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Abiotic conversion of extracellular NH$_2$OH contributes to N$_2$O emission during ammonia oxidation

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Abstract

Abiotic processes involving the reactive ammonia-oxidation intermediates nitric oxide (NO) or hydroxylamine (NH$_2$OH) for N$_2$O production have been indicated recently. The latter process would require the availability of substantial amounts of free NH$_2$OH for chemical reactions during ammonia (NH$_3$) oxidation, but little is known about extracellular NH$_2$OH formation by the different clades of ammonia-oxidizing microbes. Here we determined extracellular NH$_2$OH concentrations in culture media of several ammonia-oxidizing bacteria (AOB) and archaea (AOA), as well as one complete ammonia oxidizer (comammox) enrichment (Ca. Nitrospira inopinata) during incubation under standard cultivation conditions. NH$_2$OH was measurable in the incubation media of *Nitrosomonas europaea*, *Nitrosospira multiformis*, *Nitrososphaera gargensis*, and *Ca. Nitrosothenus uzonensis*, but not in media of the other tested AOB and AOA. NH$_2$OH was also formed by the comammox enrichment during NH$_3$ oxidation. This enrichment exhibited the largest NH$_2$OH:final product ratio (1.92%), followed by *N. multiformis* (0.56%) and *N. gargensis* (0.46%). The maximum proportions of NH$_4^+$ converted to N$_2$O via extracellular NH$_2$OH during incubation, estimated on the basis of NH$_2$OH abiotic conversion rates, were 0.12%, 0.08% and 0.14% for AOB, AOA and *Ca. Nitrospira inopinata*, respectively, and were consistent with published NH$_4^+$:N$_2$O conversion ratios for AOB and AOA.

Key words: hydroxylamine; greenhouse gas; biotic-abiotic; N$_2$O formation mechanism; ammonia oxidizer; reactive N, comammox

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1. Introduction

Nitrous oxide (N\textsubscript{2}O) is an important greenhouse gas and is currently the third largest contributor to global warming, after carbon dioxide (CO\textsubscript{2}) and methane (CH\textsubscript{4}). N\textsubscript{2}O also has deleterious effects in the stratosphere, where it is split photolytically and catalyzes the destruction of atmospheric ozone \textsuperscript{1}. In the past two centuries, the atmospheric N\textsubscript{2}O concentration has increased by about 20\% from pre-industrial levels of 270 ppbv to the current level of 324 ppbv \textsuperscript{2}. In addition to denitrification and dissimilatory nitrate reduction to ammonia, aerobic ammonia (NH\textsubscript{3}) oxidation contributes significantly to N\textsubscript{2}O production in soil \textsuperscript{3}. Traditionally, two different biochemical routes are proposed for N\textsubscript{2}O production during NH\textsubscript{3} oxidation in AOB. The first is the oxidation of hydroxylamine (NH\textsubscript{2}OH) to nitric oxide (NO) by hydroxylamine dehydrogenase (HAO) and subsequent reduction to N\textsubscript{2}O catalyzed by NO reductase \textsuperscript{4}. The second pathway is the so-called nitrifier-denitrification, by which nitrite (NO\textsubscript{2}\textsuperscript{-}) is reduced to NO and N\textsubscript{2}O by nitrite reductase (NIR) and NO reductase (NOR), respectively \textsuperscript{4,6}. However, recent studies revealed two other routes for the N\textsubscript{2}O production from the AOB \textit{N. europaea} under anaerobic conditions. One is the direct oxidation of NH\textsubscript{2}OH to N\textsubscript{2}O by the enzyme cytochrome (cyt) P460 \textsuperscript{7}, and nitrification intermediate NO \textsuperscript{8}. Nitrifier-denitrification has been suggested to play a crucial role in N\textsubscript{2}O formation at low O\textsubscript{2} and low pH \textsuperscript{9}, whereas pathways related to biological or chemical reactions of ammonia oxidation intermediates (NH\textsubscript{2}OH, nitroxyH (HNO), NO) and/or its product (NO\textsubscript{2}\textsuperscript{-}) may be more important for N\textsubscript{2}O production at high ammonium (NH\textsubscript{4}\textsuperscript{+}) levels and sufficient O\textsubscript{2} supply \textsuperscript{10}. However, not all AOB share the same route for N\textsubscript{2}O production. \textit{N. communis}, for example, has no homologues of genes encoding a canonical copper-containing NirK \textsuperscript{11}. Thus, it is unlikely to be able to conduct canonical nitrifier-denitrification, even though low production of N\textsubscript{2}O has been detected in a \textit{N. communis} culture \textsuperscript{12}. Most studies on AOB
N₂O production pathways have focused on *N. europaea* ATCC 19718⁴,⁵,¹³, and different biochemical routes responsible for N₂O production in other AOB cannot be excluded.

In recent years, ammonia oxidation-related N₂O production by several AOA strains has been reported¹⁴-¹⁶ and AOA abundance exceeds that of AOB by several orders of magnitude in some ecosystems¹⁷,¹⁸. However, the mechanism(s) of N₂O production by AOA appear to differ from that of AOB, as AOA lack genes encoding a canonical HAO and NOR, which are involved in N₂O production by AOB¹⁹-²¹. Recent research showed that the soil AOA *Nitrososphaera viennensis* is indeed not able to generate N₂O through nitrifier-denitrification¹⁵. Instead, for this organism hybrid N₂O formation from NH₄⁺ and NO₂⁻ was demonstrated in¹⁵N-labeling experiments¹⁵, indicating a N₂O production pathway from NO₂⁻ and an intermediate of ammonia oxidation, e.g. NH₂OH or NO. Recently, it was confirmed that N₂O formation by this AOA under anoxic conditions results from the abiotic reaction of NO with medium or cellular components²². However, the mechanism of N₂O production by AOA under oxic conditions remains unclear. Furthermore, complete bacterial nitrifiers (comammox) of the genus *Nitrospira* that perform ammonia oxidation via NO₂⁻ to nitrate (NO₃⁻) have recently been enriched²³,²⁴, but nothing is yet known about N₂O production by these microorganisms.

