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Shurong Liu, Ping Han, Linda Hink, James I. Prosser, Michael Wagner, and Nicolas Bruggemann

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1 **Abiotic conversion of extracellular NH₂OH contributes to N₂O emission during**
2 **ammonia oxidation**

3 Shurong Liu^{1*}, Ping Han², Linda Hink³, James I. Prosser³, Michael Wagner², Nicolas
4 Brüggemann¹

5

6 1. Institute of Bio- and Geosciences – Agrosphere (IBG-3), Forschungszentrum Jülich GmbH,
7 52425 Jülich, Germany

8 2. Department of Microbiology and Ecosystem Science, Division of Microbial Ecology,
9 Research Network Chemistry meets Microbiology, University of Vienna, Althanstrasse 14,
10 1090 Vienna, Austria

11 3. School of Biological Sciences, University of Aberdeen, Cruickshank Building, Aberdeen,
12 AB24 3UU, UK

13

14

15 ***Corresponding author:**

16 Shurong Liu, adresse: Wilhelm-Johnen-Straße, 52428 Jülich; phone: 02461/61-5504;

17 fax: 02461/61-2518; e-mail: s.liu@fz-juelich.de

18

19 **Abstract**

20 Abiotic processes involving the reactive ammonia-oxidation intermediates nitric oxide (NO) or
21 hydroxylamine (NH₂OH) for N₂O production have been indicated recently. The latter process would
22 require the availability of substantial amounts of free NH₂OH for chemical reactions during ammonia
23 (NH₃) oxidation, but little is known about extracellular NH₂OH formation by the different clades of
24 ammonia-oxidizing microbes. Here we determined extracellular NH₂OH concentrations in culture
25 media of several ammonia-oxidizing bacteria (AOB) and archaea (AOA), as well as one complete
26 ammonia oxidizer (comammox) enrichment (*Ca. Nitrospira inopinata*) during incubation under
27 standard cultivation conditions. NH₂OH was measurable in the incubation media of *Nitrosomonas*
28 *europaea*, *Nitrosospira multiformis*, *Nitrososphaera gargensis*, and *Ca. Nitrosotenuis uzonensis*, but
29 not in media of the other tested AOB and AOA. NH₂OH was also formed by the comammox
30 enrichment during NH₃ oxidation. This enrichment exhibited the largest NH₂OH:final product ratio
31 (1.92%), followed by *N. multiformis* (0.56%) and *N. gargensis* (0.46%). The maximum proportions of
32 NH₄⁺ converted to N₂O via extracellular NH₂OH during incubation, estimated on the basis of NH₂OH
33 abiotic conversion rates, were 0.12%, 0.08% and 0.14% for AOB, AOA and *Ca. Nitrospira inopinata*,
34 respectively, and were consistent with published NH₄⁺:N₂O conversion ratios for AOB and AOA.

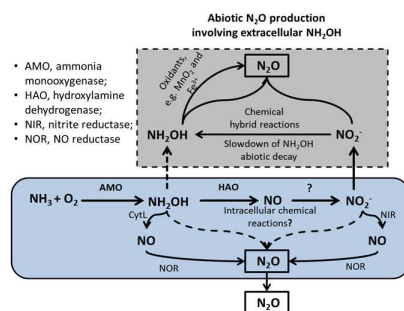
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36 **Key words:** hydroxylamine; greenhouse gas; biotic-abiotic; N₂O formation mechanism; ammonia
37 oxidizer; reactive N, comammox

38 Table of Contents (TOC)/Abstract Art

39

40



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

42 1. Introduction

43 Nitrous oxide (N₂O) is an important greenhouse gas and is currently the third largest contributor to
 44 global warming, after carbon dioxide (CO₂) and methane (CH₄). N₂O 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 N₂O 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 (NH₃) oxidation contributes significantly
 49 to N₂O production in soil ³. Traditionally, two different biochemical routes are proposed for N₂O
 50 production during NH₃ oxidation in AOB. The first is the oxidation of hydroxylamine (NH₂OH) to
 51 nitric oxide (NO) by hydroxylamine dehydrogenase (HAO) and subsequent reduction to N₂O
 52 catalyzed by NO reductase ⁴. The second pathway is the so-called nitrifier-denitrification, by which
 53 nitrite (NO₂⁻) is reduced to NO and N₂O by nitrite reductase (NIR) and NO reductase (NOR),
 54 respectively ⁴⁻⁶. However, recent studies revealed two other routes for the N₂O production from the
 55 AOB *N. europaea* under anaerobic conditions. One is the direct oxidation of NH₂OH to N₂O 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 N₂O formation at low O₂ and low pH ⁹, whereas pathways
 58 related to biological or chemical reactions of ammonia oxidation intermediates (NH₂OH, nitroxyl
 59 (HNO), NO) and/or its product (NO₂⁻) may be more important for N₂O production at high ammonium
 60 (NH₄⁺) levels and sufficient O₂ supply ¹⁰. However, not all AOB share the same route for N₂O
 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 N₂O has been detected in a *N. communis* culture ¹². Most studies on AOB

64 N₂O production pathways have focused on *N. europaea* ATCC 19718^{4,5,13}, and different biochemical
65 routes responsible for N₂O production in other AOB cannot be excluded.

