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Acetylene and oxygen as inhibitors of nitrous oxide production in *Nitrosomonas europaea* and *Nitrosospira briensis*: a cautionary tale

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Abstract

Autotrophic ammonia-oxidizing bacteria produce nitrous oxide (N_2O) as a by-product of nitrification or as an intermediate of nitrifier denitrification. In soil incubations, acetylene (C_2H_2) and large partial pressures of oxygen (O_2) are used to distinguish between these sources. C_2H_2 inhibits ammonia oxidation and should therefore inhibit N_2O production by both nitrification and nitrifier denitrification. O_2 suppresses the reduction pathway of nitrifier denitrification. However, doubts concerning the reliability of C_2H_2 and O_2 as inhibitors have arisen recently. Therefore, in this study we tested the influence of C_2H_2 and large partial pressures of O_2 alone and in combination on N_2O production in pure cultures of the ammonia oxidizers *Nitrosomonas europaea* and *Nitrosospira briensis*. C_2H_2 largely inhibited nitrite production in both ammonia oxidizers and N_2O production by *N. europaea*. Surprisingly, it did not affect the N_2O production in *N. briensis*. The variable response of ammonia oxidizers to C_2H_2 might have consequences for the use of C_2H_2 as an inhibitor of nitrification in soils. Different partial pressures of O_2 ranging from less than 10 kPa O_2 to 100 kPa O_2 were tested for their effectiveness in inhibiting N_2O production via nitrifier denitrification. The partial pressure of 100 kPa O_2 yielded minimal N_2O production by both ammonia-oxidizing species and seemed to inhibit N_2O emission from nitrifier denitrification to a large extent. However, a negative effect of 100 kPa O_2 on ammonia oxidation itself could not be excluded. The applicability of both inhibitors in determining N_2O production pathways in soils is discussed.

Keywords: Nitrous oxide; Acetylene; Oxygen; Ammonia oxidizer; Nitrifier denitrification

1. Introduction

Nitrification is one of the microbial processes leading to production of nitrous oxide (N_2O) in soils. N_2O is an important greenhouse gas with a global warming potential of 320 relative to CO_2 [1]. In the stratosphere, it is converted to nitric oxide (NO), which plays a role in the destruction of the ozone layer [2].

Autotrophic ammonia-oxidizing bacteria produce N_2O as a by-product of nitrification or as an intermediate of nitrifier denitrification. As a by-product of nitrification, N_2O is formed during the spontaneous decomposition of intermediates of ammonia oxidation, e.g. hydroxylamine (NH_2OH), or the end-product nitrous acid, under acid conditions [3]. Furthermore, incomplete oxidation of NH_2OH might lead to the development of N_2O during nitrification [4]. In nitrifier denitrification or autotrophic denitrification, ammonia oxidizers first oxidize ammonia (NH_3) to nitrite (NO_2^-) and subsequently reduce this NO_2^- to molecular nitrogen (N_2) [5–7]. N_2O can be released as an intermediate in the reduction pathway from NO_2^- to N_2 [5,8]. This has been investigated in studies with pure cultures of the ammonia-oxidizing genus *Nitrosomonas* [5,6] as well as with soil [9] and sewage sludge [8].

In addition to ammonia oxidizers, denitrifying bacteria are also involved in the production of gaseous oxidized nitrogen compounds in ecosystems such as soils and in sewage sludge. Incubations with combinations of small amounts of acetylene, C_2H_2 (10 Pa), and large concentra-

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tions of molecular oxygen, O_2 (100 kPa), have been used in soils to differentiate between nitrification, denitrification, nitrifier denitrification and other sources of N_2O production [9–11]. C_2H_2 inhibits ammonia oxidation and should therefore inhibit N_2O production by both nitrification and nitrifier denitrification. O_2 suppresses the reduction pathway of nitrifier denitrification. However, the inhibitors did not always cause the expected reduction in the production of N_2O compared to the controls. Sometimes, the production of N_2O was even larger after addition of the inhibitors [9]. Studies with cell-free extracts of the autotrophic nitrifier *Nitrosomonas eutropha* [12] showed that a complete inhibition of the ammonia monooxygenase of this organism required addition of 80 Pa C_2H_2, which is four to eight times more than is normally used in soil incubations. Furthermore, these studies suggest that ammonia mono-oxygenase is also negatively affected by O_2; a partial inhibition occurred at ambient concentrations of O_2 [12].

