Isotopomeric characterization of nitrous oxide produced by reaction of enzymes extracted from nitrifying and denitrifying bacteria

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Abstract

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas and produced in denitrification and nitrification in environmental nitrogen cycle by various microorganism. Site preference (SP) of \textsuperscript{15}N in N\textsubscript{2}O, which is defined as the difference in the natural abundance of isotopomers \textsuperscript{14}N\textsuperscript{15}NO and \textsuperscript{15}N\textsuperscript{14}NO relative to \textsuperscript{14}N\textsuperscript{14}NO, has been reported to be a useful tool to quantitatively distinguish N\textsubscript{2}O production pathway. To determine representative SP value for each microbial process, we firstly measured SP of N\textsubscript{2}O produced in the enzyme reaction of hydroxylamine oxidoreductase (HAO) purified from two species of ammonia oxidizing bacteria (AOB), \textit{Nitrosomonas europaea} and \textit{Nitrosococcus oceani}, and that of nitric oxide reductase (NOR) from \textit{Paracoccus denitrificans}, respectively. The SP value for NOR reaction (−5.9 ± 2.1 ‰) showed nearly the same value as that reported for N\textsubscript{2}O produced by \textit{P. denitrificans} in pure culture. In contrast, SP value for HAO reaction (36.3 ± 2.3 ‰) was a little higher than the values reported for N\textsubscript{2}O produced by AOB in aerobic pure culture. Using the SP values obtained by HAO and NOR reactions, we calculated relative contribution of the nitrite (NO\textsubscript{2} \textsuperscript{−}) reduction (which is followed by NO reduction) to N\textsubscript{2}O production by \textit{N. oceani} incubated under different O\textsubscript{2} availability. Our calculations revealed that previous in vivo studies might have underestimated the SP value for NH\textsubscript{2}OH oxidation pathway possibly due to a small contribution of NO\textsubscript{2} \textsuperscript{−} reduction pathway. Further evaluation of isotopomer signatures of N\textsubscript{2}O using common enzymes of other processes related to N\textsubscript{2}O would improve the isotopomer analysis of N\textsubscript{2}O in various environments.

1 Introduction

Nitrous oxide (N\textsubscript{2}O) is a potent greenhouse gas and contributes indirectly to destruction of ozone layer in the stratosphere (Ravishankara et al., 2009). Production of the N\textsubscript{2}O on the earth is mainly controlled by microbial processes that include nitrification and denitrification (Stein and Yung, 2003). In the nitrification, autotrophic microorganisms...
like ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite (NO$_2^-$) and produce N$_2$O as a byproduct under mainly aerobic environment (Casciotti et al., 2011). On the other hand, various heterotrophic denitrifying microorganisms such as archaea, bacteria, and fungi reduce nitrate (NO$_3^-$) or NO$_2^-$ to denitrogen (N$_2$) as electron acceptors for anaerobic respiration and produce the N$_2$O as an intermediate under mainly anaerobic environment (Hayastu et al., 2008). Thus, the production and consumption of N$_2$O consist of many microbiological functions including autotrophic/heterotrophic and oxic/anoxic processes. The investigation of N$_2$O production pathways is useful to understand the nitrogen cycle and relevant microorganisms in various environments.

Natural abundance ratios of isotopomers, a set of molecules containing various stable isotopes, have been used to analyze the N$_2$O production pathways and several studies reported the isotopomer ratios of each N$_2$O production pathway using several strains belonging to bacteria, archaea, and fungi (Casciotti et al., 2011; Frame and Casciotti, 2010; Santoro et al., 2011; Sutka et al., 2008, 2006; 2004; Toyoda et al., 2005; Yoshida, 1988). These studies showed that the site preference (SP), which was defined as the difference of $^{15}$N enrichment between the center (α) and the terminal (β) N atoms in N$_2$O molecule, is a powerful tool to quantitatively distinguish the production pathways such as bacterial NO$_2^-$ reduction and bacterial hydroxylamine (NH$_2$OH) oxidation because the SP value for each production pathway was found to be independent of concentrations or isotope ratios of substrates (Toyoda et al., 2005; Sutka et al., 2003, 2004, 2006). To date, the isotope analysis has been applied to N$_2$O produced in various ecosystems including natural and agricultural soils, oceans, rivers, wastewater treatment plants (Goldberg et al., 2010; Koba et al., 2009 ; Park et al., 2011; Sasaki et al., 2011; Toyoda et al., 2011; Well et al., 2008; Yoshida and Toyoda, 2000). However, as argued by Baggs et al. (2008), the isotopomer signatures were reported for only a few bacterial strains and the variation of the signatures among various species was not fully evaluated. In addition, previous reports on the SP value of N$_2$O produced by NH$_2$OH oxidation might have been biased by simultaneous N$_2$O production
by NO$_2^-$ reduction because some AOB have denitrifying enzymes and produce N$_2$O by NO$_2$ reduction in addition to NH$_2$OH oxidation (Arp et al., 2003). In consideration of this problem, Frame and Casciotti (2010) estimated the SP value of N$_2$O produced by NH$_2$OH oxidation using the relationship between SP and oxygen isotope ratios in N$_2$O obtained in incubation experiments with _Nitrosomonas marina_ C-113a, a marine ammonia-oxidizing bacterium, under various oxygen concentrations. Clearly more information on SP produced by other strains should be obtained according to Frame and Casciotti (2010) for the better use of SP and precise estimation of the contribution from each N$_2$O production pathway.

In this study, we report the isotopomer ratios of N$_2$O produced in vitro using enzymes extracted and purified from two strains of AOB (_Nitrosococcus oceani_ and _Nitrosomonas europaea_) and one species of denitrifying bacteria (_Paracoccus denitrificans_). Our experiments have an advantage that isotope effects related to the N$_2$O production by enzymes (hydroxylamine oxidoreductase (HAO) in AOB and nitric oxide reductase (NOR) in denitrifier) can be directly determined and that the effects related to other processes such as diffusion of substrate/product through cell membrane and reactions mediated by other enzymes can be excluded. We also measure isotopomer ratios of N$_2$O produced in vivo by _N. oceani_ under different oxygen concentrations and estimated the relative contribution of NH$_2$OH oxidation and NO$_2^-$ reduction using the isotopomer signatures obtained in vitro.

