Biogeosciences Discuss., 10, 16615–16643, 2013
www.biogeosciences-discuss.net/10/16615/2013/
doi:10.5194/bgd-10-16615-2013

Biogeosciences Discussions
Open Access

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

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Received: 26 September 2013 – Accepted: 12 October 2013 – Published: 25 October 2013

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Published by Copernicus Publications on behalf of the European Geosciences Union.

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas and produced in denitrification and nitrification in environmental nitrogen cycle by various microorganism. Site preference (SP) of ^{15}N in N_2O, which is defined as the difference in the natural abundance of isotopomers $^{14}N^{15}NO$ and $^{15}N^{14}NO$ relative to $^{14}N^{14}NO$, has been reported to be a useful tool to quantitatively distinguish N_2O production pathway. To determine representative SP value for each microbial process, we firstly measured SP of N_2O 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*, respectively. The SP value for NOR reaction (-5.9 ± 2.1 ‰) showed nearly the same value as that reported for N_2O 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 N_2O 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_2^-) reduction (which is followed by NO reduction) to N_2O production by *N. oceani* incubated under different O_2 availability. Our calculations revealed that previous in vivo studies might have underestimated the SP value for NH_2OH oxidation pathway possibly due to a small contribution of NO_2^- reduction pathway. Further evaluation of isotopomer signatures of N_2O using common enzymes of other processes related to N_2O would improve the isotopomer analysis of N_2O in various environments.

1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and contributes indirectly to destruction of ozone layer in the stratosphere (Ravishankara et al., 2009). Production of the N_2O on the earth is mainly controlled by microbial processes that include nitrification and denitrification (Stein and Yung, 2003). In the nitrification, autotrophic microorganisms

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like ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite (NO_2^-) and produce N_2O 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_2O as an intermediate under mainly anaerobic environment (Hayastu et al., 2008). Thus, the production and consumption of N_2O consist of many microbiological functions including autotrophic/heterotrophic and oxic/anoxic processes. The investigation of N_2O 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_2O production pathways and several studies reported the isotopomer ratios of each N_2O 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_2O molecule, is a powerful tool to quantitatively distinguish the production pathways such as bacterial NO_2^- reduction and bacterial hydroxylamine (NH_2OH) 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_2O 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_2O produced by NH_2OH oxidation might have been biased by simultaneous N_2O production

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by NO_2^- reduction because some AOB have denitrifying enzymes and produce N_2O by NO_2^- reduction in addition to NH_2OH oxidation (Arp et al., 2003). In consideration of this problem, Frame and Casciotti (2010) estimated the SP value of N_2O produced by NH_2OH oxidation using the relationship between SP and oxygen isotope ratios in N_2O 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_2O production pathway.

In this study, we report the isotopomer ratios of N_2O produced in vitro using enzymes extracted and purified from two strains of AOB (*Nitorosococcus oceani* and *Nitrosomonas europaea*) and one species of denitrifying bacteria (*Paracoccus denitrificans*). Our experiments have an advantage that isotope effects related to the N_2O 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_2O produced in vivo by *N. oceani* under different oxygen concentrations and estimated the relative contribution of NH_2OH 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 $(NH_4)_2SO_4$-supplemented

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artificial seawater (37.8 mM 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 *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.

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 *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 *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 NH_2OH-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 µM NH_2OH, and 100 µM 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

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of ferricyanide and that of ferrocyanide ($\Delta\varepsilon_{mM}$) 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 NH_2OH is oxidized in a minute), was obtained and used for the assay of N_2O-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 N_2O produced during oxidation of NH_2OH 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 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_2OH were conducted on four different dates (A–D). In experiments C and D, lot number of NH_2OH 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 NO_2^- was determined spectrophotometrically by a diazo-coupling method (Nicholas and Mason, 1957).

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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_2O produced by enzymatic reduction of NO

In a same glass vial that had been used for NH_2OH oxidation experiments, a 10 mL solution was prepared so that it contain 10 mM sodium phosphate buffer (pH6.0), 2.9 µM

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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 N_2O generated was done as mentioned below.