With the introduction of molecular techniques, it has become evident that representatives of the genus *Nitrosospira*, and especially of cluster 3, are the dominant ammonia-oxidizing bacteria in fertilized agricultural soils [13]. *Nitrosospira briensis* is a well-known representative of this cluster [14]. Hence, it seemed timely to study the effects of C_2H_2 and large partial O_2 pressures on the production of NO_2^- and N_2O by this species to find a clue for the remarkable results of the soil incubations.

In this study, we investigated the effects of C_2H_2 and large partial pressures of O_2 on NO_2^- and N_2O production by *N. briensis* using *Nitrosomonas europaea* as a control. Combinations of O_2 and C_2H_2 according to the method of Webster and Hopkins [11] were tested. This should show whether the concentrations of C_2H_2 normally applied in soils can inhibit ammonia oxidation and whether C_2H_2 and O_2 interfere when used in combination. In a second experiment, different partial pressures of O_2 (<10, 20, 40, 60, 80, and 100 kPa) were tested for their effectiveness in inhibiting N_2O production by nitrifier denitrification. NO_2^- was measured as an indicator for influences of large partial pressures of O_2 on ammonia oxidation itself.

2. Materials and methods

2.1. Microorganisms

The two ammonia oxidizers used were *N. europaea* ATCC 19178 and *N. briensis* ATCC 25971. The cultures were grown on a mineral medium containing (per liter) 660 mg of $(NH_4)_2SO_4$, 585 mg of NaCl, 49 mg of $MgSO_4 \cdot 7H_2O$, 147 mg of $CaCl_2 \cdot 2H_2O$, 75 mg of KCl, 54 mg of KH_2PO_4, 10 g of HEPES, and 1 ml trace element solution (after [15]). As a pH indicator, 5 ml l^{-1} of a 0.4% (w/v) bromothymol blue stock solution was added. The pH was adjusted to 7.8 with 5 M NaOH before autoclaving (121°C, 30 min). *N. europaea* was grown in this medium for 7 days, *N. briensis* for 14 days, due to a lower specific growth rate. The cells were then in the stationary phase. The cultures were kept at 20°C in the dark. The cultures were not shaken in order to imitate conditions in the soil. The surface–volume ratio in the incubation bottles was large (100 ml culture in 250-ml Erlenmeyer flasks) to allow for a good exchange of gases.

The ammonia oxidizers were harvested by centrifugation ($32\,583 \times g$, 15 min, 4°C) and washed once in fresh medium. They were quantified by measuring the optical density at 436 nm (spectrophotometer model 100-20 by Hitachi Scientific Instruments, Tiel, The Netherlands). The results of this quantification method were consistent with counting after DAPI (4,6-diamidino-2-phenylindole) staining. This was tested with dilutions of 1- and 3-week-old cultures of *N. europaea* and *N. briensis* ($r^2 = 0.99$ for 0 to 3×10^7 *N. europaea* cells per ml, $r^2 = 0.96$ for 0 to 1.5×10^7 *N. briensis* cells per ml).