### 2 Materials and methods

#### 2.1 Cultivation of the bacterial strains

_Nitrosococcus oceani_ strain NS58 was kindly supplied by H. Urakawa (Florida Gulf Coast Univ.) and used for the experiments. Phylogenetic and morphological analyses indicated a close systematic relationship of the bacterium with _N. oceani_ ATCC19707 (Hozuki et al., 2010). The bacterium was cultivated in the (NH$_4$)$_2$SO$_4$-supplemented
artificial seawater (37.8 mM \( \text{NH}_4^+ \)), of which the pH was buffered to 7.8 by 50 mM MOPS (3-morpholinopropanesulfonic acid) as described in detail in the previous report (Hozuki et al. 2010). The inoculated medium in the conical flask was shaken reciprocally at 120 rpm at 25°C in a dark condition. After cultivation for 7–10 days and the concentration of NO\(_2^-\) in the medium had reached 25–30 mM, the culture of the bacterium in the late-log or stationary phase was used for the in vivo experiments as described below. Large scale cultivation of the bacterium to prepare the starting material for purification of HAO was carried out with the same procedure but using a glass bottle of 10 L in volume with vigorous air ventilation through the sterilized air filter.

Cultivated cells of \textit{Nitrosomonas europaea} ATCC19718, which was obtained by large scale cultivation in accordance to the previous study (Yamanaka and Shinra, 1974), were kindly supplied by Y. Fukumori (Graduate School of Natural Science and Technology, Kanazawa University) and were used as a starting material for the purification of HAO.

\textit{Paracoccus denitrificans} ATCC35512 was cultivated in the denitrifying condition and used for preparing a purified NOR according to the previous report by Fujiwara and Fukumori (1996).

### 2.2 Purification of hydroxylamine oxidoreductase (HAO)

Purification of HAO from \textit{N. oceani} NS58 was carried out according to Hozuki et al. (2010) with a slight modification. About 3 g (wet weight) of the pellet of \textit{N. oceani} NS58 cell was obtained from a 60 L culture and used as the starting material for purification. Catalytic activity of HAO was analyzed by spectrophotometrical measurement of the \( \text{NH}_2\text{OH} \)-dependent reduction of potassium ferricyanide as reported previously (Hozuki et al., 2010). The reaction was started by mixing the enzyme with 1 mL of the reaction solution containing 0.1 M sodium phosphate buffer (pH 7.8), 20 \( \mu \text{M} \) \( \text{NH}_2\text{OH} \), and 100 \( \mu \text{M} \) potassium ferricyanide, then the decreasing rate of absorbance at 420 nm was monitored in a 1 cm light-path cuvette by using an MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan). The difference between the millimolar extinction coefficient
of ferricyanide and that of ferrocyanide (ΔεₘM) at 420 nm was 1.02 mM⁻¹ cm⁻¹. Finally 1.8 mg of the purified HAO, that showed the enzymatic activity as 37 unit mg protein⁻¹ (1 unit is equivalent to the activity where 1 µmol of NH₂OH is oxidized in a minute), was obtained and used for the assay of N₂O-generating activity. HAO was also purified from *N. europaea* according to the method of Yamanaka et al. (1979) with some modifications. A low catalytic activity (6.7 unit mg protein⁻¹) of the enzyme purified from *N. europaea* in this study was probably due to using the old stock of the cultivated bacterial cells, that has been kept in a freezing container at −30°C for about 10 yr, as the starting material for purification.

2.3 Quantitative and isotopomeric analysis of N₂O produced during oxidation of NH₂OH with HAO

In a 69 mL glass vial (Maruemu Corp., Osaka, Japan), 10 mL of substrate solution was prepared so that it contains 0.1–3 mM hydroxylamine, 1 mM potassium ferycyanide as electron acceptor, and 10 mM sodium phosphate as buffer (pH = 7.8). After the vial was sealed with a butyl rubber stopper and an aluminum cap, air in the headspace was replaced with pure N₂ (Shizuoka Sanso Co., Shizuoka, Japan), and then the reaction was started by injecting 0.1 unit of the HAO extracted from *N. oceani* or *N. europaea*. Experiments with 3 mM NH₂OH were conducted on four different dates (A–D). In experiments C and D, lot number of NH₂OH reagent (hydroxylamine hydrochloride, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was different from that used in experiments A and B. After incubating the vial for 2 h at 25°C or for 12 h on ice, gas sample was extracted and analyzed as described below. Concentration of NO₂⁻ was determined spectrophotometrically by a diazo-coupling method (Nicholas and Mason, 1957).
2.4 Purification of nitric oxide reductase (NOR) from Paracoccus denitrificans

NorBC-type NO reductase was purified from the cultivated P. denitrificans cells according to the previous report (Fujiwara and Fukumori, 1996) with some modification. The membrane fraction was prepared from the bacterial cells that had cultivated anaerobically in the presence of NO$_3^-$. NO reductase was extracted from the membrane fraction by treating with the 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Dojindo Lab., Kumamoto, Japan), then was fractionated by anion-exchange chromatography using DEAE-Toyopearl 650M gel (Tosoh, Tokyo, Japan).

