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ORIGINAL ARTICLE

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

Michaela Stieglmeier[1], Maria Mooshammer[2], Barbara Kitzler[3], Wolfgang Wanek[2], Sophie Zechmeister-Boltenstern[3,4], Andreas Richter[2] and Christa Schleper[1]

[1]*Department of Ecogenomics and Systems Biology, Archaea Biology and Ecogenomics Division, University of Vienna, Vienna, Austria;* [2]*Department of Microbiology and Ecosystem Science, Division of Terrestrial Ecosystem Research, University of Vienna, Vienna, Austria;* [3]*Federal Research and Training Centre for Forests, Natural Hazards and Landscape, Unit of Soil Biology, Vienna, Austria and* [4]*University of Natural Resources and Life Sciences, Institute of Soil Research, Vienna, Austria*

Soil emissions are largely responsible for the increase of the potent greenhouse gas nitrous oxide (N_2O) 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 N_2O 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 N_2O and compare its activity with that of a marine AOA and an ammonia-oxidizing bacterium (AOB) from soil. *N. viennensis* produced N_2O at a maximum yield of 0.09% N_2O per molecule of nitrite under oxic growth conditions. N_2O production rates of 4.6 ± 0.6 amol N_2O cell^{-1} h^{-1} and nitrification rates of 2.6 ± 0.5 fmol NO_2^- 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, N_2O production of the two archaeal strains did not increase when the oxygen concentration was reduced, suggesting that they are not capable of denitrification. In ^{15}N-labeling experiments we provide evidence that both ammonium and nitrite contribute equally via hybrid N_2O formation to the N_2O produced by *N. viennensis* under all conditions tested. Our results suggest that archaea may contribute to N_2O 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 (N_2O) 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 N_2O 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 N_2O emissions accounted for 15.8 Tg N_2O-N year^{-1}, in which 5.6–6.5 Tg N_2O-N year^{-1} could be assigned to an anthropogenic source and 4.3–5.8 Tg N_2O-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 N_2O by dissimilatory nitrate (or nitrite) reduction mostly under oxygen-limiting or anoxic conditions, whereas ammonia-oxidizing bacteria (AOB) can additionally produce

Correspondence: A Richter, Department of Microbiology and Ecosystems Research, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.
E-mail: andreas.richter@univie.ac.at
or C Schleper, Department of Ecogenomics and Systems Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.
E-mail: christa.schleper@univie.ac.at
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N_2O 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 N_2O emissions, depending on soil type and environmental conditions (Mummey *et al.*, 1994; Webster and Hopkins, 1996; Gödde and Conrad, 1999; Pihlatie *et al.*, 2004). In addition, they have an indirect influence on denitrification and thus N_2O 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 N_2O 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 N_2O 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 N_2O through a side reaction of ammonia oxidation, because they lack genes for a homolog of hydroxylamine oxidoreductase known to be responsible for N_2O 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 N_2O 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 N_2O production (Santoro *et al.*, 2011; Loescher *et al.*, 2012). However, it has remained unclear under which conditions AOA produce N_2O and if they are able to perform nitrifier-denitrification, the process that contributes most to direct N_2O 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 N_2O 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 ^{15}N-labeling experiments, designed to shed light on the mechanisms of formation and the environmental conditions under which N_2O 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 Na_2CO_3. 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 N_2O 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 biomedicals, 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 Na_2SO_3, 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 µl reactions containing 10 µl QuantiFast SYBR Green PCR Mix (Qiagen, Hilden, Germany), 0.5 µM of each primer and 2 µl DNA template. For the standard curve a serial dilution of the linearized *16 S 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.

N_2O quantification

Cultures for the quantification of N_2O 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 N_2O 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 N_2 (0% oxygen in headspace). To achieve a concentration of 10% and 3% oxygen in the gaseous phase a defined amount of N_2 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 O_2 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 O_2 concentrations were approximately achieved: 217 ± 1 µM in the aqueous phase (corresponds to 21% O_2 in gas phase), 114 ± 4 µM (corresponds to 10% O_2 in gas phase), 48 ± 7 µM (corresponds to 3% O_2 in gas phase) and 28 ± 8 µM (corresponds to 0% O_2 in gas phase). Owing to residual O_2 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: N_2) connected to an automatic sample-injection system (DANI HSS 86.50, Headspace-Sampler, Sprockhövel, Germany). N_2O concentration was detected with a 63Ni-electron-capture detector. Standard gases (Inc. Linde Gas, Vienna, Austria) contained 0.5, 1 and 2.5 µl l^{-1} N_2O. 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% O_2 in N_2 or only N_2) in order to prevent reduced pressure. Furthermore, samples (220 µl) to determine nitrite and ammonium concentration were taken and analyzed photometrically as described above.

