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Abiotic conversion of extracellular NH_2OH contributes to N_2O emission during ammonia oxidation

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Abstract

Abiotic processes involving the reactive ammonia-oxidation intermediates nitric oxide (NO) or hydroxylamine (NH_2OH) for N_2O production have been indicated recently. The latter process would require the availability of substantial amounts of free NH_2OH for chemical reactions during ammonia (NH_3) oxidation, but little is known about extracellular NH_2OH formation by the different clades of ammonia-oxidizing microbes. Here we determined extracellular NH_2OH 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_2OH was measurable in the incubation media of *Nitrosomonas europaea, Nitrosospira multiformis, Nitrososphaera gargensis,* and *Ca.* Nitrosotenuis uzonensis, but not in media of the other tested AOB and AOA. NH_2OH was also formed by the comammox enrichment during NH_3 oxidation. This enrichment exhibited the largest NH_2OH: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_2O via extracellular NH_2OH during incubation, estimated on the basis of NH_2OH 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_2O conversion ratios for AOB and AOA.

Key words: hydroxylamine; greenhouse gas; biotic-abiotic; N_2O formation mechanism; ammonia oxidizer; reactive N, comammox

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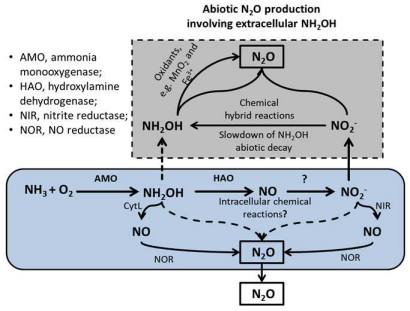

41 • Please note that the schematic cell drawing includes the periplasm

42 **1. Introduction**

43 Nitrous oxide (N_2O) is an important greenhouse gas and is currently the third largest contributor to
44 global warming, after carbon dioxide (CO_2) and methane (CH_4). N_2O also has deleterious effects in the
45 stratosphere, where it is split photolytically and catalyzes the destruction of atmospheric ozone [1]. In
46 the past two centuries, the atmospheric N_2O concentration has increased by about 20% from pre-
47 industrial levels of 270 ppbv to the current level of 324 ppbv [2]. In addition to denitrification and
48 dissimilatory nitrate reduction to ammonia, aerobic ammonia (NH_3) oxidation contributes significantly
49 to N_2O production in soil [3]. Traditionally, two different biochemical routes are proposed for N_2O
50 production during NH_3 oxidation in AOB. The first is the oxidation of hydroxylamine (NH_2OH) to
51 nitric oxide (NO) by hydroxylamine dehydrogenase (HAO) and subsequent reduction to N_2O
52 catalyzed by NO reductase [4]. The second pathway is the so-called nitrifier-denitrification, by which
53 nitrite (NO_2^-) is reduced to NO and N_2O by nitrite reductase (NIR) and NO reductase (NOR),
54 respectively [4-6]. However, recent studies revealed two other routes for the N_2O production from the
55 AOB *N. europaea* under anaerobic conditions. One is the direct oxidation of NH_2OH to N_2O by the
56 enzyme cytochrome (cyt) P460 [7], and nitrification intermediate NO [8]. Nitrifier-denitrification has
57 been suggested to play a crucial role in N_2O formation at low O_2 and low pH [9], whereas pathways
58 related to biological or chemical reactions of ammonia oxidation intermediates (NH_2OH, nitroxyl
59 (HNO), NO) and/or its product (NO_2^-) may be more important for N_2O production at high ammonium
60 (NH_4^+) levels and sufficient O_2 supply [10]. However, not all AOB share the same route for N_2O
61 production. *N. communis*, for example, has no homologues of genes encoding a canonical copper-
62 containing NirK [11]. Thus, it is unlikely to be able to conduct canonical nitrifier-denitrification, even
63 though low production of N_2O has been detected in a *N. communis* culture [12]. Most studies on AOB

64 N_2O production pathways have focused on *N. europaea* ATCC 19718 [4, 5, 13], and different biochemical
65 routes responsible for N_2O production in other AOB cannot be excluded.

66 In recent years, ammonia oxidation-related N_2O production by several AOA strains has been reported
67 [14-16] and AOA abundance exceeds that of AOB by several orders of magnitude in some ecosystems [17,
68 18]. However, the mechanism(s) of N_2O production by AOA appear to differ from that of AOB, as
69 AOA lack genes encoding a canonical HAO and NOR, which are involved in N_2O production by AOB
70 [19-21]. Recent research showed that the soil AOA *Nitrososphaera viennensis* is indeed not able to
71 generate N_2O through nitrifier-denitrification [15]. Instead, for this organism hybrid N_2O formation from
72 NH_4^+ and NO_2^- was demonstrated in ^{15}N-labeling experiments [15], indicating a N_2O production pathway
73 from NO_2^- and an intermediate of ammonia oxidation, e.g. NH_2OH or NO. Recently, it was confirmed
74 that N_2O formation by this AOA under anoxic conditions results from the abiotic reaction of NO with
75 medium or cellular components [22]. However, the mechanism of N_2O production by AOA under oxic
76 conditions remains unclear. Furthermore, complete bacterial nitrifiers (comammox) of the genus
77 *Nitrospira* that perform ammonia oxidation via NO_2^- to nitrate (NO_3^-) have recently been enriched [23, 24],
78 but nothing is yet known about N_2O production by these microorganisms.

79 Hydroxylamine has long been known as an important intermediate of chemolithoautotrophic AOB [25]
80 and was reported to be an intermediate of the marine AOA *Nitrosopumilus maritimus* [26]. Surprisingly,
81 genes homologous to those encoding HAO in AOB have not been found in AOA genomes [20, 21],
82 indicating that AOA either encode a novel enzyme for NH_2OH oxidation or form during NH_3
83 oxidation an initial oxidation product other than NH_2OH, e.g. HNO [21]. Recent research showed that
84 NO_2^- can be formed after addition of NH_2OH in *N. viennensis*, leading to the proposal of a novel
85 enzymatic mechanism for the production of NO_2^- involving NH_2OH and NO in AOA [22].

86 Hydroxylamine may play a crucial role in N_2O production from soils under oxic conditions [27-30], as
87 indicated by the close relationship between NH_2OH concentration and N_2O formation observed in
88 forest soil [29, 30]. Further support for this hypothesis comes from the intramolecular distribution of ^{15}N
89 within the linear, asymmetric NNO molecule, the so-called ^{15}N site preference (SP) [31], which is
90 distinctly different between N_2O produced via denitrification and nitrification [32]. In pure cultures of

different nitrifiers and denitrifiers, Sutka et al. [33] found SP values near 0‰ for N_2O formed by NO_2^- and NO_3^- reduction (via classical denitrification and nitrifier denitrification), while SP values were 30.8–35.6‰ for N_2O produced during aerobic NH_3 and NH_2OH oxidation, which is similar to SP values reported by Heil et al. [34] for N_2O produced by chemical reactions of NH_2OH with Fe^{3+}, Cu^{2+} and NO_2^-. Santoro et al. [16] also reported an SP value of ~30‰ for N_2O produced by an enrichment culture of a marine AOA, although soil AOA showed different SP values with a range of 13-30‰ [14]. Recently, Soler-Jofra et al. [35] observed a significant contribution of the abiotic reaction between NH_2OH and NO_2^- to N_2O formation in a full-scale nitrification reactor. All these findings indicate that chemical reactions involving NH_2OH may play an important role in N_2O production during chemolithoautotrophic NH_3 oxidation. However, this would require the availability of free NH_2OH, either in the growth medium or, potentially, in the periplasm, for abiotic N_2O formation through chemical reactions with substances such as NO_2^-, MnO_2 and Fe^{3+}. Quantitative data on extracellular NH_2OH production by AOB, AOA and comammox are therefore urgently required in order to better estimate the importance of coupled biotic–abiotic N_2O production during microbial NH_3 oxidation.

