Preferential temperature and ammonia concentration for *in-situ* growth of *Candidatus* Nitrosocosmicus ammonia oxidising archaea

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Abstract

Among cultivated and characterised ammonia oxidising archaea (AOA), representatives of *Candidatus* Nitrosocosmicus are unique in their ability to grow at high ammonia concentration (up to 100 mM), at concentrations that are tolerated by many ammonia oxidising bacteria (AOB). These strains also grow at a wide range of incubation temperatures (4 - 45°C), with highest ammonia oxidation rates at relatively high temperature (28 - 40°C). In addition, ammonia oxidiser growth is often promoted by reduced competition for ammonia, such as in the presence of a specific inhibitor against ammonia oxidiser competitors. Therefore, this study aimed at assessing the optimal conditions (temperature and ammonia concentration) of *Ca.* Nitrosocosmicus in soil by determining the nitrification rate and the growth of *Ca.* Nitrosocosmicus AOA and AOB in pH 7.5 soil microcosms amended with inorganic ammonia and octyne and incubated at a range of temperatures (15 to 35°C). It demonstrated that growth

of *Ca.* Nitrosocosmicus AOA increases with incubation temperature in soil, with an optimum of 25°C. In addition, growth of *Ca.* Nitrosocosmicus is greater when AOB are inhibited, especially under high NH_4^+ concentration. This study indicates that *Ca.* Nitrosocosmicus is tolerant to high NH_4^+ concentration in soils, which contradicts the accepted belief that AOA growth is inhibited in soil with high NH_4^+ concentration, and it also confirms the role of a near-neutrophilic AOA in nitrification activity in soil with higher nitrogen content. This study also shows the relevance and limitations of cultivated strains in predicting microbial growth in natural environments.

Keywords: ammonia oxidation, nitrification, temperature, octyne, *Candidatus* Nitrosocosmicus, ammonium concentration.

Highlights

- *Ca.* Nitrosocosmicus AOA growth is not inhibited in soil with high NH_4^+ concentration
- Near-neutrophilic AOA are active nitrifiers in soil with high nitrogen content
- Cultivated strains inform prediction of microbial growth in natural environment, but not for all parameters (e.g. temperature)
- growth of *Ca.* Nitrosocosmicus AOA increases with incubation temperature in soil
- growth of *Ca.* Nitrosocosmicus is greater when AOB are inhibited

1. Introduction

Ammonia oxidisers are ubiquitous in terrestrial ecosystems but niche specialisation and differentiation lead to differences in relative abundance and contributions of archaeal (AOA) and bacterial (AOB) ammonia oxidisers (Prosser, 2011), with greater abundance of AOA in many unfertilised soils (Prosser and Nicol, 2012). One cause of niche differentiation between, and within, AOA and AOB is ammonia supply, with evidence that high rates of inorganic ammonia fertilisation select for AOB growth, while AOA are often selected for in soils supplied with continued but low levels of ammonia (Di *et al.*, 2009; Di *et al.*, 2010; Verhamme *et al.*, 2011), through mineralisation of organic nitrogen or slow release fertilisers (Stopnisek *et al.*, 2010; Levičnik-Höfferle *et al.*, 2012; Hink et al., 2018). These observations have been explained in terms of differences in NH_3 concentration, with suggestions of higher NH_3 tolerance of AOB and higher NH_3 affinity of AOA (Martens-Habbena *et al.*, 2009). However, the discovery of AOA belonging to the *Ca.* Nitrosocosmicus genus, which have ≥10-fold greater NH_3 tolerance than AOB (Lehtovirta-Morley *et al.*, 2016), indicates that high NH_3 concentration does not limit growth of all AOA (Lehtovirta-Morley *et al.*, 2016; Jung *et al.*, 2016; Sauder *et al.,* 2017; Liu et al., 2021). In addition, kinetic analyses suggest similar NH_3 affinities for terrestrial AOA and AOB (Hink et al., 2017; Kits et al., 2017; Jung et al., 2021). Moreover, specific inhibition of AOA or AOB in soil amended with either low or high NH_4^+ concentration provided evidence for NH_3 oxidation activity and growth of AOA and AOB at high and low NH_4^+ concentration, respectively, when the competitor group is inhibited (Hink et al., 2018; Zhao et al., 2020).