NO reducing activity of the purified enzyme was measured spectrometrically by monitoring NO-dependent oxidation of horse ferrocytochrome c (Nacalai Tesque, Kyoto, Japan). Experimental procedure was detailed in the previous report (Fujiwara and Fukumori, 1996). For preventing the disappearance of NO by the reaction with oxygen, dissolved oxygen in the solution was enzymatically removed using D-glucose oxidase/catalase system before starting the assay of NO reducing activity. NO-saturated ethanol, of which the concentration of NO was 11.9 mM, was prepared by treating ethanol with pure NO gas (Sumitomo Seika Chemicals Co., Ltd. Osaka, Japan) and used for the stock solution of NO (Seidell and Linke, 1965). Oxidation rate of ferrocytochrome c was measured by monitoring the decrease in the absorbance at 550 nm with a spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan), whereby the difference of millimolar extinction coefficient ($\Delta\varepsilon_{mM}$, 21.0 mM$^{-1}$ cm$^{-1}$) at 550 nm between reduced and oxidized form of horse cytochrome c was used. NO reducing activity of the purified enzyme was estimated to be 35 unit mg protein$^{-1}$ (1 unit is equivalent to the activity where 1 µmol of NO is reduced in a minute.

2.5 Isotopomeric analysis of N$_2$O produced by enzymatic reduction of NO

In a same glass vial that had been used for NH$_2$OH oxidation experiments, a 10 mL solution was prepared so that it contain 10 mM sodium phosphate buffer (pH6.0), 2.9 µM
horse cytochrome c, 1.0 mM sodium ascorbate and 0.1 mM N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD). The reaction was started by injecting the NO-saturated ethanol to yield final concentration of 50 µM, and the purified NO reductase (0.005 unit) by using a gastight syringe (VICI Precision Sampling Inc., Baton Rouge, LA). Experiments were conducted on three different dates (A, C, and D). After incubating the vial for 2 h at 25°C or for 12 h on ice, the gas phase in the headspace of the reaction vial was extracted and the isotopomeric analysis of the N₂O generated was done as mentioned below.

2.6 Quantitative and isotopomeric analysis of N₂O produced from cultivated N. oceani cells

The 70 mL culture of the N. oceani NS58 was centrifuged at 9800 × g for 60 min at 4°C (refrigerated centrifugator model 3700, Kubota Corp., Tokyo, Japan). The pelleted cells obtained were suspended in the 70 mL of the pH-buffered and (NH₄)₂SO₄-supplemented artificial seawater (see Sect. 2.1), then were incubated with gentle stirring at 25°C in dark for 30 min to remove NO₂⁻ that accumulate in the medium during the cultivation. The resulting cell suspension was centrifuged again with the same condition, then the cell pellet obtained was resuspended in the same volume of the freshly prepared culture medium and used as the washed cell suspension.

The washed cell suspension of 10 mL in volume was put into the glass vial, and the vial was sealed by a butyl rubber stopper and an aluminum cap. After sealing, the headspace (59 mL in volume) of the vial was replaced with O₂/N₂ mixture that contained 2% (v/v) O₂ (Shizuoka Sanso Co., Shizuoka, Japan) or pure N₂ by gently bubbling for 15 min. The reaction solution was prepared in a glass vial by mixing 1.0 mL of the washed cell suspension and 9.0 mL of the cultivation medium. In addition to the anaerobic (0% O₂) and the microaerobic (2% O₂) vials, an aerobic vial without gas replacement was prepared. Incubation of the bacterial cells in the vials was carried out by gentle shaking at 25°C in dark. After 24 or 48 h from starting the incubation, 50 µL of 10 M NaOH solution was added into the reactors to stop the microbial reaction.
Quantification and measurement of isotopomer ratios of N\textsubscript{2}O gas released into the headspace of the incubation vial were performed as described below. Concentration of NO\textsuperscript{2–} that was accumulated in the reaction solution was also measured as explained above.

2.7 Measurement of N\textsubscript{2}O concentration and isotopomer ratios

Concentrations and isotopomer ratios of N\textsubscript{2}O were measured using an on-line analytical system that originally developed for N\textsubscript{2}O dissolved in water samples (Toyoda et al., 2002). The system consists of a gas extraction chamber with a septum for syringe injection, traps made of stainless-steel tubing or glass, a gas chromatograph (Agilent 6890, Agilent Technologies Japan, Ltd., Tokyo) and an isotope-ratio monitoring mass spectrometer (MAT 252, ThermoFisher Scientific KK, Yokohama) equipped with GC interface.

Using a gas-tight syringe, 0.1 to 1.5 mL of the gas was extracted from the headspace of sample vial and was injected into the gas extraction chamber. The sample gas was then transferred with He carrier gas to chemical traps (Mg(ClO\textsubscript{4})\textsubscript{2} and NaOH on support) to remove H\textsubscript{2}O and CO\textsubscript{2}, and N\textsubscript{2}O was concentrated on glass beads packed in a U-shaped trap at liquid N\textsubscript{2} temperature. After further purification on GC, N\textsubscript{2}O was introduced into the mass spectrometer for isotope ratio monitoring. Site-specific nitrogen isotope analysis was conducted with ion detectors modified for mass analysis of fragment ion of N\textsubscript{2}O (NO\textsuperscript{+}) that contains N atom in the center position of N\textsubscript{2}O molecules (Toyoda and Yoshida, 1999). Concentration and bulk nitrogen and oxygen isotope ratio of N\textsubscript{2}O was determined with analysis of N\textsubscript{2}O molecule ion (N\textsubscript{2}O\textsuperscript{+}). Pure N\textsubscript{2}O was used as a reference gas for isotopomer ratios. Notation of isotopomer ratios of N\textsubscript{2}O is shown below.

\[
\delta^{15}\text{N}_{\text{sample}}^i = \frac{^{15}R_{\text{sample}}^i}{^{15}R_{\text{standard}}^i} - 1 \quad (1)
\]

\[
\delta^{18}\text{O}_{\text{sample}}^i = \frac{^{18}R_{\text{sample}}^i}{^{18}R_{\text{standard}}^i} - 1 \quad (2)
\]
In Eqs. (1) and (2), $^{15}\text{R}$ and $^{18}\text{R}$ represent $^{15}\text{N}/^{14}\text{N}$ ratio and $^{18}\text{O}/^{16}\text{O}$ ratio, respectively. Subscript “Sample” and “Standard” indicate isotope ratios for sample and the standard (atmospheric N$_2$ for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for oxygen), respectively. Superscript $i$ is $\alpha$, $\beta$, or bulk which respectively designates central, peripheral, or average isotope ratios in nitrogen atom(s) in N$_2$O molecule. We also define the $^{15}\text{N}$-site preference (SP) as an illustrative parameter of intramolecular distribution of $^{15}\text{N}$ (Yoshida and Toyoda, 1999). The precision of measurement is better than 0.5‰ for $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{18}\text{O}$, and better than 1.0‰ for $\delta^{15}\text{N}_{\alpha}$ and $\delta^{15}\text{N}_{\beta}$.

