

ORIGINAL ARTICLE

Isotopic signatures of N₂O produced by ammonia-oxidizing archaea from soils

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N₂O gas is involved in global warming and ozone depletion. The major sources of N₂O are soil microbial processes. Anthropogenic inputs into the nitrogen cycle have exacerbated these microbial processes, including nitrification. Ammonia-oxidizing archaea (AOA) are major members of the pool of soil ammonia-oxidizing microorganisms. This study investigated the isotopic signatures of N₂O produced by soil AOA and associated N₂O production processes. All five AOA strains (I.1a, I.1a-associated and I.1b clades of *Thaumarchaeota*) from soil produced N₂O and their yields were comparable to those of ammonia-oxidizing bacteria (AOB). The levels of site preference (SP), $\delta^{15}\text{N}^{\text{bulk}}$ and $\delta^{18}\text{O}$ -N₂O of soil AOA strains were 13–30%, –13 to –35% and 22–36%, respectively, and strains MY1–3 and other soil AOA strains had distinct isotopic signatures. A ¹⁵N-NH₄⁺-labeling experiment indicated that N₂O originated from two different production pathways (that is, ammonia oxidation and nitrifier denitrification), which suggests that the isotopic signatures of N₂O from AOA may be attributable to the relative contributions of these two processes. The highest N₂O production yield and lowest site preference of acidophilic strain CS may be related to enhanced nitrifier denitrification for detoxifying nitrite. Previously, it was not possible to detect N₂O from soil AOA because of similarities between its isotopic signatures and those from AOB. Given the predominance of AOA over AOB in most soils, a significant proportion of the total N₂O emissions from soil nitrification may be attributable to AOA.

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Introduction

Nitrous oxide (N₂O) is a long-lived greenhouse gas, which is also involved in ozone depletion. The atmospheric abundance of N₂O is approximately one-thousandth of CO₂ but its radiative efficiency (Wm⁻² ppb⁻¹) is 216 times greater than that of CO₂ (Rahn and Wahlen, 1997). Soils are major sources of N₂O (Conrad, 1996) and are believed to contribute approximately 65% of the total N₂O emitted to the atmosphere (Seitzinger *et al.*, 2000). The atmospheric concentrations of N₂O have been increasing from pre-industrial levels of 0.27 ppmv to current concentrations approaching 0.32 ppmv (Conrad, 1996). Nitrogen deposition and nitrogen fertilization

(Nevison and Holland, 1997) due to agricultural expansion and intensification have contributed to the conspicuous post-industrial increase in atmospheric N₂O loading via increased soil nitrogen availability.

The pathways of biological N₂O production include partial dissimilatory nitrate or nitrite reduction (denitrification), nitrifier denitrification (Wrage *et al.*, 2001), ammonia (hydroxylamine) oxidation and NO_x detoxification (also known as the ‘nitrosative stress’ pathway) (Hendriks *et al.*, 2000). Nitrification-related pathways (nitrifier denitrification and ammonia oxidation) mediated by ammonia-oxidizing bacteria (AOB) are known to be a major source of N₂O production from terrestrial environments (Gödde and Conrad, 1999). The first N₂O-yielding route in AOB is related to the activity of hydroxylamine oxidoreductase (HAO), which mediates the conversion of hydroxylamine to nitrite, the second step in ammonia oxidation. The second N₂O-yielding route is through a denitrification pathway in AOB, the so-called nitrifier denitrification,

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where nitrite is reduced to NO and N₂O (Wrage *et al.*, 2001; Arp and Stein, 2003), as in the classical heterotrophic denitrification pathway.

Isotopologue analysis of N₂O has many advantages over inhibitor- and tracer-based methods for the determination of production pathways. In addition to $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ and $\delta^{18}\text{O}\text{-N}_2\text{O}$, Yoshida and Toyoda (2000) suggested that analyses of the intramolecular distributions of ¹⁵N in N₂O (N^β=N^α=O) (isotopomers), which are often expressed as the site preference (SP) ($\delta^{15}\text{N}^{\alpha}-\delta^{15}\text{N}^{\beta}$), may provide critical information that could help identify the precise sources and sinks of this greenhouse gas. Site preference has emerged as a potential conservative tracer for microbial N₂O production because 1) it is independent of the isotopologue composition of the substrates and 2) it does not exhibit changes during the course of production. Distinct site preference values for N₂O production due to bacterial denitrification, including nitrifier denitrification, relative to hydroxylamine oxidation and fungal denitrification provide a fundamental basis that facilitates the resolution of production pathways in the natural environment (Sutka *et al.*, 2006, 2008).

Strains of soil ammonia-oxidizing archaea (AOA) in the clade *Thaumarchaeota*, which were cultivated in laboratories, were shown to be capable of growing as autotrophic ammonia oxidizers (Jung *et al.*, 2011; Lehtovirta-Morley *et al.*, 2011; Kim *et al.*, 2012). The thaumarchaeotal I.1a, I.1a-associated and I.1b clades are frequently recognized as the dominant ammonia-oxidizing organisms in terrestrial environments, possibly because of their high affinity for NH₃ (Jung *et al.*, 2011; Kim *et al.*, 2012). Thus,

studies on the isotopic signatures of N₂O and the pathways of N₂O production by AOA are crucial for tracking the sources of N₂O emitted from soils and estimating the soil N₂O budget. AOA are highly fastidious and hence the ecophysiological characterization of AOA using cultured representatives is largely hindered. Recently, an AOA strain enriched from the ocean was shown to produce N₂O with distinct isotopic signatures (Santoro *et al.*, 2011). N₂O production by AOA enriched from agricultural soils has been reported (Jung *et al.*, 2011; Kim *et al.*, 2012), but there has been no isotopic analysis of N₂O produced by soil AOA. The aim of this study was to investigate the isotopomeric signatures of N₂O produced by AOA strains enriched from soils and their pathways of N₂O production. This study may provide insights into the N₂O production mechanisms of AOA, thereby facilitating the tracking of archaeal sources of N₂O in terrestrial environments.

Materials and methods

Cultivation of AOA

Soil AOA strains (Table 1) were enriched from various soils. The enrichment culture procedure was basically the same as that described by Jung *et al.* (2011) and Kim *et al.* (2012), and various soil sources were used as inocula (see the Supplementary Materials and Methods). Initial enrichment cultures were set up in allylthiourea (ATU) (20 μM) and chlorate (50 μM) to selectively inhibit the growth of AOB and nitrite oxidizing bacteria (NOB), respectively. The enriched AOA cultures contained heterotrophic bacteria, and

Table 1 Ammonia-oxidizing microorganisms used in this study and their N₂O production

