
</tbody>
</table>

na: not applicable.

*Set point value around which actual value was kept constant.
*Measured initial value with target value in parentheses.
*Measured initial value. Target value was 1.4.
*Measured initial value. Target value was 1.5.

Table 1. Experimental conditions applied in oxic and anoxic batch tests
different C/N ratios (C/N ratio of 0.68, 1.0, 1.4, and 1.7).

For concentration analyses of dissolved inorganic nitrogen (DIN) species, water was sampled from the incubation chamber at 15 min intervals until 60 min passed, and then at 30 min intervals until 240 min. Sampling for concentration and isotopic analyses of dissolved N2O was conducted less frequently for logistic reasons (e.g., available time for mass spectrometric analysis within the period for which collected samples can be stored without qualitative change), and sampling procedure was similar to those of N2O decomposition experiment. Six samplings were conducted in experiments D1, D3 and D4 whereas two samplings for experiment D2, respectively. For isotopic analysis of NO3–, three samplings were done in experiment D4 (Table 2).

**Oxic experiments (N1–N4)** The experimental conditions of the oxic N2O production experiments are presented in Table 1. Activated sludge was taken from an oxic reaction tank and was diluted to 30 L. Then it was put into the incubation reactor. The operating parameters (air flow, temperature and pH) were optimized to keep DO at a designated level before starting the experiment. Oxic conditions were confirmed by the ORP measurement. They were, respectively, +70–150 mV and about +200 mV at 25°C and 18°C. Experiments (N1–N4) were conducted under different oxygen concentrations (DO of 0.5, 1.0, 2.0 and 3.0 mg L−1). Although we tried to control the N-loading and MLSS concentration at the target values, they were difficult to control in some cases probably because of inhomogeneity of the activated sludge (target value of N-load and MLSS are shown in the footnotes of Table 1). The experiments were started by adding a 100-mL solution of NH4Cl (6 g-N L−1) as a substrate so that the initial NH4+ concentration became the target value. The pH was initially adjusted by adding NaHCO3 solution of >7.5 and >7, respectively, at 25°C and 18°C. Sampling procedures and frequency for concentration analyses of DIN species were similar to those of anoxic experiments. Sampling procedures for concentration and isotopic analysis of dissolved N2O are similar to those of anoxic experiments, and number of the collected samples were six or seven for experiments N1 and N3, and two for experiments N2 and N4. For isotopic measurement of NH4+, samplings were done three times for experiments N1 and N3 (Table 2).

**Analysis** The concentration of dissolved NH4+ was measured using a coulometric ammonia meter (MT-1; Central Kagaku Corp., Tokyo, Japan, and MM-60R; DKK-TOA Corp., Tokyo, Japan). The NO3– and NO2– were measured using an ion chromatograph equipped with a conductivity detector (DX-320; Dionex Corp., Osaka, Japan). Then N2O analysis was performed using an isotope-ratio mass spectrometer (IRMS, MAT252; Thermo Fisher Scientific K.K., Yokohama, Japan) with an on-line analytical system comprising a glass-made gas extraction chamber in which the water is sparged with ultrapure helium, a stainless steel gas transfer line, a pre-concentration trap, and chemical traps for removal of H2O and CO2 (Fujii et al., 2013; Yamagishi et al., 2007).

Site-specific nitrogen isotope analysis in N2O was conducted using ion detectors that had been modified for mass analysis of the N2O fragment ions (NO+), containing the N atoms in the central positions (α) of precursor N2O molecules, whereas bulk (average) nitrogen and oxygen isotope ratios were determined from molecular ions (N2O+∗). An aliquot of the water sample containing 1–5 nmol of N2O was measured gravimetrically and introduced to the gas extraction cham-
Isotopocule characterization of N₂O dynamics during simulated wastewater treatment

Pure N₂O gas (purity >99.999%; Showa Denko K.K., Japan) that had been calibrated previously with international standards was used as a laboratory standard for isotopocule ratios. The notation of the isotopocule ratio is the following.

\[ \delta X = \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 10^3 \]  

In that equation, \( X \) is \( ^{15}\text{N}a \), \( ^{15}\text{N}b \) or \( ^{18}\text{O} \), and \( R \) denotes the \( ^{15}\text{N}/^{14}\text{N} \) ratios at the center or end N atoms in NNO molecules or \( ^{18}\text{O}/^{16}\text{O} \) ratio. Subscripts “sample” and “standard” respectively signify isotope ratios of the sample and the standard. The \( \delta \) value is expressed as permil (‰).

Standards are atmospheric N₂ for N and Vienna Standard Mean Ocean water (VSMOW) for O. In addition, instead of \( \delta ^{15}\text{N}a \) and \( \delta ^{15}\text{N}b \), \( \delta ^{15}\text{N}_{\text{bulk}} \) and 15N-site preference (SP) are defined as illustrative parameters for N₂O (Toyoda and Yoshida, 1999).

\[ \delta ^{15}\text{N}_{\text{bulk}} = \left( \delta ^{15}\text{N}a + \delta ^{15}\text{N}b \right)/2 \]  

\[ \text{SP} = \delta ^{15}\text{N}a - \delta ^{15}\text{N}b. \]  

Measurement precision was typically better than 1% for concentration, +0.1‰ for \( \delta ^{15}\text{N}_{\text{bulk}} \), +0.5‰ for \( \delta ^{18}\text{O} \), and better than +0.4‰ for \( \delta ^{15}\text{N}a \) and \( \delta ^{15}\text{N}b \). The N₂O concentration was obtained simultaneously with the isotopocule ratios from the peak area of the major ions (masses 44 and 30 in molecular ion analysis and fragment ion analysis, respectively) measured in the sample water and standard gas (8.82 ppm N₂O in He).