$$^{15}\text{N} - \text{Site preference (SP)} = \delta^{15}\text{N}_{\alpha} - \delta^{15}\text{N}_{\beta} \quad (3)$$

The $\delta^{15}\text{N}$ of NH$_2$OH was measured by an elemental analyzer coupled with isotope ratio mass spectrometer. Statistical analysis was performed using Excel 2011 (Microsoft, USA). The statistical difference was determined by two-side Student’s $t$ test. Difference with $p < 0.05$ was considered significant.

3 Results

3.1 Concentrations and isotopomer ratios of N$_2$O produced during oxidation of NH$_2$OH with HAO

Figure 1 shows the amount of NO$_2^-$ and N$_2$O produced during the reaction catalyzed by HAO from $N$. oceani with different initial concentration of NH$_2$OH (0.1–3 mM). About 5.1–29.0 % of NH$_2$OH was converted to NO$_2^-$ or N$_2$O after the reaction, and the ratio of produced NO$_2^-$ and N$_2$O decreased with initial NH$_2$OH concentration. With high initial NH$_2$OH concentrations (1 and 3 mM), production of N$_2$O in negative control runs without HAO (HAO−) for 1 mM and 3 mM was 5.9 %, 7.1 % of that in the presence of HAO (HAO+), respectively (Table 1). With low NH$_2$OH concentrations (0.1 and 0.3 mM),
however, production of $N_2O$ in the HAO− runs was 32.2 % and 179.1 % of that in the HAO+ runs for 0.3 mM and 0.1 mM, respectively (Table 1). For this reason, further experiments were conducted at initial NH$_2$OH concentration of 3 mM to examine repeatability of the reaction and effect of bacterial strain and N isotope ratio of NH$_2$OH. The $\delta^{15}N_{\text{bulk}}$ and $\delta^{18}O$ of N$_2$O showed lower values at high (3 mM) NH$_2$OH concentration compared to low (1 mM) concentration (Table 1). SP value was independent of substrate concentration or the degree of the reaction since the difference of SP value between sample with 1 mM and 3 mM was insignificant ($p > 0.05$).

With initial NH$_2$OH concentration of 3 mM (see Table 2), $\delta^{15}N_{\text{bulk}}$ for *N. oceani* and *N. europaea* was almost constant in experiments A and B, and was about 10‰ lower than $\delta^{15}N$ of NH$_2$OH (−7.0‰). In experiment D of *N. oceani*, the difference of $\delta^{15}N_{\text{bulk}}$ from $\delta^{15}N$ of NH$_2$OH used in the experiment (−43.9‰) was similar to those in experiments A and B (−9‰). In experiment C, however, $\delta^{15}N$ of N$_2$O was higher than $\delta^{15}N$ of NH$_2$OH by 1.8–9.9‰ for both strains (Table 2). The $\delta^{18}O$ also showed a variation among experiments A–D of *N. oceani*, although it is not clear whether the $\delta^{18}O$ of NH$_2$OH was different between experiments A/B and C/D. When we compare the results from experiment A, the difference in $\delta^{15}N_{\text{bulk}}$ and $\delta^{18}O$ of N$_2$O was not significant between the two strains ($p > 0.05$ and $p > 0.05$, respectively).

In contrast, SP value of N$_2$O produced by NH$_2$OH oxidation with HAO was independent of initial concentration or degree of reaction process and $\delta^{15}N$ value of NH$_2$OH (Tables 1 and 2). Moreover, difference in SP between experiments with HAO extracted from *N. oceani* in experiments A, B, C and D (average: 36.2 ± 1.7‰, $n = 7$) and *N. europaea* in experiments A and C (average: 36.6 ± 3.3‰, $n = 4$) was insignificant ($p > 0.05$).
3.2 Concentration and isotopomer ratios of N\textsubscript{2}O produced during reduction of NO with NOR

Table 3 shows concentration and isotopomer ratios of N\textsubscript{2}O produced by the reduction of NO with NOR. About 66.0–151.6 % of NO was converted to N\textsubscript{2}O. The concentration of N\textsubscript{2}O was 17 times and 7.3 times higher in the presence of NOR (NOR+) compared to the control runs without NOR (NOR−). SP value of N\textsubscript{2}O from the enzymatic reaction showed little variation (−5.9 ± 2.1 ‰) and was lower than SP values observed in the control runs (15.1–16.8 ‰) (p < 0.05).

3.3 Isotopomer ratio of N\textsubscript{2}O produced by \textit{N. oceani} under different initial O\textsubscript{2} concentration

The amount of N\textsubscript{2}O produced by \textit{N. oceani} was 2.9 ± 0.9 (n = 6), 8.9 ± 0.5 (n = 2), and 18.1 ± 3.6 (n = 5) nmol for O\textsubscript{2} concentration of 0, 2, and 21 %, respectively. Production of both N\textsubscript{2}O and NO\textsubscript{−} was much higher in aerobic condition than in anaerobic condition (p < 0.05, Fig. 2). Isotopomer ratios of N\textsubscript{2}O showed a strong dependence on initial O\textsubscript{2} concentration (Fig. 3). They were more enriched under aerobic condition compared to anaerobic conditions, and positive correlation between the isotopomer ratios was observed (Fig. 3, \(R^2 = 0.89\), p < 0.05).