2.6 Quantitative and isotopomeric analysis of N_2O 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 $(NH_4)_2SO_4$-supplemented artificial seawater (see Sect. 2.1), then were incubated with gentle stirring at 25 °C in dark for 30 min to remove NO_2^- 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 O_2/N_2 mixture that contained 2 % (v/v) O_2 (Shizuoka Sanso Co., Shizuoka, Japan) or pure N_2 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 % O_2) and the microaerobic (2 % O_2) 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.

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Quantification and measurement of isotopomer ratios of N_2O gas released into the headspace of the incubation vial were performed as described below. Concentration of NO_2^- that was accumulated in the reaction solution was also measured as explained above.

2.7 Measurement of N_2O concentration and isotopomer ratios

Concentrations and isotopomer ratios of N_2O were measured using an on-line analytical system that originally developed for N_2O 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 ($Mg(ClO_4)_2$ and NaOH on support) to remove H_2O and CO_2, and N_2O was concentrated on glass beads packed in a U-shaped trap at liquid N_2 temperature. After further purification on GC, N_2O 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_2O (NO^+) that contains N atom in the center position of N_2O molecules (Toyoda and Yoshida, 1999). Concentration and bulk nitrogen and oxygen isotope ratio of N_2O was determined with analysis of N_2O molecule ion (N_2O^+). Pure N_2O was used as a reference gas for isotopomer ratios. Notation of isotopomer ratios of N_2O is shown below.

$$\delta^{15}N^{i}_{sample} = {}^{15}R^{i}_{sample} / {}^{15}R_{standard} - 1 \quad (1)$$

$$\delta^{18}O^{i}_{sample} = {}^{18}R^{i}_{sample} / {}^{18}R_{standard} - 1 \quad (2)$$

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In Eqs. (1) and (2), ^{15}R and ^{18}R represent $^{15}N/^{14}N$ ratio and $^{18}O/^{16}O$ 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 α, β, or bulk which respectively designates central, peripheral, or average isotope ratios in nitrogen atom(s) in N_2O molecule. We also define the ^{15}N-site preference (SP) as an illustrative parameter of intramolecular distribution of ^{15}N (Yoshida and Toyoda, 1999). The precision of measurement is better than 0.5 ‰ for $\delta^{15}N^{bulk}$ and $\delta^{18}O$, and better than 1.0 ‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$.

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

The $\delta^{15}N$ of NH_2OH 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_2O produced during oxidation of NH_2OH with HAO

Figure 1 shows the amount of NO_2^- and N_2O produced during the reaction catalyzed by HAO from *N. oceani* with different initial concentration of NH_2OH (0.1–3 mM). About 5.1–29.0 % of NH_2OH was converted to NO_2^- or N_2O after the reaction, and the ratio of produced NO_2^- and N_2O decreased with initial NH_2OH concentration. With high initial NH_2OH concentrations (1 and 3 mM), production of N_2O 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_2OH concentrations (0.1 and 0.3 mM),

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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_2OH concentration of 3 mM to examine repeatability of the reaction and effect of bacterial strain and N isotope ratio of NH_2OH. The $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of N_2O showed lower values at high (3 mM) NH_2OH 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_2OH concentration of 3 mM (see Table 2), $\delta^{15}N^{bulk}$ 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_2OH (−7.0 ‰). In experiment D of *N. oceani*, the difference of $\delta^{15}N^{bulk}$ from $\delta^{15}N$ of NH_2OH 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_2O was higher than $\delta^{15}N$ of NH_2OH 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_2OH was different between experiments A/B and C/D. When we compare the results from experiment A, the difference in $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of N_2O was not significant between the two strains ($p > 0.05$ and $p > 0.05$, respectively).

In contrast, SP value of N_2O produced by NH_2OH oxidation with HAO was independent of initial concentration or degree of reaction process and $\delta^{15}N$ value of NH_2OH (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 ($p > 0.05$).