2.2. General set-up of incubation experiments

To allow comparison we used a general set-up of the incubation experiments that was similar to the commonly used set-up in soil incubations. Serum bottles (250 ml) with 10 ml mineral medium (see above) without KH_2PO_4 were autoclaved (121°C, 30 min). While still hot, they were closed to create negative pressure in the bottles during cooling. Then, the headspace was filled up with the gas that formed the main constituent of the respective incubation atmosphere (see below). When the headspace was filled (recognizable by the movement of the septum and the decrease of gas flow to the bottles), flushing was continued for another 10 s (flux: 1 l min^{-1}) by piercing a second needle through the septum. This flushing method was chosen as a compromise between methods normally used in pure culture studies (e.g. [16–18]) and those used in soil incubation studies (e.g. [9,19]). The incubation atmospheres were completed by adding other gases as appropriate (see below). To avoid contamination with other microorganisms, gases (including flushing) were added via 0.2-μm filters (Schleicher and Schuell, Dassel, Germany). After addition of all gases, the septa of the serum bottles were briefly pierced to make sure that no pressure had built up in the bottles. The harvested and washed microorganisms were added to these prepared bottles using a syringe. The bottles were then incubated at 20°C in the dark. To imitate conditions in soil incubations, the bottles were not shaken. In both experiments, all treatments were carried out in four replicates. Both experiments were repeated three times.

This batch-like set-up was chosen since *N. briensis* cannot yet be grown in continuous culture (Bollmann and Laanbroek, personal communication). Preliminary experiments had shown that due to the small production rate of N_2O, an accumulation over 24 h was necessary. Therefore,

continuous flushing of the headspace of the incubation bottles was not possible.

Gas measurements were carried out after the addition of microorganisms to the bottles and one more time after an additional 24 h. Preliminary experiments had shown that the gas production in the cultures proceeded linearly in this time period (r^2 between 0.97 and 0.99). N_2O was analyzed in 5-ml samples with a gas chromatograph with electron capture detector (PU 4400 Unicam Analytical Systems, Philips, The Netherlands). The system was calibrated with 4.5 μl l^{-1} N_2O in N_2 (Hoek Loos, Schiedam, The Netherlands). The detection limit was 8 nl l^{-1}. Preliminary tests had shown that multiple gas samples up to a total of 40 ml could be taken from 250-ml serum bottles without influencing the linearity of gas measurements.

The culture media were analyzed colorimetrically for nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_4^+) contents [20]. Samples (1.5 ml) of the culture medium were taken from each bottle at the beginning and end of the inhibition experiment. To inhibit microbial activity before these samples could be analyzed, they were centrifuged (27 793 × *g*, 15 min) and the supernatants frozen (−13°C).

2.3. Inhibition experiments

To test the influence of O_2 and/or C_2H_2 alone and in combination, pure cultures of *N. europaea* and *N. briensis* were incubated with 100 kPa O_2 and 0.02 kPa C_2H_2 in four combinations. For a control treatment (C) and a treatment with 0.02 kPa C_2H_2 (A), the incubation bottles were flushed with N_2. O_2 and CO_2 were added to re-establish ambient concentrations. The bottles for treatments with 100 kPa O_2 (O) and with 100 kPa O_2 plus 0.02 kPa C_2H_2 (AO) were flushed with O_2, and CO_2 was added to re-establish ambient concentrations. C_2H_2 was added to treatments A and AO.

To test the influence of elevated partial pressures of O_2 on ammonia oxidizers, pure cultures of *N. europaea* and *N. briensis* were incubated under six different incubation atmospheres. The incubation atmospheres contained 20, 40, 60, 80 or 100 kPa O_2 in N_2. Furthermore, flushing with N_2 created a suboxic treatment with less than 10 kPa O_2. All treatments were supplied with CO_2 to re-establish ambient air concentrations. The given partial pressures of O_2 are only approximate to the conditions in the bottles, due to the flushing method, use of O_2 by ammonia oxidizers during the incubation, and diffusion constraints of gases in liquids.

2.4. Statistics

All experiments were set up using four replicates for each treatment. Changes in gas concentration and mineral N concentrations were calculated for every bottle by subtracting the amount measured at time 0 from that measured after 24 h. Results are presented as means with standard deviations ($n = 4$). Since the standard deviations appeared very large, each experiment was repeated three times. These repetitions were also analyzed statistically. Treatment effects within each repetition and between repetitions were analyzed statistically with SPSS for Windows [21] and with Excel. Gaussian distribution of the data was tested with the Kolmogorov–Smirnov test. When the data was not distributed normally, the Kruskal–Wallis test was used to find differences between treatments. In case of significant effects ($\alpha = 0.05$), Schaich–Hamerle analysis was used for multiple comparison of means. When the data followed a normal distribution, homogeneity of variance was tested with Levene's test. Comparisons between treatments were then done using analysis of variance (ANOVA) ($\alpha = 0.05$) with subsequent LSD test as post-hoc test. When variances were not homogeneous, the data was transformed before ANOVA. In most cases, ANOVA was used to compare means between replicates within each repetition of the experiments, while the Kruskal–Wallis test was used to compare means between repetitions.