If the N₂O reduction process is assumed to be negligible, the contributions of NO₂⁻ reduction (\( x \)) and NH₂OH oxidation (1 – \( x \)) to N₂O production are estimated using the SP value as presented below:

\[ \text{SP}_{\text{sample}} = x \text{SP}_{\text{NO₂⁻ reduction}} + (1 - x) \text{SP}_{\text{NH₂OH oxidation}} \]  

Therein, \( \text{SP}_{\text{NO₂⁻ reduction}} \) and \( \text{SP}_{\text{NH₂OH oxidation}} \) respectively, signify the SP values when N₂O is produced only by NO₂⁻ reduction and when N₂O is produced only by NH₂OH oxidation.

The \( \delta ^{15}\text{N} \) of NH₄⁺ was measured using the diffusion method (Holmes et al., 1998), where ammonium absorbed onto a glass fiber filter containing H₂SO₄ was converted to N₂, and analyzed using an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS) system (EA1110; Thermo Fisher Scientific K.K.). The \( \delta ^{15}\text{N} \) of NO₃⁻ was...
measured using the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001), where N₂O produced by *Pseudomonas aureofaciens* (NBRC 3521) from NO₃⁻ was analyzed as described above. The isotopic fractionation associated with possible incomplete diffusion or denitrification was canceled out by processing field samples and standards under identical conditions. The analytical precisions based on standard measurements were 0.3‰ and 0.2‰, respectively, for δ¹⁵N of NH₄⁺ and NO₃⁻.

Isotopic discrimination during consumption of N₂O, NH₄ or NO₃⁻ in a closed system is defined as isotope enrichment factor, ε(X) using a Rayleigh isotope fractionation model (Mariotti et al., 1981).

\[ \delta X = \delta_0 X + \varepsilon(X) \times \ln(C/C_0) \]  

Therein, C denotes the concentration. Subscript 0 represents the initial value.

**RESULTS**

**Effect of MLSS concentration on N₂O reduction**

Results of the N₂O decomposition experiments R1 and R2 are presented in Fig. 2. The dissolved N₂O concentrations were 25880 nmol kg⁻¹ and 26680 nmol kg⁻¹ in experiments R1 and R2, respectively, after the bubbling of standard N₂O gas (Fig. 2a). In both experiments, the concentration of N₂O sharply decreased during the 30 min time course of the experiment. The reduction rate in experiment R1 (850 nmol kg⁻¹ min⁻¹) with high MLSS concentration was higher than in R2 (533 nmol kg⁻¹ min⁻¹). The isotopocule ratios (δ¹⁵Nbulk, δ¹⁸O, and SP) of N₂O showed an intensive increment that ranged in –2.7 – 24.5‰ for δ¹⁵Nbulk, +28.6 – +87.0‰ for δ¹⁸O and –2.7 – +20.9‰ for SP in experiment R1 whereas it was –2.9 – 7.6‰, +24.2 – +55.0‰ and –6.7 – +3.3‰, respectively in experiment R2 (Figs. 2b–d).

**Effect of C/N ratio on N₂O production under anoxic conditions**

The time courses of the NO₃⁻ and NO₂⁻ concentrations at 25°C and 18°C are portrayed in Fig. 3. The initial NO₃⁻ concentrations (1008–1159 mmol L⁻¹) in all experiments at both temperatures showed a monotonic decrease, which suggests the occurrence of heterotrophic denitrification (Figs. 3a and 3c). In experiments D1–D4, the NO₂⁻ concentration increased gradually from 0 to 212.4–397.5 mmol L⁻¹ until 150 min at 25°C. However, it was decreased to 40 mmol L⁻¹ at 240 min in experiments D3–D4. At 18°C, the NO₂⁻ concentrations in the experiments D1, D3, and D4 presented a slight increase along time courses, although it was almost constant in experiment D2 (Figs. 3b and 3d). The δ¹⁵N values of NO₃⁻ at several timings in all experiments are presented in Table 2. The measurements of the δ¹⁵N of NO₃⁻ were done
Fig. 4. Time course of the concentration and isotopocule ratios ($\delta^{15}$N$^{\text{bulk}}$, $\delta^{18}$O and SP) of N$_2$O under anoxic condition (experiments D1–D4) at 25°C (a–d) and 18°C (e–h). The reference (control) experiment is D4 (stars). Effects of the C/N ratio were examined at these experiments.

Figure 4 presents results of dissolved N$_2$O measurements associated with factors controlling the production and consumption of N$_2$O. Experiments D1, D3, and D4 at both temperatures provide a time series of N$_2$O measurements showing a change in concentration and isotopocule ratios. The difference between D1, D3, and D4 was the C/N ratio. Unfortunately, we really do not only in the experiment D4 at 25°C, but we estimated the $\delta^{15}$N values in other experiments using Eq. (5) based on measured NO$_3^-$ concentrations and the parameters ($\delta_0^{15}$N = +23.4‰ and $\epsilon^{(15)}N$ = −2.3‰) obtained from the fitting.
know how concentrations and isotopocule ratios of N\textsubscript{2}O were changed in experiment D2 because they were measured only at the beginning and then at the end of the time course.