Strain	Isolation site	Growth temperature (°C)	Growth pH	Taxonomic group ^a	Cell abundance (ml ⁻¹) ^{b,c}	Cell yield after ammonia oxidation (cells ml ⁻¹ μM ⁻¹)	N ₂ O production ^d		
							N ₂ O (N ₂ O-N; μmol) ^e	NO ₂ ⁻ (NO ₂ ⁻ -N; μmol) ^e	N ₂ O yield (N ₂ O-N/NO ₂ ⁻ -N; %)
AOA^a									
MY1	Agricultural soil	25	6.5	I.1a	1.2 × 10 ⁸ (1.0 × 10 ⁷)	1.1 × 10 ⁵	0.0555 (0.003)	51.21 (3.31)	0.11
MY2	Agricultural soil	25	6.5	I.1a	1.4 × 10 ⁸ (1.3 × 10 ⁷)	1.3 × 10 ⁵	0.0551 (0.002)	50.83 (2.89)	0.11
MY3	Tar-contaminated soil	30	7.0	I.1b	9.3 × 10 ⁷ (4.4 × 10 ⁶)	8.8 × 10 ⁴	0.0411 (0.003)	50.45 (3.73)	0.08
JG1	Agricultural soil	37	6.5	I.1b	1.8 × 10 ⁸ (1.3 × 10 ⁷)	1.7 × 10 ⁵	0.4191 (0.023)	51.14 (4.01)	0.82
AR	Marine sediment	25	7.5	I.1a	1.3 × 10 ⁸ (1.1 × 10 ⁷)	1.2 × 10 ⁵	0.0458 (0.004)	50.02 (3.89)	0.09
CS	Acid mine	25	5.0	I.1a-associated	5.1 × 10 ⁷ (2.8 × 10 ⁶)	4.8 × 10 ⁵	0.1783 (0.009)	4.16 (0.21)	4.28
AOB									
<i>N. europaea</i> ATCC 19718	Soil	25	7.5	Beta-proteobacteria	9.7 × 10 ⁷ (4.2 × 10 ⁶)	9.2 × 10 ⁴	0.1675 (0.011)	50.22 (4.12)	0.33

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria.

^aThe classification of thaumarchaeotal groups is based on Ochsenreiter *et al.* (2003).

^bCell counting was performed using real-time quantitative PCR after the depletion of 1 mM ammonia, except with strain CS. Strain CS was analyzed after the oxidation of 0.1 mM ammonia.

^cThe numbers in parentheses indicate the standard deviation for triplicate experiments.

^dThe yield of N₂O was determined after complete ammonia oxidation.

^eMY1 (Jung *et al.*, 2011), JG1 (Kim *et al.*, 2012) and AR (Park *et al.*, 2010) strains were described previously. MY2, MY3 and CS strains were obtained in this study.

attempts were made to further enrich AOA by successive culture in artificial freshwater medium (AFM) containing a mixture of ampicillin and kanamycin (each at 50 µg ml⁻¹). The composition of the culture media is described in the Supplementary Materials and Methods. After ca. 3 years (strains MY1, MY2 and JG1) and 2 years (strains MY3 and CS) of tri-weekly transfer, the cultures were serially diluted (10-fold) to extinction, and the highest dilution showing nitrifying activity was selected for isolating a single archaeal strain.

Soil AOA strains and a reference strain of AOB, *Nitrosomonas europaea* ATCC 19718, were incubated in an AFM. A marine AOA strain AR used as a reference strain in this study was incubated in an artificial seawater medium (ASM), which was modified from the study by Jung *et al.* (2013). For the N₂O studies, cultures of AOA and a strain of *N. europaea* were incubated with 50 ml of AFM or ASM in 120 ml serum bottles sealed with a butyl rubber stopper (Bellco Glass, Vineland, NJ, USA). All of the cultures were incubated in the dark at their optimum growth temperatures (Table 1) with unmodified ambient air in the head space. Sampling for gas and chemical analysis and supplementation with chemicals were conducted using syringes. The resistance of the butyl rubber stopper to the leakage of N₂O gas for 1 month was confirmed using standard gas. The cultures were routinely supplemented with 1 mM ammonium chloride as the sole energy source. After the ammonia oxidation was completed (in ca three weeks), 5% of the total culture was routinely transferred to fresh medium. The maximum ammonia oxidized by strain CS was ca 0.1 mM and hence strain CS was transferred when no further oxidation of ammonia occurred. The ammonia and nitrite concentrations were determined using an ion chromatograph (ICS-1500, Dionex, Sunnyvale, CA, USA) with an OnGuard II Ag Cartridge (Dionex) and colorimetrically, respectively (Solorzano, 1969). The N₂O yield after the completion of ammonia oxidation was determined on the basis of the nitrite produced from the same cultures. Cell growth was determined as described in the Supplementary Materials and Methods.

Isotopic signature of N₂O

N₂O was analyzed after the ammonia was depleted by oxidation. The ammonia level was monitored by measuring ammonia in the media and was depleted in 3 weeks. Strain CS was analyzed after the oxidation of 0.1 mM ammonium chloride. The headspaces of the 120 ml serum bottles were used for the isotopic N₂O analysis. The dual isotope and isotopomer signatures of N₂O were analyzed, that is, δ¹⁸O of N₂O (δ¹⁸O-N₂O), average δ¹⁵N (δ¹⁵N^{bulk}) and δ¹⁵N from the central N-position (δ¹⁵N^α), after cryofocusing by isotope ratio mass spectrometry (IRMS) using a Delta V IRMS (Thermo-Fisher, Bremen, Germany), which allowed simultaneous detection

of m/z 44, 45 and 46 (Well and Flessa, 2008). The IRMS was connected to a gas chromatograph (GC) (Trace GC Ultra, Thermo-Fisher, Bremen, Germany) and a modified Precon (Thermo-Fisher) equipped with an autosampler (model Combi-PAL, CTC-Analytix, Zwingen, Switzerland) (Well and Flessa, 2008). The ¹⁵N SP was determined as follows: δ¹⁵N^β = 2 × δ¹⁵N^{bulk} - δ¹⁵N^α. The dual isotope and isotopomer ratios of a sample (*R*_{sample}) were expressed as the ‰ deviation from ¹⁵N/¹⁴N and ¹⁸O/¹⁶O ratios of the reference standard materials (*R*_{std}), atmospheric N₂ and standard mean ocean water (SMOW):

$$\delta X = (R_{\text{sample}}/R_{\text{std}} - 1) \times 1000$$

where *X* = ¹⁵N^{bulk}, ¹⁵N^α, ¹⁵N^β or ¹⁸O. The typical analytical precision levels were 0.2, 0.4 and 0.3‰ for δ¹⁵N^{bulk}, δ¹⁵N^α and δ¹⁸O, respectively.

Isotopic signatures of H₂O, NO₂⁻, and NH₄⁺

The δ¹⁸O values of the medium water were analyzed using a laser spectrometer (model L 1115-I, Picarro, Santa Clara, CA, USA), which was suitable for analyzing water as a liquid or vapor. To determine δ¹⁵N and δ¹⁸O values for NO₂⁻ in the media, the denitrifier method was used to transform NO₂⁻ quantitatively to N₂O (Casciotti *et al.*, 2002). The subsequent isotope analysis was conducted as described above. The δ¹⁵N value of NH₄⁺ and NO₂⁻ was measured by combustion of the pure salts (ammonium chloride and sodium nitrite, Sigma-Aldrich) using an elemental analyzer coupled to an IRMS (DeltaPlus, Thermo-Finnigan, Bremen, Germany).

