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

T. Yamazaki¹,*, T. Hozuki², K. Arai², S. Toyoda¹, K. Koba⁴, T. Fujiwara², and N. Yoshida¹,³,⁵

¹Department of Environmental Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan
²Department of Biological Science, Graduate School of Science, Shizuoka University, 836 Oh-ya, Suruga-ku, Shizuoka 422-8529, Japan
³Department of Environment Chemistry and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan
⁴Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-city, Tokyo 183-8509, Japan
⁵Earth-Life Science Institute, Tokyo Institute of Technology, Meguro, Tokyo, 152-8551, Japan

*present address: LSI Medience Corporation, 1000 Kamoshita-chou, Aoba-ku, Yokohama 227-0033, Japan

Correspondence to: S. Toyoda (toyoda.s.aa@m.titech.ac.jp)

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Abstract. Nitrous oxide (N₂O) is a potent greenhouse gas and produced in denitrification and nitrification by various microorganisms. Site preference (SP) of ¹⁵N in N₂O, which is defined as the difference in the natural abundance of isotopomers ¹⁴N¹⁵NO and ¹⁵N¹⁴NO relative to ¹⁴N¹⁴NO, has been reported to be a useful tool to quantitatively distinguish N₂O production pathways. To determine representative SP values for each microbial process, we firstly measured SP of N₂O produced in the enzyme reaction of hydroxylamine oxidoreductase (HAO) purified from two species of ammonia oxidizing bacteria (AOB), Nitrosomonas europaea and Nitrosococcus oceani, and that of nitric oxide reductase (NOR) from Paracoccus denitrificans. The SP value for NOR reaction (−5.9 ± 2.1 ‰) showed nearly the same value as that reported for N₂O produced by 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 N₂O produced by AOB in aerobic culture. Using the SP values obtained by HAO and NOR reactions, we calculated relative contribution of the nitrite (NO₂⁻) reduction (which is followed by NO reduction) to N₂O production by N. oceani incubated under different O₂ availability. Our calculations revealed that previous in vivo studies might have underestimated the SP value for the NH₂OH oxidation pathway possibly due to a small contribution of NO₂⁻ reduction pathway. Further evaluation of isotopomer signatures of N₂O using common enzymes of other processes related to N₂O would improve the isotopomer analysis of N₂O in various environments.

1 Introduction

Nitrous oxide (N₂O) is a potent greenhouse gas and contributes indirectly to the destruction of the ozone layer in the stratosphere (Ravishankara et al., 2009). Production of N₂O on earth is mainly controlled by microbial processes that include nitrification and denitrification (Stein and Yung, 2003). In nitrification, autotrophic microorganisms like ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB), Nitrosomonas europaea and Nitrosococcus oceani, and that of nitric oxide reductase (NOR) from Paracoccus denitrificans. The SP value for NOR reaction (−5.9 ± 2.1 ‰) showed nearly the same value as that reported for N₂O produced by 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 N₂O produced by AOB in aerobic culture. Using the SP values obtained by HAO and NOR reactions, we calculated relative contribution of the nitrite (NO₂⁻) reduction (which is followed by NO reduction) to N₂O production by N. oceani incubated under different O₂ availability. Our calculations revealed that previous in vivo studies might have underestimated the SP value for the NH₂OH oxidation pathway possibly due to a small contribution of NO₂⁻ reduction pathway. Further evaluation of isotopomer signatures of N₂O using common enzymes of other processes related to N₂O would improve the isotopomer analysis of N₂O in various environments.

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autotrophic/heterotrophic and oxic/anoxic processes. Investigation of \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) production pathways, and several studies have reported isotopomer ratios of each \( \text{N}_2\text{O} \) 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 in \( ^{15}\text{N} \) enrichment between the center (\( \alpha \)) and the terminal (\( \beta \)) \( \text{N} \) atoms in \( \text{N}_2\text{O} \) molecules, is a powerful tool to quantitatively distinguish the production pathways such as bacterial \( \text{NO}_2^- \) reduction and bacterial hydroxylamine (\( \text{NH}_2\text{OH} \)) oxidation, as 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, isotope analysis has been applied to \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) produced by \( \text{NH}_2\text{OH} \) oxidation might have been biased by simultaneous \( \text{N}_2\text{O} \) production by \( \text{NO}_2^- \) reduction, since some \( \text{AOB} \) have denitrifying enzymes and produce \( \text{N}_2\text{O} \) by \( \text{NO}_3^- \) reduction in addition to \( \text{NH}_2\text{OH} \) oxidation (Arp et al., 2003). In consideration of this problem, Frame and Casciotti (2010) estimated the SP value of \( \text{N}_2\text{O} \) produced by \( \text{NH}_2\text{OH} \) oxidation using the relationship between SP and oxygen isotope ratios in \( \text{N}_2\text{O} \) obtained in incubation experiments with \( \text{Nitrosomonas marina} \) C-113a, a marine ammonia-oxidizing bacterium, under various oxygen concentrations. Clearly, more information on SP produced by other strains should be obtained for the better use of SP and precise estimation of the contribution from each \( \text{N}_2\text{O} \) production pathway.