4 Discussion

The characteristic SP value of N\textsubscript{2}O produced during the in vitro oxidation of NH\textsubscript{2}OH with HAO from the two strains of AOB (average SP for the two strains: 36.3 ± 2.3 ‰) indicates that this parameter is determined by the enzymatic reaction step and not affected by other factors such as concentration or the degree of the reaction and nitrogen isotope ratios of substrate (Table 2). This study first demonstrates the direct evidence of isotopomeric fractionation during the enzymatic reaction and showed the similar SP
value (36.3 ± 2.4 ‰) as previous studies predicted and its robustness during the bacterial oxidation of NH₂OH (e.g., Frame and Casciotti, 2010).

Observed dependence of the product concentration ratio between NO₂⁻ and N₂O on initial NH₂OH concentration could be caused by the availability of electron acceptor. The oxidation of NH₂OH with HAO mainly gives NO₂⁻ as a product when enough electron acceptor is supplied, while the reaction is likely to produce N₂O when the amount of electron acceptor is not enough to complete the reaction as proposed by Yamanaka and Sakano (1980). In the present study, electron acceptor (potassium ferrocyanide) might have been depleted under the condition with 1 and 3 mM NH₂OH because its initial concentration was kept constant. With low NH₂OH concentrations (0.1 and 0.3 mM), rate of N₂O production was smaller than that of NO₂⁻ production and the amount of produced N₂O became closer to that produced in the control experiments (HAO⁻ runs). The amount of N₂O produced in HAO⁻ runs were almost constant (70–90 nmol; note that the “yield” listed in Table 1 shows the amount of N₂O relative to initial NH₂OH), and it must have been produced by non-catalytic reactions.

The yield for N₂O produced during the reduction of NO with NOR exceeded 100 %, which could be caused by the difference in the temperature between the reservation and the preparation of the NO-saturated ethanol. The NO-saturated ethanol was prepared by purging the ethanol with NO gas in a sealed vial at the room temperature, although the solution was reserved in a refrigerator. Therefore, it would cause the high yield because it is known that the solubility of a gas depends on the temperature.

The SP value of N₂O produced during the in vitro reduction of NO with NOR from P. denitrificans (−5.9 ± 2.1 ‰) agrees with that value reported for N₂O produced in vivo from NO₃⁻ reduction by the same species (−5.1 ± 1.8 ‰, Toyoda et al., 2005). Therefore, ours result proves that the factor controlling SP value of N₂O produced in NO₃⁻ reduction is the reaction with NOR rather than other reaction steps including diffusion of substrate and product through cell membranes. However, the SP value is slightly lower than SP values reported for N₂O produced from NO₃⁻ or NO₂⁻ reduction by other denitrifying bacteria or some species of AOB (−0.8 to +0.1 ‰) (Sutka et al.,
2004, 2006), and slightly higher than the value estimated for N₂O produced from NO₂⁻ reduction by *N. marina 113a*, oceanic AOB (−10.7±2.9 ‰; Frame and Casciotti, 2010). This implies that previously reported SP for N₂O from NO₃⁻/NO₂⁻ reduction could have been affected by other processes like NH₂OH oxidation or that SP might depend on a small structural difference in NOR of studied species.

It is known that AOB produces N₂O as a byproduct during the oxidation of NH₂OH to NO₂⁻ with HAO and that some species of AOB can also reduce NO₂⁻ to N₂O with NO₂⁻ reductase (NIR) and NOR (Klotz et al., 2006; Arp et al., 2003). The latter pathway is often referred to as nitrifier-denitrification (Wrage et al., 2001) and is believed to occur under anaerobic condition. Although the reason why those AOB have a function of nitrifier-denitrification is still uncertain, detoxification of accumulated NO₂⁻ has been proposed as a possible explanation (Beaumont et al., 2004). In our experiments, production of both NO₂⁻ and N₂O are enhanced under aerobic condition because O₂ is required for ammonium oxidation, the first step of the successive reaction to NO₂⁻ (Fig. 2). Observed co-variation of SP value of N₂O and oxygen concentration (Fig. 3) implies that relative contributions from the two pathways are sensitive to oxygen availability; NH₂OH oxidation becomes dominant N₂O production pathway under aerobic condition while NO₂⁻ reduction is dominant under anaerobic condition (Fig. 3). It is noteworthy that even in aerobic condition (20 % O₂), SP value of N₂O produced by *N. oceani* is lower than the value obtained by in vitro NH₂OH oxidation with HAO from the same bacteria (Fig. 3), which suggests NO₂⁻ reduction pathway is not negligible under aerobic condition and thus previous studies based on pure culture incubation of AOB underestimated the SP value for N₂O from NH₂OH oxidation.

We further show quantitative estimation of the contribution of NO₂⁻ reduction to N₂O production by using following equation.

\[
SP_{\text{measured}} = SP_{\text{NOR}} \times X_{\text{NOR}} + SP_{\text{HAO}} \times (1 - X_{\text{NOR}})
\]

(4)

In Eq. (4), \(SP_{\text{measured}}\), \(SP_{\text{NOR}}\), and \(SP_{\text{HAO}}\) represent SP values of observed N₂O, N₂O produced in enzymatic reaction with NOR and HAO, respectively, and \(X_{\text{NOR}}\) indicates
relative contribution of the NOR–mediated pathway. As shown in Table 4, average $X_{\text{NOR}}$ is calculated as $82 \pm 18\%$, $42 \pm 10\%$, and $22 \pm 4\%$ under 0\%, 2\%, and 21\% initial $O_2$ concentration, respectively. These values were similar to those measured by Frame and Casciotti (2010). Using the total amount of $N_2O$ produced in each experiment, the $N_2O$ produced from $NO_2^-$ reduction is estimated at $2.5 \pm 1.0\,nmol\,(n=6)$, $3.8 \pm 0.7\,nmol\,(n=2)$, $3.9 \pm 0.7\,nmol\,(n=5)$ under 0, 2, and 21\% initial $O_2$ concentration, respectively. This means that the rate of $N_2O$ production via $NO_2^-$ reduction pathway does not depend on the amount of $NO_2^-$ produced which showed an increase of more than 20 times under aerobic condition than under anaerobic condition. Although the accumulation of $NO_2^-$ inactivates an ammonia monooxygenase (AMO) enzyme activity and $nirK$ was expressed in response to the accumulation of $NO_2^-$ for detoxification of $NO_2^-$ (Beaumont et al., 2004), activities of NIR or NOR seems to have been not enhanced in this study.