N₂O tracer experiment

The N₂O tracer experiment was performed using the same culture conditions used for the isotope signature analysis. However, 0.5 mM 99 atm% ¹⁵NH₄Cl (Cambridge Isotope laboratories, Tewksbury, MA, USA) was used as the sole electron donor in the presence of excess unlabeled nitrite (2 mM NaNO₂), which was added as a background to determine the N₂O fractions derived from preexisting nitrite via nitrifier denitrification. The same cultures used in the trace experiment with unlabeled substrates (0.5 mM unlabeled ammonium chloride with 2 mM unlabeled nitrite) were also used for comparative SP analysis. The amount of N₂O gas in the headspace was measured using a GC with a quadrupole mass spectrometer (QMS) (6890A (G1540A)/5973N, Agilent, Santa Clara, CA, USA). The quadrupole mass spectrometer recorded the relative quantitative intensity of ions according to their mass-to-charge ratios (*m/e*). From ¹⁵N-labeled NH₄⁺ and unlabeled NO₂⁻, there were three possible combinations of N isotopes for N₂O: ^{14,14}N₂O (*m/e* 44), ^{14,15}N₂O (*m/e* 45) and ^{15,15}N₂O (*m/e* 46). The N₂O ion peaks produced by electron impact ionization

were measured in these three *m/e* positions and their background in the mass spectrometer was subtracted. Gas samples were taken from the headspace of culture bottles using a 5 ml gas-tight syringe and injected via a gas sampling valve using a 1 ml sample loop in the GC. The GC was fitted with a 4 m stainless steel column packed with Porapak Q (80/100 mesh, Restek, Bellefonte, PA, USA) to separate N₂O and CO₂ from the sample gas. The oven was isothermal at 50 °C and the N₂O ion peaks were measured three times for each sample. We used six certified reference gas mixtures of N₂O in nitrogen, that is, 0.198, 0.331, 0.550, 0.798, 12.0, and 94.9 N₂O μmol mol⁻¹, to calibrate the GC/QMS. These reference gas mixtures were prepared gravimetrically by the Korea Research Institute for Standards and Science and verified by international comparisons, that is, CCQM-K68 in 2010 (Lee *et al.*, 2011).

Effect of PTIO on nitrification

To evaluate the crucial role of NO as an intermediate in nitrification and N₂O production by soil AOA, the effect of 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxide-3-oxyl (100 μM, PTIO), a NO scavenger (Amano and Noda, 1995; Ellis *et al.*, 2001), was

tested on the growth and ammonia oxidation activities of AOA. The PTIO experiment was performed using the same growth conditions as those used for the isotopic signature studies. Cell growth and ammonia oxidation were determined during and after incubation.

Results and discussion

AOA strains

The soil AOA strains used in this study are shown in Table 1. An AOA strain (AR) from a marine sediment (Park *et al.*, 2010) and an AOB strain were also included as references. The phylogenetic analysis performed using archaeal 16S rRNA and *amoA* genes as described in Supplementary Materials and Methods indicated that I.1a (MY1, MY2 and AR), I.1a-associated (CS) and I.1b (MY3 and JG1) clades within *Thaumarchaeota* were included (Figure 1). The phylogenetic information regarding enriched AOA strains (based on 16S rRNA and *amoA* gene sequences) is described in the Supplementary Results.

All five soil AOA cultures used in this study grew on minimal medium, with ammonia as the sole electron donor. Each AOA culture was highly

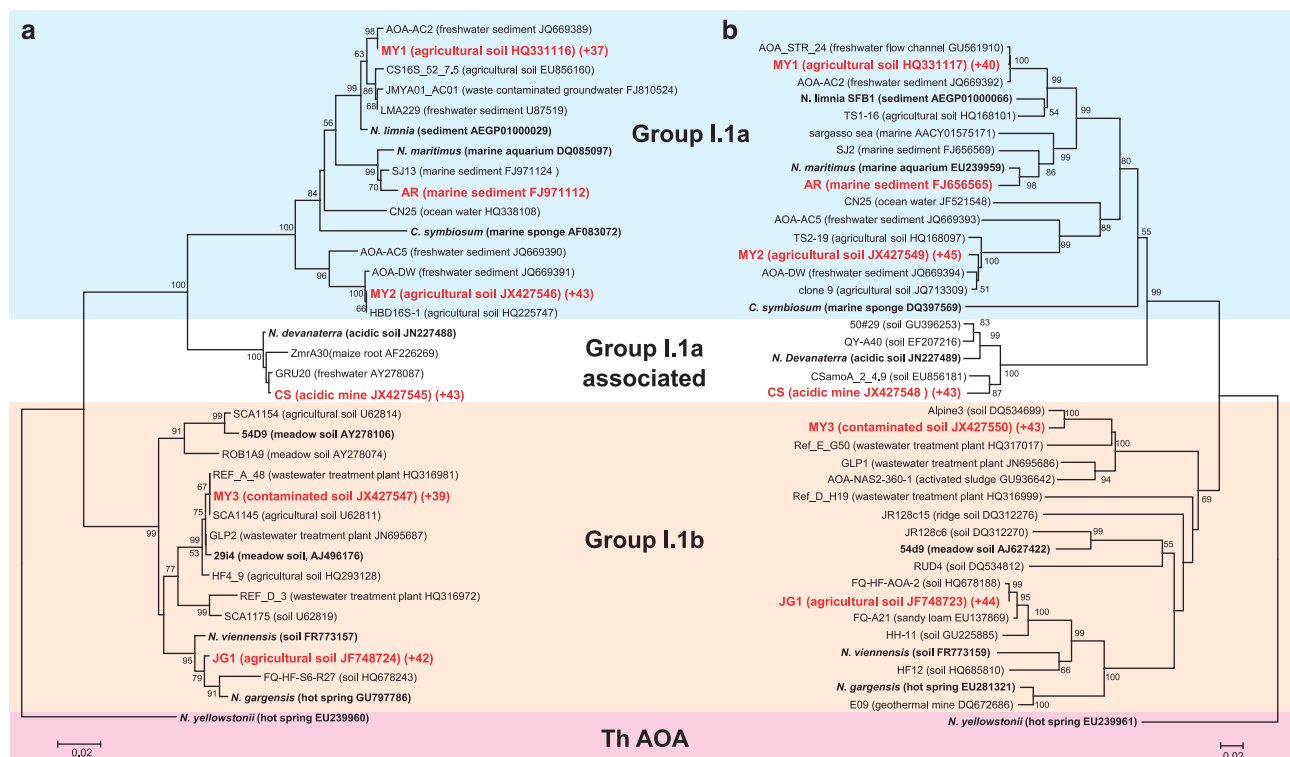


Figure 1 Comparative phylogenetic analysis of (a) 16S rRNA and (b) *amoA* gene sequences from *Thaumarchaeota* strains used in this study. The classification of thaumarchaeotal groups is based on Ochsenreiter *et al.* (2003). Branching patterns supported by bootstrap values (1000 iterations) > 50% according to the neighbor-joining method are denoted by their bootstrap values. Strains indicated in red were used in this study. Strains marked in bold are reference strains or fosmid clones of *Thaumarchaeota*. The origins of each clone or strain are indicated in parentheses with their accession numbers. The number of 16S rRNA and *amoA* gene sequences used for checking the purity of AOA cultures is indicated in the second set of parentheses.

enriched and contained a single archaeal strain, as revealed by a single band corresponding to that of archaeal 16S rRNA gene sequence in a denaturing gradient gel electrophoresis. As described previously, clone libraries derived from the enrichment cultures confirmed that only one unique sequence (>99.7% identity within the error rate expected for PCR amplification (von Wintzingerode *et al.*, 1997; Könneke *et al.*, 2005)) for the archaeal 16S rRNA and *amoA* genes, respectively, was identified in each culture (Jung *et al.*, 2011; Kim *et al.*, 2012). The number of clones (>37 per library) that were sequenced and analyzed is shown in Figure 1. FISH with domain-specific probes and real-time quantitative PCR analysis confirmed that the archaeal abundance was >90% of the minor bacterial cells co-cultured. In this study, AOB were selectively inhibited at 20 μM ATU as reported previously (Bedard and Knowles, 1989; Ginestet *et al.*, 1998; Jung *et al.*, 2011). As reported by Santoro *et al.* (2011) and Shen *et al.* (2013), the AOA used in this study were not inhibited at 20 μM ATU (apart from strain CS, which was partially inhibited at 20 μM ATU, as previously reported in Lehtovirta-Morley *et al.* (2013)). The absence of AOB contamination was confirmed by the inability to amplify the bacterial 16S rRNA gene from betaproteobacterial AOB using specific nested PCR primers, and the *amoA* genes from gamma and betaproteobacterial AOB using specific PCR primers (Supplementary Table S1). The PCR results were also supported by the analysis of bacterial 16S rRNA gene clone libraries (Supplementary Table S2). Only the archaeal growth was tightly coupled to ammonia oxidation, as shown in other studies (Jung *et al.*, 2011; Kim *et al.*, 2012). These results suggested that ammonia-oxidizing archaea were solely responsible for the observed ammonia oxidation.