In this study, we report the isotopomer ratios of \( \text{N}_2\text{O} \) produced in vitro using enzymes extracted and purified from two strains of AOB (\( \text{Nitrosococcus oceani} \) and \( \text{Nitrosomonas europaea} \)) and one species of denitrifying bacteria (\( \text{Paracoccus denitrificans} \)). Our experiments have the advantage that isotope effects related to the \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) produced in vivo by \( \text{N. oceani} \) under different oxygen concentrations and estimate the relative contribution of \( \text{NH}_2\text{OH} \) oxidation and \( \text{NO}_3^- \) reduction using the isotopomer signatures obtained in vitro.

2 Materials and methods

2.1 Cultivation of the bacterial strains

\( \text{Nitrosococcus oceani} \) strain NS58 was kindly supplied by Dr. H. Uraka (Florida Gulf Coast University). Phylogenetic and morphological analyses indicated a close systematic relationship of the bacterium with \( \text{N. oceani} \) ATCC19707 (Hozuki et al., 2010). The bacterium was cultivated in the (\( \text{NH}_4\text{)SO}_4 \)-supplemented artificial seawater (37.8 mM \( \text{NH}_4^+ \)), of which the pH was buffered to 7.8 by 50 mM MOPS (3-morpholinopropansulfonic acid) as described in detail in a 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 \( \text{NO}_3^- \) 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 10 L in volume with vigorous air ventilation through a sterilized air filter.

Cultivated cells of \( \text{Nitrosomonas europaea} \) ATCC19718, which was obtained by large-scale cultivation in accordance to a previous study (Yamanaka and Shinra, 1974), were kindly supplied by Dr. Y. Fukumori (Graduate School of Natural Science and Technology, Kanazawa University) and were used as a starting material for the purification of HAO. \( \text{Paracoccus denitrificans} \) ATCC35512 was cultured under denitrifying conditions and used for preparing a purified NOR according to the previous report by Fujiwara and Fukumori (1996).

2.2 Purification of hydroxylamine oxidoreductase (HAO)

HAO was purified from the cultivated NS58 cells through three preparative steps, including (\( \text{NH}_4\text{)SO}_4 \) fractionation, gel-filtration and hydrogen-bonding chromatography according to a previous report (Hozuki et al., 2010). Catalytic activity of HAO was analyzed by spectrophotometrical measurement of the \( \text{NH}_2\text{OH} \)-dependent reduction of potassium ferrocyanide as reported previously (Hozuki et al., 2010). Briefly, the purified enzyme was mixed with 1 mM of the reaction solution containing 0.1 M sodium phosphate buffer (pH 7.8), 20 µM \( \text{NH}_2\text{OH} \) and 100 µM potassium ferrocyanide. Then, the decreasing rate of absorbance at 420 nm of the solution was monitored using a cuvette with an optical path length of 1 cm and a spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan).
Japan). The difference in the millimolar extinction coefficient ($\Delta \varepsilon_{\text{mM}}$) at 420 nm between ferricyanide and ferrocyanide was 1.02 mM$^{-1}$ cm$^{-1}$. We obtained 1.8 mg of the purified HAO with enzymatic activity of $37 \text{ mg protein}^{-1}$ (1 unit is equivalent to the activity where 1 µmol of NH$_2$OH is oxidized in a minute) and used it for the assay of N$_2$O-generating activity. HAO was also purified from $N$. europaea according to the method of Yamanaka et al. (1979) with some modifications. We found lower catalytic activity for the enzyme purified from $N$. europaea ($6.7 \text{ unit mg protein}^{-1}$); this is probably because we used an old stock of the cultivated bacterial cells which had been kept for about 10 years at $-30^\circ\text{C}$.

2.3 Quantitative and isotopomeric analysis of N$_2$O produced during oxidation of NH$_2$OH 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 ferricyanide as an 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 N$_2$ (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 NH$_2$OH were conducted on four different dates (A–D). In experiments C and D, the lot number of NH$_2$OH 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 $25^\circ\text{C}$ or 12 h on ice, gas samples were extracted and analyzed as described below. Concentration of NO$_3^-$ 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 a previous report (Fujiwara and Fukumori 1996) with some modification. The membrane fraction was prepared from the bacterial cells that had been 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). It was then 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 a previous report (Fujiwara and Fukumori 1996). Before starting the assay of NO-reducing activity, dissolved oxygen in the solution was enzymatically removed using D-glucose oxidase/catalase system in order to prevent its reaction with NO. A stock solution of NO-saturated ethanol (11.9 mM) was prepared by purging ethanol with pure NO gas (Sumitomo Seika Chemicals Co., Ltd. Osaka, Japan) (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 in millimolar extinction coefficient ($\Delta \varepsilon_{\text{mM}}$, 21.0 mM$^{-1}$ cm$^{-1}$) at 550 nm between the reduced and oxidized form of horse ferro- and ferriocytochrome $c$ 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 the same glass vial that had been used for NH$_2$OH oxidation experiments, a 10 mL solution was prepared so that it contained 10 mM sodium phosphate buffer (pH 6.0), 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 $25^\circ\text{C}$ or 12 h on ice, the gas phase in the headspace of the reaction vial was extracted and the isotopomeric analysis of the produced N$_2$O was conducted as mentioned below.

