

ORIGINAL ARTICLE

Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea

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Soil emissions are largely responsible for the increase of the potent greenhouse gas nitrous oxide (N₂O) in the atmosphere and are generally attributed to the activity of nitrifying and denitrifying bacteria. However, the contribution of the recently discovered ammonia-oxidizing archaea (AOA) to N₂O production from soil is unclear as is the mechanism by which they produce it. Here we investigate the potential of *Nitrososphaera viennensis*, the first pure culture of AOA from soil, to produce N₂O and compare its activity with that of a marine AOA and an ammonia-oxidizing bacterium (AOB) from soil. *N. viennensis* produced N₂O at a maximum yield of 0.09% N₂O per molecule of nitrite under oxic growth conditions. N₂O production rates of 4.6 ± 0.6 amol N₂O cell⁻¹ h⁻¹ and nitrification rates of 2.6 ± 0.5 fmol NO₂⁻ cell⁻¹ h⁻¹ were in the same range as those of the AOB *Nitrosospira multiformis* and the marine AOA *Nitrosopumilus maritimus* grown under comparable conditions. In contrast to AOB, however, N₂O production of the two archaeal strains did not increase when the oxygen concentration was reduced, suggesting that they are not capable of denitrification. In ¹⁵N-labeling experiments we provide evidence that both ammonium and nitrite contribute equally via hybrid N₂O formation to the N₂O produced by *N. viennensis* under all conditions tested. Our results suggest that archaea may contribute to N₂O production in terrestrial ecosystems, however, they are not capable of nitrifier-denitrification and thus do not produce increasing amounts of the greenhouse gas when oxygen becomes limiting.

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Introduction

Nitrous oxide (N₂O) is a greenhouse gas with 298 times the global warming potential of carbon dioxide over a 100-year period (IPCC, 2007). It contributes to the destruction of the stratospheric ozone layer (Conrad, 1996) and is even predicted to remain the dominant ozone-depleting substance of the twenty first century (Ravishankara *et al.*, 2009). The increasing food demand of the human population

has led to an excessive use of fertilizers in agriculture, which consequently increased N₂O emissions considerably in the last century (Skiba and Smith, 2000; Galloway *et al.* 2008; Smith *et al.*, 2012). As summed up by Smith *et al.* (2012) already in the year 2000 total N₂O emissions accounted for 15.8 Tg N₂O-N year⁻¹, in which 5.6–6.5 Tg N₂O-N year⁻¹ could be assigned to an anthropogenic source and 4.3–5.8 Tg N₂O-N year⁻¹ to a land or coastal biological source.

The main processes responsible for gaseous nitrogen emissions from soil are microbial transformations of ammonium, nitrite, nitrate and to a lesser extent chemodenitrification (Colliver and Stephenson, 2000; Baggs, 2008, 2011; Campbell *et al.*, 2011). Both ammonia-oxidizing and denitrifying microorganisms produce N₂O by dissimilatory nitrate (or nitrite) reduction mostly under oxygen-limiting or anoxic conditions, whereas ammonia-oxidizing bacteria (AOB) can additionally produce

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N₂O via hydroxylamine oxidation under oxic conditions, albeit to a lower extent (Hooper and Terry, 1979; Arp and Stein, 2003; Stein, 2011). It has been estimated that ammonia oxidizers can contribute considerably to direct terrestrial N₂O emissions, depending on soil type and environmental conditions (Mummey *et al.*, 1994; Webster and Hopkins, 1996; Gødde and Conrad, 1999; Pihlatie *et al.*, 2004). In addition, they have an indirect influence on denitrification and thus N₂O production through the production of the oxidized N-compound nitrite, the substrate for nitrite-oxidizing bacteria to produce nitrate, which in turn is used as a substrate by denitrifying microorganisms (Zhu *et al.*, 2013).

The various N-transforming processes in soils that lead to N₂O production are complex and the contributing microbial partners and environmental factors that influence its production are little understood (Baggs, 2011; Schreiber *et al.*, 2012). For estimations or models of future greenhouse gas production and for the development of mitigation strategies it is therefore of great importance to identify all biological sources of N₂O production and to characterize the environmental factors that influence their activity.

Recently, a novel group of ammonia oxidizers of the domain Archaea has been discovered to be widespread in marine and terrestrial environments, often outnumbering their bacterial counterparts by orders of magnitude (Leininger *et al.*, 2006; Wuchter *et al.*, 2006). The energy metabolism and general physiology of these ammonia-oxidizing archaea (AOA) is still not fully understood. Although it has been shown recently that hydroxylamine is an intermediate of ammonia oxidation in AOA (Vajrala *et al.*, 2013) it has been argued that they might not have the capacity to produce N₂O through a side reaction of ammonia oxidation, because they lack genes for a homolog of hydroxylamine oxidoreductase known to be responsible for N₂O formation in AOB (Hooper and Terry, 1979) and in methanotrophic bacteria (Campbell *et al.*, 2011). Furthermore, although AOA contain homologous genes of a nitrite reductase (NIR; Bartossek *et al.*, 2010), they lack genes encoding a potential NO-reductase (NOR), which is involved in nitrifier-denitrification and thus N₂O production in bacteria (Walker *et al.*, 2010; Campbell *et al.*, 2011; Stein, 2011; Tourna *et al.*, 2011). Nevertheless, stable isotope-labeling experiments of marine enrichment cultures and measurements of a marine isolate have recently shown that AOA are indeed capable of N₂O production (Santoro *et al.*, 2011; Loescher *et al.*, 2012). However, it has remained unclear under which conditions AOA produce N₂O and if they are able to perform nitrifier-denitrification, the process that contributes most to direct N₂O production of AOB in soils (Shaw *et al.*, 2006). The characterization of the first AOA from soil obtained in a pure laboratory culture (Tourna *et al.*, 2011) now allows studying the extent of N₂O production in this group of organisms and to test the

activity under varying environmental conditions, in particular under different oxygen concentrations. Here we present data from extensive laboratory incubations and a range of ¹⁵N-labeling experiments, designed to shed light on the mechanisms of formation and the environmental conditions under which N₂O is produced by AOA.

Materials and methods

Strains and cultures

The AOA *Nitrososphaera viennensis* EN76 was maintained at 37 °C in fresh water medium according to Tourna *et al.* (2011). The AOA *Nitrosopumilus maritimus* SCM1 was incubated at 28 °C in SCM medium according to Könneke *et al.* (2005). *N. viennensis* and *N. maritimus* cultures were supplied with 1 mM ammonium and in addition with 0.1 mM pyruvate and 0.1 mM oxaloacetate, respectively. The media of *N. viennensis* and *N. maritimus* cultures were buffered with HEPES to a pH of 7.5. The AOB *Nitrosospira multiformis* ATCC 25196^T (supplied by Jim Prosser, Aberdeen) was cultivated at 28 °C in Skinner and Walker (S+W) medium (Skinner and Walker, 1961) containing 1 mM ammonium and phenol red (0.5 mg) as pH indicator at a pH of 7.5–8. The pH was regularly adjusted by adding Na₂CO₃. Cultures were inoculated with 10% volumes of culture.