At 25°C, the dissolved N\textsubscript{2}O concentration increased as soon as the substrate was added. High N\textsubscript{2}O concentrations were observed between 15 and 30 min, especially, reaching the maximum value of 920.5 nmol kg\textsuperscript{-1} at the low C/N ratio of 0.8 (experiment D1) and 623.1 nmol kg\textsuperscript{-1} at the middle C/N ratio of 1.5 (experiment D3). Subsequently, it became almost constant around 200 nmol kg\textsuperscript{-1} in all experiments after 60 min had passed. However, the highest N\textsubscript{2}O (1250.3 nmol kg\textsuperscript{-1}) was observed in experiment D4, which had the highest C/N ratio of 2.4 (Fig. 4a). The $\delta^{15}$N\textsubscript{bulk} of N\textsubscript{2}O was increased in experiments D1, D3, and D4 when N\textsubscript{2}O concentrations became almost stable after 60 min. In contrast, the $\delta^{18}$O values in these experiments were decreased slightly from +5.5‰ to +4.4‰ after a great increase in 15–30 min (Figs. 4b–d). The large SP values were observed as +17.5 – +20.2‰ for D1, +16.0 – +19.8‰ for D3 and D4, respectively.

At 18°C, the N\textsubscript{2}O concentrations in experiments D1, D3 and D4 were increased slightly after substrate addition at 15 min. However, the concentration was about a quarter of the concentration observed at 25°C, and showed nearly stable patterns until the end of incubation. The $\delta^{15}$N\textsubscript{bulk} in experiments D1, D3, and D4 overlapped at 15 min, showing slightly greater (ca. –5‰) values compared to those observed at 25°C (ca. –10‰). They then increased to +5.8 – +20.0‰ except experiment D1 (Figs. 4e and 4f). The SP increased rapidly to +15.3 – +15.8‰ at 15 min in experiments D1 and D3. Thereafter, it fluctuated widely during incubation, although it became almost stable for experiment D4. Generally, the SP values at 18°C
Isotopocule characterization of N$_2$O dynamics during simulated wastewater treatment

were slightly lower than those at 25°C (Fig. 4h). The $\delta^{18}$O were high between 15 and 30 min followed by a decrease, but with large variations (Fig. 4g).

Effect of DO concentration on N$_2$O production under oxic conditions

Time course of the DIN concentrations in the oxic experiments N1–N4 are portrayed in Fig. 5. The initial NH$_4^+$ concentrations (1078–2200 μmol L$^{-1}$) showed a monotonic decrease, whereas NO$_2^-$ and NO$_3^-$ concentrations are built up in all experiments. In experiments N2–N4 (DO of 1.0, 2.0 and 3.0 mg L$^{-1}$) at 25°C, the NO$_2^-$ concentration was increased as high as 450 μmol L$^{-1}$. However, NO$_2^-$ showed declines with continuous NO$_3^-$

Fig. 6. Time course of the concentration and isotopocule ratios ($\delta^{15}$N$_{\text{bulk}}$, $\delta^{18}$O and SP) of N$_2$O under oxic conditions (experiments N1–N4) at 25°C (a–d) and 18°C (e–h). The reference (control) experiment is N3 (stars). Effects of DO were examined at these experiments.
increase at 180 min. The δ¹⁵N values of NH₄⁺ at several timing in all the experiments are presented in Table 2. Although we measured the δ¹⁵N of NH₄⁺ only in the experiments N1 (at 25°C and 18°C) and N3 at 60 min and 150 min (at 25°C), the data fit equation (5) quite well ($R^2 = 0.973$). Therefore, the δ¹⁵N values for experiments N2 and N4 and those for N3 (at 18°C and at 210 min at 25°C) were estimated using Eq. (5), as in the case of the δ¹⁵N of NO₃⁻ in anoxic experiments. Fitting parameters used for the estimation were, respectively, +4.2‰ and −14.1‰ for δ¹⁵N and ε¹⁸O. These values are useful to estimate the δ¹⁵N values of N₂O that might be produced from NH₄⁺ (see Section “Discussion”).

The results of dissolved N₂O measurements associated with key factor of DO that affect N₂O production or consumption are shown in Fig. 6. Experiments N1 (DO of 0.5 mg L⁻¹) and N3 (DO of 2.0 mg L⁻¹) at both temperatures provide a full-time series of N₂O measurements showing the change in concentration and isotopocule ratios. At 25°C, the N₂O concentration increased to a maximum value of 1790 nmol kg⁻¹ around 15–30 min with a simultaneous decrease in δ¹⁵Nbulk in experiment N1. Then the concentration decreased gradually until the end of incubation, whereas the δ¹⁵Nbulk, δ¹⁸O and SP remained almost constant. In contrast, the N₂O concentration in experiment N3 was almost constant until 150 min (400–500 nmol kg⁻¹). However, it decreased with large increases in δ¹⁵Nbulk, δ¹⁸O, and SP of N₂O at 210 min (Figs. 6a, 6b, and 6d). At 18°C, a slight increase in N₂O concentration and a great decline in δ¹⁵Nbulk were observed up to $t = 60$ min in experiment N1. They showed almost constant behavior. The SP of N₂O showed less variation. The N₂O concentration in experiment N3 was constant during the incubation. It was nearly two thirds of that observed in N1 between 60 and 210 min. The δ¹⁵Nbulk varied widely, whereas SP was constant (+7.0–+8.6‰) at 0–60 min after starting the experiment (Figs. 6c, 6f, and 6h).