N₂O production by AOA

We observed N₂O production in all AOA cultures where ammonia oxidation occurred (Table 1). The inocula (early stationary phase cultures) had initial cell densities of ca 1 × 10⁶ to 5 × 10⁶ 16S rRNA gene copies ml⁻¹ for all AOA cultures and *N. europaea*. The cultures of AOA had stationary phase cell densities of ca 5 × 10⁷ to 2 × 10⁸ 16S rRNA gene copies ml⁻¹ after the oxidation of 1 mM ammonia, except strain CS (Table 1). The N₂O production by the AOA strains ranged from 0.041 to 0.419 μmol (N₂O-N) after the exhaustion of 1 mM ammonia. The yields (N₂O/NO₂⁻ produced) from AOA ranged from 0.11 to 4.28%, which were comparable to those from *N. europaea* (Table 1) and other AOB (Colliver and Stephenson, 2000). The total amount and production yield of N₂O from strains JG1 and CS were at the upper end of the range. The highest N₂O production yield from the acidophilic strain CS might have been related to its high sensitivity to nitrite toxicity (Supplementary Figure S1). Indeed, ca 0.1 mM nitrite

was the maximum tolerable concentration for the CS cultures, as found with *Nitrosotalea devanattera* (Lehtovirta-Morley *et al.*, 2011).

The archaeal strains were not pure isolates, but the involvement of minor contaminant (denitrifying) bacteria with N₂O production could be rejected based on the following experimental evidence. (1) N₂O production and ammonia oxidation were tightly coupled and N₂O production ceased after ammonia oxidation was completed, as found using strain MY1 (Jung *et al.*, 2011) and JG1 (Kim *et al.*, 2012) (see Supplementary Figure S2). This indicated that the contribution of bacterial denitrification to N₂O production from the nitrite and organic debris produced by AOA was negligible. (2) Chlorite (50 μM; ClO₂⁻), an ammonia oxidation inhibitor that does not inhibit bacterial denitrification, completely inhibited N₂O production in the presence of 1 mM nitrite (Jung *et al.*, 2011; Kim *et al.*, 2012). Even when yeast extract (10 mg l⁻¹) was spiked in the medium containing chlorite, N₂O production was still not observed in the presence of 1 mM nitrite (data not shown). In this condition, the only bacterial cell growth (up to 10⁸ cells ml⁻¹) that occurred corresponded to the final bacterial cell concentrations in the ammonia-oxidizing enrichment culture. (3) Heterotrophic bacterial strains with a denitrification capability (*Pseudomonas* sp. from culture JG1; *Acinetobacter* sp. from culture MY1; *Rhodococcus* sp. from culture MY3) were isolated from the ammonia-oxidizing cultures and cultivated with 10 mg l⁻¹ yeast extract and 0.5 mM nitrite using the same medium and incubation conditions. The amount of N₂O produced after 2 weeks was negligible and was close to the atmospheric background level (0.003–0.005 N₂O-N μmol) (Supplementary Figure S3). These results showed clearly that the cultivation conditions were sufficiently oxidic that N₂O production from bacterial denitrification was negligible.

Isotopic signatures of N₂O

In addition to bulk isotopic information (δ¹⁵N-N₂O and δ¹⁸O-N₂O), the N₂O molecule contains position-dependent isotopic information from nitrogen (δ¹⁵N^α and δ¹⁵N^β). SP, the position-dependent isotope value of N₂O, is an isotopic signature that is commonly used to interpret the source of N₂O production (Toyoda and Yoshida, 1999; Sutka *et al.*, 2003; Ostrom and Ostrom, 2011). Studies using pure AOB cultures have indicated that a positive SP (30–38‰) is consistent with a N₂O source from hydroxylamine oxidation (ammonia oxidation), whereas nitrifier denitrification (and denitrification) is thought to produce N₂O with a SP value near or below zero (–10 to 0‰) (Frame and Casciotti, 2010; Sutka *et al.*, 2006). The N₂O emissions from various soils have positive (0–35‰) SP signatures (Pérez *et al.*, 2001; Park *et al.*, 2011, 2012; Toyoda *et al.*, 2011), which indicates that ammonia oxidation is an

important source of soil N₂O. The SP values of soil AOA strains (MY1-3 and JG1) were distinct and they ranged from 20 to -30‰, except strain CS (Table 2). The SP values of strain AR and *N. europaea* were at the upper end of the range. Intriguingly, the SP value of strain CS was 13‰, which was much lower than other ammonia-oxidizing microorganisms. Nitrite toxicity increased the nitrifier denitrification activity required for detoxification, which might have been attributable to the low SP and the high N₂O production by strain CS (Tables 1 and 2). A future SP analysis of AOB at acidic pH might be useful to verify whether the low SP value can be attributed to the increase in nitrifier denitrification required for nitrite detoxification under acidic conditions. The results of the present study indicated that SP analysis might not be a definitive method for distinguishing archaeal and bacterial nitrification sources of N₂O production.

The $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ values for all AOA strains were -13 to -17‰, except strain CS (-35.54‰), whereas the value for *N. europaea* was -19.88‰ (Table 2). The end-member mixing model was not applicable to the CS culture because of the partial oxidation of ammonia and hence the $^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ produced by strain CS did not reflect the isotopic shifts of NH_4^+ . The ranges of $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ from soil AOA were similar to those (-10 to -50‰) from agricultural soils and forest soils (Kim and Craig, 1993; Rahn and Wahlen, 2000; Pérez *et al.*, 2001; Rock *et al.*, 2007). Previous studies found that the $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ values of marine AOB (*Nitrosomonas marina* C-113) were about -10‰ with 20% O₂ concentration (Frame and Casciotti, 2010), whereas the $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ values of

N. europaea (ATCC 19718) in ammonia-oxidizing and nitrite-reducing conditions were -46.5‰ and -34.0‰, respectively (Sutka *et al.*, 2003, 2006). Surprisingly, the $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ values of a marine AOA strain CN25 were positive (8.7‰) (Santoro *et al.*, 2011), which differed significantly from the ideal isotope fractionation effect and from those of the soil AOA strains. These values were similar to those from surface oceans and the troposphere (5‰ to 10‰) (Kim and Craig, 1993; Rahn and Wahlen, 2000; Popp *et al.*, 2002). Strain AR was also from a marine environment but its $\delta^{15}\text{N}^{\text{bulk}}$ value (-12.91‰) was similar to those of the soil AOA strains and marine AOB (Frame and Casciotti, 2010). The 'apparent' (observable) isotope effect calculated from the data obtained by Santoro *et al.* (2012) would be $\epsilon_{\text{NH}_4\text{N}_2\text{O}} = \delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O} - \delta^{15}\text{N}\text{-NH}_4 = 8.7 - 2.5 = 6.2\%$. As the intrinsic isotope effects of N₂O formation are always negative, that is, $\delta^{15}\text{N}$ of product is depleted when compared with $\delta^{15}\text{N}$ of substrate, our value of $\epsilon_{\text{NH}_4\text{N}_2\text{O}} = \delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O} - \delta^{15}\text{N}\text{-NH}_4 = -17 - (-1) = -16\%$ is much closer to the expected value. The deviation of the apparent isotope effect from the intrinsic isotope effect (ideal Rayleigh behavior) could be caused by variations in the N₂O yield of nitrification over time. For example, if (1) the N₂O yield is higher at the end when the residual substrate (NH_4^+) is already substantially $\delta^{15}\text{N}$ -enriched, or if (2) clumps of cells in the culture media create local areas of $\delta^{15}\text{N}$ -enriched NH_4^+ , the apparent isotope effect could be reversed (that is, positive).