66 In recent years, ammonia oxidation-related N₂O production by several AOA strains has been reported
67¹⁴⁻¹⁶ and AOA abundance exceeds that of AOB by several orders of magnitude in some ecosystems¹⁷,
68¹⁸. However, the mechanism(s) of N₂O 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 N₂O production by AOB
70¹⁹⁻²¹. Recent research showed that the soil AOA *Nitrososphaera viennensis* is indeed not able to
71 generate N₂O through nitrifier-denitrification¹⁵. Instead, for this organism hybrid N₂O formation from
72 NH₄⁺ and NO₂⁻ was demonstrated in ¹⁵N-labeling experiments¹⁵, indicating a N₂O production pathway
73 from NO₂⁻ and an intermediate of ammonia oxidation, e.g. NH₂OH or NO. Recently, it was confirmed
74 that N₂O formation by this AOA under anoxic conditions results from the abiotic reaction of NO with
75 medium or cellular components²². However, the mechanism of N₂O production by AOA under oxic
76 conditions remains unclear. Furthermore, complete bacterial nitrifiers (comammox) of the genus
77 *Nitrospira* that perform ammonia oxidation via NO₂⁻ to nitrate (NO₃⁻) have recently been enriched^{23,24},
78 but nothing is yet known about N₂O production by these microorganisms.

79 Hydroxylamine has long been known as an important intermediate of chemolithoautotrophic AOB²⁵
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 NH₂OH oxidation or form during NH₃
83 oxidation an initial oxidation product other than NH₂OH, e.g. HNO²¹. Recent research showed that
84 NO₂⁻ can be formed after addition of NH₂OH in *N. viennensis*, leading to the proposal of a novel
85 enzymatic mechanism for the production of NO₂⁻ involving NH₂OH and NO in AOA²².

86 Hydroxylamine may play a crucial role in N₂O production from soils under oxic conditions²⁷⁻³⁰, as
87 indicated by the close relationship between NH₂OH concentration and N₂O formation observed in
88 forest soil^{29,30}. Further support for this hypothesis comes from the intramolecular distribution of ¹⁵N
89 within the linear, asymmetric NNO molecule, the so-called ¹⁵N site preference (SP)³¹, which is
90 distinctly different between N₂O produced via denitrification and nitrification³². In pure cultures of

91 different nitrifiers and denitrifiers, Sutka et al.³³ found SP values near 0% for N₂O formed by NO₂⁻
92 and NO₃⁻ reduction (via classical denitrification and nitrifier denitrification), while SP values were
93 30.8–35.6% for N₂O produced during aerobic NH₃ and NH₂OH oxidation, which is similar to SP
94 values reported by Heil et al.³⁴ for N₂O produced by chemical reactions of NH₂OH with Fe³⁺, Cu²⁺
95 and NO₂⁻. Santoro et al.¹⁶ also reported an SP value of ~30% for N₂O produced by an enrichment
96 culture of a marine AOA, although soil AOA showed different SP values with a range of 13–30%¹⁴.
97 Recently, Soler-Jofra et al.³⁵ observed a significant contribution of the abiotic reaction between
98 NH₂OH and NO₂⁻ to N₂O formation in a full-scale nitrification reactor. All these findings indicate that
99 chemical reactions involving NH₂OH may play an important role in N₂O production during
100 chemolithoautotrophic NH₃ oxidation. However, this would require the availability of free NH₂OH,
101 either in the growth medium or, potentially, in the periplasm, for abiotic N₂O formation through
102 chemical reactions with substances such as NO₂⁻, MnO₂ and Fe³⁺. Quantitative data on extracellular
103 NH₂OH production by AOB, AOA and comammox are therefore urgently required in order to better
104 estimate the importance of coupled biotic–abiotic N₂O production during microbial NH₃ oxidation.

105 In this study, we aimed to answer several important questions regarding N₂O formation by ammonia
106 oxidizing microbes: (1) What are the extracellular concentrations of NH₂OH during NH₃ oxidation by
107 different ammonia oxidizers? (2) If these concentrations are significant, what is the NH₂OH:final
108 product ratio for AOB, AOA, and comammox? (3) Can we estimate the contribution of extracellular
109 NH₂OH to abiotic N₂O production during NH₃ oxidation? (4) What is the role of NO₂⁻ in stabilizing
110 NH₂OH and in abiotic conversion of NH₂OH to N₂O? To address these questions, temporal changes in
111 NH₂OH concentration were determined during incubation of pure and enriched cultures of
112 chemolithoautotrophic AOB, AOA and comammox (obtained from soil and aquatic environments) at
113 two NH₄⁺ concentrations, 2 mM and 0.5 mM. These experiments were complemented by measurement
114 of abiotic NH₂OH decay rates and abiotic N₂O production involving NH₂OH in different media and at
115 different incubation temperatures and NO₂⁻ concentrations. These analyses were performed to
116 calculate extracellular NH₂OH production ratios on a final product basis, to quantify the coupled
117 biotic–abiotic NH₄⁺–NH₂OH–N₂O conversion rate of AOB, AOA and comammox, and to explore the
118 role of NO₂⁻ in the abiotic NH₄⁺–NH₂OH–N₂O conversion. We hypothesize that the coupled biotic–

119 abiotic N₂O production is an important mechanism of N₂O production during NH₄⁺ 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 soil⁶,
129 ^{20, 36, 37}; *N. nitrosa* Nm90 was isolated from industrial sewage³⁶; *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 well²³.