The δ¹⁸O of N₂O showed a great decline from +31.8‰ ($t = 0$) to +16.1‰ ($t = 150$ min) before a sudden increase at the end of the time course in experiment N3 at 25°C, although it increased slightly between $t = 0$ and 15 min, with a subsequent decrease that slows after 60 min at 18°C. In contrast, δ¹⁸O-N₂O in experiment N1 was nearly constant around +23‰ after a small fluctuation at the beginning of incubation at high temperatures, although it was decreased slightly to +15.9‰ at 210 min at low temperatures (Figs. 6c and 6g).

**DISCUSSION**

**N₂O reduction under anoxic conditions**

The reduction rate of N₂O in experiment R1 (850 nmol kg⁻¹ min⁻¹) was higher than in R2 (533 nmol kg⁻¹ min⁻¹), but the rate per unit MLSS was nearly the same, indicating that the microbes capable of N₂O reduction are distributed homogeneously in the suspended matter and that their activity is similar. The simultaneous increase in isotopocule ratios agrees with reports from pure culture incubation experiments of denitrifying bacteria in which residual N₂O becomes enriched in ¹⁵N and ¹⁸O during N₂O reduction (Ostrom et al., 2007).

This report is the first of a study of the estimation of isotope enrichment factors for N₂O reduction ($e_{O}$) during wastewater treatment applying Eq. (5). Fundamentally, the isotopic enrichment factor corresponds to the ratio of reaction rates for heavy-isotope-containing and light-isotope-containing molecules, during a simple unidirectional reaction or the rate limiting reaction of a multi-step reaction. The values of $e_{O}$ estimated using the combined dataset from both R1 and R2 experiments were −9.5 ± 1.0‰ for the bulk N, −28.7 ± 3.7‰ for the oxygen isotopes, and −10.0 ± 2.2‰ for the SP of N₂O, although data obtained at $t = 30$ min in R1 was excluded because significant loss of N₂O by evasion to the gas phase was suspected (Fig. 7, Table 3). The $e_{O}$ of bulk ¹⁵N and ¹⁸O were within the range of reported values obtained in an oceanic environment (−11.6 ± 1.0‰ for bulk N and −30.5 ± 3.2‰ for ¹⁸O) by Yamagishi et al. (2007). The $e_{O}$ of SP was slightly lower than the value estimated using pure cultures of denitrifier Pseudomonas denitrificans (−6.8‰) (Ostrom et al., 2007). The reduction of N₂O prior to its emission to the atmosphere has the potential to result in changes in isotopocule ratios of N₂O that is often used to

114 A. Tumendelger et al.
partition the production pathways. Therefore, the enrichment factors obtained in this study are expected to be useful parameters for further studies on N₂O reduction occurred in a complex bacterial system such as activated sludge.

We also examined the relation between δ¹⁵O and δ¹⁵Nbulk and between SP and δ¹⁵Nbulk isotope fractionation as a potential means for identifying N₂O reduction. Observed linear relation between δ¹⁵O and δ¹⁵Nbulk using combined datasets at both R1 and R1 experiments is defined by slopes of 2.2, which is remarkably consistent with the slopes of 2.7 obtained in pure culture (Ostrom et al., 2007) and slopes of 2.0 in marine environment (Yoshinari et al., 1997). In other words, N₂O reductase has about a two-times-greater effect on oxygen isotopes than nitrogen isotopes. This might be used as a unique signature for this process. Furthermore, SP is known to increase in parallel with δ¹⁵N with a slope of 0.9 ± 0.1 (Koba et al., 1999), which confirmed the slope acquired in groundwater (Koba et al., 2009), was also observed in this study.

N₂O dynamics under anoxic conditions

Concentrations and isotopic signatures of DIN and N₂O The monotonic decrease of initial NO₃⁻ concentrations (1008–1159 μmol L⁻¹) in all experiments at both temperatures suggests the occurrence of heterotrophic denitrification. Approximately 83.7–99.8% of NO₃⁻ was reduced to NO₂⁻ with slightly high reduction rate of 4.2–5.2 μmol L⁻¹ min⁻¹ in experiments D1–D4 (different C/N ratios) along the time course of incubation at 25°C, whereas a smaller fraction of NO₂⁻ is reduced (45–77.4%) with the rate of 1.6–3.9 μmol L⁻¹ min⁻¹ at 18°C (Figs. 3a and 3c).

As shown in Fig. 4, the dissolved N₂O concentration was always higher than that of atmospheric equilibrium concentration (about 7.8 and 9.6 nmol kg⁻¹ at 25°C and 18°C, respectively) although the gas phase was purged continuously with N₂. This difference implies that N₂O production occurred at the range of C/N ratios tested in this study. At 25°C, the temporal accumulation of N₂O at low C/N ratio of 0.8 between 15 and 30 min agrees with studies by Chung and Chung (2000) and Hanaki et al. (1992) who found that limited availability of biodegradable organic carbon increases N₂O emissions in heterotrophic denitrification. However, the highest N₂O (1250.3 nmol kg⁻¹) was observed at the highest C/N ratio of 2.4, which might be caused the effects of other factors enhancing N₂O emission, such as NO₃⁻ accumulation or unbalanced activity of nitrogen reducing enzymes (Fig. 4a).