3. Results

3.1. Influence of combinations of O_2 and C_2H_2

The two ammonia oxidizers, *N. europaea* and *N. briensis*, produced comparable amounts of N_2O in the control treatments (Table 1). The production tended to be somewhat larger (not significantly) by *N. europaea* than by *N. briensis*. The two ammonia oxidizers reacted differently to the inhibitors. The production of N_2O by *N. europaea* was clearly influenced by all added inhibitors (Table 1). In the pooled data of the three repetitions, N_2O production was significantly smaller in the treatments with inhibitors present (A, O, and AO) than in the control treatments ($P \leq 0.001$, Kruskal–Wallis test). In treatments A, O, and AO, the amount of N_2O produced was often close to or below the detection limit. On average, the N_2O production in treatment A was only 30% of that in the controls. In contrast, *N. briensis* did not react to C_2H_2 addition with a significant decrease in N_2O production (Table 1). The production in treatment A was on average still 92% of that in the controls. In treatments O and AO, the N_2O production by *N. briensis* was significantly decreased compared to the controls ($P \leq 0.001$, Kruskal–Wallis test). The production in these treatments was again below or not significantly different from the detection limit.

The pattern of NO_2^- production was comparable for *N. europaea* and *N. briensis* (Table 1), but the amounts of NO_2^- produced by *N. briensis* were at the lower end of the range observed for *N. europaea*. Both ammonia-oxidizing organisms produced similar amounts of NO_2^- in treatments C and O. In the presence of C_2H_2, NO_2^-

Table 1
Amounts of N_2O and NO_2^- produced by *N. europaea* and *N. briensis* in incubations with C_2H_2 and O_2

Repetition	N_2O (fmol cell^{-1} 24 h^{-1})						NO_2^- (pmol cell^{-1} 24 h^{-1})					
	N. europaea						*N. europaea*					
	C	A	O	AO	St	R.e.	C	A	O	AO	St	R.e.
1	128 ± 69^{a}	39 ± 2^{ab}	30 ± 10^{ab}	30 ± 2^{b}	K	a	18.5 ± 13.8^{a}	$1.1 \pm 0.2^{*ab}$	7.7 ± 2.7^{a}	$1.1 \pm 4.5^{*b}$	A	a
2	60 ± 38^{a}	30 ± 6^{a}	$23 \pm 23^{*ab}$	$8 \pm 4^{*b}$	A	ab	1.7 ± 10.2^{a}	$0.5 \pm 0.3^{*a}$	6.4 ± 4.2^{a}	$-0.1 \pm 0.2^{*a}$	A	a
3	188 ± 123^{a}	$16 \pm 15^{*ab}$	$-5 \pm 32^{*ab}$	$-16 \pm 4^{*b}$	K	b	32.0 ± 25.5^{a}	$0.6 \pm 0.1^{*a}$	13.3 ± 9.0^{a}	$0.4 \pm 0.2^{*a}$	K	a
Treatment effect	a	b	b	b	K		a	ab	a	b	K	
	N. briensis						*N. briensis*					
1	89 ± 29^{a}	89 ± 20^{a}	$20 \pm 36^{*b}$	$22 \pm 25^{*b}$	A	a	2.8 ± 2.6^{a}	$0.2 \pm 1.7^{*a}$	2.3 ± 1.8^{a}	$1.4 \pm 0.3^{*a}$	A	a
2	33 ± 10^{a}	32 ± 7^{a}	$5 \pm 3^{*b}$	$9 \pm 5^{*b}$	A	a	10.4 ± 4.4^{a}	$0.2 \pm 0.2^{*b}$	7.7 ± 1.4^{a}	$0.5 \pm 0.1^{*b}$	A	a
3	68 ± 6^{a}	53 ± 5^{a}	$24 \pm 18^{*b}$	$21 \pm 9^{*b}$	A	a	5.7 ± 5.8^{a}	$0.8 \pm 0.2^{*a}$	5.7 ± 5.8^{a}	$0.9 \pm 0.1^{*a}$	K	a
Treatment effect	a	a	b	b	K		a	b	a	ab	K	