2.6 Quantitative and isotopomeric analysis of N$_2$O 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). Obtained pellet cells were suspended in the 70 mL of the pH-buffered and (NH$_4$)$_2$SO$_4$-supplemented artificial seawater (see Sect. 2.1.), then were incubated with gentle stirring at $25^\circ\text{C}$ in dark for 30 min to remove NO$_3^-$ that accumulate in the medium during the cultivation. The resulting cell suspension was centrifuged again under the same condition, and the cell pellet was resuspended in the same volume of the freshly prepared culture medium and used as the washed cell suspension.

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. Cell density of the medium in the reaction vial was estimated at $5.8 \times 10^6$ cells mL$^{-1}$ by direct counting of the fixed and 4′,6-diamidino-2-phenylindole (DAPI)-stained cells using an epifluorescent microscope according to...
2.7 Measurement of \( \text{N}_2\text{O} \) concentration and isotopomer ratios

Concentrations and isotopomer ratios of \( \text{N}_2\text{O} \) were measured using an online analytical system that originally was developed for \( \text{N}_2\text{O} \) 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 gastight syringe, 0.1 to 1.5 mL of the gas was extracted from the headspace of the sample vial and was injected into the gas extraction chamber. The sample gas was then transferred by He carrier gas to chemical traps (\( \text{Mg(ClO}_4\text{)}_2 \) and NaOH on support) to remove \( \text{H}_2\text{O} \) and \( \text{CO}_2 \), and \( \text{N}_2\text{O} \) was concentrated on glass beads packed in a U-shaped trap at liquid \( \text{N}_2 \) temperature. After further purification on GC, \( \text{N}_2\text{O} \) was introduced into the mass spectrometer for isotope ratio monitoring. Site-specific nitrogen isotopomer analysis was conducted with ion detectors modified for isotope ratio monitoring. Site-specific nitrogen isotope analysis was conducted with ion detectors modified for isotope ratio monitoring. Site-specific nitrogen isotopomer analysis was conducted with ion detectors modified for isotope ratio monitoring. Site-specific nitrogen isotopomer analysis was conducted with ion detectors modified for isotope ratio monitoring. Site-specific nitrogen isotopomer analysis was conducted with ion detectors modified for isotope ratio monitoring. Site-specific nitrogen isotopomer analysis was conducted with ion detectors modified for isotope ratio monitoring.

Site preference (SP) was determined by the following equation:

\[
\delta^{15}\text{N}_{\text{sample}} = \delta^{15}\text{N}_{\text{standard}} - 1
\]

\[
\delta^{18}\text{O}_{\text{sample}} = \delta^{18}\text{O}_{\text{standard}} - 1
\]

In Eqs. (1) and (2), \( ^{15}\text{R} \) and \( ^{18}\text{O} \) represent \( ^{15}\text{N} / ^{14}\text{N} \) ratio and \( ^{18}\text{O} / ^{16}\text{O} \) ratio, respectively. Subscript “sample” and “standard” indicate isotopomer ratios for sample and the standard (atmospheric \( \text{N}_2 \) for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for oxygen), respectively. Superscript \( t \) is \( \alpha \), \( \beta \), or bulk, which respectively designates central, peripheral, or average isotopomer ratios in nitrogen atom(s) in \( \text{N}_2\text{O} \) molecules. 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 \( \delta^{15}\text{N}_{\text{u}} \) and \( \delta^{15}\text{N}_{\beta} \).

\[
\delta^{15}\text{N} - \text{Site preference (SP)} = \delta^{15}\text{N}_{\text{u}} - \delta^{15}\text{N}_{\beta}
\]  

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

3 Results

3.1 Concentrations and isotopomer ratios of \( \text{N}_2\text{O} \) produced during oxidation of \( \text{NH}_2\text{OH} \) with HAO

The purity of HAO obtained from \( \text{N. oceani} \) NS58 was confirmed by the SDS-PAGE technique. As shown in Supplement Fig. S1, SDS-PAGE results of the purified sample revealed a single protein band with a molecular weight of approximately 182 000, following pretreatment with SDS (Lane 2). Upon treatment of the enzyme with both SDS and \( \beta \)-mercaptoethanol, the major band accompanied by minor protein bands with molecular weights of 140 000 and 60 000 appeared on the gel (Lane 1). The chemical cleavage of the thioester bond between heme c and a Cys residue in HAO resulted in the appearance of a protein band of molecular weight 60 000 (Lane 3), suggesting that the enzyme is composed of a homotrimmer of subunit proteins covalently linked together and mediated by prosthetic heme c molecules, as previously reported for \( \text{N. europaea} \) HAO (Terry and Hooper, 1981; Igarashi et al., 1997). The two minor protein bands observed in Lane 1 possibly correspond to the monomeric and dimeric configurations of the HAO subunit. In general, AOB possesses two types of hydroxylamine-oxidizing enzymes: HAO, with a trimeric configuration of the octaheme c subunit and cytochrome P460, a monoheme protein with a molecular weight of only approximately 18 000 (Numata et al. 1990). Gel filtration was used for purification of \( \text{N. oceani} \) NS58 HAO; thus, contamination of the cytochrome P460 in the enzyme preparation should be improbable. Results of electrophoretic analysis are consistent with the absence of components that may interfere with the correct interpretation of our experimental findings (Lanes 1 and 2, Fig. S1).