Investigation of niche differentiation in soil AO has focused on differences between AOA and AOB, and differences in ammonia tolerance between specific phylotypes within AOA and AOB has received less attention (Shen et al., 2008; Verhamme *et al.*, 2011; Prosser and Nicol, 2012; Chen et al., 2014). Phylogenetic diversity in both AOA (Gubry-Rangin *et al.*,

2011; Vico Oton *et al.*, 2016; Alves et al., 2018) and AOB (Aigle et al., 2019) is high, with numerous families of soil AOA. Among them, *Ca.* Nitrosocosmicus represents an entire family within the Nitrososphaerales order based on a phylogenomic approach (Herbold *et al.,* 2017; Sheridan et al., 2020). While cultures of *Ca.* Nitrosocosmicus have been obtained from diverse environments, including sediments (Sauder *et al.,* 2017) and wastewater treatment plants (Jung *et al.,* 2016), most have been enriched from soil (Lehtovirta-Morley *et al.,* 2016; Alves et al., 2019; Liu et al., 2021). However, *Ca.* Nitrosocosmicus represent a relatively low proportion of AOA in soils, typically <2% of the total thaumarchaeotal community (Gubry-Rangin *et al.,* 2011; Pester *et al.,* 2012; Alves et al., 2018; Wang et al., 2019). Successful cultivation despite low relative abundance in the environment might be explained by several factors, including relatively rapid growth or preferential laboratory incubation conditions (Gubry-Rangin et al., 2018), and it is therefore important to assess whether the optimal and distinctive properties of isolates in culture reflect physiology within the natural environment and environmental distributions.

Available cultivated representatives of *Ca.* Nitrosocosmicus grow at a wide range of incubation temperatures (4 - 45°C), with highest ammonia oxidation rate at relatively high temperature (28 - 40°C). These characteristics, coupled with greater tolerance of high ammonia concentrations, suggest that growth of *Ca.* Nitrosocosmicus in soil will be favoured in this range of temperature. Temperature influences many ecosystem processes including nitrification (Booth et al., 2005) and net nitrification is often optimal at ≤30°C (e.g. Avrahami et al., 2003; Tourna et al., 2008; Gubry-Rangin *et al.,* 2017; Taylor et al., 2017). These data suggest that growth of *Ca.* Nitrosocosmicus in soil will be highest at 30°C and following amendment of soil with high inorganic NH_4^+ concentration. Any selective advantage provided by these conditions is also likely to be promoted by reduced competition for ammonia in the presence of octyne, a specific inhibitor of AOB (Hink et al., 2018; Zhao et al., 2020). While

i*n-situ* growth of, and NH_3 oxidation by *Ca.* Nitrosocosmicus have been demonstrated in soil (Wang et al., 2019), the influence of temperature and ammonia concentration have not been assessed. Therefore, this study aimed to test the hypotheses that (a) growth of *Ca.* Nitrosocosmicus increases with incubation temperature in soil, with an optimum of 30°C, (b) enrichment of *Ca.* Nitrosocosmicus is greater when AOB are inhibited and (c) high NH_4^+ concentration leads to enrichment of *Ca.* Nitrosocosmicus in soil. These hypotheses were tested by determination of growth and ammonia oxidation rate by AOB and *Ca.* Nitrosocosmicus in soil microcosms amended with inorganic ammonia and octyne and incubated at a range of temperatures.

2. Material and Methods

2.1 Soil microcosms

Microcosms were constructed using a sandy loam soil from a pH 7.5 agricultural plot rotating between potatoes and crop cultures (Craibstone Estate, Aberdeen, Scotland, grid reference NJ872104). Soil physicochemical parameters are described by Kemp *et al.* (1992). The air-dried soil was sieved (3.35-mm mesh) and stored at 4 °C for 2 weeks before use. Triplicate soil microcosms were established in 250-ml, sterile glass bottles containing 50 g equivalent dry soil and initial moisture content was adjusted to 30% (g water g^{-1} dry soil) with sterile distilled water. Moisture content was estimated as the weight loss of triplicate soil samples (approximately 5 g wet weight) following oven-drying at 103°C for 48 h. Each microcosm was covered with a butyl rubber stopper tightened with a metal crimp top. Microcosm experiments were performed using a multifactorial design with NH_4^+ concentration, nitrification inhibitor and temperature as factors. Microcosms were either amended to 100 µg N g^{-1} soil with $(NH_4)_2SO_4$ (referred to as high NH_4^+ concentration), or without NH_4^+ amendment (referred to as low NH_4^+ concentration). The second factor was

supplementation (or not) with the specific nitrification inhibitor 1-octyne (Taylor *et al*., 2013), which targets AOB but not AOA, at a concentration of 0.03% (v v^{-1}) per microcosm headspace as described by Hink *et al*. (2017). Microcosms were incubated at five temperatures (15, 20, 25, 30 and 35 ^{o}C) for 28 days. A non-destructive 2-g soil sample was taken twice weekly to determine NH_4^+, NO_2^- and NO_3^- concentrations (see below). NH_4^+ concentration in microcosms amended with high-NH_4^+ was maintained by addition of NH_4^+ that had been converted to NO_2^- + NO_3^- twice weekly (implying that a variable amount of NH_4^+ was supplemented depending on the amount converted into nitrite and nitrate), while microcosms with low NH_4^+ concentration received an equal volume of sterile distilled water. The moisture content increased from 30% to 34% at the end of the incubation. At each sampling point, microcosms were immediately re-capped after aeration and partial pressure of 1-octyne was re-established in microcosms incubated with AOB inhibitor. Soil samples (1 g) were also taken at the initial and final time points and immediately stored at -80 ^{o}C prior to nucleic acid extraction.