132 *N. europaea* and *N. multiformis* were maintained at 30°C in modified Skinner and Walker (S&W)
133 medium⁴⁰, containing 0.2 g KH₂PO₄, 0.04 g CaCl₂·2 H₂O, 0.04 g MgSO₄·7 H₂O, 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 (NH₄)₂SO₄ L⁻¹. The pH was regularly adjusted to 7.7 by addition of sterilized 5%
136 (w/v) Na₂CO₃. 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 NH₄⁺ concentration was kept at 0.5 mM by routinely
139 adding NH₄Cl 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 NH₄Cl 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 KH₂PO₄, 584 mg NaCl, 50 mg MgSO₄ · 7 H₂O, 1 ml of trace element solution (AOA-TES), 1 ml of

145 selenium-tungsten solution (SWS), 4 g CaCO₃ (mostly undissolved, acting as a solid buffer reservoir
146 and growth surface) and 5 ml of NH₄Cl (from an autoclaved 0.2 M stock solution). For a detailed
147 description of the composition of TES and SWS please refer to Widdel⁴¹.

148 2.2 Incubation experiments

149 Metabolically active cultures were concentrated and washed twice using fresh medium without NH₄⁺
150 by centrifugation (Table S1), and resuspended in fresh medium containing 0.5 or 2 mM NH₄⁺. Note
151 that the added NH₄⁺ 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 N₂O
153 formation in the various growth media. *Ca. N. sp. Nd2* was incubated with 0.5 mM NH₄⁺ 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 NH₄⁺ treatment) or 160 mM (for 0.5 mM NH₄⁺ treatment) sulfanilamide in 0.8 M HCl
164 for quantification of NH₂OH (see below). Another 0.2 ml supernatant was transferred to a 1.5-ml
165 Eppendorf tube for NH₄⁺ and NO₂⁻ 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 NH₂OH 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) Na₂CO₃. *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 CaCO₃, pH was stable at ~8.2
171 throughout the incubation period.

172 2.3 Determination of abiotic NH_2OH decay rates under ambient air conditions

173 Abiotic NH_2OH decay was quantified in S&W (with HEPES buffer) and modified AOA (with CaCO_3
174 buffer) media used in this study at the respective growth temperatures. The freshwater medium for *Ca.*
175 *N. sp. Nd2* and *N. viennensis* was not tested for abiotic NH_2OH decay since no extracellular NH_2OH
176 was observed during NH_3 oxidation by these cultures. Well-aerated medium (40 ml) was added to
177 120-ml glass serum bottles followed by different amounts (4, 8, 20 and 40 μl) of 5 mM NH_2OH to
178 reach final concentrations of 0.5, 1, 2.5 and 5 μM , respectively. Subsequently, 1.6 ml 50 mM NO_2^-
179 was added to give a final concentration of 2 mM to simulate abiotic NH_2OH decay in the presence of
180 NO_2^- . Bottles were then capped with aluminum foil and incubated at 30, 37 and 46°C. Samples (1.2 ml)
181 were taken after 0, 1, 2, 5 and 8 h and transferred to 1.5-ml Eppendorf tubes containing 75 μl 480 mM
182 (for 2 mM NO_2^- treatment) or 160 mM (for the treatment without NO_2^- addition) sulfanilamide in 0.8
183 M HCl. Samples were frozen at -20°C until quantification of NH_2OH (see below).

184 2.4 Chemical assays

185 Hydroxylamine concentration was determined according to the method of Liu et al.³⁰. Briefly, 1.2 ml
186 of sample, thawed at room temperature, was transferred to a 22-ml glass vial and 4.8 ml deionized
187 water was added, yielding a pH of ~2. Then, 0.6 ml of 25 mM FeCl_3 was added to the vial, which was
188 immediately closed gas-tight with a crimping tool. Control vials contained sample and water only to
189 assess N_2O in the headspace and dissolved in the sample. The vials were shaken for 3 h at 200 rpm and
190 then transferred to an autosampler for N_2O analysis by a gas chromatograph (GC) with an electron
191 capture detector (ECD) as described in Liu et al.³⁰. NH_2OH calibration in the range 0–1 μM was
192 performed before each measurement. Since N_2O background increased by about 10 ppb in the control
193 vials for the culture samples of *N. communis* and *N. nitrosa* during NH_2OH determination, NH_2OH
194 concentrations <0.06 μM were defined as not detectable. NO_2^- and NH_4^+ concentrations were
195 determined colorimetrically in 96-well plates using sulfanilamide and N-(1-naphthyl)ethylenediamine
196 dihydrochloride for NO_2^- ⁴², and the indophenol method described by Kandeler and Gerber⁴³ for NH_4^+ .
197 Protein concentration was determined with the Pierce BCA protein assay kit (Thermo Fisher
198 Scientific).

199 2.5 Calculation of the NH_2OH :final product ratio

200 Total extracellular NH_2OH concentrations by AOB, AOA and comammox during consumption of
 201 available NH_4^+ was evaluated as the NH_2OH :final product ratios (final product was NO_3^- in the case of
 202 comammox and NO_2^- in all other cases), taking into account the abiotic decay rate of the very reactive
 203 NH_2OH , which followed first-order reaction kinetics:

$$204 \quad C = C_0 e^{-kt} \quad (1)$$

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

207 The NH_2OH :final product ratio was calculated as:

$$208 \quad r = \frac{[C_{t_2} - C_{t_1} + \sum_{i=t_1}^{t_2-1} C_i \cdot (1 - e^{-k \cdot 1})]}{C'_{t_2} - C'_{t_1}} \quad (2)$$