Hydroxylamine has long been known as an important intermediate of chemolithoautotrophic AOB²⁵ and was reported to be an intermediate of the marine AOA *Nitrosopumilus maritimus*²⁶. Surprisingly, genes homologous to those encoding HAO in AOB have not been found in AOA genomes²⁰,²¹, indicating that AOA either encode a novel enzyme for NH₂OH oxidation or form during NH₃ oxidation an initial oxidation product other than NH₂OH, e.g. HNO²¹. Recent research showed that NO₂⁻ can be formed after addition of NH₂OH in *N. viennensis*, leading to the proposal of a novel enzymatic mechanism for the production of NO₂⁻ involving NH₂OH and NO in AOA²².

Hydroxylamine may play a crucial role in N₂O production from soils under oxic conditions²⁷-³⁰, as indicated by the close relationship between NH₂OH concentration and N₂O formation observed in forest soil²⁹,³⁰. Further support for this hypothesis comes from the intramolecular distribution of¹⁵N within the linear, asymmetric NNO molecule, the so-called¹⁵N site preference (SP)³¹, which is distinctly different between N₂O produced via denitrification and nitrification³². In pure cultures of
different nitrifiers and denitrifiers, Sutka et al. \textsuperscript{33} found SP values near 0‰ for N\textsubscript{2}O formed by NO\textsuperscript{2−} and NO\textsuperscript{3−} reduction (via classical denitrification and nitrifier denitrification), while SP values were 30.8–35.6‰ for N\textsubscript{2}O produced during aerobic NH\textsubscript{3} and NH\textsubscript{2}OH oxidation, which is similar to SP values reported by Heil et al. \textsuperscript{34} for N\textsubscript{2}O produced by chemical reactions of NH\textsubscript{2}OH with Fe\textsuperscript{3+}, Cu\textsuperscript{2+} and NO\textsubscript{2−}. Santoro et al. \textsuperscript{16} also reported an SP value of ~30‰ for N\textsubscript{2}O produced by an enrichment culture of a marine AOA, although soil AOA showed different SP values with a range of 13-30‰. \textsuperscript{14} Recently, Soler-Jofra et al. \textsuperscript{35} observed a significant contribution of the abiotic reaction between NH\textsubscript{2}OH and NO\textsubscript{2−} to N\textsubscript{2}O formation in a full-scale nitrification reactor. All these findings indicate that chemical reactions involving NH\textsubscript{2}OH may play an important role in N\textsubscript{2}O production during chemolithoautotrophic NH\textsubscript{3} oxidation. However, this would require the availability of free NH\textsubscript{2}OH, either in the growth medium or, potentially, in the periplasm, for abiotic N\textsubscript{2}O formation through chemical reactions with substances such as NO\textsubscript{2−}, MnO\textsubscript{2} and Fe\textsuperscript{3+}. Quantitative data on extracellular NH\textsubscript{2}OH production by AOB, AOA and comammox are therefore urgently required in order to better estimate the importance of coupled biotic–abiotic N\textsubscript{2}O production during microbial NH\textsubscript{3} oxidation.

In this study, we aimed to answer several important questions regarding N\textsubscript{2}O formation by ammonia oxidizing microbes: (1) What are the extracellular concentrations of NH\textsubscript{2}OH during NH\textsubscript{3} oxidation by different ammonia oxidizers? (2) If these concentrations are significant, what is the NH\textsubscript{2}OH:final product ratio for AOB, AOA, and comammox? (3) Can we estimate the contribution of extracellular NH\textsubscript{2}OH to abiotic N\textsubscript{2}O production during NH\textsubscript{3} oxidation? (4) What is the role of NO\textsubscript{2−} in stabilizing NH\textsubscript{2}OH and in abiotic conversion of NH\textsubscript{2}OH to N\textsubscript{2}O? To address these questions, temporal changes in NH\textsubscript{2}OH concentration were determined during incubation of pure and enriched cultures of chemolithoautotrophic AOB, AOA and comammox (obtained from soil and aquatic environments) at two NH\textsubscript{4+} concentrations, 2 mM and 0.5 mM. These experiments were complemented by measurement of abiotic NH\textsubscript{2}OH decay rates and abiotic N\textsubscript{2}O production involving NH\textsubscript{2}OH in different media and at different incubation temperatures and NO\textsubscript{2−} concentrations. These analyses were performed to calculate extracellular NH\textsubscript{2}OH production ratios on a final product basis, to quantify the coupled biotic–abiotic NH\textsubscript{4+}–NH\textsubscript{2}OH–N\textsubscript{2}O conversion rate of AOB, AOA and comammox, and to explore the role of NO\textsubscript{2−} in the abiotic NH\textsubscript{4+}–NH\textsubscript{2}OH–N\textsubscript{2}O conversion. We hypothesize that the coupled biotic–
abiotic N\textsubscript{2}O production is an important mechanism of N\textsubscript{2}O production during NH\textsubscript{4}\textsuperscript{+} oxidation, at least in some ammonia oxidizers.

2. Materials and methods

2.1 Strains and cultivation

This study involved four AOB (\textit{Nitrosomonas europaea} ATCC 19718, \textit{Nitrosospira multiformis} ATCC 25196, \textit{Nitrosomonas nitrosa} Nm90, \textit{Nitrosomonas communis} Nm2), three AOA (\textit{Nitrososphaera gargensis}, \textit{Nitrososphaera viennensis} and Ca. Nitrosotalea sp. Nd2), one AOA enrichment (Ca. Nitrosotenuis uzonensis) and one comammox enrichment (Ca. Nitrospira inopinata). \textit{N. europaea}, \textit{N. multiformis}, \textit{N. communis}, \textit{N. viennensis} and Ca. N. sp. Nd2 were isolated from soil \textsuperscript{6}, \textsuperscript{20, 36, 37}; \textit{N. nitrosa} Nm90 was isolated from industrial sewage \textsuperscript{36}; \textit{N. gargensis} and Ca. N. uzonensis were isolated from thermal springs \textsuperscript{38, 39}; Ca. N. inopinata was enriched from a hot water outflow of a deep oil exploration well \textsuperscript{23}.

