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Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities 1

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Man-Young Jung^{1,2,3,#,*}, Christopher J. Sedlacek ^{1,4,#,*}, K. Dimitri Kits¹, Anna J. Mueller¹, Sung-3 Keun Rhee⁵, Linda Hink^{6,&}, Graeme W. Nicol⁶, Barbara Bayer^{1,7,\$}, Laura Lehtovirta-Morley⁸, 4 Chloe Wright⁸, Jose R. de la Torre⁹, Craig W. Herbold¹, Petra Pjevac^{1,10}, Holger Daims^{1,4}, Michael 5 Wagner^{1,4,11} 6 7 ¹University of Vienna, Centre for Microbiology and Environmental Systems Science, Department 8 of Microbiology and Ecosystem Science, Althanstrasse 14, A-1090 Vienna, Austria 9 ²Jeju National University, Department of Biology Education, 102 Jejudaehak-ro, Jeju 63243,

- 10
- 11 Republic of Korea
- 12 ³Jeju National University, Interdisciplinary Graduate Program in Advance Convergence
- 13 Technology and Science, 102 Jejudaehak-ro, Jeju 63243, Republic of Korea
- 14 ⁴The Comammox Research Platform, University of Vienna, Althanstrasse 14, A-1090 Vienna,
- 15 Austria
- 16 ⁵Chungbuk National University, Department of Microbiology, 1 Chungdae-ro, Seowon-Gu,
- 17 Cheongju 28644, South Korea
- ⁶Environmental Microbial Genomics Group, Laboratoire Ampere, École Centrale de Lyon, 18
- Université de Lyon, 69134 Ecully cedex, France 19
- 20 ⁷University of Vienna, Division of Bio-Oceanography, Department of Limnology and Bio-
- 21 Oceanography, Center of Functional Ecology, Althanstrasse 14, A-1090 Vienna, Austria
- 22 ⁸University of East Anglia, School of Biological Sciences, Norwich Research Park, Norwich NR4
- 23 7TJ, UK

24	⁹ San Francisco State University, Department of Biology, San Francisco, CA 94132, United States
25	¹⁰ Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna,
26	Austria
27	¹¹ Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg
28	University, Aalborg, Denmark
29	
30	^{&} Present address: Leibniz University Hannover, Institute of Microbiology, 30419 Hannover,
31	Germany
32	^{\$} Present address: Department of Ecology, Evolution and Marine Biology, University of California,
33	Santa Barbara, USA
34	
35	[#] M-Y.J and C.J.S contributed equally to this work.
36	*To whom correspondence should be addressed. E-mail: <u>sedlacekc88@univie.ac.at</u> ,
37	myjung@jejunu.ac.kr
38	
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42 Ammonia-oxidizing archaea, substrate kinetics, niche differentiation, comammox, nitrification

43 Abstract

44 Nitrification, the oxidation of ammonia to nitrate, is an essential process in the biogeochemical 45 nitrogen cycle. The first step of nitrification, ammonia oxidation, is performed by three, often co-46 occurring guilds of chemolithoautotrophs: ammonia-oxidizing bacteria (AOB), archaea (AOA), 47 and complete ammonia oxidizers (comammox). Substrate kinetics are considered to be a major niche-differentiating factor between these guilds, but few AOA strains have been kinetically 48 49 characterized. Here, the ammonia oxidation kinetic properties of 12 AOA representing all major 50 phylogenetic lineages were determined using microrespirometry. Members of the genus 51 *Nitrosocosmicus* have the lowest substrate affinity of any characterized AOA, which are similar 52 to previously determined affinities of AOB. This contrasts previous assumptions that all AOA 53 possess much higher substrate affinities than their comammox or AOB counterparts. The substrate 54 affinity of ammonia oxidizers correlated with their cell surface area to volume ratios. In addition, 55 kinetic measurements across a range of pH values strongly supports the hypothesis that – like for AOB – ammonia and not ammonium is the substrate for the ammonia monooxygenase enzyme of 56 57 AOA and comammox. Together, these data will facilitate predictions and interpretation of 58 ammonia oxidizer community structures and provide a robust basis for establishing testable 59 hypotheses on competition between AOB, AOA, and comammox.

60 Introduction

61 Nitrification, the microbially mediated oxidation of ammonia (NH_3) to nitrate (NO_3) via nitrite (NO_2) , is a key process of the biogeochemical nitrogen cycle ^{1,2} and is mostly driven by 62 63 autotrophic microorganisms that are capable of growing with NH_3 and/or NO_2^- as sole energy generating substrates. For more than a century, ammonia-oxidizing bacteria (AOB) were 64 65 considered the lone drivers of aerobic ammonia oxidation by autotrophs, as ammonia-oxidizing archaea (AOA) ^{3,4} and complete ammonia oxidizers (comammox) ⁵⁻⁷ eluded discovery until 66 relatively recently. Our present-day understanding of ammonia oxidation is quite different: AOA 67 frequently outnumber AOB in oligotrophic habitats⁸⁻¹⁰, while AOB often dominate in eutrophic 68 environments¹¹⁻¹⁴. Comammox have been shown to be abundant and even dominant in various 69 natural and engineered environments ¹⁵⁻¹⁹, although the habitat range and ecophysiology of 70 71 comammox remains less well resolved. Notably, in the majority of ecosystems – with the 72 exception of the marine environment, where no comammox has been detected – AOA, AOB, and 73 comammox often co-occur.

Many environmental and physiological factors are known to affect the niche differentiation and habitat selection of ammonia-oxidizing microorganisms (AOM) ^{20,21}. In fact, AOM species display differential responses to factors such as pH, oxygen concentrations, light conditions, temperature, metal and organic compounds, and substrate concentrations ²²⁻²⁷. These differential responses are frequently used to explain the co-occurence of AOM across environments. However, the cellular properties underlying these niche-differentiating physiological characterstics of AOM often remain unclear.