In this study, we aimed to answer several important questions regarding N_2O formation by ammonia oxidizing microbes: (1) What are the extracellular concentrations of NH_2OH during NH_3 oxidation by different ammonia oxidizers? (2) If these concentrations are significant, what is the NH_2OH:final product ratio for AOB, AOA, and comammox? (3) Can we estimate the contribution of extracellular NH_2OH to abiotic N_2O production during NH_3 oxidation? (4) What is the role of NO_2^- in stabilizing NH_2OH and in abiotic conversion of NH_2OH to N_2O? To address these questions, temporal changes in NH_2OH 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_4^+ concentrations, 2 mM and 0.5 mM. These experiments were complemented by measurement of abiotic NH_2OH decay rates and abiotic N_2O production involving NH_2OH in different media and at different incubation temperatures and NO_2^- concentrations. These analyses were performed to calculate extracellular NH_2OH production ratios on a final product basis, to quantify the coupled biotic–abiotic NH_4^+–NH_2OH–N_2O conversion rate of AOB, AOA and comammox, and to explore the role of NO_2^- in the abiotic NH_4^+–NH_2OH–N_2O conversion. We hypothesize that the coupled biotic–

119 abiotic N_2O production is an important mechanism of N_2O production during NH_4^+ oxidation, at least
120 in some ammonia oxidizers.

121

122 **2. Materials and methods**

123 *2.1 Strains and cultivation*

124 This study involved four AOB (*Nitrosomonas europaea* ATCC 19718*, Nitrosospira multiformis*
125 ATCC 25196*, Nitrosomonas nitrosa* Nm90*, Nitrosomonas communis* Nm2), three AOA
126 (*Nitrososphaera gargensis, Nitrososphaera viennensis* and *Ca.* Nitrosotalea sp. Nd2), one AOA
127 enrichment (*Ca.* Nitrosotenuis uzonensis) and one comammox enrichment (*Ca.* Nitrospira inopinata).
128 *N. europaea*, *N. multiformis*, *N. communis*, *N. viennensis* and *Ca.* N. sp. Nd2 were isolated from soil [6,
129 20, 36, 37]; *N. nitrosa* Nm90 was isolated from industrial sewage [36]; *N. gargensis* and *Ca.* N. uzonensis
130 were isolated from thermal springs [38, 39]; *Ca.* N. inopinata was enriched from a hot water outflow of a
131 deep oil exploration well [23].

132 *N. europaea* and *N. multiformis* were maintained at 30°C in modified Skinner and Walker (S&W)
133 medium [40], containing 0.2 g KH_2PO_4, 0.04 g $CaCl_2 \cdot 2\ H_2O$, 0.04 g $MgSO_4 \cdot 7\ H_2O$, 1 ml FeNaEDTA
134 (7.5 mM), 1 ml phenol red (0.05%) as pH indicator, 10 ml l^{-1} HEPES buffer (1 M HEPES, 0.6 M
135 NaOH) and 4 mM $(NH_4)_2SO_4$ L^{-1}. The pH was regularly adjusted to 7.7 by addition of sterilized 5%
136 (w/v) Na_2CO_3. The acidophilic AOA *Ca.* N. sp. Nd2 and the AOA *N. viennensis* were maintained in
137 freshwater medium at 35 and 37°C, respectively, according to Tourna et al. [20]. The pH for the *Ca.* N.
138 sp. Nd2 was adjusted to 5.0-5.3 by HCl and the NH_4^+ concentration was kept at 0.5 mM by routinely
139 adding NH_4Cl stock solution. The pH for *N. viennensis* was adjusted to 7.5 by addition of 10 ml l^{-1}
140 HEPES buffer (1 M HEPES, 0.6 M NaOH). *N. viennensis* was supplied with 1 mM NH_4Cl and 0.1
141 mM pyruvate. The AOB *N. nitrosa* and *N. communis*, the AOA *N. gargensis*, and the enrichments
142 containing *Ca.* N. uzonensis and *Ca.* N. inopinata were maintained at 37, 28, 46, 46 and 37°C,
143 respectively, in AOA medium modified from Lebedeva et al. [38] containing (L^{-1}) 75 mg KCl, 50 mg
144 KH_2PO_4, 584 mg NaCl, 50 mg $MgSO_4 \cdot 7\ H_2O$, 1 ml of trace element solution (AOA-TES), 1 ml of

145 selenium-tungsten solution (SWS), 4 g $CaCO_3$ (mostly undissolved, acting as a solid buffer reservoir
146 and growth surface) and 5 ml of NH_4Cl (from an autoclaved 0.2 M stock solution). For a detailed
147 description of the composition of TES and SWS please refer to Widdel [41].

148 *2.2 Incubation experiments*

149 Metabolically active cultures were concentrated and washed twice using fresh medium without NH_4^+
150 by centrifugation (Table S1), and resuspended in fresh medium containing 0.5 or 2 mM NH_4^+. Note
151 that the added NH_4^+ concentrations were not optimal for all strains tested, but use of the same
152 concentrations for all strains maximized comparability of the chemical factors contributing to N_2O
153 formation in the various growth media. *Ca.* N. sp. Nd2 was incubated with 0.5 mM NH_4^+ only, as this
154 culture grew extremely slowly and is inhibited by high nitrous acid concentration formed under acidic
155 conditions. Cultures were incubated under different conditions and for different periods depending on
156 their different growth characteristics (Table S1). All treatments were carried out with 4-6 replicates.
157 Only *N. communis* (90 rpm, New Brunswick™ Innova® 42 Shaker) and *N. nitrosa* (90 rpm, GFL
158 3019 shaker) cultures were shaken during incubation. Before each sampling, bottles of all cultures
159 were mixed by shaking by hand. Samples (3 ml) for chemical and protein analyses were taken at 0, 2,
160 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
161 autoclaved Eppendorf tubes, respectively. The tubes were centrifuged immediately at 8000 *g* (4°C) for
162 10 min, and 1.2 ml of supernatant was transferred to two 1.5 ml Eppendorf tubes containing 75 µl 480
163 mM (for 2 mM NH_4^+ treatment) or 160 mM (for 0.5 mM NH_4^+ treatment) sulfanilamide in 0.8 M HCl
164 for quantification of NH_2OH (see below). Another 0.2 ml supernatant was transferred to a 1.5-ml
165 Eppendorf tube for NH_4^+ and NO_2^- analyses (see below) and the remaining liquid and pellet were
166 frozen at -20°C for protein quantification (see below). To prevent any potential effect of phenol red on
167 NH_2OH analysis, *N. europaea* and *N. multiformis* were grown in parallel in media buffered with
168 HEPES without and with phenol red to facilitate maintenance of pH between pH 7.5 and 8 by addition
169 of sterilized 5% (w/v) Na_2CO_3. *Ca.* N. sp. Nd2 cultures were not buffered and pH was determined
170 daily by pH measurement of 2-ml samples. For cultures buffered with $CaCO_3$, pH was stable at ~8.2
171 throughout the incubation period.

2.3 Determination of abiotic NH_2OH decay rates under ambient air conditions

Abiotic NH_2OH 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_2OH decay since no extracellular NH_2OH 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_2OH 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_2OH 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_2OH (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_2O 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_2O analysis by a gas chromatograph (GC) with an electron capture detector (ECD) as described in Liu et al. [30]. NH_2OH calibration in the range 0–1 µM was performed before each measurement. Since N_2O background increased by about 10 ppb in the control vials for the culture samples of *N. communis* and *N. nitrosa* during NH_2OH determination, NH_2OH 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_2OH:final product ratio

Total extracellular NH_2OH concentrations by AOB, AOA and comammox during consumption of available NH_4^+ was evaluated as the NH_2OH: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_2OH, which followed first-order reaction kinetics:

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

where C is the NH_2OH concentration (μM) at decay time t (h), C_0 is the initial NH_2OH concentration (μM) and k is the first-order rate constant.