Growth was followed via photometric determination of ammonium consumption and nitrite production using a salicylic acid assay (Kandeler and Gerber, 1988) or a Griess reagent system (Promega, Madison, WI, USA) for the latter. Screenings for contaminations were done regularly using light microscopy and PCR. Late exponential cultures were used to inoculate cultures for the determination of N₂O production (10% inoculum), which have been set up in serum bottles (122 ml total; 20–30 ml medium; sealed with butyl rubber stoppers).

DNA extraction

Nucleic acids were extracted based on a modified protocol of Griffiths *et al.* (2000) using 2-ml Lysing Matrix E tubes (MP biomedical, Eschwege, Germany) containing a mixture of silica, ceramic and glass beads in combination with the BIO101/Savant FastPrepFP120A Instrument (Qbiogene, Illkirch, France) for bead beating. Briefly, 1 ml of culture was harvested and the cell pellet was dissolved in 0.5 ml SDS extraction buffer (0.7 M NaCl, 0.1 M Na₂SO₃, 0.1 M Tris/HCl (pH 7.5), 0.05 M EDTA (pH8), 1% SDS). The further extraction was performed as described in the study by Nicol *et al.* (2005) with a DNA precipitation over night at 4 °C.

Quantitative PCR

Archaeal 16S rRNA genes were quantified using the primers Cren771F and Cren957R (Ochsenreiter

et al., 2003). Amplification was performed in 20 μ l reactions containing 10 μ l QuantiFast SYBR Green PCR Mix (Qiagen, Hilden, Germany), 0.5 μ M of each primer and 2 μ l DNA template. For the standard curve a serial dilution of the linearized *16S rRNA* gene of *N. viennensis* was used with an efficiency of 101% and a slope of -3.3 . The qPCR was performed in a realplex cycler (Mastercycler ep realplex, Eppendorf, Vienna, Austria) with the following PCR conditions: 95 °C for 15 min, 40 cycles of 30 s at 95 °C, 30 s at 54 °C and 30 s at 72 °C followed by a melting curve analysis at the end of the run to indicate the amplification of specific products. qPCR data were generated from independent DNA extractions of quadruplicate cultures with duplicated PCR amplifications.

N₂O quantification

Cultures for the quantification of N₂O were set up in replicates (3–5 cultures each) in serum bottles containing 20 ml fresh water medium. In addition, one blank with medium only and another one with dead cells (autoclaved culture) as inoculum were prepared. Production of N₂O was tested under one fully aerated condition with 21% oxygen in the headspace and three oxygen limited conditions. To achieve this, reduced pressure was applied for 30 s followed by flushing with sterile filtered N₂ (0% oxygen in headspace). To achieve a concentration of 10% and 3% oxygen in the gaseous phase a defined amount of N₂ was replaced by sterile filtered air. Initial oxygen concentrations in the aqueous phase of the *N. viennensis* cultures (37 °C) were measured with an oxygen microsensor (Presens, Regensburg, Germany). Initial O₂ concentrations in *N. maritimus* and *N. multiformis* cultures (28 °C) were calculated according to Henry's law. Oxygen concentrations measured in the aqueous phase revealed that the aimed gaseous O₂ concentrations were approximately achieved: 217 \pm 1 μ M in the aqueous phase (corresponds to 21% O₂ in gas phase), 114 \pm 4 μ M (corresponds to 10% O₂ in gas phase), 48 \pm 7 μ M (corresponds to 3% O₂ in gas phase) and 28 \pm 8 μ M (corresponds to 0% O₂ in gas phase). Owing to residual O₂ dissolved in the medium measured values were slightly higher than expected.

Acetylene, an inhibitor of the ammonia monooxygenase, was added during exponential growth in a final concentration of 0.01%, which is sufficient to inhibit AOB as well as AOA (Hynes and Knowles, 1978; Offre *et al.*, 2009).

Gas samples were taken at several time points during growth and 12 ml were transferred to 10-ml evacuated and sealed glass containers which were stored at 4 °C until analysis by GC (AGILENT 6890 N, Vienna, Austria; injector: 120 °C, detector: 350 °C, oven: 35 °C, carrier gas: N₂) connected to an automatic sample-injection system (DANI HSS 86.50, Head-space-Sampler, Sprockhövel, Germany). N₂O concentration was detected with a 63Ni-electron-capture

detector. Standard gases (Inc. Linde Gas, Vienna, Austria) contained 0.5, 1 and 2.5 μ l l⁻¹ N₂O. Further details are described elsewhere (Schaufler *et al.*, 2010).

The removed gas in the cultures was replaced immediately by the respective gas phase (as described above; air, 10% and 3% O₂ in N₂ or only N₂) in order to prevent reduced pressure. Furthermore, samples (220 μ l) to determine nitrite and ammonium concentration were taken and analyzed photometrically as described above.

¹⁵N-labeling experiments

For *N. viennensis*, experiments with added ¹⁵NH₄⁺ (1 mM ¹⁵NH₄Cl; 5.05 at%) or ¹⁵NO₂⁻ (0.2 mM Na¹⁵NO₂⁻; 9.69 at%) were carried out under oxic and oxygen-limited conditions (3% O₂). In order to obtain comparable conditions between both ¹⁵N-labeling experiments we also added 0.2 mM of unlabeled NaNO₂⁻ to the cultures with ¹⁵NH₄⁺-label. Bottles containing 0.2 mM NaNO₂⁻ and 1 mM NH₄Cl but no inoculum were set up as media blanks. The precise isotopic composition of the label was determined by elemental analyzer (EA 1110, CE Instruments, Wigan, UK) coupled to an IRMS system (Finnigan ConFlo III interface and Finnigan Delta^{PLUS} isotope ratio mass spectrometer, Thermo Fisher, Vienna, Austria). Additionally, we performed an N₂O isotope pool dilution assay for *N. viennensis* under oxygen-limited conditions (3% O₂) by applying exogenously ¹⁵N-labeled N₂O (~300 nM, ~49at%). ¹⁵N-labeled N₂O was produced by the reduction of ¹⁵NO₂⁻ (98at%) to ¹⁵N₂O by using azide (see below). Gross rates of N₂O production and consumption were calculated based on isotope pool dilution theory (Kirkham and Bartholomew, 1954).