\textit{N. europaea} and \textit{N. multiformis} were maintained at 30°C in modified Skinner and Walker (S&W) medium \textsuperscript{40}, containing 0.2 g KH\textsubscript{2}PO\textsubscript{4}, 0.04 g CaCl\textsubscript{2}:2 H\textsubscript{2}O, 0.04 g MgSO\textsubscript{4}:7 H\textsubscript{2}O, 1 ml FeNaEDTA (7.5 mM), 1 ml phenol red (0.05%) as pH indicator, 10 ml l\textsuperscript{-1} HEPES buffer (1 M HEPES, 0.6 M NaOH) and 4 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} L\textsuperscript{-1}. The pH was regularly adjusted to 7.7 by addition of sterilized 5% (w/v) Na\textsubscript{2}CO\textsubscript{3}. The acidophilic AOA Ca. N. sp. Nd2 and the AOA \textit{N. viennensis} were maintained in freshwater medium at 35 and 37°C, respectively, according to Tourna et al. \textsuperscript{20}. The pH for the Ca. N. sp. Nd2 was adjusted to 5.0-5.3 by HCl and the NH\textsubscript{4}\textsuperscript{+} concentration was kept at 0.5 mM by routinely adding NH\textsubscript{4}Cl stock solution. The pH for \textit{N. viennensis} was adjusted to 7.5 by addition of 10 ml l\textsuperscript{-1} HEPES buffer (1 M HEPES, 0.6 M NaOH). \textit{N. viennensis} was supplied with 1 mM NH\textsubscript{4}Cl and 0.1 mM pyruvate. The AOB \textit{N. nitrosa} and \textit{N. communis}, the AOA \textit{N. gargensis}, and the enrichments containing Ca. N. uzonensis and Ca. N. inopinata were maintained at 37, 28, 46, 46 and 37°C, respectively, in AOA medium modified from Lebedeva et al. \textsuperscript{38} containing (L\textsuperscript{-1}) 75 mg KCl, 50 mg KH\textsubscript{2}PO\textsubscript{4}, 584 mg NaCl, 50 mg MgSO\textsubscript{4}:7 H\textsubscript{2}O, 1 ml of trace element solution (AOA-TES), 1 ml of...
selenium-tungsten solution (SWS), 4 g CaCO$_3$ (mostly undissolved, acting as a solid buffer reservoir and growth surface) and 5 ml of NH$_4$Cl (from an autoclaved 0.2 M stock solution). For a detailed description of the composition of TES and SWS please refer to Widdel $^{41}$.

2.2 Incubation experiments

Metabolically active cultures were concentrated and washed twice using fresh medium without NH$_4^+$ by centrifugation (Table S1), and resuspended in fresh medium containing 0.5 or 2 mM NH$_4^+$. Note that the added NH$_4^+$ concentrations were not optimal for all strains tested, but use of the same concentrations for all strains maximized comparability of the chemical factors contributing to N$_2$O formation in the various growth media. Ca. N. sp. Nd2 was incubated with 0.5 mM NH$_4^+$ only, as this culture grew extremely slowly and is inhibited by high nitrous acid concentration formed under acidic conditions. Cultures were incubated under different conditions and for different periods depending on their different growth characteristics (Table S1). All treatments were carried out with 4-6 replicates. Only *N. communis* (90 rpm, New Brunswick™ Innova® 42 Shaker) and *N. nitrosa* (90 rpm, GFL 3019 shaker) cultures were shaken during incubation. Before each sampling, bottles of all cultures were mixed by shaking by hand. Samples (3 ml) for chemical and protein analyses were taken at 0, 2, 5, 8 and 13 h on the first day, and thereafter every 12 or 24 h, and transferred to 2-ml and 1.5-ml autoclaved Eppendorf tubes, respectively. The tubes were centrifuged immediately at 8000 g (4°C) for 10 min, and 1.2 ml of supernatant was transferred to two 1.5 ml Eppendorf tubes containing 75 µl 480 mM (for 2 mM NH$_4^+$ treatment) or 160 mM (for 0.5 mM NH$_4^+$ treatment) sulfanilamide in 0.8 M HCl for quantification of NH$_2$OH (see below). Another 0.2 ml supernatant was transferred to a 1.5-ml Eppendorf tube for NH$_4^+$ and NO$_2^-$ analyses (see below) and the remaining liquid and pellet were frozen at -20°C for protein quantification (see below). To prevent any potential effect of phenol red on NH$_2$OH analysis, *N. europaea* and *N. multiformis* were grown in parallel in media buffered with HEPES without and with phenol red to facilitate maintenance of pH between pH 7.5 and 8 by addition of sterilized 5% (w/v) Na$_2$CO$_3$. Ca. N. sp. Nd2 cultures were not buffered and pH was determined daily by pH measurement of 2-ml samples. For cultures buffered with CaCO$_3$, pH was stable at ~8.2 throughout the incubation period.
2.3 Determination of abiotic NH$_2$OH decay rates under ambient air conditions

Abiotic NH$_2$OH decay was quantified in S&W (with HEPES buffer) and modified AOA (with CaCO$_3$ buffer) media used in this study at the respective growth temperatures. The freshwater medium for Ca. N. sp. Nd2 and N. viennensis was not tested for abiotic NH$_2$OH decay since no extracellular NH$_2$OH was observed during NH$_3$ oxidation by these cultures. Well-aerated medium (40 ml) was added to 120-ml glass serum bottles followed by different amounts (4, 8, 20 and 40 µl) of 5 mM NH$_2$OH to reach final concentrations of 0.5, 1, 2.5 and 5 µM, respectively. Subsequently, 1.6 ml 50 mM NO$_2^-$ was added to give a final concentration of 2 mM to simulate abiotic NH$_2$OH decay in the presence of NO$_2^-$. Bottles were then capped with aluminum foil and incubated at 30, 37 and 46°C. Samples (1.2 ml) were taken after 0, 1, 2, 5 and 8 h and transferred to 1.5-ml Eppendorf tubes containing 75 µl 480 mM (for 2 mM NO$_2^-$ treatment) or 160 mM (for the treatment without NO$_2^-$ addition) sulfanilamide in 0.8 M HCl. Samples were frozen at -20°C until quantification of NH$_2$OH (see below).