Figure 1 shows the amount of \( \text{NO}_2^- \) and \( \text{N}_2\text{O} \) produced during the reaction catalyzed by HAO from \( \text{N. oceani} \) with different initial concentration of \( \text{NH}_2\text{OH} \) (0.1–3 mM). About 5.1–29.0 % of \( \text{NH}_2\text{OH} \) was converted to \( \text{NO}_2^- \) or \( \text{N}_2\text{O} \) after the reaction, and the ratio of produced \( \text{NO}_2^- \) and \( \text{N}_2\text{O} \) decreased with initial \( \text{NH}_2\text{OH} \) concentration. Production of \( \text{N}_2\text{O} \) in the blank runs with 1 and 3 mM \( \text{NH}_2\text{OH} \) were 5.9...
and 7.1 % of that in the presence of HAO (HAO +), respectively (Table 1). With lower NH$_2$OH concentrations of 0.1 and 0.3 mM, however, production of N$_2$O in the blank runs was 32.2 and 179.1 % of that observed in the HAO+ runs, respectively (Table 1). For this reason, further experiments were conducted at an 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 in SP values between experiments with 1 and 3 mM NH$_2$OH was insignificant ($p > 0.05$).

Results obtained with an initial NH$_2$OH concentration of 3 mM showed that $\delta^{15}$N$_{\text{bulk}}$ of N$_2$O produced by N. oceanii (experiments A and B) and N. europaea (experiment A) was almost constant and about 10 ‰ lower than $\delta^{15}$N of NH$_2$OH ($\sim$7.0 ‰) (Table 2). Similar $\delta^{15}$N difference (9 ‰) between N$_2$O and NH$_2$OH was observed in experiment D, in which NH$_2$OH with lower $\delta^{15}$N value (−43.9 ‰) was used. In experiment C of both N. europaea and N. oceanii, however, $\delta^{15}$N of N$_2$O was higher than that of NH$_2$OH by 1.8–9.9 ‰ (Table 2). The $\delta^{18}$O also showed a variation among experiments A–D of N. oceanii, 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 $\delta^{15}$N$_{\text{bulk}}$ and $\delta^{18}$O of N$_2$O were not significantly different between the two strains.

In contrast, the SP value of N$_2$O produced by NH$_2$OH oxidation with HAO was independent of initial concentration and $\delta^{15}$N value of NH$_2$OH or degree of reaction process (Tables 1 and 2). Moreover, the difference in SP between experiments with HAO extracted from N. oceanii 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$_2$O produced during reduction of NO with NOR

Table 3 shows concentration and isotopomer ratios of N$_2$O produced by the reduction of NO with NOR. About 65.0–149.2 % of NO was converted to N$_2$O. The concentration of N$_2$O was 17 times and 7.3 times higher in the presence of NOR (HAO+) compared to the blank runs without NOR. SP values of N$_2$O from the enzymatic reaction showed little variation (−5.9 ± 2.1 ‰) and were lower than SP values observed in the control runs (15.1–16.8 ‰) ($p < 0.05$).

### 3.3 Isotopomer ratio of N$_2$O produced by N. oceanii under different initial O$_2$ concentration

The amount of N$_2$O produced by N. oceanii was 2.9 ± 0.9 ($n = 6$), 8.9 ± 0.5 ($n = 2$), and 18.1 ± 3.6 ($n = 5$) nmol for O$_2$ concentrations of 0, 2, and 21 %, respectively. Production of both N$_2$O and NO$_2$ was much higher under aerobic conditions than under anaerobic conditions ($p < 0.05$, Fig. 2). Isotopomer ratios of N$_2$O showed a strong dependence on initial O$_2$ concentration (Fig. 3). They were more enriched under aerobic conditions 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$_2$O produced during the in vitro oxidation of NH$_2$OH with HAO from the two strains of AOB (36.3 ± 2.3 ‰) indicates that this parameter is determined by the enzymatic reaction step and is not affected by other factors such as concentration, the extent of reaction, and nitrogen isotope ratios of substrate (Table 2). This study demonstrates the first direct evidence of isotopomeric fractionation of N$_2$O produced by enzymatic reactions, confirming the robustness of SP of N$_2$O as predicted by previous studies (e.g., Frame and Casciotti, 2010).

Observed dependence of product NO$_2$ /N$_2$O ratio on initial NH$_2$OH concentration could be caused by the availability of electron acceptors. The oxidation of NH$_2$OH with HAO mainly provides NO$_2$ as a product when enough electron acceptors are supplied, while the reaction is likely to produce N$_2$O when the amount of electron acceptors is not enough to complete the reaction as proposed by Yamanaka and Sakano (1980). In the present study, electron acceptors (potassium...
Table 1. Concentration and isotopomer ratios of N\textsubscript{2}O produced during NH\textsubscript{2}OH oxidation by HAO enzyme extracted from \textit{N. oceanii} with different concentrations of NH\textsubscript{2}OH.