2.2 Chemical analysis

NH_4^+, NO_2^- and NO_3^- concentrations were determined in soil solution by mixing 2 g soil with 10 ml 1 M KCl solution, collecting the supernatant after centrifugation at 3,000 rpm for 15 min. Concentrations were measured colorimetrically using 96-well plates as described by Catão *et al.* (2016). The nitrification rate was estimated as the linear increase of nitrate concentration per unit of time during incubation (µg N g^{-1} day^{-1}).

2.3 Design and testing of amoA *primers specific to the* Ca. *Nitrosocosmicus clade*

A pair of specific *amoA* primers (C13_42F (GCTTACWATCAAYGCAGGAGATT) and C13_294R (AGCMGAVGGTATCCAAAC)) targeting the thaumarcheotal *Ca*. Nitrosocosmicus clade was designed using the published archaeal *amoA* sequence alignment (Gubry-Rangin et al., 2015) including *amoA* sequences from three *Ca*. Nitrosocosmicus

cultivated strains, *Ca.* N. oleophilus, *Ca.* N. exaquare and *Ca.* N. franklandus (Lehtovirta-Morley *et al.*, 2016; Jung *et al.*, 2016; Sauder *et al.*, 2017). These primers amplified a 252-bp amplicon in *Ca.* Nitrosocosmicus clade and discriminated against other thaumarchaeotal groups in *in-silico* analysis.

Specificity of the assay was determined by amplifying triplicate 25-µl PCR products from Craibstone pH 7.5 soil (see DNA extraction protocol below) using the high-accuracy KAPA Taq PCR assay (KAPABiosystems, USA). The PCR mixture contained 12.5 µl KAPA enzyme mixture, 2 µM of each primer and 2.5 µl of 20 ng of DNA extracted from Craibstone soil. The cycling conditions were 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 60 °C for 15 s, 72 °C for 15 s, followed by 72 °C for 5 min. PCR products were sequenced using Illumina MiSeq V3 sequencing (Mr DNA, www.mrdnalab.com). Raw reads were trimmed, filtered and size-selected using trim galore (www.bioinformatics.babraham.ac.uk/projects/trim_galore) and usearch (-fastx_truncate) (www.drive5.com/usearch/manual/cmd_fastx_truncate.html). The paired-end read assembly was done using the FLASH assembler (Magoč and Salzberg, 2011) and clustered at 100% homology (usearch-cluster_fast -id 1) with associated abundance. Validation of the nucleotide reads was first assessed by their translation into amino acids and then by their homology to the previously published *amoA* sequence alignment (available from Gubry-Rangin *et al.*, 2015) using a BLASTn approach (cut-off of similarity ≥ 89%) (Altschul et al., 1990).

2.4 Growth of archaeal and bacterial ammonia oxidisers

Growth was determined by increases in abundance of *amoA* genes, determined by qPCR. DNA was extracted from 0.5 g soil as described in Griffiths et al. (2000) and DNA concentration and purity were measured using a Nanodrop ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). *Ca.* Nitrosocosmicus *amoA*

standards for quantitative PCR (qPCR) were prepared using DNA extracted from a pure culture of *Ca.* N. franklandus as template following a general archaeal *amoA* PCR (primer pair amoA23F/amoA616R (Tourna *et al*., 2008)). This 593-bp PCR product was purified using the Nucleospin Clean-Up Kit (Macherey-Nagel, Düren, Germany) and diluted to give abundances of 10^1 –10^7 *amoA* genes for the *Ca.* Nitrosocosmicus-specific qPCR standard. Each qPCR reaction was performed in a 25-µL volume containing 0.4 mg mL^{-1} bovine serum albumin (BSA), 1.2 µM of each primer, 10 µL QuantiFast™ qPCR Master Mix (Qiagen, Crawley, UK) and 5 µL of 10-fold diluted nucleic acid extract. Cycling conditions were: 95 °C for 5 min, 35 cycles of 95 °C for 15 s, 60 °C for 30 s, 80 °C for 8 s followed by a melting curve analysis between 60 and 95°C with 0.2 °C increment.

General bacterial *amoA* standards and qPCR assays were prepared as described in Thion and Prosser (2014) using the amoA1F and amoA2R primers (Rotthauwe et al., 1997). All qPCR assays were run on an Eppendorf Mastercycler Realplex Real-Time PCR System (Hamburg, Germany). Specificity of amplification was assessed by melting curve analysis and agarose gel electrophoresis. The qPCR efficiencies were 88 – 91 % and 87 – 89 % for *Ca.* Nitrosocosmicus and AOB assays, respectively, and r^2 values were 0.99. The growth of *Ca.* Nitrosocosmicus and AOB were determined by estimating changes in *amoA* gene abundance over time.