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3.2 Concentration and isotopomer ratios of N_2O produced during reduction of NO with NOR

Table 3 shows concentration and isotopomer ratios of N_2O produced by the reduction of NO with NOR. About 66.0–151.6 % of NO was converted to N_2O. The concentration of N_2O 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_2O 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_2O produced by *N. oceani* under different initial O_2 concentration

The amount of N_2O produced by *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_2 concentration of 0, 2, and 21 %, respectively. Production of both N_2O and NO_2^- was much higher in aerobic condition than in anaerobic condition ($p < 0.05$, Fig. 2). Isotopomer ratios of N_2O showed a strong dependence on initial O_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_2O produced during the in vitro oxidation of NH_2OH 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

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value (36.3 ± 2.4 ‰) as previous studies predicted and its robustness during the bacterial oxidation of NH_2OH (e.g., Frame and Casciotti, 2010).

Observed dependence of the product concentration ratio between NO_2^- and N_2O on initial NH_2OH concentration could be caused by the availability of electron acceptor. The oxidation of NH_2OH with HAO mainly gives NO_2^- as a product when enough electron acceptor is supplied, while the reaction is likely to produce N_2O 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 ferycyanide) might have been depleted under the condition with 1 and 3 mM NH_2OH because its initial concentration was kept constant. With low NH_2OH concentrations (0.1 and 0.3 mM), rate of N_2O production was smaller than that of NO_2^- production and the amount of produced N_2O became closer to that produced in the control experiments (HAO− runs). The amount of N_2O produced in HAO− runs were almost constant (70–90 nmol; note that the "yield" listed in Table 1 shows the amount of N_2O relative to initial NH_2OH), and it must have been produced by non-catalytic reactions.

The yield for N_2O 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 N_2O produced during the in vitro reduction of NO with NOR from *P. denitirifcans* (−5.9 ± 2.1 ‰) agrees with that value reported for N_2O produced in vivo from NO_3^- reduction by the same species (−5.1 ± 1.8 ‰, Toyoda et al., 2005). Therefore, ours result proves that the factor controlling SP value of N_2O produced in NO_3^- 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 N_2O produced from NO_3^- or NO_2^- reduction by other denitrifying bacteria or some species of AOB (−0.8 to +0.1 ‰) (Sutka et al.,

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2004, 2006), and slightly higher than the value estimated for N_2O produced from NO_2^- reduction by *N. marina 113a*, oceanic AOB (−10.7±2.9 ‰; Frame and Casciotti, 2010). This implies that previously reported SP for N_2O from NO_3^-/NO_2^- reduction could have been affected by other processes like NH_2OH oxidation or that SP might depend on a small structural difference in NOR of studied species.

It is known that AOB produces N_2O as a byproduct during the oxidation of NH_2OH to NO_2^- with HAO and that some species of AOB can also reduce NO_2^- to N_2O with NO_2^- 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 NO_2^- has been proposed as a possible explanation (Beaumont et al., 2004). In our experiments, production of both NO_2^- and N_2O are enhanced under aerobic condition because O_2 is required for ammonium oxidation, the first step of the successive reaction to NO_2^- (Fig. 2). Observed co-variation of SP value of N_2O and oxygen concentration (Fig. 3) implies that relative contributions from the two pathways are sensitive to oxygen availability; NH_2OH oxidation becomes dominant N_2O production pathway under aerobic condition while NO_2^- reduction is dominant under anaerobic condition (Fig. 3). It is noteworthy that even in aerobic condition (20 % O_2), SP value of N_2O produced by *N. oceani* is lower than the value obtained by in vitro NH_2OH oxidation with HAO from the same bacteria (Fig. 3), which suggests NO_2^- reduction pathway is not negligible under aerobic condition and thus previous studies based on pure culture incubation of AOB underestimated the SP value for N_2O from NH_2OH oxidation.

We further show quantitative estimation of the contribution of NO_2^- reduction to N_2O production by using following equation.