The NH_2OH:final product ratio was calculated as:

$$r = \frac{[C_{t2} - C_{t1} + \sum_{i=t1}^{t2-1} C_i \cdot (1 - e^{-k \cdot 1})]}{C'_{t2} - C'_{t1}} \quad (2)$$

where r (dimensionless) is the NH_2OH:final product ratio between t_1 and t_2, C_{t1} and C_{t2} (μM) are the measured NH_2OH concentrations at t_1 and t_2, respectively, C_i (μM) is the interpolated NH_2OH concentration between times t_1 and t_2 (t_2 - t_1 = 1 hour), C_{t1}' and C_{t2}' (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_2OH 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_2OH 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_2OH was calculated using an average value of k determined at 0 or 2 mM NO_2^- when 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_2OH when NO_2^- concentration was <1 mM or >1 mM, respectively. However, the total NH_2OH:final product ratio was very likely underestimated since higher NH_2OH 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_2OH:final product ratio.

224 *2.6 Calculation of the fraction of NH_4^+ converted to N_2O during incubation*

225 The fraction of NH_4^+ converted to N_2O through incubation was calculated by determining the overall
226 abiotic N_2O product ratios (r_i in equation 3) at different NH_2OH and NO_2^- concentrations for different
227 media and incubation temperatures. For this, 1.2 ml of HEPES and $CaCO_3$ medium, respectively, was
228 added to 22-ml glass vials, followed by 0, 12 and 24 µl of 100 mM NO_2^- and 12, 24 and 60 µl of 50
229 µM NH_2OH. The final NO_2^- concentrations were 0, 1 and 2 mM and final NH_2OH concentrations were
230 0.5, 1 and 2.5 µM. Vials were then incubated for 24 h at 30, 37 and 46°C according to the cultivation
231 conditions of the respective microorganisms and headspace gas was analyzed for N_2O by GC. The
232 fraction of NH_4^+ converted to N_2O over the whole NH_3 oxidation process (R) was then calculated as
233 follows:

234 $$R = \frac{\sum_{i=1}^{n} C_i \cdot r_i}{C} \qquad (3)$$

235 where C_i is the concentration of NH_2OH during the i^{th} and $(i+1)^{th}$ sampling, r_i is the theoretical abiotic
236 N_2O production ratio determined as described in section 2.6, and C is the concentration of NH_4^+
237 consumed during incubation. Note that r_i was strongly dependent on NO_2^- concentration. Abiotic N_2O
238 production within a certain time period when NO_2^- concentration was <1 mM, 1–1.5 mM and >1.5
239 mM was calculated using r_i values for NO_2^- concentrations of 0, 1 and 2 mM, respectively. As r_i
240 increased with increasing NO_2^- concentration, this definition of r_i may have led to underestimation or
241 overestimation of abiotic N_2O production when NO_2^- concentration was < or >1.5 mM, respectively.

242 *2.7 Data analyses*

243 Abiotic NH_2OH decay was fitted to first-order reaction equations by the R software package (version
244 3.1.0). The coefficients of determination (R^2) were larger than 0.99. Paired t-tests (R, version 3.1.0)
245 were used to identify significant differences in NH_2OH concentrations between two time points during
246 culture incubation.

247

248

3. Results and discussion

3.1 Extracellular NH_2OH from autotrophic ammonia oxidizers

The NH_2OH 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_2OH release was also observed for *N. europaea*, albeit at lower concentrations than for *N. multiformis*. No NH_2OH was detectable for *N. nitrosa* Nm90 or *N. communis* at both tested NH_4^+ concentrations. Initial increases in NH_2OH 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_2OH 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_2OH 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_2OH during NH_3 oxidation (10 mM) and Yu and Chandran [13] reported 0.2–3.2 μM NH_2OH during growth of *N. europaea* 19718 on 20 mM NH_4^+. These findings are consistent with the NH_2OH concentrations detected for *N. europaea* in our study, where NH_2OH concentrations were about three orders of magnitude smaller than those of the produced NO_2^-, although they did not specify whether they measured NH_2OH in supernatant (as in our study) or in untreated cultures. In our experiments, *N. multiformis* NH_2OH 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_2OH release. However, the *N. communis* biomass in the batch experiments showed no detectable NH_2OH 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_2OH 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_2OH in contrast to tolerance of 250 μM NH_2OH by *N. europaea* and *N. multiformis* [12], which may relate to the absence

of NH_2OH in the medium of *N. communis*, although the exact mechanism for the low tolerance of NH_2OH 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_2OH release.

Among the three AOA pure cultures, NH_2OH 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_2OH release by *N. gargensis* differed from that of AOB, with a small but rather constant increase in NH_2OH during incubation on 2 mM NH_4^+, resulting in a final NH_2OH 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_2OH 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_2OH (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_m552 that are considered critical for energy conversion [21], initially casting some doubt on the role of NH_2OH as an intermediate in NO_2^- formation by AOA [21]. However, Vajrala et al. [26] reported the production of NH_2OH in the marine AOA *N. maritimus* during NH_3 oxidation. Furthermore, Kozlowski et al. [22] showed that the addition of NH_2OH 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_2OH. Also, in a preliminary experiment, *N. gargensis* could convert NH_2OH to NO_2^- biotically, especially at lower NH_2OH levels (Experiment S1, Fig. S1). Stieglmeier et al. [15] observed aerobic N_2O production by *N. viennensis* and attributed this to hybrid formation of N_2O via an N-nitrosating reaction. Kozlowski et al. [22] later reported that N_2O 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_2O in *N. viennensis* could also stem from the well-known chemical reaction between

303 NH_2OH and NO_2^-. However, we failed to observe NH_2OH in the medium of *N. viennensis*, which
304 could reflect (i) lack of NH_2OH release by this culture (indicating that the coupling between AMO and
305 the archaeal NH_2OH-converting enzyme is more efficient than in some AOB) or (ii) rapid chemical
306 NH_2OH conversion in the medium (which could mask small amounts of released NH_2OH), as the
307 medium response of *N. viennensis* was different from that of *N. gargensis* in terms of the nitrogenous
308 gas production from abiotic NH_2OH decay (Experiment S2, Fig. S2). Also for *Ca.* N. sp. Nd2, NH_2OH
309 was not detectable, possibly due to low NH_3 oxidation rates.

310 The comammox organism *Ca.* N. inopinata oxidized NH_4^+ to NO_3^- (Fig. 3). After 48 h of incubation,
311 *Ca.* N. inopinata produced 0.46 mM NO_3^- with 2 mM initial NH_4^+ concentration, while it produced
312 0.27 mM NO_3^- when fed with 0.5 mM NH_4^+. Release of the NH_2OH into the medium by *Ca.* N.
313 inopinata was similar for both NH_4^+ levels, but unlike the other cultures, increasing mainly at the
314 beginning of the incubation, decreasing and then increasing again in parallel with increasing NO_3^-
315 concentration to reach 0.43 µM at the end of the incubation period. This decreasing and increasing
316 trend was significant ($P < 0.025$) for the culture growing on 2 mM NH_4^+ initial concentration.
317 Consistent with the detection of NH_2OH, previous genomic analysis had shown that *Ca.* N. inopinata
318 encodes a predicted octaheme cytochrome c protein resembling the HAO of AOB, cytochromes c554
319 and c_m552, and an AMO that is relatively closely related to the AMO of the betaproteobacterial AOB
320 [23]. *Ca.* N. inopinata lacks canonical NO reductases but encodes enzymes for dissimilatory nitrate
321 reduction to ammonia [46]. Whether the latter enzymes are also expressed and active under aerobic
322 conditions and might contribute to N_2O formation has not yet been investigated.