<table>
<thead>
<tr>
<th>HAO</th>
<th>NH\textsubscript{2}OH</th>
<th>Concentration (µmol L\textsuperscript{-1})</th>
<th>Yield (%)</th>
<th>(\delta^{15})N\textsubscript{bulk} (%)</th>
<th>(\delta^{15})N\textsubscript{α} (%)</th>
<th>(\delta^{15})N\textsubscript{β} (%)</th>
<th>(\delta^{18})O (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>3 mM</td>
<td>Average ± SD</td>
<td>23.9 ± 2.2</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>Blank</td>
<td>3 mM</td>
<td></td>
<td>1.7</td>
<td>0.4</td>
<td>−17.0</td>
<td>−2.9</td>
<td>−31.1</td>
<td>32.3</td>
</tr>
<tr>
<td>+</td>
<td>1 mM</td>
<td>Average ± SD</td>
<td>26.1 ± 6.1</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>Blank</td>
<td>1 mM</td>
<td></td>
<td>1.5</td>
<td>1.0</td>
<td>−15.4</td>
<td>1.0</td>
<td>−31.8</td>
<td>37.7</td>
</tr>
<tr>
<td>+</td>
<td>0.3 mM</td>
<td>Average ± SD</td>
<td>4.9 ± 0.3</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>Blank</td>
<td>0.3 mM</td>
<td></td>
<td>1.6</td>
<td>3.4</td>
<td>−15.1</td>
<td>2.9</td>
<td>−33.1</td>
<td>47.7</td>
</tr>
<tr>
<td>+</td>
<td>0.1 mM</td>
<td>Average ± SD</td>
<td>1.1 ± 0.2</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>Blank</td>
<td>0.1 mM</td>
<td></td>
<td>1.9</td>
<td>12.3</td>
<td>−15.5</td>
<td>0.5</td>
<td>−31.5</td>
<td>50.4</td>
</tr>
</tbody>
</table>

Concentration is that in the gas phase (head space) in the vial. N\textsubscript{2}O yield represents the fraction of N atoms converted to N\textsubscript{2}O from substrate NH\textsubscript{2}OH ([N\textsubscript{2}O-N]/[NH\textsubscript{2}OH-N]). Isotopomer ratios for “HAO+” were corrected for the blank. Average and SD (standard deviation) were calculated for samples, including HAO in each condition (n = 3).

Table 2. Concentration and isotopomer ratios of N\textsubscript{2}O produced during NH\textsubscript{2}OH oxidation by HAO enzyme extracted from \textit{Nitrosooccus oceanii} and \textit{Nitrosomonas europaea} with 3 mM of NH\textsubscript{2}OH.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HAO</th>
<th>Concentration (µmol L\textsuperscript{-1})</th>
<th>Yield (%)</th>
<th>(\delta^{15})N\textsubscript{bulk} (%)</th>
<th>(\delta^{15})N\textsubscript{α} (%)</th>
<th>(\delta^{15})N\textsubscript{β} (%)</th>
<th>(\delta^{18})O (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosooccus oceanii (HAO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>31.0</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>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>28.1</td>
<td>12.1</td>
<td>−17.1</td>
<td>1.8</td>
<td>−36.0</td>
<td>41.3</td>
<td>37.8</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>29.6 ± 2.1</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>
</tr>
<tr>
<td>A</td>
<td>Blank</td>
<td>4.7</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>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>25.0</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>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>21.4</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>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>25.5</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>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>23.9 ± 2.2</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>
</tr>
<tr>
<td>B</td>
<td>Blank</td>
<td>1.7</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>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>38.7</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>
</tr>
<tr>
<td>C</td>
<td>Blank</td>
<td>0.6</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>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>24.7</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>
</tr>
<tr>
<td>D</td>
<td>Blank</td>
<td>2.6</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>
</tr>
<tr>
<td>D</td>
<td>Blank</td>
<td>0.1</td>
<td>0.03</td>
<td>−46.2</td>
<td>−27.0</td>
<td>−65.5</td>
<td>55.3</td>
<td>38.5</td>
</tr>
</tbody>
</table>

Nitrosomonas europaea (HAO) | | | | | | | | |
| A          | +     | 32.8                                       | 14.1      | −14.4                                    | 4.9                             | −33.7                           | 42.5             | 38.6  |
| A          | +     | 36.4                                       | 15.6      | −13.8                                    | 4.6                             | −32.2                           | 42.9             | 36.8  |
| A          | +     | 34.7                                       | 14.9      | −17.1                                    | 2.4                             | −36.5                           | 42.8             | 38.9  |
| Average    |       | 34.6 ± 1.8                                 | 14.9 ± 0.9| −15.0 ± 1.5                              | 2.9 ± 1.2                       | −33.0 ± 1.9                   | 41.8 ± 0.2       | 35.9 ± 0.9 |
| A          | Blank | 4.7                                        | 2.0       | −14.8                                    | −4.0                            | −25.6                           | 36.3             | 21.6  |
| C          | +     | 5.0                                        | 2.2       | −34.0                                    | −18.1                           | −49.9                           | 37.4             | 31.8  |
| C          | Blank | 0.3                                        | 0.1       | −39.7                                    | −24.2                           | −55.2                           | 33.1             | 31.1  |