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

In Eq. (4), $SP_{measured}$, SP_{NOR}, and SP_{HAO} represent SP values of observed N_2O, N_2O produced in enzymatic reaction with NOR and HAO, respectively, and X_{NOR} indicates

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relative contribution of the NOR–mediated pathway. As shown in Table 4, average X_{NOR} is calculated as 82 ± 18 %, 42 ± 10 %, and 22 ± 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 ± 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 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 *nir*K 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 (ε, which is approximately equal to $\delta^{15}N_{product} - \delta^{15}N_{substrate}$ 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_{NOR} = 0), $SP_{measured} = SP_{HAO}$ and $\delta^{15}N^{bulk}$ of N_2O is estimated at −32.9 ‰ from the linear relationship between SP and $\delta^{15}N^{bulk}$ (Fig. 3). On the other hand, if N_2O is produced only by NO_2^- reduction pathway (X_{NOR} = 1), $SP_{measured} = SP_{NOR}$ and $\delta^{15}N^{bulk}$ 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 ε_{HAO} = −32.6 ‰ and ε_{NOR} = −67.2 ‰. The ε_{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 ε is significantly different between NH_2OH oxidation pathway and NO_2^- reduction pathway.

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Finally, we discuss the mechanisms that control SP value of N_2O 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 NH_2OH molecule binds to the center and is oxidized to NO_2^-. If substrate NH_2OH is supplied in excess or electron acceptor for the reaction is lacking, however, another NH_2OH molecule would bind to the same center to form N-N bond. In this case, the primary-binding NH_2OH molecule could be more depleted in ^{15}N than secondary-binding NH_2OH molecule by the kinetic isotope effect. The observed positive SP might indicate that the peripheral (β) and central (α) N atoms in product N_2O derive from the primary and secondary NH_2OH, 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 (Fe_b) and heme b_3 (Hino et al., 2010), and it is proposed that two NO molecules bind at each center simultaneously to form N_2O (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 ^{15}N within the intermediate molecule, then SP value of N_2O would be nearly 0 ‰.

5 Conclusions

We presented the direct evidence that SP values of N_2O produced by bacterial nitrification and denitrification are controlled by enzymatic reaction of HAO and NOR during NH_2OH oxidation and NO reduction, respectively. The SP value does not depend

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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_2O production by AOB in pure culture, and showed that they are sensitive to oxygen concentration and that NO_2^- reduction could occur under aerobic condition.

Although further studies are required, this study demonstrates that isotopomer analysis constitutes a powerful tool to investigate N_2O 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, isotopomeric 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_2OH oxidation and fungal denitrification because SP value of N_2O from the two pathways has been found to be nearly the same (Sutka et al., 2008). As for the characterization of various microbial N_2O 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 N_2O 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.

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Table 1. Isotopomer ratios of N_2O produced during NH_2OH oxidation by HAO enzyme extracted from *N. oceani* with different concentration of NH_2OH.

HAO	NH_2OH		Yield (%)	$\delta^{15}N^{bulk}$ (‰)	$\delta^{15}N^{\alpha}$ (‰)	$\delta^{15}N^{\beta}$ (‰)	$\delta^{18}O$ (‰)	SP (‰)
+	3 mM	Average ± SD	5.1 ± 0.4	−17.9 ± 0.2	0.1 ± 0.2	−36.0 ± 0.2	37.8 ± 0.1	36.1 ± 0.2
−	3 mM		0.4	−17.0	−2.9	−31.1	32.3	28.2
+	1 mM	Average ± SD	16.8 ± 3.9	−13.4 ± 0.2	4.7 ± 0.4	−31.6 ± 0.3	42.6 ± 0.2	36.3 ± 0.6
−	1 mM		1.0	−15.4	1.0	−31.8	37.7	32.8
+	0.3 mM	Average ± SD	10.6 ± 0.3	−5.8 ± 0.2	12.0 ± 0.3	−23.6 ± 0.2	44.5 ± 0.1	35.6 ± 0.3
−	0.3 mM		3.4	−15.1	2.9	−33.1	47.7	36.0
+	0.1 mM	Average ± SD	6.9 ± 1.2	−10.7 ± 0.5	6.6 ± 0.4	−28.1 ± 0.4	45.1 ± 0.8	34.7 ± 0.9
−	0.1 mM		12.3	−15.5	0.5	−31.5	50.4	32.0

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−").