81 The substrate affinity of a microorganism can be expressed with Michaelis-Menten kinetic 82 equations, analogous to enzyme kinetics, defined by an apparent-half-saturation activity ($K_{m(app)}$)

and a maximal activity rate (V_{max}). In addition, the specific substrate affinity (a^{o}) takes into account 83 84 both the cellular $K_{m(app)}$ and V_{max} , and is thus an appropriate measure for comparing interspecies competitiveness ²⁸. Based on whole cell kinetic properties, AOM were observed to have different 85 86 survival or lifestyle strategies. The first study investigating the whole cell kinetics of an AOA 87 revealed that *Nitrosopumilus maritimus* SCM1 displayed a low maximum NH₃ oxidation activity rate, but a very high substrate affinity for NH₃ and a° , compared with AOB ²⁹. Based on these 88 89 findings with a single AOA strain, substrate affinity was postulated as a major niche-differentiating factor between AOA and AOB ^{20,29}. However, recently it was shown that (i) the only comammox 90 91 isolate *Nitrospira inopinata* has a $K_{m(app)}$ for NH₃ lower than that of all characterized AOB and (ii) that the $K_{m(app)}$ for NH₃ in a few non-marine AOA strains is not always orders of magnitude lower 92 93 than that of AOB⁵. Nevertheless, the AOA with comparatively high $K_{m(aDD)}$ for NH₃ (low affinity) 94 still possess a significantly higher a° than AOB, indicating that these AOA are still more efficient substrate scavengers⁵. Furthermore, temperature and pH, which are known niche-differentiating 95 factors ³⁰⁻³², have previously been shown to affect the substrate affinity of AOB ³³⁻³⁵, but the 96 97 influence of these parameters on the substrate affinity of AOA and comammox remains to be determined. 98

In this study, the whole cell kinetic properties of twelve AOA species were determined through instantaneous substrate-dependent microrespirometry (MR) experiments. These include representatives from all four major AOA phylogenetic lineages, isolated or enriched from various habitats (i.e. marine, terrestrial, and geothermal) and possessing a wide variety of pH and temperature growth optima. In these analyses, we also explored the links between the cellular $K_{m(app)}$ and a° of AOM with their cell surface area to volume ratio. Furthermore, by performing

- 105 MR experiments at different pH values we investigated whether the undissociated NH₃ or
- 106 ammonium (NH_4^+) is the substrate for AOA and comammox.

107

109 Results and Discussion

110 AOA kinetic properties

111 In this study we investigated the kinetic properties of twelve AOA strains, including 112 representatives from all four described AOA phylogenetic lineages: *Nitrosopumilales* (Group I.1a), 113 'Ca. Nitrosotaleales' (Group I.1a-associated), Nitrososphaerales (Group I.1b), and 'Ca. Nitrosocaldales' (thermophilic AOA clade) ^{36,37} (Fig. 1). These AOA isolates and enrichments 114 115 were obtained from a variety of habitats (marine, soil, sediment, hot spring) and have optimal 116 growth pH and temperatures ranging from 5.3-7.8 and 25-72°C, respectively (Supplementary 117 Table 2). The substrate-dependent oxygen consumption rates for all AOA tested followed 118 Michaelis-Menten kinetics. Below, the kinetic properties of these AOA are put into a broader 119 context with comparisons to previously characterized AOM.

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121 *Nitrosopumilales* (Group I.1a). From this lineage, three mesophilic marine (*N. piranensis* D3C, *N. adriaticus* NF5, and *N. maritimus* SCM1)^{3,38}, two agricultural soil (*N. koreense* MY1 and '*Ca.* 122 N. chungbukensis' MY2) 39,40 and one thermal spring isolate ('*Ca*. N. uzonensis' N4)⁴¹ were 123 124 kinetically characterized (Supplementary Fig. 1). These AOA all displayed a high substrate affinity 125 for NH₃, ranging from ~2.2 to 24.8 nM. Thus, all characterized *Nitrosopumilales*, and not just 126 marine isolates, are adapted to oligotrophic conditions. All possess substrate affinities several 127 orders of magnitude higher (lower $K_{m(app)}$) than any characterized AOB, with the exception of the 128 recently characterized acidophilic gammaproteobacterial AOB 'Ca. Nitrosacidococcus tergens' ⁴² 129 (Fig. 2a). This finding appears to support the widely reported hypothesis that regardless of the environment, AOA in general are adapted to lower substrate concentrations than AOB ^{22,29,30}. 130 131 However, as described later, this trend does not apply to all AOA.

132 As the substrate oxidation kinetics of the marine AOA strain, N. maritimus SCM1, originally characterized by Martens-Habbena et al.²⁹ have recently been disputed ⁴³, they were 133 134 revisited in this study (Supplementary Fig. 2). With the same strain of *N. maritimus* used in Hink et al., ⁴³ (directly obtained by the authors), we were able to reproduce (Supplementary Figs. 1 and 135 2) the original kinetic properties of *N. maritimus* SCM1 reported in Martens-Habbena *et al.*²⁹ 136 137 ruling out strain domestication during lab propagation as cause for the observed discrepancy. 138 Therefore, the reported differences in the literature possibly reflect the measurements of two distinct cellular properties, $K_{m(app)}^{29}$ and K_s^{43} , representing the half saturation of activity and 139 140 growth, respectively. In addition, differences in pre-measurement cultivation and growth conditions could also contribute to these unexpected differences ^{43,44}. More details are provided in 141 142 the Supplementary Results and Discussion.

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144 '*Ca.* Nitrosotaleales' (Group I.1a-associated). The only isolated AOA strains in this lineage '*Ca.* 145 Nitrosotalea devanaterra' Nd1 and 'Ca. Nitrosotalea sinensis' Nd2, are highly adapted for survival in acidic environments and grow optimally at pH 5.3 ^{25,45}. Both display a relatively low affinity 146 147 for total ammonium ($K_{m(app)} = 3.41$ to 11.23 μ M), but their affinity for NH₃ is among the highest 148 calculated of any AOA characterized ($K_{m(app)} = \sim 0.6$ to 2.8 nM) (Fig. 2a,c, and Supplementary Fig. 149 3). This seemingly drastic difference in substrate affinity for total ammonium versus NH_3 is due 150 to the high acid dissociation constant of ammonium (pKa = 9.25). The very limited availability of 151 NH₃ under acidic conditions has led to the hypothesis that these acidophilic AOA should be highly 152 adapted to very low NH₃ concentrations and possess a high substrate affinity (low $K_{m(aDD)}$) for NH₃ ^{46,47}. Our data corroborate this hypothesis. 153