323

324 *3.2 NH_2OH abiotic decay and NH_2OH:final product ratios during NH_3 oxidation*

325 To better understand the presence of extracellular NH_2OH during ammonia oxidation of the tested
326 organisms, a series of NH_2OH abiotic decay and formation experiments were conducted with different
327 media, incubation temperatures and NO_2^- concentrations (Fig. 4). All three factors, i.e., medium type,
328 temperature and NO_2^- concentration, had strong effects on the rate of abiotic NH_2OH decay. The decay

rate was faster in $CaCO_3$ than in HEPES-buffered media: 0.5 to 2.5 μM NH_2OH 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_2OH 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_2OH 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_2OH decay in both media tested (Figure 4, Table S2), although NO_2^- is known to oxidize NH_2OH to N_2O, albeit preferentially at low pH (e.g., Heil et al., 2014 [34]). 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_2OH 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_2OH occurred (Experiment S3). An additional ^{15}N-NO_2^- experiment showed that NO_2^- did not interfere with the NH_2OH analysis (Experiment S4, Table S3). Under alkaline conditions, one product of NH_2OH abiotic decay is NO_2^- [47], which has been also observed in abiotic NH_2OH decay experiments in $CaCO_3$-buffered medium in this study (Experiment S5, Fig. S3). In addition to NO_2^- and N_2O, nitrogen dioxide (NO_2), but almost no NO, was observed during the abiotic NH_2OH decay (Fig. S2). The presence of NO_2 may explain the observation of abiotic NH_2OH-to-NO_2^- conversion as NO_2 is highly reactive and can hydrolyze to nitric acid (HNO_2) and nitrous acid (HNO_3) in aqueous solution. Consequently, NO_2^-, N_2O and NO_2 comprised approximately 18.5%, 9.8% and 32.1%, respectively, of the abiotically decayed NH_2OH in the $CaCO_3$-buffered medium (Fig. S2, S3). Therefore, a possible reason for the inhibitory effect of NO_2^- on the abiotic NH_2OH decay could be that the presence of NO_2^- slowed down the transformation of NH_2OH to NO_2^- by inhibiting the disproportionation of NO_2, one of the primary decay products of NH_2OH, to HNO_3 and HNO_2.

The effect of temperature on abiotic NH_2OH decay was as expected, as NH_2OH is extremely unstable and reactive, especially at higher temperatures [47]. The exact reason for the difference of abiotic

357 NH_2OH decay between the two media (HEPES- and $CaCO_3$-buffered) is not obvious. The media differ
358 mainly in terms of pH, the composition and concentrations of the trace metals and the buffer (HEPES
359 vs. $CaCO_3$). Both pH and redox active trace metals are known to have a strong effect on abiotic
360 NH_2OH decay. Acidic pH stabilizes NH_2OH in the absence of redox active trace metals, while trace
361 metals such as Cu^{2+}, Fe^{3+} and Mn^{4+} can stimulate NH_2OH decomposition [47]. Therefore, higher pH and
362 the presence of trace metals could lead to greater abiotic NH_2OH decay in the $CaCO_3$-buffered
363 medium than in HEPES-buffered medium.

364 First-order kinetic rate constants and Equation 2 were used to estimate both instantaneous and total
365 NH_2OH:final product ratios during NH_3 oxidation by those cultures producing relatively high NH_2OH
366 concentrations, i.e. *N. europaea*, *N. multiformis*, *N. gargensis* and *Ca.* N. inopinata (Fig. S4 and Table
367 1). For the three pure cultures (*N. europaea*, *N. multiformis* and *N. gargensis*), instantaneous
368 NH_2OH:final product ratios were in the range 0.1 to 0.6% during early phases of the incubation
369 experiments, but several-fold higher as the substrate NH_4^+ was nearly consumed, e.g., as high as about
370 4% for *N. multiformis* (Fig. S4). For the comammox organism *Ca.* N. inopinata, instantaneous
371 NH_2OH: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
372 NH_4^+ concentration, respectively, also with higher values at the end of incubation (Fig. S4). Generally,
373 *Ca.* N. inopinata had the largest total NH_2OH:final product ratio of all cultures tested, with ratios of
374 0.63% and 1.92% after incubation for 60 h at 0.5 and 2 mM initial NH_4^+ concentration, respectively
375 (Table 1). In contrast, *N. gargensis* had a total NH_2OH:NO_2^- ratio of 0.46% at 2 mM initial NH_4^+
376 concentration after 60 h, whereas *N. multiformis* and *N. europaea* had total NH_2OH:final product
377 ratios of 0.34–0.56% and 0.24–0.33%, respectively, depending on the initial NH_4^+ concentration.

378 *3.3 Estimating the fraction of NH_4^+ converted to N_2O during NH_3 oxidation under ambient air*
379 *conditions*

380 For an informed estimate of the fraction of NH_4^+ that was converted to N_2O by the different ammonia
381 oxidizers under ambient air conditions over the whole incubation period, it is essential to consider
382 abiotic N_2O production from different NH_2OH concentrations, at different incubation temperatures,
383 and at different concentrations of NO_2^-. 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_2O conversion ratio from NH_2OH [27, 29]. The abiotic N_2O:NH_2OH 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_2OH concentration range from 0.5 to 2.5 μM. The contribution of NO_2^- to N_2O production involving NH_2OH was even larger at higher temperature, e.g. 46°C (Table 2). The stimulated conversion of NH_2OH to N_2O by NO_2^- was likely caused by the hybrid reaction of NO_2^- and NH_2OH. However, another mechanism could be inhibition of NH_2OH conversion to NO_2/NO_2^- by NO_2^-, thereby channeling NH_2OH to N_2O indirectly via other mechanisms.

The total fraction of NH_4^+ converted to N_2O through extracellular NH_2OH 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_2O 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_2O (0.05–0.1%) during aerobic incubation of a *Nitrosospira* strain [6, 48]. The fraction of NH_4^+ converted to N_2O by *N. europaea* was lower than that of *N. multiformis*, but still consistent with that converted to N_2O 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_2O than *N. europaea* at greater dissolved O_2 concentrations, while *N. europaea* produced much more N_2O during nitrifier-denitrification than *N. multiformis*, which is consistent with our finding that the fraction of NH_4^+ converted to N_2O 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_2O via hybrid formation, as revealed by ^{15}N-labeling [15]. In the present study, potential abiotic N_2O 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_2O by the comammox

411 organism *Ca.* N. inopinata was even higher (in the range of 0.06–0.14%), but no measured data on
412 N_2O emissions from comammox organisms are yet available for comparison.

413 In summary, we show that extracellular NH_2OH is formed in growth media during aerobic NH_3
414 oxidation in batch incubations by AOB, AOA and comammox cultures, but with large differences
415 between the different organisms and incubation conditions. The calculated fraction of NH_4^+ converted
416 to N_2O by abiotic reactions between extracellular NH_2OH and substances in the growth medium
417 during aerobic NH_3 oxidation, was in the range of values reported previously for the conversion of
418 substrate to N_2O for various AOB and AOA. The presence of NO_2^- in the medium not only offers a
419 reactant for hybrid N_2O formation from NH_2OH, but also delays overall NH_2OH abiotic decay, further
420 stimulating the conversion of NH_2OH to N_2O. In view of the new results presented here and in recent
421 studies [15, 22, 52-54], it is tempting to speculate that at least for some strains extracellular NH_2OH might
422 contribute to aerobic ammonia-oxidizer-associated N_2O formation. In others, e.g. *N. viennensis,* no
423 extracellular NH_2OH was observed during NH_3 oxidation but aerobic N_2O production has been
424 reported [15], indicating a different mechanism, e.g. the abiotic reactions between intracellular NH_2OH
425 and periplasmic substances.