For all ¹⁵N-labeling experiments, we used serum bottles inoculated with 10% volumes of culture to a final volume of 30 ml. For each sampling during the growing phase (four times) quadruplicate bottles were prepared and triplicate un-inoculated media served as controls. We followed changes in concentration of NH₄⁺, NO₂⁻ and N₂O as well as isotopic composition of NO₂⁻ and N₂O over time. Headspace samples were transferred to helium-flushed and pre-evacuated vials (12-ml exetainers) for N₂O determination. Liquid samples for NH₄⁺ and NO₂⁻ analysis were immediately frozen to -20 °C until used.

Concentrations of NH₄⁺ and NO₂⁻ were measured as described above. Isotopic composition of NO₂⁻ was determined by a method based on the reduction of NO₂⁻ to N₂O by using azide under acidified conditions following the protocol of Lachouani *et al.* (2010). Briefly, 1 ml sample or standard was transferred to 12-ml exetainer and 1 ml 1 M HCl was added. After purging the vials with helium to eliminate air-N₂O in the sample headspace, 150 μ l 1 M sodium azide buffer (in 10% acetic acid solution) were injected and the vials were placed on a shaker at 37 °C for 18 h. The reaction was

stopped by injecting 250 µl of 10 M NaOH. For mass calibration, NO₂⁻ standards ranging from natural abundance to 8at% were analyzed. N₂O concentration and isotopic ratio of the azide conversion as well as the headspace samples were determined using a purge-and-trap GC/IRMS system (PreCon, GasBench II headspace analyzer, Delta Advantage V; Thermo Fischer, Vienna, Austria). Isotopic ratios of N₂O of the headspace samples were corrected for blanks.

Calculations

The N₂O concentration was calculated per l culture at 25 °C and was corrected for the ambient concentration of N₂O in air (or the respective gas atmosphere of the cultures). The N₂O yield is the average ratio of µmol N₂O per µmol NO₂⁻ produced and was generated from values of 2–4 time points during exponential growth. For the calculation of the nitrification rate (fmol cell⁻¹ h⁻¹) and N₂O production rate (amol cell⁻¹ h⁻¹) the average cell density between two time points during exponential growth as approximated by qPCR was used.

The ¹⁵N-labeling experiments are closed systems where we can assume that there are only two possible sources for the formation of N₂O: NH₄⁺ (or an intermediate stemming from NH₄⁺) and NO₂⁻. We used a two-pool mixing model to determine the percentage contribution of each source pool to the product pool. Because of temporal changes in concentration and therefore in isotopic composition of NO₂⁻ through the input by ammonia oxidation calculations were performed for time intervals along the growing phase. The isotopic ratio of N₂O produced in a certain time interval (Δ at %) was calculated as follows:

$$\Delta \text{ at } \% = \frac{(C_{t2} \cdot \text{at}\%_{t2} - C_{t1} \cdot \text{at}\%_{t1})}{\Delta C} \quad (1)$$

where C_{t1} , C_{t2} , $\text{at}\%_{t1}$ and $\text{at}\%_{t2}$ are N₂O concentrations and atom% of N₂O at $t1$ and $t2$ representing sampling time. ΔC is the increase in N₂O concentration from $t1$ to $t2$. In this time interval the contribution of NO₂⁻ (*source1*) to N₂O production was estimated by a two-pool mixing model:

$$\text{proportion derived from source 1} = \frac{(\text{at}\%_{\text{product}} - \text{at}\%_{\text{source2}})}{(\text{at}\%_{\text{source1}} - \text{at}\%_{\text{source2}})} \cdot 100 \quad (2)$$

where $\text{at}\%_{\text{product}}$ is the isotopic ratio of N₂O (according to Equation (1)). As the atom% of the NO₂⁻ pool changed in the course of time due to the input from ammonia oxidation we used the mean isotopic composition of NO₂⁻ between $t1$ and $t2$ as the atom% of NO₂⁻ ($\text{at}\%_{\text{source1}}$). At enrichment levels, as applied here, the discrimination between isotopes is negligible, and we therefore assumed that the isotopic composition of the NH₄⁺ pool is constant over time. Thus, we used natural abundance (0.3663 atom%) and 5.05 atom% (which was determined as described above) for the unlabeled and labeled

substrate addition experiments, respectively, as the source for N₂O which derives from the NH₄⁺ pool or an intermediate stemming from NH₄⁺ ($\text{at}\%_{\text{source2}}$).

Following up on the results of the two-pool mixing model, we generated a probability model in order to distinguish whether the produced N₂O was derived from hybrid formation or a combination of nitrifier-denitrification and ammonia oxidation. Owing to different labeled N sources (NH₄⁺ and/or NO₂⁻) different N₂O-forming processes will yield a distinctive fraction of N₂O, which is double-labeled (¹⁵N¹⁵NO). Therefore, the model predicts the concentration of double-labeled N₂O (¹⁵N¹⁵NO) as a function of N₂O concentration (that is, sum of N₂O with mass 44, 45 and 46). It is based on the theoretical probability of the occurrence of N₂O with mass 46 (⁴⁶N₂O; including natural abundance of oxygen isotopes). The probability of the occurrence of ⁴⁶N₂O is the sum of the probabilities of four isotopologs:

$$P(^{46}\text{N}_2\text{O}) = P(^{15}\text{N}^{15}\text{N}^{16}\text{O}) + P(^{14}\text{N}^{15}\text{N}^{17}\text{O}) + P(^{15}\text{N}^{14}\text{N}^{17}\text{O}) + P(^{14}\text{N}^{14}\text{N}^{18}\text{O}) \quad (3)$$

To determine the probability of each isotopolog, we multiplied the respective relative natural abundance of the O isotope (¹⁶O, ¹⁷O or ¹⁸O), the relative ¹⁵N abundance [$P(^{15}\text{N})$] and/or the relative abundance of ¹⁴N of the N source [$P(^{14}\text{N}) = 1 - P(^{15}\text{N})$]. In the case that N₂O is solely produced during ammonia oxidation, $P(^{46}\text{N}_2\text{O})$ is based on the isotopic composition of the NH₄⁺ pool. Assuming that only nitrifier-denitrification occurs, $P(^{46}\text{N}_2\text{O})$ is calculated from the isotopic composition of the NO₂⁻ pool. If those two processes occur simultaneously, $P(^{46}\text{N}_2\text{O})$ is the sum of their relative contributions. In case of hybrid N₂O formation (that is, one N atom stems from NO₂⁻ and one from NH₄⁺ or an intermediate of ammonia oxidation), the model considers that one N atom of each isotopolog derives from NH₄⁺ and the other one from NO₂⁻, which are combined to form hybrid N₂O. For the ¹⁵NO₂⁻-labeling experiments, we computed $P(^{46}\text{N}_2\text{O})$ for the different scenarios based on the isotopic composition of NH₄⁺, which was constant at natural abundance, and NO₂⁻, which varied between ~2–4.4 at% due to input by ammonia oxidation. We accounted for this variability in the NO₂⁻ isotopic composition by considering the ¹⁵N relative abundance of the NO₂⁻ pool as a function of N₂O concentration. For each scenario using the respective $P(^{46}\text{N}_2\text{O})$ function, we calculated the cumulative ⁴⁶N₂O concentration (that is, ¹⁵N¹⁵NO) as a function of N₂O produced according to Equation 4.