2.5 Statistical analyses

Statistical analyses were performed using Sigmaplot 13.0. Within each ammonia concentration and inhibitor treatment condition, data for nitrification rate, pH and both AOB and *Ca.* Nitrosocosmicus growth were analysed independently using one-way ANOVA (or one-way Kruskal-Wallis when normality and homoscedasticity were not respected) with temperature as a fixed factor. Holm-Sidak (or Student-Newman-Keuls for non-parametric

tests) multiple *post-hoc* tests were used to assess the significance of differences among the means. In addition, a two-way ANOVA using ammonia concentration and inhibitor treatment as fixed factors was performed to determine the effect of AOB growth on the *Ca*. Nitrosocosmicus AOA growth.

3. Results

3.1 Nitrification rate

The concentration of NH_4^+ was below the detection limit (0.1 µg N-NH_4^+ g^{-1} of soil) throughout incubation of all low-NH_4^+ (unamended) microcosms at all incubation temperatures except 35 ºC and in the absence of the AOB inhibitor octyne (Figs. 1A-E). At 35 ºC, NH_4^+ concentration increased from 0 to 76 µg N g^{-1} in the presence of octyne, as mineralisation rate exceeded the rate of ammonia oxidation by AOA alone. In the absence of octyne at 35 ºC, NH_4^+ concentration increased from <1 to 34 µg N g^{-1}, within 10 days, when mineralisation again exceeded ammonia oxidation, but then steadily decreased below the detection limit during the remaining 18 days (Fig. 1E) as ammonia oxidation rate increased. During incubation of high-NH_4^+ microcosms (supplemented with inorganic NH_4^+), NH_4^+ concentration decreased at all temperatures, except at 35 ºC in the presence of octyne, where NH_4^+ increased from 100 to 162 µg N g^{-1} (Fig. 1A-E), as mineralisation exceeded ammonia oxidation. The addition of octyne reduced NH_4^+ oxidation in high-NH_4^+ microcosms, at all incubation temperatures. NO_3^- concentration increased during incubation in all treatments, except at 35 ºC in the presence of octyne (Fig. 1F-J). NO_3^- production rate was higher in high-NH_4^+ microcosms than in low-NH_4^+ microcosms and was reduced in the presence of octyne in high-NH_4^+ microcosms. In our study, the rate of denitrification was considered negligible due to the soil being incubated under aerobic conditions and to the neutral pH of the soil.

Net nitrification rate, rather than gross nitrification rate (which includes effects of mineralisation and nitrate conversion), were determined in low- and high-NH_4^+ microcosms during periods of linear increases in NO_3^- concentration, representing NH_4^+-limited and -unlimited rates, respectively. However, nitrification rate could not be determined in many high-NH_4^+ microcosms due to complete oxidation of NH_4^+ between sample points, and calculated rates for high-NH_4^+ microcosms therefore represent minimum nitrification rate at all temperatures except 35 ^oC in the absence of octyne, and at 15, 30 and 35 ^oC in the presence of octyne. Nitrification rate was lower in low-NH_4^+ microcosms than in high-NH_4^+ microcosms in both presence and absence of octyne (*t*-test; $p = 6 \times 10^{-8}$) (Fig. 2). In low-NH_4^+ microcosms, nitrification rate increased with incubation temperature from 15 to 35 ^oC in the absence of octyne and increased from 15 to 30 ^oC and decreased at 35 ^oC in the presence of octyne (Fig. 2). In high-NH_4^+ microcosms, minimum nitrification rates in the absence of octyne at 15, 20, 25 and 30 ^oC were ≥27 μg N g^{-1} day^{-1} and were significantly higher than the rate at 35 ^oC (Fig. 2). In the presence of octyne, nitrification rate increased with temperature from 15 to 25 ^oC and decreased from 25 to 35 ^oC but are estimates of minimum rate at 20 and 25 ^oC (Fig. 2).

The initial pH in all microcosms was not significantly different, regardless of moisture content and amendment with NH_4^+, but soil pH decrease was proportional to the NO_3^- production during the incubation (Fig S1).

3.2 Ammonia oxidiser growth

The specificity of *Ca.* Nitrosocosmicus-specific *amoA* primers was determined by triplicate *Ca.* Nitrosocosmicus *amoA* Illumina MiSeq sequencing of *Ca.* Nitrosocosmicus *amoA* genes in DNA extracted from Craibstone pH 7.5 soil, generating 372,719 reads, and 287,351 cleaned assembled reads. The majority of reads (96%) affiliated to *Ca.* Nitrosocosmicus phylogenetic clades, while 1.2% and 2.9% affiliated to other thaumarchaeotal

237 *amoA* clades and to non *amoA* sequences, respectively, confirming the high specificity of the
238 *Ca.* Nitrosocosmicus *amoA* primers to the *Ca.* Nitrosocosmicus phylogenetic clade.