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Table 2. Concentration in gas phase and isotopomer ratios of N_2O produced during NH_2OH oxidation by HAO enzyme extracted from *Nitrosococcus oceani* and *Nitrosomonas europaea* with 3 mM of NH_2OH.

Experiment	HAO	Yield (%)	$\delta^{15}N^{bulk}$ (‰)	$\delta^{15}N^{\alpha}$ (‰)	$\delta^{15}N^{\beta}$ (‰)	$\delta^{18}O$ (‰)	SP (‰)
Nitrosococcus oceani (HAO)							
A	+	13.3	−16.5	2.1	−35.1	41.6	37.1
A	+	12.1	−17.1	1.8	−36.0	41.3	37.8
	Average	12.7 ± 0.9	−16.8 ± 0.4	1.9 ± 0.2	−35.5 ± 0.6	41.5 ± 0.2	37.4 ± 0.5
A	–	2.0	−14.8	−4.0	−25.6	36.3	21.6
B	+	10.7	−17.7	0.0	−35.4	37.2	35.3
B	+	9.2	−18.0	0.0	−16.4	37.6	35.8
B	+	10.9	−18.1	−0.2	−16.5	37.3	35.5
	Average	10.3 ± 1.0	−17.9 ± 0.2	−0.1 ± 0.1	−35.7 ± 0.2	37.4 ± 0.2	35.6 ± 0.3
B	–	0.7	−17.0	−2.9	−31.1	32.3	28.2
C	+	16.6	−42.1	−25.4	−58.8	33.9	33.3
C	–	0.3	−39.7	−24.2	−55.2	33.1	31.1
D	+	10.6	−52.7	−33.5	−71.9	56.8	38.4
D	–	1.1	−49.4	−38.2	−60.5	54.7	22.3
D	–	0.0	−46.2	−27.0	−65.5	55.3	38.5
Nitrosomonas europaea (HAO)							
A	+	14.1	−14.4	4.9	−33.7	42.5	38.6
A	+	15.6	−13.8	4.6	−32.2	42.9	36.8
A	+	14.9	−17.1	2.4	−36.5	42.8	38.9
	Average	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	–	2.0	−14.8	−4.0	−25.6	36.3	21.6
C	+	2.2	−34.0	−18.1	−49.9	37.4	31.8
C	–	0.1	−39.7	−24.2	−55.2	33.1	31.1

Experiments A, B, C and D were conducted on different date. Isotopomer ratios for "HAO+" were corrected for the blank "HAO−". The initial $\delta^{15}N$ value of NH_2OH was −7.0 ‰ for A and B, −43.9 ‰ for C and D. Average and standard deviation were calculated for samples including HAO.

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Table 3. Concentration and isotopomer ratios of N_2O during NO reduction by NOR extracted from *Paracoccus denitrificans*.

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

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 $\delta^{18}O$ value obtained in experiment C was significantly different from that obtained in experiments A and D.

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Table 4. Estimated contribution of NO_2^- reduction catalyzed by NOR to N_2O production by *N. oceani* under different initial O_2 concentration.

Initial O_2 (%)	N_2O (nmol)	N_2O yield (10^{-5})	X_{NOR} [a]	N_2O_{NOR} [b] (nmol)
0 %	2.9 ± 0.9	1.5 ± 0.5	0.82 ± 0.1	2.5 ± 1.0
2 %	8.9 ± 0.5	4.7 ± 0.3	0.42 ± 0.1	3.8 ± 0.7
21 %	18.1 ± 3.6	9.5 ± 1.9	0.22 ± 0.0	3.9 ± 0.7

N_2O yield represents the fraction of N-atoms converted to N_2O from NH_4^+ as substrate; $N_2O - N/NH_4^+ - N$.