$$^{46}\text{N}_2\text{O}(x) = \int_{xt0}^x P(^{46}\text{N}_2\text{O}) dx + ^{46}\text{N}_2\text{O}_{xt0} \quad (4)$$

where x is the N₂O concentration, $xt0$ is the N₂O concentration at the first sampling time and $^{46}\text{N}_2\text{O}_{xt0}$ is the concentration of ⁴⁶N₂O at the first sampling time.

Statistical analyses

In case of using independent variables for calculation (that is, independent samples of two samplings during the growth phase) the standard error was estimated by propagation of error. Analysis of variance, Holm–Sidak *post hoc* tests and *t*-tests ($\alpha=0.05$) were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA).

Results

Quantification of N₂O production

N₂O production was measured in *N. viennensis* cultures amended with different initial headspace oxygen concentrations (21%, 10%, 3% and 0%) and compared with N₂O production from the AOA *N. maritimus* and the AOB *N. multiformis*, grown under the same initial ammonia and oxygen concentrations. N₂O accumulation, nitrite production and ammonia consumption are shown in Figure 1.

N₂O production paralleled nitrite production in all strains over the incubation period. N₂O production of *N. viennensis* and *N. maritimus* was dependent on

ammonia oxidation and was not significantly affected by the varying oxygen concentrations (Figure 1, Supplementary Table S1) with stable N₂O yields at all tested oxygen concentrations. There was no increase in N₂O production with decreasing oxygen. In contrast, both strains reached slightly higher maximal N₂O concentrations and yields at higher oxygen concentrations (Table 1). Independent of the oxygen concentration *N. viennensis* produced almost twice as much N₂O as *N. maritimus*. For example, *N. viennensis* had a maximal yield of 0.09 (± 0.00) % N₂O/NO₂⁻ and a maximal N₂O concentration of 0.80 (± 0.08) μM N₂O at 21% O₂ in the headspace, whereas *N. maritimus* produced at maximum 0.44 (± 0.04) μM N₂O with an N₂O yield of 0.05 (± 0.02) % N₂O/NO₂⁻ at the same oxygen level.

Different from that of *N. viennensis* and *N. maritimus*, N₂O production and yields of the AOB *N. multiformis* increased 3–4-fold under decreasing oxygen concentrations, which is in line with earlier studies (Goreau *et al.*, 1980; Anderson and Levine, 1986). *N. multiformis* showed a maximal N₂O production of 3.32 (± 0.30) μM N₂O and the highest

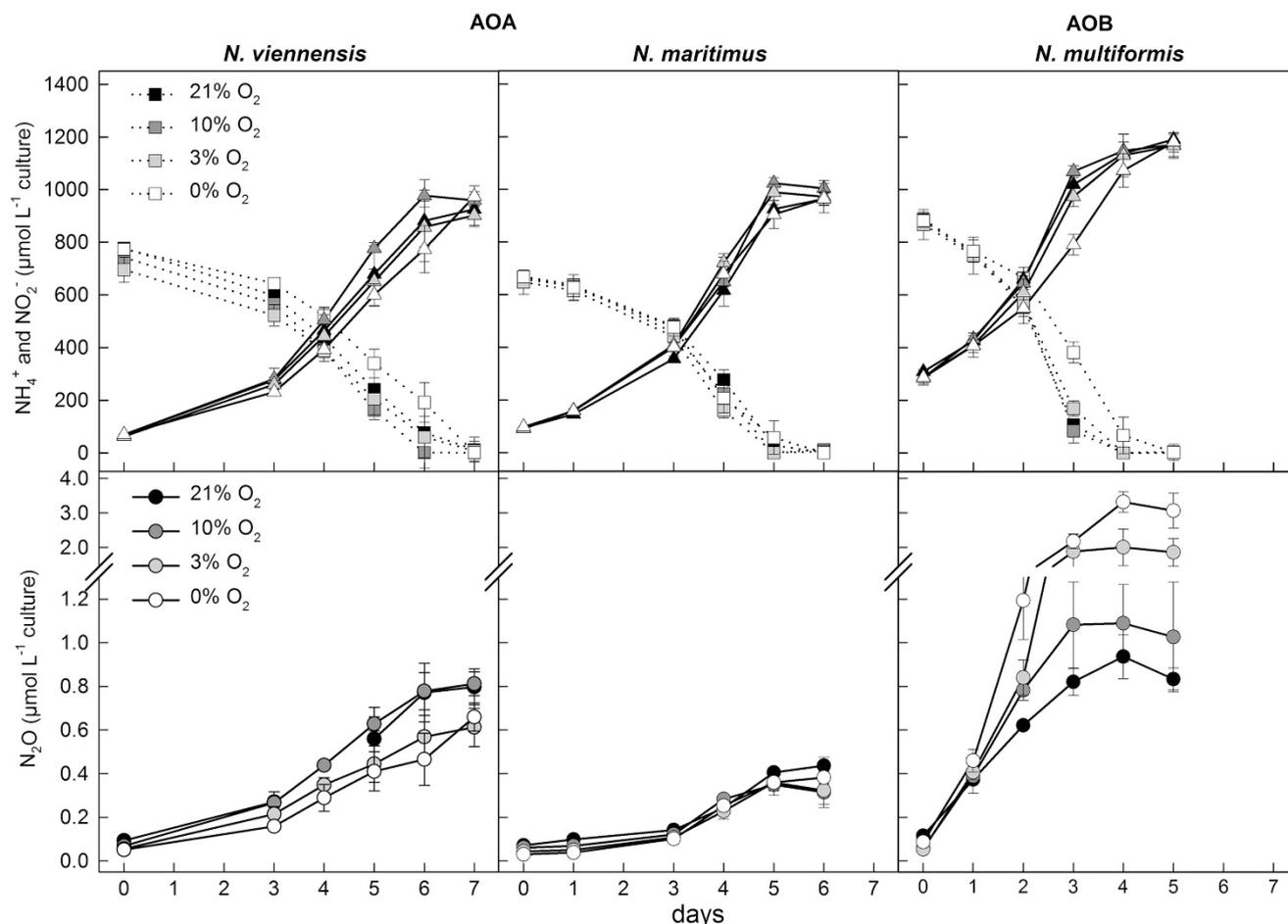


Figure 1 Near stoichiometric conversion of ammonium (squares, dotted lines) to nitrite (triangles, solid lines; upper plots) and concurrent N₂O production (circles; lower plots) during the growth of the AOA *N. viennensis* and *N. maritimus* as well as the AOB *N. multiformis*. The strains have been cultivated under four different oxygen concentrations (21%: black; 10%: dark gray; 3%: light gray; 0%: white). Mean values of triplicate or quadruplicate experiments, respectively, are shown with standard deviations plotted.