155 Nitrososphaerales (Group I.1b). The AOA strains 'Ca. N. nevadensis' GerE (culture information provided in Supplementary Results and Discussion), 'Ca. N. oleophilus' MY3⁴⁸ and 'Ca. N. 156 franklandus' C13⁴⁹ were kinetically characterized, and contextualized with the previously 157 158 published kinetic characterization of Nitrososphaera viennensis EN76 and 'Ca. Nitrososphaera 159 gargensis' ⁵. Together, the Nitrososphaerales AOA possess a wide range of affinities for NH₃ 160 $(K_{m(app)} = ~0.14 \text{ to } 31.5 \ \mu\text{M})$ (Fig. 2a and Supplementary Fig. 4). Although this range of NH₃ 161 affinities spans more than two orders of magnitude, none of the Nitrososphaerales AOA possess 162 an affinity for NH₃ as high as any *Nitrosopumilales* or 'Ca. Nitrosotaleales' AOA (Fig. 2a).

163 The moderately thermophilic enrichment culture 'Ca. N. nevadensis' GerE displayed a higher substrate affinity (lower $K_{m(app)}$) for NH₃ (0.17 ± 0.03 µM) than the other characterized 164 165 AOA strains within the genus Nitrososphaera (Fig. 2a). In contrast, 'Ca. N. oleophilus' MY3 and 166 'Ca. N. franklandus' C13, which belong to the genus Nitrosocosmicus, had the lowest affinity 167 (highest $K_{m(app)}$) for NH₃ (12.37 ± 6.78 µM and 16.32 ± 14.11 µM, respectively) of any AOA 168 characterized to date. In fact, their substrate affinity is comparable to several characterized AOB 169 (Fig. 2a). In this context it is interesting to note that several *Nitrosocosmicus* species have been shown to tolerate very high ammonium concentrations ⁴⁸⁻⁵⁰, a trait usually associated with AOB 170 171 ^{24,51}. The low substrate affinity observed in *Nitrosocosmicus* AOA correlates with the absence of 172 a putative Amt-type high affinity ammonium transporter in the genome of any sequenced *Nitrosocosmicus* species to date ^{48,49,52}. 173

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175 *'Ca.* Nitrosocaldales' (Thermophilic AOA lineage). The thermophilic AOA enrichment cultures, 176 *'Ca.* Nitrosocaldus yellowstonensis' HL72 ⁵³ and *'Ca.* N. tenchongensis' DRC1 (culture 177 information provided in Supplementary Results and Discussion), possess affinities for NH_3 ($K_{m(app)}$ 178 = $\sim 1.36 \pm 0.53 \mu$ M and $\sim 0.83 \pm 0.01 \mu$ M) for NH₃ comparable to AOA within the genus 179 *Nitrososphaera* (Fig. 2a). Notably, the substrate oxidation rate of these two AOA quickly dropped 180 with increasing substrate concentrations after V_{max} was reached (Supplementary Fig. 5). This trend 181 was not observed with any other AOA tested here and may reflect an increased susceptibly to NH₃ 182 stress at high temperatures, as the free NH₃ concentration increases with increasing temperatures 183 ³³.

184

185 Together, these results highlight that the substrate affinity for NH_3 among AOA species is much 186 more variable than previously hypothesized, spanning several orders of magnitude and in some 187 cases overlapping with the substrate affinity values of characterized non-oligotrophic AOB. In 188 addition, the substrate affinity of AOA is related, to a certain degree, to their phylogenetic placement within each of the four AOA phylogenetic lineages mentioned above (Fig. 2). Although 189 190 the substrate affinity ranges of these AOA lineages overlap, the link between AOA phylogeny and kinetic properties provides deeper insights into the physiological and evolutionary differences 191 192 among AOA species. While substrate affinity is certainly one of multiple factors that contribute to 193 niche differentiation between AOM, it is more complex than the prevailing dogma that all AOA 194 have a higher substrate affinity than their AOB counterparts and may be as significant in 195 differentiating the niches of different AOA species as between AOA and AOB.

196

197 **Maximum substrate oxidation rates** (V_{max}). The normalized maximum substrate oxidation rate 198 of all the AOA characterized to date only span about one order of magnitude from 4.27 to 54.68 199 µmol N mg protein⁻¹ h⁻¹. These normalized AOA V_{max} values are in the same range as the recorded 200 V_{max} for the comammox *N. inopinata* (~12 µmol N mg protein⁻¹ h⁻¹) and the marine AOB strain

Nitrosococcus oceani ATCC 19707 (~38 µmol N mg protein⁻¹ h⁻¹) but are lower than the 201 202 normalized V_{max} of the AOB Nitrosomonas europaea ATCC 19718 (average of 84.2 µmol N mg protein⁻¹ h⁻¹; Fig. 2e). The high V_{max} value for N. europaea is the only real outlier among the AOM 203 204 characterized to date and it remains to be determined whether other AOB related to N. europaea 205 also possess such a high V_{max} or if members of the *Nitrosomonadales* possess a broad range of 206 V_{max} values. Similarly, as additional comammox strains become available as pure cultures their 207 kinetic characterization will be vital in understanding the variability of these ecologically 208 important parameters within this guild.

209

210 **Specific substrate affinity** (a°). Although the $K_{m(app)}$ and V_{max} of AOM can be compared by 211 themselves and provide useful information on cellular properties, the ability of an AOM to 212 scavenge (and compete for) substrate from a dilute solution is most appropriately represented by the a° , which takes into account both the cellular $K_{m(app)}$ and V_{max}^{28} . In previous studies, the a° of 213 214 AOM has been calculated using the $K_{m(app)}$ value for total ammonium (NH₃ + NH₄⁺) and not the 215 $K_{\rm m(app)}$ value for NH₃^{5,29}. Calculating the $a^{\rm o}$ based on the $K_{\rm m(app)}$ value for total ammonium allows 216 for the a° of AOM to be compared with the a° of microorganisms that do not use NH₃ as a sole 217 energy generating substrate, such as ammonium assimilating heterotrophic bacteria or diatoms²⁹. 218 While this is useful when evaluating competition for total ammonium in mixed communities or 219 environmental settings, an a° calculated using the $K_{m(app)}$ value for NH₃ may be more useful when 220 directly comparing the interspecies competitiveness of AOM for the following reasons: i) the 221 substrate for all AOM is NH₃ and not NH₄⁺ (see below) and ii) the $K_{m(app)}$ value for total ammonium 222 is more dependent on the environmental factors it was measured at (e.g. pH, temperature, salinity) 223 than the $K_{m(app)}$ for NH₃.