^{15}N-labeling experiments

For *N. viennensis*, experiments with added $^{15}NH_4^+$ (1 mM $^{15}NH_4Cl$; 5.05 at%) or $^{15}NO_2^-$ (0.2 mM $Na^{15}NO_2^-$; 9.69 at%) were carried out under oxic and oxygen-limited conditions (3% O_2). In order to obtain comparable conditions between both ^{15}N-labeling experiments we also added 0.2 mM of unlabeled $NaNO_2^-$ to the cultures with $^{15}NH_4^+$-label. Bottles containing 0.2 mM $NaNO_2^-$ and 1 mM NH_4Cl 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 N_2O isotope pool dilution assay for *N. viennensis* under oxygen-limited conditions (3% O_2) by applying exogenously ^{15}N-labeled N_2O (~300 nM, ~49at%). ^{15}N-labeled N_2O was produced by the reduction of $^{15}NO_2^-$ (98at%) to $^{15}N_2O$ by using azide (see below). Gross rates of N_2O production and consumption were calculated based on isotope pool dilution theory (Kirkham and Bartholomew, 1954).

For all ^{15}N-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 NH_4^+, NO_2^- and N_2O as well as isotopic composition of NO_2^- and N_2O over time. Headspace samples were transferred to helium-flushed and pre-evacuated vials (12-ml exetainers) for N_2O determination. Liquid samples for NH_4^+ and NO_2^- analysis were immediately frozen to −20 °C until used.

Concentrations of NH_4^+ and NO_2^- were measured as described above. Isotopic composition of NO_2^- was determined by a method based on the reduction of NO_2^- to N_2O 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-N_2O in the sample headspace, 150 µl 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, NO_2^- standards ranging from natural abundance to 8at% were analyzed. N_2O 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 N_2O of the headspace samples were corrected for blanks.

Calculations

The N_2O concentration was calculated per l culture at 25 °C and was corrected for the ambient concentration of N_2O in air (or the respective gas atmosphere of the cultures). The N_2O yield is the average ratio of µmol N_2O per µmol NO_2^- 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 N_2O 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 ^{15}N-labeling experiments are closed systems where we can assume that there are only two possible sources for the formation of N_2O: NH_4^+ (or an intermediate stemming from NH_4^+) and NO_2^-. 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 NO_2^- through the input by ammonia oxidation calculations were performed for time intervals along the growing phase. The isotopic ratio of N_2O produced in a certain time interval (Δ *at %*) was calculated as follows:

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

where C_{t1}, C_{t2}, $at\%_{t1}$ and $at\%t_2$ are N_2O concentrations and atom% of N_2O at *t1* and *t2* representing sampling time. ΔC is the increase in N_2O concentration from *t1* to *t2*. In this time interval the contribution of NO_2^- (*source1*) to N_2O production was estimated by a two-pool mixing model:

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

where $at\%_{product}$ is the isotopic ratio of N_2O (according to Equation (1)). As the atom% of the NO_2^- pool changed in the course of time due to the input from ammonia oxidation we used the mean isotopic composition of NO_2^- between *t1* and *t2* as the atom% of NO_2^- ($at\%_{source1}$). At enrichment levels, as applied here, the discrimination between isotopes is negligible, and we therefore assumed that the isotopic composition of the NH_4^+ 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 N_2O which derives from the NH_4^+ pool or an intermediate stemming from NH_4^+ ($at\%_{source2}$).