2.4 Chemical assays

Hydroxylamine concentration was determined according to the method of Liu et al. $^{30}$ Briefly, 1.2 ml of sample, thawed at room temperature, was transferred to a 22-ml glass vial and 4.8 ml deionized water was added, yielding a pH of ~2. Then, 0.6 ml of 25 mM FeCl$_3$ was added to the vial, which was immediately closed gas-tight with a crimping tool. Control vials contained sample and water only to assess N$_2$O in the headspace and dissolved in the sample. The vials were shaken for 3 h at 200 rpm and then transferred to an autosampler for N$_2$O analysis by a gas chromatograph (GC) with an electron capture detector (ECD) as described in Liu et al. $^{30}$ NH$_2$OH calibration in the range 0–1 µM was performed before each measurement. Since N$_2$O background increased by about 10 ppb in the control vials for the culture samples of N. communis and N. nitrosa during NH$_2$OH determination, NH$_2$OH concentrations <0.06 µM were defined as not detectable. NO$_2^-$ and NH$_4^+$ concentrations were determined colorimetrically in 96-well plates using sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride for NO$_2^-$ $^{42}$, and the indophenol method described by Kandeler and Gerber $^{43}$ for NH$_4^+$. Protein concentration was determined with the Pierce BCA protein assay kit (Thermo Fisher Scientific).
2.5 Calculation of the NH$_2$OH:final product ratio

Total extracellular NH$_2$OH concentrations by AOB, AOA and comammox during consumption of available NH$_4^+$ was evaluated as the NH$_2$OH:final product ratios (final product was NO$_3^-$ in the case of comammox and NO$_2^-$ in all other cases), taking into account the abiotic decay rate of the very reactive NH$_2$OH, which followed first-order reaction kinetics:

$$C = C_0 e^{-kt}$$  \hspace{1cm} (1)

where $C$ is the NH$_2$OH concentration (µM) at decay time $t$ (h), $C_0$ is the initial NH$_2$OH concentration (µM) and $k$ is the first-order rate constant.

The NH$_2$OH:final product ratio was calculated as:

$$r = \frac{[C_{t2} - C_{t1} + \sum_{i=1}^{t2-t1} C_i (1-e^{-k t})]}{C_{t2} - C_{t1}'}$$  \hspace{1cm} (2)

where $r$ (dimensionless) is the NH$_2$OH:final product ratio between $t_1$ and $t_2$, $C_i$ (µM) is the interpolated NH$_2$OH concentration between times $t_1$ and $t_2$, respectively, $C_{i1}$ (µM) and $C_{i2}$ (µM) are the NO$_2^-$ or (for comammox) NO$_3^-$ concentrations at $t_1$ and $t_2$, and $k$ is the average value of the measured kinetic constant for abiotic NH$_2$OH decay in the range of 0.5–2.5 (for HEPES buffered medium) or 0.5–5 (for CaCO$_3$ buffered medium) µM initial NH$_2$OH concentrations. Note that the presence of NO$_2^-$ in the medium would also decrease $k$. As $k$ was determined in the absence or presence of 2 mM NO$_2^-$, loss of NH$_2$OH was calculated using an average value of $k$ determined at 0 or 2 mM NO$_2^-$, concentration in the medium was <1 mM or >1 mM, respectively. As NO$_2^-$ concentration increased gradually with time, this definition of $k$ would have led to overestimation or underestimation of NH$_2$OH when NO$_2^-$ concentration was <1 mM or >1 mM, respectively. However, the total NH$_2$OH:final product ratio was very likely underestimated since higher NH$_2$OH concentration was detected during late growth when NO$_2^-$ concentration was mostly >1 mM. For the comammox, NO$_2^-$ concentration was low (<0.033 mM) at all time points and had negligible effect on calculation of NH$_2$OH:final product ratio.
2.6 Calculation of the fraction of NH$_4^+$ converted to N$_2$O during incubation

The fraction of NH$_4^+$ converted to N$_2$O through incubation was calculated by determining the overall abiotic N$_2$O product ratios ($r_i$ in equation 3) at different NH$_2$OH and NO$_3^-$ concentrations for different media and incubation temperatures. For this, 1.2 ml of HEPES and CaCO$_3$ medium, respectively, was added to 22-ml glass vials, followed by 0, 12 and 24 µl of 100 mM NO$_3^-$ and 12, 24 and 60 µl of 50 µM NH$_2$OH. The final NO$_3^-$ concentrations were 0, 1 and 2 mM and final NH$_2$OH concentrations were 0.5, 1 and 2.5 µM. Vials were then incubated for 24 h at 30, 37 and 46°C according to the cultivation conditions of the respective microorganisms and headspace gas was analyzed for N$_2$O by GC. The fraction of NH$_4^+$ converted to N$_2$O over the whole NH$_3$ oxidation process ($R$) was then calculated as follows:

$$ R = \frac{\sum_{i=1}^{n} C_i r_i}{C} $$

(3)

where $C_i$ is the concentration of NH$_2$OH during the $i^{th}$ and $(i+1)^{th}$ sampling, $r_i$ is the theoretical abiotic N$_2$O production ratio determined as described in section 2.6, and $C$ is the concentration of NH$_4^+$ consumed during incubation. Note that $r_i$ was strongly dependent on NO$_3^-$ concentration. Abiotic N$_2$O production within a certain time period when NO$_3^-$ concentration was <1 mM, 1–1.5 mM and >1.5 mM was calculated using $r_i$ values for NO$_3^-$ concentrations of 0, 1 and 2 mM, respectively. As $r_i$ increased with increasing NO$_3^-$ concentration, this definition of $r_i$ may have led to underestimation or overestimation of abiotic N$_2$O production when NO$_3^-$ concentration was < or >1.5 mM, respectively.

2.7 Data analyses

Abiotic NH$_2$OH decay was fitted to first-order reaction equations by the R software package (version 3.1.0). The coefficients of determination (R$^2$) were larger than 0.99. Paired t-tests (R, version 3.1.0) were used to identify significant differences in NH$_2$OH concentrations between two time points during culture incubation.
3. Results and discussion

3.1 Extracellular NH$_2$OH from autotrophic ammonia oxidizers

The NH$_2$OH concentration in the medium during NH$_3$ oxidation differed significantly among AOB cultures (Fig. 1) and was highest for *N. multiformis* on initial NH$_4^+$ concentrations of 0.5 and 2 mM. NH$_2$OH release was also observed for *N. europaea*, albeit at lower concentrations than for *N. multiformis*. No NH$_2$OH was detectable for *N. nitrosa* Nm90 or *N. communis* at both tested NH$_4^+$ concentrations. Initial increases in NH$_2$OH concentration in cultures of *N. multiformis* and *N. europaea* were associated with increases in NO$_2^-$ concentration, but eventually reached a plateau or decreased before NO$_2^-$ concentration reached a maximum. The largest measured NH$_2$OH concentrations in the medium were 2.2 and 0.78 µM, from *N. multiformis* and *N. europaea*, respectively, during incubation with 2 mM NH$_4^+$. 