The experiment was repeated three times with each ammonia oxidizer. Shown are means and standard deviations ($n = 4$) of every repetition of the experiment. Results of the statistical analyses are shown as superscript letters for each repetition ($\alpha = 0.05$). The applied statistical test is shown in column 'St': A: ANOVA, K: Kruskal–Wallis test. Different letters in the columns under heading 'R.e.' (= repetition effects) show differences between repetitions, those in the rows 'Treatment effect' show differences between treatments for pooled data from three repetitions. C: control; A: with 0.02 kPa C_2H_2; O: with 100 kPa O_2; and AO: with both 0.02 kPa C_2H_2 and 100 kPa O_2.
Asterisks indicate values that were below or not significantly different from the detection limit. Differences in the detection limit between repetitions are due to variable numbers of cells in the experiment and the expression of the values per cell here.

production was significantly reduced. On average, C_2H_2 reduced the NO_2^- production of *N. europaea* by 88% and that of *N. briensis* by 92%. NO_3^- could not be detected. Only very little NH_4^+ was consumed during the incubation period, so that changes in NH_4^+ concentration could not be measured against the large background concentrations (10 mM). The ratio of N_2O:NO_2^- produced by *N. europaea* and by *N. briensis* in the controls and in the C_2H_2 treatments shifted from 1.6% to 4.1% and from 1.6% to 22.4%, respectively.

3.2. Influence of elevated partial pressures of O_2

The amounts of N_2O produced at different partial pressures of O_2 were comparable to those in the experiment with different inhibitors described above. Higher levels of O_2 significantly decreased N_2O production by both *N. europaea* and *N. briensis* ($P \leq 0.001$, Kruskal–Wallis test; Table 2). While 80 kPa O_2 almost totally inhibited N_2O production in *N. europaea*, *N. briensis* still produced some N_2O in incubations with 100 kPa O_2. Same as

Table 2
Amounts of N_2O and NO_2^- produced by *N. europaea* and *N. briensis* in incubations with different partial pressures of O_2 (< 10, 20, 40, 60, 80, and 100 kPa O_2)

Repetition	N_2O (fmol cell^{-1} 24 h^{-1})								NO_2^- (pmol cell^{-1} 24 h^{-1})							
	N. europaea								*N. europaea*							
	< 10	20	40	60	80	100	St	R.e.	< 10	20	40	60	80	100	St	R.e.
1	123 ± 64^{a}	88 ± 12^{a}	53 ± 47^{ab}	$23 \pm 18^{*b}$	$6 \pm 6^{*c}$	$0 \pm 0^{*c}$	A	a	19 ± 30^{a}	50 ± 9^{a}	31 ± 33^{a}	18 ± 9^{a}	17 ± 5^{a}	8 ± 9^{a}	A	a
2	36 ± 12^{a}	42 ± 42^{a}	30 ± 6^{ab}	18 ± 6^{bc}	$6 \pm 0^{*c}$	$0 \pm 0^{*d}$	A	a	1 ± 2^{a}	6 ± 10^{a}	1 ± 8^{a}	3 ± 1^{a}	$1 \pm 1^{*a}$	2 ± 1^{a}	A	b
3	96 ± 40^{a}	116 ± 52^{a}	66 ± 35^{ab}	$28 \pm 11^{*bc}$	$17 \pm 9^{*cd}$	$8 \pm 11^{*d}$	A	a	14 ± 17^{a}	28 ± 14^{a}	13 ± 8^{a}	3 ± 10^{a}	9 ± 4^{a}	7 ± 5^{a}	A	a
Treatment effect	a	a	ab	abc	bc	c	K		a	a	a	a	a	a	K	
	N. briensis								*N. briensis*							
1	123 ± 14^{a}	96 ± 31^{a}	103 ± 26^{a}	47 ± 6^{b}	53 ± 20^{b}	36 ± 6^{b}	A	a	5 ± 3^{a}	3 ± 3^{a}	7 ± 4^{a}	4 ± 3^{a}	3 ± 2^{a}	4 ± 1^{a}	A	a
2	92 ± 10^{a}	57 ± 7^{bc}	70 ± 14^{b}	59 ± 3^{bc}	56 ± 7^{c}	54 ± 5^{c}	A	a	7 ± 7^{a}	5 ± 3^{a}	9 ± 8^{a}	4 ± 14^{a}	12 ± 5^{a}	$1 \pm 7^{*a}$	A	a
3	74 ± 32^{abc}	50 ± 8^{ab}	44 ± 2^{a}	$31 \pm 3^{*bc}$	30 ± 2^{bc}	$24 \pm 5^{*c}$	A	b	3 ± 3^{a}	7 ± 2^{a}	5 ± 3^{a}	6 ± 1^{a}	8 ± 2^{a}	4 ± 2^{a}	A	a
Treatment effect	a	ab	ab	b	b	b	K		a	a	a	a	a	a	A	