209 where r (dimensionless) is the NH_2OH :final product ratio between t_1 and t_2 , C_{t_1} and C_{t_2} (μM) are the
 210 measured NH_2OH concentrations at t_1 and t_2 , respectively, C_i (μM) is the interpolated NH_2OH
 211 concentration between times t_1 and t_2 ($t_2 - t_1 = 1$ hour), C'_{t_1} and C'_{t_2} (μM) are the NO_2^- or (for
 212 comammox) NO_3^- concentrations at t_1 and t_2 , and k is the average value of the measured kinetic
 213 constant for abiotic NH_2OH decay in the range of 0.5–2.5 (for HEPES buffered medium) or 0.5–5 (for
 214 CaCO_3 buffered medium) μM initial NH_2OH concentrations. Note that the presence of NO_2^- in the
 215 medium would also decrease k . As k was determined in the absence or presence of 2 mM NO_2^- , loss of
 216 NH_2OH was calculated using an average value of k determined at 0 or 2 mM NO_2^- when NO_2^-
 217 concentration in the medium was <1 mM or >1 mM, respectively. As NO_2^- concentration increased
 218 gradually with time, this definition of k would have led to overestimation or underestimation of
 219 NH_2OH when NO_2^- concentration was <1 mM or >1 mM, respectively. However, the total
 220 NH_2OH :final product ratio was very likely underestimated since higher NH_2OH concentration was
 221 detected during late growth when NO_2^- concentration was mostly >1 mM. For the comammox, NO_2^-
 222 concentration was low (<0.033 mM) at all time points and had negligible effect on calculation of
 223 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:

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

235 where C_i is the concentration of NH_2OH during the i^{th} and $(i+1)^{\text{th}}$ 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

249 **3. Results and discussion**250 *3.1 Extracellular NH₂OH from autotrophic ammonia oxidizers*

251 The NH₂OH concentration in the medium during NH₃ oxidation differed significantly among AOB
252 cultures (Fig. 1) and was highest for *N. multiformis* on initial NH₄⁺ concentrations of 0.5 and 2 mM.
253 NH₂OH release was also observed for *N. europaea*, albeit at lower concentrations than for *N.*
254 *multiformis*. No NH₂OH was detectable for *N. nitrosa* Nm90 or *N. communis* at both tested NH₄⁺
255 concentrations. Initial increases in NH₂OH concentration in cultures of *N. multiformis* and *N.*
256 *europaea* were associated with increases in NO₂⁻ concentration, but eventually reached a plateau or
257 decreased before NO₂⁻ concentration reached a maximum. The largest measured NH₂OH
258 concentrations in the medium were 2.2 and 0.78 μM, from *N. multiformis* and *N. europaea*,
259 respectively, during incubation with 2 mM NH₄⁺.

260 Several studies have determined NH₂OH concentrations in the medium during NH₃ oxidation by pure
261 cultures of the AOB *N. europaea*. Stüven et al.⁴⁴ observed 0.2–1.7 μM NH₂OH during NH₃ oxidation
262 (10 mM) and Yu and Chandran¹³ reported 0.2–3.2 μM NH₂OH during growth of *N. europaea* 19718
263 on 20 mM NH₄⁺. These findings are consistent with the NH₂OH concentrations detected for *N.*
264 *europaea* in our study, where NH₂OH concentrations were about three orders of magnitude smaller
265 than those of the produced NO₂⁻, although they did not specify whether they measured NH₂OH in
266 supernatant (as in our study) or in untreated cultures. In our experiments, *N. multiformis* NH₂OH
267 concentrations were even larger than for *N. europaea*. The exact reason for this phenomenon remains
268 unclear. One possible explanation is that *N. multiformis* biomass consumed NH₄⁺ faster (for the 0.5
269 mM NH₄⁺ treatment) than *N. europaea*, and faster NH₃ oxidation might have led to higher NH₂OH
270 release. However, the *N. communis* biomass in the batch experiments showed no detectable NH₂OH
271 release into the medium even though it had the highest NH₃ oxidation rates. Although *N. communis*
272 prefers higher concentrations of NH₄⁺ (10–50 mM)⁴⁵, the absence of NH₂OH could be due to
273 complete consumption by HAO and conversion to NO₂⁻, assuming that HAO activity in *N. communis*
274 is larger than in other AOB. Moreover, *N. communis* is unable to tolerate >100 μM NH₂OH in contrast
275 to tolerance of 250 μM NH₂OH by *N. europaea* and *N. multiformis*¹², which may relate to the absence

276 of NH_2OH in the medium of *N. communis*, although the exact mechanism for the low tolerance of
277 NH_2OH by *N. communis* is still not clear. NH_3 oxidation by *N. nitrosa* Nm90 was lower than by the
278 other tested AOB strains, possibly explaining the lack of detectable NH_2OH release.

279 Among the three AOA pure cultures, NH_2OH release was detected from the thermal spring isolate *N.*
280 *gargensis* growing on 2 mM initial NH_4^+ , but not on 0.5 mM NH_4^+ (Fig. 2). The pattern of NH_2OH
281 release by *N. gargensis* differed from that of AOB, with a small but rather constant increase in NH_2OH
282 during incubation on 2 mM NH_4^+ , resulting in a final NH_2OH concentration of 0.33 μM in the medium
283 after 58 hours. In contrast, NH_3 oxidation by the soil AOA *N. viennensis* and *Ca. N. sp. Nd2* was not
284 associated with detectable NH_2OH release (Fig. 2). The NO_2^- production rate by the AOA enrichment
285 *N. uzonensis* (~0.3 mM NO_2^- produced within 104 hours) was similar at the two initial NH_4^+
286 concentrations, but more NH_2OH (0.34 μM) was observed at the end of the incubation at 2 than 0.5
287 mM NH_4^+ initial concentration.