239 Growth of *Ca.* Nitrosocosmicus AOA and AOB was estimated by measuring temporal
240 changes in *amoA* abundance during incubation (i.e. the difference in *amoA* abundance between
241 day 0 and day 28). *Ca.* Nitrosocosmicus AOA growth was detected in both low- and high-NH_4^+
242 microcosms and in the presence and absence of octyne at incubations from 15 to 30 ^oC. In the
243 presence of octyne, growth in both low- and high-NH_4^+ microcosms increased significantly
244 with incubation temperature with an optimum at 25 ^oC (Fig. 2). Therefore, temperature
245 significantly affected growth of *Ca.* Nitrosocosmicus in soil during incubation for 28 days in
246 the presence of octyne. Growth of AOB was not detected in presence of octyne, except for
247 small increases in abundance at low- and high-NH_4^+ microcosms incubated at 25 ^oC. In the
248 absence of octyne, AOB growth was greatest in high-NH_4^+ microcosms (1-way ANOVA,
249 $p(NH_4$ treatment) < 0.001)), where it increased significantly with temperature from 15 to 30 ^oC,
250 with lower growth at 35 ^oC, while some AOB growth was detected in low-NH_4^+ microcosms,
251 especially at 15 ^oC (Fig. 2). Such high nitrification activity likely reduced the soil pH (Fig. S1),
252 which, in turn, could affect AOA and AOB activities. In addition, growth of *Ca.*
253 Nitrosocosmicus AOA was higher when AOB growth was inhibited with octyne (2-way
254 ANOVA, p(inhibitor treatment) = 0.004) and this effect was not significantly different in low-
255 and high-NH_4^+ microcosms (2-way ANOVA, p(ammonia concentration) = 0.174).

256 **4. Discussion**

257 The relatively low abundance but widespread distribution of *Ca*. Nitrosocosmicus AOA
258 in a range of soils (Gubry-Rangin *et al.,* 2011; Pester *et al.,* 2012; Alves et al., 2019; Wang et
259 al., 2019) has led to investigation of their activity and potential role in soil nitrification. Their
260 autotrophic growth in soil has been demonstrated using stable-isotope probing (Wang et al.,

2019) but temperature and ammonia concentration preferences have not been investigated. Here, we tested predictions of three hypotheses regarding growth and activity of *Ca.* Nitrosocosmicus in soil on the basis of assumptions derived from physiological characteristics of soil isolates.

The first hypothesis predicted optimal growth of *Ca*. Nitrosocosmicus at 30 °C, as soil nitrification rates are often optimal at ≤30 °C (Booth et al., 2005; Avrahami et al., 2003; Tourna et al., 2008; Gubry-Rangin *et al.*, 2017; Taylor et al., 2017). This is within, but towards the lower end, of the range for optimal growth temperatures for *Ca*. Nitrosocosmicus isolates (28 - 40˚C), with optimal growth rates reported at 28, 30, 33, 38 and 40˚C for *Ca.* N. arcticus (Alves *et al*., 2019), *Ca.* N. oleophilus (Jung *et al.,* 2016), *Ca.* N. exaquare (Sauder *et al.*, 2017), *Ca*. N. agrestis (Liu et al., 2021) and *Ca.* N. franklandus (Lehtovirta-Morley *et al*., 2016), respectively. Therefore, it is significantly lower than the optimal temperature for growth of *Ca*. Nitrosocosmicus franklandus (40 ^{o}C), which was isolated from this soil. Across the range of soil incubation temperature (15 to 35°C), optimal temperature for growth of *Ca*. Nitrosocosmicus was 25°C, with lower growth observed at 30°C and no detectable growth at 35°C, suggesting that the characteristics of the *Ca*. Nitrosocosmicus strains in culture did not predict well conditions for growth in the soil environment. However, a temperature of 25°C is consistent with previously reported optimal temperature for growth of neutrophilic AOA in soil (Gubry-Rangin et al., 2017; Taylor et al., 2017).

The difference of optimal growth temperature between cultivated strains and environmental sources probably results from methodological biases. While enrichment and isolation would ideally be performed at temperatures typical of the source environment, cultivation approaches often use higher incubation temperatures to increase the speed of enrichment and isolation. This potentially selects for organisms with higher temperature optima, while strains with these higher optima may be at lower relative abundance in the natural

soils because those constant high temperatures occur rarely. It is indeed assumed that a large proportion of the microbial community is adapted to recurrent environmental conditions, including temperature, to the exception of dormant strains required when environmental change occurs (Lennon and Jones, 2011). Therefore, strains with lower temperature optima may be more abundant in a Scottish soil (latitude 57°11'17"N) than strains with higher temperature optima and would dominate ammonia oxidation process in such soil. However, the incubation period used in short-term microcosm studies (i.e. 28 days) does not allow sufficient time for selection and growth of the presumably most abundant strains at low temperatures, which often decreases growth rate. This is particularly true in soils, in which strains have limited resources compared to culture conditions. Altogether, it is not surprising that strains isolated at higher temperature (e.g., 40 °C) are not a good predictor of optimal temperature of related ecosystem function in soil containing these strains. Many other factors may explain such discrepancy between optimal temperature in soil and in culture, but there is only limited evidence for culture behaviour being a good predictor of activity in soil concerning the temperature, which is not the case for other culture conditions, such as nutrient composition, pH or osmotic pressure for example.