Following up on the results of the two-pool mixing model, we generated a probability model in order to distinguish whether the produced N_2O was derived from hybrid formation or a combination of nitrifier-denitrification and ammonia oxidation. Owing to different labeled N sources (NH_4^+ and/or NO_2^-) different N_2O-forming processes will yield a distinctive fraction of N_2O, which is double-labeled ($^{15}N^{15}NO$). Therefore, the model predicts the concentration of double-labeled N_2O ($^{15}N^{15}NO$) as a function of N_2O concentration (that is, sum of N_2O with mass 44, 45 and 46). It is based on the theoretical probability of the occurrence of N_2O with mass 46 ($^{46}N_2O$; including natural abundance of oxygen isotopes). The probability of the occurrence of $^{46}N_2O$ is the sum of the probabilities of four isotopologs:

$$\begin{aligned} P(^{46}N_2O) = {} & P(^{15}N^{15}N^{16}O) + P(^{14}N^{15}N^{17}O) \\ & + P(^{15}N^{14}N^{17}O) + P(^{14}N^{14}N^{18}O) \end{aligned} \quad (3)$$

To determine the probability of each isotopolog, we multiplied the respective relative natural abundance of the O isotope (^{16}O, ^{17}O or ^{18}O), the relative ^{15}N abundance [$P(^{15}N)$] and/or the relative abundance of ^{14}N of the N source [$P(^{14}N) = 1 - P(^{15}N)$]. In the case that N_2O is solely produced during ammonia oxidation, $P(^{46}N_2O)$ is based on the isotopic composition of the NH_4^+ pool. Assuming that only nitrifier-denitrification occurs, $P(^{46}N_2O)$ is calculated from the isotopic composition of the NO_2^- pool. If those two processes occur simultaneously, $P(^{46}N_2O)$ is the sum of their relative contributions. In case of hybrid N_2O formation (that is, one N atom stems from NO_2^- and one from NH_4^+ or an intermediate of ammonia oxidation), the model considers that one N atom of each isotopolog derives from NH_4^+ and the other one from NO_2^-, which are combined to form hybrid N_2O. For the $^{15}NO_2^-$-labeling experiments, we computed $P(^{46}N_2O)$ for the different scenarios based on the isotopic composition of NH_4^+, which was constant at natural abundance, and NO_2^-, which varied between ~2–4.4 at% due to input by ammonia oxidation. We accounted for this variability in the NO_2^- isotopic composition by considering the ^{15}N relative abundance of the NO_2^- pool as a function of N_2O concentration. For each scenario using the respective $P(^{46}N_2O)$ function, we calculated the cumulative $^{46}N_2O$ concentration (that is, $^{15}N^{15}NO$) as a function of N_2O produced according to Equation 4.

$$^{46}N_2O(x) = \int_{xt0}^{x} P(^{46}N_2O)\,dx + {}^{46}N_2O_{xt0} \quad (4)$$

where x is the N_2O concentration, $xt0$ is the N_2O concentration at the first sampling time and $^{46}N_2O_{xt0}$ is the concentration of $^{46}N_2O$ 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 N_2O production
N_2O production was measured in *N. viennensis* cultures amended with different initial headspace oxygen concentrations (21%, 10%, 3% and 0%) and compared with N_2O production from the AOA *N. maritimus* and the AOB *N. multiformis*, grown under the same initial ammonia and oxygen concentrations. N_2O accumulation, nitrite production and ammonia consumption are shown in Figure 1.

N_2O production paralleled nitrite production in all strains over the incubation period. N_2O 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 N_2O yields at all tested oxygen concentrations. There was no increase in N_2O production with decreasing oxygen. In contrast, both strains reached slightly higher maximal N_2O concentrations and yields at higher oxygen concentrations (Table 1). Independent of the oxygen concentration *N. viennensis* produced almost twice as much N_2O as *N. maritimus*. For example, *N. viennensis* had a maximal yield of 0.09 ($\pm$0.00) % N_2O/NO_2^- and a maximal N_2O concentration of 0.80 ($\pm$0.08) μM N_2O at 21% O_2 in the headspace, whereas *N. maritimus* produced at maximum 0.44 ($\pm$0.04) μM N_2O with an N_2O yield of 0.05 ($\pm$0.02) % N_2O/NO_2^- at the same oxygen level.