288 No published AOA genome contains an obvious homologue of the HAO of AOB, or of cytochromes
289 c_{554} and c_{m552} that are considered critical for energy conversion²¹, initially casting some doubt on
290 the role of NH_2OH as an intermediate in NO_2^- formation by AOA²¹. However, Vajjala et al.²⁶
291 reported the production of NH_2OH in the marine AOA *N. maritimus* during NH_3 oxidation.
292 Furthermore, Kozłowski et al.²² showed that the addition of NH_2OH to a culture of *N. viennensis*
293 resulted in respiration and NO_2^- formation and thus the most current model of AOA physiology
294 postulates a yet undiscovered novel hydroxylamine-converting enzyme²¹. The data from the *N.*
295 *uzonensis* enrichment culture, that does not contain any known AOB³⁸, confirms the *N. gargensis* data
296 in showing that some AOA release NH_2OH . Also, in a preliminary experiment, *N. gargensis* could
297 convert NH_2OH to NO_2^- biotically, especially at lower NH_2OH levels (Experiment S1, Fig. S1).
298 Stieglmeier et al.¹⁵ observed aerobic N_2O production by *N. viennensis* and attributed this to hybrid
299 formation of N_2O via an N-nitrosating reaction. Kozłowski et al.²² later reported that N_2O formation
300 from *N. viennensis* could be attributed to abiotic reactions between NO and medium substances during
301 growth, especially under anoxic conditions. It is tempting to speculate that the aerobic hybrid
302 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

329 rate was faster in CaCO_3 than in HEPES-buffered media: 0.5 to 2.5 μM NH_2OH decayed abiotically at
330 30°C within ~ 8 h and ~ 30 h in the CaCO_3 and HEPES-buffered media, respectively. Consequently, the
331 first-order rate constants for abiotic NH_2OH decay were much higher in the CaCO_3 than in the
332 HEPES-buffered media, with an average value approximately fourfold larger in the former (0.71 vs.
333 0.16) (Table S2). Temperature increased the rate of abiotic NH_2OH decay (with a single exception, see
334 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
335 average first-order rate constant was $\sim 80\%$ greater at 46°C (1.31) than at 30°C (0.71). Nitrite, however,
336 unexpectedly inhibited abiotic NH_2OH decay in both media tested (Figure 4, Table S2), although NO_2^-
337 is known to oxidize NH_2OH to N_2O , albeit preferentially at low pH (e.g., Heil et al., 2014³⁴). This
338 stabilizing effect of NO_2^- was particularly pronounced at higher temperatures for the CaCO_3 medium,
339 where the first-order rate constant decreased by 52% for 2 mM NO_2^- at 46°C compared to the absence
340 of NO_2^- . To exclude the possibility of abiotic conversion of NO_2^- to NH_2OH by components of the
341 medium, an additional test was conducted using the more active CaCO_3 -buffered medium (compared
342 to the HEPES-buffered medium) at the highest culture incubation temperature, but no abiotic
343 conversion of NO_2^- to NH_2OH occurred (Experiment S3). An additional ^{15}N - NO_2^- experiment showed
344 that NO_2^- did not interfere with the NH_2OH analysis (Experiment S4, Table S3). Under alkaline
345 conditions, one product of NH_2OH abiotic decay is NO_2^- ⁴⁷, which has been also observed in abiotic
346 NH_2OH decay experiments in CaCO_3 -buffered medium in this study (Experiment S5, Fig. S3). In
347 addition to NO_2^- and N_2O , nitrogen dioxide (NO_2), but almost no NO , was observed during the abiotic
348 NH_2OH decay (Fig. S2). The presence of NO_2 may explain the observation of abiotic NH_2OH -to- NO_2^-
349 conversion as NO_2 is highly reactive and can hydrolyze to nitric acid (HNO_2) and nitrous acid (HNO_3)
350 in aqueous solution. Consequently, NO_2^- , N_2O and NO_2 comprised approximately 18.5%, 9.8% and
351 32.1%, respectively, of the abiotically decayed NH_2OH in the CaCO_3 -buffered medium (Fig. S2, S3).
352 Therefore, a possible reason for the inhibitory effect of NO_2^- on the abiotic NH_2OH decay could be
353 that the presence of NO_2^- slowed down the transformation of NH_2OH to NO_2^- by inhibiting the
354 disproportionation of NO_2 , one of the primary decay products of NH_2OH , to HNO_3 and HNO_2 .

355 The effect of temperature on abiotic NH_2OH decay was as expected, as NH_2OH is extremely unstable
356 and reactive, especially at higher temperatures⁴⁷. 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 decomposition⁴⁷. 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

384 content, pH and content of suitable oxidants like MnO_2 and Fe^{3+} will also affect the chemical N_2O
385 conversion ratio from NH_2OH ^{27, 29}. The abiotic $\text{N}_2\text{O}:\text{NH}_2\text{OH}$ conversion ratio was 12–14% for the
386 HEPES-buffered medium at 30°C in the absence of NO_2^- , and between 18% and 37% for the same
387 medium with 1 and 2 mM NO_2^- , respectively (Table 2). The ratio in CaCO_3 -buffered medium at 30°C
388 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,
389 for the NH_2OH concentration range from 0.5 to 2.5 μM . The contribution of NO_2^- to N_2O production
390 involving NH_2OH was even larger at higher temperature, e.g. 46°C (Table 2). The stimulated
391 conversion of NH_2OH to N_2O by NO_2^- was likely caused by the hybrid reaction of NO_2^- and NH_2OH .
392 However, another mechanism could be inhibition of NH_2OH conversion to $\text{NO}_2/\text{NO}_2^-$ by NO_2^- , thereby
393 channeling NH_2OH to N_2O indirectly via other mechanisms.