[a] Contribution of NO_2^- reduction to N_2O production.

[b] Amount of N_2O produced by NO_2^- reduction.

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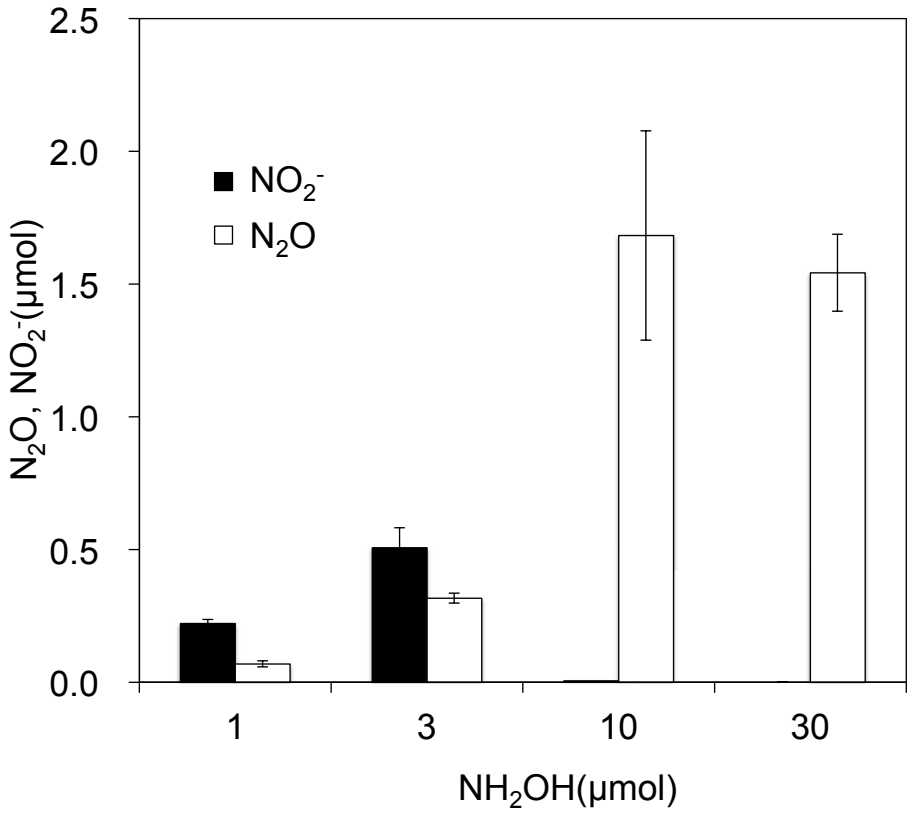

Fig. 1. Amount of N_2O and NO_2^- produced by HAO extracted from *N. oceani* under different substrate (NH_2OH) concentration (expressed as amount in the reaction vessel). The error bar indicates standard deviation for three replicates.