Table 1 Maximal N₂O production and N₂O yields of *N. viennensis*, *N. maritimus* and *N. multiformis* under different oxygen conditions

Strain	NH ₄ ⁺ (mM)	O ₂ (%)	N ₂ O max (μM) ^a	N ₂ O yield (%) ^b
AOA <i>N. viennensis</i>	1	21	0.80 ± 0.08	0.09 ± 0.00
		10	0.81 ± 0.05	0.09 ± 0.01
		3	0.61 ± 0.04	0.08 ± 0.01
		0	0.66 ± 0.06	0.07 ± 0.00
<i>N. maritimus</i>	1	21	0.44 ± 0.04	0.05 ± 0.02
		10	0.35 ± 0.03	0.04 ± 0.01
		3	0.36 ± 0.06	0.03 ± 0.00
		0	0.36 ± 0.04	0.03 ± 0.01
AOB <i>N. multiformis</i>	1	21	0.94 ± 0.10	0.09 ± 0.01
		10	1.09 ± 0.18	0.10 ± 0.02
		3	2.00 ± 0.53	0.14 ± 0.05
		0	3.32 ± 0.30	0.27 ± 0.05

^aMaximal N₂O value (μM) measured during growth.

^bN₂O/NO₂⁻ ratio (%). Yields are calculated for the exponential growth phase only. Data represent average values of triplicate or quadruplicate experiments with standard deviations.

Table 2 N₂O and NO₂⁻ production rates of *N. viennensis* at two different oxygen concentrations

	21% oxygen	3% oxygen
Net NO ₂ ⁻ production μmol l ⁻¹ h ⁻¹	7.7 ± 0.6	6.2 ± 0.5
fmol cell ⁻¹ h ⁻¹	2.6 ± 0.5	2.8 ± 0.5
Net N ₂ O production nmol l ⁻¹ h ⁻¹	13.6 ± 1.2	9.3 ± 1.1
amol cell ⁻¹ h ⁻¹	4.6 ± 0.6	4.2 ± 0.1
Cell density ^a Cells × 10 ⁹ l ⁻¹	2.9 ± 1.0	2.2 ± 0.7

Data represent average values of quadruplicate experiments with standard deviations.

^aMeasured by qPCR.

N₂O yield of 0.27 (± 0.05) % N₂O/NO₂⁻ under 0% O₂ in the headspace. In comparison with both AOA the maximal N₂O production of *N. multiformis* (AOB) was significantly higher at all tested oxygen concentrations (see Supplementary Table S1 for statistical tests).

Nitrification and N₂O production rates were determined for *N. viennensis* by relating production to cell numbers estimated by quantitative PCR of the 16S rRNA gene, which occurs only once in the genome (Tournai *et al.*, 2011). The N₂O production rates were 4.6 (± 0.6) amol cell⁻¹ h⁻¹ under ambient oxygen and 4.2 (± 0.1) amol cell⁻¹ h⁻¹ under reduced oxygen (3% O₂ in headspace), with nitrification rates of 2.6 (± 0.5) and 2.8 (± 0.5) fmol nitrite cell⁻¹ h⁻¹, respectively (Table 2).

When 0.01% of the ammonia oxidation inhibitor acetylene was supplied to an exponentially growing culture of *N. viennensis*, both nitrite production and N₂O production ceased immediately, indicating that N₂O production was linked to the process of ammonia oxidation as has been shown for AOB (Supplementary Figure S1). Furthermore, controls with inactivated cells or media blanks without cell inoculum but supplemented with nitrite did not show any increase in N₂O concentration over the incubation period (not shown).

Contribution of ammonia-N and nitrite-N to N₂O

To elucidate the potential mechanism of N₂O production in *N. viennensis* we conducted ¹⁵N-labeling experiments using either ¹⁵N-labeled ammonium plus unlabeled nitrite or *vice versa*. When exogenous ¹⁵NH₄⁺ was supplied, a continuous increase in the ¹⁵N/¹⁴N ratio of the NO₂⁻ pool over time was observed, reflecting the enrichment of labeled NO₂⁻ from ammonia oxidation (Figures 2a and b and Supplementary Figures S2A and B). The ¹⁵N/¹⁴N ratio of the concurrently produced N₂O was higher compared with NO₂⁻ throughout the experiment. The addition of ¹⁵N-labeled NO₂⁻ (together with unlabeled NH₄⁺) resulted in a decrease of the ¹⁵N/¹⁴N ratio of the NO₂⁻ pool over time due to the input of unlabeled NO₂⁻ from ammonia oxidation (Figures 2c and d and Supplementary Figures S2C and D). In this case the concurrently produced N₂O had a lower ¹⁵N/¹⁴N ratio compared with NO₂⁻ at both oxygen concentrations. Thus, in both labeling experiments the differences between the isotopic composition of NO₂⁻ and N₂O indicated that both NO₂⁻ and NH₄⁺ contributed to the production of N₂O.

The ¹⁵N-labeling experiments were closed system experiments with only two possible N-sources for the formation of N₂O: NH₄⁺ (or an intermediate of ammonia oxidation stemming from NH₄⁺) and NO₂⁻. Therefore, a two-pool mixing model was used to elucidate the contribution of NO₂⁻ to the formation of N₂O. The contribution of NO₂⁻ to the formation of N₂O under ambient oxygen concentrations was 40.2% and 40.8% in the ¹⁵NH₄⁺ and ¹⁵NO₂⁻-labeling experiments, respectively, and under reduced oxygen conditions 46.6% and 45.1%, respectively (Figure 3a). These results show a nearly equal contribution of NH₄⁺ and NO₂⁻ to the N₂O production at both oxygen levels tested.