394 The total fraction of NH_4^+ converted to N_2O through extracellular NH_2OH and substances in the
395 medium over the whole incubation period was then calculated according to Equation 3 (Table 3). The
396 total fraction of NH_4^+ converted to N_2O by this mechanism was 0.05% and 0.12% for *N. multiformis*
397 incubated at 0.5 and 2 mM initial NH_4^+ , respectively, which is consistent with that emitted as N_2O
398 (0.05–0.1%) during aerobic incubation of a *Nitrosospira* strain ^{6, 48}. The fraction of NH_4^+ converted to
399 N_2O by *N. europaea* was lower than that of *N. multiformis*, but still consistent with that converted to
400 N_2O by *N. europaea* reported by other studies, e.g., 0.05–1.95% ⁴⁹ and 0.05–0.15% ⁵⁰. Dundee and
401 Hopkins ⁵¹ also reported that *N. multiformis* produced more N_2O than *N. europaea* at greater dissolved
402 O_2 concentrations, while *N. europaea* produced much more N_2O during nitrifier-denitrification than *N.*
403 *multiformis*, which is consistent with our finding that the fraction of NH_4^+ converted to N_2O was larger
404 for *N. multiformis* than for *N. europaea* under ambient air conditions.

405 The AOA *N. viennensis* and *N. maritimus* are reported to be incapable of canonical nitrifier-
406 denitrification at reduced O_2 concentration, but produce N_2O via hybrid formation, as revealed by ¹⁵N-
407 labeling ¹⁵. In the present study, potential abiotic N_2O production was approximately 0.08% of the
408 total substrate turnover during aerobic NH_3 oxidation by AOA. Albeit this value was found only in *N.*
409 *gargensis*, it was close to the values reported for *N. viennensis* (0.09%) and *N. maritimus* (0.05%) by
410 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 N₂O emissions from comammox organisms are yet available for comparison.

413 In summary, we show that extracellular NH₂OH is formed in growth media during aerobic NH₃
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 NH₄⁺ converted
416 to N₂O by abiotic reactions between extracellular NH₂OH and substances in the growth medium
417 during aerobic NH₃ oxidation, was in the range of values reported previously for the conversion of
418 substrate to N₂O for various AOB and AOA. The presence of NO₂⁻ in the medium not only offers a
419 reactant for hybrid N₂O formation from NH₂OH, but also delays overall NH₂OH abiotic decay, further
420 stimulating the conversion of NH₂OH to N₂O. 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 NH₂OH might
422 contribute to aerobic ammonia-oxidizer-associated N₂O formation. In others, e.g. *N. viennensis*, no
423 extracellular NH₂OH was observed during NH₃ oxidation but aerobic N₂O production has been
424 reported¹⁵, indicating a different mechanism, e.g. the abiotic reactions between intracellular NH₂OH
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 NO_x 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.

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597 **Tables**

598 **Table 1** Total NH₂OH:final product (NO₂⁻ or NO₃⁻) ratios after incubation for 58 h for different
 599 ammonia oxidizers. [§] For *Ca. N. inopinata* (a comammox organism), NO₃⁻ is the final product of NH₃
 600 oxidation. [#] The NH₂OH concentration here is the total extracellular NH₂OH including the calculated
 601 concentration of NH₂OH that was abiotically converted during incubation.

Cultures	Initial NH ₄ ⁺ concentration (mM)	Final NO ₂ ⁻ or NO ₃ ⁻ [§] concentration (μM)	NH ₂ OH [#] concentration (μM)	NH ₂ OH:final product ratio (%)
<i>N. multiformis</i>	0.5	516	1.8	0.34
	2	1955	11.0	0.56
<i>N. europaea</i>	0.5	537	1.8	0.33
	2	1930	4.7	0.24
<i>N. gargensis</i>	2	1860	7.1	0.46
<i>Ca. N. inopinata</i>	0.5	280	1.8	0.63
	2	490	9.4	1.92

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605 **Table 2** Fraction (%) of N₂O abiotically produced from the added NH₂OH in the different media at
 606 various levels of NH₂OH (0.5, 1 and 2.5 μM) and NO₂⁻ (0, 1 and 2 mM).

NH ₂ OH (μM)	0 mM NO ₂ ⁻			1 mM NO ₂ ⁻			2 mM NO ₂ ⁻		
	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

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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 (%)
<i>N. multiformis</i>	500	0.05
	2000	0.12
<i>N. europaea</i>	500	0.05
	2000	0.07
<i>N. gargensis</i>	2000	0.08
<i>Ca. N. inopinata</i>	500	0.06
	2000	0.14

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615 **Figure captions**

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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 \pm 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 \pm standard error (SE).