The δ¹⁵Nbulk of N₂O was increased perhaps because of either N₂O production or reduction in experiments D1, D3, and D4 when N₂O concentrations became almost stable after 60 min. In contrast, the δ¹⁵O values in these experiments were decreased slightly from +55‰ to +44‰, which are within the ranges of atmospheric values (+45–+50‰, Yoshida and Toyoda, 2000) after a great increase in 15–30 min (Figs. 4b–d). The occurrence of N₂O reduction is suggested by observed large SP values (+17.5 – +20.2‰ for D1, +16.0 – +19.8‰ for D3 and D4) because N-O bond breakage during N₂O reduction enriches ¹⁵N in the alpha (ε) position in the remaining N₂O molecules attributable to a primary kinetic isotope effect (i.e., the bonds of the light N₂O isotopocules break faster than those containing heavy isotopes) (Ostrom et al., 2007). However, neither a temporal increase in SP values at the initial time (t = 0–60 min) nor a monotonic increase in δ¹⁵Nbulk throughout the incubation can give us full information explaining whether the reduction of N₂O alone occurred. Therefore, we attempted to check the occur-

Table 3. Reported and estimated enrichment factors for bacterial N₂O reduction process

<table>
<thead>
<tr>
<th>Process</th>
<th>ε¹⁵O (‰)</th>
<th>ε¹⁸O (‰)</th>
<th>SP</th>
<th>Co-variation in</th>
<th>Experimental condition/sample</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂O → N₂</td>
<td>-26.0 ± 5.0</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Ps. denitrificans</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-12.9</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Ps. aeruginosa</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-4.1</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Pa. denitrificans</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-6.6</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Ps. denitrificans</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-11.6 ± 1.0</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Eastern Tropical North Pacific</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>2.4</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Groundwater</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-2.0</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Eastern Tropical North Pacific</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-3.0</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>nm</td>
<td>Arabian Sea</td>
</tr>
<tr>
<td>N₂O → N₂</td>
<td>-9.5 ± 1.0</td>
<td>-28.7 ± 3.7</td>
<td>-10.0 ± 2.2</td>
<td>2.2</td>
<td>0.9</td>
<td>Wastewater incubation</td>
</tr>
</tbody>
</table>

nm: not measured.

Isotopocule characterization of N₂O dynamics during simulated wastewater treatment
rence of N₂O reduction by the correlation between SP and δ¹⁵N of N₂O. We found a poor correlation that might result from the simultaneous occurrence of N₂O production and reduction in this system. At 18°C, the N₂O concentrations in experiments D1, D3 and D4 were about a quarter of the concentration observed at 25°C, and showed nearly stable patterns until the end of incubation. In general, the SP values at 18°C were slightly lower than those at 25°C, which suggests that the rate of N₂O reduction was slow under low-temperature conditions (Fig. 4h).

**Source-partitioning of N₂O** We infer that the N₂O net production (=production – consumption) and its emission from the water to the head space were balanced after 60 min because the N₂O concentration was stable in this system. Consequently, the isotopocule ratios after 60 min were regarded as values for N₂O net production.

In Figs. 8a and 8b, the data obtained at t = 60 min or later are shown, respectively, in SP-δ¹⁵Nbulk and SP-δ¹⁸O diagrams. Experiments D1–D4 were conducted under anoxic conditions. Therefore, N₂O should have been produced by heterotrophic denitrification as we expected. Reportedly SP of N₂O produced by the two pathways is independent of isotope ratios of the substrates (Sutka et al., 2004, 2006; Toyoda et al., 2005). We define the range of SP values for each pathway as +33 ± 4‰ for NH₂OH oxidation and +1.0 ± 5.5‰ for NO₂⁻ reduction according to estimations based on values from the literature (Toyoda et al., 2011b and references therein). The range of SP values for N₂O produced by denitrification (i.e., nitrite reduction) is shown by the vertical side of the gray rectangles in Figs. 8a and 8b. On the other hand, the range of δ¹⁵N of N₂O produced by in each experiment can be estimated from δ¹⁵N of substrate (NO₃⁻) and isotopic enrichment factor for the pathway using the following equation.

\[ \delta^{15}N_{N_2O} = \delta^{15}N_{substrate} + \epsilon(15N)_{substrate \rightarrow N_2O} \]  

The value of δ¹⁵N–NO₃⁻ is taken from Table 2. The range of ε(¹⁵N)NO₃⁻–N₂O obtained by studies incubating pure culture of denitrifying bacteria under anaerobic conditions (~37 to ~15‰, Toyoda et al., 2011a). It is shown by a horizontal capped segment in the gray rectangle in Fig. 8a (solid and dashed lines respectively correspond to experiments conducted at 25°C and 18°C, respectively). As indicated by arrows in Fig. 8a, the δ¹⁵N and SP are expected to show co-variation with a slope of 0.9 during N₂O reduction according to the results of experiments R1 and R2. In Fig. 8b, however, only the range of SP is shown for the same reason discussed in oxic experiments (see oxic experiment).

The measured SP of N₂O in experiments D1–D4 (+16‰ ~ +20‰ at 25°C and +11‰ ~ +17‰ at 18°C) with different C/N ratios were higher than the SP of N₂O produced by NO₂⁻ reduction (~1.0 to +5.5‰). Consequently, the measured N₂O cannot be explained solely by the NO₂⁻ reduction. In Fig. 8a, the observed data are distributed in the region located upward and slightly rightward of the
gray box. They are bounded by the slanted arrows drawn from the box, which strongly suggests the occurrence of simultaneous N\(_2\O\) production by NO\(_3\) reduction and N\(_2\O\) reduction to N\(_2\). In summary, results show that N\(_2\O\) was produced mainly by heterotrophic denitrification. It was then partially reduced to dinitrogen gas in all experiments tested under various C/N ratios at different temperatures. The difference in C/N ratios had no effect on the production pathway.