Different from that of *N. viennensis* and *N. maritimus*, N_2O 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 N_2O production of 3.32 ($\pm$0.30) μM N_2O and the highest

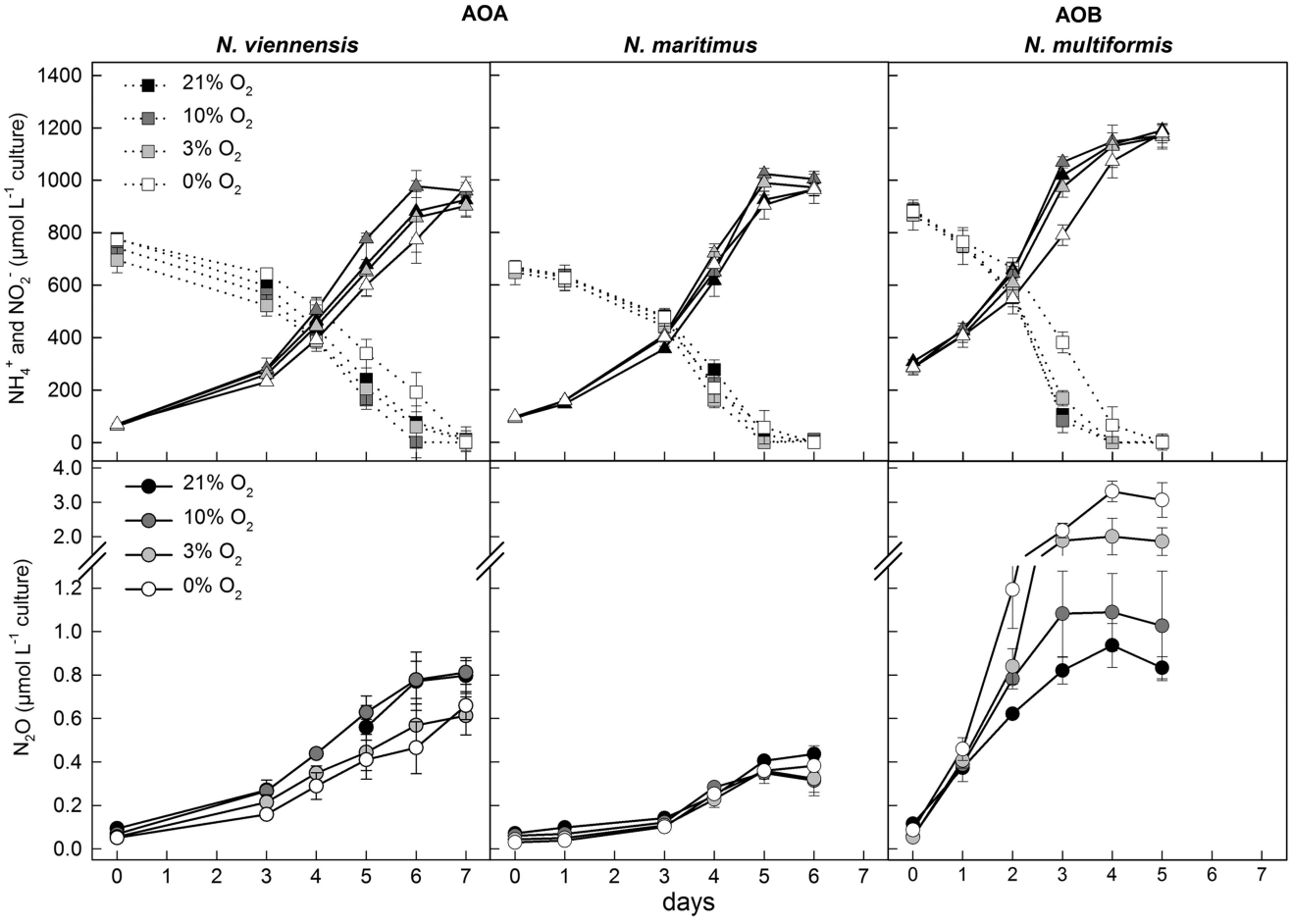

Figure 1 Near stoichiometric conversion of ammonium (squares, dotted lines) to nitrite (triangles, solid lines; upper plots) and concurrent N_2O production (circles; lower plots) during the growth of the AOA *N. viennensis* and *N. maritimus* as well as the AOB *N. multiformis*. The strains have been 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 N_2O production and N_2O yields of *N. viennensis*, *N. maritimus* and *N. multiformis* under different oxygen conditions