Several studies have determined NH$_2$OH concentrations in the medium during NH$_3$ oxidation by pure cultures of the AOB *N. europaea*. Stüven et al. $^{44}$ observed 0.2–1.7 µM NH$_2$OH during NH$_3$ oxidation (10 mM) and Yu and Chandran $^{13}$ reported 0.2–3.2 µM NH$_2$OH during growth of *N. europaea* 19718 on 20 mM NH$_4^+$. These findings are consistent with the NH$_2$OH concentrations detected for *N. europaea* in our study, where NH$_2$OH concentrations were about three orders of magnitude smaller than those of the produced NO$_2^-$, although they did not specify whether they measured NH$_2$OH in supernatant (as in our study) or in untreated cultures. In our experiments, *N. multiformis* NH$_2$OH concentrations were even larger than for *N. europaea*. The exact reason for this phenomenon remains unclear. One possible explanation is that *N. multiformis* biomass consumed NH$_4^+$ faster (for the 0.5 mM NH$_4^+$ treatment) than *N. europaea*, and faster NH$_3$ oxidation might have led to higher NH$_2$OH release. However, the *N. communis* biomass in the batch experiments showed no detectable NH$_2$OH release into the medium even though it had the highest NH$_3$ oxidation rates. Although *N. communis* prefers higher concentrations of NH$_4^+$ (10–50 mM) $^{45}$, the absence of NH$_2$OH could be due to complete consumption by HAO and conversion to NO$_2^-$, assuming that HAO activity in *N. communis* is larger than in other AOB. Moreover, *N. communis* is unable to tolerate >100 µM NH$_2$OH in contrast to tolerance of 250 µM NH$_2$OH by *N. europaea* and *N. multiformis* $^{12}$, which may relate to the absence
of NH$_3$OH in the medium of *N. communis*, although the exact mechanism for the low tolerance of NH$_3$OH by *N. communis* is still not clear. NH$_3$ oxidation by *N. nitrosa* Nm90 was lower than by the other tested AOB strains, possibly explaining the lack of detectable NH$_3$OH release.

Among the three AOA pure cultures, NH$_3$OH release was detected from the thermal spring isolate *N. gargensis* growing on 2 mM initial NH$_4^+$, but not on 0.5 mM NH$_4^+$ (Fig. 2). The pattern of NH$_3$OH release by *N. gargensis* differed from that of AOB, with a small but rather constant increase in NH$_3$OH during incubation on 2 mM NH$_4^+$, resulting in a final NH$_3$OH concentration of 0.33 µM in the medium after 58 hours. In contrast, NH$_3$ oxidation by the soil AOA *N. viennensis* and *Ca. N. sp. Nd2* was not associated with detectable NH$_3$OH release (Fig. 2). The NO$_2^-$ production rate by the AOA enrichment *N. uzonensis* (~0.3 mM NO$_2^-$ produced within 104 hours) was similar at the two initial NH$_4^+$ concentrations, but more NH$_3$OH (0.34 µM) was observed at the end of the incubation at 2 than 0.5 mM NH$_4^+$ initial concentration.

No published AOA genome contains an obvious homologue of the HAO of AOB, or of cytochromes c554 and c$_m$552 that are considered critical for energy conversion $^{21}$, initially casting some doubt on the role of NH$_3$OH as an intermediate in NO$_2^-$ formation by AOA $^{21}$. However, Vajrala et al. $^{26}$ reported the production of NH$_3$OH in the marine AOA *N. maritimus* during NH$_3$ oxidation. Furthermore, Kozlowski et al. $^{22}$ showed that the addition of NH$_3$OH to a culture of *N. viennensis* resulted in respiration and NO$_2^-$ formation and thus the most current model of AOA physiology postulates a yet undiscovered novel hydroxylamine-converting enzyme $^{21}$. The data from the *N. uzonensis* enrichment culture, that does not contain any known AOB $^{38}$, confirms the *N. gargensis* data in showing that some AOA release NH$_3$OH. Also, in a preliminary experiment, *N. gargensis* could convert NH$_3$OH to NO$_2^-$ biotically, especially at lower NH$_3$OH levels (Experiment S1, Fig. S1). Stieglmeier et al. $^{15}$ observed aerobic N$_2$O production by *N. viennensis* and attributed this to hybrid formation of N$_2$O via an N-nitrosating reaction. Kozlowski et al. $^{22}$ later reported that N$_2$O formation from *N. viennensis* could be attributed to abiotic reactions between NO and medium substances during growth, especially under anoxic conditions. It is tempting to speculate that the aerobic hybrid formation of N$_2$O in *N. viennensis* could also stem from the well-known chemical reaction between...
NH$_2$OH and NO$_3^-$). However, we failed to observe NH$_2$OH in the medium of *N. viennensis*, which could reflect (i) lack of NH$_2$OH release by this culture (indicating that the coupling between AMO and the archaeal NH$_2$OH-converting enzyme is more efficient than in some AOB) or (ii) rapid chemical NH$_2$OH conversion in the medium (which could mask small amounts of released NH$_2$OH), as the medium response of *N. viennensis* was different from that of *N. gargensis* in terms of the nitrogenous gas production from abiotic NH$_2$OH decay (Experiment S2, Fig. S2). Also for Ca. N. sp. Nd2, NH$_2$OH was not detectable, possibly due to low NH$_3$ oxidation rates.

The comammox organism Ca. N. inopinata oxidized NH$_4^+$ to NO$_3^-$ (Fig. 3). After 48 h of incubation, Ca. N. inopinata produced 0.46 mM NO$_3^-$ with 2 mM initial NH$_4^+$ concentration, while it produced 0.27 mM NO$_3^-$ when fed with 0.5 mM NH$_4^+$. Release of the NH$_2$OH into the medium by Ca. N. inopinata was similar for both NH$_4^+$ levels, but unlike the other cultures, increasing mainly at the beginning of the incubation, decreasing and then increasing again in parallel with increasing NO$_3^-$ concentration to reach 0.43 µM at the end of the incubation period. This decreasing and increasing trend was significant (*P* < 0.025) for the culture growing on 2 mM NH$_4^+$ initial concentration.