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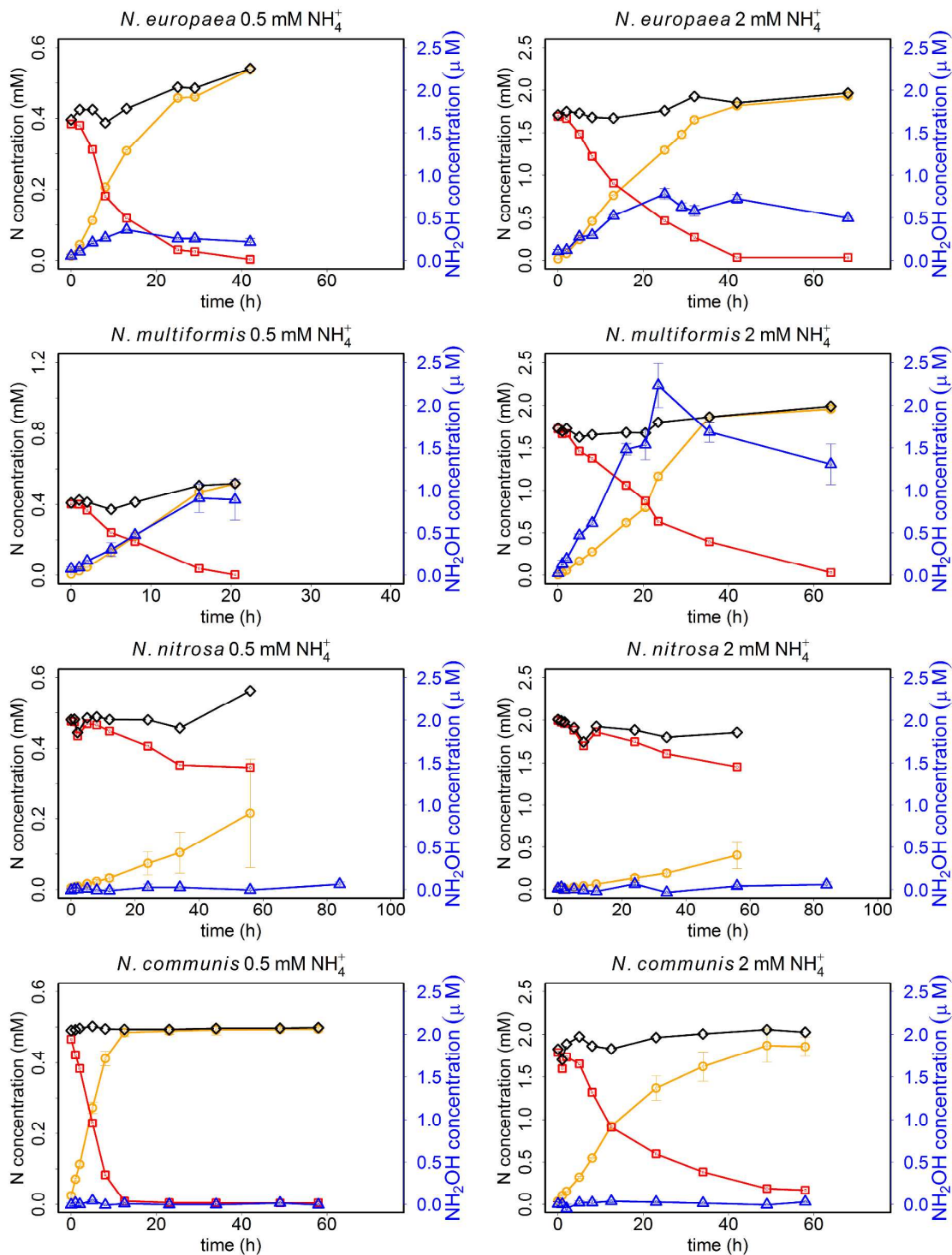
629 **Figure 3** Dynamics of NH_4^+ (red squares), NO_3^- (yellow circles), NH_2OH (blue triangles) and total N
630 (sum of NO_3^- and NH_4^+ , black diamonds) concentrations during the incubation of the comammox
631 organism *Ca. N. inopinata*. NH_4^+ , NO_3^- and total N are plotted using the left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

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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.

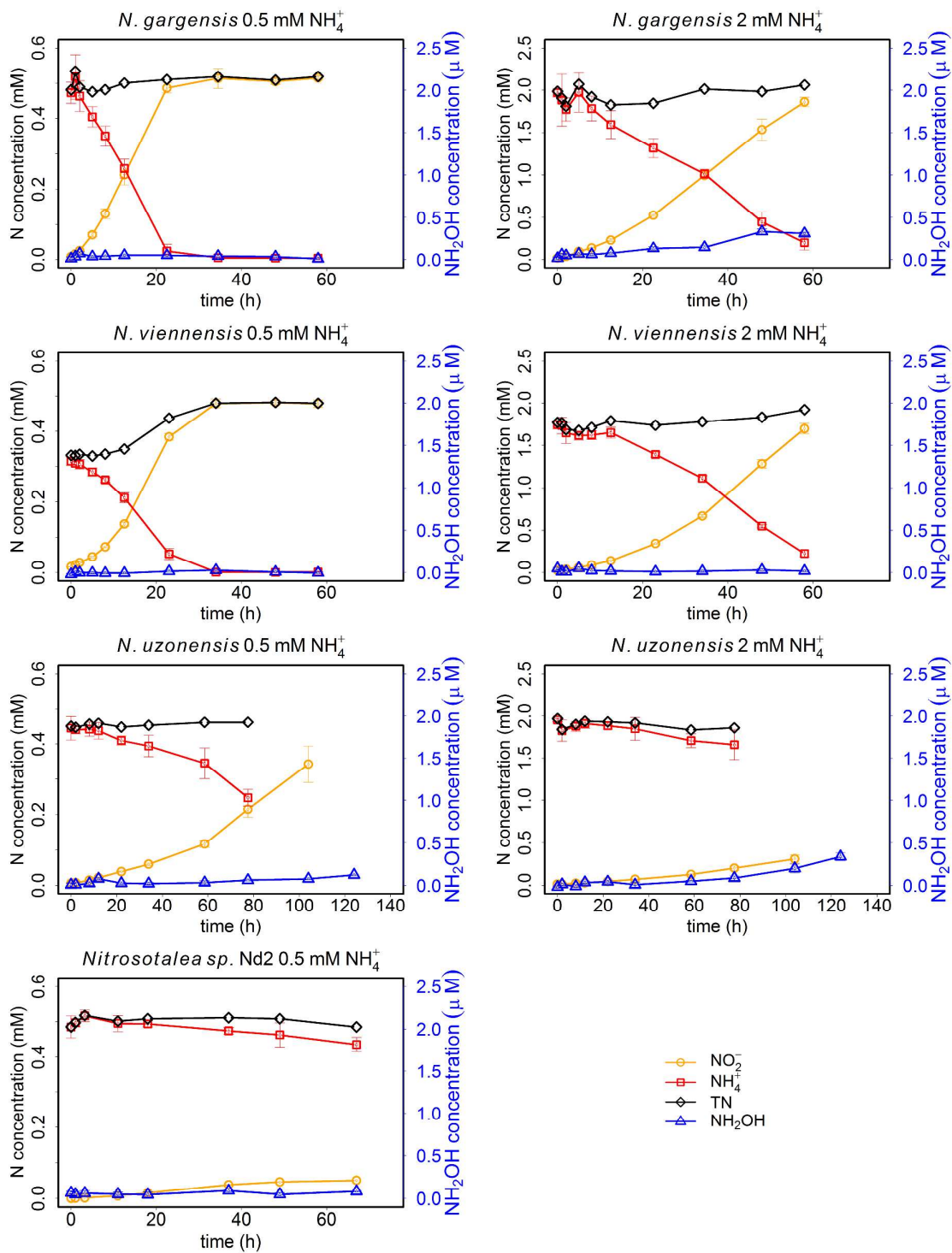
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641 **Figures**