Strain	NH_4^+ (mM)	O_2 (%)	*N_2O max* (μM)[a]	*N_2O yield* (%)[b]
AOA				
N. viennensis	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
N. maritimus	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				
N. multiformis	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

[a]Maximal N_2O value (μM) measured during growth.
[b]N_2O/NO_2^- ratio (%). Yields are calculated for the exponential growth phase only. Data represent average values of triplicate or quadruplicate experiments with standard deviations.

Table 2 N_2O and NO_2^- 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_2O 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.
[a]Measured by qPCR.

N_2O yield of 0.27 (± 0.05) % N_2O/NO_2^- under 0% O_2 in the headspace. In comparison with both AOA the maximal N_2O production of *N. multiformis* (AOB) was significantly higher at all tested oxygen concentrations (see Supplementary Table S1 for statistical tests).

Nitrification and N_2O 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 (Tourna *et al.*, 2011). The N_2O 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% O_2 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 N_2O production ceased immediately, indicating that N_2O 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 N_2O concentration over the incubation period (not shown).

Contribution of ammonia-N and nitrite-N to N_2O

To elucidate the potential mechanism of N_2O production in *N. viennensis* we conducted ^{15}N-labeling experiments using either ^{15}N-labeled ammonium plus unlabeled nitrite or *vice versa*. When exogenous $^{15}NH_4^+$ was supplied, a continuous increase in the $^{15}N/^{14}N$ ratio of the NO_2^- pool over time was observed, reflecting the enrichment of labeled NO_2^- from ammonia oxidation (Figures 2a and b and Supplementary Figures S2A and B). The $^{15}N/^{14}N$ ratio of the concurrently produced N_2O was higher compared with NO_2^- throughout the experiment. The addition of ^{15}N-labeled NO_2^- (together with unlabeled NH_4^+) resulted in a decrease of the $^{15}N/^{14}N$ ratio of the NO_2^- pool over time due to the input of unlabeled NO_2^- from ammonia oxidation (Figures 2c and d and Supplementary Figures S2C and D). In this case the concurrently produced N_2O had a lower $^{15}N/^{14}N$ ratio compared with NO_2^- at both oxygen concentrations. Thus, in both labeling experiments the differences between the isotopic composition of NO_2^- and N_2O indicated that both NO_2^- and NH_4^+ contributed to the production of N_2O.

The ^{15}N-labeling experiments were closed system experiments with only two possible N-sources for the formation of N_2O: NH_4^+ (or an intermediate of ammonia oxidation stemming from NH_4^+) and NO_2^-. Therefore, a two-pool mixing model was used to elucidate the contribution of NO_2^- to the formation of N_2O. The contribution of NO_2^- to the formation of N_2O under ambient oxygen concentrations was 40.2% and 40.8% in the $^{15}NH_4^+$ and $^{15}NO_2^-$-labeling experiments, respectively, and under reduced oxygen conditions 46.6% and 45.1%, respectively (Figure 3a). These results show a nearly equal contribution of NH_4^+ and NO_2^- to the N_2O production at both oxygen levels tested.

We found no significant difference in the contribution of NO_2^- to N_2O between ambient and reduced oxygen condition, which was corroborated by two independent ^{15}N-labeling approaches with $^{15}NH_4^+$ and $^{15}NO_2^-$.

In order to distinguish whether the produced N_2O by *N. viennensis* was derived from hybrid formation (that is, one N atom stems from NO_2^- and one from NH_4^+ or an intermediate of ammonia oxidation) or a combination of two simultaneous processes (that