All characterized AOA (with the exception of representatives of the genus *Nitrosocosmicus*) and the comammox bacterium *N. inopinata* possess much higher a° for total ammonium or NH₃ (~10 to 3000×) than the AOB, *N. oceani* or *N. europaea* (Fig. 2b,d), indicating that they are highly competitive in environments limited in either total ammonium or only NH₃. However, due to the low number of published normalized V_{max} values for AOB, a° could only be calculated for these two AOB representatives. Thus, extrapolations to the a° of all AOB species, based solely on these observations should be approached with caution.

231 The low variation in experimentally measured V_{max} values (Fig. 2e) across all measured 232 AOM in combination with the high variation in $K_{m(app)}$ values leads to a strong relationship 233 between cellular a° and the reciprocal of $K_{m(app)}$ (Fig. 3) according to equation 2 (see Materials and 234 Methods). AOM adapted to oligotrophic (low substrate) conditions should possess both a high substrate affinity (low $K_{m(app)}$) and a high $a^{\circ 28}$. Therefore, the AOM best suited for environments 235 236 limited in total ammonium are the AOA belonging to the Nitrosopumilales and the comammox 237 isolate N. inopinata, (top right corner of Fig. 3a). Overall, when looking at solely NH_3 or total 238 ammonium, the separation of species in these plots remains similar, with the exception that the 239 acidophilic AOA belonging to the 'Ca. Nitrosotaleales' are predicted to be best suited for life in 240 environments limited in NH₃ (Fig. 3b). The adaptation correlates well with the fact the AOA 'Ca. 241 Nitrosotalea devanaterra' Nd1 and 'Ca. Nitrosotalea sinensis' Nd2 were isolated from acidic soils with a pH of 4.5 and 4.7, respectively ^{25,45}, where NH₃ is limiting even when total ammonium is 242 243 not.

In either case, when looking at NH₃ or total ammonium, the AOA belonging to the genus *Nitrosocosmicus* (*Ca.* N. oleophilus' MY3 and *Ca.* N. franklandus' C13) and AOB populate the lower left section of these plots, indicating that they are not strong substrate competitors in NH₃ or total ammonium limited environments (Fig. 3). If the cellular kinetic property of V_{max} really is so similar across all AOB, AOA, and comammox species (Fig. 2e), then substrate competitiveness can be predicted from an AOMs $K_{\text{m(app)}}$ for either NH₃ or total ammonium (Fig. 2a,c). This is especially helpful when characterizing enrichment cultures, where normalizing ammoniaoxidizing activity to cellular protein in order to obtain a comparable V_{max} value is not possible. However, there is also a need for more kinetically characterized AOB and comammox species to confirm this hypothesis.

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255 The effect of environmental and cellular factors on AOA kinetic properties

256 The concentration of NH_3 present in a particular growth medium or environment can vary by orders of magnitude, based solely on the pH, temperature, or salinity of the system ⁵⁴. This is 257 258 notable because at a given total ammonium concentration, the concentration of NH_3 is ~10 times 259 higher at 70°C versus 30°C and ~1000 times lower at pH 5.3 versus pH 8.4 (representative of 260 maximum ranges tested). While it should be recognized that in our dataset no AOM were included 261 that have a pH optimum between 5.3 and 7.0, the effect of pH and temperature on the ammonia 262 oxidation kinetics of AOM must be considered in order to understand their ecophysiological niches. 263 However, there was no correlation between the kinetic properties of AOM ($K_{m(app)}$, V_{max} , and a°) 264 measured in this study and their optimal growth temperature or pH. This lack of correlation 265 between AOM species kinetic properties and growth conditions does not imply that the cellular 266 kinetic properties of an individual AOM species will remain the same over a range of pH and 267 temperature conditions. Therefore, we investigated the effect of pH and temperature variation on the substrate-dependent kinetic properties of the AOA strain 'Ca. N. oleophilus' MY3, and the 268 269 effect of pH on the comammox strain N. inopinata.

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271 The effect of temperature. The effects of short-term temperature changes on the substrate-272 dependent kinetic properties of 'Ca. N. oleophilus' MY3 were determined. Temperature shifts of 273 5°C above and below the optimal growth temperature (30°C) had no effect on the $K_{m(app)}$ for total 274 ammonium. However, the $K_{m(app)}$ for NH₃, V_{max} , and a° of 'Ca. N. oleophilus' MY3 all increased 275 with increasing temperatures (Supplementary Fig. 6). Therefore, as temperature increased, 'Ca. N. 276 oleophilus' MY3 displayed a lower substrate affinity (higher $K_{m(app)}$ for NH₃) but would be able to 277 turnover substrate with a higher V_{max} and better compete for substrate with a higher a° . Increasing 278 AOA $K_{m(app)}$ values for NH₃ with increasing temperatures have also been observed across studies 279 with N. viennensis EN76 (Supplementary Fig. 2). These cases are discussed in more detail in the 280 Supplemental Results and Discussion. In addition, similar observations have previously been made for AOB strains belonging to the genus *Nitrosomonas* 33,34 . The increase in V_{max} and a° can be 281 282 explained in terms of the Van't Hoff rule, which states that reaction velocity increases with temperature ⁵⁵. While there is no theoretical upper limit to the reaction rate increase, the instability 283 284 of proteins at elevated temperatures eventually becomes limiting.