Consistent with the detection of NH$_2$OH, previous genomic analysis had shown that Ca. N. inopinata encodes a predicted octaheme cytochrome c protein resembling the HAO of AOB, cytochromes c554 and c$_m$552, and an AMO that is relatively closely related to the AMO of the betaproteobacterial AOB 23. Ca. N. inopinata lacks canonical NO reductases but encodes enzymes for dissimilatory nitrate reduction to ammonia 46. Whether the latter enzymes are also expressed and active under aerobic conditions and might contribute to N$_2$O formation has not yet been investigated.

### 3.2 NH$_2$OH abiotic decay and NH$_2$OH:final product ratios during NH$_3$ oxidation

To better understand the presence of extracellular NH$_2$OH during ammonia oxidation of the tested organisms, a series of NH$_2$OH abiotic decay and formation experiments were conducted with different media, incubation temperatures and NO$_3^-$ concentrations (Fig. 4). All three factors, i.e., medium type, temperature and NO$_3^-$ concentration, had strong effects on the rate of abiotic NH$_2$OH decay. The decay
rate was faster in CaCO$_3$ than in HEPES-buffered media: 0.5 to 2.5 µM NH$_2$OH decayed abiotically at 30°C within ~8 h and ~30 h in the CaCO$_3$ and HEPES-buffered media, respectively. Consequently, the first-order rate constants for abiotic NH$_2$OH decay were much higher in the CaCO$_3$ than in the HEPES-buffered media, with an average value approximately fourfold larger in the former (0.71 vs. 0.16) (Table S2). Temperature increased the rate of abiotic NH$_2$OH decay (with a single exception, see Table S2). The decay time at 46°C (~4 h) was half that at 30°C (~8 h) for the CaCO$_3$ medium, and the average first-order rate constant was ~80% greater at 46°C (1.31) than at 30°C (0.71). Nitrite, however, unexpectedly inhibited abiotic NH$_2$OH decay in both media tested (Figure 4, Table S2), although NO$_2^-$ is known to oxidize NH$_2$OH to N$_2$O, albeit preferentially at low pH (e.g., Heil et al., 2014). This stabilizing effect of NO$_2^-$ was particularly pronounced at higher temperatures for the CaCO$_3$ medium, where the first-order rate constant decreased by 52% for 2 mM NO$_2^-$ at 46°C compared to the absence of NO$_2^-$.

To exclude the possibility of abiotic conversion of NO$_2^-$ to NH$_2$OH by components of the medium, an additional test was conducted using the more active CaCO$_3$-buffered medium (compared to the HEPES-buffered medium) at the highest culture incubation temperature, but no abiotic conversion of NO$_2^-$ to NH$_2$OH occurred (Experiment S3). An additional $^{15}$N-NO$_2^-$ experiment showed that NO$_2^-$ did not interfere with the NH$_2$OH analysis (Experiment S4, Table S3). Under alkaline conditions, one product of NH$_2$OH abiotic decay is NO$_2^-$. This, which has been also observed in abiotic NH$_2$OH decay experiments in CaCO$_3$-buffered medium in this study (Experiment S5, Fig. S3). In addition to NO$_2^-$ and N$_2$O, nitrogen dioxide (NO$_2$), but almost no NO, was observed during the abiotic NH$_2$OH decay (Fig. S2). The presence of NO$_2$ may explain the observation of abiotic NH$_2$OH-to-NO$_2^-$ conversion as NO$_2$ is highly reactive and can hydrolyze to nitric acid (HNO$_3$) and nitrous acid (HNO$_2$) in aqueous solution. Consequently, NO$_2^-$, N$_2$O and NO$_2$ comprised approximately 18.5%, 9.8% and 32.1%, respectively, of the abiotically decayed NH$_2$OH in the CaCO$_3$-buffered medium (Fig. S2, S3). Therefore, a possible reason for the inhibitory effect of NO$_2^-$ on the abiotic NH$_2$OH decay could be that the presence of NO$_2^-$ slowed down the transformation of NH$_2$OH to NO$_2^-$ by inhibiting the disproportionation of NO$_2$, one of the primary decay products of NH$_2$OH, to HNO$_3$ and HNO$_2$.

The effect of temperature on abiotic NH$_2$OH decay was as expected, as NH$_2$OH is extremely unstable and reactive, especially at higher temperatures. The exact reason for the difference of abiotic...
NH$_2$OH decay between the two media (HEPES- and CaCO$_3$-buffered) is not obvious. The media differ
mainly in terms of pH, the composition and concentrations of the trace metals and the buffer (HEPES
vs. CaCO$_3$). Both pH and redox active trace metals are known to have a strong effect on abiotic
NH$_2$OH decay. Acidic pH stabilizes NH$_2$OH in the absence of redox active trace metals, while trace
metals such as Cu$^{2+}$, Fe$^{3+}$ and Mn$^{4+}$ can stimulate NH$_2$OH decomposition.$^{47}$ Therefore, higher pH and
the presence of trace metals could lead to greater abiotic NH$_2$OH decay in the CaCO$_3$-buffered
medium than in HEPES-buffered medium.

First-order kinetic rate constants and Equation 2 were used to estimate both instantaneous and total
NH$_2$OH:final product ratios during NH$_3$ oxidation by those cultures producing relatively high NH$_2$OH
concentrations, i.e. *N. europaea*, *N. multiformis*, *N. gargensis* and Ca. *N. inopinata* (Fig. S4 and Table
1). For the three pure cultures (*N. europaea*, *N. multiformis* and *N. gargensis*), instantaneous
NH$_2$OH:final product ratios were in the range 0.1 to 0.6% during early phases of the incubation
experiments, but several-fold higher as the substrate NH$_4^+$ was nearly consumed, e.g., as high as about
4% for *N. multiformis* (Fig. S4). For the comammox organism Ca. *N. inopinata*, instantaneous
NH$_2$OH:final product ratios were in the range 0.1 to 2.6% and 0.9 to 5.7% at 0.5 and 2 mM initial
NH$_4^+$ concentration, respectively, also with higher values at the end of incubation (Fig. S4). Generally,
Ca. *N. inopinata* had the largest total NH$_2$OH:final product ratio of all cultures tested, with ratios of
0.63% and 1.92% after incubation for 60 h at 0.5 and 2 mM initial NH$_4^+$ concentration, respectively
(Table 1). In contrast, *N. gargensis* had a total NH$_2$OH:NO$_2^-$ ratio of 0.46% at 2 mM initial NH$_4^+
concentration after 60 h, whereas *N. multiformis* and *N. europaea* had total NH$_2$OH:final product
ratios of 0.34–0.56% and 0.24–0.33%, respectively, depending on the initial NH$_4^+$ concentration.