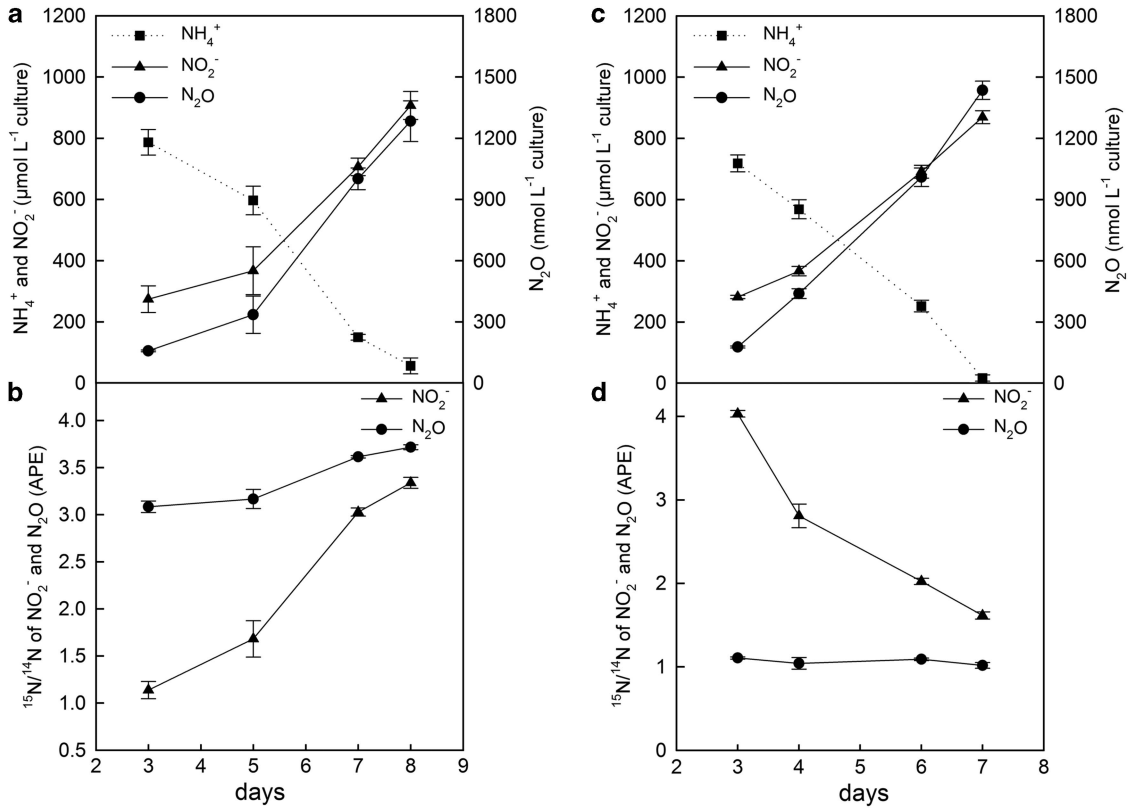

Figure 2 15N-labeling experiment of *N. viennensis* under ambient oxygen concentration (21%) with an addition of $^{15}NH_4^+$ (**a**, **b**; 1mM $^{15}NH_4^+$ and 0.2mM $^{14}NO_2^-$) and $^{15}NO_2^-$ (**c** and **d**; 0.2mM $^{15}NO_2^-$ and 1mM $^{14}NH_4^+$). The concentrations of NH_4^+, NO_2^- and N_2O were followed during the growth phase (**a**, **c**). The NO_2^- concentrations presented here were corrected for the exogenously supplied NO_2^-. The isotopic composition of NO_2^- and N_2O are atom percent excess (APE; **b**, **d**). When $^{15}NO_2^-$ was exogenously supplied, no label was recovered as NH_4^+. 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 N_2O ($^{15}N^{15}NO$) for different N_2O-forming processes. Each process, or a combination of them, will yield a distinctive fraction of double-labeled N_2O ($^{46}N_2O$). 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 N_2O production via hybrid formation or only via ammonia oxidation, whereas the latter case can be excluded because we detected $^{15}N_2O$ while labeling the nitrite pool. Taken together, the results of the two-pool mixing model and the probability model indicate hybrid N_2O formation by *N. viennensis*.

To clarify whether the produced N_2O was further metabolized, for example to N_2, we used an isotope pool dilution assay in which we labeled the N_2O pool with $^{15}N_2O$ enabling us to calculate gross and net production rates of the greenhouse gas under oxygen-limiting conditions in *N. viennensis*. The gross N_2O production (14.4 nmol l^{-1} h^{-1} ± 1.1) was not significantly different from net N_2O production (12.1 nmol l^{-1} h^{-1} ± 1.2), indicating that N_2O was neither reduced further to N_2 nor re-assimilated.