**N\(_2\O\) dynamics underoxic conditions**

**Concentrations and isotopic signatures of DIN and N\(_2\O\)**
Based on monotonic decrease of initial NH\(_4\)\(^+\) concentrations following to increments of NO\(_3\) and NO\(_2\)\(^-\) concentrations, we found that approximately 98.3–99.1% of initial NH\(_4\)\(^+\) was converted into NO\(_2\) and NO\(_3\), by the end of incubation with the NH\(_4\)\(^+\) oxidation rate of 5.3–7.6 \(\mu\)mol L\(^{-1}\) min\(^{-1}\) in experiments N2–N4 (DO of 1.0, 2.0 and 3.0 mg L\(^{-1}\) at 25°C) (Fig. 5). The NO\(_2\)\(^-\) concentration in these experiments was high as 450 \(\mu\)mol L\(^{-1}\). Such an accumulation of NO\(_3\) during the oxidation of NH\(_4\)\(^+\) by activated sludge has also been described in reports of previous studies (Itoh et al., 2001; Kampschreur et al., 2006). However, NO\(_3\)\(^-\) showed declines with continuous NO\(_3\) increase at 180 min, which indicates NO\(_3\)\(^-\) oxidation to NO\(_\infty\) by nitrifiers. In contrast, the fraction of NH\(_4\)\(^+\) oxidized was low in experiment N1 with low DO (0.5 mg L\(^{-1}\)). It was about 60.5% with the rate of 5.9 \(\mu\)mol L\(^{-1}\) min\(^{-1}\) at 25°C, whereas it was 60.8% with the rate of 3.0 \(\mu\)mol L\(^{-1}\) min\(^{-1}\) at 18°C, which indicates that NH\(_4\)\(^+\) oxidation is affected by the insufficient amount of oxygen concentration. This trend confirms the presence of ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Moreover, it indicates no significant heterotrophic activity. The NH\(_4\)\(^+\) oxidation rates at 25°C were slightly higher than those of 18°C, indicating that AOB can be more active in warmer conditions.

Because the liquid phase and the gas phase were purged continuously with air and N\(_2\), respectively, the dissolved N\(_2\O\) concentration was controlled not only by the rates of production and reduction by microbes, but also by the rate of diffusion to the gas phase. Nevertheless, it was always higher than the concentration expected under atmospheric equilibrium (approximately 7.8 and 9.6 \(\mu\)mol kg\(^{-1}\) at 25°C and 18°C, respectively (Weiss and Price, 1980)), indicating that significant N\(_2\O\) production occurred at the range of DO tested in this study (Fig. 6a). At 25°C, the N\(_2\O\) concentration increased with simultaneous decrease in \(\delta^{15}\)N\(_\infty\) around 15–30 min in experiment N1 with the lowest DO condition (0.5 mg L\(^{-1}\)). The accumulation of N\(_2\O\) in this low condition means a higher N\(_2\O\) production rate relative to the rate of diffusive loss, which is consistent with previous works reporting that lower DO concentrations engender higher N\(_2\O\) emissions during nitrification (Kampschreur et al., 2008; Tallec et al., 2006). The decrease in \(\delta^{15}\)N\(_\infty\) during the N\(_2\O\) increasing phase is explained by addition of isotopically light N\(_2\O\) produced by nitrification, as described later. Thereafter, the concentration decreased gradually until the end of incubation when the isotopocule ratios of N\(_2\O\) remained almost constant, which suggest that the accumulated N\(_2\O\) was emitted gradually to the gas phase, with only a marginal isotope effect (Inoue and Moo-ko, 1994).

For experiment N3 with high DO concentration, almost constant N\(_2\O\) concentration until 150 min implies that the N\(_2\O\) production and its emission to the gas phase were balanced. However, it decreased with large increases in \(\delta^{15}\)N\(_\infty\), \(\delta^{18}\)O, and SP of N\(_2\O\) at the end of incubation (Figs. 6a–d), which might be caused by occurrence of N\(_2\O\) reduction by denitrifiers only locally existed in suspended matter. In experiment N3, N\(_2\O\) concentrations were low compared to those of experiment N1 in which the DO concentration was lower than in experiment N3. This result is consistent with findings by Zheng et al. (1994) who reported that the high DO level can minimize N\(_2\O\) production from nitrification.

Results of experiment N1 show that the N\(_2\O\) concentration was lower at 18°C than at 25°C by a factor of two or three. This would have been caused not only by the temperature difference but also by unexpectedly high N-loading at 25°C (Figs. 5a and 5d). Although the difference was not as large as in the case of N1, a similar temperature effect on N\(_2\O\) concentration was also observed in experiment N3. The SP values at both temperatures were almost nearby which have almost no temperature influence as well. This suggests N\(_2\O\) production process was not significantly affected by the temperature difference of 7°C, although N\(_2\O\) production rate can be increased. In experiment N3, notable increases in \(\delta^{15}\)N\(_\infty\), \(\delta^{18}\)O, and SP observed between t = 150 and 210 min at 25°C were not found at 18°C, which indicates that N\(_2\O\) reduction might have been promoted at higher temperatures. For experiments N2 and N4, unfortunately, it remains unclear how the concentration and isotopocule ratios of N\(_2\O\) were changed because they were measured only at the beginning and end of the time course.