3.3 Estimating the fraction of NH$_4^+$ converted to N$_2$O during NH$_3$ oxidation under ambient air
conditions

For an informed estimate of the fraction of NH$_4^+$ that was converted to N$_2$O by the different ammonia
oxidizers under ambient air conditions over the whole incubation period, it is essential to consider
abiotic N$_2$O production from different NH$_2$OH concentrations, at different incubation temperatures,
and at different concentrations of NO$_3^-$ In the environment, additional factors such as organic matter
content, pH and content of suitable oxidants like MnO$_2$ and Fe$^{3+}$ will also affect the chemical N$_2$O conversion ratio from NH$_2$OH$^{27, 29}$. The abiotic N$_2$O:NH$_2$OH conversion ratio was 12–14% for the HEPES-buffered medium at 30°C in the absence of NO$_2^-$, and between 18% and 37% for the same medium with 1 and 2 mM NO$_2^-$, respectively (Table 2). The ratio in CaCO$_3$-buffered medium at 30°C was larger, with values of 15–28%, 32.2–46.9%, and 37.6–48.9% at 0, 1 and 2 mM NO$_2^-$, respectively, for the NH$_2$OH concentration range from 0.5 to 2.5 µM. The contribution of NO$_2^-$ to N$_2$O production involving NH$_2$OH was even larger at higher temperature, e.g. 46°C (Table 2). The stimulated conversion of NH$_2$OH to N$_2$O by NO$_2^-$ was likely caused by the hybrid reaction of NO$_2^-$ and NH$_2$OH. However, another mechanism could be inhibition of NH$_2$OH conversion to NO$_2$/NO$_2^-$ by NO$_2^-$, thereby channeling NH$_2$OH to N$_2$O indirectly via other mechanisms.

The total fraction of NH$_4^+$ converted to N$_2$O through extracellular NH$_2$OH and substances in the medium over the whole incubation period was then calculated according to Equation 3 (Table 3). The total fraction of NH$_4^+$ converted to N$_2$O by this mechanism was 0.05% and 0.12% for *N. multiformis* incubated at 0.5 and 2 mM initial NH$_4^+$, respectively, which is consistent with that emitted as N$_2$O (0.05–0.1%) during aerobic incubation of a *Nitrosospira* strain$^{6, 48}$. The fraction of NH$_4^+$ converted to N$_2$O by *N. europaea* was lower than that of *N. multiformis*, but still consistent with that converted to N$_2$O by *N. europaea* reported by other studies, e.g., 0.05-1.95%$^{49}$ and 0.05-0.15%$^{50}$. Dundee and Hopkins$^{51}$ also reported that *N. multiformis* produced more N$_2$O than *N. europaea* at greater dissolved O$_2$ concentrations, while *N. europaea* produced much more N$_2$O during nitrifier-denitrification than *N. multiformis*, which is consistent with our finding that the fraction of NH$_4^+$ converted to N$_2$O was larger for *N. multiformis* than for *N. europaea* under ambient air conditions.

The AOA *N. viennensis* and *N. maritimus* are reported to be incapable of canonical nitrifier-denitrification at reduced O$_2$ concentration, but produce N$_2$O via hybrid formation, as revealed by $^{15}$N-labeling$^{15}$. In the present study, potential abiotic N$_2$O production was approximately 0.08% of the total substrate turnover during aerobic NH$_3$ oxidation by AOA. Albeit this value was found only in *N. gargensis*, it was close to the values reported for *N. viennensis* (0.09%) and *N. maritimus* (0.05%) by Stieglmeier et al.$^{15}$ The calculated fraction of NH$_4^+$ to be converted to N$_2$O by the comammox
organism *Ca. N. inopinata* was even higher (in the range of 0.06–0.14%), but no measured data on N\textsubscript{2}O emissions from comammox organisms are yet available for comparison.

In summary, we show that extracellular NH\textsubscript{2}OH is formed in growth media during aerobic NH\textsubscript{3} oxidation in batch incubations by AOB, AOA and comammox cultures, but with large differences between the different organisms and incubation conditions. The calculated fraction of NH\textsubscript{4}\textsuperscript{+} converted to N\textsubscript{2}O by abiotic reactions between extracellular NH\textsubscript{2}OH and substances in the growth medium during aerobic NH\textsubscript{3} oxidation, was in the range of values reported previously for the conversion of substrate to N\textsubscript{2}O for various AOB and AOA. The presence of NO\textsubscript{2}\textsuperscript{-} in the medium not only offers a reactant for hybrid N\textsubscript{2}O formation from NH\textsubscript{2}OH, but also delays overall NH\textsubscript{2}OH abiotic decay, further stimulating the conversion of NH\textsubscript{2}OH to N\textsubscript{2}O. In view of the new results presented here and in recent studies\textsuperscript{15, 22, 52-54}, it is tempting to speculate that at least for some strains extracellular NH\textsubscript{2}OH might contribute to aerobic ammonia-oxidizer-associated N\textsubscript{2}O formation. In others, e.g. *N. viennensis*, no extracellular NH\textsubscript{2}OH was observed during NH\textsubscript{3} oxidation but aerobic N\textsubscript{2}O production has been reported\textsuperscript{15}, indicating a different mechanism, e.g. the abiotic reactions between intracellular NH\textsubscript{2}OH and periplasmic substances.