285 The increase in $K_{\rm m(app)}$ for NH₃ (lower NH₃ affinity) with increasing temperature is less 286 straightforward to interpret. As this is a whole cell measurement, the observed differences may 287 result from either broad cellular changes or from changes in individual enzymes involved in the 288 ammonia oxidation pathway specifically. At the cellular level, changes in the proteinaceous 289 surface layer (S-layer) or lipid cell membrane could affect substrate movement/transport and 290 enzyme complex stability. It has been suggested that the negatively charged AOA S-layer proteins 291 act as a substrate reservoir, trapping NH₄⁺ and consequently increasing the NH₃ concentration in the AOA pseudo-periplasmic space ⁵⁶. It is interesting to note that sequenced representatives from 292

the genus 'Ca. Nitrosocosmicus' lack the main S-layer protein (slp1) found in all Nitrosopumilales, 293 294 Nitrososphaerales, and 'Ca. Nitrosotaleales' sequenced isolates ⁵². In addition, it has been 295 demonstrated that elevated temperatures significantly alter the lipid composition in the AOA cell membrane ^{57,58}. However, it is unclear how changes in the S-layer or cell membrane affect whole 296 297 cell substrate affinity. On the single enzyme level, previous studies have shown the same trend of 298 decreasing substrate affinity and increasing maximal reaction velocity with increasing 299 temperatures, due to altered protein structures and an increased enzyme-substrate dissociation constant 59,60. 300

Notably, differing optimum growth and activity conditions were previously determined for the marine AOB strain *Nitrosomonas cryotolerans* ³⁴. These observations raise interesting, albeit unanswered, questions about why the growth and activity temperature optima are or can be uncoupled in AOM, and what this means for AOM niche differentiation and their competitiveness *in-situ*. Moving forward, investigations into the growth and cellular kinetic properties of AOM across a range of environmental factor gradients will be essential in understanding competition between AOM in engineered and environmental systems.

308

The effect of pH. The effects of short-term pH changes on the substrate-dependent kinetics of '*Ca*. N. oleophilus' MY3 and *N. inopinata* were determined. The V_{max} of both '*Ca*. N. oleophilus MY3' and *N. inopinata* were stable at 37.3 ± 6.6 µmol N mg protein⁻¹ h⁻¹ and 11.2 ± 2.5 µmol N mg protein⁻¹ h⁻¹, respectively, in medium with a pH between ~6.5 to ~8.5 (Supplementary Table 3). However, the $K_{\text{m(app)}}$ for total ammonium of '*Ca*. N. oleophilus MY3' and *N. inopinata* decreased by more than an order of magnitude (~11×) across this pH range, while the $K_{\text{m(app)}}$ for NH₃ remained more stable, increasing only 3 to 4 times (Fig. 4). As an increase of pH shifts the NH₃ +

NH₄⁺ equilibrium towards NH₃, this suggests that the actual substrate used by AOA and comammox is indeed the undissociated form (NH₃) rather than the ammonium ion (NH₄⁺), as previously demonstrated for AOB ^{34,35,51,61}. Interestingly, the only exception to this rule to date is the gammaproteobacterial marine AOB *Nitrosococcus oceani*. The reported $K_{m(app)}$ for total ammonium of *N. oceani* remained more stable (~2.3×) than the $K_{m(app)}$ for NH₃ (78×) when the pH was shifted from 6.3-8.6 ⁶². With this exception in mind, it is likely that all AOA, AOB, and comammox compete for NH₃ as their substrate in the environment.

It is important to note that the substrate affinities reported here represent whole cell 323 324 affinities and not the substrate affinity of ammonia monooxygenase (AMO) enzymes. Therefore, 325 further experimental investigation with purified AMO and ammonia/ammonium transporter 326 proteins is warranted. Although NH₃ can freely diffuse passively into AOM, this does not mean 327 that the cellular affinity reported here is necessarily unrelated to the transporter-mediated 328 movement of NH₃/NH₄⁺ into AOM cells. For example, AOB have previously been shown to accumulate very high (1 M) intracellular NH₄⁺ concentrations ⁶³. This high intracellular NH₄⁺ 329 330 concentration may provide a concentrated substrate reservoir, indirectly increasing the 331 concentration of NH₃ around the AMO enzyme complex. However, it is unknown if such a 332 concentration mechanism would be more important for an AOB with a low substrate affinity (e.g. 333 N. europaea) or for an AOA living in extremely substrate-limited environments (e.g. N. maritimus).

334

335 *The effect of cell morphology.* All AOM share the primary enzyme involved in ammonia oxidation, 336 AMO, which is located in the cytoplasmic membrane with its substrate-binding site most likely 337 facing the outside of the cell ⁵⁶. Therefore, a higher cellular surface area to volume (SA/V) ratio 338 likely contributes to an increase in a° , as it increases the space available for AMO and the chance

339 to bind NH₃ at very low concentrations. This assumption is based on the hypothesis that an increased abundance of uptake enzymes (e.g., permeases) leads to a higher $a^{\circ 28,64}$. In fact, the 340 341 SA/V ratio of AOM (Supplementary Table 4) correlates to the log of their observed $K_{m(app)}$ for NH₃ ($R^2 = 0.82$), $K_{m(app)}$ for total ammonium ($R^2 = 0.74$), a° for NH₃ ($R^2 = 0.77$), and a° for total 342 ammonium ($R^2 = 0.81$; Fig. 5). Therefore, the SA/V ratio of newly cultured AOM might be a useful 343 344 indicator for these cellular kinetic properties. Consequently, AOM with a high SA/V ratio will 345 likely outcompete other AOM in many natural aquatic and terrestrial environments, such as the 346 pelagic marine water column that has a very low standing total ammonium pool. Consistently, 347 these oligotrophic environments have already been postulated to select for organisms with a high SA/V ratio, enhancing their nutrient uptake capabilities ^{65,66}. 348

349 The correlation between the SA/V ratio and cellular kinetic properties of AOM sheds some 350 light on the unusual kinetic properties of the AOA belonging to the genus Nitrosocosmicus. Both 351 'Ca. N. oleophilus' MY3 and 'Ca. N. franklandus' C13 possess a very low SA/V ratio compared to other AOA isolates and they both possess several characteristics normally associated with AOB 352 - high substrate tolerances $^{48-50}$, low affinities for NH₃, and a low a° for NH₃ - that are not 353 354 consistent with the long-held convention that all AOA are much stronger competitors for NH₃ than 355 AOB in substrate-limited environments. Therefore, the individual cell morphology of AOM may 356 have a direct relationship with their cellular kinetic properties. Although this is only a correlation-357 based observation, it highlights that further investigation into these characteristics is warranted.