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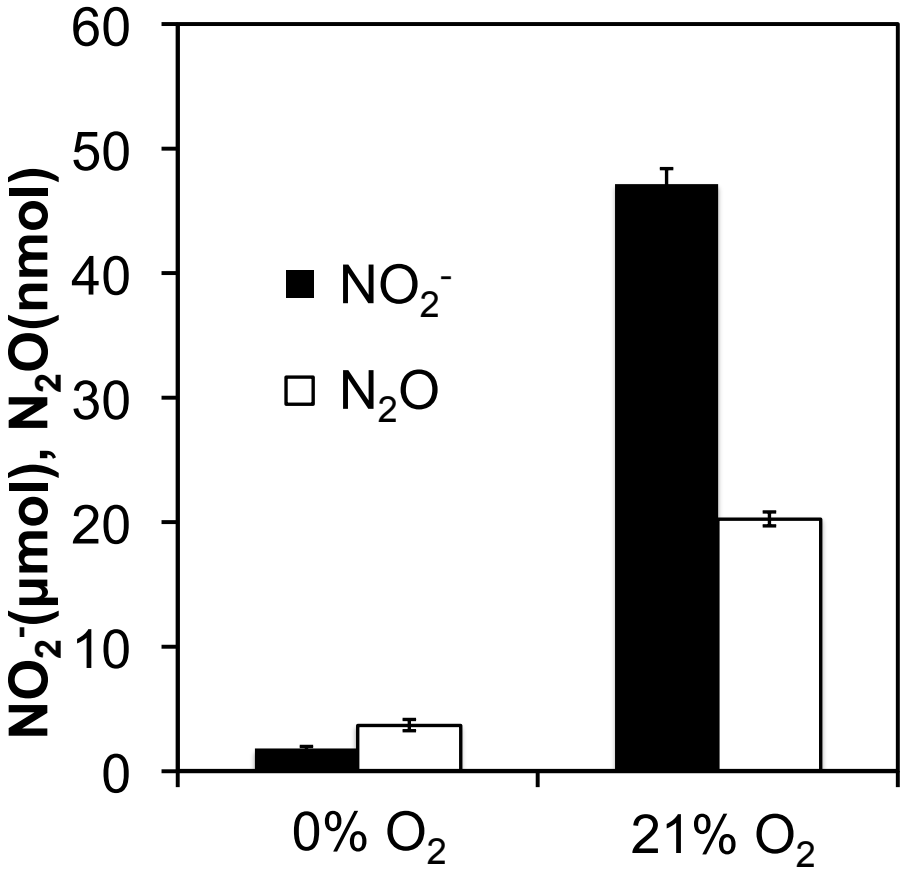

Fig. 2. Amount of NO_2^- and N_2O produced from 378 μmol of NH_4^+ in concentrated cell suspensions of *N. oceani* under different initial O_2 concentration ($n = 2$).

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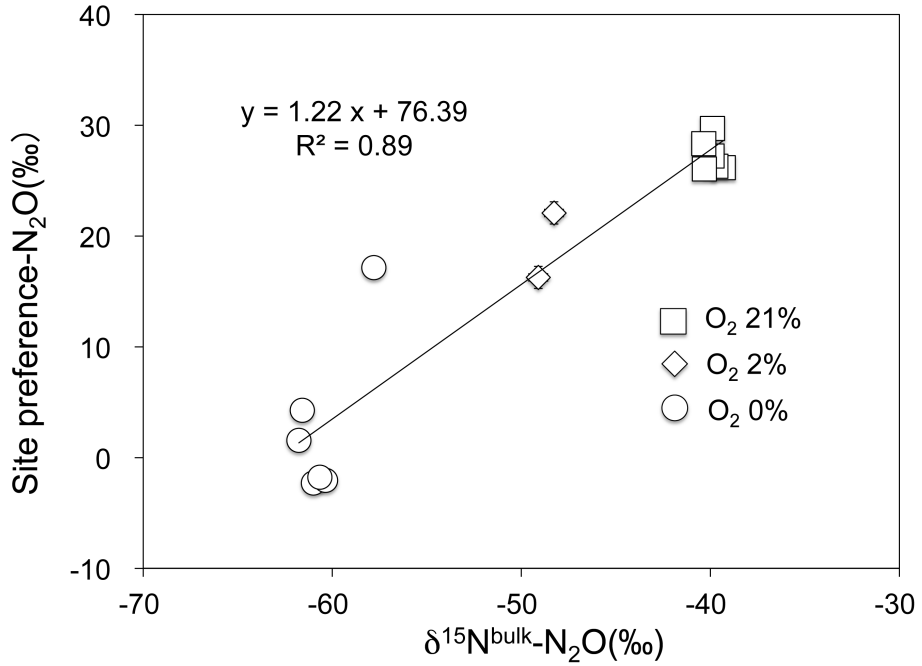

Fig. 3. Site preference and $\delta^{15}N$ of N_2O in concentrated cell suspensions of *N. oceani* under different initial O_2 concentration. Error bar indicates the measurement error.

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