Different N₂O production pathways incorporate elemental O from isotopically distinct sources

Table 2 Isotopic characteristics of N₂O produced during ammonia oxidation by soil AOA strains

Strain	$\delta^{18}\text{O}\text{-N}_2\text{O}$ (‰)	$\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ (‰)	$\delta^{15}\text{N}^{\text{z}}$ (‰)	$\delta^{15}\text{N}^{\text{b}}$ (‰)	SP (‰)
AOA					
MY1	30.91 (2.06)	-13.53 (2.12)	-1.55 (3.63)	-25.42 (1.68)	23.87 (3.75)
MY2	29.26 (2.47)	-16.96 (1.81)	-6.04 (3.44)	-27.80 (1.33)	21.76 (3.75)
MY3	33.56 (3.27)	-16.49 (2.18)	-3.89 (4.15)	-28.98 (1.40)	25.09 (4.39)
JG1	21.59 (0.34)	-15.32 (0.16)	-5.48 (0.28)	-25.11 (0.03)	19.62 (0.24)
AR	36.11 (0.67)	-12.91 (1.50)	2.07 (1.33)	-27.77 (1.70)	29.83 (0.67)
CS ^a	22.44 (0.72)	-35.54 (0.89)	-28.97 (1.48)	-42.07 (0.32)	13.10 (1.20)
AOB					
<i>N. europaea</i> ATCC 19718	26.58 (0.18)	-19.88 (0.39)	-5.37 (0.58)	-34.31 (0.50)	28.94 (1.01)
Addition of 2 mM nitrite					
MY1	22.22 (3.23)	-17.67 (0.79)	-11.48 (3.10)	-23.86 (2.29)	12.38 (5.22)
<i>N. europaea</i> ATCC 19718	21.85 (0.49)	-27.39 (0.39)	-19.57 (1.37)	-35.14 (0.58)	15.57 (1.95)

Abbreviations: AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; ATCC, American Type Culture Collection; SP, site preference.

All experiments used air-equilibrated media with an assumed $\delta^{18}\text{O}\text{-O}_2$ of ca 23.5‰ versus VSMOW, and the initial 1 mM ammonia concentration was oxidized completely during each experiment. The $\delta^{15}\text{N}$ values are reported as ‰ versus AIR, while the $\delta^{18}\text{O}$ values are reported as ‰ versus VSMOW. The isotope values for N₂O are the isotopic composition of the N₂O produced after correcting for background material. $\delta^{18}\text{O}\text{-H}_2\text{O}$ was -7.97 (0.06)‰. $\delta^{15}\text{N}\text{-NH}_4^+$ was -1.20 (0.10)‰. The $\delta^{15}\text{N}\text{-NO}_2^-$ value of the nitrite added in the high nitrite background was -2.7 (0.3)‰. The standard deviation values were calculated from replicate experiments (refer to Supplementary Table S3 for the raw data) and are shown in parentheses. ^aOnly 10% of the ammonia (0.1 mM) was oxidized in this condition, possibly because of high sensitivity to the accumulated nitrite. Thus, the end-member mixing model did not explain the CS cultures (the $\delta^{15}\text{N}^{\text{bulk}}\text{-N}_2\text{O}$ and $\delta^{18}\text{O}\text{-N}_2\text{O}$ values produced by strain CS reflect isotopic shifts in substrates, i.e., Rayleigh effects (Mariotti *et al.*, 1981)).

(for example, H₂O or O₂) and they have unique fractionation factors (Ostrom *et al.*, 2000). The $\delta^{18}\text{O}\text{-N}_2\text{O}$ values of surface oceans and the troposphere were 20–40‰, whereas those of agricultural soils and forest soils were 0–60‰ (Kim and Craig, 1993; Pérez *et al.*, 2001; Rock *et al.*, 2007). The $\delta^{18}\text{O}\text{-N}_2\text{O}$ values of the AOA strains were 29–34‰ for MY1, 2, and 3, ca 22‰ for CS and JG1 and 36‰ for AR. The $\delta^{18}\text{O}\text{-N}_2\text{O}$ value of *N. europaea* was 27‰. The oxygen isotope branching effects that affect $\delta^{18}\text{O}$ enrichment from oxygen sources during N₂O production may also be applicable to soil AOA strains as suggested for AOB and marine AOA (Frame and Casciotti, 2010; Santoro *et al.*, 2011). The $\delta^{18}\text{O}\text{-N}_2\text{O}$ signature was not significantly different from those of AOB and hence the ratio of oxygen atom sources (H₂O to O₂) incorporated into nitrite (1:1) appears to hold for AOA and AOB. The $\delta^{18}\text{O}\text{-N}_2\text{O}$ values of marine AOA (strain CN25) were also similar to those of soil AOA. The shift in $\delta^{18}\text{O}\text{-N}_2\text{O}$ reflected changes in the proportion of N₂O derived from two different pathways, that is, ammonia oxidation and nitrifier denitrification; hence, the covariance of SP and $\delta^{18}\text{O}\text{-N}_2\text{O}$ was observed in AOB. The covariance of SP and $\delta^{18}\text{O}\text{-N}_2\text{O}$ for soil AOA may be inferred from the plot of SP versus $\delta^{18}\text{O}\text{-N}_2\text{O}$, including other ammonia-oxidizing AOA and AOB, as shown in Figure 2. Although the proportion of nitrifier denitrification varied between replicates (Supplementary Table 3 and Supplementary Figure S4), there was a distinct covariance between SP and $\delta^{18}\text{O}\text{-N}_2\text{O}$ for each strain in the incubation conditions used in this study. This indicated that combined isotopic signatures can be used to improve the process identification, whereas individual signatures had limitations when trying to identify sources (Snider *et al.*, 2012).

There were similar covariance results from N₂O reduction to N₂ during denitrification (Jinuntuya-Nortman *et al.*, 2008; Well and Flessa, 2009). The SP/ $\delta^{18}\text{O}\text{-N}_2\text{O}$ slope of N₂O emitted from soil AOA (approx. 1) (Figure 2) was within the slope range reported for residual N₂O after partial reduction to N₂ (Well *et al.*, 2012). This similarity might further complicate the distinction of N₂O from nitrification and denitrification in environments.

¹⁵N-labeling experiments

The mixing of two different N₂O production pathways by AOB (that is, ammonia oxidation and nitrifier denitrification) results in different SP values, which are well established using various approaches. The SP signatures of N₂O can be used to calculate the magnitude of each pathway's contribution to the total N₂O output based on its SP signature for AOB (Sutka *et al.*, 2003; Sutka *et al.*, 2006).