We found no significant difference in the contribution of NO₂⁻ to N₂O between ambient and reduced oxygen condition, which was corroborated by two independent ¹⁵N-labeling approaches with ¹⁵NH₄⁺ and ¹⁵NO₂⁻.

In order to distinguish whether the produced N₂O by *N. viennensis* was derived from hybrid formation (that is, one N atom stems from NO₂⁻ and one from NH₄⁺ or an intermediate of ammonia oxidation) or a combination of two simultaneous processes (that

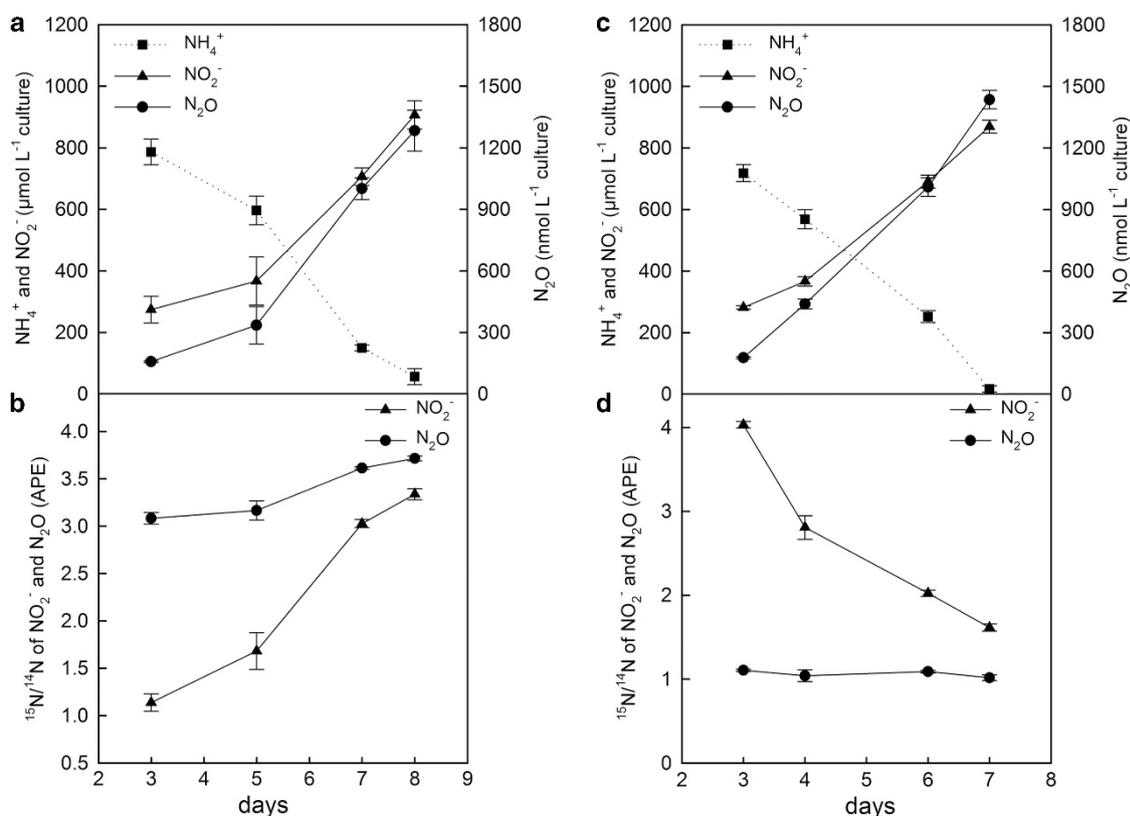


Figure 2 ¹⁵N-labeling experiment of *N. viennensis* under ambient oxygen concentration (21%) with an addition of ¹⁵NH₄⁺ (a, b; 1 mM ¹⁵NH₄⁺ and 0.2 mM ¹⁴NO₂⁻) and ¹⁵NO₂⁻ (c and d; 0.2 mM ¹⁵NO₂⁻ and 1 mM ¹⁴NH₄⁺). The concentrations of NH₄⁺, NO₂⁻ and N₂O were followed during the growth phase (a, c). The NO₂⁻ concentrations presented here were corrected for the exogenously supplied NO₂⁻. The isotopic composition of NO₂⁻ and N₂O are atom percent excess (APE; b, d). When ¹⁵NO₂⁻ was exogenously supplied, no label was recovered as NH₄⁺. At each sampling day samples were harvested from independent flasks. Each data point represents the mean value of four replicates (± 1 s.e.).

is, nitrifier-denitrification and ammonia oxidation) we calculated the concentration of double-labeled N₂O (¹⁵N¹⁵NO) for different N₂O-forming processes. Each process, or a combination of them, will yield a distinctive fraction of double-labeled N₂O (⁴⁶N₂O). The probability model shows that a combination of nitrifier-denitrification and ammonia oxidation with a relative contribution between 40 and 60% as indicated by the two-pool mixing model was unlikely, under both oxygen conditions tested (Figures 3b and c). The model fitted to the measured data suggests under oxic conditions a relative contribution of ~20% and 80% by nitrifier-denitrification and ammonia oxidation, respectively, and of ~14% and 86% under reduced oxygen conditions, which stands in contrast to the results of the two-pool mixing model. The results of the probability model point to N₂O production via hybrid formation or only via ammonia oxidation, whereas the latter case can be excluded because we detected ¹⁵N₂O while labeling the nitrite pool. Taken together, the results of the two-pool mixing model and the probability model indicate hybrid N₂O formation by *N. viennensis*.

To clarify whether the produced N₂O was further metabolized, for example to N₂, we used an isotope

pool dilution assay in which we labeled the N₂O pool with ¹⁵N₂O enabling us to calculate gross and net production rates of the greenhouse gas under oxygen-limiting conditions in *N. viennensis*. The gross N₂O production (14.4 nmol l⁻¹ h⁻¹ ± 1.1) was not significantly different from net N₂O production (12.1 nmol l⁻¹ h⁻¹ ± 1.2), indicating that N₂O was neither reduced further to N₂ nor re-assimilated.

Discussion

The growth of *N. viennensis* in pure culture allowed us to determine, for the first time, gross nitrification and N₂O production rates of an AOA from soil.