**Acknowledgements**

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Supporting Information

Centrifugation and incubation conditions for the ammonia-oxidizing strains tested; first-order rate constant (k) of abiotic NH$_2$OH decay in different media at different NH$_2$OH (0.5, 1, 2.5 and 5 µM) and NO$_2^-$ (0 and 2 mM) concentrations; biotic (N. gargensis) and abiotic conversion of NH$_2$OH (30 or 80 µM) to NO$_2^-$; N$_2$O and NOx emissions from CaCO$_3$-buffered medium (A) and fresh water medium (FWM) (B) after addition of 0.08 mM NH$_2$OH; test for the abiotic conversion of NO$_2^-$ to NH$_2$OH in the growth medium; $^{15}$N-NO$_2^-$ labeling experiment to quantify the effect of NO$_2^-$ on the NH$_2$OH assay; abiotic conversion of NH$_2$OH to NO$_2^-$ in CaCO$_3$ medium at different NH$_2$OH concentrations (0.03, 0.08 and 0.2 mM) and two temperatures (37 and 46°C); NH$_2$OH:final product ratios (%) during incubation at two different initial NH$_4^+$ concentrations (0.5 mM, square; 2 mM, circle) for four different cultures of ammonia-oxidizers.
References


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(36) Koops, H. P.; Böttcher, B.; Möller, U. C.; Pomerening-Röser, A.; Stehr, G., Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. and *Nitrosomonas halophila* sp. nov. *Microbiology* 1991, 137, (7), 1689-1699.


Table 1  Total NH$_2$OH:final product (NO$_2^-$ or NO$_3^-$) ratios after incubation for 58 h for different ammonia oxidizers. § For Ca. N. inopinata (a comammox organism), NO$_3^-$ is the final product of NH$_3$ oxidation. a The NH$_2$OH concentration here is the total extracellular NH$_2$OH including the calculated concentration of NH$_2$OH that was abiotically converted during incubation.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Initial NH$_4^+$ concentration (mM)</th>
<th>Final NO$_2^-$ or NO$_3^-$ concentration (µM)</th>
<th>NH$_2$OH concentration (µM)</th>
<th>NH$_2$OH:final product ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. multiformis</td>
<td>0.5</td>
<td>516</td>
<td>1.8</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1955</td>
<td>11.0</td>
<td>0.56</td>
</tr>
<tr>
<td>N. europaea</td>
<td>0.5</td>
<td>537</td>
<td>1.8</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1930</td>
<td>4.7</td>
<td>0.24</td>
</tr>
<tr>
<td>N. gargensis</td>
<td>2</td>
<td>1860</td>
<td>7.1</td>
<td>0.46</td>
</tr>
<tr>
<td>Ca. N. inopinata</td>
<td>0.5</td>
<td>280</td>
<td>1.8</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>490</td>
<td>9.4</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Table 2  Fraction (%) of N$_2$O abiotically produced from the added NH$_2$OH in the different media at various levels of NH$_2$OH (0.5, 1 and 2.5 µM) and NO$_2^-$ (0, 1 and 2 mM).

<table>
<thead>
<tr>
<th>NH$_2$OH (µM)</th>
<th>0 mM NO$_2^-$</th>
<th>1 mM NO$_2^-$</th>
<th>2 mM NO$_2^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (30°C)</td>
<td>14.1 13.7 12.0</td>
<td>29.3 20.0 18.4</td>
<td>36.6 33.1 23.4</td>
</tr>
<tr>
<td>CaCO$_3$ (30°C)</td>
<td>15.0 20.9 28.0</td>
<td>33.2 32.2 46.9</td>
<td>45.0 37.6 48.9</td>
</tr>
<tr>
<td>CaCO$_3$ (37°C)</td>
<td>6.7 5.6 6.7</td>
<td>36.2 31.0 43.7</td>
<td></td>
</tr>
<tr>
<td>CaCO$_3$ (46°C)</td>
<td>6.3 4.6 12.5</td>
<td>29.5 22.4 36.1</td>
<td>38.8 46.0 57.1</td>
</tr>
</tbody>
</table>
Table 3 Estimated fraction of NH$_4^+$ converted to N$_2$O from the abiotic reactions between the biologically produced extracellular NH$_2$OH and substances in the medium for different ammonia oxidizers.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Initial NH$_4^+$ concentration (µM)</th>
<th>Estimated fraction of NH$_4^+$ converted to N$_2$O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. multiformis</em></td>
<td>500</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0.12</td>
</tr>
<tr>
<td><em>N. europaea</em></td>
<td>500</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0.07</td>
</tr>
<tr>
<td><em>N. gargensis</em></td>
<td>2000</td>
<td>0.08</td>
</tr>
<tr>
<td><em>Ca. N. inopinata</em></td>
<td>500</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>0.14</td>
</tr>
</tbody>
</table>
**Figure captions**

**Figure 1** Dynamics of NH$_4^+$ (red squares), NO$_2^-$ (yellow circles), NH$_2$OH (blue triangles) and total N (sum of NO$_2^-$ and NH$_4^+$, black diamonds) concentrations during incubation of four ammonia-oxidizing bacteria. NH$_4^+$, NO$_2^-$ and total N are plotted using the left y-axis, while NH$_2$OH is plotted using the right y-axis. Please note that the left y-axes and the x-axes, respectively, are not always scaled identically to improve data presentation. The values are presented as mean ± standard error (SE).

**Figure 2** Dynamics of NH$_4^+$ (red squares), NO$_2^-$ (yellow circles), NH$_2$OH (blue triangles) and total N (sum of NO$_2^-$ and NH$_4^+$, black diamonds) concentrations in the batch experiments with four ammonia-oxidizing archaea. NH$_4^+$, NO$_2^-$ and total N are plotted using the left y-axis, while NH$_2$OH is plotted using the right y-axis. Please note that the left y-axes and the x-axes, respectively, are not always scaled identically to improve data presentation. The values are present as mean ± standard error (SE).

**Figure 3** Dynamics of NH$_4^+$ (red squares), NO$_3^-$ (yellow circles), NH$_2$OH (blue triangles) and total N (sum of NO$_3^-$ and NH$_4^+$, black diamonds) concentrations during the incubation of the comammox organism Ca. N. inopinata. NH$_4^+$, NO$_3^-$ and total N are plotted using the left y-axis, while NH$_2$OH is plotted using the right y-axis. The values are present as mean ± standard error (SE).

**Figure 4** Abiotic decay of NH$_2$OH in the absence (open symbols) or presence (closed symbols) of 2 mM NO$_2^-$ in HEPES-buffered and CaCO$_3$-buffered media at different incubation temperatures. The NH$_2$OH concentrations were 0.5 (square), 1 (circle), 2.5 (triangle), and 5 (diamond) µM. Mean values of three replicates are presented. The relative standard deviation (RSD) of all data is smaller than 10%. Please note that the x-axes are not always scaled identically to improve data presentation.
Figures

Figure 1
Figure 2
Figure 3
Figure 4