Growth of *Ca*. Nitrosocosmicus AOA was also detected at 15 °C, but was lower than that to 30 °C. This is consistent with reductions in soil nitrification rate at lower temperatures (Booth et al., 2005; Gubry-Rangin et al., 2017; Taylor et al., 2017), and with undetectable growth of most *Ca.* Nitrosocosmicus strains at 15 °C (Ca. N. oleophilus, Ca. N. exaquare, Ca. N. agrestis and Ca. N. franklandus (Jung et al., 2016; Sauder et al., 2017; Liu et al., 2021; Lehtovirta-Morley *et al*., 2016)), although *Ca*. N. arcticus growth occurs at low temperature, even if decoupled from ammonia oxidation (Alves et al., 2019). This suggests that growth observed in the present soil at 15 °C could be due to the presence of *Ca.* Nitrosocosmicus AOA strains with physiology similar to that of *Ca*. N. arcticus. Another explanation is that isolates

311 are not representative of natural populations with respect to their temperature optima, as
312 discussed above.

313 Surprisingly, *Ca.* Nitrosocosmicus growth was not detected at 35 °C irrespective of
314 NH_4^+ and octyne treatment, while cultures would predict both nitrification and growth of *Ca.*
315 Nitrosocosmicus at this temperature. Such discrepancy could be because strains with high
316 temperature optima in soil are at very low relative abundance but were selected in cultures
317 incubated at high temperature. In contrast, AOB growth was detected at this temperature in
318 high-NH_4^+ microcosms in the absence of octyne, confirming a difference in temperature
319 sensitivity between AOA and AOB (Taylor et al., 2017).

320 The second hypothesis predicted that *Ca*. Nitrosocosmicus growth is greater when AOB
321 are inhibited and was strongly supported. *Ca.* Nitrosocosmicus growth was significantly
322 greater when AOB were inhibited in both low- and high-NH_4^+ microcosms. This provides
323 further evidence for competition between AOA and AOB for ammonia, regardless of NH_4^+
324 concentration, as indicated in previous studies with AOA-specific (simvastatin) and AOB-
325 specific (octyne) inhibitors (Hink et al., 2017; Hink et al., 2018; Wang et al., 2019; Zhao et al.,
326 2020).

327 The third hypothesis predicted that high NH_4^+ concentration leads to growth of *Ca.*
328 Nitrosocosmicus in soil. There is no clear evidence for such selection of *Ca.* Nitrosocosmicus
329 in high-NH_4^+ microcosms, even though *Ca.* Nitrosocosmicus growth was higher when AOB
330 were inhibited. This provided further support that *Ca.* Nitrosocosmicus can tolerate high NH_4^+
331 concentration. Therefore, the suggestion that selection of AOB in highly fertilised soils is due
332 to inhibition of AOA by high ammonia concentration does not apply in soil containing *Ca.*
333 Nitrosocosmicus or other ammonia-tolerant AOA. Growth of *Ca.* Nitrosocosmicus under high
334 NH_4^+ concentration corroborates the low ammonia affinity demonstrated for *Ca*. N.

335 franklandus, which is the similar range to most AOB (Wright et al., 2020; Jung et al., 2021).
336 Nevertheless, similar growth of *Ca.* Nitrosocosmicus occurred in both low- and high-NH_4^+
337 microcosms, while nitrification rate was >10-fold greater in high than in low NH_4^+
338 concentration soils when AOB were inhibited, suggesting that other AOA (non-*Ca.*
339 Nitrosocosmicus) are also tolerant to high NH_4^+ concentration.

340 This study demonstrates the relevance of laboratory cultures to predict functioning of
341 ecosystems, but also highlight their limitations and the need to test these culture-based
342 predictions in soil because of highly selective cultivation conditions.

343

344 **5. Acknowledgements**

345 MOB was supported by a University of Aberdeen Elphinstone Scholarship and by
346 TETFund through Adekunle Ajasin University Akungba (AAUA) Nigeria. CGR was funded
347 by a Royal Society University Research Fellowship (UF150571).