The mass of N₂O produced during the oxidation of ¹⁵N-labeled NH₄⁺ (0.5 mM) in the presence of excess unlabeled NO₂⁻ (2 mM) was determined to trace the sources of N₂O. The tracer experiment with

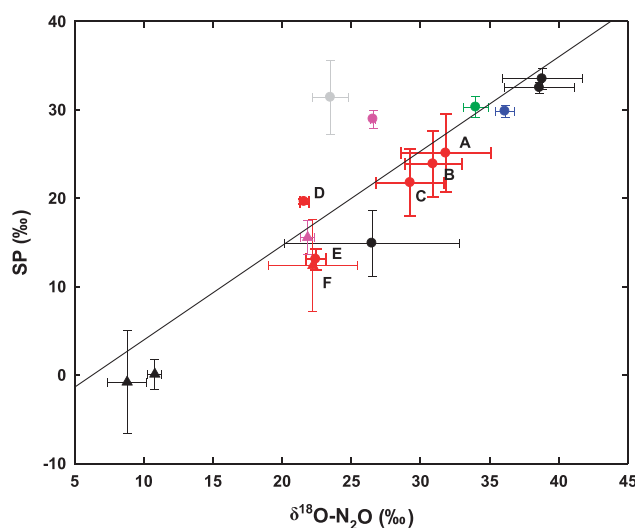


Figure 2 Covariance of SP and $\delta^{18}\text{O}\text{-N}_2\text{O}$. Symbols shown in red (soil AOA), blue (marine AOA strain AR), and pink (*N. europaea*) correspond to the data from this study (circles indicate values from ammonia-oxidizing cultures without nitrite addition; triangles indicate values from ammonia-oxidizing cultures with 2 mM nitrite): A, MY3; B, MY1; C, MY2; D, JG1; E, CS; F, MY1 with 2 mM nitrite. The green circle indicates marine AOA strain CN25 (Santoro *et al.*, 2011). The black circles and triangles indicate *N. europaea* under hydroxylamine-oxidizing and denitrifying conditions, respectively, whereas the gray circle indicates *N. europaea* under ammonia-oxidizing conditions (Sutka *et al.*, 2003; Sutka *et al.*, 2006). The error bars are based on replicate experiments. The raw data used in this plot are presented in Supplementary Table S3.

N. europaea (Ritchie and Nicholas, 1972) suggested that the dimerization of ¹⁵NOH, derived from ¹⁵NH₂OH, and ¹⁴NOH, derived from nitrite, might subsequently lead to the formation of ^{14,15}N₂O. Three possible combinations of the N isotopes in N₂O were also possible for soil AOA via two different processes: ^{14,14}N₂O (nitrifier denitrification, *m/e* 44), ^{14,15}N₂O (mixing of nitrifier denitrification and ammonia oxidation, *m/e* 45) and ^{15,15}N₂O (ammonia oxidation, *m/e* 46) (Figure 3a). Strain CS could not be included in this experiment because of its high sensitivity to NO₂⁻. The N₂O mass ratios varied, depending on the strains (Figure 3b). N₂O produced by the mixed process was dominant in JG1 and *N. europaea*, where the three different mass ratios of N₂O (*m/e* 46:45:44) from JG1 and *N. europaea* were 20.4%:71.2%:8.4% and 28.8%:62.8%:8.4%, respectively. Most of the N₂O produced by strains MY1, MY2, MY3 and AR had *m/e* values of 45 and 44, which indicated that N₂O derived from the mixed process and nitrifier denitrification were co-dominant (up to 90%) (Figure 3b). The proportions of mixed and pure ammonia-derived N₂O were not correlated with the SP values shown in Table 2. For example, SP was not high in strain JG1 despite the high proportion of mixed and pure ammonia-derived N₂O. The mixing process might be a key step that contributes to the high SP of ammonia-derived N₂O. The contribution made by

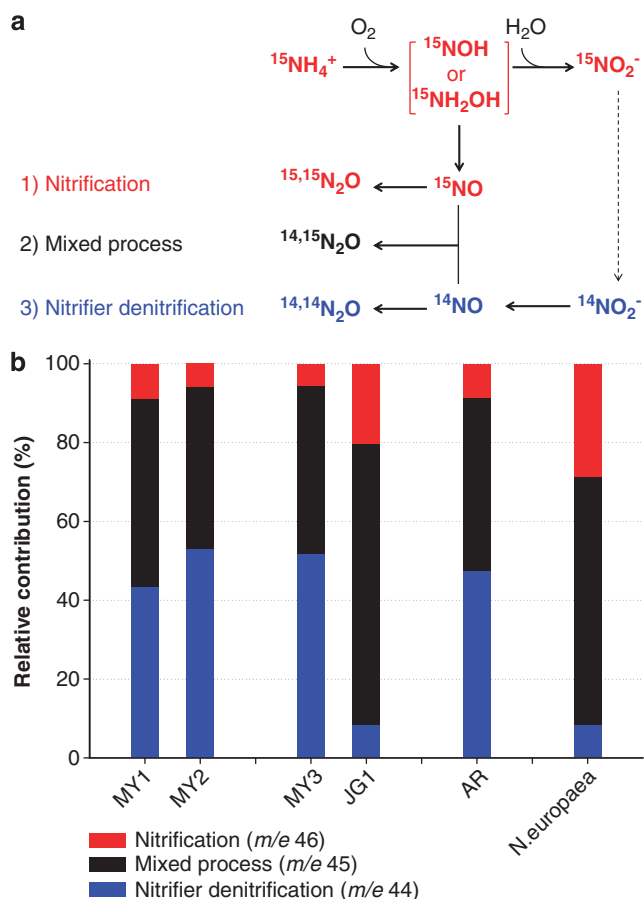


Figure 3 (a) Proposed biochemical processes during archaeal nitrification, showing the pathways leading to N₂O production in the tracer experiment. Two different NO sources permitted three possible combinations for N₂O production. HNO (nitroxyl) or hydroxylamine has been proposed as an intermediate during the oxidation of ammonia to nitrite (Walker *et al.*, 2010). (b) The composition of labeled N₂O produced during the tracer experiment. Soil AOA strains were incubated in the presence of 0.5 mM ¹⁵N-labeled NH₄⁺ and 2 mM nitrite.

nitrifier denitrification to N₂O production might be overestimated due to the high background (2 mM) nitrite level. In the present study, we observed an increased N₂O yield and reduced SP values in strain MY1 and in *N. europaea* in the presence of 2 mM nitrite (Figure 2, Table 2, and Supplementary Table S3), which indicated enhanced nitrifier denitrification; similar findings were reported for AOB (Frame and Casciotti, 2010). Therefore, the contribution made by the direct conversion of ¹⁵N-labeled NH₄⁺ to N₂O production, that is, via ammonia oxidation (*m/e* 46) and the mixed process (*m/e* 45), might be more significant in the absence of background nitrite. Thus, the tracer experiment was not applicable for determining the exact contributions made by the different processes in the isotopomer study. Furthermore, the fraction of N₂O produced via nitrifier denitrification by AOA in soil might be much lower because of the presence of nitrite-oxidizing bacteria. This study indicated that

the isotopic signatures of N₂O produced by AOA might have been due to combinations of two different N₂O production processes (see Figure 3), similar to AOB (Ritchie and Nicholas, 1972). In a similar experiment using marine AOA with ¹⁵N-labeled NH₄⁺ addition, at least some of the N₂O was shown to be produced directly from the ¹⁵N-enriched NH₃ pool without passing through the nitrite pool (Santoro *et al.*, 2011).