In addition to cellular morphology, the size of cell aggregates can affect the kinetic properties of AOM ⁶⁷. Cell aggregates have a lower SA/V ratio than individual cells, which can decrease diffusion rates and create microscale substrate/oxygen gradients within aggregates ⁶⁸. In order to ensure that the large differences in substrate affinity among AOA are not caused by

362 differences in cell aggregation, the aggregate size of 'Ca. N. uzonensis' N4, 'Ca. N. oleophilus' 363 MY3, and N. piranensis D3C cultures were inspected before and after MR experiments 364 (Supplementary Fig. 7). No aggregation pattern was observed that would explain the multiple 365 orders of magnitude differences in substrate affinity between these AOA. In fact, of the three AOA 366 investigated, the only strain to form large cell aggregates either before or after MR experiments 367 was N. piranensis D3C, which has one of the highest measured substrate affinities (lowest $K_{m(app)}$) 368 for NH₃). In contrast, the cell aggregate size of 'Ca. N. oleophilus' MY3 and 'Ca. N. uzonensis' 369 N4 were unaffected by the MR experiment and remained relatively small (Supplementary Fig. 7). 370 As '*Ca*. N. oleophilus' MY3 has one of the lowest substrate affinities (highest $K_{m(app)}$ for NH₃) and 371 formed only small cell aggregates, the low substrate affinity of 'Ca. N. oleophilus' MY3 was not 372 an artefact caused by cell aggregation.

373

Taken together, both environmental (pH and temperature) and AOM cellular (SA/V ratio) factors affect or are related to the observable cellular kinetic properties of individual AOM species. These factors need to be considered when investigating AOM competition or niche differentiation *in-situ*, as they are often in flux in environmental settings. This can be especially true considering cell morphology, which is often dependent on growth conditions ⁶⁹. However, the plasticity of the cellular kinetic properties within individual AOM species does not explain the larger trends observed here across AOA lineages or between AOM.

381

382 *Concluding remarks*

In this study we substantially extended the set of available substrate oxidation kinetic parametersfor AOA by the analysis of pure cultures or enrichments from various lineages within this guild.

385 Furthermore, our kinetic data obtained at different pH values supports the hypothesis that, like for 386 AOB, the substrate for AOA and comammox is NH₃. Together, our findings provide novel insights 387 for our understanding of niche differentiation among AOM and demonstrate a surprising 388 variability of the inferred kinetic parameters among AOA. Thus, the long-standing and recently 389 questioned⁵ hypothesis that all AOA have extremely high substrate affinities and specific substrate 390 affinities, can no longer be maintained. The observed links between AOA kinetic properties, 391 phylogeny, and cell morphology also enables the formulation of testable hypotheses on 392 nitrification kinetics in systems thus far characterized solely with molecular (e.g. amplicon 393 sequencing or metagenomic) tools.

As environmental factors such as temperature and pH influence kinetic parameters of AOA including their cellular affinity for NH₃, future analyses of kinetic parameters of AOM should not only be performed at their optimal growth conditions, but also over a range of conditions that reflect their environmental niches. Such experiments will generate a more informative picture on AOM competition and niche differentiation.

399

400 Newly isolated Nitrosotenuis species

401 The isolated strain N4 is a novel species of the genus *Nitrosotenuis* of the order *Nitrosopumilales*,
402 and we propose the following candidate status:

403 Taxonomy. (i) Etymology. The taxonomy for '*Candidatus* Nitrosotenuis uzonensis' sp. nov. is as
404 follows: Nitrosus (Latin masculine adjective), nitrous; tenuis (Latin masculine adjective),
405 small/slender; uzonensis (Latin neutrum genitive), from Uzon.

406 (ii) Locality. A terrestrial thermal spring located in the Uzon caldera on the Kamchatka peninsula,
407 Russia.

- 408 (iii) **Diagnosis.** A chemolithoautotrophic ammonia oxidizer of the phylum Thaumarchaeota, which
- 409 is straight and rod-shaped, with a diameter of 0.2-0.3 μm and a length of 0.4-1.7 μm. Growth over
- 410 a period of several years has been maintained in a medium with a pH of 7.5 at 37°C. It belongs to
- 411 the AOA order Nitrosopumilales (group I.1a). AOA with almost identical 16S rRNA and amoA
- 412 gene sequences have been detected in various environments, including soil and groundwater^{22,40,41}.

413

415 Materials and Methods

416 *Cultivation of ammonia oxidizers*

417 Several previously described growth media were used to cultivate the AOM used in this study. A 418 comprehensive guide with medium components and cultivation conditions is provided in the 419 Supplementary Materials and Methods, Supplementary Tables 1, and 2. Briefly, all cultures were 420 grown without shaking, in the dark, at their optimum growth temperature and pH, unless otherwise 421 stated. Ammonium (NH4Cl) from pre-sterilized stocks was added as substrate as needed. The 422 growth medium of Nitrosarchaeum koreense MY1, 'Ca. Nitrosotenuis chungbukensis' MY2, 'Ca. 423 Nitrosotenuis uzonensis' N4, N. maritimus SCM1, Nitrosopumilus piranensis D3C, and 424 *Nitrosopumilus adriaticus* NF5 was supplemented with sodium pyruvate (0.5 mM) at all times. 425 The pH of all growth media were adjusted when necessary by addition of sterile NaHCO₃. 426 Ammonia oxidation activity was determined by measuring ammonium, nitrite, and nitrate concentrations photometrically ⁷⁰⁻⁷³ using an Infinite 200 Pro M Nano+ spectrophotometer (Tecan 427 428 Group AG, Switzerland).

429

430 Novel AOA enrichments and pure culture

The sampling site, enrichment process, and initial strain characterization details for the two novel thermophilic AOA enrichment cultures '*Ca.* Nitrosofervidus tenchongensis' DRC1, and '*Ca.* Nitrososphaera nevadensis' GerE used in this study are provided in the Supplementary Materials and Methods. In addition, in this study, '*Ca.* N. uzonensis' N4 was isolated as a pure culture from a previously described geothermal spring enrichment culture ⁴¹. Further details are provided below and in the Supplementary Materials and Methods.