Experiments A, B, C and D were conducted on different dates. The initial \(\delta^{15}\)N value of NH\textsubscript{2}OH was −7.0 % for A and B, −43.9 % for C and D. Concentration is that in the gas phase (head space) in the vial. N\textsubscript{2}O yield represents the fraction of N atoms converted to N\textsubscript{2}O from substrate NH\textsubscript{2}OH ([N\textsubscript{2}O-N]/[NH\textsubscript{2}OH-N]). Isotopomer ratios for “HAO+” were corrected for the blank. Average and standard deviation were calculated for samples, including HAO.

Ferricyanide might have been depleted under conditions of 1 and 3 mM NH\textsubscript{2}OH because its initial concentration was kept constant. With lower NH\textsubscript{2}OH concentrations (0.1 and 0.3 mM), the production rate of N\textsubscript{2}O was smaller than that of NO\textsubscript{3}−, and the amount of produced N\textsubscript{2}O became closer to that produced in the blank experiments. The amount of blank N\textsubscript{2}O was almost constant (70–90 nmol; note that the “yield” listed in Table 1 shows the amount of N\textsubscript{2}O-N relative
The yield of N₂O produced by the reduction of NO with NOR exceeded 100 %, which could be caused by underestimation of initial NO concentration. Since the NO-saturated ethanol solution was prepared by purging the ethanol with pure NO gas in a sealed vial at room temperature (25 °C), we calculated the NO concentration based on NO solubility at 25 °C and 1 atm. However, there is a possibility that the vial inside was pressurized and the excess NO dissolved in ethanol during the preservation at 4 °C.

The SP value of N₂O produced during the in vitro reduction of NO with NOR from Paracoccus denitrificans (−5.9 ± 2.1 ‰) agrees with the value reported for N₂O produced in vivo from NO₃⁻ reduction by the same species (−5.1 ± 1.8 ‰, Toyoda et al. 2005). Therefore, our results prove 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 SP might depend on a small structural difference in the active center of NOR between the studied species, or that previously reported SP for N₂O from NO₂⁻ / NO₃⁻ reduction could have been affected by other processes like NH₂OH oxidation.

It is known that AOB produce N₂O as a byproduct during the oxidation of NH₂OH to NO₃⁻ with HAO and that some species of AOB can also produce N₂O from nitrite by the sequential reduction of nitrite to NO (using NIR) followed by reduction of NO (using 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 conditions. Although elucidating the reason why those AOB function as nitrifier-denitrification requires study of a wider spectrum of strains, detoxification of accumulated nitrite, co-respiration of oxygen and nitrite, and other mechanisms have been proposed (Beaumont et al., 2004; Stein, 2011).

In our bacterial culture experiments, production of both NO₂⁻ and N₂O are enhanced under aerobic conditions (Fig. 2) because O₂ is required for ammonium oxidation, the first step of the successive reaction to NO₃⁻. Although we did not measure O₂ concentration during the aerobic incubation (21 % O₂), oxygen concentration must have been

Table 3. Concentration and isotopomer ratios of N₂O during NO reduction by NOR extracted from Paracoccus denitrificans.

<table>
<thead>
<tr>
<th>NOR</th>
<th>Concentration (µmol L⁻¹)</th>
<th>Yield (%)</th>
<th>¹⁵Nbulk (%)</th>
<th>¹⁵Nβ (%)</th>
<th>¹⁸O (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>5.1</td>
<td>129.4</td>
<td>17.1</td>
<td>14.3</td>
<td>19.9</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>5.0</td>
<td>127.9</td>
<td>17.1</td>
<td>15.1</td>
<td>19.1</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>5.9</td>
<td>149.2</td>
<td>17.1</td>
<td>14.6</td>
<td>19.6</td>
</tr>
<tr>
<td>A</td>
<td>Blank</td>
<td>0.3</td>
<td>7.6</td>
<td>14.5</td>
<td>22.0</td>
<td>6.9</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>2.6</td>
<td>65.0</td>
<td>11.0</td>
<td>6.5</td>
<td>15.5</td>
</tr>
<tr>
<td>C</td>
<td>Blank</td>
<td>0.3</td>
<td>7.4</td>
<td>11.7</td>
<td>19.9</td>
<td>3.5</td>
</tr>
<tr>
<td>D</td>
<td>Blank</td>
<td>0.4</td>
<td>8.9</td>
<td>16.3</td>
<td>24.7</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Experiments A, C and D were conducted on different dates. Concentration is that in the gas phase (head space) in the vial. N₂O yield represents the fraction of N atoms converted to N₂O from substrate NO ([N₂O-N/NO-N]). Isotopomer ratios for NOR+ were corrected for the blank and were averaged for each experiment. SD represents standard deviation for each experiment. For experiment C, results of experiment D were adopted as the blank, because the blank δ¹⁸O value obtained in experiment C was significantly different from that obtained in experiments A and D.