The experiment was repeated three times with each ammonia oxidizer. Shown are means and standard deviations ($n = 4$) of every repetition. Results of the statistical analyses are shown as superscript letters for each repetition ($\alpha = 0.05$). The applied statistical test is shown in column 'St': A: ANOVA, K: Kruskal–Wallis test. Different letters in the columns 'R.e.' (= repetition effects) show differences between repetitions, those in the rows 'Treatment effect' show differences between treatments for pooled data from the three repetitions.
Asterisks indicate values that were below or not significantly different from the detection limit. Differences in the detection limit between repetitions are due to variable numbers of cells in the experiment and the expression of the values per cell here.

above, changes in NH_4^+ concentrations could not be measured due to its large background concentration. NO_3^- was not detected either. Increased O_2 concentrations were apparently tied in with a decrease in NO_2^- production by *N. europaea* (Table 2), but this trend was not statistically significant. No trend could be found either for *N. briensis*, where the production of NO_2^- was small (Table 2). Generally, *N. briensis* and *N. europaea* produced similar amounts of N_2O per NO_2^- produced ($1.33 \pm 0.72\%$ for *N. briensis* as opposed to $0.98 \pm 1.06\%$ for *N. europaea*). No significant trend in this percentage could be found with increasing O_2 concentrations.

4. Discussion

This study was carried out to investigate the influence of C_2H_2 and large partial pressures of O_2 on pure cultures of ammonia oxidizers. The most important finding was that addition of C_2H_2 inhibited N_2O production to a large extent in *N. europaea*, but had only a minor effect on *N. briensis*. No N_2O was produced in bottles incubated without ammonia oxidizers. Therefore, we can exclude purely chemical sources for the production of N_2O in experiments with *N. briensis*. In experiments with ammonia oxidizers, small amounts of NO_2^- were still produced in the presence of C_2H_2. We assume that this ongoing ammonia oxidation made possible the production of N_2O. Since the N_2O production was significantly decreased in incubations with both C_2H_2 and large partial pressures of O_2, we suppose that nitrifier denitrification was suppressed under this condition, but might have contributed to N_2O production in the incubations with C_2H_2 under ambient O_2 conditions. Under the influence of C_2H_2, *N. briensis* produced more N_2O per NO_2^- formed than *N. europaea*. We therefore conclude that *N. briensis* is less sensitive to C_2H_2 as an inhibitor of ammonia oxidation. This is the first study where the effect of C_2H_2 on N_2O production by *N. briensis* is tested. Ammonia oxidation by *N. briensis* isolated from a Finnish ombrotrophic peat soil, measured as NO_2^- production, was successfully inhibited by 1 kPa C_2H_2 [22]. Unfortunately, the authors did not measure the N_2O production in that study. To see whether larger concentrations of C_2H_2 could inhibit N_2O production by *N. briensis*, we tested C_2H_2 concentrations up to 1 kPa. To date, a consistent inhibition of N_2O production by *N. briensis* at these larger C_2H_2 concentrations could not be measured (unpublished data).