426

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435

436

437 **Supporting Information**

438 Centrifugation and incubation conditions for the ammonia-oxidizing strains tested; first-order rate
439 constant (k) of abiotic NH_2OH decay in different media at different NH_2OH (0.5, 1, 2.5 and 5 μM) and
440 NO_2^- (0 and 2 mM) concentrations; biotic (*N. gargensis*) and abiotic conversion of NH_2OH (30 or 80
441 μM) to NO_2^-; N_2O and NOx emissions from $CaCO_3$-buffered medium (A) and fresh water medium
442 (FWM) (B) after addition of 0.08 mM NH_2OH; test for the abiotic conversion of NO_2^- to NH_2OH in
443 the growth medium; ^{15}N-NO_2^- labeling experiment to quantify the effect of NO_2^- on the NH_2OH assay;
444 abiotic conversion of NH_2OH to NO_2^- in $CaCO_3$ medium at different NH_2OH concentrations (0.03,
445 0.08 and 0.2 mM) and two temperatures (37 and 46°C); NH_2OH:final product ratios (%) during
446 incubation at two different initial NH_4^+ concentrations (0.5 mM, square; 2 mM, circle) for four
447 different cultures of ammonia-oxidizers.

References

(1) IPCC, Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change In Stocker, T. F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley, Ed. Cambridge University Press: Cambridge, United Kingdom and New York, USA, **2013**; p 1552.

(2) WMO, WMO Greenhouse gas bulletin: the state of greenhouse gases in the atmosphere based on observations through 2009. *See* http://www.wmo.int/gaw/ **2010**.

(3) Huang, T.; Gao, B.; Hu, X.-K.; Lu, X.; Well, R.; Christie, P.; Bakken, L. R.; Ju, X.-T., Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous Fluvo-aquic soil. *Sci. Rep.* **2014,** *4*, 3950.

(4) Ritchie, G.; Nicholas, D., Identification of the sources of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. *Biochem. J.* **1972,** *126*, (5), 1181-1191.

(5) Poth, M.; Focht, D. D., ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. *Appl. Environ. Microb.* **1985,** *49*, (5), 1134-1141.

(6) Shaw, L. J.; Nicol, G. W.; Smith, Z.; Fear, J.; Prosser, J. I.; Baggs, E. M., *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ. Microbiol.* **2006,** *8*, (2), 214-222.

(7) Caranto, J. D.; Vilbert, A. C.; Lancaster, K. M., *Nitrosomonas europaea* cytochrome P460 is a direct link between nitrification and nitrous oxide emission. *Proc. Natl. Acad. Sci. U. S. A.* **2016,** *113*, 14704-14709.

(8) Caranto, J. D.; Lancaster, K. M., Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc. Natl. Acad. Sci. U. S. A.* **2017,** *114*, (31), 8217-8222.

(9) Wrage, N.; Velthof, G.; Van Beusichem, M.; Oenema, O., Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biochem.* **2001,** *33*, (12), 1723-1732.

(10) Wunderlin, P.; Mohn, J.; Joss, A.; Emmenegger, L.; Siegrist, H., Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions. *Water Res.* **2012,** *46*, (4), 1027-1037.

(11) Kozlowski, J. A.; Kits, K. D.; Stein, L. Y., Genome sequence of *Nitrosomonas communis* strain Nm2, a mesophilic ammonia-oxidizing bacterium isolated from mediterranean soil. *Genome Announc.* **2016,** *4*, (1), e01541-15.

(12) Kozlowski, J. A.; Kits, K. D.; Stein, L. Y., Comparison of nitrogen oxide metabolism among diverse ammonia-oxidizing bacteria. *Front. Microbiol.* **2016,** *7*.

(13) Yu, R.; Chandran, K., Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiol.* **2010,** *10*, (1), 1-11.

(14) Jung, M.-Y.; Well, R.; Min, D.; Giesemann, A.; Park, S.-J.; Kim, J.-G.; Kim, S.-J.; Rhee, S.-K., Isotopic signatures of N_2O produced by ammonia-oxidizing archaea from soils. *ISME J.* **2014,** *8*, (5), 1115-1125.

(15) Stieglmeier, M.; Mooshammer, M.; Kitzler, B.; Wanek, W.; Zechmeister-Boltenstern, S.; Richter, A.; Schleper, C., Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J.* **2014,** *8*, (5), 1135-1146.

(16) Santoro, A. E.; Buchwald, C.; McIlvin, M. R.; Casciotti, K. L., Isotopic Signature of N_2O Produced by Marine Ammonia-Oxidizing Archaea. *Science* **2011,** *333*, (6047), 1282-1285.

(17) He, J. z.; Shen, J. p.; Zhang, L. m.; Zhu, Y. g.; Zheng, Y. m.; Xu, M. g.; Di, H., Quantitative analyses of the abundance and composition of ammonia‐oxidizing bacteria and ammonia‐oxidizing archaea of a Chinese upland red soil under long‐term fertilization practices. *Environ. Microbiol.* **2007,** *9*, (9), 2364-2374.

(18) Leininger, S.; Urich, T.; Schloter, M.; Schwark, L.; Qi, J.; Nicol, G.; Prosser, J.; Schuster, S.; Schleper, C., Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **2006,** *442*, (7104), 806-809.

(19) Spang, A.; Poehlein, A.; Offre, P.; Zumbrägel, S.; Haider, S.; Rychlik, N.; Nowka, B.; Schmeisser, C.; Lebedeva, E. V.; Rattei, T.; Böhm, C.; Schmid, M.; Galushko, A.; Hatzenpichler, R.; Weinmaier, T.; Daniel, R.; Schleper, C.; Spieck, E.; Streit, W.; Wagner, M., The genome of the ammonia-oxidizing