Figure 2. Amount of NO₂⁻ and N₂O produced from 378 µmol of NH₃ in concentrated cell suspensions of N. oceani under different initial O₂ concentrations (n = 2).
kept high enough to maintain the aerobic condition for the following reasons: (1) it is reported that the measured ratio of consumed O$_2$ and produced NO$_2^-$ during aerobic incubation of *N. oceani* (1.35–1.43) is consistent with the ratio calculated from the stoichiometry of ammonia oxidation to nitrite (1.50) (Gundersen, 1966); (2) oxygen consumption by respiration using endogenous organics is low (Gundersen, 1966); and (3) a quantitative estimate based on the stoichiometry and the amount of the product (NO$_2^-$ and N$_2$O) in this study indicates that the oxygen consumption was relatively small (e.g., oxygen concentration should have decreased from 21% to ca. 18%).

Furthermore, observed co-variation of SP values of N$_2$O and oxygen concentrations (Fig. 3) implies that relative contributions from the two production pathways are sensitive to oxygen availability. It has been shown that SP of N$_2$O produced by nitrifier-denitrification and by NH$_2$OH oxidation are approximately 0 and 30 ‰, respectively (Sutka et al., 2006). Consequently, the lower SP values produced during nitrification in this study are most likely the result of the addition of N$_2$O from nitrifier-denitrification. It turns out that NH$_2$OH oxidation is the dominant N$_2$O production pathway under aerobic conditions, while NO$_2^-$ reduction is dominant under anaerobic conditions (Fig. 3). We note that even in aerobic conditions (21% O$_2$), the SP value of N$_2$O produced by *N. oceani* is lower than that of N$_2$O produced by in vitro NH$_2$OH oxidation with HAO extracted from the same bacteria (Fig. 3). This suggests the NO$_2^-$ reduction pathway is not negligible under aerobic conditions and thus previous studies based on pure culture incubation of AOB underestimated the SP value for N$_2$O from NH$_2$OH oxidation.

We further show quantitative estimation of the contribution of NO$_2^-$ reduction to N$_2$O production. Here we assume that only NH$_2$OH oxidation and nitrifier-denitrification were the N$_2$O production pathways, although there was a possibility that N$_2$O was produced by other unknown processes. This assumption allows a simple interpretation of the relative contribution of hydroxylamine oxidation and nitrifier-denitrification to N$_2$O production on the basis of SP as follows.

$$\text{SP}_{\text{measured}} = \text{SP}_{\text{NOR}} \times X_{\text{NOR}} + \text{SP}_{\text{HAO}} \times (1 - X_{\text{NOR}}) \quad (4)$$

In Eq. (4), SP$_{\text{measured}}$, SP$_{\text{NOR}}$, and SP$_{\text{HAO}}$ represent SP values of observed N$_2$O and N$_2$O produced in enzymatic reaction with NOR and HAO, respectively, and $X_{\text{NOR}}$ indicates the relative contribution of the NOR-mediated pathway. As shown in Table 4, average $X_{\text{NOR}}$ is calculated as 82 ± 18 ‰, 42 ± 10 ‰, and 22 ± 4 ‰ under 0, 2, and 21% initial O$_2$ concentrations, respectively. These values were similar to those measured by Frame and Casciotti (2010). Using the total amount of N$_2$O produced in each experiment, the N$_2$O produced from NO$_2^-$ reduction is estimated at 2.5 ± 1.0 nmol ($n = 6$), 3.8 ± 0.7 nmol ($n = 2$), 3.9 ± 0.7 nmol ($n = 5$) under 0, 2, and 21% initial O$_2$ concentrations, respectively. This means that the rate of N$_2$O production via the 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 conditions compared to anaerobic conditions. This might indicate that activities of NIR or NOR were not enhanced in this study, although we did not directly measure the regulation and activity of the enzymes. The process information provided by SP values enables us to estimate bulk 15N-enrichment factors ($\epsilon$, which is approximately equal to $\delta^{15}N_{\text{product}} - \delta^{15}N_{\text{substrate}}$ under the excess supply of substrate) for N$_2$O production from NH$_3^+$ by NOR- and HAO-mediated pathways. If N$_2$O is produced only by a NH$_2$OH oxidation pathway ($X_{\text{NOR}} = 0$), SP$_{\text{measured}} = \text{SP}_{\text{HAO}}$ and $\delta^{15}N_{\text{bulk}}$ of N$_2$O is estimated at −32.9 ‰ from the linear relationship between SP and $\delta^{15}N_{\text{bulk}}$ (Fig. 3). However, if N$_2$O is produced only by a NO$_2^-$ reduction pathway ($X_{\text{NOR}} = 1$), SP$_{\text{measured}} = \text{SP}_{\text{NOR}}$ and $\delta^{15}N_{\text{bulk}}$ of N$_2$O is estimated at −67.5 ‰. Combining these values with $\delta^{15}N$ of (NH$_4$)$_2$SO$_4$ used in this study (±0.34 ‰), we obtain $\epsilon_{\text{HAO}} = -32.6$ ‰ and $\epsilon_{\text{NOR}} = -67.2$ ‰. The $\epsilon_{\text{NOR}}$ 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 ($\delta^{15}N_{\text{NO}} = -56.9$ ‰). The cause of the difference could be different experimental approaches (with/without enzymatic reactions) or different species studied. However, it is evident that $\epsilon$ is significantly different between the NH$_2$OH oxidation pathway and the NO$_2^-$ reduction pathway.