Effect of PTIO on nitrification

Bacterial ammonia oxidation is traditionally defined as the oxidation of ammonia to nitrite via hydroxylamine as an intermediate. In addition to hydroxylamine, the reactive intermediate nitroxyl (nitroxyl hydride; HNO) has been proposed as a key intermediate during archaeal ammonia oxidation (Walker *et al.*, 2010). The present study showed that archaeal ammonia oxidation was very sensitive to PTIO, a scavenger of free radical nitric oxide (NO) and nitroxyl (Amano and Noda, 1995; Ellis *et al.*, 2001; Samuni *et al.*, 2010), and neither ammonia oxidation nor archaeal growth was observed at 100 μM PTIO (Supplementary Figure S5, and Yan *et al.* (2012)). In addition, the inhibitory effects of 100 μM PTIO on AOB were negligible (Supplementary Figure S5 and Shen *et al.* (2013)). Recently, Vajrala *et al.* (2013) demonstrated hydroxylamine-induced oxygen consumption and ATP production by the marine AOA strain, *Nitrosopumilus maritimus*. In this case, NO was suggested to act as an electron shuttling factor in the same way as the quinone moiety in AOB. High expression of *nirK* gene was observed in strain MY1 during ammonia oxidation (Jung *et al.*, 2011). A more plausible explanation may be that the inhibitory effect of PTIO is related to NO-based electron shuttling in the hydroxylamine pathway rather than to the production of nitroxyl intermediates. Our results suggest that the ammonia oxidation mechanism in AOA and the N₂O production mechanism in AOA via ammonia oxidation might be distinct from those of AOB. By contrast, the isotopic signatures of δ¹⁵N-N₂O and δ¹⁸O-N₂O, the SP, and the results of the labeled tracer experiment did not differ greatly from those of the AOB strains.

Conclusions

Soil is the most important source of global N₂O emissions, and a significant but unknown fraction of the soil N₂O is produced by nitrification. The recent discovery of AOA in soils implies that it is essential to revise the current assumption that AOB are the sole sources of N₂O from soil nitrification. The present study showed that the isotopic signatures (SP, δ¹⁵N^{bulk}-N₂O, and δ¹⁸O-N₂O) of soil AOA strains were similar to those of AOB strains and also similar to those from agricultural soils and forest soils. Thus, the activities and roles of soil AOA during

N₂O emission from soil have been hidden in previous isotopic studies of N₂O. The current study, based on soil AOA cultures, indicates that a significant amount of N₂O could be produced from archaeal nitrification. However, there is controversy regarding AOA's contribution to autotrophic nitrification in terrestrial systems, despite their numerical dominance over AOB (Schleper, 2010). Global climate change and the upturn in atmospheric N₂O imply that it is imperative to incorporate archaeal N₂O production processes into global N₂O models to accurately predict the response of the N₂O flux in future soils. Selective inhibitors of nitrification, such as ATU for AOB and PTIO for AOA, could provide an important tool for differentiating the activity of soil AOA from that of AOB with respect to N₂O emissions. These results may provide fundamental information for facilitating future studies on the N₂O production pathway in AOA and the tracking of N₂O emission sources in soils.

Conflict of Interest

The authors declare no conflict of interest.

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References

- Amano F, Noda T. (1995). Improved detection of nitric oxide radical (NO●) production in an activated macrophage culture with a radical scavenger, carboxy PTIO and Griess reagent. *FEBS Lett* **368**: 425–428.
- Arp DJ, Stein LY. (2003). Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Crit Rev Biochem Mol Biol* **38**: 471–495.
- Bedard C, Knowles R. (1989). Physiology, biochemistry, and specific inhibitors of CH₄, NH₄⁺, and CO oxidation by methanotrophs and nitrifiers. *Microbiol Rev* **53**: 68–84.
- Casciotti KL, Sigman DM, Hastings MG, Bohlke JK, Hilkert A. (2002). Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal Chem* **74**: 4905–4912.
- Colliver BB, Stephenson T. (2000). Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. *Biotechnol Adv* **18**: 219–232.
- Conrad R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). *Microbiol Rev* **60**: 609–640.
- Ellis A, Lu H, Li CG, Rand MJ. (2001). Effects of agents that inactivate free radical NO (NO●) on nitroxyl anion-mediated relaxations, and on the detection of NO● released from the nitroxyl anion donor Angeli's salt. *Br J Clin Pharmacol* **134**: 521–528.
- Frame CH, Casciotti KL. (2010). Biogeochemical controls and isotopic signatures of nitrous oxide production by a marine ammonia-oxidizing bacterium. *Biogeosciences* **7**: 2695–2709.
- Gödde M, Conrad R. (1999). Immediate and adaptational temperature effects on nitric oxide production and nitrous oxide release from nitrification and denitrification in two soils. *Biol Fertil Soils* **30**: 33–40.
- Ginestet P, Audic JM, Urbain VV, Block JC. (1998). Estimation of nitrifying bacterial activities by measuring oxygen uptake in the presence of the metabolic inhibitors allylthiourea and azide. *Appl Environ Microbiol* **64**: 2266–2268.
- Hendriks J, Oubrie A, Castresana J, Urbani A, Gemeinhardt S, Saraste M. (2000). Nitric oxide reductases in bacteria. *Biochim Biophys Acta* **1459**: 266–273.
- Jinuntuya-Nortman M, Sutka RL, Ostrom PH, Gandhi H, Ostrom NE. (2008). Isotopologue fractionation during microbial reduction of N₂O within soil mesocosms as a function of water-filled pore space. *Soil Biol Biochem* **40**: 2273–2280.
- Jung MY, Park SJ, Min D, Kim JS, Rijpstra WI, Sinninghe Damste JS *et al.* (2011). Enrichment and characterization of an autotrophic ammonia-oxidizing archaeon of mesophilic crenarchaeal group I.1a from an agricultural soil. *Appl Environ Microbiol* **77**: 8635–8647.
- Jung MY, Shin KS, Kim S, Kim SJ, Park SJ, Kim JG *et al.* (2013). *Hoeflea halophila* sp. nov., a novel bacterium isolated from marine sediment of the East Sea, Korea. *Antonie Van Leeuwenhoek* **103**: 971–978.
- Könneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB, Stahl DA. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.
- Kim JG, Jung MY, Park SJ, Rijpstra WI, Sinninghe Damste JS, Madsen EL *et al.* (2012). Cultivation of a highly enriched ammonia-oxidizing archaeon of thaumarchaeotal group I.1b from an agricultural soil. *Environ Microbiol* **14**: 1528–1543.
- Kim KR, Craig H. (1993). Nitrogen-15 and oxygen-18 characteristics of nitrous oxide: a global perspective. *Science* **262**: 1855–1857.
- Lee JB, Lee JS, Moon DM, Kim JS, Wessel R, Aoki N *et al.* (2011). Final report on international comparison CCQM-K68: nitrous oxide in synthetic air. *Metrologia* **48**: 08004.
- Lehtovirta-Morley LE, Stoecker K, Vilcinskas A, Prosser JI, Nicol GW. (2011). Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc Natl Acad Sci USA* **108**: 15892–15897.
- Lehtovirta-Morley LE, Verhamme DT, Nicol GW, Prosser JI. (2013). Effect of nitrification inhibitors on the growth and activity of *Nitrosotalea devanattera* in culture and soil. *Soil Biol Biochem* **62**: 129–133.