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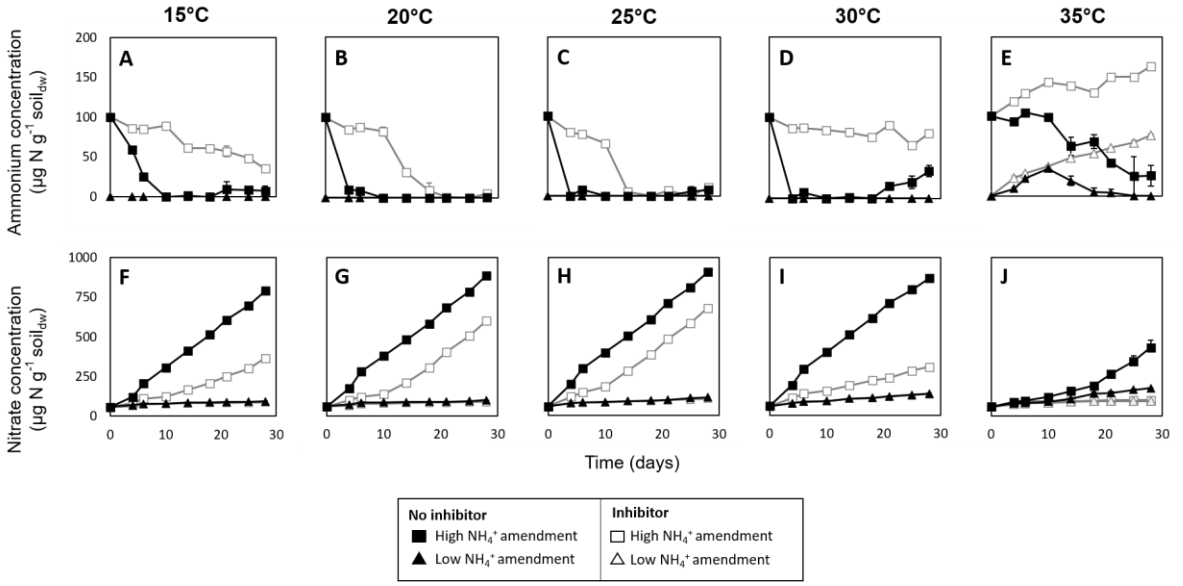
7. Figure legends

Figure 1. Temporal changes in NH_4^+ (A - E) and NO_3^- (F - J) concentrations during incubation of soil microcosms following amendment with water (low-NH_4^+) or 100 µg N g^{-1} soil (high-NH_4^+) at five temperatures in the absence or presence of a specific AOB inhibitor (octyne). Plotted points and error bars represent means and standard errors of triplicate measurements.

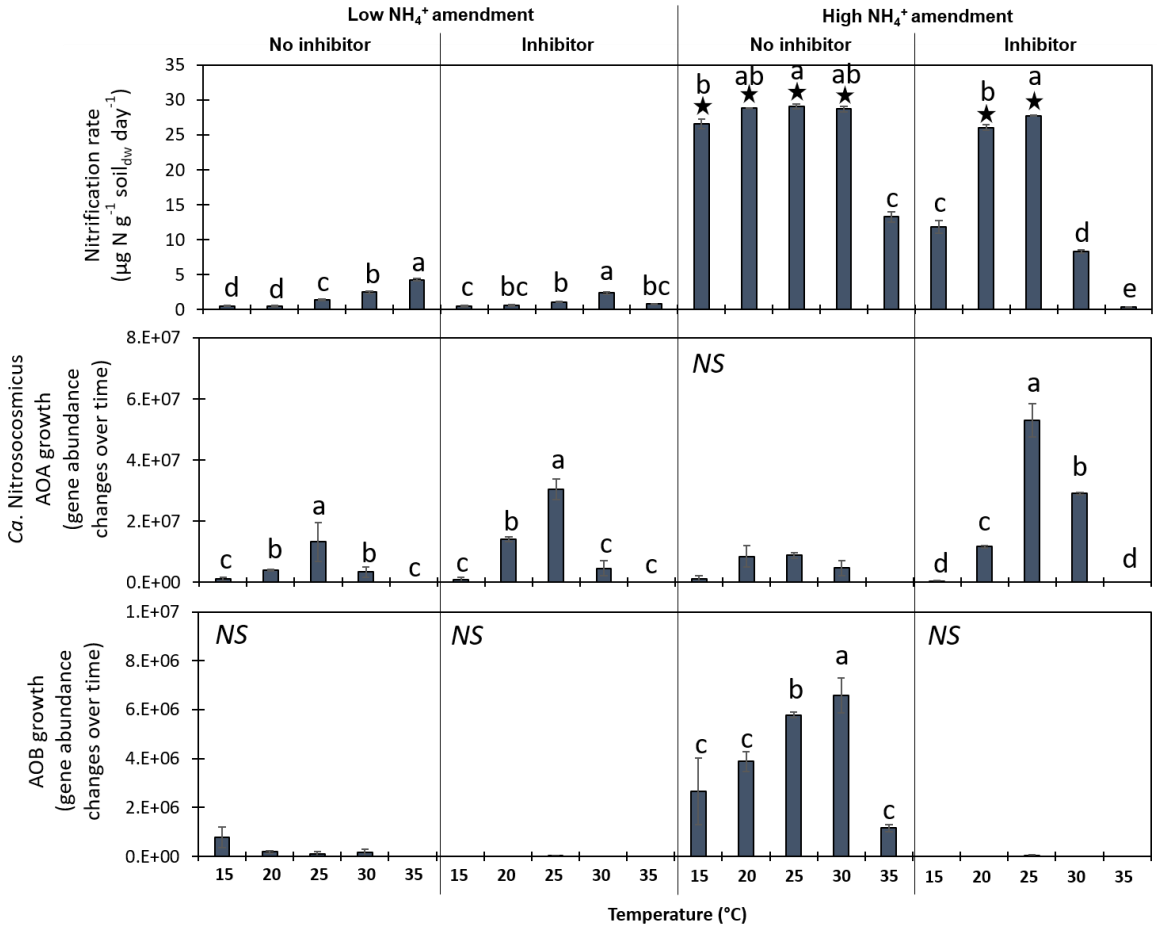
Figure 2: Nitrification rate (µg N g^{-1} dry soil day^{-1}), growth of *Ca.* Nitrosocosmicus AOA and AOB growth during incubation of low- and high-NH_4^+ soil microcosms at five temperatures, in the absence or presence of the AOB-inhibitor octyne. Stars indicate minimum estimated nitrification rates, due to complete oxidation of NH_4^+ between sample points. Plotted points and error bars represent means and standard errors of triplicate measurements. Different letters indicate significant differences (p<0.05) between means within each ammonia x inhibitor combination and NS indicates non-significant mean difference.