Finally, we discuss the mechanisms that control SP values of N$_2$O produced during the enzymatic reactions. Both HAO and NOR enzymes are known to have Fe atoms as active centers, but their structures 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 NH$_2$OH molecule binds to the center and is oxidized.
Table 4. Estimated contribution of NO$_2^-$ reduction catalyzed by NOR to N$_2$O production by *N. oceanii* under different initial O$_2$ concentrations.

<table>
<thead>
<tr>
<th>Initial O$_2$ (%)</th>
<th>N$_2$O (nmol)</th>
<th>N$_2$O yield (mmol)</th>
<th>$X_{NOR}$</th>
<th>N$<em>2$O$</em>{NOR}$ $^\ast\ast$ (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 %</td>
<td>2.9 ± 0.9</td>
<td>1.5 ± 0.5</td>
<td>0.82 ± 0.18</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 ± 0.10</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 ± 0.04</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 substrate NH$_4^+$ ([N$_2$O-N]/[NH$_4^+$]).

$^\ast$ Contribution of NO$_2^-$ reduction to N$_2$O production.

$^\ast\ast$ Amount of N$_2$O produced by NO$_2^-$ reduction.

To explain the high SP values of the N$_2$O generated by HAO reactions, we propose a two-step reaction mechanism as indicated in Fig. 4.

If substrate NH$_2$OH is supplied in excess or the electron acceptor for the reaction is lacking, however, another NH$_2$OH molecule will bind to the same center to form an N-N bond. In this case, the primary-binding NH$_2$OH molecule could be more depleted in $^{15}$N than the secondary-binding NH$_2$OH molecule by the kinetic isotope effect. The observed positive SP might indicate that the peripheral (β) and central (α) N atoms in the product N$_2$O derive from the primary and secondary NH$_2$OH, respectively.

The catalytic center of the *N. europaea* HAO for hydroxylamine oxidation is formed by the P460 molecule and the side-chains of three amino acids (Asp291, His292, Tyr358; *N. europaea* HAO numbering) that surround the distal side of the heme (Igarashi et al. 1996). Although two of the three residues (Asp291 and His292) are completely conserved among all the HAOs, the other (Tyr358) is replaced by Asn in the enzymes of the gamma-AOBs, including the NS58. Recently, the crystal structure of the *N. europaea* HAO in the substrate-binding state was reported by Maalcke et al. (2014). They proposed that the side-chain of Tyr358 would participate in the second step of the two-step reaction shown in Fig. 4, namely the addition of water molecules to the intermediate species bound on the P460. The replacement of the Tyr to Asn in *N. oceanii* NS58 HAO (Asn368) may obstruct the addition of H$_2$O to the intermediate in the HAO reaction. For that reason, it is probable that the N$_2$O-generating pathway shown in Fig. 4 would be more likely to occur in the case of *N. oceanii* NS58 HAO compared to the case of *N. europaea* HAO. In addition, this explanation is consistent with the present result that *N. oceanii* NS58 generates more N$_2$O under the aerobic condition than the anaerobic condition (Fig 3), showing a contrast to the case of *N. europaea* (Poth and Focht., 1985).

In the case of NOR, three types are known for bacteria: cNOR that accepts electrons from cytochrome c, and qNOR and qCuNOR that accept electrons from quinols (Zumft et al. 2005). Bacterial denitrification is considered to be catalyzed by cNOR, although qNOR and 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 (Fe$b_1$) and heme $b_3$ (Hino et al. 2010), and it is proposed that two NO molecules respectively bind to the two centers simultaneously to form N$_2$O (Watmough et al. 2009). This parallel binding mechanism could bring about nearly the same isotope effect for the two N atoms in intermediate ONNO, and if the elimination of the O atom from N-O bonding does not fractionate $^{15}$N within the intermediate molecule, then the SP value of N$_2$O would be nearly 0‰.

5 Conclusions

We presented direct evidence that SP values of N$_2$O produced by bacterial nitrification and denitrification are controlled by the enzymatic reactions of HAO and NOR during NH$_2$OH 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 N$_2$O production by AOB in pure culture, and showed that they are sensitive to oxygen concentration and that NO$_2^-$ reduction could occur under aerobic conditions.

Although further studies are required, this study demonstrates that isotopomer analysis constitutes a powerful tool to investigate N$_2$O production pathways in various environments. It will be important to expand these observations 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 co-denitrification are unknown. Combined analysis of several isotopomer ratios should be developed to distinguish NH$_2$OH oxidation and fungal denitrification, because SP values of N$_2$O from the two pathways have 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 reactions would be effective, as shown in this work. Although the enzymatic pathway for N₂O production by AOA is still uncertain, combined analysis of genome sequences, physiology, and isotopomer ratios would promise further understanding of microbial N₂O production mechanisms.

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References


T. Yamazaki et al.: Isotopomeric characterization of nitrous oxide