Discussion

The growth of *N. viennensis* in pure culture allowed us to determine, for the first time, gross nitrification and N_2O production rates of an AOA from soil.

Despite its relatively small cell size, N_2O production rates (4.2–4.6 amol N_2O-N h^{-1} $cell^{-1}$) of *N. viennensis* were comparable to many bacterial ammonia-oxidizing soil strains (*N. multiformis* ATCC 25196: 7.6 amol N_2O-N h^{-1} $cell^{-1}$, *Nitrosospira* sp. strain 40KI: 4.6 amol N_2O-N h^{-1} $cell^{-1}$, *Nitrosospira* sp. strain NpAV: 3.9 amol N_2O-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 N_2O (expressed as a percentage of moles of

NO_2^- produced) were of the same order of magnitude as those reported for many AOB (Jiang and Bakken, 1999; Shaw *et al.*, 2006).

N_2O yields of *N. viennensis* (0.07–0.09% N_2O/NO_2^-) were higher compared with group I.1a AOA enrichment cultures (0.0022–0.055% N_2O/NO_2^- (Santoro *et al.*, 2011), 0.065% N_2O/NO_2^- (Jung *et al.*, 2011)) and to the pure culture of *N. maritimus* (0.03–0.05% N_2O/NO_2^- (this study), 0.002–0.026% N_2O/NH_4^+ (Loescher *et al.*, 2012)). The measured N_2O 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 N_2O yields for *N. maritimus* under limited oxygen concentrations (initial concentration: 112 μM O_2; in line with 10% O_2 culture in our study) and reported decreasing N_2O concentrations with increasing oxygen levels. At ambient oxygen concentrations Loescher *et al.* (2012) have shown 20 times lower N_2O 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 N_2O 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 N_2O production.

The equally high production of N_2O by *N. viennensis* and *N. maritimus* under different oxygen levels and especially the lack of an increase in N_2O production under oxygen limitation indicate that AOA are not capable of nitrifier-denitrification (N_2O 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 N_2O indicated that it is mainly not produced via nitrifier-denitrification (Santoro *et al.*, 2011; Loescher *et al.*, 2012).