The difference in \(\delta^{18}\)O-N\(_2\O\) in N1 between 25°C and 18°C was approximately 10%. This fact might be explained by a different degree of O-exchange between NO\(_3\)\(^-\) and H\(_2\O\). The production rate of N\(_2\O\) was lower at 18°C than at 25°C (Figs. 6a and 6c). Therefore, the O-exchange can be enhanced if the rate-limiting step is NO\(_3\)\(^-\) reduction. No report in the literature to date has described the temperature dependence of O-exchange rate during microbiological N\(_2\O\) production. Further studies must be conducted to elucidate this point. **Source-partitioning of N\(_2\O\)** Here we discuss the N\(_2\O\) production/consumption processes under a steady state as...
assuming that (i) $N_2O$ production and its emission to the gas phase were balanced, (ii) $N_2O$ reduction was sufficiently small, except at $t = 150–210 \text{ min}$ in experiment N3 at $25^\circ\text{C}$, and (iii) production processes were unchanged at $t = 60 \text{ min}$ or later. These assumptions are based on full time series measurements in experiments N1 and N3, although temporal variation of $N_2O$ concentration and isotopocule ratios were not negligible in N3 at $25^\circ\text{C}$. We can regard the isotopocule ratios after $60 \text{ min}$ as values for $N_2O$ produced in the system because isotope fractionation associated with emission of dissolved $N_2O$ to the gas phase is sufficiently small compared to that related to $N_2O$ production (Inoue and Mook, 1994).

In Figs. 9a and 9b, the data obtained at $t = 60 \text{ min}$ or later are shown, respectively, in SP-$\delta^{15}N_{\text{bulk}}$ and SP-$\delta^{18}O$ diagrams. Experiments N1–N4 were conducted under oxic conditions. Therefore, $N_2O$ should have been produced by $\text{NH}_2\text{OH}$ oxidation (nitrification) or $\text{NO}_2^–$ reduction (nitrifier-denitrification) pathways. The range of $\delta^{15}N_{\text{bulk}}$ for $N_2O$ produced by $\text{NH}_2\text{OH}$ oxidation (nitrification) or $\text{NO}_2^–$ reduction (nitrifier-denitrification) is shown by the vertical sides of the gray rectangles in Figs. 9a and 9b. The resulting range of $\delta^{15}N_{\text{bulk}}$ for $N_2O$ produced by $\text{NH}_2\text{OH}$ oxidation in each experiment is shown by a horizontal capped segment in the upper gray rectangle in Fig. 9a (solid and dashed lines correspond to experiments conducted at $25^\circ\text{C}$ and $18^\circ\text{C}$, respectively). $\delta^{15}N$-$\text{NO}_2^–$ was not measured individually in this study. Therefore, we estimate $\delta^{15}N_{\text{bulk}}$ for $N_2O$ produced by $\text{NH}_4^+$ oxidation to $\text{NO}_2^–$ followed by $\text{NO}_2^–$ reduction using $\delta^{15}N$-$\text{NH}_4^+$ and $\epsilon^{15}N_{\text{NH}_4^+\rightarrow\text{NO}_2^-\rightarrow\text{N}_2O}$. The $\epsilon^{15}N_{\text{NH}_4^+\rightarrow\text{NO}_2^-\rightarrow\text{N}_2O}$ is estimated from $\epsilon^{15}N_{\text{NH}_4^+\rightarrow\text{NO}_2^-\rightarrow\text{N}_2O}$ and $\epsilon^{15}N_{\text{NO}_2^-\rightarrow\text{N}_2O}$ reported in the literature (–76 to –11‰, Toyoda et al., 2011a). The range of $\epsilon^{15}N_{\text{NH}_4^+\rightarrow\text{NO}_2^-\rightarrow\text{N}_2O}$ obtained by studies incubating pure culture of nitrifying bacteria under aerobic conditions (–60 to –48‰, Toyoda et al., 2011a) is assumed to represent $^{15}N$-enrichment factor for $N_2O$ production from $\text{NH}_4^+$ via $\text{NH}_2\text{OH}$. The calculated range of $\epsilon^{15}N_{\text{NH}_4^+\rightarrow\text{NO}_2^-\rightarrow\text{N}_2O}$ for $N_2O$ produced by $\text{NO}_2^–$ reduction in each experiment is shown as a horizontal capped segment in the bottom gray rectangle in Fig. 9a. The SP values of measured $N_2O$ in experiments N1–N4 with different DO concentrations at both temperatures (+5.0 – +5.6‰ at $25^\circ\text{C}$ and +4.1 – +8.5‰ at $18^\circ\text{C}$) are close to the range of SP for $\text{NO}_2^–$ reduction source. Observed SP values show that $N_2O$ production in most of the oxic experiments is consistent with nitrifier-denitrification (Fig. 9a), which dominantly contributed to $N_2O$ production as about 74–87% for experiment N1, 92–96% for experiment N2, 74–92% for experiment N3 and 79–82% for experiment N4, respectively, from Eq. (4). Our results agree with those from studies by Wunderlin et al. (2013), who found that nitrifier-denitrification was the dominant $N_2O$ production process in an experiment exploring the multiple steps of $\text{NH}_4^+$ oxidation. This finding can also be confirmed by the progressive depletion of $\text{NH}_4^+$ and $\text{NO}_2^–$ accumulation (Figs. 5a, 5c, 5d, and 5f). The SP values of $N_2O$ observed in