Despite its relatively small cell size, N₂O production rates (4.2–4.6 amol N₂O-N h⁻¹ cell⁻¹) of *N. viennensis* were comparable to many bacterial ammonia-oxidizing soil strains (*N. multiformis* ATCC 25196: 7.6 amol N₂O-N h⁻¹ cell⁻¹, *Nitrosospira* sp. strain 40KI: 4.6 amol N₂O-N h⁻¹ cell⁻¹, *Nitrosospira* sp. strain NpAV: 3.9 amol N₂O-N h⁻¹ cell⁻¹ (Shaw *et al.*, 2006)) but lower than those measured for strains of the genus *Nitrosomonas* (Goreau *et al.*, 1980; Hynes and Knowles, 1984; Anderson and Levine, 1986; Remde and Conrad, 1990; Shaw *et al.*, 2006). Similarly, molar yields of N₂O (expressed as a percentage of moles of

NO₂⁻ produced) were of the same order of magnitude as those reported for many AOB (Jiang and Bakken, 1999; Shaw *et al.*, 2006).

N₂O yields of *N. viennensis* (0.07–0.09% N₂O/NO₂⁻) were higher compared with group I.1a AOA enrichment cultures (0.0022–0.055% N₂O/NO₂⁻ (Santoro *et al.*, 2011), 0.065% N₂O/NO₂⁻ (Jung *et al.*, 2011)) and to the pure culture of *N. maritimus* (0.03–0.05% N₂O/NO₂⁻ (this study), 0.002–0.026% N₂O/NH₄⁺ (Loescher *et al.*, 2012)). The measured N₂O yields for *N. maritimus* were thus in

accordance with reported values and slightly higher than reported earlier (Loescher *et al.*, 2012). However, Loescher *et al.* (2012) have obtained maximal N₂O yields for *N. maritimus* under limited oxygen concentrations (initial concentration: 112 μM O₂; in line with 10% O₂ culture in our study) and reported decreasing N₂O concentrations with increasing oxygen levels. At ambient oxygen concentrations Loescher *et al.* (2012) have shown 20 times lower N₂O yields for *N. maritimus* compared with yields obtained in our study for the same organism. In this study, we could not observe a significant difference ($P < 0.001$) in maximal N₂O concentrations produced by *N. maritimus* at the four different oxygen concentrations tested. Different from the study by Loescher *et al.* (2012) we have added oxaloacetate to the culture medium, which led to an increased growth rate and also higher cell numbers for *N. maritimus* and this might have also caused higher N₂O production.

The equally high production of N₂O by *N. viennensis* and *N. maritimus* under different oxygen levels and especially the lack of an increase in N₂O production under oxygen limitation indicate that AOA are not capable of nitrifier-denitrification (N₂O production from nitrite alone). This is supported by the absence of genes for *bona fide* nitric oxide reductase (NOR) in the genomes of AOA (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012) and is also in agreement with earlier isotopic studies in which the site preferences of N₂O indicated that it is mainly not produced via nitrifier-denitrification (Santoro *et al.*, 2011; Loescher *et al.*, 2012).

Hybrid N₂O formation in *N. viennensis*

Stable isotope-labeling experiments with *N. viennensis* showed a nearly equal contribution of nitrogen from ammonia and nitrite to the N₂O production

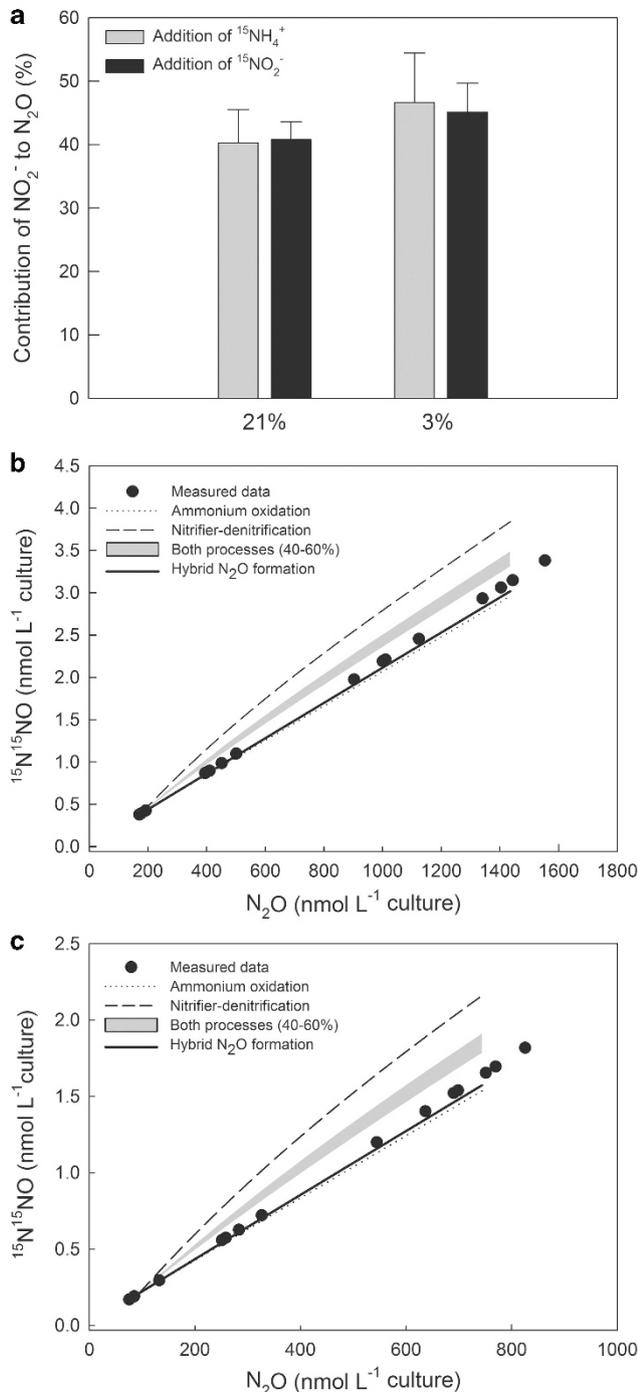


Figure 3 Two-pool mixing model (a) showing the comparison of percentage contribution of NO₂⁻ to the N₂O formation between ¹⁵N-labeling experiments (addition of ¹⁵NH₄⁺ and ¹⁵NO₂⁻) at each O₂-treatment (21% and 3%) for *N. viennensis*. Data presented are means of all time intervals along the growth phase for each experiment (± 1 s.e.). We found no significant difference in the mean of the percentage contribution of NO₂⁻ to the N₂O formation between ¹⁵NH₄⁺- and ¹⁵NO₂⁻-labeling experiments at each O₂ treatment (t -test, 21% oxygen, $t_4 = -0.0868$, $P = 0.935$; 3% oxygen, $t_4 = 0.167$, $P = 0.876$). Within each labeling experiment, there is also no significant difference between the mean of the O₂-treatments (t -test, ¹⁵NH₄⁺-labeling, $t_4 = -0.677$, $P = 0.536$; ¹⁵NO₂⁻-labeling, $t_4 = -0.810$, $P = 0.463$). Probability models (b, c) showing predicted double-labeled N₂O (¹⁵N¹⁵NO; based on the theoretical probability of the occurrence of N₂O with mass 46) produced by different possible pathways compared with measured data of the ¹⁵NO₂⁻-labeling experiment under oxic (b) and reduced oxygen (c; 3% O₂ in headspace) conditions. The grey shaded area represents a combination of ammonia oxidation and nitrifier-denitrification with a contribution of each process between 40 and 60%. The upper border of the grey shaded area represents a contribution of nitrifier-denitrification with 60% and ammonia oxidation with 40% and *vice versa* for the lower border.