The process information provided by SP value enables us to estimate bulk $^{15}N$-enrichment factors ($\varepsilon$, which is approximately equal to $\delta^{15}N_{\text{product}} - \delta^{15}N_{\text{substrate}}$ under the excess supply of substrate) for $N_2O$ production from $NH_4^+$ by NOR and HAO–mediated pathways. If $N_2O$ is produced only by $NH_2OH$ oxidation pathway ($X_{\text{NOR}} = 0$), $SP_{\text{measured}} = SP_{\text{HAO}}$ and $\delta^{15}N_{\text{bulk}}$ of $N_2O$ is estimated at $-32.9\%$ from the linear relationship between SP and $\delta^{15}N_{\text{bulk}}$ (Fig. 3). On the other hand, if $N_2O$ is produced only by $NO_2^-$ reduction pathway ($X_{\text{NOR}} = 1$), $SP_{\text{measured}} = SP_{\text{NOR}}$ and $\delta^{15}N_{\text{bulk}}$ of $N_2O$ is estimated at $-67.5\%$. Combining these values with $\delta^{15}N$ of $(NH_4)_2SO_4$ used in this study ($= -0.34\%$), we obtain $\varepsilon_{HAO} = -32.6\%$ and $\varepsilon_{NOR} = -67.2\%$. The $\varepsilon_{NOR}$ value is about 10\% lower than the value estimated from pure culture incubation of *Nitrosomonas marina* C-113a (Frame and Casciotti, 2010) under several $O_2$ concentrations ($^{15}\varepsilon_{ND} = -56.9\%$). The cause of the difference could be different experimental approach (with/without enzymatic reactions) or different species studied. However, it is proved that $\varepsilon$ is significantly different between $NH_2OH$ oxidation pathway and $NO_2^-$ reduction pathway.
Finally, we discuss the mechanisms that control SP value of N₂O produced during the enzymatic reactions. Both HAO and NOR enzymes are known to have Fe atoms as active centers, but their structure are different according to functional types. Because the catalytic site of HAO has a single Fe atom (one nuclear center) (Igarashi et al., 1997), it is likely that a single NH₂OH molecule binds to the center and is oxidized to NO⁻. If substrate NH₂OH is supplied in excess or electron acceptor for the reaction is lacking, however, another NH₂OH molecule would bind to the same center to form N-N bond. In this case, the primary-binding NH₂OH molecule could be more depleted in ¹⁵N than secondary-binding NH₂OH molecule by the kinetic isotope effect. The observed positive SP might indicate that the peripheral (β) and central (α) N atoms in product N₂O derive from the primary and secondary NH₂OH, respectively.

In the case of NOR, three types are known for bacteria: cNOR that accepts electrons from cytochrome c, qNOR and qCuNOR that accept electrons from quinols (Zumft et al., 2005). Bacterial denitrification is considered to be catalyzed by cNOR, although qNOR, qCuNOR may be responsible for detoxification of NO produced in environments (Hendriks et al., 2000). The active site of NOR enzymes has two Fe atoms (binuclear center) and have similarity among NOR types. In the case of cNOR, the binuclear center consists of non-heme iron (Feₐ) and heme b₃ (Hino et al., 2010), and it is proposed that two NO molecules bind at each center simultaneously to form N₂O (Watmough et al., 2009). This parallel binding mechanism could bring about nearly the same isotope effect for the two N atoms in intermediate like ONNO, and if the elimination of O atom from N-O bonding does not fractionate ¹⁵N within the intermediate molecule, then SP value of N₂O would be nearly 0 ‰.

5 Conclusions

We presented the direct evidence that SP values of N₂O produced by bacterial nitrification and denitrification are controlled by enzymatic reaction of HAO and NOR during NH₂OH oxidation and NO reduction, respectively. The SP value does not depend
on factors like concentration and isotope ratios of substrate and degree of reaction progress. Using the distinct SP values for HAO and NOR related processes, we evaluated the relative contributions from the two pathways of N₂O production by AOB in pure culture, and showed that they are sensitive to oxygen concentration and that NO₂⁻ reduction could occur under aerobic condition.

Although further studies are required, this study demonstrates that isotopomer analysis constitutes a powerful tool to investigate N₂O production pathways in various environments. It will be important to expand these observation beyond nitrifying and denitrifying bacteria to examine the generality of these results. For example, isotopomer characteristics on fungal denitrification and archaeal nitrification have been reported only recently (Santro et al., 2011; Sutka et al., 2008), and those on fungal codenitrification are unknown. Combined analysis of several isotopomer ratios should be developed to distinguish NH₂OH oxidation and fungal denitrification because SP value of N₂O from the two pathways has been found to be nearly the same (Sutka et al., 2008). As for the characterization of various microbial N₂O production processes, studies focused on enzymatic reaction would be effective as shown in this work. Combined analysis of genome sequence, physiology, and isotopomer ratios would promise further understanding of microbial N₂O production mechanisms.

Acknowledgements. We thank members of Yoshida Laboratory at Tokyo Tech for fruitful discussion and kind technical assistance. F. Breider is acknowledged for proofreading of the manuscript. This work was supported by the Global Environmental Research Fund (A-0904) of the Ministry of the Environment, Japan, and by KAKENHI (23224013) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References


### Table 1. Isotopomer ratios of N$_2$O produced during NH$_2$OH oxidation by HAO enzyme extracted from *N. oceani* with different concentration of NH$_2$OH.