Candidatus *Nitrososphaera gargensis*: insights into metabolic versatility and environmental adaptations. *Environ. Microbiol.* **2012,** *14*, (12), 3122-3145.
(20) Tourna, M.; Stieglmeier, M.; Spang, A.; Könneke, M.; Schintlmeister, A.; Urich, T.; Engel, M.; Schloter, M.; Wagner, M.; Richter, A.; Schleper, C., *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc. Natl. Acad. Sci. U. S. A.* **2011,** *108*, (20), 8420-8425.
(21) Walker, C.; De La Torre, J.; Klotz, M.; Urakawa, H.; Pinel, N.; Arp, D.; Brochier-Armanet, C.; Chain, P.; Chan, P.; Gollabgir, A., *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** *107*, (19), 8818-8823.
(22) Kozlowski, J. A.; Stieglmeier, M.; Schleper, C.; Klotz, M. G.; Stein, L. Y., Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota. *ISME J.* **2016,** *10*, (8), 1836-1845.
(23) Daims, H.; Lebedeva, E. V.; Pjevac, P.; Han, P.; Herbold, C.; Albertsen, M.; Jehmlich, N.; Palatinszky, M.; Vierheilig, J.; Bulaev, A., Complete nitrification by *Nitrospira* bacteria. *Nature* **2015,** *528*, (7583), 504-509.
(24) van Kessel, M. A.; Speth, D. R.; Albertsen, M.; Nielsen, P. H.; den Camp, H. J. O.; Kartal, B.; Jetten, M. S.; Lücker, S., Complete nitrification by a single microorganism. *Nature* **2015,** *528*, (7583), 555-559.
(25) Lees, H., Hydroxylamine as an intermediate in nitrification. *Nature* **1952,** *169*, 156-157.
(26) Vajrala, N.; Martens-Habbena, W.; Sayavedra-Soto, L. A.; Schauer, A.; Bottomley, P. J.; Stahl, D. A.; Arp, D. J., Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea. *Proc. Natl. Acad. Sci. U. S. A.* **2013,** *110*, (3), 1006-1011.
(27) Bremner, J. M., Sources of nitrous oxide in soils. *Nutr. Cycl. Agroecosys* **1997,** *49*, (1), 7-16.
(28) Heil, J.; Liu, S.; Vereecken, H.; Brüggemann, N., Abiotic nitrous oxide production from hydroxylamine in soils and their dependence on soil properties. *Soil Biol. Biochem.* **2015,** *84*, 107-115.
(29) Liu, S.; Herbst, M.; Bol, R.; Gottselig, N.; Pütz, T.; Weymann, D.; Wiekenkamp, I.; Vereecken, H.; Brüggemann, N., The contribution of hydroxylamine content to spatial variability of N_2O formation in soil of a Norway spruce forest. *Geochim. Cosmochim. Ac* **2016,** *178*, 76-86.
(30) Liu, S.; Vereecken, H.; Brüggemann, N., A highly sensitive method for the determination of hydroxylamine in soils. *Geoderma* **2014,** *232*, 117-122.
(31) Toyoda, S.; Yoshida, N., Determination of nitrogen isotopomers of nitrous oxide on a modified isotope ratio mass spectrometer. *Anal. Chem.* **1999,** *71*, (20), 4711-4718.
(32) Ostrom, N. E.; Ostrom, P. H., The isotopomers of nitrous oxide: analytical considerations and application to resolution of microbial production pathways. In *Handbook of Environmental Isotope Geochemistry, Advances in Isotope Geochemistry*, Baskaran, M., Ed. Springer Berlin Heidelberg: **2011**; pp 453-476.
(33) Sutka, R. L.; Ostrom, N.; Ostrom, P.; Breznak, J.; Gandhi, H.; Pitt, A.; Li, F., Distinguishing nitrous oxide production from nitrification and denitrification on the basis of isotopomer abundances. *Appl. Environ. Microb.* **2006,** *72*, (1), 638-644.
(34) Heil, J.; Wolf, B.; Brüggemann, N.; Emmenegger, L.; Tuzson, B.; Vereecken, H.; Mohn, J., Site-specific ^{15}N isotopic signatures of abiotically produced N_2O. *Geochim. Cosmochim. Ac* **2014,** *139*, 72-82.
(35) Soler-Jofra, A.; Stevens, B.; Hoekstra, M.; Picioreanu, C.; Sorokin, D.; van Loosdrecht, M. C. M.; Pérez, J., Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water. *Chem. Eng. J.* **2016,** *287*, 720-726.
(36) Koops, H. P.; Böttcher, B.; Möller, U. C.; Pommerening-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.

549 (37) Lehtovirta-Morley, L. E.; Ge, C.; Ross, J.; Yao, H.; Nicol, G. W.; Prosser, J. I., Characterisation of
550 terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic
551 compounds. *Fems Microbiol. Ecol.* **2014,** *89*, (3), 542-552.
552 (38) Lebedeva, E. V.; Hatzenpichler, R.; Pelletier, E.; Schuster, N.; Hauzmayer, S.; Bulaev, A.;
553 Grigor'eva, N. V.; Galushko, A.; Schmid, M.; Palatinszky, M., Enrichment and genome sequence of the
554 group I. 1a ammonia-oxidizing Archaeon "*Ca.* Nitrosotenuis uzonensis" representing a clade globally
555 distributed in thermal habitats. *PLoS One* **2013,** *8*, (11), e80835.
556 (39) Palatinszky, M.; Herbold, C.; Jehmlich, N.; Pogoda, M.; Han, P.; von Bergen, M.; Lagkouvardos,
557 I.; Karst, S. M.; Galushko, A.; Koch, H.; Berry, D.; Daims, H.; Wagner, M., Cyanate as an energy source
558 for nitrifiers. *Nature* **2015,** *524*, (7563), 105-108.
559 (40) Skinner, F. A.; Walker, N., Growth of Nitrosomonas europaea in batch and continuous culture.
560 *Archiv für Mikrobiologie* **1961,** *38*, (4), 339-349.
561 (41) Widdel, F., Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten
562 Sulfat-reduzierender Bakterien. *Dissertation, Universität Göttingen* **1980**, 1-443.
563 (42) Strickland, J. D.; Parsons, T. R., *A practical handbook of seawater analysis*. 2nd ed.; Fisheries
564 Research Board of Canada: Ottawa, **1972**.
565 (43) Kandeler, E.; Gerber, H., Short-term assay of soil urease activity using colorimetric
566 determination of ammonium. *Biol. Fertil. Soils* **1988,** *6*, (1), 68-72.
567 (44) Stüven, R.; Vollmer, M.; Bock, E., The impact of organic matter on nitric oxide formation by
568 *Nitrosomonas europaea*. *Arch. Microbiol.* **1992,** *158*, (6), 439-443.
569 (45) Prosser, J. I.; Head, I. M.; Stein, L. Y., The Family Nitrosomonadaceae. In *The Prokaryotes:*
570 *Alphaproteobacteria and Betaproteobacteria*, Rosenberg, E.; DeLong, E. F.; Lory, S.; Stackebrandt, E.;
571 Thompson, F., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, **2014**; pp 901-918.
572 (46) Kits, K. D.; Sedlacek, C. J.; Lebedeva, E. V.; Han, P.; Bulaev, A.; Pjevac, P.; Daebeler, A.;
573 Romano, S.; Albertsen, M.; Stein, L. Y.; Daims, H.; Wagner, M., Kinetic analysis of a complete nitrifier
574 reveals an oligotrophic lifestyle. *Nature* **2017,** *549*, (7671), 269-272.
575 (47) Butler, J. H.; Gordon, L. I., An improved gas chromatographic method for the measurement of
576 hydroxylamine in marine and fresh waters. *Mar. Chem.* **1986,** *19*, (3), 229-243.
577 (48) Jiang, Q.; Bakken, L. R., Nitrous oxide production and methane oxidation by different
578 ammonia-oxidizing bacteria. *Appl. Environ. Microb.* **1999,** *65*, (6), 2679-2684.
579 (49) Remde, A.; Conrad, R., Production of nitric oxide in *Nitrosomonas europaea* by reduction of
580 nitrite. *Arch. Microbiol.* **1990,** *154*, (2), 187-191.
581 (50) Hynes, R. K.; Knowles, R., Production of nitrous oxide by *Nitrosomonas europaea*: effects of
582 acetylene, pH, and oxygen. *Can. J. Microbiol.* **1984,** *30*, (11), 1397-1404.
583 (51) Dundee, L.; Hopkins, D. W., Different sensitivities to oxygen of nitrous oxide production by
584 *Nitrosomonas europaea* and *Nitrosolobus multiformis*. *Soil Biol. Biochem.* **2001,** *33*, (11), 1563-1565.
585 (52) Heil, J.; Vereecken, H.; Brüggemann, N., A review of chemical reactions of nitrification
586 intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *Eur. J. Soil Sci.*
587 **2016,** *67*, (1), 23-39.
588 (53) Terada, A.; Sugawara, S.; Hojo, K.; Takeuchi, Y.; Riya, S.; Harper, W. F.; Yamamoto, T.; Kuroiwa,
589 M.; Isobe, K.; Katsuyama, C.; Suwa, Y.; Koba, K.; Hosomi, M., Hybrid Nitrous Oxide Production from a
590 Partial Nitrifying Bioreactor: Hydroxylamine Interactions with Nitrite. *Environ. Sci. Technol.* **2017,** *51*,
591 (5), 2748-2756.
592 (54) Liu, S.; Berns, A. E.; Vereecken, H.; Wu, D.; Brüggemann, N., Interactive effects of MnO_2,
593 organic matter and pH on abiotic formation of N_2O from hydroxylamine in artificial soil mixtures. *Sci.*
594 *Rep.* **2017,** *7*, 39590.

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Tables

Table 1 Total NH_2OH: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. [#] The NH_2OH concentration here is the total extracellular NH_2OH including the calculated concentration of NH_2OH that was abiotically converted during incubation.