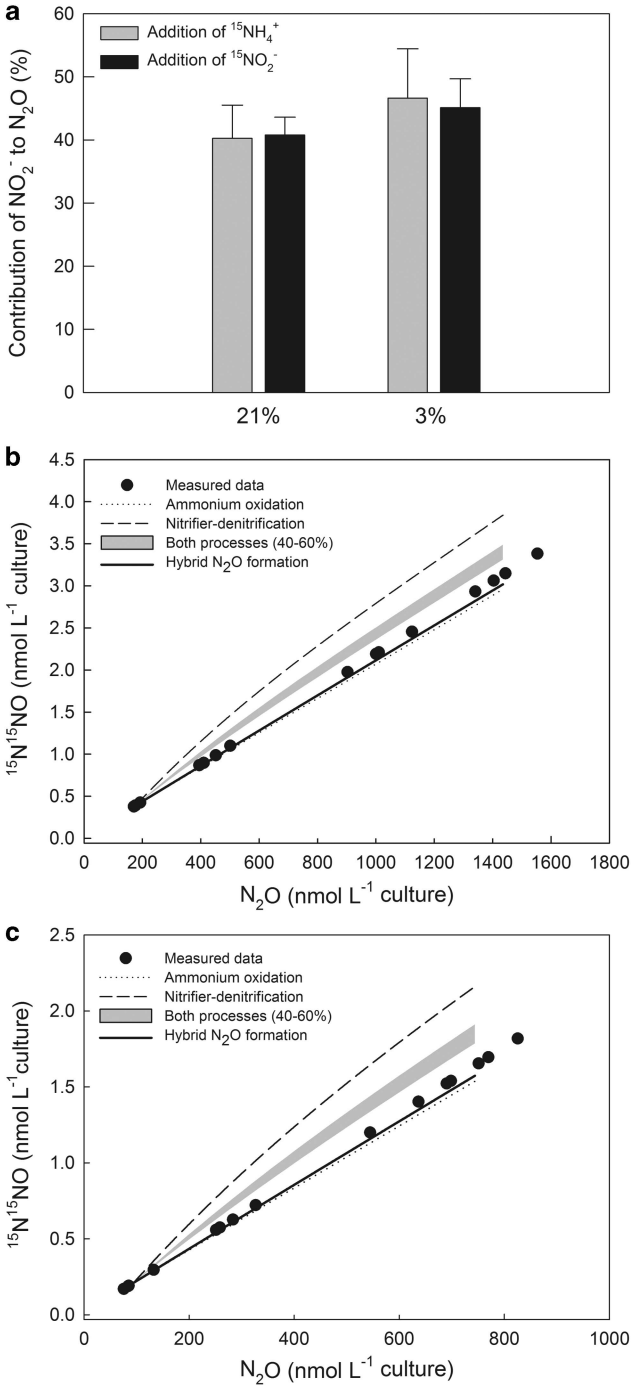

Figure 3 Two-pool mixing model (**a**) showing the comparison of percentage contribution of NO_2^- to the N_2O formation between ^{15}N-labeling experiments (addition of $^{15}NH_4^+$ and $^{15}NO_2^-$) at each O_2-treatment (21% and 3%) for *N. viennensis*. Data presented are means of all time intervals along the growth phase for each experiment (± 1s.e.). We found no significant difference in the mean of the percentage contribution of NO_2^- to the N_2O formation between $^{15}NH_4^+$- and $^{15}NO_2^-$-labeling experiments at each O_2 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 O_2-treatments (*t*-test, $^{15}NH_4^+$-labeling, $t_4=-0.677$, $P=0.536$; $^{15}NO_2^-$-labeling, $t_4=-0.810$, $P=0.463$). Probability models (**b**, **c**) showing predicted double-labeled N_2O ($^{15}N^{15}NO$; based on the theoretical probability of the occurrence of N_2O with mass 46) produced by different possible pathways compared with measured data of the $^{15}NO_2^-$-labeling experiment under oxic (**b**) and reduced oxygen (**C**; 3% O_2 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.

Hybrid N_2O formation in N. viennensis

Stable isotope-labeling experiments with *N. viennensis* showed a nearly equal contribution of nitrogen from ammonia and nitrite to the N_2O production

at all oxygen levels tested. This was further supported, when we modeled the amount of dually labeled N_2O molecules (^{15}N-^{15}N-O) to determine the different possible pathways that could be used to synthesize N_2O and compared the calculated values to the actual measurements obtained from our experiments with ^{15}N-labeled nitrite under ambient and reduced oxygen concentrations (Figures 3b and c). Thus, *N. viennensis* seems to produce N_2O during aerobic ammonia oxidation, from nitrite and an intermediate of ammonia oxidation mostly via a hybrid formation mechanism. Such a mechanism of N_2O 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 N_2O production in our AOA study.

There are two main N_2O production mechanisms described for bacterial ammonia oxidizers. Under oxic conditions AOB oxidize hydroxylamine by hydroxylamine oxidoreductase to NO, which is further oxidized to N_2O 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 N_2O is produced via the process of nitrifier-denitrification, which is the reduction of nitrite to NO by NIR and a further reduction to N_2O 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 N_2O 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 N_2O (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 N_2O via the yet unidentified second enzyme of ammonia oxidation cannot be excluded. However, we can also not exclude an abiotic formation of hybrid N_2O 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 N_2O 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. ^{15}N-labeling studies performed with *N. viennensis* indicate N_2O 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 N_2O emissions, albeit at low rates, comparable to those of AOB under oxic conditions and low ammonia supply. As AOB might produce more N_2O under higher ammonia concentrations than supplied in our experiments their relative contribution to N_2O emissions in the environment is certainly higher than that of AOA on a per-cell

basis. A bigger contribution to global N_2O production through AOA might occur rather indirectly through the production of oxidized nitrogenous compounds (mostly NO_2^-) that are converted into substrates for denitrifying organisms.

Conflict of Interest

The authors declare no conflict of interest.

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