<table>
<thead>
<tr>
<th>HAO</th>
<th>NH$_2$OH</th>
<th>Yield (%)</th>
<th>$\delta^{15}$N$_{\text{bulk}}$ (%)</th>
<th>$\delta^{15}$N$^\alpha$ (%)</th>
<th>$\delta^{15}$N$^\beta$ (%)</th>
<th>$\delta^{18}$O (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>3 mM</td>
<td>Average ± SD</td>
<td>5.1 ± 0.4</td>
<td>−17.9 ± 0.2</td>
<td>0.1 ± 0.2</td>
<td>−36.0 ± 0.2</td>
<td>37.8 ± 0.1</td>
</tr>
<tr>
<td>−</td>
<td>3 mM</td>
<td>0.4</td>
<td>−17.0</td>
<td>−2.9</td>
<td>−31.1</td>
<td>32.3</td>
<td>28.2</td>
</tr>
<tr>
<td>+</td>
<td>1 mM</td>
<td>Average ± SD</td>
<td>16.8 ± 3.9</td>
<td>−13.4 ± 0.2</td>
<td>4.7 ± 0.4</td>
<td>−31.6 ± 0.3</td>
<td>42.6 ± 0.2</td>
</tr>
<tr>
<td>−</td>
<td>1 mM</td>
<td>1.0</td>
<td>−15.4</td>
<td>1.0</td>
<td>−31.8</td>
<td>37.7</td>
<td>32.8</td>
</tr>
<tr>
<td>+</td>
<td>0.3 mM</td>
<td>Average ± SD</td>
<td>10.6 ± 0.3</td>
<td>−5.8 ± 0.2</td>
<td>12.0 ± 0.3</td>
<td>−23.6 ± 0.2</td>
<td>44.5 ± 0.1</td>
</tr>
<tr>
<td>−</td>
<td>0.3 mM</td>
<td>3.4</td>
<td>−15.1</td>
<td>2.9</td>
<td>−33.1</td>
<td>47.7</td>
<td>36.0</td>
</tr>
<tr>
<td>+</td>
<td>0.1 mM</td>
<td>Average ± SD</td>
<td>6.9 ± 1.2</td>
<td>−10.7 ± 0.5</td>
<td>6.6 ± 0.4</td>
<td>−28.1 ± 0.4</td>
<td>45.1 ± 0.8</td>
</tr>
<tr>
<td>−</td>
<td>0.1 mM</td>
<td>12.3</td>
<td>−15.5</td>
<td>0.5</td>
<td>−31.5</td>
<td>50.4</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Average and SD (Standard Deviation) were calculated for samples including HAO in each conditions ($n = 3$). Isotopomer ratios for “HAO+” were corrected for the blank “HAO−”).
Table 2. Concentration in gas phase and isotopomer ratios of N₂O produced during NH₂OH oxidation by HAO enzyme extracted from *Nitrosococcus oceani* and *Nitrosomonas europaea* with 3 mM of NH₂OH.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HAO</th>
<th>Yield (%)</th>
<th>δ¹⁵N&lt;sub&gt;bulk&lt;/sub&gt; (%)</th>
<th>δ¹⁵N&lt;sub&gt;α&lt;/sub&gt; (%)</th>
<th>δ¹⁵N&lt;sub&gt;β&lt;/sub&gt; (%)</th>
<th>δ¹⁸O (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitrosococcus oceani</em> (HAO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A +</td>
<td>13.3</td>
<td>−16.5</td>
<td>2.1</td>
<td>−35.1</td>
<td>41.6</td>
<td>37.1</td>
<td></td>
</tr>
<tr>
<td>A +</td>
<td>12.1</td>
<td>−16.1</td>
<td>1.8</td>
<td>−36.0</td>
<td>41.3</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>12.7 ± 0.9</td>
<td>−16.8 ± 0.4</td>
<td>1.9 ± 0.2</td>
<td>−35.5 ± 0.6</td>
<td>41.5 ± 0.2</td>
<td>37.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>A −</td>
<td>2.0</td>
<td>−14.8</td>
<td>−4.0</td>
<td>−25.6</td>
<td>36.3</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td>B +</td>
<td>10.7</td>
<td>−17.7</td>
<td>0.0</td>
<td>−35.4</td>
<td>37.2</td>
<td>35.3</td>
<td></td>
</tr>
<tr>
<td>B +</td>
<td>9.2</td>
<td>−18.0</td>
<td>0.0</td>
<td>−16.4</td>
<td>37.6</td>
<td>35.8</td>
<td></td>
</tr>
<tr>
<td>B +</td>
<td>10.9</td>
<td>−18.1</td>
<td>−0.2</td>
<td>−16.5</td>
<td>37.3</td>
<td>35.5</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>10.3 ± 1.0</td>
<td>−17.9 ± 0.2</td>
<td>−0.1 ± 0.1</td>
<td>−35.7 ± 0.2</td>
<td>37.4 ± 0.2</td>
<td>35.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>B −</td>
<td>0.7</td>
<td>−17.0</td>
<td>−2.9</td>
<td>−31.1</td>
<td>32.3</td>
<td>28.2</td>
<td></td>
</tr>
<tr>
<td>C +</td>
<td>16.6</td>
<td>−42.1</td>
<td>−25.4</td>
<td>−58.8</td>
<td>33.9</td>
<td>33.3</td>
<td></td>
</tr>
<tr>
<td>C −</td>
<td>0.3</td>
<td>−39.7</td>
<td>−24.2</td>
<td>−55.2</td>
<td>33.1</td>
<td>31.1</td>
<td></td>
</tr>
<tr>
<td>D +</td>
<td>10.6</td>
<td>−52.7</td>
<td>−33.5</td>
<td>−71.9</td>
<td>56.8</td>
<td>38.4</td>
<td></td>
</tr>
<tr>
<td>D −</td>
<td>1.1</td>
<td>−49.4</td>
<td>−38.2</td>
<td>−60.5</td>
<td>54.7</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>D −</td>
<td>0.0</td>
<td>−46.2</td>
<td>−27.0</td>
<td>−65.5</td>
<td>55.3</td>
<td>38.5</td>
<td></td>
</tr>
<tr>
<td><em>Nitrosomonas europaea</em> (HAO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A +</td>
<td>14.1</td>
<td>−14.4</td>
<td>4.9</td>
<td>−33.7</td>
<td>42.5</td>
<td>38.6</td>
<td></td>
</tr>
<tr>
<td>A +</td>
<td>15.6</td>
<td>−13.8</td>
<td>4.6</td>
<td>−32.2</td>
<td>42.9</td>
<td>36.8</td>
<td></td>
</tr>
<tr>
<td>A +</td>
<td>14.9</td>
<td>−17.1</td>
<td>2.4</td>
<td>−36.5</td>
<td>42.8</td>
<td>38.9</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>14.9 ± 0.9</td>
<td>−15.0 ± 1.5</td>
<td>2.9 ± 1.2</td>
<td>−33.0 ± 1.9</td>
<td>41.8 ± 0.2</td>
<td>35.9 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>A −</td>
<td>2.0</td>
<td>−14.8</td>
<td>−4.0</td>
<td>−25.6</td>
<td>36.3</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td>C +</td>
<td>2.2</td>
<td>−34.0</td>
<td>−18.1</td>
<td>−49.9</td>
<td>37.4</td>
<td>31.8</td>
<td></td>
</tr>
<tr>
<td>C −</td>
<td>0.1</td>
<td>−39.7</td>
<td>−24.2</td>
<td>−55.2</td>
<td>33.1</td>
<td>31.1</td>
<td></td>
</tr>
</tbody>
</table>