438 Phylogenetic analysis

A phylogenetic tree for all strains used in this study as well as select reference strains were calculated using IQ-TREE v 1.6.2 ⁷⁴. Automatic model determination using modelFinder ⁷⁵ identified LG+F+R5 as the best-fit model according to the Bayesian Information Criterion (BIC). Bipartition support was determined with ultrafast bootstraps (UFboots ⁷⁶). The tree was based on an alignment of 34 universal genes (43 markers) extracted from genomes, aligned and concatenated using CheckM ⁷⁷.

445

446 Substrate-dependent oxygen uptake measurements

447 Cellular substrate oxidation kinetics were determined from instantaneous substrate-dependent 448 oxygen uptake measurements as previously described ^{5,29,78}. Briefly, measurements were 449 performed with a microrespirometry (MR) system, equipped with a PA 2000 picoammeter and a 450 500 μm tip diameter OX-MR oxygen microsensor (Unisense, Denmark), polarized continuously 451 for at least 24 hours before use.

452 Active AOA, AOB, and N. inopinata cells were harvested $(4000 \times g, 10 \text{ min}, 20^{\circ}\text{C})$ from ammonium replete active cultures, using 10 kDa-cutoff, Amicon Ultra-15 centrifugal filter units 453 454 (Merck Millipore, Germany). Concentrated cells were washed with and resuspended in substrate-455 free medium appropriate for the respective cultures. Exceptions were 'Ca. Nitrosocosmicus 456 franklandus' C13 and the marine AOA, N. maritimus SCM1, N. piranensis D3C, and N. adriaticus 457 NF5. These four AOA strains were not active in the MR chambers after attempts to concentrate 458 their biomass. Therefore, ammonium concentrations were monitored daily for these four cultures, and cells were used without concentration for MR promptly upon substrate depletion ²⁹. AOM 459 460 harvested cells for MR experiments were incubated for at least 30 min in a recirculating water bath

set to the experimental temperature (Supplementary Tables 2 and 3) prior to being transferred to
the MR chambers (~2 ml).

463 In addition to MR experiments at optimal growth temperature and pH (Supplementary 464 Table 2), MR experiments were also performed at non optimal growth temperatures and medium 465 pH (Supplementary Table 3). 'Ca. N. oleophilus' MY3 was cultivated at 30°C, harvested with 466 centrifugal filter units (see above), and incubated for ~2 hours in substrate-free medium across a 467 range of temperatures (25, 30, and 35°C). MR experiments were then performed at the respective 468 preincubation temperature. Likewise, N. inopinata and 'Ca. N. oleophilus' MY3 cells were 469 harvested with centrifugal filter units (see above) and resuspended in substrate-free medium 470 containing 10 mM HEPES (pH 7.4). The pH was adjusted to 6.5-8.4 with 1 M HCl or 1 M NaOH 471 (Supplementary Table 3). These cultures were then incubated at their optimum growth temperature 472 for ~1 hour prior to cellular kinetic measurements. Culture pH was determined before and after 473 oxygen uptake measurements to confirm the pH did not change during MR. Substrate-dependent 474 oxygen uptake measurements were performed as described below.

475 For all MR experiments, glass MR chambers containing glass-coated magnetic stir bars 476 were filled headspace-free, sealed with MR injection lids, and submerged in a recirculating water 477 bath. An OX-MR microsensor was inserted into each MR chamber and left to equilibrate (300 rpm, 478 ~1 h). Exact temperatures used for each culture and experiment are provided in Tables S2 and S3. 479 Stable background sensor signal drift was measured for at least 15 min prior to initial substrate 480 injections, and the background oxygen consumption rate was subtracted from the measured oxygen 481 uptake rates. Hamilton syringes (10 or 50 μ l; Hamilton, USA) were used to inject NH₄Cl stock 482 solutions into MR chambers. Both single and multiple trace oxygen uptake measurements were 483 performed. For single trace measurements, a single substrate injection was performed, and oxygen

484 uptake was recorded until substrate depletion. For multiple trace measurements, multiple injections 485 of varying substrate concentration were performed in a single MR chamber. Once stable, discrete 486 slopes of oxygen uptake were calculated following each substrate injection. Immediately following 487 oxygen uptake measurements, the total ammonium concentration and pH of the MR chamber 488 contents were determined. The cells were stored at -20°C for protein analysis. Cells were lysed 489 with the Bacterial Protein Extraction Reagent (BPER, Thermo Scientific) and the total protein 490 content was determined photometrically with the Pierce bicinchoninic acid (BCA) Protein Assay 491 Kit (Thermo Scientific) as per the manufacturer's instructions. Before and after MR assays of N. 492 piranensis D3C, 'Ca. N. uzonensis' N4, and 'Ca. N. oleophilus' MY3, an aliquot of culture was 493 filtered onto membranes (0.2 µm polycarbonate GTTP membranes; Merck Milipore, Germany) 494 and DAPI (4',6-diamidino-2-phenylindole; 10 µg ml⁻¹; 5 min; room temperature) stained prior to microscopic measurement of biomass cell aggregate size, as described previously ^{67,79}. 495

496

497 Calculation of kinetic properties

498 $K_{\rm m(app)}$ and $V_{\rm max}$ were calculated from both single and multiple trace substrate-dependent oxygen 499 uptake measurements. Total ammonium (NH₃ + NH₄⁺) oxidation rates were calculated from 500 oxygen uptake measurements using a substrate to oxygen consumption ratio of 1:1.5 ^{5,29}. Total 501 ammonium uptake rates were fitted to a Michaelis-Menten model using the equation:

502 (1)
$$V = (V_{\max} \times [S]) \times (K_{\max} + [S])^{-1}$$

where *V* is the reaction rate (μ M h⁻¹), V_{max} is the maximum reaction rate (μ M h⁻¹), *S* is the total ammonium concentration (μ M), and $K_{m(app)}$ is the reaction half saturation concentration (μ M). A nonlinear least squares regression analysis was used to estimate $K_{m(app)}$ and V_{max} ⁸⁰. The $K_{m(app)}$ for NH₃ for each strain was calculated based on the $K_{m(app)}$ for total ammonium, incubation temperature, pH, and salinity ⁸¹. $K_{m(app)}$ values for AOM not determined in this study were compiled from the literature ^{5,29,33,34,42,51,82-84}. If only total ammonium information was given by the authors for $K_{m(app)}$, the corresponding NH₃ values were calculated based on the reported experimental temperature, pH, and salinity values. V_{max} values of pure cultures were normalized to culture protein content. The specific substrate affinity (a° ; 1 g wet cells⁻¹ h⁻¹) of each pure culture strain was calculated using the equation:

513 (2)
$$a^o = \left(\frac{V_{\text{max}}}{\text{cellular protein x 5.7}}\right) \times K_{\text{m(app)}}^{-1}$$

514 Where the V_{max} is normalized to the protein concentration (g l⁻¹) of the culture in the MR chamber 515 and the factor of 5.7 g wet cell weight per g of protein was used for all AOM ^{5,29,64}. The a° for NH₃ 516 or total ammonium were calculated using the respective $K_{\text{m(app)}}$ for NH₃ or total ammonium.

517

518 Cell surface area to volume ratio calculation

Approximate cell surface area to volume ratios were determined using cell dimensions provided by or calculated from previously published phase contrast, transmission electron, or scanning electron microscopy images (Supplementary Table 4). The following equations for the surface (SA) area and volume (V) of a sphere (3) and rod (4) were used:

523 (3) SA = $4\pi r^2$; V = $4/3\pi r^3$

524 (4)
$$SA = 2\pi r(h+r); V = \pi r^2 h$$

525 where r is the cell radius (μ m) and h is the cell length (μ m). The cell size and volume from 526 published phase contrast images were verified using MicrobeTracker⁸⁵.

527

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753 Author contributions

M-YJ, CJS HD, and MW designed the study and wrote the manuscript with the help of all
authors. M-YJ and CJS performed the kinetic experiments with the help of KDK, LH, BB, L LM, and CW. Additional data analysis was performed by AM, S-KR, PP, GWN, JRT, and CWH.

758 **Competing Interests**

759 The authors declare no competing interests.

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762 Figures:

Fig. 1. A phylogenetic reconstruction of the AOA used in this study. AOA kinetically characterized for the first time are shown in black. '*Ca.* N. uzonensis' N4 is also labeled in black as its previous kinetic characterization occurred as an enrichment culture ⁵. An unrooted approximate maximum likelihood tree was calculated using IQ-TREE under model LG+F+R5 using an alignment of 34 universal genes (43 markers). Support values (UFboot) greater than 95% for bipartitions are shown with a filled circle. All other bipartitions received <80% UFboot support. The tree was arbitrarily rooted on the branch leading to the *Nitrosocaldaceae* and order designations reflect lineages proposed by Alves *et al.*, 2018. The scale bar indicates amino acids changes per site.



774	Fig. 2. Substrate-dependent oxidation kinetics of ammonia-oxidizing microorganisms. The (a) apparent substrate affinity $(K_{m(app)})$
775	for NH ₃ , (b) specific substrate affinity (a°) for NH ₃ , (c) $K_{m(app)}$ for total ammonium, (d) a° for total ammonium, and (e) maximum
776	oxidation rate (V_{max}), of AOA (red), comammox (blue), and AOB (black) are provided. Symbols filled with light grey represent
777	previously published values from reference studies (references provided in Materials and Methods). The four different gradations of red
778	differentiate the four AOA phylogenetic lineages: (I) Nitrosopumilales, (II) 'Ca. Nitrosotaleales', (III) Nitrososphaerales, and (IV) 'Ca.
779	Nitrosocaldales'. Measurements were performed with either pure (circles) or enrichment (diamonds) cultures. Multiple symbols per
780	strain represent independent measurements performed in this study and/or in the literature. The individual Michaelis-Menten plots for
781	each AOM determined in this study are presented in Supplementary Figs. 1, 3-5, and 8. Note the different scales.
782	



Fig. 3. The reciprocal relationship between the substrate affinity ($K_{m(app)}$) and specific substrate affinity (a°) of ammoniaoxidizing microorganisms (AOM). Reciprocal plots for both (a) total ammonium and (b) NH₃ are depicted. The $K_{m(app)}$ and a° values correspond to the values presented for pure AOM isolates in Fig. 2. Data for AOA (red), comammox (blue), and AOB (black) are shown. The correlation (\mathbb{R}^2) indicates the linear relationship between the logarithmically transformed data points.





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793	Fig. 4. The effect of medium pH on the substrate affinity of 'Ca. N. oleophilus MY3' and N. inopinata. The substrate affinities for
794	both (a,b) NH_3 and (c,d) total ammonium ($NH_3 + NH_4^+$) are provided. Individual substrate affinity values determined at each pH are
795	shown as single points (circles). The boxes represent the first and third quartiles (25% to 75%) of the substrate affinity range under each
796	condition. The median (line within the boxes) and mean substrate affinity (black diamonds) values are also indicated. The whiskers
797	represent the most extreme values within 1.58× of quartile range. The variation of the substrate affinity values across the entire tested
798	pH range are indicated in each panel. In all four instances there was a significant difference between the affinity at the lowest pH and
799	the highest pH, as determined by a Student's t-test (p <0.005). The average substrate affinity values for ' <i>Ca</i> . N. oleophilus MY3' and <i>N</i> .
800	inopinata at each pH are provided in Supplementary Table 3.



Fig. 5. Logarithmic correlation of (a) substrate affinity ($K_{m(app)}$) and (b) specific substrate affinity (a^{0}) with the cellular surface area to volume ratio of ammonia-oxidizing microorganisms (AOM). All $K_{m(app)}$ and a^{0} values correspond to values presented in Fig. 2. The surface area to volume (SA/V) ratio calculations for each AOM are provided in Supplementary Table 4. Data for AOA (red), comammox (blue), and AOB (black) are shown. The three different gradations of red differentiate three distinct AOA phylogenetic lineages. The error bars represent the standard deviation of replicate kinetic experiments or SA/V ratio measurements of each AOM strain. The logarithmic correlation (\mathbb{R}^{2}) value was calculated from the average values of each AOM and is presented on a semi-log axis.