Cultures	Initial NH_4^+ concentration (mM)	Final NO_2^- or NO_3^- [§] concentration (μM)	NH_2OH[#] concentration (μM)	NH_2OH:final product ratio (%)
N. multiformis	0.5	516	1.8	0.34
	2	1955	11.0	0.56
N. europaea	0.5	537	1.8	0.33
	2	1930	4.7	0.24
N. gargensis	2	1860	7.1	0.46
Ca. N. inopinata	0.5	280	1.8	0.63
	2	490	9.4	1.92

Table 2 Fraction (%) of N_2O abiotically produced from the added NH_2OH in the different media at various levels of NH_2OH (0.5, 1 and 2.5 μM) and NO_2^- (0, 1 and 2 mM).

	0 mM NO_2^-			1 mM NO_2^-			2 mM NO_2^-		
NH_2OH (μM)	0.5	1	2.5	0.5	1	2.5	0.5	1	2.5
HEPES (30°C)	14.1	13.7	12.0	29.3	20.0	18.4	36.6	33.1	23.4
$CaCO_3$ (30°C)	15.0	20.9	28.0	33.2	32.2	46.9	45.0	37.6	48.9
$CaCO_3$ (37°C)	6.7	5.6	6.7	36.2	31.0	43.7			
$CaCO_3$ (46°C)	6.3	4.6	12.5	29.5	22.4	36.1	38.8	46.0	57.1

611 **Table 3** Estimated fraction of NH_4^+ converted to N_2O from the abiotic reactions between the
612 biologically produced extracellular NH_2OH and substances in the medium for different ammonia
613 oxidizers.

Cultures	Initial NH_4^+ concentration (μM)	Estimated fraction of NH_4^+ converted to N_2O (%)
N. multiformis	500	0.05
	2000	0.12
N. europaea	500	0.05
	2000	0.07
N. gargensis	2000	0.08
Ca. N. inopinata	500	0.06
	2000	0.14

614

615 **Figure captions**

616

617 **Figure 1** Dynamics of NH_4^+ (red squares), NO_2^- (yellow circles), NH_2OH (blue triangles) and total N
618 (sum of NO_2^- and NH_4^+, black diamonds) concentrations during incubation of four ammonia-oxidizing
619 bacteria. NH_4^+, NO_2^- and total N are plotted using the left y-axis, while NH_2OH is plotted using the
620 right y-axis. Please note that the left y-axes and the x-axes, respectively, are not always scaled
621 identically to improve data presentation. The values are presented as mean ± standard error (SE).

622

623 **Figure 2** Dynamics of NH_4^+ (red squares), NO_2^- (yellow circles), NH_2OH (blue triangles) and total N
624 (sum of NO_2^- and NH_4^+, black diamonds) concentrations in the batch experiments with four ammonia-
625 oxidizing archaea. NH_4^+, NO_2^- and total N are plotted using the left y-axis, while NH_2OH is plotted
626 using the right y-axis. Please note that the left y-axes and the x-axes, respectively, are not always
627 scaled identically to improve data presentation. The values are present as mean ± standard error (SE).

628

629 **Figure 3** Dynamics of NH_4^+ (red squares), NO_3^- (yellow circles), NH_2OH (blue triangles) and total N
630 (sum of NO_3^- and NH_4^+, black diamonds) concentrations during the incubation of the comammox
631 organism Ca. N. inopinata. NH_4^+, NO_3^- and total N are plotted using the left y-axis, while NH_2OH is
632 plotted using the right y-axis. The values are present as mean ± standard error (SE).

633

634 **Figure 4** Abiotic decay of NH_2OH in the absence (open symbols) or presence (closed symbols) of 2
635 mM NO_2^- in HEPES-buffered and $CaCO_3$-buffered media at different incubation temperatures. The
636 NH_2OH concentrations were 0.5 (square), 1 (circle), 2.5 (triangle), and 5 (diamond) μM. Mean values
637 of three replicates are presented. The relative standard deviation (RSD) of all data is smaller than 10%.
638 Please note that the x-axes are not always scaled identically to improve data presentation.

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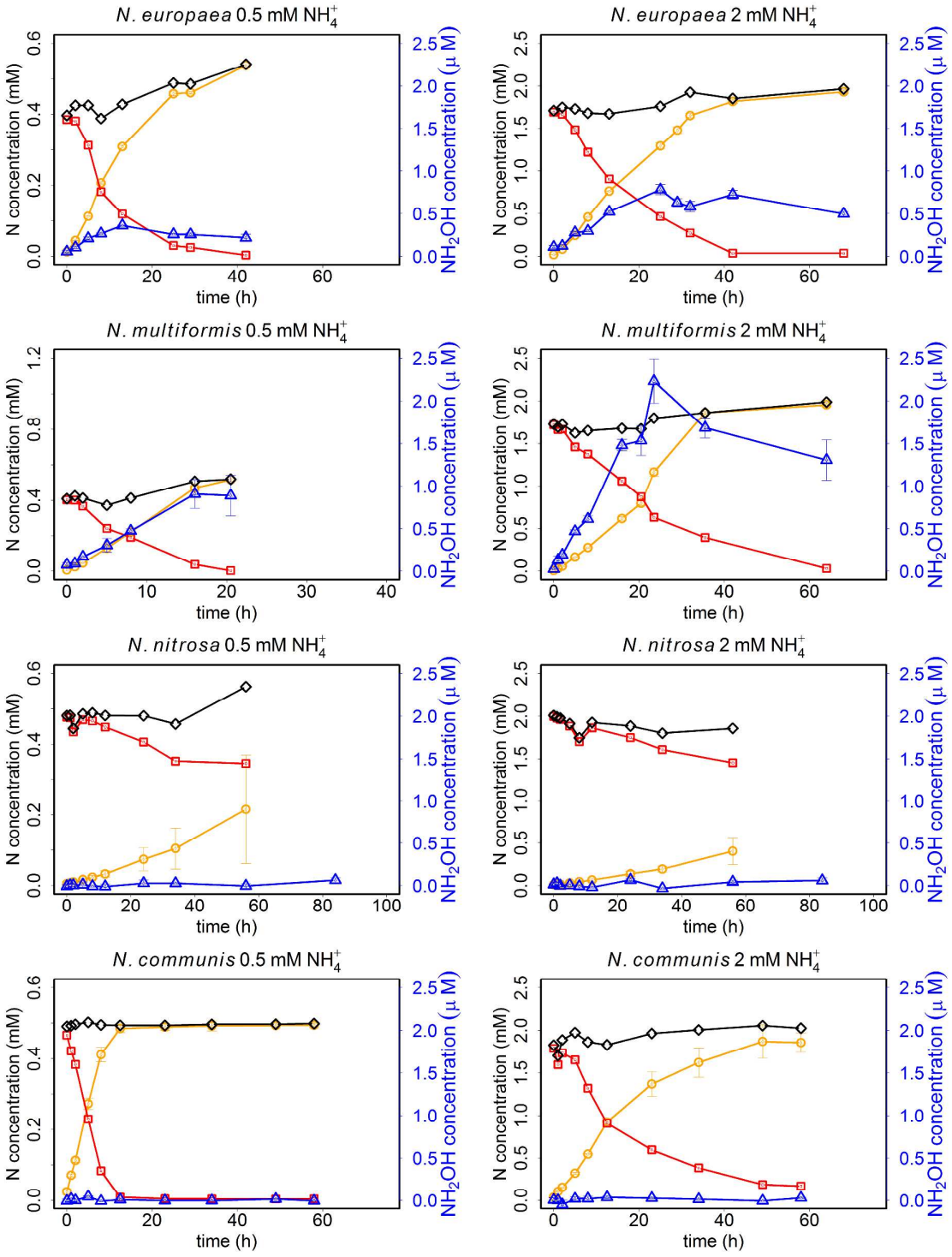
641 **Figures**