Supplementary Figure 1: Soil pH measured during incubation of low- and high-NH_4^+ soil microcosms at five temperatures, in the absence or presence of the AOB-inhibitor octyne. Plotted points and error bars represent means and standard errors of triplicate measurements. Different letters indicate significant differences (p<0.05) within each ammonia x inhibitor combination.


15°C
20°C
25°C
30°C
35°C
A
B
C
D
E
F
G
H
I
J
Ammonium concentration (µg N g-1 soildw)
Nitrate concentration (µg N g-1 soildw)
Time (days)
No inhibitor
High NH4+ amendment
Low NH4+ amendment
Inhibitor
High NH4+ amendment
Low NH4+ amendment

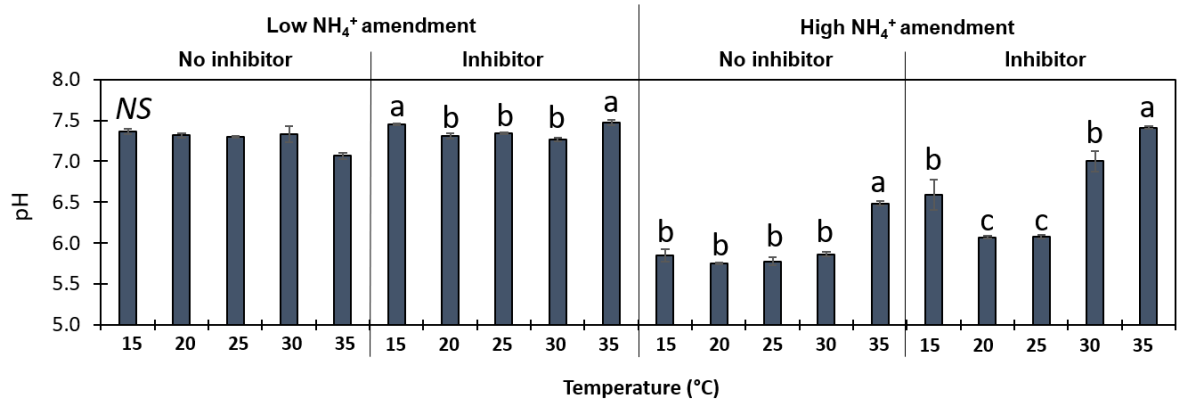
509

510


Low NH4+ amendment
High NH4+ amendment
No inhibitor
Inhibitor
No inhibitor
Inhibitor
Nitrification rate (μg N g-1 soildw day-1)
Ca. Nitrosocosmicus AOA growth (gene abundance changes over time)
AOB growth (gene abundance changes over time)
NS
Temperature (°C)

511

512


Low NH_4^+ amendment
High NH_4^+ amendment
No inhibitor
Inhibitor
No inhibitor
Inhibitor
pH
8.0
7.5
7.0
6.5
6.0
5.5
5.0
NS
a b b b a
b b b b a
b c c b a
15 20 25 30 35 15 20 25 30 35 15 20 25 30 35 15 20 25 30 35
Temperature (°C)

513