Insensitivity of *N. briensis* to C_2H_2 in terms of N_2O production would have severe implications for the use of C_2H_2 as an inhibitor of N_2O production by ammonia oxidizers in soils. *Nitrosospira* species are the most common ammonia oxidizers found in most soils [13]. It needs to be investigated whether insensitivity to C_2H_2 is more widespread in this group of ammonia oxidizers. Problems with the inhibition of ammonia oxidation have also been reported for *N. eutropha* [12]. Insensitivity of a number of ammonia oxidizers to C_2H_2 might be the reason for observed problems with C_2H_2 inhibition of N_2O production by nitrifiers in soil (e.g. [9]). Most authors regard N_2O produced after addition of small concentrations of C_2H_2 as derived from denitrification and the reciprocal, inhibited part as that from nitrification. If C_2H_2 does not totally inhibit N_2O production by all nitrifiers, the contribution of nitrifiers to N_2O production might have been underestimated.

Large partial pressures of O_2 inhibited N_2O production in both ammonia oxidizers studied. The inhibition of N_2O production was almost complete in *N. europaea* at 80 kPa O_2, while *N. briensis* still produced some N_2O at 100 kPa O_2. We conclude that partial pressures of O_2 less than 100 kPa are probably not sufficient for inhibiting nitrifier denitrification in soils. It is almost impossible to establish such large concentrations of O_2 in soil incubations. Even after extensive flushing, there may still be microhabitats with smaller O_2 concentrations inside aggregates. These problems might be more serious in wet soils and undisturbed soil columns than in dry and crumbled soil. Furthermore, such large partial pressures of O_2 might have negative effects on ammonia oxidation itself and on other soil processes. In cell-free extracts, a sensitivity of ammonia mono-oxygenase to ambient concentrations of O_2 was found [12]. In our study, the NO_2^- production did not differ significantly between treatments with different O_2 concentrations. However, at O_2 concentrations larger than ambient, *N. europaea* tended to produce less NO_2^-, suggesting that ammonia oxidation in this organism was negatively affected by large O_2 concentrations. The NO_2^- production in *N. briensis* was not influenced by different partial pressures of O_2. Thus, the ammonia oxidation in this organism does not seem to have been negatively affected by large concentrations of O_2.

The variation between replicates and between repetitions was large, although all experiments were carried out in a similar way and under semi-controlled conditions. The microorganisms were always grown in the same way under constant conditions. However, the numbers of microorganisms used for the experiments varied between 5 and 17×10^6 cells l^{-1}. Replicate cultures might have been different with respect to the time needed to start full N_2O production. Due to methodological constraints, gas measurements could only be carried out twice during the incubation time. If N_2O production did not in all cases proceed linearly between these measurements, this will have contributed to large variability. However, the trends observed were the same in the three repetitions of the experiments and the amounts of N_2O and NO_2^- produced did not differ significantly between the repetitions of the experiments in most cases.

We conclude that O_2 acted as an inhibitor of nitrifier denitrification in both ammonia oxidizers studied. Since O_2 concentrations of 100 kPa seem necessary to inhibit

production of N_2O via nitrifier denitrification, the applicability of this method to soils may be limited. Furthermore, side effects of large concentrations of O_2 on ammonia oxidation and on other soil processes cannot be excluded. C_2H_2 effectively inhibited N_2O production in *N. europaea*, but not in *N. briensis*. NO_2^- production was largely inhibited by C_2H_2 in both *N. europaea* and *N. briensis*. Screening of different ammonia oxidizers for their sensitivity to C_2H_2 is needed to clarify whether insensitivity is a more common phenomenon in ammonia oxidizers with respect to N_2O production.

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