![Fig. 9. Relation between SP and $\delta^{15}N_{\text{bulk}}$ and between SP and $\delta^{18}O$ of dissolved $N_2O$ at 60–210 min during oxic experiments at both 25°C and 18°C. Gray rectangles show overall ranges for $N_2O$ produced by nitrification ($\text{NH}_2\text{OH}$-oxidation, upper boxes) and by nitrifier-denitrification ($\text{NO}_2^–$ reduction, bottom boxes). The ranges of $\delta^{15}N_{\text{bulk}}$ for each experiment (N1–N4) were estimated using Eq. (6); they are shown by horizontal capped segments with solid (25°C) and dashed (18°C) lines (a). The SP of $N_2O$ produced by $\text{NH}_2\text{OH}$ oxidation was assigned as 29–37‰, whereas those by $\text{NO}_2^–$ reduction were $-1.0 \pm 5.5‰$. In (b), only the expected ranges of SP are shown by horizontal belts. The $N_2O$ produced in each experiment is shown with closed/half-closed (25°C) and open (18°C) symbols.](image-url)
experiments with low DO (N1 and N2) were close to the values obtained in experiment N3, which was conducted under reference conditions. This result suggests that the changes in DO did not alter the N₂O production pathway (NO₂⁻ reduction by AOB). However, high SP values were observed at 25°C in experiment N3 at 210 min (+18.3‰). This is explainable by the partial contribution (ca. 55%) of N₂O produced via NH₂OH oxidation. Nevertheless, at least for N3, the N₂O reduction could also be the cause (see below).

The δ¹⁸O of N₂O produced either by NH₂OH oxidation or NO₂⁻ reduction by AOB is not well constrained to date. Although the δ¹⁸O also has process-dependent and substrate dependent signatures like δ¹⁵Nbulk, it is difficult to estimate the range of δ¹⁸O of N₂O produced by each process using an equation similar to Eq. (5) because several substrates are involved (e.g., O₂, H₂O, NO₂⁻). Moreover, δ¹⁸O for each production process has not been well characterized. Therefore we showed no range of δ¹⁸O of N₂O in Fig. 9b: the horizontal belt shows the range of SP for N₂O production process.

We found a significant correlation between SP and δ¹⁸O of N₂O during the "steady state" phase (t = 60 min or later) in experiment N3 at 25°C (slope 1.619, R² = 0.988, Fig. 6b). This might indicate that N₂O reduction happened to be enhanced during the "steady state", because it is known that isotopocule ratios show co-variation during N₂O reduction (Table 3). However, no significant correlation was observed between δ¹⁵Nbulk and δ¹⁸O nor between δ¹⁵Nbulk and SP, and apparent correlation could be obtained not only N₂O reduction process but also mixing of different N₂O production processes. Further studies are needed to explore the temporal change in N₂O production/consumption processes using the concentration and isotopocule ratios.

CONCLUSION

We conducted batch incubation experiments using activated sludge under oxic and anoxic conditions to investigate the main factors underpinning N₂O production and consumption. Our results emphasize the usefulness of measurements of N₂O isotopocouples together with the isotopic signature of NH₄⁺ and NO₃⁻ for identifying N₂O production and consumption mechanisms in a lab-scale simulation of biological wastewater treatment. Understanding of N₂O production mechanism which interpreted by these experiments can induce well management on the mitigation strategy of N₂O emission through effective ways to control key factors affecting N₂O production. The main findings obtained in this study are summarized as presented below.

- Under the condition in which N₂O production is negligible, increased concentration of MLSS enhances N₂O reduction to N₂ in anoxic treatment. Enrichment factors (εR’s) for N₂O reduction by activated sludge are first estimated as −9.5 ± 1.0‰ for δ¹⁵Nbulk, −28.7 ± 3.7‰ for δ¹⁸O and −10.0 ± 2.2‰ for SP.

- During N₂O reduction, strong linear relations were found between δ¹⁸O and δ¹⁵Nbulk with slope of 2.2, and between SP and δ¹⁵Nbulk with slope of 0.9. The slopes obtained in this study would be more applicable to the studies of N₂O dynamics in wastewater treatment than εR obtained from pure culture of denitrifying bacteria.

- N₂O production can be enhanced under decreased DO conditions during oxic treatment. However, the N₂O production mechanism is not sensitive to DO. Moreover, the nitrifier-denitrification by AOB during NH₄⁺ oxidation is the main pathway for N₂O production in most cases examined in this study.

- During anoxic treatment, N₂O is dominantly produced by NO₂⁻ reduction (heterotrophic denitrification); N₂O reduction to N₂ occurs simultaneously. The N₂O production mechanism is not sensitive to the temperature or the C/N ratio.

Acknowledgments—This work was partly funded by Global Environmental Research Fund (A-0904) of the Ministry of Environment, Japan and JSPS KAKENHI Grant Number 23224013. We thank the technical staff of the Bureau of Sewerage, Tokyo Metropolitan Government for sampling and analysis. We also thank three anonymous reviewers and S. Hattori for valuable comments on this manuscript. A. Tumendelger was supported by Global COE program “From the Earth to Earths” project of MEXT.

REFERENCES


Isotopocule characterization of N₂O dynamics during simulated wastewater treatment


120 A. Tumendelger et al.