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643 **Figure 1**

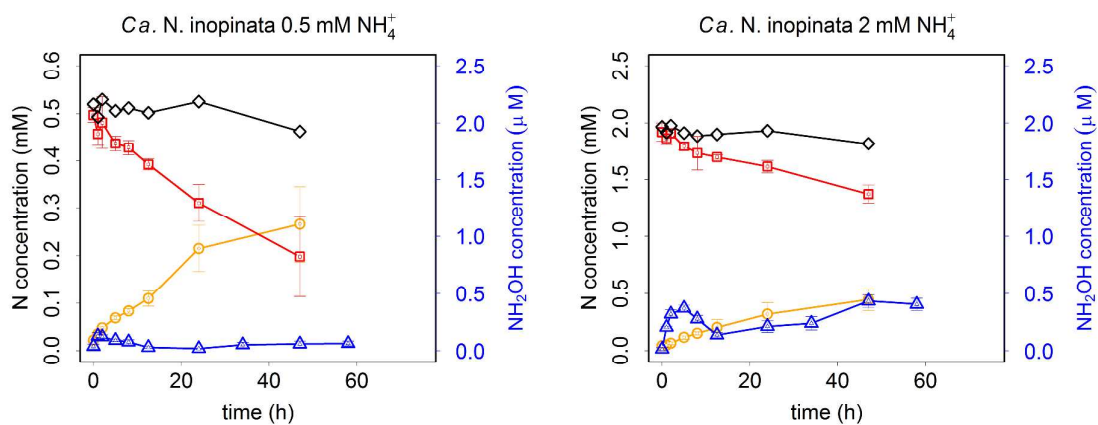


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645 **Figure 2**

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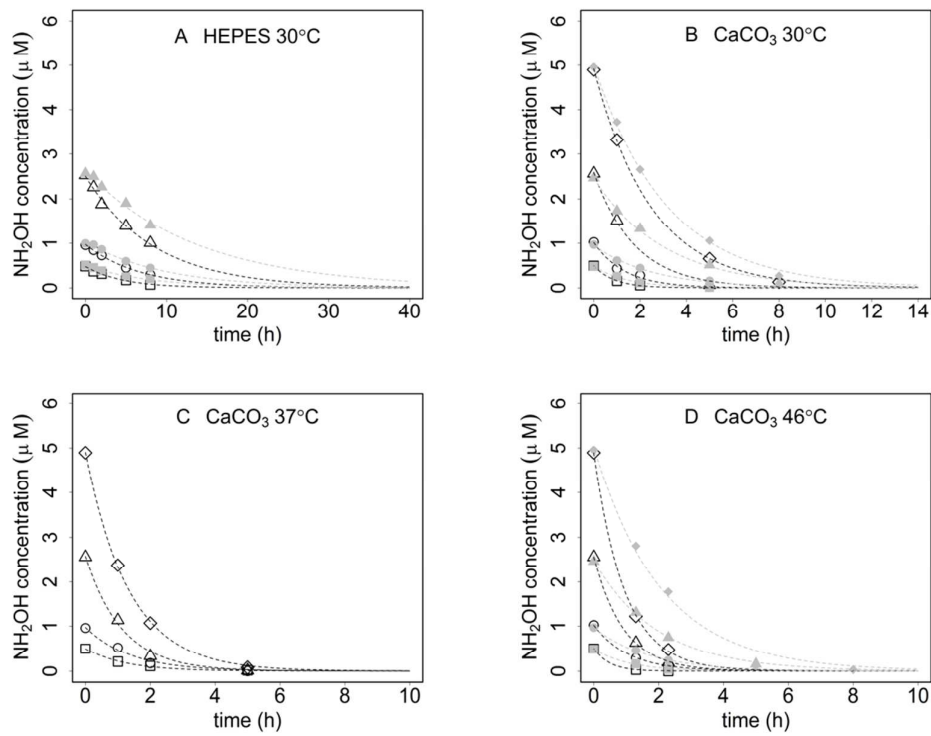


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649 **Figure 3**

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653 **Figure 4**

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