- Mariotti A, Germon JC, Hubert P, Kaiser P, Letolle R, Tardieux A *et al.* (1981). Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. *Plant and Soil* **62**: 413–430.
- Nevison C, Holland E. (1997). A reexamination of the impact of anthropogenically fixed nitrogen on atmospheric N₂O and the stratospheric O₃ layer. *J Geophys Res* **102**: 25519–25536.
- Ochsenreiter T, Selezi D, Quaiser A, Bonch-Osmolovskaya L, Schleper C. (2003). Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* **5**: 787–797.
- Ostrom N, Ostrom P. (2011). The isotopomers of nitrous oxide: analytical considerations and application to resolution of microbial production pathways. In: Baskaran M (ed). *Handbook Environ Isot Geochem*. Springer: Berlin Heidelberg, pp 453–476.
- Ostrom NE, Russ ME, Popp B, Rust TM, Karl DM. (2000). Mechanisms of nitrous oxide production in the subtropical North Pacific based on determinations of the isotopic abundances of nitrous oxide and di-oxygen. *Chemosphere—Glob Change Sci* **2**: 281–290.
- Pérez T, Trumbore SE, Tyler SC, Matson PA, Ortiz-Monasterio I, Rahn T *et al.* (2001). Identifying the agricultural imprint on the global N₂O budget using stable isotopes. *J Geophys Res* **106**: 9869–9878.
- Park BJ, Park SJ, Yoon DN, Schouten S, Sinninghe Damsté JS, Rhee SK. (2010). Cultivation of autotrophic ammonia-oxidizing archaea from marine sediments in co-culture with sulfur-oxidizing bacteria. *Appl Environ Microbiol* **76**: 7575–7587.
- Park S, Pérez T, Boering KA, Trumbore SE, Gil J, Marquina S *et al.* (2011). Can N₂O stable isotopes and isotopomers be useful tools to characterize sources and microbial pathways of N₂O production and consumption in tropical soils? *Glob Biogeochem Cycle* **25**: GB1001.
- Park S, Croteau P, Boering KA, Etheridge DM, Ferretti D, Fraser PJ *et al.* (2012). Trends and seasonal cycles in the isotopic composition of nitrous oxide since 1940. *Nat Geosci* **5**: 261–265.
- Popp BN, Westley MB, Toyoda S, Miwa T, Dore JE, Yoshida N *et al.* (2002). Nitrogen and oxygen isotopomeric constraints on the origins and sea-to-air flux of N₂O in the oligotrophic subtropical North Pacific gyre. *Glob Biogeochem Cycle* **16**: 1064.
- Rahn T, Wahlen M. (1997). Stable isotope enrichment in stratospheric nitrous oxide. *Science* **278**: 1776–1778.
- Rahn T, Wahlen M. (2000). A reassessment of the global isotopic budget of atmospheric nitrous oxide. *Glob Biogeochem Cycle* **14**: 537–543.
- Ritchie GA, Nicholas DJ. (1972). Identification of the sources of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. *Biochem J* **126**: 1181–1191.
- Rock L, Ellert BH, Mayer B, Norman AL. (2007). Isotopic composition of tropospheric and soil N₂O from successive depths of agricultural plots with contrasting crops and nitrogen amendments. *J Geophys Res* **112**: D18303.
- Samuni U, Samuni Y, Goldstein S. (2010). On the distinction between nitroxyl and nitric oxide using nitronyl nitroxides. *J Am Chem Soc* **132**: 8428–8432.
- Santoro AE, Buchwald C, McIlvin MR, Casciotti KL. (2011). Isotopic signature of N₂O produced by marine ammonia-oxidizing archaea. *Science* **333**: 1282–1285.
- Schleper C. (2010). Ammonia oxidation: different niches for bacteria and archaea?. *ISME J* **4**: 1092–1094.
- Seitzinger SP, Kroeze C, Styles RV. (2000). Global distribution of N₂O emissions from aquatic systems: natural emissions and anthropogenic effects. *Chemosphere—Global Change Sci* **2**: 267–279.
- Shen T, Stieglmeier M, Dai J, Urich T, Schleper C. (2013). Responses of the terrestrial ammonia-oxidizing archaea *Nitrososphaera viennensis* and the ammonia-oxidizing bacterium *Nitrososphaera multiformis* to nitrification inhibitors. *FEMS Microbiol Lett* **344**: 121–129.
- Snider DM, Venkiteswaran JJ, Schiff SL, Spoelstra J. (2012). Deciphering the oxygen isotope composition of nitrous oxide produced by nitrification. *Glob Change Biol* **18**: 356–370.
- Solorzano L. (1969). Determination of ammonia in natural waters by the phenylhypochlorite method. *Limnol Oceanogr* **14**: 799–801.
- Sutka RL, Ostrom NE, Ostrom PH, Gandhi H, Breznak JA. (2003). Nitrogen isotopomer site preference of N₂O produced by *Nitrosomonas europaea* and *Methylococcus capsulatus* Bath. *Rapid Commun Mass Spectrom* **17**: 738–745.
- Sutka RL, Ostrom NE, Ostrom PH, Breznak JA, Gandhi H, Pitt AJ *et al.* (2006). Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances. *Appl Environ Microbiol* **72**: 638–644.
- Sutka RL, Adams GC, Ostrom NE, Ostrom PH. (2008). Isotopologue fractionation during N₂O production by fungal denitrification. *Rapid Commun Mass Spectrom* **22**: 3989–3996.
- Toyoda S, Yoshida N. (1999). Determination of nitrogen isotopomers of nitrous oxide on a modified isotope ratio mass spectrometer. *Anal Chem* **71**: 4711–4718.
- Toyoda S, Yano M, Nishimura S-i, Akiyama H, Hayakawa A, Koba K *et al.* (2011). Characterization and production and consumption processes of N₂O emitted from temperate agricultural soils determined via isotopomer ratio analysis. *Glob Biogeochem Cycle* **25**: GB2008.
- Vajrala N, Martens-Habbena W, Sayavedra-Soto LA, Schauer A, Bottomley PJ, Stahl DA *et al.* (2013). Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proc Natl Acad Sci USA* **110**: 1006–1011.
- von Wintzingerode F, Gobel UB, Stackebrandt E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21**: 213–229.
- Walker CB, de la Torre JR, Klotz MG, Urakawa H, Pinel N, Arp DJ *et al.* (2010). *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc Natl Acad Sci USA* **107**: 8818–8823.
- Well R, Flessa H. (2008). Isotope fractionation factors of N₂O diffusion. *Rapid Commun Mass Spectrom* **22**: 2621–2628.
- Well R, Flessa H. (2009). Isotopologue enrichment factors of N₂O reduction in soils. *Rapid Commun Mass Spectrom* **23**: 2996–3002.

- Well R, Eschenbach W, Flessa H, von der Heide C, Weymann D. (2012). Are dual isotope and isotopomer ratios of N₂O useful indicators for N₂O turnover during denitrification in nitrate-contaminated aquifers? *Geochim Cosmochim Acta* **90**: 265–282.
- Wrage N, Velthof GL, van Beusichem ML, Oenema O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol Biochem* **33**: 1723–1732.
- Yan J, Haaijer SC, Op den Camp HJ, van Niftrik L, Stahl DA, Konneke M *et al.* (2012). Mimicking the oxygen minimum zones: stimulating interaction of aerobic archaeal and anaerobic bacterial ammonia oxidizers in a laboratory-scale model system. *Environ Microbiol* **14**: 3146–3158.
- Yoshida N, Toyoda S. (2000). Constraining the atmospheric N₂O budget from intramolecular site preference in N₂O isotopomers. *Nature* **405**: 330–334.

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