Experiments A, B, C and D were conducted on different date. Isotopomer ratios for “HAO+” were corrected for the blank “HAO−”. The initial δ¹⁵N value of NH₂OH was −7.0 ‰ for A and B, −43.9 ‰ for C and D. Average and standard deviation were calculated for samples including HAO.
Table 3. Concentration and isotopomer ratios of $\text{N}_2\text{O}$ during NO reduction by NOR extracted from *Paracoccus denitrificans*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NOR</th>
<th>Yield (%)</th>
<th>$\delta^{15}\text{N}_{\text{bulk}}$ (%)</th>
<th>$\delta^{15}\text{N}^{a}$ (%)</th>
<th>$\delta^{15}\text{N}^{b}$ (%)</th>
<th>$\delta^{18}\text{O}$ (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>131.5</td>
<td>17.1</td>
<td>14.3</td>
<td>19.9</td>
<td>24.0</td>
<td>−5.7</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>129.9</td>
<td>17.1</td>
<td>15.1</td>
<td>19.1</td>
<td>24.1</td>
<td>−4.0</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>151.6</td>
<td>17.1</td>
<td>14.6</td>
<td>19.6</td>
<td>22.9</td>
<td>−5.0</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>137.6 ± 12.1</td>
<td>17.1 ± 0.0</td>
<td>14.6 ± 0.4</td>
<td>19.5 ± 0.4</td>
<td>23.7 ± 0.6</td>
<td>−4.9 ± 0.8</td>
</tr>
<tr>
<td>A</td>
<td>−</td>
<td>7.7</td>
<td>14.5</td>
<td>22.0</td>
<td>6.9</td>
<td>−7.5</td>
<td>15.1</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>66.0</td>
<td>11.0</td>
<td>6.5</td>
<td>15.5</td>
<td>18.7</td>
<td>−9.0</td>
</tr>
<tr>
<td>C</td>
<td>−</td>
<td>7.5</td>
<td>11.7</td>
<td>19.9</td>
<td>3.5</td>
<td>13.5</td>
<td>16.4</td>
</tr>
<tr>
<td>D</td>
<td>−</td>
<td>9.0</td>
<td>16.3</td>
<td>24.7</td>
<td>8.0</td>
<td>−6.5</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Experiments A, C and D were conducted on different date. Isotopomer ratios for NOR+ was corrected for the blank (NOR−) and were averaged for each experiment. SD represents standard deviation for each experiments. For experiment C, results of experiment D were adopted as the blank because the blank $\delta^{18}\text{O}$ value obtained in experiment C was significantly different from that obtained in experiments A and D.
Table 4. Estimated contribution of NO$_2^-$ reduction catalyzed by NOR to N$_2$O production by *N. oceani* under different initial O$_2$ concentration.

<table>
<thead>
<tr>
<th>Initial O$_2$ (%)</th>
<th>N$_2$O (nmol)</th>
<th>N$_2$O yield ($10^{-5}$)</th>
<th>$X_{NOR}$ (^a)</th>
<th>N$<em>2$O$</em>{NOR}$ (^b) (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>2.9 ± 0.9</td>
<td>1.5 ± 0.5</td>
<td>0.82 ± 0.1</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>2 %</td>
<td>8.9 ± 0.5</td>
<td>4.7 ± 0.3</td>
<td>0.42 ± 0.1</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>21 %</td>
<td>18.1 ± 3.6</td>
<td>9.5 ± 1.9</td>
<td>0.22 ± 0.0</td>
<td>3.9 ± 0.7</td>
</tr>
</tbody>
</table>

N$_2$O yield represents the fraction of N-atoms converted to N$_2$O from NH$_4^+$ as substrate; N$_2$O – N/NH$_4^+$ – N.

\(^a\) Contribution of NO$_2^-$ reduction to N$_2$O production.

\(^b\) Amount of N$_2$O produced by NO$_2^-$ reduction.
Fig. 1. Amount of $\text{N}_2\text{O}$ and $\text{NO}_2^-$ produced by HAO extracted from *N. oceani* under different substrate ($\text{NH}_2\text{OH}$) concentration (expressed as amount in the reaction vessel). The error bar indicates standard deviation for three replicates.
Fig. 2. Amount of NO$\textsubscript{2}^-$ and N$\textsubscript{2}$O produced from 378 µmol of NH$\textsubscript{4}^+$ in concentrated cell suspensions of *N. oceani* under different initial O$\textsubscript{2}$ concentration (*n* = 2).
Fig. 3. Site preference and $\delta^{15}$N of N$_2$O in concentrated cell suspensions of *N. oceani* under different initial O$_2$ concentration. Error bar indicates the measurement error.