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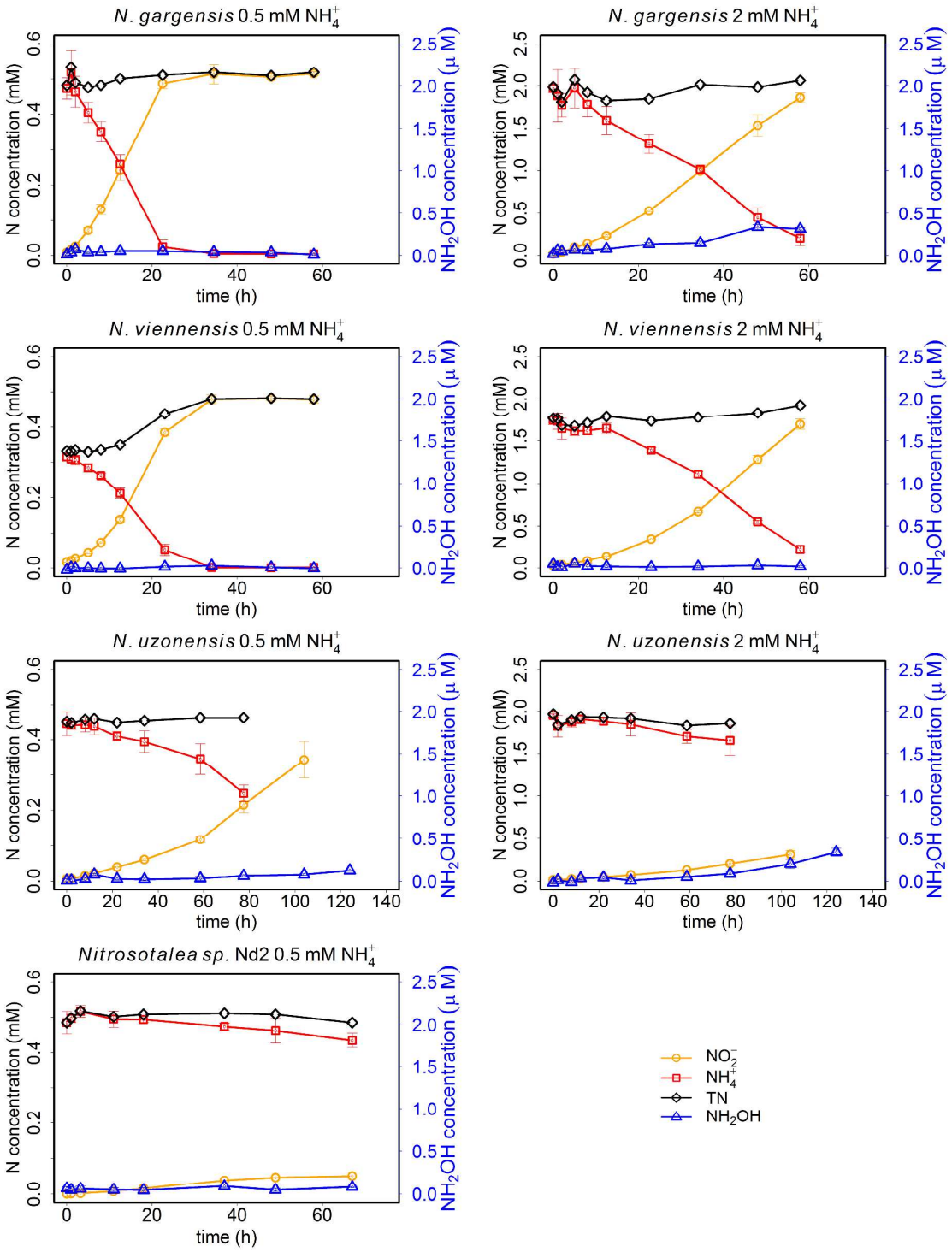
643 **Figure 1**



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645 **Figure 2**

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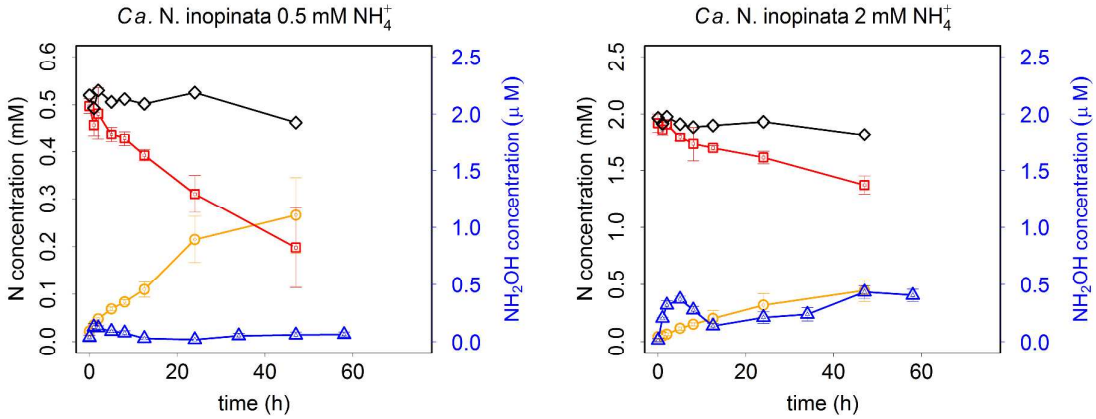

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649 **Figure 3**

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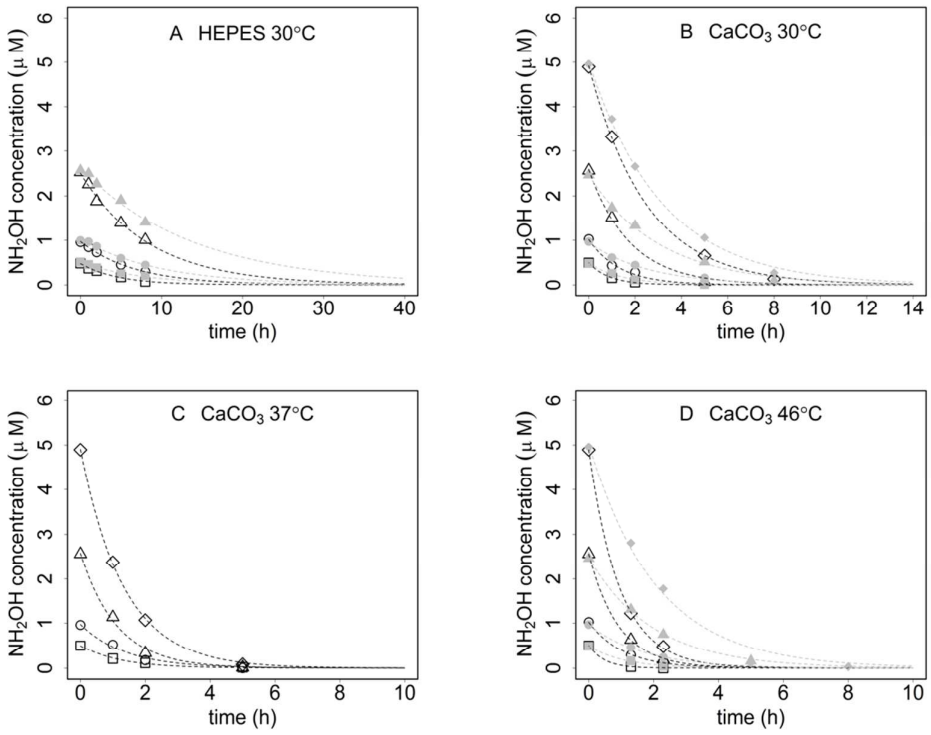

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653 **Figure 4**

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