at all oxygen levels tested. This was further supported, when we modeled the amount of dually labeled N₂O molecules (¹⁵N-¹⁵N-O) to determine the different possible pathways that could be used to synthesize N₂O and compared the calculated values to the actual measurements obtained from our experiments with ¹⁵N-labeled nitrite under ambient and reduced oxygen concentrations (Figures 3b and c). Thus, *N. viennensis* seems to produce N₂O during aerobic ammonia oxidation, from nitrite and an intermediate of ammonia oxidation mostly via a hybrid formation mechanism. Such a mechanism of N₂O formation is also known from denitrifying fungi and bacteria mainly under anoxic or reduced oxygen conditions, where it is described as co-metabolic denitrification (that is, co-denitrification) through a biotically mediated N-nitrosation reaction (Spott *et al.*, 2011). In this process one N from nitrite or NO is combined in an enzymatic reaction with one N from a co-substrate (ammonium, hydroxylamine, amines, and so on). NIR and NOR have been suggested as possible enzyme candidates catalyzing this reaction (Spott *et al.*, 2011). As all published thaumarchaeal genomes (except that of *Cenarchaeum symbiosum*) contain a *nirK* homolog (encoding NIR) (Bartossek *et al.*, 2010) and as it has been shown in metatranscriptomic studies that this thaumarchaeal gene is highly expressed in planktonic samples (Frias-Lopez *et al.*, 2008; Hollibaugh *et al.*, 2011), sponge tissues (Radax *et al.*, 2012) and in soil (Urich *et al.*, 2008), it might be a good candidate for performing this reaction. However, one has to note that the term co-denitrification has so far been used for a process that increases with decreasing oxygen concentrations (Spott *et al.*, 2011), which was not the case for N₂O production in our AOA study.

There are two main N₂O production mechanisms described for bacterial ammonia oxidizers. Under oxic conditions AOB oxidize hydroxylamine by hydroxylamine oxidoreductase to NO, which is further oxidized to N₂O by a yet unknown enzyme (Hooper and Terry, 1979; Schreiber *et al.*, 2012). However, cytochrome c554 and NorS have been discussed as potential candidates for this reaction in AOB (Stein, 2011), whereas CytS has been described to have a role in NO-detoxification in methane-oxidizing bacteria (Poret-Peterson *et al.*, 2008; Campbell *et al.*, 2011). Under reduced oxygen conditions N₂O is produced via the process of nitrifier-denitrification, which is the reduction of nitrite to NO by NIR and a further reduction to N₂O by NOR (Goreau *et al.*, 1980; Arp and Stein, 2003). However, some nitrifier-denitrification of AOB has also been demonstrated under oxic conditions (Shaw *et al.*, 2006). In addition, it has been discussed that aerobic N₂O production in AOB might proceed via a different and unknown pathway including HNO as a further intermediate of ammonia oxidation, which might react abiotically to N₂O (Schreiber *et al.*, 2012). Further, a recent study has

shown the emission of HONO and NO by the AOB *Nitrosomonas europaea* (Oswald *et al.*, 2013).

The pathway of ammonia oxidation in AOA is still not fully understood. Although it has been shown recently that hydroxylamine is an intermediate of ammonia oxidation in *N. maritimus* (Vajrala *et al.*, 2013) homologous genes of hydroxylamine oxidoreductase have not been identified in their genomes (Walker *et al.*, 2010; Kim *et al.*, 2011; Tourna *et al.*, 2011; Spang *et al.*, 2012). Thus, biotic production of hybrid N₂O via the yet unidentified second enzyme of ammonia oxidation cannot be excluded. However, we can also not exclude an abiotic formation of hybrid N₂O via an N-nitrosation reaction of nitrite and an intermediate of ammonia oxidation, for example, hydroxylamine, HNO or NO (Zollinger, 1988; Spott *et al.*, 2011), which have been discussed to be possible intermediates of AOA (Schleper and Nicol, 2010; Walker *et al.*, 2010; Vajrala *et al.*, 2013). Noteworthy, recent studies by Yan *et al.* (2012) and our laboratory (Shen *et al.*, 2013) have demonstrated inhibition of ammonia oxidation by carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), a scavenger of NO (Amano and Noda, 1995; Akaike and Maeda, 1996), indicating that NO does indeed have an important role in the energy metabolism of AOA as postulated earlier (Schleper and Nicol, 2010; Walker *et al.*, 2010).

Conclusion

N. viennensis and *N. maritimus*, the only available pure cultures of AOA, produced N₂O under oxic conditions at similar yields and rates as bacterial ammonia oxidizers grown under similar conditions (for example, same ammonia supply). However, both AOA are not capable of nitrifier-denitrification like AOB and thus do not produce increasing amounts of the greenhouse gas when oxygen becomes limiting. ¹⁵N-labeling studies performed with *N. viennensis* indicate N₂O production that results in hybrid formation independent of the oxygen concentration.

Extrapolating from our data obtained with two representatives of the two major clades of AOA (soil and marine clade) and considering the vast numbers of AOA (Karner *et al.*, 2001; Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Adair and Schwartz, 2008; Shen *et al.*, 2008) and their ammonia-oxidizing activity in both terrestrial and oceanic environments (Martens-Habbena *et al.*, 2009; Offre *et al.*, 2009; Di *et al.*, 2010; Verhamme *et al.*, 2011) one can assume that AOA contribute directly to continuous persistent N₂O emissions, albeit at low rates, comparable to those of AOB under oxic conditions and low ammonia supply. As AOB might produce more N₂O under higher ammonia concentrations than supplied in our experiments their relative contribution to N₂O emissions in the environment is certainly higher than that of AOA on a per-cell

basis. A bigger contribution to global N₂O production through AOA might occur rather indirectly through the production of oxidized nitrogenous compounds (mostly NO₂⁻) that are converted into substrates for denitrifying organisms.

Conflict of Interest

The authors declare no conflict of interest.

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